POLLEN, EMBRYO AND ENDOSPERM DEVELOPMENT FOLLOWING CROSS-POLLINATION WITHIN AND BETWEEN THE CROP SPECIES *BRASSICA CAMPESTRIS*, *BRASSICA OLERACEA*, *BRASSICA NAPUS* AND *RAPHANUS SATIVUS*

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Ph.D.

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1985

Abstract

A number of features concerning pollen, embryo and endosperm development after all the possible cross combinations of *Brassica campestris*, *B. oleracea*, *B. napus* and *Raphanus sativus* were examined. Two cultivars, one diploid and one tetraploid cultivar, were investigated from each species with the exception of *B. napus* where two different plant types, a leafy forage rape and a swede, were examined.

It was found that *Brassica* pollen could be stored at low temperature and low humidity and still remain viable for more than a year. In some cases, the effect was to increase the percentage of ovules that were fertilised, while with other species, the opposite was observed.

The standard bud pollination technique produced a lower seed set when compared to normal insect pollination. The percentage of seed set varied according to the species. Optimum pollination conditions for bud self pollinations, intraspecific and interspecific hybridisations were examined and were found to vary according to the different pollination types.

Embryo and endosperm development after bud self pollination was observed. All the cultivars examined followed the same pattern of development but each was found to have varying lag periods between pollination and the beginning of embryo development. The time taken to complete development also varied depending on species and cultivar.

Following interspecific hybridisation many combinations produced a relatively high percentage of developing ovules, most of the crosses produced embryos which developed to the later stages. Other crosses produced a few developing ovules, which tended to abort in the early stages of development. It was found that there are critical stages of

embryo development where abortion is likely to occur, and by choosing parents with a similar lag period between pollination and the commencement of development, may increase the success of hybrid production.

The endosperm failure and embryo abortion occurring after hybridisation was discussed in relation to the genomic constitution of the parents. All the cross combinations were based on three genomes (A, C and R), therefore it was possible to examine the effect of hybridity, genome number, ratio and composition and tissue types of the embryo and endosperm in relation to their success in hybrid production.

Dedicated

to my son,

Mark.

Acknowledgements

I would like to express my sincere thanks to Dr. Adrian Dyer for his advice and encouragement throughout the work reported in this thesis. I am also indebted to Professor M.M. Yeoman for the use of the facilities in the Botany Department; and to Dr I.H. McNaughton for his advise and co-operation with the practical aspects of the work carried out at the Scottish Crop Research Institute at Pentlandfield.

I am also grateful to my parents for their encouragement and support throughout this work. Finally, I would like to thank my husband for his moral support, for providing computer software for the analysis of some of the data and for typing this manuscript.

This work was carried out during the term of an SERC CASE studentship.

Declaration

This thesis has been composed by myself and the work reported herein is my own.

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Introduction.

The nature of human society produces ever changing demands for new and improved agricultural and horticultural products. The aim of the plant breeder is to satisfy these demands via the creation of improved genotypes. His aim is to increase the yield of a given crop. This can be achieved through improving the quality and disease resistance of a crop. The emphasis made on a particular character depends largely on the market at which the end product is aimed.

The coupling of Darwin's idea on the breeding of better adapted individuals, and Mendel's analysis of hereditary traits, saw the birth of Neo-Darwinian concept at the beginning of this century (Simmonds, 1979). In addition, the demonstration of the continuity of the germplasm and the phenotype-genotype relationship help provide the basis for a scientific approach to plant breeding.

Up until about 200 years ago, the improvement of crops was achieved mainly by farmers. Early improvements in agricultural worth were therefore achieved by natural selection, plus a conscious selection by the farmers to grow only the most productive plants. In contrast, today's plant breeder improves the productivity of a crop primarily by introducing genetic variation, usually by artificial hybridisation of genetically different parents, then exerting selection pressure on the population, retaining the superior genotypes while those of a lower agricultural worth are discarded.

1.2 The contribution of wide hybridisation to plant breeding.

Successful interspecific hybridisations are advantageous to plant breeding since it is a means by which genes for disease resistance, and other important genetic traits, can be transferred from other

related crops or from more primitive species. It can also widen the genetic base and thereby increase the variation in a species from which to select desirable characters, for example in Brassica. The breeding barriers which naturally isolate these species or populations often make it difficult to produce desirable hybrids. Development of techniques such as bud pollination (Pearson, 1921), embryo culture (Harberd, 1969 and 1971; Nishi, Kawata & Toda, 1959 and Snell, 1977), ovule culture (Inomata, 1977, 1978a, 1978b and 1979; Kameya & Hinata, 1970 and Takeshita, Kata & Tokumasu, 1980), and more recently, techniques such as the isolation and fusion of protoplasts from somatic tissues and the generation of embryos from them (Gleba & Hoffman, 1979 and Kemeya & Takahashi, 1972) and finally irradiation of pollen (Borrino, Caligari, Powell, McNaughton & Hayter, 1985) can all be used to increase the possible range of hybrids produced.

1.3 Concept of interspecific hybridisation.

Hybridisation between two species involves the exchange and fusion of gametes between them. Breeding populations can remain isolated by the use of pre-zygotic and post-zygotic incompatibility mechanisms. The most common pre-zygotic mechanism (ie. the prevention of pollination or fertilisation) includes spatial separation, where the two populations are too far apart for pollen to be transferred. Separation can be temporal, the two populations may flower at different times of the year, or different times of the day, or the pollen may be shed before the stigmas are receptive. Biological separation may be present where the pollination vector is different for each population. Stigma/style barriers may be effective where pollen tubes are prevented from fertilising the egg cell.

Post-zygotic mechanisms (ie. the prevention of normal offspring

development) operate at various stages. The incompatibility of zygotic or embryonic tissues with that of the female plant or the incompatibility of genotypes within the zygote leads to seed abortion. F_1 hybrids may be completely inviable, or they may be vigorous but sterile. The F_1 generation may be fertile but successive generations are often weak or sterile. Hybrids do occur in nature as a result of introgressive hybridisation. The successful hybrids are often a result of backcrossing between the original F_1 hybrid and either, or both, of the parental types. Whether hybridisation is intergeneric, interspecific or intraspecific is therefore really a consequence of taxonomic decisions where hybridisation of the latter type is between two populations which are more closely related and leads to less disturbance than the former cases. Some intraspecific hybridisation will lead to the same developmental difficulties of pollen and embryo as interspecific crosses, while some interspecific hybrids will develop without difficulty. It is this that causes the differences between biological and taxonomic definitions of species.

1.4 Domestication of *Brassica* crops, with particular reference to the British species.

Domesticated *Brassica* crops are grown in many parts of the world, but the species discussed here are pertinent to Britain with their origins based in Europe. Three amphidiploid species have been recognised, based on hybridisation between the three species *B.nigra*, *B.oleracea* and *B.campestris* (Figure 1.1). In general the amphidiploids are self-compatible, whereas the parent derivatives are self-incompatible. The relationship between the species *B.campestris*, *B.oleracea*, *B.napus* and *R.sativus* and the potential amphidiploid products are shown in Figure 1.2.

Figure 1.1 The species relationship between Brassica nigra, Brassica oleracea and Brassica napus and the amphidiploids they produce (From McNaughton & Ross, 1978).

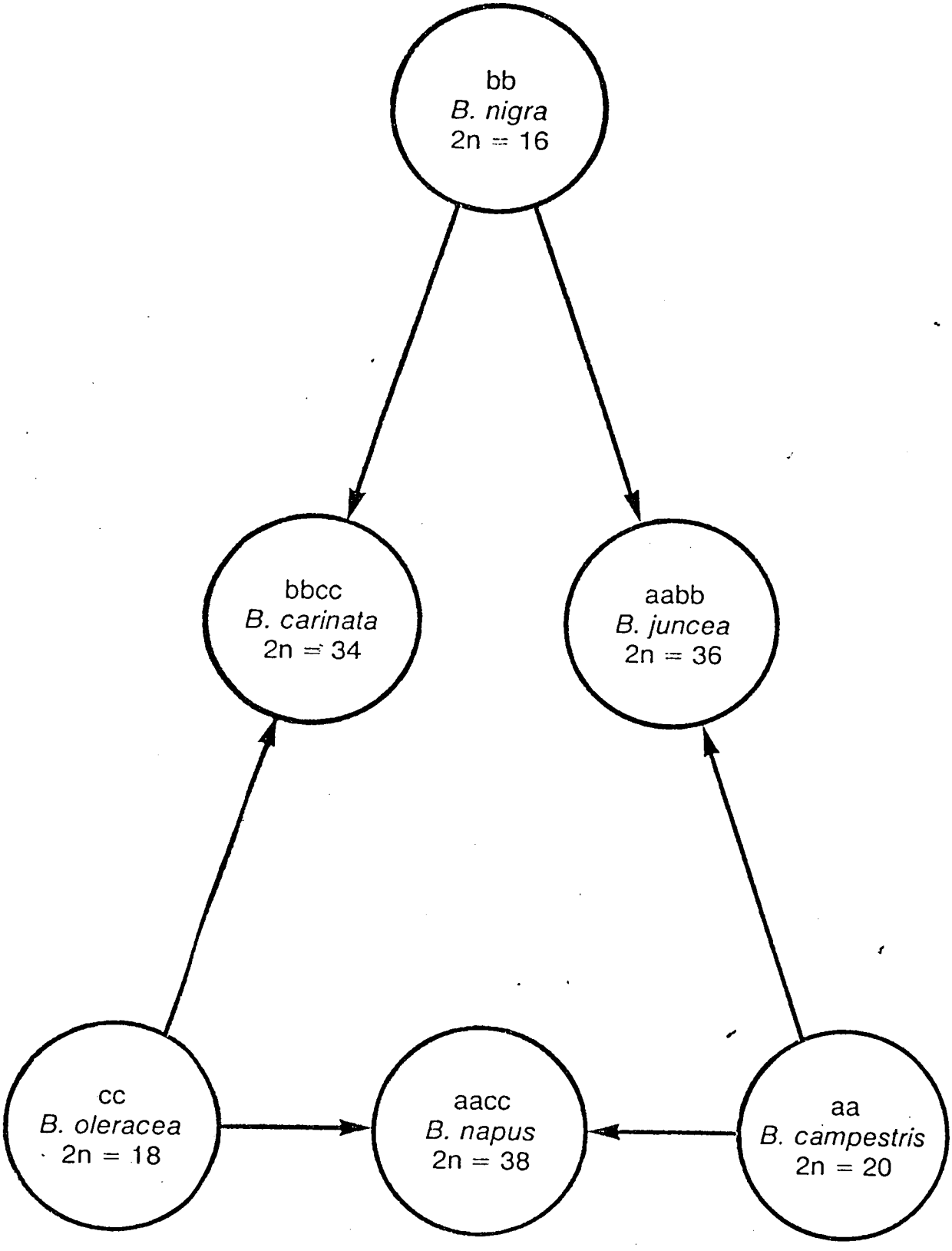
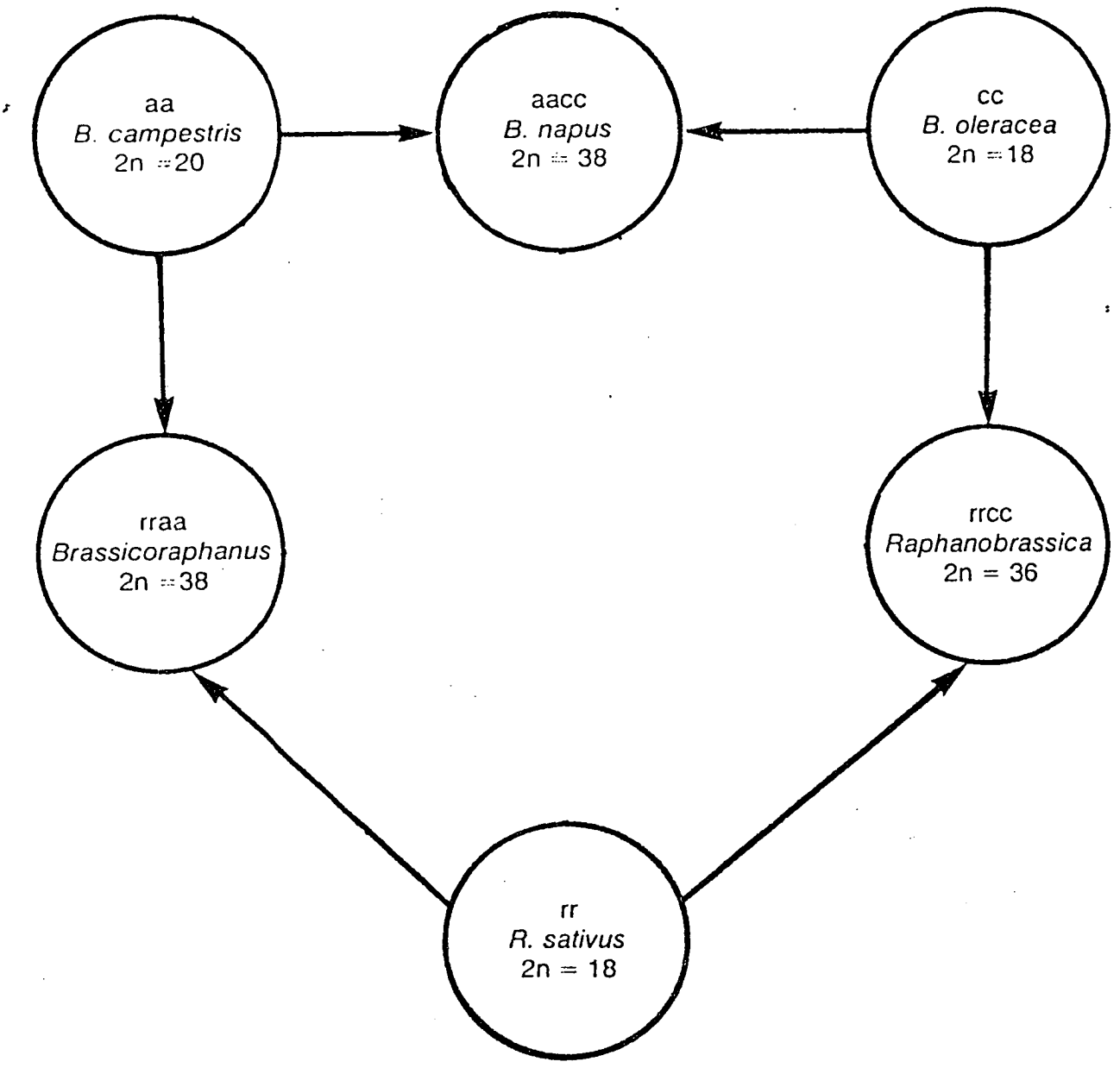


Figure 1.2 The relationship between the species Brassica campestris, Brassica oleracea, B.napus and Raphanus sativus and the amphidiploids they produce (From McNaughton & Ross, 1978).



1.4.1 Brassica campestris, 2n : 2x = AA = 20

The Mediterranean area is thought to be the primary centre of the origin for the European forms (Simmonds, 1976). Annual oil-seed forms are thought to have been domesticated since about two-millenia BC. Cultivation of oil-seed turnip rape is thought to have started in Europe in the thirteenth century. The biennial oil-seed forms were thought to be the source of turnips, with origins in the cooler parts of Europe. Stubble-turnip on the other hand is a more recent development, originating in Europe probably around the fifteenth to eighteenth centuries. Forms resembling the modern Dutch cultivars were being grown in Britain in the early nineteenth century. In the nineteenth century various forms of turnip were reported to have been grown side by side in Britain. More recently, heterosis for dry matter yield of turnips has been shown (Wit, 1966). Synthetic varieties based on inbred lines of good combining ability have outyielded some of the best commercial cultivars. Tetraploid cultivars have a marginally higher dry matter yield than their diploid counterparts. Breeding of oil-seed turnip rape has produced strains with low erucic acid content.

Future work improving the different varieties includes the development of leafier forms of stubble-turnip, with good leaf retention for use as forage. This character can be introduced from the leafy oriental species, although these subspecies are often susceptible to clubroot. Hybrids between different subspecies of B.campestris have produced yields which are superior to either parent.

1.4.2 Brassica oleracea, 2n : 2x = CC = 18

The early domestication of this species is thought to be the Mediterranean area and/or Asia Minor. The different varieties found

in this polymorphic species probably took place in several different areas. The Greeks grew kales as early as 600 BC, and several other types, including heading cabbage and possibly kohlrabi were described by writers in ancient Rome. Wild species in the Mediterranean areas are annuals, which grow in the warm moist winter. As the early cultivars spread northwards to the cooler climate of Europe, biennial types were probably selected with greater winter-hardiness and a requirement for cold vernalisation treatment for flowering. Less fibrous stems, and more succulent storage organs, were other early selection criteria. B.oleracea var capitata, cabbage, was known to be cultivated in Germany about 1150AD and in England by the fourteenth century. Cauliflower, (B.oleracea var botrytis), in its present form was unknown before the early Middle Ages. It reached Germany, France and England around the seventeenth century. Sprouting broccoli, (B.oleracea var italica), originated in the Cyprus region to Italy, where is was first mentioned in 1660. B.oleracea var gemmifera, Brussels sprout, appeared in Belgium around 1750, and reached France and England about 1800. Its earlier history is uncertain. Marrow stem kale, B.oleracea var acephala was first recorded in the Vendée region of France in the early nineteenth century. Its origin is however not known.

The breeding emphasis of varieties which are harvested mechanically is for uniformity of heading, curding or sprouting production, depending which is considered. With marrow-stem kale, which is grazed in situ, uniformity is of lesser importance than for shorter, less fibrous cultivars. Future improvements are aimed at reducing the goitrogenic thiocyanate content of the leaf and also S-methyl cysteine sulphoxide, responsible for producing kale anemia in livestock.

1.4.3 Brassica napus, the amphidiploid of B.campestris and B.oleracea,

2n : 4x = AACC = 38

B.napus provides two forage types - (i) the swede, which is important animal fodder in Northern Europe, Russia and New Zealand as well as being used for human consumption and (ii) the leafy forage rape, which provides fodder for sheep in Northern Europe and New Zealand. The biennial form of oil seed rape is used for oil-seed production in Europe. The oil is used in a variety of food and industrial products.

It is uncertain is these species existed in truly "wild" forms. If they do, they must be European-Mediterranean in origin, where there is an overlap between B.campestris and B.oleracea. The biennial oil-seed form was known to be growing in Britain in the early nineteenth century. Swedes were first recorded in Europe in 1620. They were introduced to Britain around 1780. Introgression between B.napus and B.campestris has been reported in Britain since the middle of the nineteenth century. Artifical swedes obtained from crossing B.campestris and B.oleracea has provided useful breeding material.

Work is in progress towards the breeding of F_1 hybrid varieties of swede, as these show heterosis for high quality fodder production. Interspecific transfer of genes for self incompatible alleles and Plasmodiophora resistance have been achieved for B.campestris. Artifically produced B.napus could also widen the genetic variation within the species.

1.4.4 Raphanus sativus, 2n : 2x = RR = 18

It is supposed that some area east of the Mediterranean was the probable source of this crop, but exact definition of the area has not been made. The niger type of radish was known here about 2500BC. At the end of the sixteenth century a long white form of the radicula type appeared in Europe. In the eighteenth century white and red

globular forms were developed.

Most current work is aimed towards further adaptation to different growth conditions and improved resistance to pests and diseases. The success of fodder radish in Western Europe is a result of German breeding work.

1.5 Hybridisation of British forage brassicas.

Much of the earlier hybridisisation work has been carried out to establish the phylogenetic relationship between the brassica species. The present emphasis in Brassica crossing programmes is on the introduction of characters from related species to improve the yield, general vigour and the spectrum of disease resistance of agricultural crops. For example, Plasmodiophora brassicae (causing clubroot) and Erysiphe cruciferarum (causing powdery mildew) are both important pathogens of B.napus (rape and swede). These diseases can seriously reduce yield and palatability of the crop. B.campestris shows a high level of resistance to Plasmodiophora and B.oleracea shows tolerance to both club-root and powdery mildew. These two species can be hybridised to produce new synthetic B.napus which shows high levels of resistance to both these diseases. Disease resistance may also be introduced into existing B.napus by crossing with either parent.

Considerable heterosis has been demonstrated in F_1 hybrids as a result of crossing cultivars of B.napus. Self-incompatibility alleles may be introduced from either B.campestris or B.oleracea into this virtually self-compatible species by hybridisation.

Resistance to Plasmodiophora and some degree of tolerance for Erysiphe cruciferarum is also found in R.sativus. This species may be effective in adding to the genetic variability in brassica species

mentioned if the disease resistance can be transferred from it.

Successful hybridisation between B.oleracea and R.sativus is possible and produces the recognised species of Raphanobrassica. However, there is doubt about its potential as a seed crop because of the problems with harvesting and threshing the siliques. The yields produced are insufficient to merit it as a commercial crop.

It is also possible to improve the nutritional value and digestability of the leafy forms of rape and kale by hybridisation within the species.

1.5.1 Intraspecific hybridisation of Brassica - hybridisation of distinct subspecies with cultivars of the same species.

1.5.1.1 Intraspecific hybridisation of B.campestris.

Olsson (1954) has reported that some oriental types of B.campestris intercross easily and produce hybrids. Most of these are fertile. Nishiyama & Inomata (1966) studied the embryo and endosperm development after reciprocal crosses between diploid B.campestris ssp chinensis and tetraploid B.campestris ssp pekinesis. When the diploid was pollinated by the tetraploid, many ovules showed slight development, but in most crosses the seed collapsed at the young embryonic stages. The reciprocal cross produced a few viable triploid seeds. The diploid and tetraploid parents have a different rate of endosperm development. Nishiyama & Inomata (1966) have suggested that the embryo development was probably arrested at the early stages of growth in the hybrid cross due to poor development of the endosperm. The fact that triploid hybrids can be produced by the use of embryo culture tends to substantiate this claim.

1.5.1.2 Intraspecific hybridisation of B.oleracea.

This species consists of a number of distinct botanical varieties, for example, cauliflower (var botrytis), cabbage (var capitata), broccoli (var italica), Brussels sprouts (var gemmifera) and marrow stem kale (var acephala). Mackiewicz (1973) investigated the cross between B.oleracea var capitata (cabbage) and B.oleracea var acephala (marrow-stem kale). The cross was made at the tetraploid level and hybrids were produced. Hakansson (1956) carried out reciprocal crosses of diploid and tetraploid B.oleracea var acephala. Triploids were produced from the tetraploid by diploid cross, but not in the reciprocal. The endosperm failed to become cellular but instead contained giant nuclei, unlike normally developing endosperms.

1.5.1.3 Intraspecific hybridisation of R.sativus.

The development of the embryo and endosperm after reciprocal crosses between diploid and tetraploid forms of radish have been reported by Inomata (1970). Many triploid hybrids were found when the tetraploid plants were the female parent. In the reciprocal crosses many ovules showed slight development, but most collapsed in the young embryonic stages. This was associated with the appearance of abnormalities in the endosperm development.

Reciprocal crosses of triploid and diploid radish were investigated by Tokumasu (1965). Most seed was formed when the female parent had the higher chromosome number.

1.5.2 Interspecific hybridisation of Brassica species.

1.5.2.1 Hybridisation between B.campestris and B.oleracea

These two species have been recognised as being difficult to cross (Hosoda, 1961; Hosoda, Nama & Gotho, 1963; Hosoda, Sarashima & Namai,

1969; McNaughton, 1968; Sarashima, 1964). Hakansson (1956) looked at the seed development of reciprocal crosses between kale and turnip at the diploid and tetraploid level. She concluded that one of the main barriers in the production of hybrids was endosperm deficiency. Cell formation may be inhibited and the endosperm remains nuclear; enlarged endosperm nuclei were often found in large numbers. Chiang, Chiang & Grant, 1977; Ellerstorm, 1978; Homma & Heeckt, 1960; and Olsson, 1960 all recognised this as a unilateral cross, succeeding only when B.campestris is the female parent. The frequency of mature plants obtained is very low.

Others have obtained hybrids with B.oleracea as the maternal parent, but again seed production was low (Yarnell, 1956). Embryo culture can be used to increase the frequency of hybrids (Nishi, Kawata & Toda, 1962 and Snell, 1977). Inomata (1980) improved the production of hybrids by employing the technique of ovule culture. A large proportion of non-hybrid maternal offspring ("matromorphs") is often observed with this species combination (Mackay, 1972 and Yarnell, 1956).

When the re-synthesis of B.napus (2n=38) is successful the hybrid is often indistinguishable from the natural material. The fertility of the hybrid is frequently poor, but may be improved in later generations. Artificial B.napus hybridises readily with natural B.napus to produce fully fertile hybrids (Lammerink, 1970 and Snell, 1977) and can therefore be used as a bridge to bring in desirable characters from the parent species.

1.5.2.2 Hybridisation between B.napus and B.campestris.

These two species hybridise readily in either direction to produce an allotriploid (2n=29) genome constitution AAC. There is no strong

incompatibility barrier between them, and they will hybridise when grown together in the field (Palmer, 1962). Quite often more hybrid seed is produced when B.napus is the maternal parent (Frandsen & Winge, 1932; McNaughton, 1963a, 1968 and 1973). Sometimes a hexaploid plant is produced spontaneously, otherwise full fertility can be restored with colchicine.

1.5.2.3 Hybridisation between B.napus and B.oleracea.

These two species have been reported as being difficult to cross successfully (Chiang, Chiang & Grant, 1977; Gowers, 1974 and Yarnell, 1956). When B.napus is used as the maternal parent, the cross is more productive than after the reciprocal combination. Success is enhanced by healthy maternal plants, given favourable environmental conditions throughout the growing period after the inflorescence has emerged (Chiang et.al., 1977).

1.5.3 Intergeneric hybridisation between Raphanus and Brassica species.

1.5.3.1 Hybridisation between R.sativus and B.campestris.

When B.campestris is used as the female parent, few developing hybrid seeds result (Dolstra & Zuidgeest, 1979a and 1979b). This combination is most successful when Raphanus is used as the maternal parent. The frequency of hybrids produced is however low (McNaughton & Ross, 1978; U, 1937 and Yarnell, 1956). The hybrids have very low fertility and poor vigour after crosses at the tetraploid level (Ellerstrom, 1973 and 1978).

1.5.3.2 Hybridisation between R.sativus and B.oleracea.

These two species are recognised as a unilateral cross combination,

succeeding when Raphanus is female (Ellerstorm, 1978; Kakizaki, 1927; Karpechenko, 1924; McNaughton, 1973b and Sampson, 1962). Seed setting and pollen fertility are originally low in the hybrid, but can be improved by selection over a number of generations. The prospect of Raphanobrassica as a seed crop is thwarted by seed yield, which is too low for commercial production (McNaughton, 1979b). Hybrids produced at the diploid level are sterile. Direct synthesis of the hybrid from tetraploid parents depends largely on the meiotic stability of the parents.

1.5.3.3 Hybridisation between R.sativus and B.napus.

Turesson & Nordenskiold (1943) obtained a few sterile hybrids when they crossed B.napus with tetraploid Raphanus. McNaughton & Ross (1978) found that the cross in either direction produced only a few hybrids.

1.6 Failure of hybrid crosses.

Except for a few cultivars, B.campestris, B.oleracea and R.sativus may be considered as outbreeding species, possessing well defined sporophytic self-incompatibility systems. B.napus with few exceptions, is self compatible.

In a sporophytically controlled system, the prevention of self pollination can be due to either of two factors.

i) the total inhibition of pollen tube growth.
ii) the failure of germinated pollen tubes to penetrate the stigmatic papillae.

Considerable variation exists in the degree of pollen tube

penetration. Material which is weakly self compatible shows coiling of the pollen tubes on the stigma. Some pollen tubes may grow through the stigmatic surface with inhibition sometimes occuring in the style (Gowers, 1979). With bud self pollination the pollen is applied to the stigma before the self-incompatibility system is developed. Pollen tube penetration is similar to that for a compatible pollination.

With the cross B.campestris x R.sativus, Dolstra & Zuidgeeest (1979b) found that most of the pollen grains stopped on the stigma. Many of the pollen grains possessed very short pollen tubes, characteristic of a self incompatible reaction. A low number of pollen tubes penetrated the ovary but often appeared disorientated. Subramanyam (1954) also observed poor pollen tube growth in the cross Brassica x Raphanus.

When pollen germination occurs, the sequence of the main events in the pattern of the seed development in angiosperms is:

i) double fertilisation.

ii) initiation of the mitotic division of the fertilised egg and primary endosperm nucleus, associated with an increase of cytoplasmic endosperm.

iii) the differentiation of the embryo and endosperm, followed by physiological and structural changes of several tissues of the ovule.

The failure of development of the hybrid seed can occur at any of these stages of development. Reports in the literature (Cooper & Brink, 1940; Hakansson, 1956; Nishiyama & Inomata, 1966) strongly suggest the embryo abortion in general may be due to occlusion and/or degeneration of the endosperm. Once F_1 plants are produced they may differ in fertility, the more fertile plants often show

sib-relationships, as for example in Raphanobrassica. McNaughton (1973) and Ellerstrom (1977) have both agreed that the varied fertility is due to variation in the genetic combinations produced, because the hybrids show very little cytological abnormalities. It was suggested that incompatibility between the nucleus and cytoplasm could result in sterility (McNaughton, 1973).

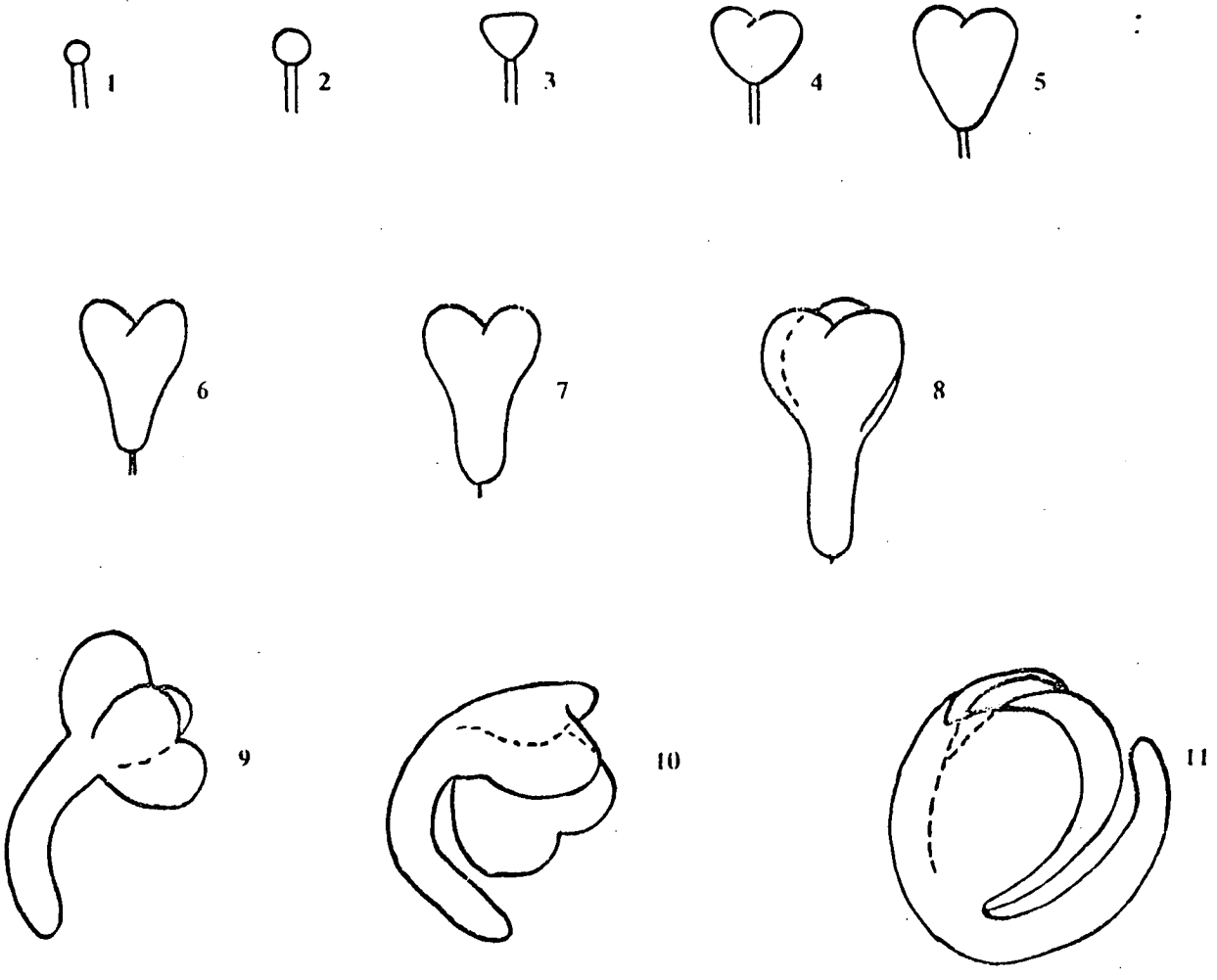
1.7 General description of embryo development in the Cruciferae.

1.7.1 Morphological stages of embryogenesis.

The morphological stages of embryo development in the Cruciferae, as represented by Capsella bursa-pastoris, has been well documented (Rijven, 1952 and Wardlaw, 1955). The stages of development as arbitrarily defined are shown in Figure 1.3. Schulz & Jensen (1968a and 1968b) have also produced a series of papers outlining changes in ultrastructure of the egg and embryo after fertilisation in Capsella bursa-pastoris. The general sequence of development found in Cruciferae is described below.

After fertilisation, the zygote divides to produce an embryo cell and a suspensor cell. The first few divisions of the embryo are synchronous. Globular shaped embryos are produced. The shape of the embryo is altered as the primordia of the two cotyledons begin to grow to produce a triangular shape as the 'pre-heart' stage is established. As these grow the 'heart' shape embryo is established. The embryo elongates to produce the 'torpedo' stage. The growing cotyledons curve to adopt the shape of the embryo sac and produce the 'walking-stick' shape. The cotyledons enlarge and grow towards the hypocotyl, 'mature I', until eventually the embro sac is filled, 'mature II' and 'mature III' embryos. The difference between the mature II and mature III stages of development is the final

Figure 1.3 The different stages of embryo development as arbitrarily defined, and described in the text. The final size of the embryos are similar in the three Brassica species and are approximately twice the size in Raphanus sativus. The measurements given are for B.napus cv Lair. 1= early globular (22µm); 2= globular (125µm); 3= pre-heart (216µm); 4=heart (216µm); 5= late heart (425µm); 6=early torpedo (476µm); 7= torpedo (770µm); 8= late torpedo (1467µm); 9= walking stick (1710µm); 10= mature I & II (2626µm); 11= mature II & III (3940µm).



disappearance of the endosperm. The size of the embryos at the various stages of development will depend on the species involved. The suspensor cell divides to produce a uniseriate suspensor.

1.7.2 Changes in the carbohydrates, proteins and nucleic acids during seed development.

There is not much literature available on the changes which occur during development *in situ*. Johri & Maheshwari (1966) have reported the changes which occur in the opium poppy. The type of seed development is similar to *Brassica* so the changes may be relevant. Once endosperm development had begun, the embryo and endosperm contained starch granules until the change from coenocytic to cellular endosperm, after which the starch granules began to disappear from the endosperm. In the mature seeds starch grains were present only in the cells of the embryo and nucellus.

Soluble nitrogen (as amide nitrogen) also decreased with maturation and proteins were formed in the endosperm or embryo. The significant increase in protein was noted at about the heart to torpedo stage of development. During the accumulation of proteins, the carbohydrates decrease. Johri & Maheshwari (1966) suggested that the starch reserves may have been utilised in the manufacture of the proteins, since increased activity of the enzyme glutamic-alanine transaminase was noted during this period of protein accumulation. RNA and DNA also increased rapidly during the first half of development. The maximum amounts were found almost at the same time as the maximum ovule size (ie. about the torpedo to walking stick stage). Huang (pers. comm.) found a dramatic increase in the amount of storage protein at about the 'walking stick' stage of embryogenesis in *Arabidopsis thaliana*. He feels that the increase may be present earlier but is limited by his detection techniques.

Tykarska (1982) reported starch presence during rape embryogenesis. After fertilisation, starch was noted for the first time in cells of the suspensor of the proembryo with a 2-cell apical embryo. Small starch grains were present in the proembryo body at the octant stage. Large starch grains were found in the suspensor and hypophysis. By the time the embryo had reached the heart stage, the embryos were green and there was a gradual accumulation of stored starch which lasted to the end of embryogenesis. The starch began to disappear when green colour of the seed coat began to fade. Mature seeds have hard, white embryos which contain many fats and lipids.

With the development of techniques for somatic embryogenesis over the past few years, information on the biochemical control of the stages of development *in vitro* is beginning to emerge. An increase in embryonic proteins, polyamine synthesis and enzyme activity, specific to the very early stages of somatic embryogenesis of carrot culture has been reported (Sung *et.al.*, 1984). The mechanism which causes these changes and whether they occur *in vivo* is not yet known. Sung *et.al.* (1984) also reported that, once embryogenesis is initiated, the hormone 2,4-D can prevent the transition from the globular to the heart stage if present in the culture media.

Huang (pers. comm.) also working in this field but with *Arabidopsis thaliana* feels that changes in the stage of development are under hormonal control *in vitro*, although it has yet to be established if this is so *in vivo*.

1.8 Objective of this investigation.

The objective of this investigation was to observe and record the events of embryo and endosperm development after intra- and interspecific hybridisation of the *Brassica* and *Raphanus* species

previously mentioned. From these observations the possible causes of failure to produce hybrids are hoped to be identified.

The applied objective of this project is that by identifying those areas where problems arise after hybridisation, a solution to overcome the problem may be investigated. It may then be possible to increase hybrid production, and hence the variation required by plant breeders from which to select new and improved characters.

There are many stages in the development of a hybrid where the barriers are found. The failure of pollen penetration, double fertilisation, early embryo and endosperm development and completion of the various stages of embryogenesis are all possible stages where development of the hybrid can be stopped. There are many reports in the literature of hybrid development, although very little is known about the reasons for success or failure of wide crosses between genera, species or varieties. In those cases where hybrid development has been studied the main suggested cause of failure was poor endosperm development. Only a small amount of literature (Dyer, 1963; Roupakias & Kaltsikes, 1977) has reported any suggestion of the role of genome effects. If a number of different combinations can be made, based on two or three genomes, it may be possible to determine the effect on embryo and endosperm development of different genome numbers, ratio and composition in different cytoplasmic background. This system is provided by intercrossing B. campestris, B. oleracea and B. napus, the three main forage brassicas grown in Britain. R. sativus is also used as a forage crop, but is of lesser importance.

The system used in this thesis is based on different combinations of A, C and R genomes in embryo and endosperm tissue in different cytoplasms, and offers the possibility of identifying important factors determining the development of the hybrid. For example, the

products from an AA x CC and a AAAA x CCCC combination will have the same genomic ratio and similar cytoplasm, but the ploidy level will be different in both the endosperm and the embryo. Comparisons of the cross combinations AA x CC and the reciprocal CC x AA, has the same nuclear genotype, but different cytoplasmic backgrounds in the embryo. The endosperms differ in genome ratio. Hybridisation of AACC x AA and AACC x CC would produce embryos and endosperms which have the same cytoplasm and genome representation, but differ in genome ratio in both the embryo and endosperm.

It is necessary for Brassica breeders to hybridise the different species in order to improve yield, disease resistance and palatability of potential new varieties. A better knowledge of how, when and how often different cross combinations fail may lead to ways of improving the productivity of desirable hybrid combinations.

CHAPTER 2

MATERIAL AND METHODS

2.1 Introduction.

This chapter gives the description of the relevant techniques used throughout this investigation. A detailed description of materials and methods is given in the chapters with which they are concerned.

2.2 Choice of species.

The material chosen for the work in this project was confined to the three main forage brassicas grown in Britain, Brassica campestris, B.oleracea and B.napus. Raphanus sativus was also examined. R.sativus is also used for forage, but is of lesser significance in British agriculture. Inclusion of Raphanus, has added interest in that it involves hybridisation between different genera.

For all the species, except B.napus, a diploid and tetraploid cultivar was used. For B.oleracea the diploid cultivar was Ponda and the tetraploid cultivar Taronda; for R.sativus the diploid cultivar was Slobolt and the tetraploid cultivar Crail. These tetraploid cultivars are autopolyploids of the diploid cultivars, but have undergone some selection to become commercially grown cultivars. In B.oleracea the tetraploid cultivar used is an autotetraploid of the cultivar Maris Kestrel, and has undergone very little selection. B.napus is an established amphidiploid of B.campestris and B.oleracea, and two different plant types were chosen for examination; the leafy forage rape, cultivar Lair and the cultivar Ruta Otofte, which is a swede.

By examination of diploid and tetraploid cultivars it is possible to investigate a greater range of ploidy levels and genomic ratios in the embryo and endosperm. By examination of two different genotypes with the same genomic constitution, as in B.napus, it is possible to distinguish genetic effects, when the genomic combinations and ratios

are the same after cross pollination.

2.3 Vernalisation of plant material.

All species examined are biennials and therefore require some degree of vernalisation before flowering. To produce plant material which flowered simultaneously in the spring, the different species required different artifical manipulation. Two vernalisation methods for B.campestris, B.napus and R.sativus were examined.

The first involved artificial vernalisation of imbibed seeds. In mid-December, seeds of B.campestris, B.napus, and R.sativus were germinated at 20°C on damp filter paper, in petri dishes. On appearance of the radicle, they were transferred to the dark and maintained at 4°C for eight weeks. During February, the seedlings were pricked out into John Innes compost and moved to glasshouse conditions with a minimum temperature of 13°C. Flowering commenced at the beginning of April.

The second method involved natural vernalisation of the seedlings. Seeds of the above species were sown directly into compost in early September, and placed into a cold frame. When large enough, the seedlings were pricked into two inch square pots, and left in the cold frame over winter. These young plants were potted into six inch square pots and placed in the glasshouse conditions described above, at the beginning of March. By the end of March, flowering had begun.

B.oleracea required a different flower induction treatment. If the plants are to be grown from seed, they need to have 12 to 13 leaves (approximately 13 weeks old) before they will respond to vernalisation treatment. Alternatively, cuttings from mature plants can be rooted and subjected to vernalisation treatment. These plants require a

temperature of around 4°C for 13 weeks with an eight hour daylength before vernalisation is complete. After this treatment the B.oleracea plants can then be potted up and transferred to the glasshouse. To produce flowering of both Brassica species simultaneously, seeds of B.oleracea should be sown in August, the plants then being of an age where they are responsive to vernalisation by December. If material is available from which cuttings can be taken, they should be collected in December.

2.4 Bud pollination technique.

The standard technique of bud pollination has been in use since it was introduced by Pearson (1921). It has been used for bud self pollinations, where a self incompatibility system is present and also for interspecific hybridisations. There have been suggestions of a dual self and interspecific function of the incompatibility locus (de Nettancourt, 1977). It also ensures the exclusion of unwanted pollen, an important factor for both intraspecific and interspecific pollinations.

The method of bud pollination is normally carried out in a glasshouse where plants have been shown to flower successfully. Flowers or freshly dehisced anthers of the selected pollen parent were collected. A raceme of the receiving, maternal, parent is chosen. Normally flowering should have begun and there should be a range of bud sizes about the middle of the raceme, and a cluster of buds at the top. The opened flowers are removed, and the small cluster of buds at the top of the raceme, pinched off. The number of buds remaining, and their size, will depend on the species being used. For example, there were approximately 10 to 15 buds, each 1 to 1.5cm long, if B.oleracea was being used, while if B.campestris was to be the maternal parent, there

were six to eight buds, 0.5 to 1cm long. These remaining buds are emasculated using fine tweezers which have been cleaned in alcohol to prevent contamination. Most of the corolla and calyx are also removed, leaving the stigma, style and most of the ovary exposed. The selected pollen is applied to the stigma surface by brushing the dehisced anthers against it. When all the buds have been pollinated, the raceme is enclosed in a glassine bag, to prevent uncontrolled pollination. The bag should remain in place for at least 14 days, and should be examined to check that the growth of the raceme is not restricted.

Figures 2.1 to 2.6 show the different stages of the bud pollination technique.

2.5 Fixation and staining of material after pollination.

2.5.1 Fluorescence microscopy.

To examine pollen penetration of the stigma, pollinated gynoecia were collected three days after pollination, fixed in 3:1 absolute ethanol : glacial acetic acid and stored at $4^{\circ}C$ until required for examination by fluorescence microscopy. The method used was an adaptation of the fluorescence microscopy technique that is used at the Scottish Crop Research Institute, (formerly the Scottish Society for Research into Plant Breeding Institute) at Pentlandfield. Pistils were hydrolysed initially in 1M NaOH, allowing two hours at room temperature for the penetration of NaOH into the tissue. Hydrolysis was then completed with a further 30 minutes in fresh 1M NaOH at $60^{\circ}C$. The NaOH was removed and the plant material washed with distilled water. This was replaced with 2% methyl blue for two hours, at room temperature, or overnight in a refrigerator. The stain contained two grams methyl blue and 20 grams tri-potassium orthophosphate ($K_3 PO_4$)

Figure 2.1 First of all, collect together the plant chosen to be the maternal parent, in this example Brassica oleracea cv. Maris Kestrel (2x), and flowers which are ready for pollination from the paternal parent.

Figure 2.2 Select a raceme which is to be pollinated.

Figure 2.3 Remove any open flowers and the apical buds from the raceme. With sterile tweezers, remove the anthers and most of the calyx and corolla, taking care not to damage the gynoecium.

Figure 2.4 Remove the petals from the chosen male parent flower and brush the pollen loaded anthers against the exposed stigma.

Figure 2.5 Cover the pollinated raceme with a glassine bag and tie at the base. This should be done immediately to avoid uncontrolled pollination by insects.

Figure 2.6 The bag can be removed once growth of the siliques is evident. This example is B. oleracea cv. Maris Kestrel (2x) 22 days after pollination.

per litre of water. Pistils were mounted on glass slides in methyl blue solution, squashed under a coverslip and examined using a fluorescence microscope. A Vickers Photoplan M41 microscope was used. This employs the epi-illumination technique devised by Ploem (1967). The microscope was set up with a high pressure mercury vapour lamp as the light source, with a BG12 exciter filter giving a peak transmitted wavelength of 400μm and with a barrier blue filter (OG1 and GG9) to reflect wavelengths less than 500μm. Buds which were pollinated were not uniform in length, and therefore the pistils from these buds also varied in length.

2.5.2 Early development of ovules in sectioned material.

To observe the very earliest stages of ovule development two species were examined, one species that developed quickly and another species that developed at a slower rate. Developing siliques were fixed in a 3:1 absolute ethanol: glacial acetic acid and stored in a refrigerator at 4°C. At a later time, and at room temperature, the fixative was replaced with a number of changes of absolute ethanol, the last change being left overnight. The plant material was then put through a series of xylene/ethanol mixtures (1:3, followed by 1:2 and finally 1:1), and then they were put into pure xylene. Each change was for at least one hour. Most of the xylene was decanted from the tubes, which were then filled with wax shavings and placed in an oven at 60° C. and left overnight. The following day, two changes of hot wax were made, the last of which was overnight in the oven. The following day, the plant material was embedded into wax blocks.

The sections were cut using a microtome set at 15μm thickness. The wax ribbons containing plant material were mounted onto prepared slides. The wax was removed with two changes of xylene. Slides were

then transferred to alchohol : xylene, 1:1, and then into absolute alchohol and changed, through a number of concentrations, into water. The slides were left in 1% aqueous chromic acid overnight. Slides were then rinsed with water and the sectioned material was stained with 0.5% aqueous crystal violet for 20 mins.. The slides were again rinsed with water. Working with single slides, the sectioned material was mordanted in 1% iodine and 1% potassium iodine in 80% ethanol applied to the sectioned material and left for 30-45 seconds before being rinsed in absolute ethanol for 2 seconds. The staining was then differentiated with clove oil, while being observed down a microscope. The slides were then put through three changes of xylene for 10 minutes, each change and then mounted using Canada balsam.

2.5.3. Ovule development in dissected material.

Developing siliques were harvested at regular intervals between pollination and 40 days, and fixed in 3:1 absolute ethanol: glacial acetic acid. The siliques were opened and the ovules, if any, were removed. Individual ovules were dissected open and a few drops of HCl-carmine stain (Snow, 1963) placed inside. The stain was made by boiling together 15ml. of distilled water, four grams carmine (G.T. Gurr, London) and one ml. concentrated hydrochloric acid for about 10 minutes. When cool, 95mls. of 85% ethanol were added and the ingredients mixed well. The solution was filtered and kept in a stopped bottle. After application to the dissected ovules, the stain was left a few minutes to allow penetration of the tissues, care being taken not to allow the tissues to dry out. Excess stain was rinsed away with 70% ethanol. The ethanol was then carefully removed with a piece of filter paper and replaced with 45% acetic acid which contained a few drops of glycerine. The endosperm and embryo were teased from the ovular tissue, which was then discarded. A coverslip

was gently lowered over the embryo and endosperm. This material was then examined under a Vickers light microscope, and photographs were taken using Ilford Pan F film and a Zeiss Photomicroscope. The length of the expanded embryos was measured (μm) using an eye piece micrometer. The developmental stage of the embryo and endosperm, and any abnormalities which were present noted.

CHAPTER 3

POLLEN VIABILITY AFTER LOW TEMPERATURE STORAGE IN *BRASSICA CAMPESTRIS*, *B. OLERACEA* AND *B. NAPUS*

3.1 Introduction

When making interspecific crosses, it is necessary to manipulate the parent species so that they flower at the same time. When the age and conditions for flower induction differ in the two species, this can be difficult, or at the very least inconvenient. If pollen can be collected and stored, even for a few weeks, it may help to overcome this problem.

There is little in the literature on the storage of Brassica pollen. Chiang (1974) observed the longevity of cabbage (Brassica oleracea var capitata) pollen (a) stored at room temperature, (b) refrigerated at 4. ^{0}C. and (c) stored in the freezer compartment at -13^{0}C.. Chiang did not reduce the moisture content of the pollen before he placed it in one of the three treatments. The viability of the pollen was tested by its germination on a synthetic medium. It was reported that pollen grains lost their viability entirely after 1 day when stored at -13 ^{0}C.. Pollen stored for up to 10 days at 4^{0} C showed a reasonable germination percentage (36.3%). Further storage at this temperature resulted in reduced viability. Pollen stored at room temperature for one day, or more, yielded a lower germination percentage than pollen stored at 4^{0}C.. Working with dry pollen, Ockendon (1974) found that he could store dried Brassica oleracea pollen, at -20^{0}C., for up to two years without loss of viability. The stored pollen is often used for S-allele identification studies.

The pollen storage technique used in the following investigation was developed from a freeze drying method used for the storage of potato pollen (M. DeMaine, pers. comm.).

Maintained desiccation of pollen appears to be an important factor for increasing the longevity of pollen of many species (Linskens, 1964 and

Ockendon 1974).

If Brassica pollen can be stored, it would ease the problems of inducing simultaneous flowering in both parents. Storage of Brassica pollen may also have the benefit of giving rise to mutants which may overcome incompatibility barriers. Little is known of the genetic effects of storage on pollen but it is known that the long term storage of seed may lead to an accumulation of chromosome damage (Roberts, 1975). This phenomenon is found in stored animal tissue and seeds.

The following experiment was initiated to determine the pollen viability of a range of Brassica varieties after low temperature storage of one year or more. B.campestris cvs Ponda, Civasto, Taronda and Marco, along with B.oleracea cv Maris Kestrel (2x) and B.napus cv Lair were examined.

3.2 Material and Methods

Freshly dehisced anthers of B.campestris cv's Ponda, Civasto, Taronda and Marco, B.oleracea cv Maris Kestrel and B.napus cv Lair, were collected and air dried in open petri dishes at room temperature for 20 to 24 hours. Pollen from a number of parents was bulked, in order to minimise any effect due to self-incompatibility, then collected in vials, the lids of which contained self-indicating silica gel. The vials were all placed in a larger sealed vessel which also contained silica gel, and subsequently stored at -20°C to -25°C.

In April 1981, plants of these cultivars were bud pollinated (see section 2.3) using pollen which had been stored for periods varying from 21 weeks to 72 weeks. On average, 30 pollinations were made for each cultivar (see Table 3.1). Some of the stored pollen had been

obtained from plants flowering outside the normal flowering season. In April 1982, similar bud pollinations were carried out, using a different batch of pollen which had been stored for periods ranging from 52 weeks to 130 weeks.

In each series of pollinations, fresh pollen from several plants of the same cultivars was collected and used as a control for bud pollinations carried out on these plants at the same time as stored pollen pollinations.

After pollination, any fertilised ovules were left to develop to maturity. The fruits were collected before dehiscence, enabling the number of seeds within each silique to be counted.

Viability tests could have been carried out on the pollen after storage, for example flourescein diacetate (Heslop-Harrison & Heslop Harrison, 1970), but the important feature was to estimate the performance of the pollen yielding viable seed. The viability test used therefore, was the number of seeds produced per silique after pollination with stored pollen.

3.3 Results

The results from the 1981 and 1982 pollinations are shown in Table 3.1.

3.3.1 The percentage of pollinations yielding mature siliques.

Table 3.1 shows the percentage of the total number of pollinations which yielded mature siliques when pollinated with fresh and stored pollen. Results from both 1981 and 1982 show variation in the storage ability between the different cultivars of B.campestris and between the different Brassica species. In general, the percentage of mature

Table 3.1 The percentage of pollinations which resulted in mature siliques after bud pollination with fresh and stored Brassica pollen.

Species and Cultivar	Storage period (weeks) 1981	1982	Percentage of pollinations yielding mature siliques: Fresh pollen	Stored pollen 1981	1982
B. campestris					
cv Ponda (2x)	21	52	55%	47%	68%
cv Civasto (2x)	22	-	-	70%	-
	52	52	44%	50%	42%
cv Taronda (4x)	-	52	54%	-	81%
cv Marco (4x)	50	52	55%	62%	88%
B. oleracea					
cv M.Kestrel (2x)	26	-	-	45%	-
	78	75	60%	48%	75%
	-	130	60%	-	18%
B. napus					
cv Lair	50	54	57%	12%	24%
Mean number of pollinations.			24.0±1.558	26.7±3.19	41.6±5.61

siliques formed in 1981 was less than 1982 for a given cultivar. This was probably a direct result of improved plant care, pollen collection and storage in 1982.

The four cultivars of B.campestris were in low temperature storage for a maximum of 52 weeks. All the cultivars observed, (with the exception of Civasto) showed improved silique formation with stored pollen. Both fresh and stored pollen results for Civasto were lower than the others. Both the tetraploid cultivars, Taronda and Marco, produced around 80% mature siliques when pollinated with stored pollen compared with around 50% when fresh pollen was used.

B.oleracea cv Maris Kestrel showed improved performance when pollinated with pollen that had been stored for 75 weeks, compared with fresh pollen. However, the performance of stored pollen was markedly reduced, (where 18% of pollinations produced siliques) when the pollen was stored for 130 weeks.

B.napus cv Lair was the only cultivar which showed a reduction in the percentage of mature siliques produced after pollination with stored pollen compared with fresh pollen. This result was found in both 1981 and 1982.

3.3.2 The mean number of seeds per silique.

Table 3.2 shows the mean number of seeds per silique after pollination with fresh and stored pollen. B.campestris cvs Ponda and Taronda, and B.napus cv Lair showed no significant difference, using a paired 't' test, in the number of seeds produced when pollinations were made with fresh pollen or stored pollen.

B.campestris cv Civasto, however, showed a significant reduction ($p<0.001$) in the numbers of seeds produced after pollination with

Table 3.2 The mean number of seeds per silique after bud pollination with fresh and stored Brassica pollen.

	Storage period (weeks)		Mean number of seeds per silique		
				Stored pollen	
Species and Cultivar	1981	1982	Fresh pollen 1982	1981	1982
B.campestris					
cv Ponda (2x)	21	52	19.6	10.4	20.1
cv Civasto (2x)	22	-	-	0.0	-
	52	52	11.0	0.1	0.0
cv Taronda (4x)	-	52	7.8	-	7.3
cv Marco (4x)	50	52	8.7	8.5	7.0
B.oleracea					
cv M.Kestrel (2x)	26	-	-	11.0	-
	78	75	4.5	12.0	10.1
	-	130	4.5	-	19.7
B.napus					
cv Lair	50	54	19.7	9.0	19.6

stored pollen when compared to the numbers produced by pollination with fresh pollen. When stored pollen was used, virtually no mature seeds were obtained. This result was also found in 1981 after pollination with different batches of stored pollen, stored for 22 and 52 weeks. Fresh pollen also produced significantly more seeds ($p<0.01$) than stored pollen with B.campestris cv Marco.

B.campestris cv. Ponda performed the best amongst the B.campestris cultivars examined. The mean number of seeds produced per silique was around 20. The mean ovule number for this species was found to be 22.4 ovules per silique, so fertilisation and development, in this instance, was around 100%.

In contrast, pollination of B.oleracea cv Maris Kestrel with stored pollen produced significantly more seeds per silique ($p<0.001$) than was produced by pollinating with fresh pollen. Pollinations of Maris Kestrel using fresh pollen showed a poorer performance than would have been expected. Perhaps this effect is related to Maris Kestrel's mixed parentage. Maris Kestrel is a triple cross hybrid, with a range of S-alleles to increase self-incompatibility. Fresh pollen pollinations yield 4.5 mature seeds per silique, while stored pollen pollinations yielded 10.1 seeds per silique after 75 weeks storage and 19.7 seeds per silique after 130 weeks storage. Later experiments (see Chapter 6) indicated that the expected number of seeds should have been around 18 seeds per silique after bud pollination with fresh pollen. The mean ovule number in this series was 29.4 ovules per silique. The apparent significant increase after pollination by stored pollen found in this experiment should therefore be treated with a degree of caution.

B.napus cv. Lair produced 19.7 mature ovules after bud pollination with fresh pollen and 19.6 ovules per silique after pollination with

stored pollen. The mean ovule number for this species is 26.9, hence the production of fertilised ovules is around 74%, even after pollen storage for a year.

The total number of seeds per silique, averaged over all the cultivars, showed no significant difference between fresh and stored pollen. The overall mean number of aborted ovules per silique was 0.61, when fresh pollen was used, and significantly more ($p<0.01$), 1.01, when stored pollen was used. B.campestris cv. Civasto showed a significantly higher abortion rate ($p<0.001$) of 5.5 aborted ovules per silique when stored pollen was used.

3.3.3 Stages of embryo development.

Table 3.3 shows the stage of embryo development observed at 22 days post-pollination, except for the slower developing species, B.oleracea, which was observed at 32 days after pollination, for both fresh and stored pollen. No embryo development was found in B.campestris cv Civasto after pollination with stored pollen. The other B.campestris cultivars showed no difference in embryo stage reached after pollination with either fresh or stored pollen. Embryo development was slightly slower in B.oleracea after pollination with pollen stored for 75 weeks, but fresh and stored pollen produced the same developmental stage when the pollen had been stored for 130 weeks. B.napus cv Lair showed the same stage of embryo development irrespective of whether stored or fresh pollen was used.

3.3.4 Precocious Germination.

B.campestris cv Marco and B.napus cv Lair, showed a low percentage of seed which was germinating prematurely in the silique, after pollination with fresh pollen (Table 3.4). Storage of pollen

Table 3.3 The average stage of embryo development at 22 days post pollination (*B. oleracea* was examined at 32 days post pollination) after pollination with fresh and stored *Brassica* pollen.

Species and Cultivar	Average stage of embryo development	
	Fresh pollen	Stored pollen
B. campestris		
cv Ponda (2x)	MIII	MIII
cv Civasto (2x)	MII	No development
cv Taronda (4x)	MIII	MIII
cv Marco (4x)	MII	MII
B. oleracea		
cv M.Kestrel (2x)	MI	WS/MI
B. napus		
cv Lair	MI/MII	MI

Table 3.4 The percentage of seeds which pre-germinated after bud pollination with fresh and stored Brassica pollen.

Species and Cultivar	Storage period (weeks) (1982)	Percentage of pre-germinated seeds	
		Fresh pollen	Stored pollen
B.campestris			
cv Marco (4x)	52	0.8%	36.2%
B.oleracea			
cv M.Kestrel (2x)	75	0.0%	12.8%
cv M.Kestrel (2x)	130	0.0%	5.1%
B.napus			
cv Lair	54	0.8%	1.5%

increased the level of precocious germination in these two cultivars, to 36.2% in the case of Marco. This condition was not found when B.oleracea cv Maris Kestrel was pollinated with fresh pollen, but it was observed after the pollen had been stored.

3.3.5 Germination of mature seeds formed after pollen storage.

Mature seed of B.oleracea cv Maris Kestrel (2x) was collected after bud self pollinations made with pollen that had been stored for 78 weeks. From 30 seeds that were sown in a petri dish, 29 seeds germinated. These were transplanted into pots of compost and grown in the normal manner (see section 2.2). The fully grown plants had a normal phenotype.

3.4 Discussion.

The results from bud self pollination with stored pollen have introduced some interesting aspects of post pollination development. Compared to fresh pollen, pollination with stored pollen often produced an increase in the percentage of mature siliques. The number of seeds per silique was usually similar to, or less than, the number of mature seeds found after pollination with fresh pollen. The ability to produce mature seed after pollination with stored pollen is cultivar dependent. The embryos produced after pollination with fresh or stored pollen develop at the same rate. There was an increase in the percentage of precocious germination after pollination with stored pollen.

Ockendon (1974) has stated that maintained desiccation of pollen is an important factor for increasing the longevity of pollen. Under normal growing conditions pollen is ready for dispersal at anthesis. In the

case of Brassica, the insect vector would transport the pollen to a waiting stigma, where a number of processes take place. The pollen grain must be recognised by the stigma, and undergo biochemical and physiological changes to produce a pollen tube. This tube will penetrate tissues of the style and convey the two sperm cells and generative nuclei to the endosperm mother cell and the egg cell, and hence effect double fertilisation. Desiccation of the pollen grains and storage at a low temperature can reduce the metabolic processes in the pollen grains and keep them in a suspended state for a year, perhaps not unlike the process which leads to the induction and release of dormancy in seeds.

The viability of pollen grains after storage appears to have some genetic control as shown by B.campestris cv Civasto, which showed no storage potential.

There is a growing understanding of the physiology and biochemistry of seed dormancy and germination. Dormancy is a state of suspended growth which has to be got in and out of if there is to be continuity of the organism, in this case the seed, and perhaps the pollen grain in storage. There is evidence of hormones initiating selective physiological and metabolic changes at the level of the cellular membrane, which may in turn initiate events leading to the induction and release of dormancy (Khan, 1977). It is necessary to discuss some of these changes in order to understand why there should be an increase in precocious germination of seed after pollen storage. Abscisic acid (ABA) is known to be an important substance in controlling seed dormancy (Schopfer, Bajracharya & Plachy, 1979 and Schopfer & Plachy, 1984). The levels increase as the seed develops and then decrease during desiccation (Maguire, 1984 and Walton, 1977).

ABA exogenously applied to a seed once the radicle has emerged had no

inhibitory effect. Cytokinins occur naturally in seeds and interact with growth inhibitors to control metabolic processes preceeding visible germination. Cytokinins have been shown to be involved in radicle elongation and cotyledon expansion (Thomas, 1977). Some crops show inherited dormancy characteristics, for example, lettuce and reed canary grass (Maguire, 1984). Dormancy provides a mechanism for regeneration and survival. Genetic variability also offers an evolutionary basis for diversity and hence survival.

Abscisic acid has also been found to inhibit poly(A) RNA in cotyledons. Shanon et.al. (1981, cited by Maguire, 1984) suggested that dormancy breakage may be associated with the transcription of noval mRNA's specific for germination. Perhaps the failure of dormancy is a failure to repress genetic transcription in the seed.

Storage of pollen in a suspended state must somehow affect the control of hormone production and protein biosynthesis, to produce the observed increase in precocious germination. Since the cultivars behave differently, there may be a genetically inherited factor involved.

The stimulation of fertilisation extends not only to ovules, but to other parts of the flower; tissues of the ovary wall undergo marked changes. Auxin may be involved in the development of the fruit. Exogenous auxin can induce a high percentage of seedless fruit in tomatoes if applied before pollination. The cultivars of B.campestris and B.oleracea showed an increase in the percentage of mature siliques formed after pollination with stored pollen. B.campestris cv Civasto showed silique stimulation, but contained no developing ovules. The number of seeds produced per silique was fairly constant whether pollinated with fresh or stored pollen, but it was always less than the potential number of ovules for the species. The technique of bud

pollination (section 2.3) involves pollination of immature gynoecia. Pollinations of the very young buds may contribute to the failure of some pollinations to produce mature siliques. Furthermore, fewer mature seeds are formed after bud pollination compared to normal cross pollination (Yun, Bagget & Rowe, 1981). Perhaps immaturity of some of the ovules even when stigmas have become receptive, leads to the abortion levels found after bud self pollination.

It has been known for some time that seeds accumulate chromosome damage with ageing and that this damage gives rise to mutants (Harrington, 1970; Roberts, 1975 and Wang, 1975). Chromosomal breakage and the induction of recessive mutations, manifested by increases in pollen abortion were found in plants produced from such seeds and by segregation of mutant phenotypes in subsequent generations (Roberts, 1973). There is a predictable relationship between viability percentage and the amount of chromosomal damage in the surviving seeds. Such damage is similar to that induced by X-rays. Damage to the subcellular organelles is also associated with loss of viability. Accumulated genetic damage such as that found with the storage of seeds may occur with pollen storage and may be creating another means of increasing genetic variability in a crop with a narrow genetic base.

3.5 Conclusion.

Pollen can be stored for up to one year without serious loss of viability. Pollen storage of all three species will allow interspecific crosses to be made at any time up to one year after collecting the pollen. This would therefore eliminate the need for simultaneous flowering in conventional flowering seasons. In view of the results found for B.campestris cv Civasto, individual cultivars

would need to be tested to determine their individual storage capabilities.

Storing pollen has an effect on its performance after bud pollinations. Storage of pollen appears to improve the percentage of mature siliques formed in B.oleracea and B.campestris The number of seeds per silique does not show the same increase. However, an overall increase in productivity would be obtained for a given number of pollinations. The increase in precocious germination may be a desirable character, since it would remove the need to wait for a dormant period. Storing the pollen may be a means to increase the genetic variability in a crop by increasing the frequency of mutation..

CHAPTER 4

THE EFFECT OF FLORET POSITION AND EMASCULATION ON POLLEN RETENTION AND POLLEN PERFORMANCE OF *BRASSICA OLERACEA* VAR. *ACEPHALA* IN A SELF POLLINATION, AN INTRASPECIFIC CROSS AND AN INTERSPECIFIC CROSS.

4.1 Introduction.

The effect of the sporophytic self-incompatibility system present in many Brassica species has been well documented, (Carter, Williams & McNeilly, 1975; Heslop-Harrison & Shivanna, 1977; Roberts, Stead, Ockendon & Dickinson, 1980; Roggen, 1972; Shivanna, Heslop-Harrison & Heslop-Harrison, 1978). The germination and growth of compatible pollen tubes is unaffected by stigma age, whereas pollen tubes from incompatible pollen fail to penetrate the papillae of mature stigmas. However, incompatible pollen can penetrate the immature stigmas found in buds.

The plant breeder may require to produce and maintain homozygous lines of self-incompatible plants, and therefore requires to somehow overcome the self-incompatibility system. The method of "bud pollination", where incompatible pollen is applied to immature stigmas, was adopted and has been used for many years, (Pearson, 1921; Attia, 1950). It has also been used when making crosses between different species, the main reason being to exclude unwanted pollen from the stigma (G.R. Mackay, (pers. comm.)).

The technique involves emasculation of several buds, followed by pollination of the immature stigmas, and bagging of the racemes to avoid uncontrolled pollination. In preliminary studies it was noted that not all buds pollinated set seed, and that this effect was often associated with the position of the bud on the raceme. Of the buds which showed some ovule development after bud pollination, there was always a proportion of unfertilised ovules. Yun-Fu, Baggett & Rowe, (1981) found that open-flower pollinated plants of B.oleracea var italica produced more seed than those which had been bud pollinated. Many of the table varieties of B.oleracea show a tendency towards

self-compatibility, compared to the agricultural kales, which are strongly self-incompatible. Assuming that open-flower pollination produces nearly 100% fertilisation, the present bud pollination technique must account for much of the ovule failure. There is a need to improve the success rate of pollination, not only in bud selfs and controlled intraspecific bud pollinations, but to achieve optimum pollination conditions for interspecific crosses where the seed set is low anyway. The following study was designed to investigate the pollination conditions for maximum seed set in self-pollinations, intraspecific crosses and interspecific crosses.

4.2 Material and Methods.

The following section outlines the parental material, methods and experimental design in this study.

4.2.1 Crosses made and factors examined.

Plants of two types of Brassica oleracea var. acephala were used in this study. Each has a strong self-incompatibility allele at either the S16 or S23 locus. The use of strong S-alleles lessened the risk of uncontrolled self pollination. B.oleracea was also selected as the female parent, since it has large buds, well spaced along the raceme, which allowed the proposed techniques to be carried out with relative ease. The S23 type was used as the maternal parent. The S16 type is closely related to the S23 type and used as the pollen parent in the intraspecific cross. B.campestris cv Marco was used for the interspecific cross, B.oleracea x B.campestris. This combination usually has a low proportion of ovules fertilised and an improved success rate of ovule development would be beneficial for plant breeding. For each of the three pollination types, three factors were

examined (1) the effect of bud position; (2) the effect of emasculation and (3) the effect of a time interval between emasculation and pollination.

From the plants to be emasculated the anthers were removed from the four most recent flowers and from the twelve successive preceding buds. Any other flowers and buds remaining on the raceme were removed. As a control, a second series of non-emasculated racemes was also selected. The buds of these non-emasculated series were opened so that the anthers were visible. This was done in an attempt to subject the non-emasculated buds and flowers to a wound similar to that caused by emasculation, although of course, as the anthers remained intact, the wound response is likely to be less. In the non-emasculated series, a tin foil cap was placed over the gynoecium to prevent any self-pollination. Both emasculated and non-emasculated racemes were then bagged to prevent uncontrolled pollination by insects.

To examine the effect of a time lapse between emasculation, or wounding, and pollination, four time intervals were chosen. Pollen was applied to the stigma directly after pollination (0 hours), or at 24 hours, 48 hours or 96 hours after pollination or wounding.

4.2.2 Experimental design.

The experiment was based on a factorial design with three factors (three pollination types, self, interspecific and intraspecific; emasculated and non-emasculated; and four time lapse periods after emasculation or wounding). Each combination of factors was replicated three times (in the case of pollen loading each was only replicated twice) and the whole experiment was completely randomised.

4.2.3 Variates recorded.

(i) Pollen loading.

The amount of pollen loaded onto a stigma was estimated using a Hawksley Cristalite B.S.748 Haemocytometer slide. The pollinated stigmas were placed in 0.5 mls of methyl blue solution, and agitated with a pipette. Ten samples were used to estimate the number of pollen grains per stigma, by the equation:

$$\frac{X}{9 \times 0.2 \times y} \times Z \qquad \ldots\ldots \text{ equation 4.1}$$

Where X = The original volume of liquid.

Z = The number estimated from y samples.

and y = the number of samples.

The haemocytometer slide was marked with nine, 1mm squares, and the distance between the slide and the cover glass was 0.2mm.

Two replicates were examined for each time interval in each of the three pollination combinations. Two values were calculated:

(i) the number of pollen grains retained on the stigma.

(ii) the median bud position of the distribution of pollen grains.

The median bud position is a weighted mean of the bud positions, weighted according to the distribution of the amount of pollen retained at each of the buds. By the equation:

$$\sum_{i=-4}^{12} i \times p_i \Big/ \sum_{i=-4}^{12} p_i \qquad \ldots\ldots \text{ equation 4.2}$$

where p is the performance of the ith bud.

As the median bud position is weighted according to the amount of pollen grains which were found at each bud position it gives an indication of the bud position which shows the greatest pollen retention.

(ii) Seed set.

To estimate the pollen performance in each of the three pollinations types, siliques were collected five weeks after pollination. The number of developing seeds found within each silique was counted. In the interspecific cross, B.oleracea x B.campestris, any developing seeds had aborted by five weeks after pollination. The performance of this cross was therefore estimated by the number of aborted seeds. The median bud position for the number of seeds is the weighted mean of the bud positions (see equation 4.2), weighted according to the number of seeds that had developed at each bud.

4.3 Results.

4.3.1 Pollen loading.

The mean number of pollen grains loaded onto the stigma of B.oleracea var. acephala, according to bud position on the raceme and time after pollination that pollen was applied, for each of the three pollination types are shown in Table 4.1.

The three types of pollination - bud selfs, intraspecific and interspecific - all follow the same pattern of pollen loading with respect to bud position. The newly opened flowers and the oldest buds retained the most pollen. Delaying pollination for 96 hours after emasculation can improve pollen retention in the younger buds, in the self and the intraspecific crosses. There appears to be no advantage

Table 4.1 Mean number of pollen grains loaded onto the stigma of B.oleracea var acephala for the pollination type and bud number at time delay of 0, 24, 48, and 96 hours after pollination. B1 is the oldest bud and F-1 is the youngest flower.

Bud	S23 x S23				S23 x S16				S23 x B.campestris			
number	0	24	48	96	0	24	48	96	0	24	48	96
F-4	367	186	172	556	70	375	106	153	0	97	28	153
F-3	523	181	459	384	161	361	445	384	56	189	189	172
F-2	361	334	506	473	217	445	445	459	264	222	172	245
F-1	681	227	409	695	439	361	328	367	83	153	300	245
B 1	222	200	245	439	250	270	228	389	111	89	111	250
B 2	195	181	236	200	217	242	145	245	44	42	89	200
B 3	195	89	250	411	250	120	200	328	139	125	111	97
B 4	439	200	292	467	222	270	181	272	83	78	117	70
B 5	153	245	125	245	228	325	50	328	42	33	78	97
B 6	217	70	195	172	278	92	161	172	42	97	33	101
B 7	264	125	125	250	161	167	111	278	56	70	50	78
B 8	300	133	42	153	189	175	61	195	83	78	70	50
B 9	228	209	145	172	89	259	133	217	42	56	56	61
B10	284	70	78	228	106	92	70	83	70	89	83	36
B11	217	61	22	200	153	83	70	125	0	83	97	92
B12	61	56	22	200	50	75	22	89	0	28	28	125

F = flower B = bud

of this delay in the interspecific cross.

Mean squares from the analysis of variance of the mean number of pollen grains found on each stigma (Table 4.2) showed significant differences between pollination types, ($p<0.001$), between different time intervals, ($p<0.01$), and between the two plants used in the experiment, ($p<0.001$).

Averaged over all the factors the stigma of the self pollination, S23 x S23, retained an average of 258 grains/silique, the intraspecific cross, S23 x S16, retained 213 grains/silique, while the interspecific cross S23 x Marco, retained 126 grains/silique, significantly fewer than the other types of crosses (Table 4.3). Thus, with regard to pollen retention, the self retained more pollen grains while the interspecific cross retained least.

The mean number of pollen grains/silique at the time intervals 0, 24, 48 and 96 hours after emasculation were 242, 153, 172 and 230 respectively, averaged over the other factors (Table 4.4). Similar amounts of pollen were retained on the stigma immediately after emasculation (0 hours) and after 96 hours. There was a 36.7% reduction in pollen retention when pollen was applied 24 hours after emasculation. When pollen was applied 48 hours after emasculation the stigma was more receptive than after 24 hours. Measurements were recorded from two plant (plant A and plant B) for each combination of factors. Plant B accepted significantly more pollen ($p<0.001$) than plant A, particularly when pollen was applied immediately after emasculation and after 96 hours. However, the general trend over all the treatments was the same for both plants. Significant interactions were found between times and plants, ($p<0.01$), and between times and crosses and plants, ($p<0.01$). These interactions were, in the main, the result of the differences between the two plants A and B.

Table 4.2 Mean squares from the analyses of variance of mean number of pollen grains on the stigma, and the median bud position of the number of pollen grains on the stigma of B.oleracea var acephala.

Source	d.f.	No. pollen grains	Median bud position
Pollinations (C)	2	656891 ***	0.245 ns
Times (T)	3	203017 **	3.252 ***
Plants (P)	1	540354 ***	0.037 ns
C x T	6	15457 ns	0.564 ns
C x P	2	18285 ns	0.346 ns
T x P	3	47947 **	0.116 ns
C x T x P	6	40667 *	0.576 ns
Error	24	14691	1.3006

ns=not significant

**=0.05<p<0.01

***=0.01<p<0001

Table 4.3 Mean number of pollen grains on the stigma of B.oleracea var acephala after a self, interspecific and intraspecific cross, where the time interval and bud position have not been isolated.

S23 x S23	S23 x S16	S23 x B.campestris
258	213	126

Table 4.4 Mean number of pollen grains on the stigma of each plant of B.oleracea var acephala at each time interval.

	Plant A	Plant B	Mean
0 hours	177	307	242
24 hours	135	170	153
48 hours	149	195	172
96 hours	193	266	230
Mean	164	234	

Table 4.5 Mean median bud position for the number of pollen grains on the stigma of B.oleracea var acephala at each time interval.

0 hours	24 hours	48 hours	96 hours	s.e.
2.86	2.25	1.59	2.27	0.199

Examination of the mean squares from the analyses of variance of median bud position for number of pollen grains retained (Table 4.2) shows significant differences between the time intervals ($p<0.001$). All other main effects and interactions in this analysis were not significant. The median bud position for the number of pollen grains retained was highest immediately after emasculation (2.86) (Table 4.5). It then decreased to 2.25 after 24 hours, then to 1.59 after 48 hours and increased after 96 hours to 2.27. Thus if pollen is applied immediately after emasculation, the 2.86th bud will retain most pollen. If pollination is delayed until after 48 hours, then the 1.59th bud retains most pollen. Hence for optimum pollen retention, then bud pollinations should be made using the three oldest buds.

4.3.2 Number of developing ovules.

The mean number of ovules developing at a particular bud position for a given time delay in an emasculated and non-emasculated series is given in Table 4.6 and Table 4.7. These data are also shown graphically in Figures 4.1, 4.2 and 4.3.

There is a very large difference between emasculated and non-emasculated buds and flowers in the performance of the bud self pollination S23 X S23, where the unemasculated series produces many more developing ovules from the second bud position onwards after a time delay of 0, 24, or 96 hours (Figure 4.1). When the time delay is 48 hours, an increase in performance was observed from the fourth bud position onwards. The mature flowers, -1 to -4, perform similarly in both series, with a slight increase in the number of developing ovules with an increasing time delay period. When pollinated directly after emasculation, the emasculated series shows development of a few

Table 4.6 Mean number of ovules developing in B.oleracea var acephala for three pollination types, by bud position at time 0, 24, 48 and 96 hours

Emasculated series

Bud	S23 x S23				S23 x S16				S23 x B.campestris			
position	0	24	48	96	0	24	48	96	0	24	48	96
F-4	5.0	0.7	0.8	3.0	8.0	2.0	1.7	2.7	6.7	6.0	4.0	5.7
F-3	0.0	0.3	2.3	3.3	9.3	5.5	5.7	1.0	8.7	1.7	5.0	5.3
F-2	1.3	0.0	4.3	6.3	6.7	9.5	8.0	1.7	7.3	0.3	5.0	3.7
F-1	4.3	2.0	3.3	3.7	8.7	11.5	8.0	3.3	9.7	0.0	4.7	2.0
B 1	0.6	1.0	2.7	2.0	9.3	12.0	7.0	3.7	5.0	3.0	2.7	3.7
B 2	1.7	0.0	0.3	4.7	3.3	1.0	5.7	6.3	7.3	2.3	3.3	2.0
B 3	6.3	1.3	0.7	4.0	3.0	1.5	3.7	7.6	6.7	1.7	3.0	2.3
B 4	1.7	0.0	0.7	2.0	3.0	1.5	4.3	8.3	4.7	1.7	5.0	3.3
B 5	2.0	2.7	1.3	0.3	7.0	7.3	4.7	9.7	2.7	0.3	6.5	3.7
B 6	2.7	3.3	1.7	0.7	3.5	7.0	5.3	11.0	0.0	0.0	0.0	3.3
B 7	1.3	3.0	0.0	1.3	1.5	3.0	1.0	9.3	0.3	0.0	0.5	3.0
B 8	1.0	3.7	0.0	0.0	-	0.0	-	11.0	0.0	0.0	0.0	2.0
B 9	-	3.0	1.0	0.0	-	-	-	2.5	0.6	0.0	-	1.0
B10	-	2.3	1.0	0.0	-	-	-	-	0.6	-	-	1.0
B11	-	-	3.0	0.0	-	-	-	-	-	-	-	-
B12	-	-	0.3	-	-	-	-	-	-	-	-	-

F = flower B = bud

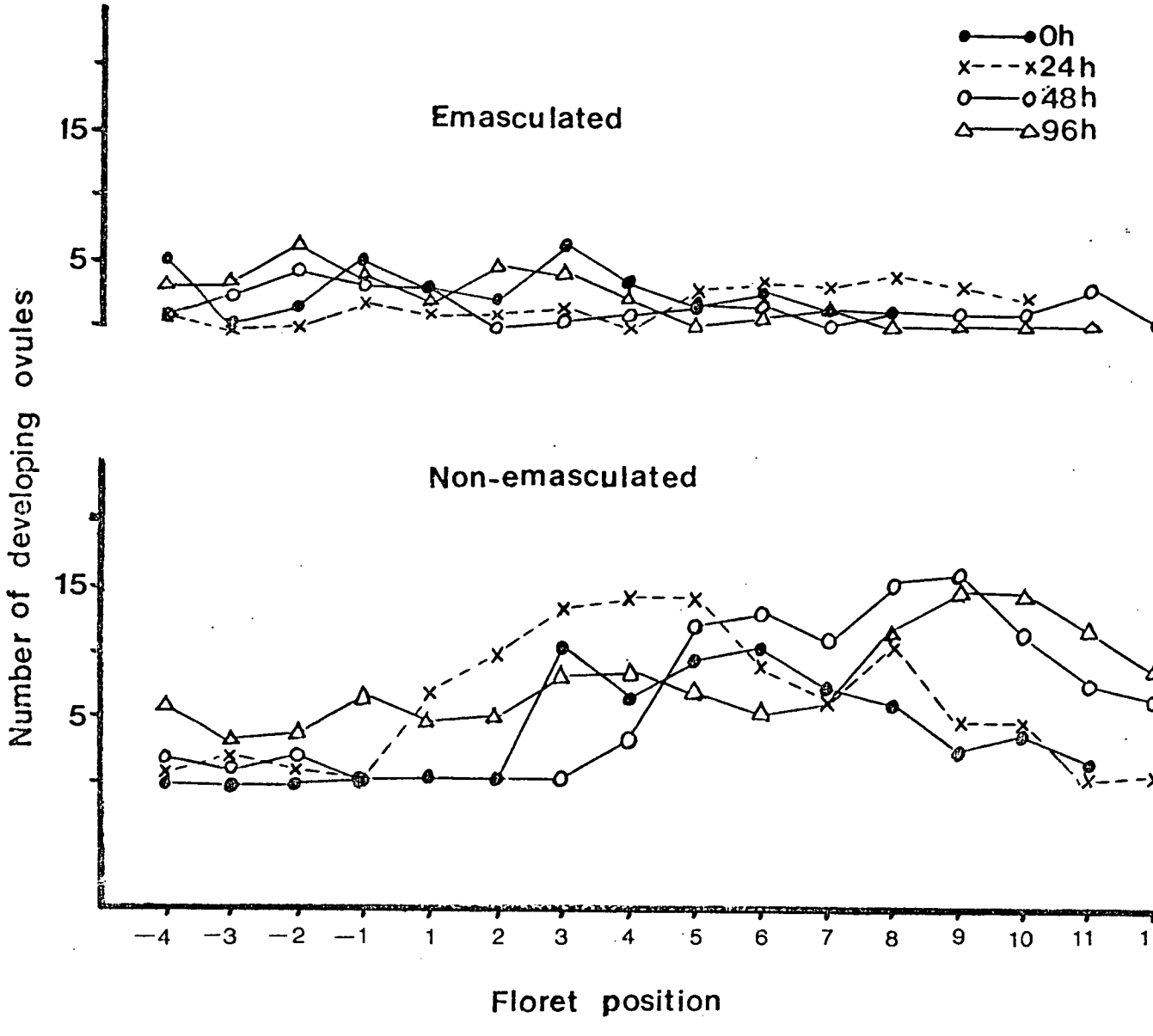
Table 4.7 Mean number of ovules developing in B.oleracea var acephala for three pollination types by bud position at time 0, 24, 48 and 96 hours.

Non-emasculated series

Bud	S23 x S23				S23 x S16				S23 x B.camestris			
Position	0	24	48	96	0	24	48	96	0	24	48	96
F-4	0.0	1.0	1.7	6.0	6.5	11.0	9.0	7.5	0.5	1.7	0.5	0.5
F-3	0.0	2.3	1.0	2.7	16.5	15.0	9.5	4.5	5.0	2.7	1.7	0.5
F-2	0.0	1.0	2.0	3.7	14.0	17.0	14.5	11.0	6.0	2.3	2.3	2.0
F-1	0.0	0.0	0.3	6.7	22.0	17.0	13.0	12.3	2.0	1.7	3.0	1.7
B 1	0.3	6.7	0.0	4.7	20.5	21.0	13.0	13.0	1.0	2.3	1.7	6.0
B 2	0.0	9.7	0.0	5.0	17.5	13.5	14.5	12.5	0.5	5.0	2.3	5.5
B 3	10.3	13.3	0.0	8.3	19.5	19.0	18.0	17.0	1.5	3.0	2.0	6.0
B 4	6.3	14.0	3.3	8.3	17.5	7.0	13.5	15.0	0.5	4.3	0.0	5.0
B 5	9.3	14.0	12.0	7.0	9.0	16.0	14.5	10.3	0.0	0.3	0.3	6.5
B 6	10.0	9.0	13.0	5.3	15.5	14.5	10.0	17.0	0.0	0.0	0.0	4.0
B 7	7.0	6.3	11.0	6.0	4.0	9.0	9.5	13.0	0.0	0.3	0.0	1.5
B 8	6.0	11.0	15.0	11.3	12.5	14.0	11.0	11.6	0.0	0.0	0.0	1.5
B 9	2.3	4.5	15.5	15.0	7.0	4.5	6.0	7.7	0.5	0.7	0.0	3.0
B10	4.3	4.0	11.5	14.5	-	3.5	8.0	12.5	0.0	0.0	0.0	1.5
B11	1.0	0.5	7.5	12.0	-	2.0	7.0	5.5	0.0	0.0	2.0	2.5
B12	-	0.5	6.5	8.5	-	4.0	8.0	5.5	0.0	0.0	0.0	1.5

F = flower B = bud

Figure 4.1 The mean number of developing ovules per silique after bud self pollination (S23 x S23) of emasculated and non-emasculated buds according to floret position on the raceme. Results are shown for four treatments of time delays of 0, 24, 48 and 96 hours between emasculation/treatment and pollination.



ovules, whereas the non-emasculated series showed no development.

With the intraspecific cross S23 x S16, the unemasculated series again performs better than the emasculated series (Figure 4.2). From bud positions two to twelve there is a gradual decline in the number of ovules developing towards the younger buds. There is a common response over the four time periods. In the emasculated series, the time delay periods of 0, 24 and 48 hours showed maximum performance around the first flower. There was then a gradual decline in performance until the second bud. Between the second and fourth buds, performance remained constant but again there was an increase in performance at the fifth bud position, after which there was a decline in performance in the buds towards the top of the raceme. With a delay of 96 hours between emasculation and pollination there was a steady increase in the number of developing ovules with maximum performance around bud position six to eight before the number declines towards the top of the raceme.

Figure 4.3 shows the number of aborted ovules after the interspecific cross B.oleracea x B.campestris. The number obtained is really the potential number of hybrids, since the developing ovules had aborted by the time the samples were collected five weeks after pollination. From Figure 4.3 the best performance over all the buds was achieved from the non-emasculated series when pollination was delayed by 96 hours after wounding. Bud positions one to six produce a constant number of fertilised ovules. In the emasculated series a good performance was achieved from buds one to four when pollinated directly after emasculation.

Overall there were significant differences ($p<0.001$) between the pollination types with respect to the number of seeds that the self or cross produced (Table 4.8). The non-emasculated buds and flowers

Figure 4.2 The mean number of developing ovules per silique after intraspecific pollination (S23 x S16) of emasculated and non-emasculated buds according to floret position on the raceme. Results are shown for four treatments of time delays of 0, 24, 48 and 96 hours between emasculation/treatment and pollination.

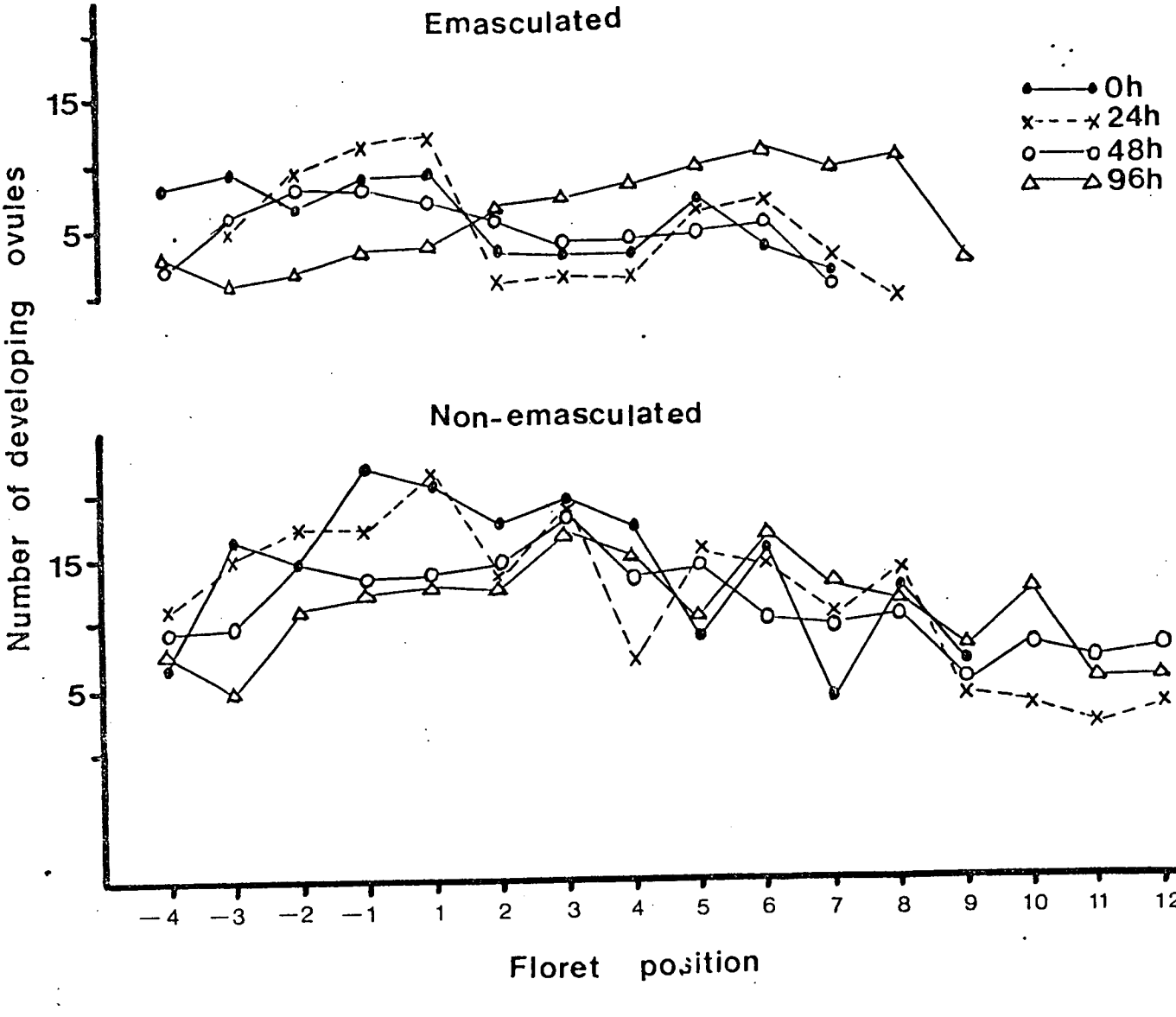


Figure 4.3 The mean number of aborted ovules per silique after interspecific pollination (S23 x Marco) of emasculated and non-emasculated buds according to floret position on the raceme. Results are shown for four treatments of time delays of 0, 24, 48 and 96 hours between emasculation/treatment and pollination.

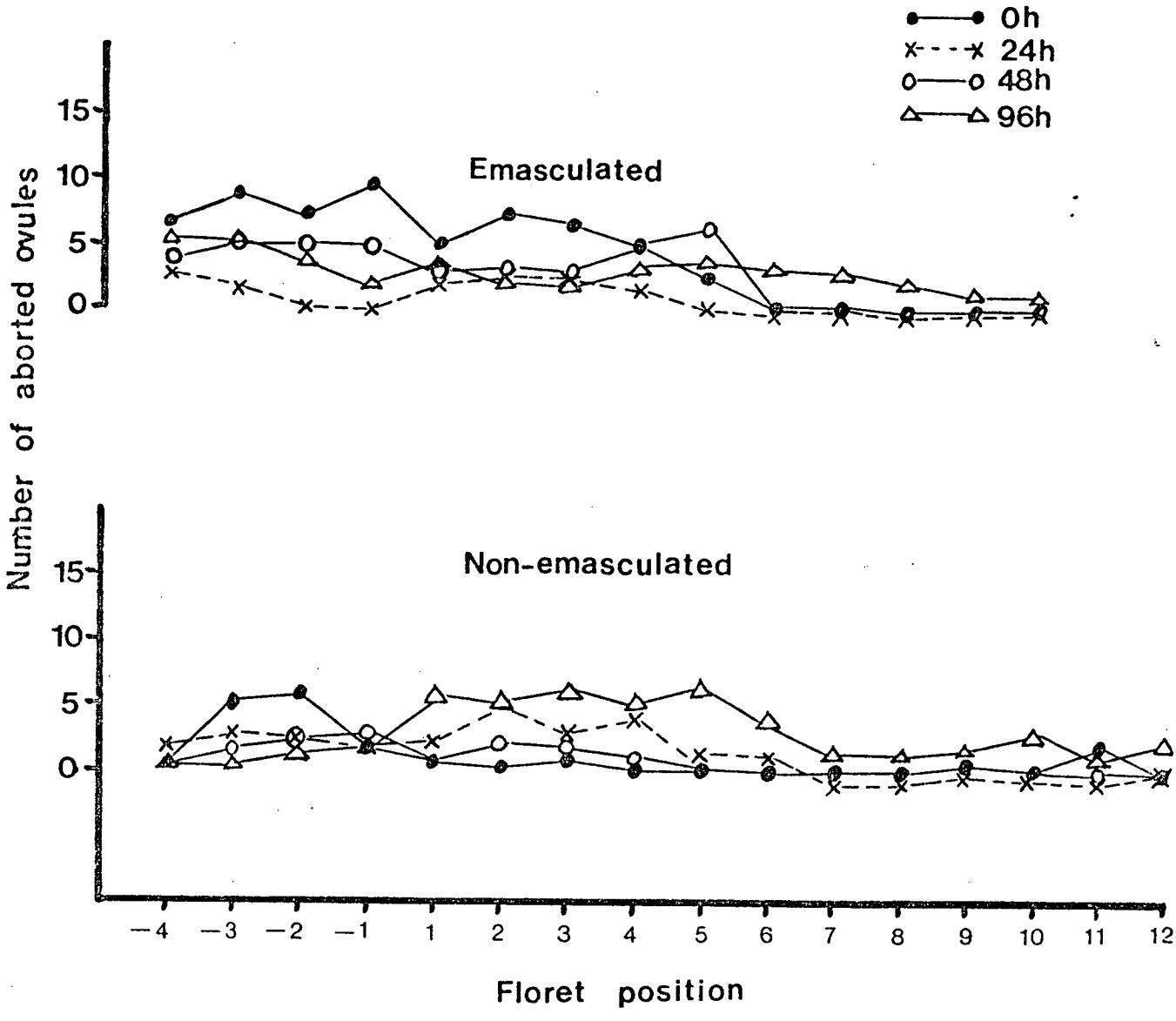


Table 4.8 Mean squares from the analyses of variance of the number of developing ovules produced and the median bud position of the number of developing ovules.

Source	d.f.	Number of seeds	Median bud position
Pollinations (C)	2	178.28 ***	37.684 ***
Emasculation (E)	1	213.52 ***	32.509 **
Times (T)	3	7.28 ns	11.355 *
C x E	2	88.18 ***	8.144 ns
C x T	6	1.40 ns	8.418 ns
E x T	3	4.81 ns	1.882 ns
C x E x T	6	0.94	1.158 ns
Error	48	10.345	3.633

ns = not significant

*=$p<0.05$

**=$0.05<p<0.01$

***=$0.01<p<0.001$

produced significantly more seed ($p<0.01$) than those buds that had been emasculated. There were significant interactions between pollination types and emasculation treatment ($p<0.001$), and between emasculated treatment and time intervals ($p<0.01$). Averaged over all other factors, S23 x S23 produced 3.51 seeds, S23 x S16 produced 7.35 seeds and S23 x B.campestris produced 2.08 seeds per silique (Table 4.9). Self pollinations and the intraspecific cross both produce most seeds when not emasculated, while the interspecific cross produced more seeds when it had been emasculated (Table 4.9). This is the major cause of the interaction between pollination types and emasculation treatments. When buds are emasculated, then most seed is produced when pollination occurs immediately after emasculation or when pollination is delayed until 96 hours after emasculation (Table 4.10). After wounding, but not emasculating, most seeds were produced when pollination was delayed until 96 hours after wounding.

Anaylsis of variance of median bud position for seeds produced showed significant differences between pollination types, ($p<0.001$) and between time intervals ($p<0.05$) (Table 4.8). The mean median bud position for the number of seeds produced in B.oleracea by each cross at each time interval can be found in Table 4.11. The non-emasculated buds produced significantly more seeds than the emasculated buds. All interactions from the analysis of variance were non-significant. The mean number of seeds produced at each bud position for each of the three pollination types are shown in Figure 4.4 The number of seeds at each bud position is averaged, by the rolling mean technique, so that it is easier to observe the general trend of the figure. For example, if x_1 ,x_2 and $x_3$are the number of seeds produced by three consecutive buds, then the rolling mean for bud $X_2=(x_1+x_2+x_3))/3.0$. From Figure 4.4, the intraspecific cross, S23 x S16, produced most seeds and the interspecific cross, S23 x B.campestris produced fewest seeds at

Table 4.9 Mean number of developing ovules produced by each pollination type when emasculated and non-emasculated buds and flowers of B.oleracea were pollinated.

Pollination	Emasculated	Non-emasculated	Mean
S23 x S23	1.67	5.36	3.51
S23 x S16	3.78	10.93	7.35
S23 x Marco	2.34	1.83	2.08
Mean	2.59	6.04	

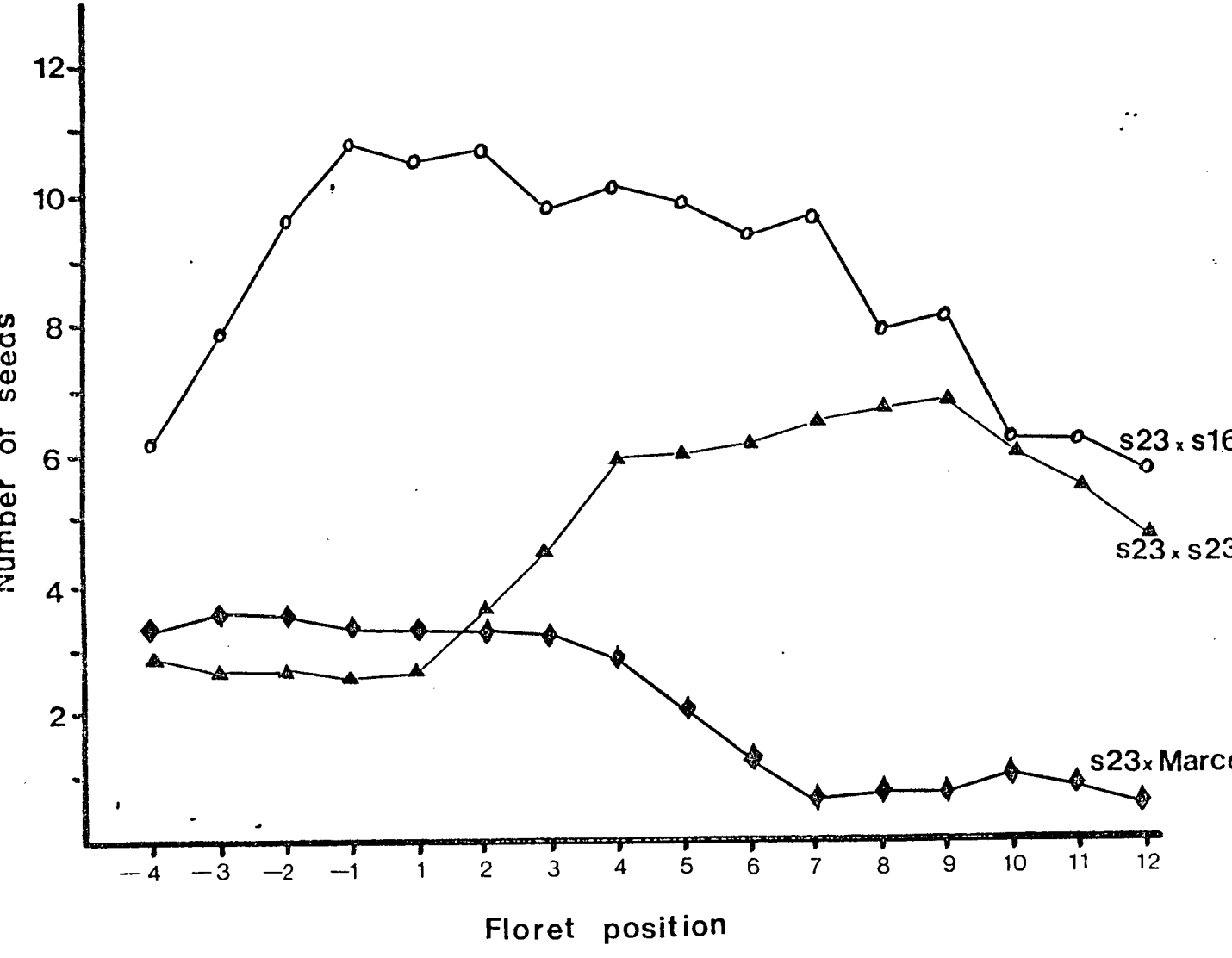
Table 4.10 Mean number of developing ovules produced at each time interval for the emasculated and non-emasculated series of flowers and buds in B.oleracea.

	Emasculated	Non-emasculated	Mean
0 hours	3.10	5.27	4.19
24 hours	1.87	6.01	3.91
48 hours	2.37	5.42	3.89
96 hours	3.04	7.46	5.25
Mean	2.59	6.04	

Table 4.11 Mean median bud position for the number of developing ovules produced in B.oleracea by each pollination type and at each time interval.

Time interval	S23 x S23	S23 x S16	S23 x B.oleracea	Mean
0 hours	3.83	0.10	1.31	1.75
24 hours	4.54	0.73	2.03	2.43
48 hours	4.75	1.91	2.31	2.99
96 hours	2.93	3.33	4.58	3.61
Mean	4.02	1.52	2.56	

Figure 4.4 Mean number of seeds per silique, averaged over four time delays and irrespective of emasculation or non-emasculation, at each floret position on the raceme for a bud self pollination (S23 x S23), an intraspecific pollination (S23 x S16) and an interspecific pollination (S23 x Marco).



almost all floret positions. Similarly, Figure 4.5 shows the rolling means at each bud position for the emasculated and the non-emasculated pollinations. From this it is seen that the non-emasculated buds are more productive than those buds that were emasculated. When the buds were emasculated or wounded, maximum numbers of seeds were produced by pollinating 96 hours after emasculation or wounding. When pollinating directly after emasculation, performance declines rapidly after the fifth bud position (Figure 4.6). When pollination is delayed until 24 hours after emasculation a similar trend is observed to that for immediate pollination. A delay of 48 hours shows increasing performance between the third and ninth bud positions, after which a decline is observed.

4.4 Discussion.

When pollinated immediately after emasculation, the self retained most pollen while the interspecific cross retained the least pollen grains per stigma on the stigma. All species examined produced elliptical pollen grains. B.oleracea, types S23 and S16 produced approximately the same size of pollen grains (48µm x 36 µm), but B.campestris cv Marco produced pollen grains three times larger in volume (72µm x 36 µm). Since fewer of the larger pollen grains will be able to adhere to the B.oleracea stigma, this explains, at least in part, why fewer B.campestris cv. Marco pollen grains were retained than in the other two pollination types. If, however, pollen grain size was the only factor affecting pollen retention, then the self pollination, S23 x S23, and the intraspecific pollination, S23 x S16, should have retained similar numbers of pollen grains on the stigma. However, this was found not to be true.

It was found that immature stigmas, which were found in buds and hence

Figure 4.5 Mean number of seeds per silique, averaged over three pollination types and four delays before pollination, at each floret position for emasculated and unemasculated buds.

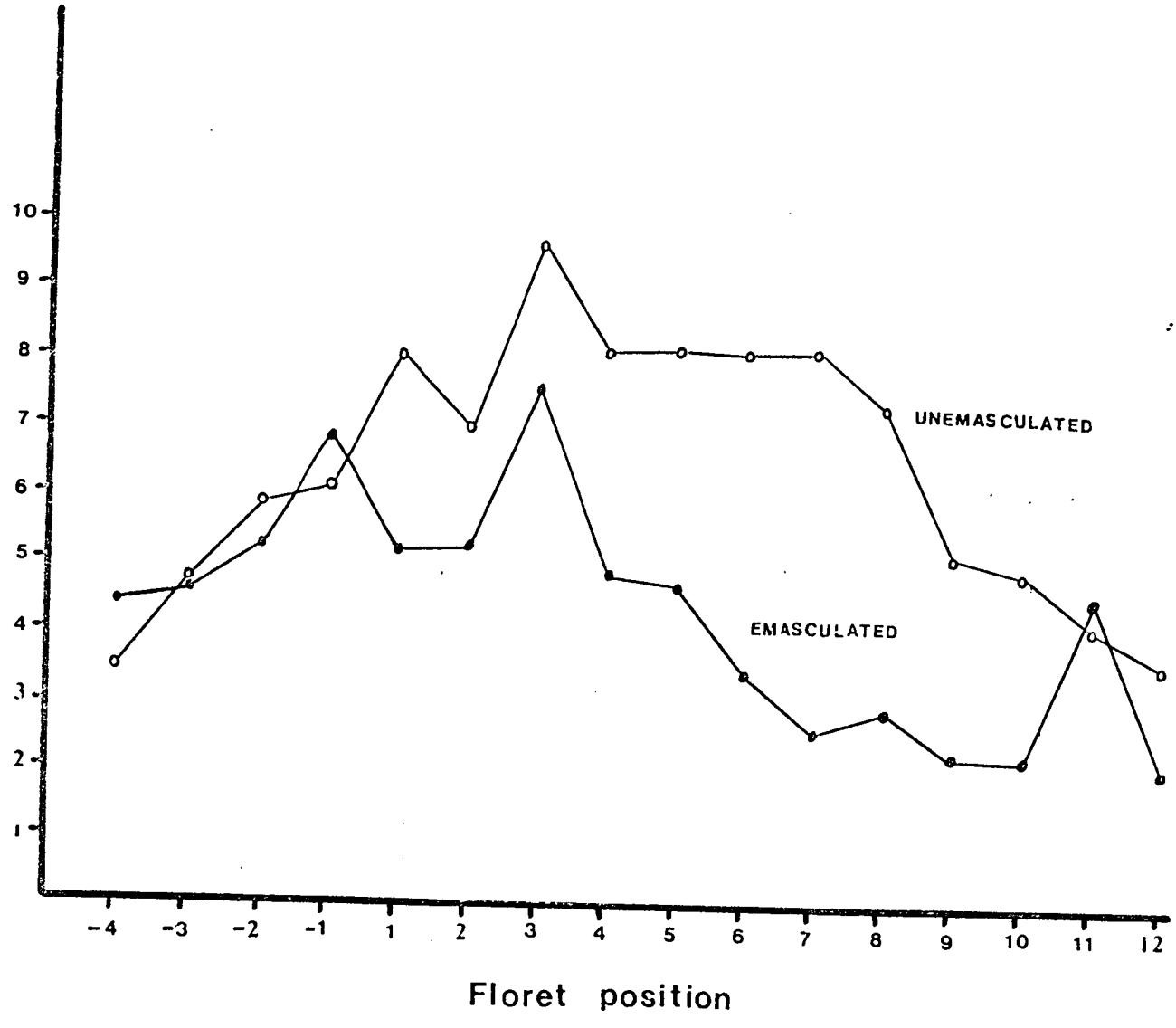
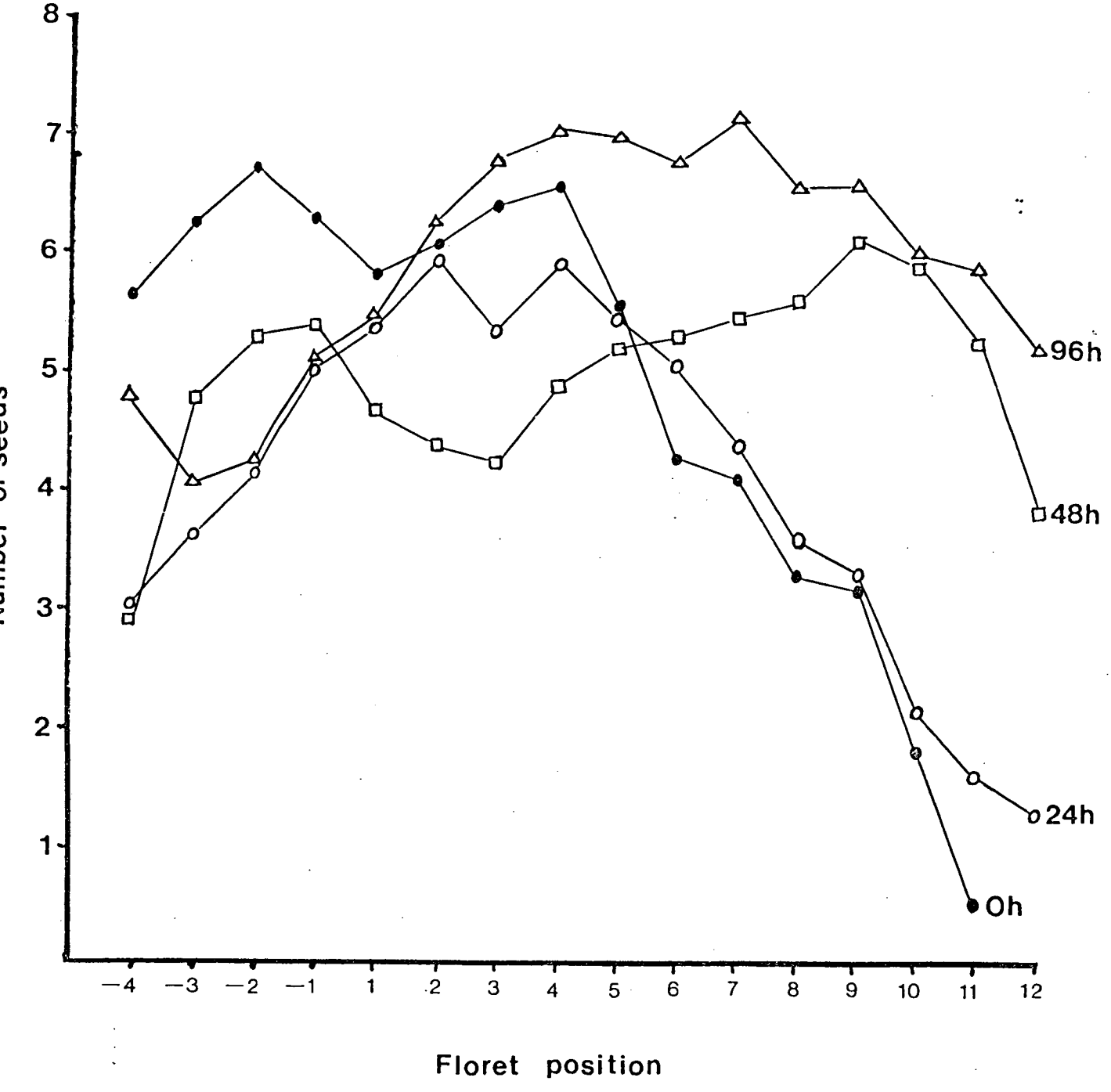


Figure 4.6 Mean number of seeds per silique, averaged over three pollination types and irrespective of emasculated or non-emasculated, at each floret position on the raceme for four time delays (0, 24, 48 and 96 hours) before pollination.



had not fully developed their incompatibility system, retain a greater number of pollen grains than mature ones after the self pollination but not after the interspecific cross. Furthermore, the analyses of the number of pollen grains retained on the stigma and the median bud position for the number of pollen grains retained both showed significant differences between the different times after emasculation that pollen was applied. The best pollen retention was obtained when pollen was applied immediately after emasculation, or when pollination was delayed until 96 hours after emasculation. Pollen applied between these times resulted in reduced pollen retention. This may be associated with the trauma experienced after emasculation. If pollination occurs directly after emasculation, the buds may not have had time to react to the wounding effect. After 96 hours, the bud may have recovered from the trauma of emasculation and the physiological conditions within the bud returned to a more balanced state, hence the stigma exhibits better pollen retention when compared with the intermediate times when emasculation is affecting the buds.

The rolling mean number of seeds that were produced by each pollination type at each bud position is shown in Figure 4.4. The pollination method to obtain the maximum number of seeds varied depending on the parental combination. Between the three pollination types examined, the intraspecific cross, S23 x S16, produced most seed. When making this cross, pollination of the most recently opened flowers, and the four oldest buds, provided most seed. From the fourth oldest bud, there was a decline in the number of seed produced in relation to the age of the buds. Similarly, the older flowers also produced fewer seeds. With the self, S23 x S23 a different pattern of seed set along the raceme was observed. The flowers and the oldest buds produced fewest seeds, while from the fourth bud, as the buds became younger, there was a gradual increase in the number of seeds

produced. The increase continued until the tenth bud, after which there was a gradual decline until the top of the raceme. Figure 4.4 suggests that the self-incompatibility system is beginning to have an effect from about the seventh bud position downwards, and it is mature at the time when the oldest bud is ready to open into a flower. The interspecific cross, S23 x B.campestris, produced least seed. The number of seed produced from the flowers and the first four bud positions was constant, after which there was a decline in the number of seed produced towards the top of the raceme.

The decline in seed set in younger buds was probably due to immaturity of the ovules and ovary. Allee & Mutschler (1983) found that in the older buds of B.campestris, pollen tubes were apparently guided to the ovule, but in the younger buds, the pollen tubes in an ovary often grew beyond the ovule to the base of the ovary rather than to the ovules. Perhaps a similar guidance mechanism works in B.oleracea and is active in more mature buds and inactive in very young buds.

Figure 4.5 clearly shows that the highest number of seeds are produced when the buds are not emasculated. This effect is from the larger number of developing ovules after bud self and intraspecific pollination. The interspecific cross produced most developing ovules in the emasculated series, although these were fewer in number than the intraspecific combination (Table 4.9). In the unemasculated series, most developing ovules per ovary were produced in buds one to seven inclusive, and in buds one to five in the emasculated series. After this, there is a continuous decline in the number of developing ovules produced towards the top of the raceme. The reduction in seed produced from the emasculated buds is probably due to increased damage by removal of the anthers. Emasculation at the open flower stage after anthesis did not affect the number of seeds produced. In both

the emasculated and non-emasculated flowers, fewer seeds were produced by the older flowers.

Figure 4.6 clearly demonstrates that over all other factors a time delay between emasculation, or wounding, and pollination increases the number of seed produced per silique. By delaying pollination until after 96 hours the maximum number of developing ovules was obtained. This effect was apparent in all three pollination types, particularly in the non-emasculated series.

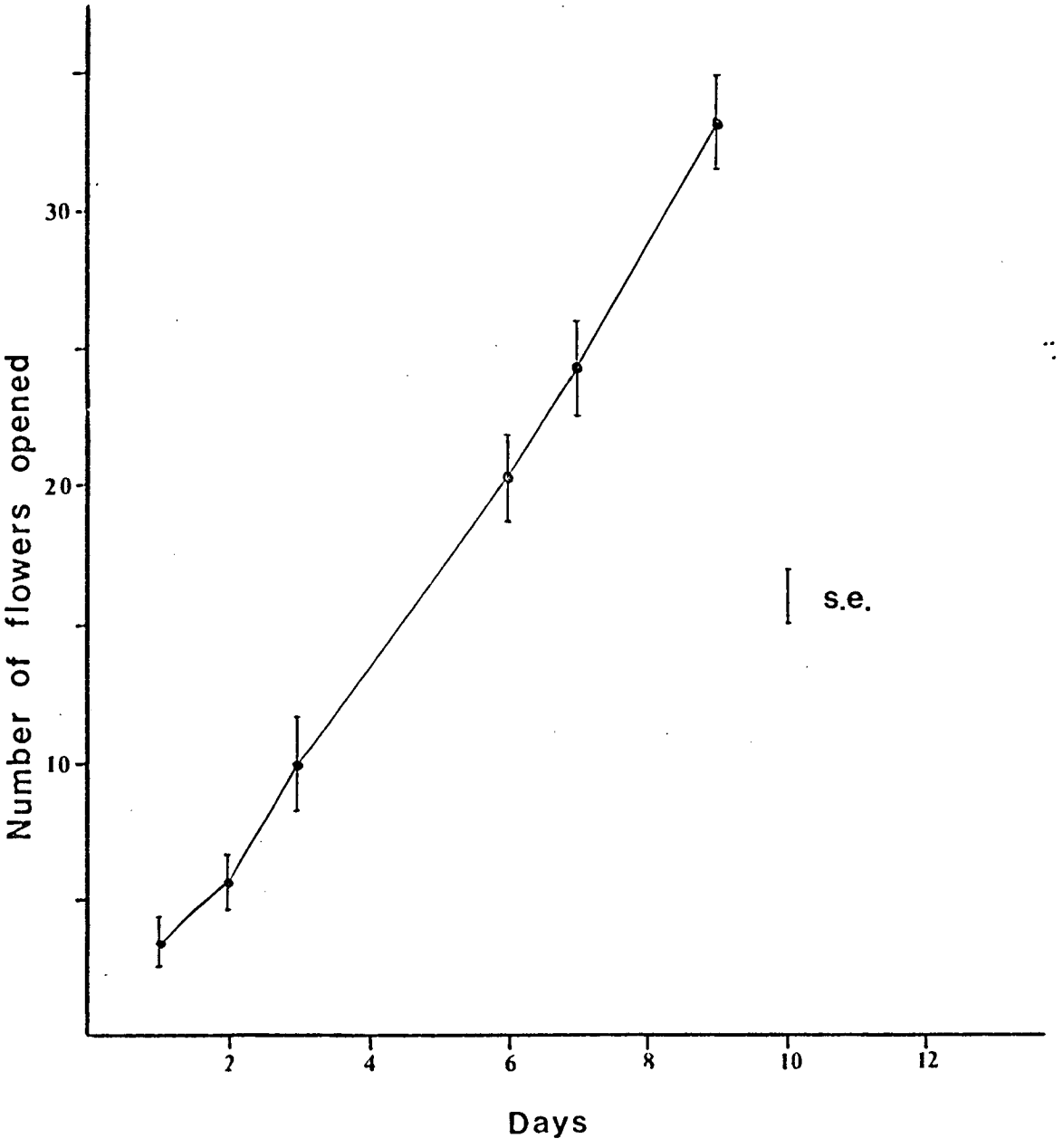
These trends may be attributable to two isolated factors, (1) upset of physiological balance in the bud by removal of the anthers and (2) the stage of development of the self-incompatibility system at the time of emasculation. The anthers and stamens are an important source of gibberellic acid in the buds. It has been reported that gibberellic acid is an important growth hormone associated with the development of the corolla (Murakami, 1975; Plack, 1958), so removal of the gibberellic acid source must disturb the physiology of the bud, at least initially. Overall, the maturity of the whole bud is important. The amount of pollen retained on the stigma does not appear to affect seed set. The older buds have reached a stage of maturity such that they can withstand the trauma of emasculation better than the younger buds. Hence the older buds respond better to pollination either directly or 24 hours after emasculation. The younger buds respond better to pollination 48 hours and 96 hours after emasculation, the physiological condition of the buds has presumably returned to a more balanced state and are therefore more responsive to pollination. Secondly, it is suspected that the self-incompatibility system is developing from the seventh bud position (see Figures 4.1, 4.2 and 4.3), increasing in intensity, towards the first flower. The rate of flower emergence was dependent on the weather conditions. One to two

flowers emerged each day, although on a cold day no new flowers may open, while two or three may open on a warm sunny day. Despite there being no data available on flower emergence of B.oleracea var acephala, evidence from B.napus showed the relationship between flower opening and time to be linear (Figure 4.7). The results from the present experiment indicate that the development of the self-incompatibility system behaves according to flower opening, and hence is also linear.

So from the time of emasculation buds 1 to 3 would be comparable with buds 2 to 5 after 24 hours, buds 3 to 7 after 48 hours, and buds 5 to 11 after 96 hours, delay after emasculation if one to two flowers open per day. From the number of seeds that were produced at each bud position, after a 96 hour delay buds 5 to 13 have not developed a self-incompatibility system, and are comparable with buds 1 to 3 when pollen is applied directly after emasculation. After a 48 hour delay, the lowest seed set was from the third bud position, so the expected development of the self-incompatibility system may be realised. However, after the third bud position, there is an increase in the number of seeds set over a wider range than would have been expected. This suggests that by delaying pollination by more than 48 hours after emasculation, the effectiveness of the incompatibility system is reduced. The ability to produce a wider range of buds with reduced incompatibility would be beneficial to improving the yield of developing ovules produced after artificial pollination.

The self-incompatibility system and the interspecific incompatibility system are apparently two different systems (Figure 4.1 and Figure 4.3). From Figure 4.3 it can be seen that the interspecific cross performs best when emasculated up to the sixth bud position, regardless of the different time intervals, whereas the

Figure 4.7 The relationship between flower opening and time of B.napus cv Lair.



self-incompatibility system is best overcome by pollination of the non-emasculated series after the third bud position over all the time intervals.

4.5 Conclusions.

To obtain maximum seed set in a bud self or intraspecific cross, the female parent should not be emasculated. In the self, most seed was retained from the second bud position onwards, with 0, 24 or 96 hours delay between wounding and pollination. A similar pattern was found after the intraspecific pollinations. The best performance, with regards to developing ovules, in the interspecific cross was found with a 96 hour delay after wounding, without emasculation, or with direct pollination after emasculation. Overall, the emasculated series produced most seed in the interspecific cross. Of the three pollination types examined, the intraspecific cross produced most seed.

The method of pollinating non-emasculated buds varied with the species depending on the presence of self incompatibility and the ease of the mechanics of artificial bud pollination. A follow up from this experiment would involve the pollination of buds after bud position three, where the S-alleles are not fully functional, without pre-wounding the buds. This would be equivalent to wounding plus a time delay. If this experiment was successful, then it may be possible to carry out the procedure of non-emasculation in species with smaller, more clustered buds, for example B.campestris.

Incompatibility mechanisms prevent self and interspecific fertilisation. The self incompatibility system limits inbreeding and the interspecific incompatibility, which prevents the development of hybrid zygotes, prevents gene flow between species.

From the results obtained in this experiment it is doubtful whether self incompatibility and interspecific incompatibility are under the same control, as suggested by some other authors. Knox, Willing & Ashford, (1972) demonstrated that in families where the self-incompatibility system is sporophytic that interspecific incompatibility was probably under similar control. His evidence came from two species of Populus and some species of the Compositae. They did suggest however that loci other than the S-locus must contribute to the control of the interspecific incompatibility. Removal of the self-incompatibility system may produce developing seeds but removal of self incompatibility, does not necessarily produce hybrid seed.. Various pre-fertilisation and post zygotic barriers may be involved. An extreme example is found Theobroma cacao, where after gametic fusion, if a certain proportion of the ovules fail to develop, the flowers bearing them are shed from the plant (Cope, 1962; Bouharmont, 1960).

In preliminary studies, it was found that different species used as maternal parents in the main crossing experiment (Chapter 5 and Chapter 6) had very different proportions of aborted ovules after bud self pollination and interspecific pollinations indicating some interspecific differences in the factors involved. These observations are discussed in the following chapters.

CHAPTER 5

POLLEN, EMBRYO AND ENDOSPERM DEVELOPMENT AFTER BUD SELF POLLINATIONS OF *BRASSICA CAMPESTRIS*, *B. OLERACEA*, *B. NAPUS* AND *RAPHANUS SATIVUS*.

5.1 Introduction.

It has been known for some time that many species of the Cruciferae family operate a self-incompatibility system (Bateman, 1955). The self-incompatibility system operating in Brassica and Raphanus is a sporophytic homomorphic system. This system is characterised by a number of different alleles which segregate at the incompatibility locus. With few exceptions, self-incompatible plants are heterozygous at the incompatibility locus. The self-incompatibility alleles may express relationships of dominance, independence and interaction in the anther or pistil (DeNettancourt, 1977). Characteristics of such systems include trinucleate pollen, dry papillate stigmas and rejection of incompatible pollen at the stigma surface (Heslop-Harrison, 1974; Heslop-Harrison & Shivanna, 1977). If incompatible pollen lands on a stigma, the grain either fails to germinate, or short twisted pollen tubes, which do not penetrate the stigma, are produced (Roggen, 1972).

The incompatibility system can be overcome in some Cruciferae by bud pollination. The incompatibility system is fully operative in the newly opened flower and the oldest bud. It is not fully developed in the immature stigmas of the younger buds. By this technique, pollen is applied to immature stigmas (Attai, 1950; DeNettancourt, 1977; Shivannai, Heslop-Harrison & Heslop-Harrison, 1978). This technique is used for both self incompatible pollinations and interspecific cross hybridisations.

There have been many attempts at interspecific hybridisation, but few successes. The reasons for the failure of so many crosses have never been fully examined. In order to examine the failure of the interspecific hybridisations it was first necessary to examine and

describe the basic events of embryo and endosperm development in the parental cultivars.

Controlled bud pollinations of two cultivars from each of the species B.campestris, B.oleracea, B.napus and R.sativus were examined for a number of characters. Bud pollination of a raceme involves emasculation and pollination of buds of various lengths (ie different stages of maturity). In order to minimise effects of bud maturity on pollen tube growth, a number of pollinated styles of different ages were examined for each species. Bud self pollination is not equivalent to natural pollination with respect to seed set. A reduced seed set is normally obtained when the method of bud pollination is employed (see Chapter 4).

Preliminary observations of development after self pollination showed that embryo and endosperm developed at a faster rate in some species than others. In all species examined after dissection of the ovules, there was an interval between pollination and the first sighting of an embryo. In order to observe the events in this early period, one species, B.napus cv Lair, which from previous observations was known to develop rapidly, and another, B.oleracea cv Maris Kestrel (2x), which developed at a slower rate, were examined.

Three characters were recorded after dissection of developing ovules. The embryo and endosperm stage of development and the length of the embryo. The different stages of development could be easily compared between the species. By measuring the length of the embryo it was possible to distinguish any quantitative differences between the species and any hybrids which may be formed (Chapter 6). In some cases the self pollinations were repeated over two or three years.

5.2 Material and Methods

5.2.1 Parental material.

Three closely related species from the Brassica genus and one species from the genus Raphanus were examined. From each, a diploid (2x) cultivar and tetraploid (4x) derived from it were bud self pollinated. To represent the A genome, B.campestris, cv Ponda (2x) and Taronda, (4x), were chosen. B.oleracea cv Maris Kestrel, diploid and tetraploid, were chosen to represent the C genome, and R.sativus cvv Slobolt (2x) and Crail (4x) were chosen to represent the R genome. In the case of B.napus, an amphidiploid of the A and C genomes, two totally different plant types were selected for examination in hybridisations, B.napus cv Lair (giant rape) and B.napus cv Ruta Otofte (swede), see Table 5.1.

Bud self pollinations were carried out as described in Chapter 2 section 3, and illustrated in Figures 2.1 to 2.6 inclusively. The number of bud pollinations per raceme depended on the species. For example, many more buds from B.oleracea could be pollinated per raceme than with B.campestris. B.oleracea has many large buds, spaced along the raceme, whereas B.campestris has smaller buds, which tend to be clustered towards the top of the raceme. Samples were collected at frequent intervals between two days and 40 days after pollination. Three replicates were collected for each sample time.

5.2.2 Parameters Measured.

(i) Gynoecium length.

In order to estimate gynoecium length at the stage when the self-incompatibility system is fully operative, 10 flowers from each of the eight cultivars were collected when the buds had newly opened and were ready for normal insect pollination. The gynoecium length

Table 5.1 The chromosome number and the genome formula of the eight cultivars examined in self pollinations (chapter 5) and in hybrid crosses (chapter 6).

Species	Cultivar	Chomosome number	Genomic formula
B. campestris	Ponda (2x)	2n=20	AA
	Taronda (4x)	2n=40	AAAA
B. oleracea	Maris Kestrel (2x)	2n=18	CC
	Maris Kestrel (4x)	2n=36	CCCC
B. napus	Lair	2n=38	AACC
	Ruta Otofte	2n=38	AACC
R. sativus	Slobolt (2x)	2n=18	RR
	Crail (4x)	2n=36	RRRR

was measured (mm) from the base of the ovary to the end of the stigma (Table 5.2).

The gynoecium length was also measured in a number of buds of varying age and their performance with regard to pollen tube penetration of the stigma and ovary estimated (Table 5.3).

(ii) The number and distribution of developing ovules per silique.

Siliques were collected at different time intervals between pollination and 40 days. Two siliques were taken and examined from each sample day. Each silique was opened to expose the ovules attached to their placentae. The ovules were scored according to three categories. (1) Number of fertilised ovules in the apical half and the number in the basal half of the silique, (2) number of aborted ovules and (3) number of unfertilised ovules.

(iii) Early development of ovules as seen in sectioned material.

To observe the very earliest stages of ovule development, two species were examined, one species that developed quickly, and another species that developed at a slower rate.

(iv) Ovule development as seen in dissected material.

Developing siliques were harvested at regular intervals between pollination and 40 days, and fixed in 1:3 glacial acetic acid : absolute ethanol. Three siliques were opened for each time interval and any developing ovules were removed. Where present, two developing ovules from each of the upper, middle and lower thirds of the silique, were selected and measured. The developmental stage of the embryo and endosperm, and any abnormalities present, were noted. The overall distribution of the six developing ovules was estimated from the apical and basal halves of the silique.

(v) Annual repeats.

In order to examine possible differences between the flowering seasons, bud self pollinations of three cultivars, B.campestris cv Taronda, B.oleracea cv Maris Kestrel (2x) and B.napus cv Lair, were repeated in 1980, 1981 and 1982. In each season, the self-pollinations were examined for all parameters described above.

The analysis of variance of the angular transformation (ARCSIN) of the percentage of fertilised ovules, examined over the three years showed no significant difference between years or significant interactions between years and cultivars (Table 5.4 and Table 5.5).

5.3 Results.

5.3.1 Pollen growth.

In order to see if the differences in bud maturity on a raceme had any effect on pollen tube growth the number of pollen tubes in the style and the ovary was estimated after bud pollination of the eight parent species. The number of pollen tubes in the style, and also the ovary, varied with the length and therefore the age of the gynoecium (Table 5.3). In all samples fewer pollen tubes were found in the ovary than were found in the style.

For B.campestris cv Ponda most pollen tubes were found when the gynoecium was 4mm long. As the length increased to 6mm, the length expected at anthesis, no pollen tubes were observed. Pollen tubes were found in the style and ovary of Taronda when the gynoecium was 5mm long, but none were found at 8mm and 9mm. For B.oleracea cv Maris Kestrel (2x) gynoecia at lengths from 6 to 9 mm all possessed a large number of pollen tubes in the style and ovary. In the case of B.napus cv Lair, over 250 pollen tubes were observed in the style when the

Table 5.2 Mean gynoecium length (mm) at anthesis of eight cultivars.

Species	Cultivar	Gynoecium length
B. campestris	Ponda	6.05 ± 0.302
	Taronda	9.80 ± 0.186
B. oleracea	Maris Kestrel (2x)	15.20 ± 0.231
	Maris Kestrel (4x)	15.70 ± 0.583
B. napus	Lair	11.80 ± 0.153
	Ruta Otofte	10.55 ± 0.174
R. sativus	Slobolt	12.20 ± 0.291
	Crail	13.70 ± 0.300

Table 5.3 Variation with gynoecium length of buds, and between the cultivars in the pollen penetration in the style and in the ovary.

Species	Cultivar	gynoecium length (mm)	number of pollen tubes in style	in ovary
B. campestris	Ponda	4	45.0	23.0
		5	12.5	11.5
		6	0.5	0.5
		7	0.0	0.0
B. campestris	Taronda	5	97.3	17.3
		6	0.0	0.0
		7	0.0	0.0
B. oleracea	Maris Kestrel (2x)	6	79.0	25.0
		7	64.0	24.3
		8	98.0	34.0
		9	124.0	35.0
B. napus	Lair	5	261.5	>100
		6	431.5	>100
		7	275.0	>100
		8	228.0	>100
R. sativus	Slobolt	8	72.0	12.0
		10	75.0	12.5
		12	3.5	2.0
	Crail	5	19.0	9.0
		7	70.5	22.5
		10	19.0	11.0
		12	2.0	1.0

Table 5.4 Percentage of ovules fertilised from three self pollinations, repeated in three years.

Species	Cultivar	1980	1981	1982	mean
B.campestris	Taronda	22.1	20.7	20.9	21.2
B.oleracea	Maris Kestrel (2x)	38.3	66.3	56.8	53.8
B.napus	Lair	80.2	68.7	78.0	75.6
Mean		46.9	51.9	51.9	

Table 5.5 Mean squares from the analyses of variance of the ARCSIN of the percentage of fertilised ovules, for three bud self pollinations examined in three years.

B. campestris Taronda

Source	d.f.	Mean square
Years	2	44.86 ns
Error	25	116.12

B. oleracea Maris Kestrel (2x)

Source	d.f.	Mean square
Years	2	720.64 ns
Error	24	168.09

B. napus Lair

Source	d.f.	Mean square
Years	2	185.18 ns
Error	28	157.10

ns = not significant.

gynoecium was between 5 and 8mm long. Almost as many were observed in the ovary. Pollen tube numbers similar to those found in B.campestris and B.oleracea were observed in the immature style and ovary of R.sativus cvv Slobolt and Crail. For Slobolt, the optimum pollination time, with respect to pollen tube penetration, was when the gynoecium length was 8 to 10mm. For Crail, most pollen tubes were observed when the gynoecium was 7mm long, the number gradually decreased as the gynoecium increased in length, with the lowest number observed at 13mm.

Figure 5.1 shows the compatible response observed after bud self pollination of a 7mm long gynoecium of R.sativus cv Crail. Figure 5.2 shows a typical incompatible response observed when the gynoecium of R.sativus cv Crail measured 14mm.

The shorter lengths of buds, when compared to the length at anthesis, corresponds to the very immature buds. In Chapter 4, it was demonstrated that the immature buds showed the poorest performance with regard to seed set after emasculation in bud self pollinations. This would correspond to floret position B5 onwards in B.oleracea (Figure 4.1). In B.campestris and B.napus fewer buds can be emasculated, purely due to the small size of the buds, and the fact that they are in a cluster at the top of the raceme, rather than spaced along its length as in B.oleracea or R.sativus.

5.3.2 Early development of the embryo and endosperm.

Tables 5.6 and 5.7 give details of the early development of the embryo and endosperm after bud selfing of B.napus cv Lair and B.oleracea cv Maris Kestrel (2x) respectively. These average stages of development were observed from sectioned material (for method see section 2.4.2).

Figure 5.1 Stigma response after bud self pollination of a 7mm long gynoecium of R.sativus cv Crail. The response is compatible.

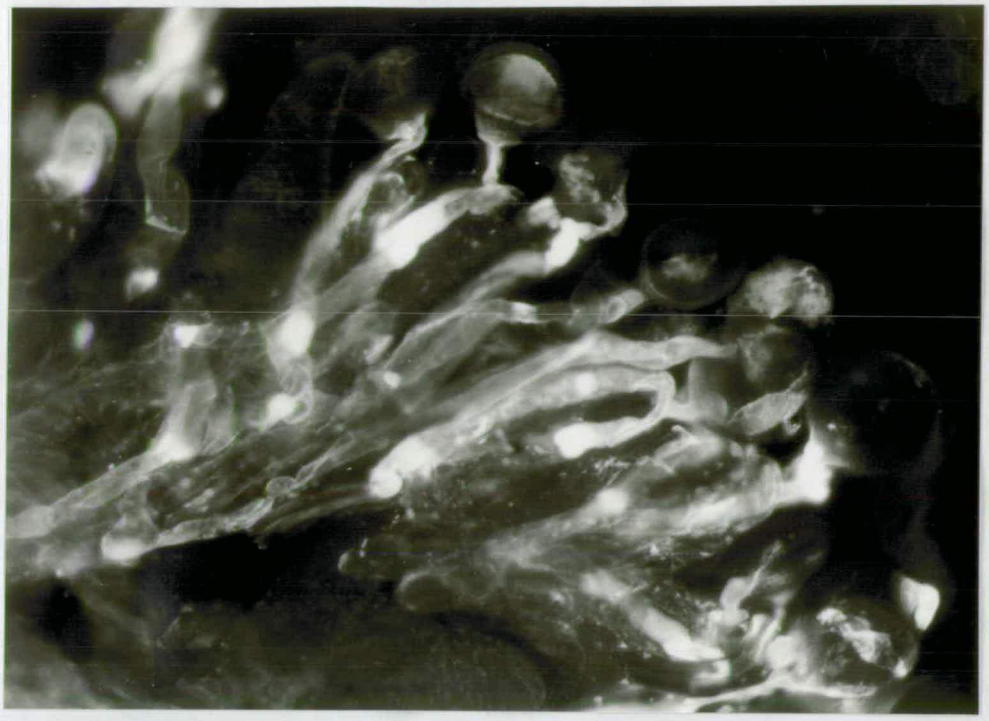


Figure 5.2 Stigma response after bud self pollination of s R.sativus cv Crail gynoecium which is 14mm long. The response is typical of an incompatible response.

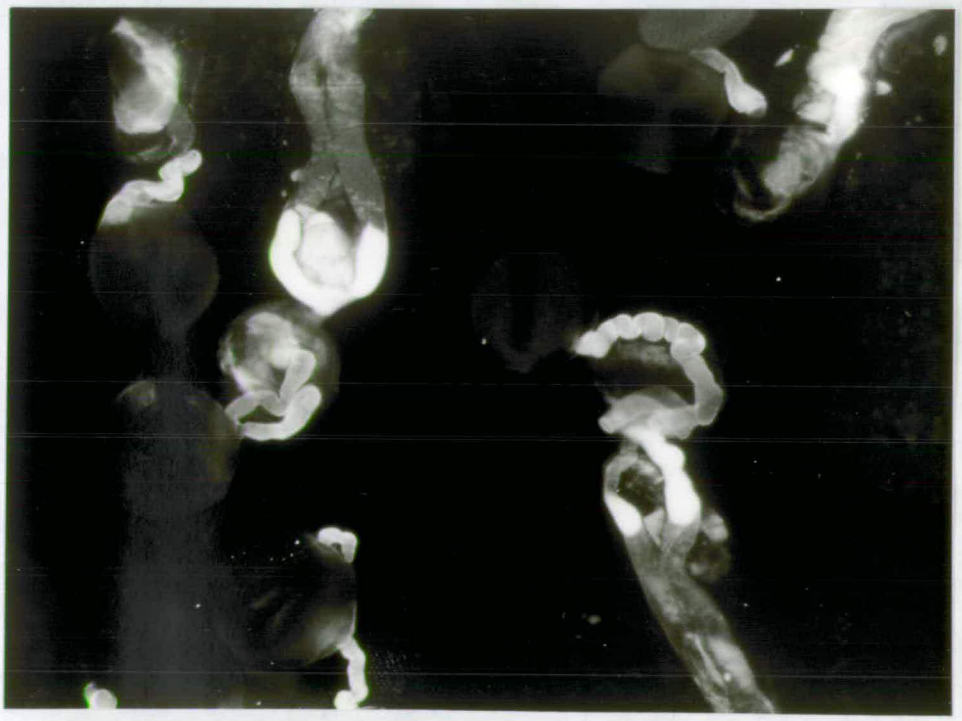


Table 5.6 The early development of embryo and endosperm after bud self pollination of B.napus cv Lair as observed in sectioned material.

Days after pollination	Embryo development	Endosperm development
2	None.	Synchronous division.
4	Pre-embryo differentiation to produce embryo and suspensor cell.	Coenocytic endosperm developing round the perimeter of the embryo.
6	2 and 4 celi embryo with developing suspensor.	" "
8	Spherical group of cells, attached to a suspensor of six cells.	Endosperm expanding to bathe suspensor.
10	Globular embryos (80-100um), the suspensor may have 9, 10 or 11 cells.	Endosperm has the appearance of a large empty sac with the embryo at the bottom.
12	Pre-heart/heart stage.	Endosperm undergoing changes to become cellular.

Table 5.7 The early development of embryo and endosperm after bud self pollination of B.oleracea cv Maris Kestrel (2x) as observed in sectioned material.

Days after pollination	Embryo development	Endosperm development
2	No fertilisation	No fertilisation.
6	None.	Synchronous division.
10	One cell embryo attached to two cell suspensor.	Endosperm developing around the perimeter of the embryo sac.
12	4 and 8 celled embryos attached to suspensors of 3 and 6 cells.	" "
16	Spherical group of cells attached to suspensor of variable cell numbers.	Endosperm has an empty sac appearance, suspensor and part of embryo bathed in endosperm.
20	Globular embryos (80-100um).	Coenocytic endosperm.
26	Pre-heart/heart stage embryo.	Endosperm undergoing changes to become cellular.

A time interval between pollination and first sighting of the embryo was two days after pollination. It was six days after pollination before the same stage was observed in B.oleracea. Pollen tubes with three nuclei in the tip have been observed in B.oleracea at two days post-pollination. This suggests that fertilisation had not taken place at that time.

Figure 5.3 shows the first divisions of the egg cell six days after pollination of R.sativus cv Slobolt. Evidence of unfertilised ovules, free polar nuclei is shown in Figure 5.4, a section of B.campestris cv Ponda four days after pollination.

5.3.3 Number and distribution of developing ovules.

Table 5.8 presents the mean number of ovules in a silique, classified according to whether they were developing, aborted or unfertilised. All four species produced a very low number of aborted ovules. A one-way analysis of variance of the eight bud self pollinations for ARCSIN of the percentage of fertilised ovules (Table 5.9) showed that there were significant differences ($p<0.001$) between the different selfs. The percentage of ovules fertilised ranged from 80.2% to 3.2%. Lair produced the highest number of ovules and also showed the highest percentage of fertilised ovules, while Maris Kestrel (4x), which produced on average, fewer than one ovule per silique, showed the lowest percentage of fertilised ovules. B.campestris cv's Ponda and Taronda, both showed strong incompatibility, producing only 5.9 and 3.7 developing ovules from a potential of 21.3 and 21.6 respectively. Both R.sativus cultivars showed high percentage fertilisation, although, because of the low ovule number, Slobolt produced on average only 3.3 ovules and Crail only 2.9 ovules per silique. In each case the tetraploid varieties produced significantly fewer

Figure 5.3 A sectioned ovule from R.sativus cv Slobolt, 6 days after bud self pollination, showing the first division of the egg cell. Scale bar 50µm.

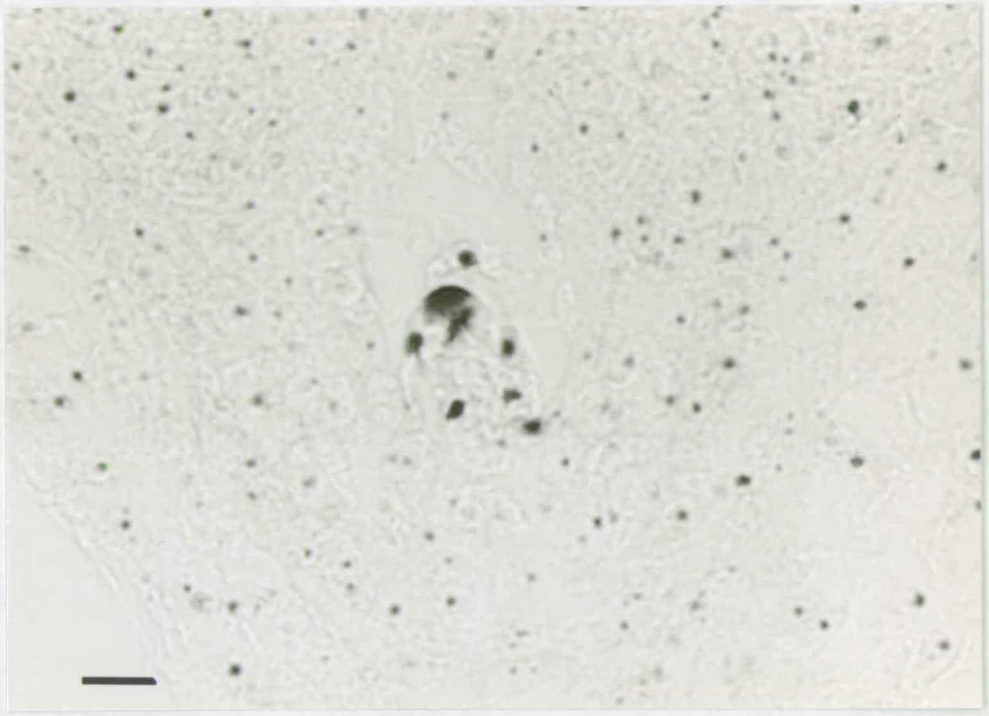


Figure 5.4 Polar nuclei in B.campestris cv Ponda, 4 days after bud self pollination as shown from a sectioned ovule. Scale bar 100µm.

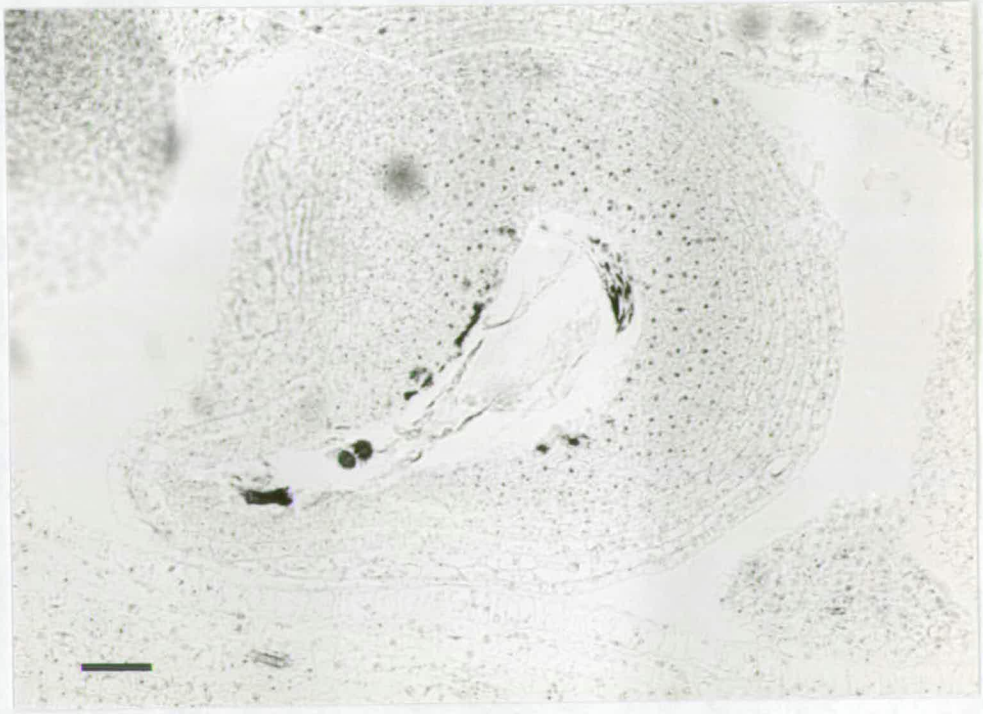


Table 5.8 Mean number of ovules per silique, grouped according to whether they were fertilised, aborted or unfertilised after bud self pollinations. Percentages are in brackets.

Species and cultivar	Mean number of ovules				No. of
	Fertilised	Aborted	Unfertilised	Total	siliques
B.campestris					
Ponda	5.94 (28)	0.06 (0.3)	15.19 (72)	21.19	16
Taronda	3.67 (22)	0.42 (2.5)	12.42 (75)	16.51	12
B.oleracea					
M.Kestrel (2x)	19.11 (57)	0.00 (0.0)	14.67 (43)	33.78	9
M.Kestrel (4x)	0.93 (3)	0.31 (1.0)	28.92 (96)	30.15	13
B.napus					
Lair	19.59 (80)	0.06 (0.2)	4.71 (19)	24.36	17
Ruta Otofte	18.56 (65)	0.22 (0.8)	9.89 (34)	28.67	9
R.sativus					
Slobolt	3.33 (63)	0.17 (3.2)	1.75 (33)	5.25	12
Crail	2.88 (58)	0.00 (0.0)	2.06 (42)	4.94	16

Table 5.9 Mean squares from the one-way analysis of variance of eight self pollinations for ARCSIN of the percentage of fertilised ovules.

Source	d.f.	Mean Square
Between cultivars	7	5975.25 ***
Error	96	183.58

***=0.01<p<0.001

($p<0.001$) fertilised ovules than their respective diploids. The difference between the different ploidy levels was most evident with Maris Kestrel, the diploid producing over 19 ovules and the tetraploid fewer than one ovule per silique.

Table 5.10 shows the overall distribution of the developing ovules in a silique, according to whether they are in the apical or basal half of the silique. There was found to be no significant difference between the number of ovules that developed in the apical end of the silique compared to the number that developed at the basal end (Table 5.11).

5.3.4 Embryo and endosperm development from dissected ovules.

(i) Stage of embryo and endosperm development.

Table 5.12 shows the mean stage of embryo development with time for all four species. There is much variation with regard to the first embryo sighting, not only between species, but also between cultivars within species.

The cultivars can be grouped into three arbitrary categories, early, intermediate and late, according to when the embryo was first sighted. B.campestris cv Ponda and B.napus cv Lair were early. The intermediate category includes B.campestris cv Taronda, B.napus cv Ruta Otofte and R.sativus cv Crail. While B.oleracea cv Maris Kestrel, both diploid and tetraploid cultivars, and R.sativus cv Slobolt were later to commence embryo development.

The duration of development also varies and is independent of commencement time. For example, Taronda and Crail began development at the same time, but Taronda produced its mature embryos much sooner than Crail.

Table 5.10 Number of developing ovules at the apical (top) end of the silique and at the basal (bottom) end of the silique of eight bud self pollinations.

Species	Cultivar	Apical	Basal
B. campestris	Ponda	3.0	2.9
	Taronda	1.8	1.8
B. oleracea	Maris Kestrel (2x)	7.5	5.1
	Maris Kestrel (4x)	0.4	0.5
B. napus	Lair	12.3	13.0
	Ruta Otofte	9.4	9.1
R. sativus	Slobolt	1.2	2.1
	Crail	3.0	3.7
Mean		4.8	4.8

Table 5.11 Mean squares from the analysis of variance of the number of developing ovules in the apical and basal ends of the silique of eight bud self pollinations.

Source	d.f.	Mean square Number of ovules
Pollination (selfs)	7	72.820 ns
Apical v basal	1	0.024 ns
Selfs x (apical v basal)	7	1.072 ns
Error	15	34.484

ns = not significant.

Table 5.12 Mean stage of embryo and endosperm development with time after bud self pollination. A diagramatic representation of the stages of development are shown in Figure 1.1.

days after pollin.	Ponda	Taronda	MK(2x)	MK(4x)	Lair	R.Otof.	Slobolt	Crail
10	EGa	....	....	....	EG a	....	....	....
12	....	EG a	....	....	....	....	....	EG a
14	G	G	....	....	G-PH	EG a	....	G
16	PH	PH	....	....	PH	....	....	G
18	H b	....	....	EG a	H b	....	EG-G a	....
20	....	LT b	G a	....	....	G-PH	....	H-ET b
22	....	....	....	....	LT-WS c	....	....	....
24	....	WS-MI c	H b	....	....	....	....	T
26	MI-II c	....	....	....	MII	....	H b	....
28	....	MII-III	LT	PH	....	WS c	....	WS-MI c
30	MIII d	....	....	....	MII	....	WS-MI c	....
32	....	....	MI c	H-ET b	....	MII	....	MII
34	....	MIII	....	....	MIII d	....	MII	....
36	....	....	....	MI c	....	....	....	....
38	....	....	....	....	....	....	MII-III	....
40	....	....	MIII d	MII	....	MIII d	....	MIII d

Stages of embryo development: EG = early globular; G= globular; PH= pre-heart; H= heart; ET= early torpedo; T= torpedo; LT= late torpedo; WS= walking stick; MI= mature I; MII= mature II; MIII= mature III.

Stages of endosperm development: a= coenocytic; b= cellular; c= gelatinous; d= dry.

Endosperm development appears to be closely linked with embryo development. In the early stages, globular embryos are associated with a coenocytic fluid endosperm. Changes in endosperm to form a cellular structure begin around the pre-heart stage of the embryo. Shrinkage of the endosperm and the gelatinous structure is associated with the "torpedo" to "walking stick" stage of the embryo. By the time the embryo has reached the mature stage the endosperm is dry and virtually non-existent.

5.3.4 (ii) Embryo length.

The lengths of the embryos were measured to give an indication of their overall size. The natural logarithm of the mean embryo length after bud self pollination is shown in Table 5.13. As expected the length of the embryo increases with time. However, the final length of the embryo, the MIII stage, varies depending on cultivar. Table 5.14 presents the final embryo lengths (mm) attained for each of the eight cultivars.

The length of the mature Brassica embryos ranged from 2.39mm to 3.94 mm. The Raphanus cultivars produced embryos which at their final size, were almost twice the size of those produced by Brassica

5.4 Discussion.

5.4.1 Pollen growth.

The number of pollen tubes in the style and ovary was found to vary within cultivars, according to the gynoecium length at the time of pollination. This indicates that the maturity of the bud at time of pollination is important to the success of the pollination assuming the same amount of pollen is presented on the stigma each time. In

Table 5.13 Mean embryo length (natural logarithm mm) with time after bud self pollination. Plateau values omitted.

days after pollin.	Ponda	Taronda	MK(2x)	MK(4x)	Lair	R.Otof.	Slobolt	Crail
10	*	*	*	*	3.29	*	3.18	*
12	4.01	3.50	*	*	*	*	4.65	3.24
14	4.48	4.81	*	*	4.90	3.66	*	4.22
16	4.90	5.12	*	*	5.12	*	5.45	4.81
18	5.08	*	*	3.78	*	*	4.22	*
20	*	7.16	4.78	*	*	4.89	7.78	5.96
22	*	*	*	*	7.44	*	*	*
24	*	7.08	6.00	*	*	*	8.48	7.26
26	7.78	*	*	*	8.29	*	*	*
28	*	7.98	6.43	5.24	*	7.53	*	8.54
30	8.00	*	*	*	*	*	*	*
32	*	*	7.86	6.30	*	7.94	*	*
36	*	*	*	7.77	*	*	*	*
Mean	5.25	6.03	6.84	6.24	5.81	6.01	6.87	6.12

Table 5.14 Final embryo length (mm), at maturity, of eight bud self pollinations.

Species	Cultivar	Embryo length
B. campestris	Ponda	2.39
	Taronda	3.28
B. oleracea	Maris Kestrel (2x)	3.34
	Maris Kestrel (4x)	3.10
B. napus	Lair	3.94
	Ruta Otofte	2.81
R. sativus	Slobolt	6.12
	Crail	6.47

most of the buds which were observed from B.campestris and B.oleracea the number of pollen tubes in the ovary was less than the total number of ovules. The percentage of fertilised ovules would therefore be expected to be less than 100%. In most of the gynoeciums of Raphanus, the number of pollen tubes observed in the ovary was greater than the number of ovules, therefore a high percentage fertilisation may be expected from the two Raphanus cultivars examined. The results confirmed these expectations.

In these species the highest number of pollen tubes were observed a few days before anthesis, when the self-incompatibility system is immature. As anthesis approaches, the incompatibility system increases in effectivness, until it is fully mature, when the flowers appear. This would explain the reduction in the number of pollen tubes found with increasing gynoecium length and also the relatively high pollen penetration where gynoecium size showed that they were least mature.

In Cruciferae the incompatibility response is mediated by sporophytically-derived materials carried in the pollen wall and the interaction is with individual stigma papillae (Heslop-Harrison et al, 1977). Generally the pollen grain fails to germinate, or if a tube is produced it is often short and twisted and makes little growth. Raphanus stigmas differ from Brassica stigmas in that the coating of the wax over the papillae is not always complete, and may sometimes be confined to small flakes scattered over the surface (Dickinson & Lewis, 1975). This may be a factor in producing a weaker self or interspecific incompatibility system. From Table 5.3, we might say that the two R.sativus cultivars examined have a weaker self-incompatibility system than B.campestris, since in R.sativus there is some pollen tube penetration in buds which have nearly the

same gynoecium length as at anthesis.

In the case of B.napus cv Lair many times more pollen tubes than ovules were observed in the style and the ovary than there were ovules. Because Lair is a self-compatible cultivar, there may be no advantage in bud pollination, unless this is done to avoid self pollination when this cultivar is to be pollinated with another. B.oleracea cv Maris Kestrel (2x) shows reasonable acceptance of pollen on buds. Some varieties of Sinapis alba, also of the Cruciferae, do not accept self pollen in buds of any age (Shivanna et al, 1978). Perhaps the increase in ploidy has had the effect of increasing the incompatible reaction on the stigma. Lewis (1979) reported that tetraploids of species with sporophytically determined incompatibility are fully self incompatible and show normal diploid behaviour of their S-alleles. Another opinion is that self-incompatibility is often weakened in polyploids derived from self-incompatible diploids (McNaughton, 1976).

5.4.2 Number and distribution of developing ovules.

The success of bud pollination varies with the species observed, although it is always less than 100%. Both diploid and tetraploid cultivars of B.campestris have shown a poor response to bud pollination, as did the tetraploid cultivar of B.oleracea. Both Raphanus cultivars with low total ovule numbers, and the diploid B.oleracea cultivar, attained about 50% success. Both types of B.napus responded favourably after bud pollination, perhaps because they are not self-incompatible.

The different responses shown by the three self-incompatible species examined suggest that different strengths of self-incompatibility is present in the three species. The lack of ovule abortion indicates

that development, once initiated, is usually regular. Hence, the incompatibility system which is operating in self-pollination is pre-fertilisation and there are no other external factors preventing development of fertilised ovules. One factor which may be important is the difference in total ovule number. In R.sativus, much fewer pollen tubes are necessary for 100% fertilisation.

The random distribution of developing ovules along the length of the silique reveals that even when the self-incompatible system is partially operative, ovules at the bottom of the ovary are as likely to be fertilised as those at the top.

5.4.3 Embryo and endosperm development.

The eight cultivars all followed the same pattern of development for both embryo and endosperm (Table 5.12). However, they do not all begin development at the same time, nor do they complete growth in the same time period, or achieve the same size of embryo at comparable morphological stages. Variation was found between species and between cultivars within species. Some cultivars began development soon after pollination, whereas others had a delay period. B.napus cv Lair showed early fertilisation and commencement of development (Table 5.6). It also developed quickly (Table 5.12). Conversely, B.oleracea cv Maris Kestrel (2x) appears to have a time lapse between pollination and the first signs of endosperm development (Table 5.7). It also took a much longer time period to complete development (Table 5.12).

The endosperm also follows a set pattern of development and is closely linked to embryo development. Cultivars with similar rates of embryo development will also have similar rates of endosperm development.

By measuring embryo length, it was possible to examine quantitative

differences between the cultivars and it gives an indication of the rate at which embryo development procedes once it has begun. Changes in embryo length with time can be seen graphically from Figure 5.5. The regression coefficients and intercept values for each cultivar from the straight line regression of the natural logarthm of embryo length against days after pollination are presented in Table 5.15. Lair showed the fastest change in embryo length with time, while Maris Kestrel (4x) developed slowest. Examination of the gradients shows that the eight cultivars fall broadly into three different groups. The first group consists of Lair, Slobolt and Crail. This group develops fastest. The second group consists of Ponda, Taronda and Ruta Otofte which develops at an intermediate rate, while the third group, Maris Kestrel 2x and 4x develops slower in comparison with cultivars from the other two groups. When the regression lines are projected back to the day of pollination, the cultivars were found to have different intercept values.

Maris Kestrel (2x) and (4x) and Slobolt, the cultivars which showed a long latent period between pollination and sighting the first embryos, showed a negative intercept. The other cultivars all showed positive intercepts, therefore these intercept values have no biological meaning. For example, Maris Kestrel (2x) was shown to have the highest intercept value, however it is not correct to state that, at the time of pollination, the natural logarithm of embryo length or this cultivar was 1.94. However ranking the cultivars, according to their intercept value or the regression of natural logarithm of embryo length against time, suggests that the intercept value provides information about the relative length of the latent period of the cultivars. Hence, the larger the intercept value, the shorter the latent period, before embryo development begins.

Figure 5.5 Regression slopes of the natural logarithm of embryo length against days after pollination for the eight bud selfs: Ponda (Po ○); Taronda (Ta ●); Maris Kestrel (2x) (MK(2x) △); Maris Kestrel (4x) (MK(4X) ▲); Lair (La □); Ruta Otofte (RO ■); Slobolt (Sl ◇) and Crail (Cr ◆).

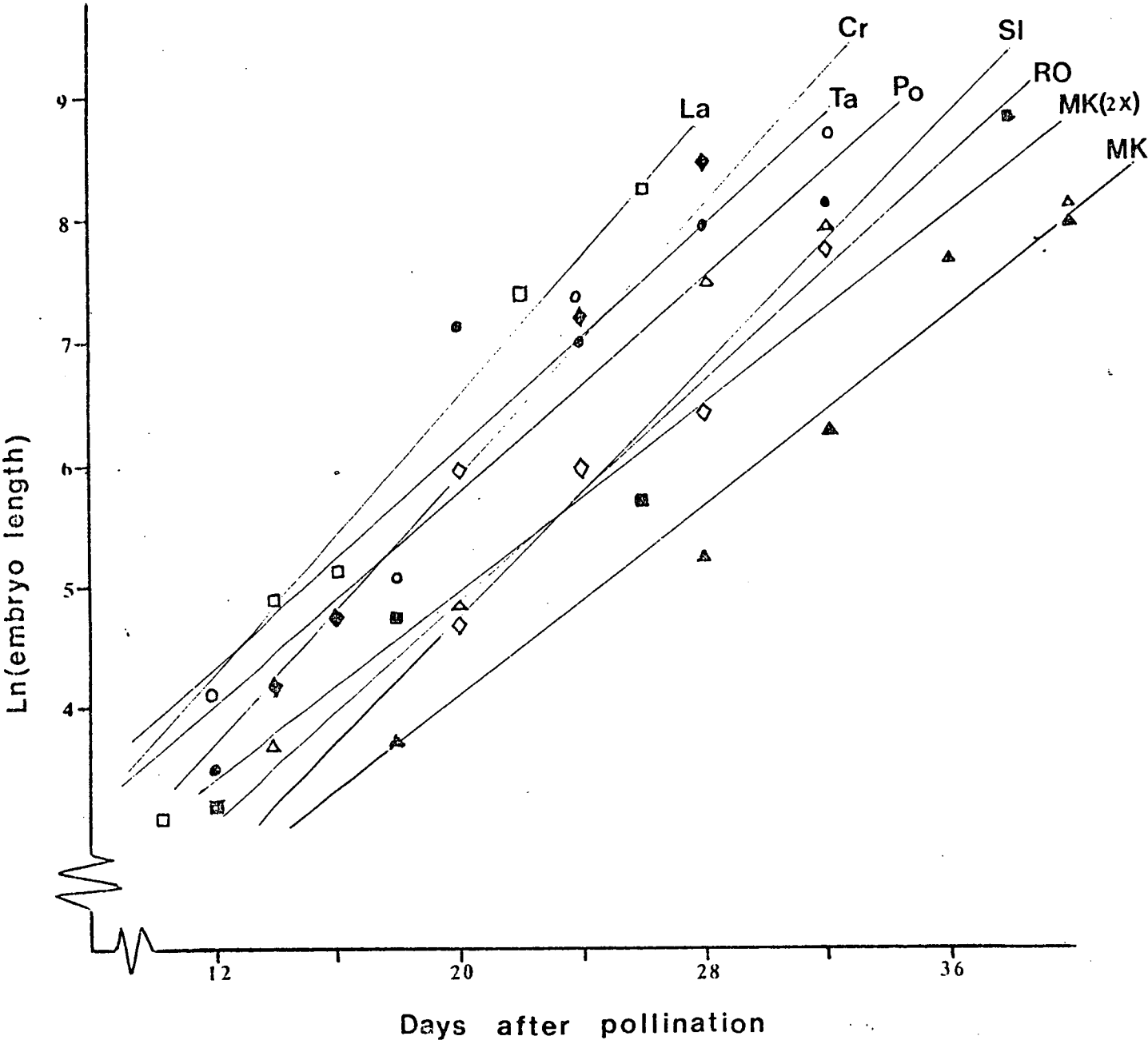


Table 5.15 Mean, regression coefficient and intercept after regression of natural logarithm of embryo length against days after pollination.

Species	Cultivar	Mean	Regression Coefficient	Intercept
B.campestris	Ponda	5.25	0.24	0.96
	Taronda	6.26	0.23	1.46
B.oleracea	Maris Kestrel (2x)	6.84	0.24	-0.76
	Maris Kestrel (4x)	6.24	0.21	-0.12
B.napus	Lair	5.88	0.31	0.34
	Ruta Otofte	6.01	0.25	0.05
R.satuvus	Slobolt	6.87	0.26	-0.49
	Crail	6.11	0.28	0.16

Examination of embryo and endosperm development after bud self pollination of these eight cultivars has highlighted several biological factors which may be related to reproduction. These factors are summerised in Table 5.16.

It may be that the rate of development and/or the delay period between pollination and the beginning of embryo development are important factors in determining the success of interspecific hybridisation. Careful choice of cultivars match the developmental rates of the two parents, would then increase the success of the hybridisation. It would also be possible to see if one parent is exerting a greater effect on its progeny than the other parent. This hypothesis is examined in the following chapter, where all the possible pollinations between these eight cultivars were examined.

5.5 Conclusions.

In order to be able to assess critically the results of interspecific hybridisation it is essential to have a basic understanding of the post-pollination development in the parent cultivars. From the present study, the self-incompatibility system has been confirmed. Reports from the literature indicate that interspecific incompatibility shows many similarities (De Nettancourt, 1977; Heslop-Harrison, 1978).

All bud self pollinations produced mature embryos and endosperm. However the percentages of ovules that were fertilised in the different self pollinations were significantly different between cultivars. B.oleracea cv Maris Kestrel (4x) showed only 3.2% ovule fertilisation. Maris Kestrel (2x), which has strong incompatibility alleles, produced significantly more fertilised ovules than its tetraploid equivalent. B.campestris was found to have 29.2% and 22.1%

Table 5.16 A summary of the developmental features; Ovule number; % of ovules that are fertilised; lag phase (days) between fertilisation and development; Rate of development (Slow, Intermediate or Slow); Duration of development (days) and Final embryo size (mm) which are characteristic to the embryo for each of the cultivars, after bud self pollination.

	Ovule number	% ovules fertilised	lag phase	Rate of development	Duration of devel.	Final size
B.campestris						
Ponda	21.2	28	10	Inter	20	2.39
Taronda	16.5	22	12	Inter	20	3.28
B.oleracea						
M.Kestrel(2x)	33.8	57	18	Slow	22	3.34
M.Kestrel(4x)	30.1	3	18	Slow	22+	3.10
B.napus						
Lair	24.4	80	10	Fast	24	3.94
Ruta Otofte	28.7	65	14	Inter	26	2.81
R.sativus						
Slobolt	5.2	63	18	Fast	22	6.12
Crail	4.9	58	12	Fast	28	6.47

ovule fertilisation in a diploid and tetraploid respectively, after bud pollination. Both these species exhibit a strong self-incompatibility system. B.napus and R.sativus both showed relatively high percentage seed set. The absolute numbers are very different although perhaps they are high for different reasons.

From Tables 5.3 and 5.8, it can be seen that there was great variation between the eight cultivars examined for the different stages of bud maturity, the number of pollen tubes that penetrated the style and ovary, and the potential ovule number. Bud self pollinations do not realise 100% seed set, therefore either incompatibility barriers are partially operating, or other pre-fertilisation factors are operating, since there is an extremely low number of aborted ovules. These factors will probably also have an effect in interspecific hybridisation. The developing ovules were found to be randomly distributed along the length of the silique.

Observations of pollen penetration showed that younger pistils allowed greater penetration of pollen tubes in the stigma and ovary. The smaller buds used in the bud pollinations have not developed their self-incompatibility system, whereas the pistils from larger buds are exhibiting self incompatibility to a larger extent. When making bud self pollinations of B.campestris, B.oleracea and R.sativus, which all have self-incompatibility systems, it would be as well to remove 1 or 2 of the largest buds as well as the flowers, before pollination. B.napus is largely a self-compatible species. The different species have different degrees of acceptance of self pollen. The percentage of ovules fertilised varied from 2.3% (Maris Kestrel (4x)) to 80.2% (Lair). It would therefore seem sensible to make more pollinations in the cases where the probability of success is low. R.sativus was least wasteful of its ovules. It produced the fewest ovules and had

the highest seed set of the incompatible species.

Observations of the sectioned material showed a delay, or latent period, between pollination and the beginning of embryo and endosperm development. The latent periods were different for the different cultivars examined. The intercept at pollination from the regression of embryo length against time, although in most cases not biologically meaningful, because negative intercepts were obtained, proved to be a good indicator for estimating the latent period. There were significant differences between the rates of embryo growth of the eight bud self pollinations. However, the different stages of embryo and endosperm development were closely linked. The final size of the embryos varied markedly between the different cultivars. Success may be greater when parents are similar in respect to some or all of these characters. Extremes in hybrid combination may be the first barrier in many unsuccessful hybridisations.

CHAPTER 6

POLLEN DEVELOPMENT, OVULE DISTRIBUTION AND STAGE OF EMBRYO ABORTION AFTER INTERSPECIFIC POLLINATIONS OF *BRASSICA CAMPESTRIS*, *B. OLERACEA*, *B. NAPUS* AND *RAPHANUS SATIVUS*

6.1 Introduction

Where a sporophytic self-incompatibility system is present, interspecific pollinations generally show the same incompatibility reaction at the stigma as self pollinations (Heslop-Harrison, 1978). Chapter 5 describes the basic embryo and endosperm development after bud self pollination of the four chosen parental species. This chapter is concerned with post pollination events after interspecific crosses.

Two cultivars from each of the four species were selected (see chapter 5). The effect of ploidy, genome ratios, genome number and composition, and cytoplasmic differences as found in reciprocal crosses, could all be examined. To examine all possible hybrid combinations of the eight cultivars, the crosses were made and scored as an eight by eight diallel. The crossing procedure and the cytological methods used were as previously described in Chapter 5. The number of developing ovules and their distribution along the silique and the embryo and endosperm stage of development reached were scored for each of the cross combinations at regular time intervals from two to 40 days after pollination. The number of fertilised and unfertilised ovules was also noted. These characters were also measured after bud self pollination of each cultivar, hence allowing comparisons to be made.

The results obtained from the self pollinations, explained in detail in the previous chapter, have been omitted from this chapter.

The eight by eight tables of results were analysed using a technique similar to that described by Griffing (1956), where general combining ability (GCA) and specific combining ability are estimated. The total variation in the eight by eight table was partitioned into the GCA of

the cultivars when used as female parents, the GCA of the cultivars when used as male parents and a remainder effect. The remainder effect is a combination of specific combining ability and reciprocal effects. The GCA of both the maternal and paternal effects was then partitioned into the GCA of the four species (between species), and for the difference between the cultivars within the four species (cultivars within species). When the "cultivars within species" effect was found to be non-significant, the "between species" effects were tested against the remainder effect. The remainder was tested for significance against the replicate error. Missing values were estimated from the relevant row and column means in the table. The row and column means were then re-estimated, to include any estimated missing values, for the purpose of the analysis of variance.

6.2 Results

The results in this chapter have been sub-divided into the same sections as the bud selfs, to allow comparisons to be made more easily. In most cases reference will be made to cultivars, unless it is relevant to discuss species effects.

6.2.1 Pollen tube growth

Data from all of the possible cross combinations are not available. The results from crosses which were scored are shown in Table 6.1. The values are estimated from counts on eight stigmas, two days after pollination.

Of those scored, all the combinations, except one, showed penetration of the stigma. The number of pollen tubes penetrating the stigma was in the range of 1 to 100. Maris Kestrel (4x) x Taronda and Ruta Otofte x Slobolt showed fewer than 10 pollen tubes on average. The

Table 6.1 Number of pollen tubes that penetrate the stigma after bud pollination between and within species.

	Ponda	Taronda	MK.(2x)	MK.(4x)	Lair	Ru.Ot	Slobolt	Crail
Ponda	-	#	#	2	#	#	2	2
Taronda	#	-	#	#	#	#	3	2
M.Kestrel (2x)	3	2	-	#	2	3	3	2
M.Kestrel (4x)	#	1	#	-	#	#	2	2
Lair	3	#	#	#	-	#	3	0
Ruta Otofte	#	#	2	#	#	-	1	1
Slobolt	2	#	3	#	3	#	-	#
Crail	2	2	#	#	3	#	#	-

0= no pollen tubes ; 1= 1 to 10 pollen tubes ; 2= 10 to 50 pollen tubes

3= 50 to 100 pollen tubes ; #= no estimate available.

combinations using Crail as a pollen parent showed consistently low pollen tube penetration of the stigma. In the cross using B.napus cv Lair as female parents no penetration was found in the stigmas examined.

6.2.2 Number and distribution of ovules

The number of unfertilised ovules for a particular cross remained fairly constant over the time period between two and 40 days after pollination. Initially the number of unfertilised and fertilised ovules compliment each other, adding up to the total number of ovules per silique. Eventually most of the fertilised ovules abort and therefore the fertilised ovules were categorised into developing and aborted ovules. Initially all the fertilised ovules are scored as developing, but in most cases, with time there is a change of emphasis towards the aborted ovules. The time taken for this change depends on the cross combination that is examined.

(i) Ovule number of the different species.

Table 6.2a shows the mean number of ovules per ovary produced by each of the species examined. The mean was estimated from all the cross combinations. The number per ovary in each of the species varies from those quoted in Chapter 5. The figures given here were calculated from a larger sample and is therefore a better estimate and will be used here. B.oleracea and B.napus produced most ovules, around 26 to 30 per silique. B.campestris was intermediate, producing around 20 to 25 ovules per silique. The two cultivars of R.sativus produced the least number of ovules per silique, with a mean of around 7. The number of ovules in each silique gave the potential number of seeds that would be possible if all the ovules were fertilised and developed to maturity. Table 6.2b presents the average gynoecium length for

Table 6.2a Mean total number of ovules per ovary for each cultivar, estimated from all the cross combinations involving that cultivar.

	B.campestris		B.oleracea		B.napus		R.sativus	
	Ponda	Taronda	MK.(2x)	MK.(4x)	Lair	Ru.Ot.	Slobolt	Crail
Mean ovule number	23.71	23.36	29.54	27.58	26.95	30.60	7.58	7.67
SE(mean)	3.058	1.440	1.747	3.497	2.221	2.229	0.748	1.053

Table 6.2b Average gynoecium length of the genotypes used (from chapter 5) at stage of insect pollination.

	B.campestris		B.oleracea		B.napus		R.sativus	
	Ponda	Taronda	MK.(2x)	MK.(4x)	Lair	Ru.Ot.	Slobolt	Crail
Mean length	6.05	9.80	15.20	15.70	11.80	10.55	12.20	13.70
SE(mean)	0.302	0.186	0.231	0.583	0.153	0.174	0.291	0.300

each of the cultivars to enable comparisons of ovule number with the length of the various gynoecia.

(ii) Percentage of unfertilised ovules.

Table 6.3 shows the percentage of unfertilised ovules per silique after crossing the eight cultivars in all possible combinations. The results for the cross Crail x Ponda were mislaid. However, from other recorded results it is known that a very low number of siliques developed after pollination and that samples were only collected up to 10 days after pollination. This suggests that either there was no fertilisation or there was very early abortion of developing ovules. The value estimated for the missing data in Table 6.3 was therefore not calculated from the row and column means but rather the mean values of Crail (which showed a low ovule number) was used in analysis.

The best and worst performances of the individual cultivars when used as male and female parents are shown in Table 6.4. The most obvious trend when considering the cultivars as female parents is that most unfertilised ovules occur after pollination with R.sativus cultivar Crail. In three cases none were fertilised. The least number of ovules left unfertilised occurred when B.napus cvv Ruta Otofte or Lair were the pollen parents.

When the cultivars are considered as pollen parents, most ovules were left unfertilised when combined with Ruta Otofte and Maris Kestrel (4x). Least were left unfertilised when combined with B.napus cv Lair or either of the R.sativus cultivars.

By estimating the mean percentage of unfertilised ovules per silique for each cultivar the overall effect of the female and male parents can be seen. Maris Kestrel (2x) , Ruta Otofte, Slobolt and Crail

Table 6.3 Percentage of unfertilised ovules per silique after interspecific pollinations between eight cultivars, the mean percentage of each cultivar as a maternal and paternal parent and the percentage unfertilised of each cultivar after bud self pollination.

	Ponda	Taronda	MK.(2x)	MK.(4x)	Lair	Ru.Ot	Slobolt	Crail	Mean
Ponda	-	68.8	40.8	79.1	39.1	32.9	92.6	94.8	64.0
Taronda	48.2	-	73.8	82.3	46.1	76.0	73.5	94.4	70.6
M.Kestrel (2x)	65.8	91.0	-	72.4	91.0	30.1	93.3	100.0	77.7
M.Kestrel (4x)	77.5	94.3	52.7	-	65.2	56.5	100.0	100.0	78.0
Lair	45.1	44.4	38.5	69.6	-	29.4	85.4	100.0	58.9
Ruta Otofte	80.5	68.2	86.8	86.9	60.5	-	95.4	99.4	82.5
Slobolt	66.7	75.4	22.0	76.0	35.0	41.4	-	37.5	50.6
Crail	(65.6)	87.2	64.8	64.1	79.6	61.9	45.8	-	67.0
Mean	64.2	75.6	54.2	75.8	59.5	46.9	83.7	89.4	
Mean selfs	71.7	75.2	43.4	95.9	19.3	34.5	33.3	41.7	

(65.6) estimated mean from overall mean of Crail as a maternal parent and Ponda as a male parent.

Table 6.4 The most and the least percentage of unfertilised ovules after interspecific pollination when each of the eight cultivars was examined as a male and female parent.

	Female Parent		Male Parent	
Cultivar	Most	Least	Most	Least
Ponda	Crail	Ruta Otofte	Ruta Otofte	Lair
Taronda	Crail	Lair	MK.(4x)	Lair
MK.(2x)	Crail*	Ruta Otofte	Ruta Otofte	Slobolt
MK.(4x)	Slo/Crail*	MK.(2x)	Ruta Otofte	Crail
Lair	Crail*	Ruta Otofte	Crail	Slobolt
Ruta Otofte	Crail	Lair	Taronda	Lair
Slobolt	Taronda	MK.(2x)	MK.(4x)*	Crail
Crail	Taronda	Slobolt	MK.(2x+4x)/Lair	Slobolt

* = 100% unfertilised ovules

showed variation in performance depending on whether they were used as the male or female parent. Maris Kestrel (2x) shows 77.7% of ovules unfertilised when used as a female parent but only 54.2% when considered as the male parent. R.sativus performs better as a female parent where 50.6% to 67.0% of the ovules per silique remain unfertilised. As a male parent, the percentage of unfertilised ovules were in the range of 80% to 90%. As a female parent, B.napus cv Ruta Otofte left a higher percentage of unfertilised ovules than Lair, while as male parents the reverse was found.

It has already been stated that the bud pollination technique does not realise 100% fertilisation. This has to be taken into account when considering the percentage of unfertilised ovules after interspecific pollination.

The percentage of unfertilised ovules for a given cultivar as the female parent varies depending on which pollinator is used. Comparison of the overall mean percentage of the cultivar compared to the results after bud self pollination may show more or less unfertilised ovules (Table 6.3). Both cultivars of B.campestris show a slightly lower percentage of unfertilised ovules after cross-pollination, ie. a higher percentage of ovules were fertilised. B.oleracea cv Maris Kestrel (2x) showed a 34% incerease in the percentage of unfertilised ovules, while Maris Kestrel (4x) showed a 17% decrease. Both B.napus cultivars had a higher percentage of unfertilised ovules, cv Lair 39%, ,cv Ruta Otofte 48% after cross-pollination. R.sativus cv Slobolt had 17% and cv Crail had 26% more unfertilised ovules after interspecific pollinations.

The percentage of ovules unfertilised were transformed, by the ARCSIN transformation before analysis. Table 6.5 shows the means squares from this analysis. Significant differences were found in the GCA of

Table 6.5 Mean squares from the analysis of variance of the ARCSIN of the percentage of unfertilised ovules. Maternal and paternal effects are both partitioned into differences between the four species (b species) and differences between cultivars within species (w species).

Source	df	MSq	
Maternal effects			
b species	3	551.41	**
w species	4	261.00	n.s.
Paternal effects			
b species	3	1833.93	***
w species	4	307.16	n.s.
Remainder	40	123.08	*
Replicate error	55	45.46	

n.s. = not significant ; * = $p<0.05$; ** = $0.05<p<0.01$; *** = $0.01<p<0.001$

the four species both as maternal ($p<0.01$) and paternal ($p<0.001$). When the cultivars were considered as female parents, most of the ovules remained unfertilised after pollination with R.sativus. The least number of unfertilised ovules was found after pollination with B.napus. When the cultivars were thought of as male parents, most of the ovules were left unfertilised when combined with B.napus cv Ruta Otofte and least when combined with B.napus cv Lair. There were no significant differences between the cultivars within species. The remainder effect was shown to be significant when tested with the replicate error, hence all the variation in the table was not accounted for by the maternal and paternal GCA.

(iii) Percentage of aborted ovules.

After cross pollination most cross combinations produced some developing ovules. With an increasing number of days after pollination, there was a change of emphasis from developing ovules to aborted ovules. How quickly abortion occurred depended on the parental combination. Since, in the majority of crosses, all of the developing ovules aborted, the percentage of aborted ovules at the final sampling time was examined. By comparing these values with the percentage of unfertilised ovules, it can be deduced whether all the developing ovules aborted, or whether some of the hybrid ovules developed to maturity. (Table 6.9). The percentage of the total number of ovules per silique which had aborted by the final sample day is presented in Table 6.6. Consideration of the cross combinations with the highest and lowest percentages of aborted ovules of each cultivar, used as a male and female parent, is shown in Table 6.7.

When considering each of the cultivars as female parents, most abortion occurred after pollination with B.oleracea and B.napus.

Table 6.6 Percentage of the total number of ovules per silique which had aborted by the last sample day after interspecific pollinations between eight cultivars and the mean percentage of each cultivar as a maternal and paternal parent.

	Ponda	Taronda	MK.(2x)	MK.(4x)	Lair	Ru.Ot	Slobolt	Crail	Mean
Ponda	-	17.6	53.7	17.3	26.5	1.9	9.4	6.8	19.0
Taronda	45.6	-	15.9	5.3	52.1	26.8	5.3	7.3	22.6
M.Kestrel (2x)	19.1	10.3	-	32.1	6.7	45.8	12.5	0.0	18.1
M.Kestrel (4x)	20.7	9.6	5.2	-	8.3	58.2	0.0	0.0	14.6
Lair	2.0	65.6	73.7	44.3	-	7.1	11.8	0.0	29.2
Ruta Otofte	7.4	13.3	11.4	5.5	0.0	-	2.9	0.0	5.8
Slobolt	0.0	31.2	12.5	12.5	61.5	25.0	-	2.9	30.4
Crail	(18.9)	0.0	41.1	33.3	7.1	28.6	22.2	-	21.6
Mean	16.2	21.1	30.5	21.5	23.2	27.6	9.2	12.1	

Value in brackets has been estimated from row and column means.

Table 6.7 The most and the least percentage aborted ovules, at the final sampling time, after interspecific pollination when each of the eight cultivars was examined as a male and female parent.

	Female Parent		Male Parent	
Cultivar	Most	Least	Most	Least
Ponda	MK.(2x)	Ruta Otofte	Taronda	Slobolt*
Taronda	Lair	Slobolt	Lair	Crail*
MK.(2x)	Ruta Otofte	Crail*	Lair	MK.(4x)
MK.(4x)	Ruta Otofte	Slo/Crail*	Lair	Taronda
Lair	MK.(2x)	Crail*	Slobolt	Ruta Otofte*
Ruta Otofte	Taronda	Lair/Crail*	MK.(4x)	Ponda
Sloblot	Crail	Ponda*	Lair	MK.(4x)*
Crail	MK.(4x)	Taronda*	Slobolt	MK.(2x/4x)/La/RuO*

* = No abortion observed

Least abortion occurred with R.sativus. When each cultivar is considered as a male parent, four of the eight cultivars showed most abortion after pollination with Lair (Table 6.6 and Table 6.7). However, Slobolt, Taronda and Maris Kestrel (4x) also contributed to high abortion when used as pollen parents. All of the cultivars were represented in producing a lower percentage of abortion when used as male parents.

The mean percentage of aborted ovules for each of the cultivars, estimated from all the cross combinations with that cultivar used as the female parent, showed no ploidy effect for B.campestris. In B.oleracea and R.sativus, the diploid cultivars had a slightly higher percentage of aborted ovules than their tetraploid counterparts. A more varied response was found within B.napus, where Lair showed a much higher percentage of aborted ovules than Ruta Otofte. When the cultivars were considered as male parents, in the same way, diploid B.oleracea and R.sativus again showed a higher percentage of aborted ovules compared to the tetraploids. In B.campestris the tetraploid cultivar showed the higher percentage of aborted ovules. B.napus cv Ruta Otofte produced a higher percentage of ovule abortion than Lair.

Table 6.8 presents the mean squares from the analysis of variance, after angular transformation (ARCSIN), of the final percentage of aborted ovules. As maternal parents there was no significant differences found between the four species or between the cultivars within species. As paternal parents, differernces between the four species was found to be just short of significance at the 95% level. A high proportion of the effect of the difference between the paternal parents was due to the R.sativus cultivars, where lower percentages of ovules were generally found to have aborted. Also, Maris Kestrel (2x), which showed 35% abortion inflated the B.oleracea average.

Table 6.8 Mean squares from the analysis of variance of the ARCSIN of the percentage of aborted ovules on the last sample day. Maternal and paternal effects are both partitioned into differences between the four species (b species) and differences between cultivars within species (w species).

Source	df	MSq	
Maternal effects			
b species	3	206.607	n.s.
w species	4	300.193	n.s.
Paternal effects			
b species	3	611.284	n.s.
w species	4	67.444	n.s.
Remainder	40	235.805	n.s.
Replicate error	55	226.753	

n.s. = not significant

(iv) Percentage of developing ovules.

Table 6.9 presents the mean percentage of developing ovules per silique over the sampling period, after interspecific pollination between the eight cultivars. In most of the cross combinations the ovules abort fairly early in development, but with some hybrid combinations, development continued to maturity. The crosses which developed to produce mature seeds are highlighted in Table 6.9. There is a dominant species effect in the examples which develop to maturity, also a strong reciprocal effect.

The percentage of developing ovules after the various cross combinations between B.campestris and B.napus shows considerable variation. Ponda x Lair, and the reciprocal, show 54.4% and 52.9%, respectively, of developing ovules, compared to Ponda x Ruta Otofte, and the reciprocal, which produced 65.8% and 15.9% of developing ovules respectively. Ponda x Ruta Otofte develops to maturity while the reciprocal cross aborted early in development.

The mature embryos found from the various cross combinations of B.napus and B.campestris frequently showed pre-germination. The hypocotyl was often long, and did not curve round between the cotyledons as in normal embryo development, The result of this protrusion was to rupture the ovule integuments and expose the embryos inside.

B.oleracea shows a cultivar effect. When crossed in the direction Maris Kestrel (2x) x Maris Kestrel (4x) only 16% of the ovules were found to be developing, while in the reciprocal cross, 45.4% were found to be developing. Ruta Otofte x Lair produced 39.5% and Lair x Ruta Otofte produced 67.5% of developing ovules. Slobolt x Maris Kestrel (2x) and Crail x Slobolt both developed to produce mature

Table 6.9 Percentage of developing ovules per silique over the sampling period after interspecific pollinations between eight cultivars, the mean percentage of each cultivar as a maternal and paternal parent and the percentage developing of each cultivar after bud self pollination. Figure in parenthesis is an estimated value as described in the text.

	Ponda	Taronda	MK.(2x)	MK.(4x)	Lair	Ru.Ot	Slobolt	Crail	Mean
Ponda	-	15.8	29.2	1.5	54.4	65.8	3.2	0.0	24.3
Taronda	14.0	-	2.8	11.0	30.8	10.7	23.5	3.2	13.7
M.Kestrel (2x)	28.8	0.8	-	16.2	5.5	59.3	0.7	0.0	15.9
M.Kestrel (4x)	17.2	2.3	45.4	-	30.4	14.7	0.0	0.0	15.7
Lair	52.9	23.9	5.6	3.8	-	67.5	3.8	0.0	22.5
Ruta Otofte	15.9	29.4	4.5	7.3	39.5	-	1.2	0.6	14.1
Slobolt	33.3	6.6	74.0	20.0	35.0	40.0	-	32.3	34.5
Crail	(25.0)	12.8	10.3	23.4	18.5	23.8	49.4	-	23.3
Mean	26.7	13.1	24.5	11.9	30.6	40.3	11.7	5.2	
Mean selfs	28.0	22.2	56.6	3.0	80.4	64.7	63.4	58.3	

Combinations which developed to produce mature embryos are underlined.

ovules whereas the reciprocals of both crosses produced no mature seeds.

The mean squares from the analysis of variance, after angular transformation (ARCSIN), of the mean percentage of developing ovules is presented in Table 6.10. Significant differences ($0.01<p<0.05$) were found between the four species used as female parents. Highly significant differences ($p<0.001$) were found between the four species when used as male parents. When the cultivars were considered as female parents, most developing ovules occurred after pollination with B.napus, sometimes the cultivar Ruta Otofte and sometimes Lair. The least percentage of developing ovules were found after pollination with R.sativus. As male parents, most developing ovules were found in combinations with B.napus or R.sativus. The lowest percentages were found in cross combinations with B.campestris and B.oleracea. Once again there was no significant difference between cultivars within species either as maternal or paternal parents. There was however greater variation between cultivars within species when used as male parents (just short of significance at the 95% level) than when used as female parents. The cross combinations Maris Kestrel (2x) x Crail, Maris Kestrel (4x) x Slobolt, Maris Kestrel (4x) x Crail and Lair x Crail showed no fertilisation, and hence did not produce any developing ovules.

(v) The distribution of developing ovules before abortion.

The numbersof developing ovules found at the apical (top) and basal (bottom) half of the silique from the cross combinations of the eight parents are presented in Table 6.11. Their distribution over the time period measured was fairly consistent. Since most of these ovules eventually abort, it was assumed that the distribution of aborted ovules was similar. Mean squares from the analysis of variance of

Table 6.10 Mean squares from the analysis of variance of the ARCSIN of the percentage of developing ovules. Maternal and paternal effects are both partitioned into differences between the four species (b species) and differences between cultivars within species (w species).

Source	df	MSq	
Maternal effects			
b species	3	444.36	*
w species	4	76.69	n.s.
Paternal effects			
b species	3	1464.65	***
w species	4	253.80	n.s.
Remainder	40	156.20	n.s.
Replicate error	55	86.32	

n.s. = not significant ; * = $p<0.05$; *** = $0.01<p<0.001$

Table 6.11 The number of developing ovules in the apical (A) and the basal (B) halves of the silique, after cross pollination of eight cultivars. Missing data are indicated by an * symbol.

		Ponda	Taronda	MK.(2x)	MK.(4x)	Lair	Ru.Ot	Slobolt	Crail
Ponda	A	-	1.71	1.42	0.20	5.33	7.50	0.17	0.00
	B	-	2.00	7.42	0.20	6.33	9.00	0.33	0.08
Taronda	A	1.55	-	0.43	1.07	2.86	1.37	4.67	0.50
	B	1.70	-	0.14	1.86	3.71	1.62	0.67	0.17
M.Kestrel (2x)	A	3.87	0.25	-	2.75	1.00	9.00	0.10	0.00
	B	2.75	0.00	-	3.12	0.70	7.75	0.10	0.00
M.Kestrel (4x)	A	3.20	0.27	6.10	-	3.50	2.43	0.00	0.00
	B	2.00	0.27	6.30	-	3.50	1.57	0.00	0.00
Lair	A	6.07	3.67	0.86	0.54	-	8.54	0.33	0.00
	B	6.27	4.00	0.71	0.69	-	10.15	0.56	0.00
Ruta Otofte	A	2.33	4.33	0.89	0.87	6.00	-	0.10	0.10
	B	2.08	3.83	0.56	1.37	5.86	-	0.30	0.10
Slobolt	A	1.60	0.12	2.57	0.67	1.20	1.50	-	1.25
	B	0.80	0.37	2.71	1.00	0.90	2.00	-	1.33
Crail	A	*	0.29	0.38	0.62	0.50	0.87	2.00	-
	B	*	0.43	0.54	1.25	0.75	1.13	1.10	-

ovule distribution between the apical and basal halves of the silique (Table 6.12) showed a significant difference between crosses ($p<0.001$). Over all crosses the basal end of the silique showed significantly more developing ovules than the apical half ($p<0.001$). There was no interaction found between crosses and distribution of developing ovules. Most of the cross combinations showed a random distribution of the developing ovules. In combinations where there was a difference of more than 1.5 ovules between the top and bottom of the silique, the distribution was considered to be non-random. The crosses which were found to be in this non-random group which had an apical bias were Taronda x Solbolt and Maris Kestrel x Ruta Otofte. Ponda x Maris Kestrel (2x), Ponda x Ruta Otofte and Lair x Ruta Otofte all showed a bias towards the basal halves of the silique. From these results B.napus cv Ruta Otofte appears to predominate in non-random distribution of developing ovules when used as a pollen parent.

6.2.3 Embryo and endosperm development

(i) Embryo development

Table 6.13 shows the number of days after pollination when embryos were first detected in dissected ovules. The different species varied with respect to the time after pollination at which development was first detected. Embryos were observed, on average after 12 days in B.campestris, on average after 20 days in B.oleracea, after 12 days in B.napus, and after 14 days in R.sativus. Within the species variation was observed depending on which cultivar was used as the female and its pollinator. With B.campestris as female the days when embryo development was first detected between 10 and 14 days after pollination. In B.oleracea this period was found to range from 14 to 24 days, in B.napus 6 to 16 days, and R.sativus 10 to 20 days.

Table 6.12 Mean squares from the analysis of variance of ovule distribution between the apical and basal halves of the silique.

Source	df	MSq	
Crosses	54	0.0654	***
Position	1	0.1116	***
Cross x Position	54	0.0049	n.s.
Replicate error	110	0.00332	

n.s. = not significant ; *** = $0.01<p<0.001$

Table 6.13 Days after pollination when embryo development was first detected in dissected ovules of all possible combinations of crosses between eight cultivars.

	Ponda	Taronda	MK.(2x)	MK.(4x)	Lair	Ru.Ot	Slobolt	Crail
Ponda	-	12	14	14	12	10	12	0
Taronda	12	-	10	14	10	10	10	0
M.Kestrel (2x)	20	24	-	20	20	20	20	0
M.Kestrel (4x)	20	24	14	-	19	16	0	0
Lair	10	10	10	10	-	6	12	0
Ruta Otofte	14	14	0	16	10	-	16	16
Slobolt	14	12	20	14	18	12	-	10
Crail	*	16	12	12	14	14	10	-
Bud self	10	12	20	18	10	14	18	12

0 indicates no embryos detected

* indicates missing datum

The time when embryo development ceased (Table 6.14) was scored as the time after pollination when the embryo failed to develop to the next stage of embryogenesis. A great deal of variation was noted for this character, not only between species but also within a cultivar, depending on the interspecific combination. In some cases the embryos ceased to develop soon after first being detected, (for example Taronda x Maris Kestrel (2x)), while other cross combinations may continue embryo development for some time (for example Ponda x Lair).

Table 6.15 presents the maximum stages of development reached by the embryos after interspecific pollination. In most cases, the stage of development reached is closely linked with the days after pollination when embryo development ceased (Table 6.14). All of the cultivars, when used as female parents, were capable of producing a mature hybrid embryo, in at least one combination. However, some crosses did not produce any embryos. Development of the embryos in 19 out of the remaining 47 crosses were stopped in the globular stage of development; 5 of the 47 crosses managed to develop to the pre-heart stage; 10 of the 47 crosses showed development as far as the torpedo stage of development. The remaining 13 combinations were able to develop into the mature stages of embryo development. B.campestris cv Ponda produced mature embryos in two combinations. B.napus cvv Lair and Ruta Otofte both produced mature stages of development in three crosses, while the remaining cultivars produced mature embryos in only one cross combination.

(ii) Endosperm development.

Table 6.16 shows the maximum stage of endosperm development reached in each of the cross combinations. In most cases the endosperm remained coenocytic, except where mature stages of embryo formation were found.

Table 6.14 Days after pollination when embryo development ceased after interspecific hybridisation of eight cultivars in all combinations.

	Ponda	Taronda	MK.(2x)	MK.(4x)	Lair	Ru.Ot	Slobolt	Crail
Ponda	-	16	18	14	40	28	14	0
Taronda	18	-	10	20	36	14	10	0
M.Kestrel (2x)	36	28	-	36	22	24	20	0
M.Kestrel (4x)	28	24	32	-	19	24	0	0
Lair	30	20	10	10	-	36	16	0
Ruta Otofte	32	28	0	20	24	-	20	16
Slobolt	20	14	32	18	30	16	-	16
Crail	*	18	16	16	28	28	35	-
Bud self	30	34	40	40+	34	40	38+	40

0 indicates no embryos detected. + indicates that embryo had not reached maturity a final sample time. * indicates missing value.

Table 6.15 Maximum stage of embryo development by 40 days post pollination, after interspecific crosses of eight cultivars in all possible combinations.

	Ponda	Taronda	MK.(2x)	MK.(4x)	Lair	Ru.Ot	Slobolt	Crail
Ponda	-	H	G	G	MII	MIII	G	0
Taronda	PH	-	EG	G	MIII	T	EG	0
M.Kestrel (2x)	T	EG	-	MII	EG	EG	PH	0
M.Kestrel (4x)	PH	EG	MIII	-	0	T	0	0
Lair	MIII	MIII	EG	EG	-	MIII	PH	0
Ruta Otofte	MIII	H	0	G	MII	-	ET	MI
Slobolt	G	EG	MII	G	T	PH	-	H
Crail	*	G	G	G	H	LT	MIII	-

0 = no embryos observed; G = globular; PH = pre-heart; H = heart; ET = early torpedo; T = torpedo; MI, MII and MIII = consecutive stages of development of the mature embryo; * = missing data.

Table 6.16 Maximum stage of endosperm development by 40 days post pollination after interspecific crosses of eight cultivars in all possible combinations.

	Ponda	Taronda	MK.(2x)	MK.(4x)	Lair	Ru.Ot	Slobolt	Crail
Ponda	-	A	A	A*	C	C/dry	A*	0
Taronda	A	-	A	B*	C/dry	B	A*	0
M.Kestrel (2x)	A	A*	-	A	A*	A	A	0
M.Kestrel (4x)	A	A*	C/dry	-	0	A	0	0
Lair	dry	dry	A*	A*	-	dry	A*	0
Ruta Otofte	dry	A	0	A*	C	-	A	B/C
Slobolt	A	A*	G	A	A	A	-	A
Crail	*	A	A	A	A	A	dry	-

A = coenocytic endosperm; A* = very small amount of coenocytic endosperm; B = cellular endosperm; B* = very small amount of cellular endosperm; C = gelatinous endosperm; dry = mature endosperm before it is totally adsorbed; 0 = no endosperm; * = missing data.

In these examples the endosperm appeared more normal and developed through the cellular and gelatinous stages to produce the dry endosperm characteristic of the mature stage of embryo development. In most of the cross combinations which aborted earlier than the mature stage of development, the endosperm remained coenocytic. In the crosses where the embryos aborted soon after beginning development, coenocytic endosperm was present only in small amounts.

(iii) The rate of hybrid embryo development.

The rate of embryo development was estimated by comparing the days after pollination when embryo development was first detected and when it stopped with the parent cultivars after bud self pollinations. Most development is at the same rate as the female parent, but in some cross combinations, slower or faster development was observed, regardless of the final stage of embryo development, depending on the cross involved.

Consider first of all the cultivars as female parents. Combinations with Lair developed at the same rate as bud self pollinations. Ruta Otofte and Slobolt either developed at the same rate or faster than the bud self. Maris Kestrel (2x) and Crial developed at the same rate, or slower, than the bud self, while Ponda, Taronda and Maris Kestrel (4x) all showed rates of development which were faster, slower or at the same rate as the bud self in different cross combinations (Table 6.17).

When the cultivars are used as pollen parents a similar pattern occurs. Those crosses with Crail which were successful in producing embryos develop at a faster rate than the bud self. Ponda, Ruta Otofte and Slobolt crosses develop either at the same rate as or faster than, the self. Maris Kestrel (4x) crosses develop at the same

Table 6.17 The rate of embryo development in comparison to the female parent, after crossing eight cultivars in all combinations. >>> indicates a faster rate of development than the female parent; <<< indicates a slower rate of development than the female parent; --- indicates the same rate of development as the female parent; 0 indicates no development; * indicates missing datum.

	Ponda	Taronda	MK.(2x)	MK.(4x)	Lair	Ru.Ot	Slobolt	Crail
Ponda		---	<<<	<<<	<<<	>>>	---	0
Taronda	---		---	<<<	---	>>>	---	0
M.Kestrel (2x)	---	<<<		---	---	---	---	0
M.Kestrel (4x)	---	<<<	>>>		---	>>>	0	0
Lair	---	---	---	---		---	---	0
Ruta Otofte	>>>	---	0	---	>>>		>>>	>>>
Slobolt	---	>>>	---	---	---	>>>		>>>
Crail	*	<<<	---	---	<<<	---	>>>	

rate or slower than selfs and the crosses of the remaining cultivars showed faster, slower and the same rate of development as the bud selfed parent depending on the female parent used.

6.3 Discussion

6.3.1 Fertilisation and ovule development.

Within each interspecific cross-combination which was examined for pollen tube penetration, the varied lengths of gynoecium represented different stages of maturity. It was observed that there was more pollen tube penetration with the smaller immature buds, but this did not result in an increase in the number of fertilised ovules. It has been found in B.campestris, that pollen tubes which show penetration of the stigma in very small buds often grow beyond the ovules to the base of the ovary (Allee & Mutshler, 1983). In the cross combination B.campestris cv Ponda x R.sativus cv Crail, no chemotactic response to the ovules was noted (Figure (6.1). An example of chemotactic response is shown after bud self pollination of R.sativus cv Slobolt, three days after pollination in Figure 6.2). Pollen tubes may penetrate the stigma regardless of the stigma age, but most or all of them are stopped in the style, depending on the cross and the length of the gynoecium. Some of the combinations show no penetration, but in some cases this may have been due to the small sample size. For example, Ruta Otofte x Crail showed no pollen penetration, but examination of the siliques at a later date showed early abortion of the ovules. There is no obvious connection between the number of pollen tubes penetrating the stigma and the number of ovules that are fertilised. In B.napus, pollen tubes more than ten times the number of ovules penetrated the stigma, but it did not lead to a correspondly high percentage of fertilised ovules.

Figure 6.1 A squashed ovary of R.sativus cv Slobolt three days after bud self pollination. A chemotactic response between the ovule and pollen tube is shown.

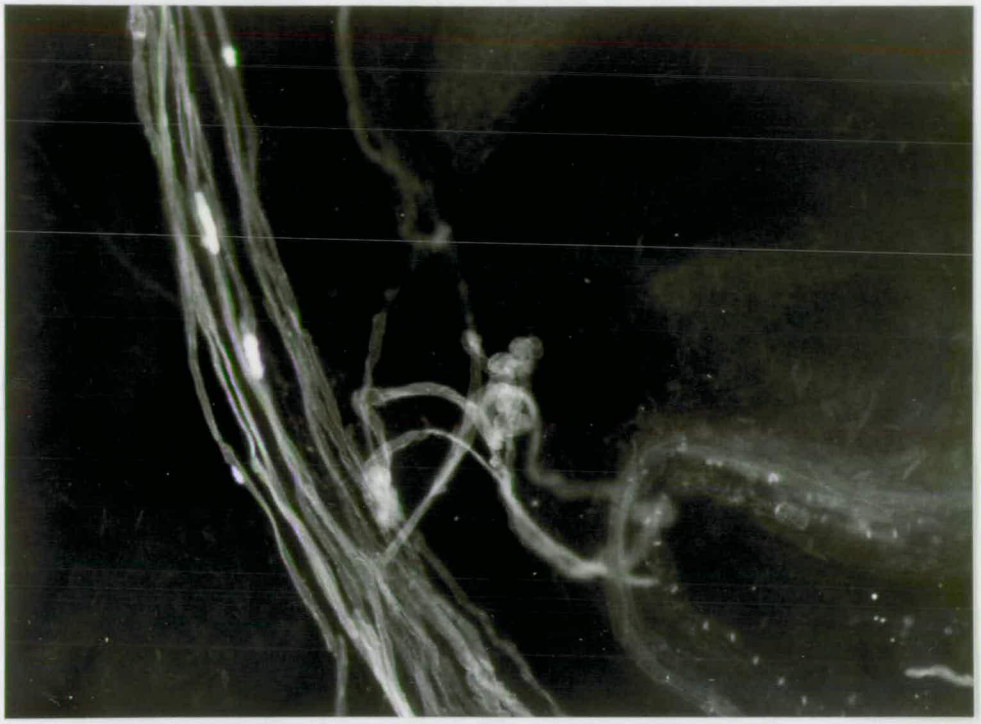
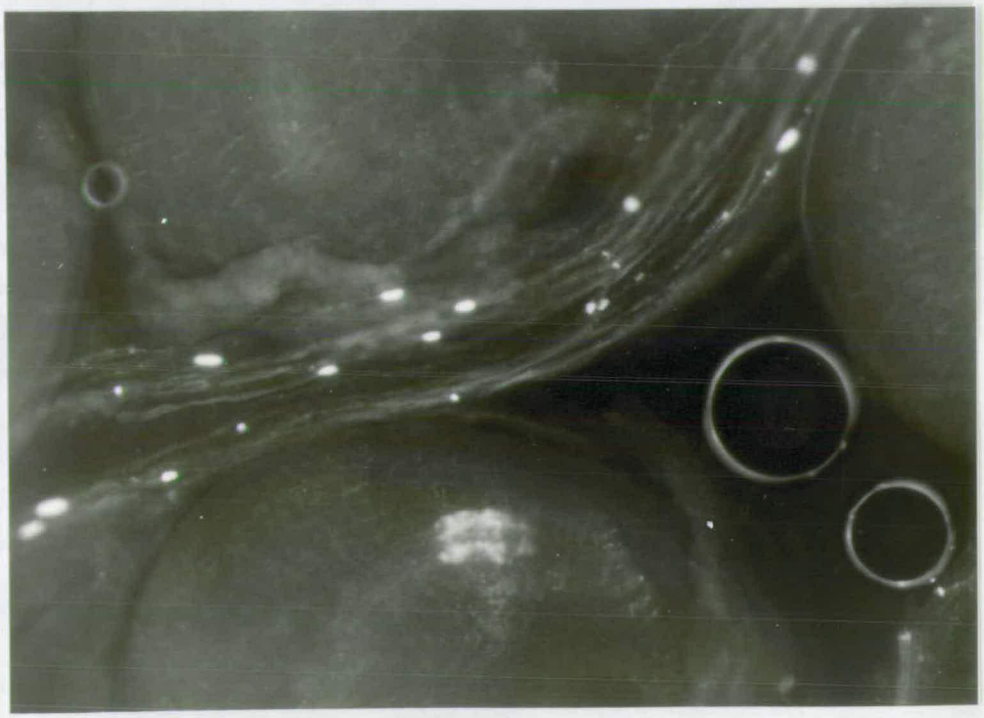


Figure 6.2 A squashed ovary of B.campestris cv Ponda one day after pollination with R.sativus cv Slobolt. The pollen tubes show no attraction to the ovules and grow to the bottom of the ovary.



All crosses, except some that were pollinated using R. sativus, showed penetration of pollen as far as the ovary and fertilisation of at least some ovules. With most of the cultivars, fewer ovules were fertilised after interspecific pollinations when compared to the percentage fertilised after bud self pollinations. In many interspecific crosses, most of the ovules which were developing had aborted by the final sample day. The speed of change from developing to aborted depends on the specific combination of parents, as does the percentage of aborted seeds. There did not appear to be any relationship between the number of pollen tubes penetrating the style and the number of ovules that eventually aborted. Lair x Ponda showed good stigma penetration and a high percentage of fertilised ovules. Most ovules from this cross developed to produce mature embryos, and the percentage of abortion was low. In contrast, Lair x Slobolt also showed good stigma penetration, but had a low percentage of fertilised ovules. This cross showed a higher proportion of aborted ovules and the most mature stage of embryo development reached was the heart stage. Ploidy effects were noted with respect to the percentage of aborted ovules. Crosses made with diploid B. oleracea and R. sativus showed a higher percentage of aborted ovules when compared to their respective tetraploids, considered as either male or female parents. Reciprocal effects were also evident. As female parents Lair had more aborted ovules than Ruta Otofte. On the other hand, Ruta Otofte as a male parent had more aborted ovules than Lair.

Between the different Brassica species, the larger gynoecia tended to contain more ovules than the shorter ones. R. sativus had a long gynoecium, which contained fewer, but larger, ovules. The gynoecium has a very long style, which contributed to its length. Overall the distribution of developing ovules shows equal numbers between the

apical and basal halves of the silique, but much fewer in total than would be obtained after bud self pollination.

Some specific combinations showed non-random distribution of ovules. The combinations Ponda x Maris Kestrel (2x), Ponda x Ruta Otofte and Lair x Ruta Otofte showed a difference of greater than 1.5 ovules between the two halves of the silique. Most ovules were found in the basal half. Under normal pollination conditions, the pollen tubes can expect to penetrate a greater distance before reaching the ovary than is found with Ponda as the female parent. It is more difficult to understand why there is such a difference between the two B.napus cultivars, Lair x Ruta Otofte. Perhaps there is no attraction of pollen tubes to the ovules and fertilisation in the lower half of the silique is teleological with respect to avoiding wastage of the pollen reserves. Taronda x Slobolt or Maris Kestrel (2x) also showed more than 1.5 ovules difference, but in this case with a bias towards the apical region of the silique. Maris Kestrel (2x) x Ruta Otofte is a long by short gynoecuim combination and limited pollen growth may explain why more fertilised ovules are found in the upper region of the fruit. Taronda x Slobolt is however more difficult to explain. Normally, pollen tubes of Slobolt have to travel down a long style before meeting any ovules, so therefore more fertilised ovules might be expected in the basal section of the silique.

6.3.2 The pattern of ovule abortion.

Over all cross combinations the percentage of aborted ovules ranged from 5.8% to 31.8%. The minimum percentage of aborted ovules was found when B.napus cv Ruta Otofte was used as the female parent. This cultivar had the highest percentage of unfertilised ovules (82.5%). The low percentage of fertilisation and low percentage of abortion

indicates pre-zygotic crossing barriers. These barriers are not exclusive to interspecific crosses as shown by less than 100% fertilisation after bud self pollination.

The maximum percentage of abortion was found when B.oleracea cv Maris Kestrel (2x) was used as a male parent. This cultivar almost had the lowest percentage of unfertilised ovules (54.2%). The higher abortion rates in some hybrid combinations, especially those with a higher fertilisation rate, in comparison with bud self pollinations, shows there is also a significant post zygotic effect. Indeed, where pollination is reasonably effective, abortion, or post zygotic incompatibility, may be, or even is the major cause of failure to produce hybrid seed. A most extreme example of this type of incompatibility is found in Theobroma cacao (Bouharmost, 1960 and Cope, 1962), where the incompatibility reaction is not initiated until after fertilisation. This incompatibility system depends on a threshold value of aborted ovules. Once this level is reached, the flower bearing them is usually shed from the plant.

Ploidy effects were observed with regard to the percentage of aborted ovules. As female and male parents B.oleracea and R.sativus showed an increase in the percentage of aborted ovules in the diploid cultivar than in their tetraploid equivalent. Perhaps the higher ploidy level is more tolerant of the introduction to new genomes. Reciprocal effects were also noted. As the male parent Ruta Otofte had a higher percentage of aborted ovules than Lair. As female parents the opposite was observed: Lair had a higher percentage of aborted ovules. This result may be due to the cytoplasmic or genetic effect. Both have the same genomic constitution, although they are both different plant types.

The pattern of ovule abortion with time after different combinations

of interspecific hybridisation can be partitioned into four types:

(i) In the early stages of development, all the fertilised ovules were developing normally, but with time a small percentage abort. The remainder develop successfully.

(ii) Abortion occurs fairly early in development, with time the percentage abortion increases, and only a few embryos develop normally to maturity.

(iii) Sporadic abortion may be present, but most of the fertilised ovules continue to develop.

(iv) There is no recorded abortion and all the fertilised ovules develop to maturity.

Of these four categories, the first was found to be most common in the hybrid crosses examined. The combinations found in this category include Ponda x Lair, Ponda x Ruta Otofte, Lair x Ponda, Lair x Ruta Otofte, Ruta Otofte x Ponda, Slobolt x Maris Kestrel (2x), and Crail x Slobolt. The second category included Taronda x Lair, Lair x Taronda and Maris Kestrel (2x) x Maris Kestrel (4x). The reciprocal cross Maris Kestrel (4x) x Maris Kestrel (2x) was found to be of category (iii), and the intra-specific combination, Ruta Otofte x Lair was of category (iv).

Table 6.18 gives a summary of the stages at which embryo abortion occurs in the various cross combinations. All the species show a range of embryo abortion. A predominant block effect can be seen from the table. Abortion in crosses between cultivars within species occurs in the later stages of embryo development. Early abortion was noted mostly in crosses between cultivars from different species. Combinations with B.napus as the female parent showed least abortion.

Table 6.18 The pattern of embryo abortion after hybrid combinations. Embryo abortion is categorised as: (i) cessation soon after beginning; (ii) development to LH stage; (iii) abortion beyond the PH stage and M mature embryos observed. 0 indicates no embryo development and # indicates a missing datum value.

	Ponda	Taronda	MK.(2x)	MK.(4x)	Lair	Ru.Ot	Slobolt	Crail
Ponda		iii	i	i	M	M	i	0
Taronda	ii		i	i	M	iii	i	0
M.Kestrel (2x)	iii	i		M	i	i	ii	0
M.Kestrel (4x)	ii	i	M		ii	iii	0	0
Lair	M	M	i	i		M	ii	0
Ruta Otofte	M	iii	0	i	M		iii	M
Slobolt	i	i	M	i	iii	ii		iii
Crail	#	i	i	i	iii	ii	M	

This may be due to the fact that it is already the produce of a selected successful interspecific cross. B.napus was found to be equally successful as a male parent when crossed with B.campestris. The mature embryos found with this combination frequently showed premature germination (phenomenon discussed in Chapter 3). With Raphanus as female in the intergeneric crosses, most combinations showed early abortion. Embryo development to the later stages was attained when crossed with B.napus. As a male parent Slobolt (2x) was most successful with B.napus, where abortion was fairly late in the development of the embryo. With Crail (4x) as a male parent however, virtually no embryo development was observed. Most combinations with Crail as a male parent showed germination of the pollen and penetration of the stigma, but no fertilisation of the ovules. This cultivar is therefore exhibiting a strong pre-zygotic hybridisation barrier.

6.3.3 Embryo and endosperm development.

Embryo and endosperm are closely linked. Those crosses which produced mature embryos show the normal stages of endosperm development. Coenocytic endosperm was maintained in those interspecific crosses where embryo development is stopped before reaching maturity. Taronda x Maris Kestrel (4x) and Taronda x Slobolt are the two exceptions where the endosperm becomes cellular. In the former, the embryo aborted early and the quantity of endosperm was much reduced. This indicates that the development of the embryo is out of step with the endosperm. In the latter combination early abortion was also noted. In some of the interspecific crosses, only a very small amount of coenocytic endosperm was present. The embryo sac was poorly defined and much reduced in size. Proliferation of nucellar tissues producing a small embryo sac, or often occluding the embryo sac, has also been

observed by other workers (Cooper & Brink, 1940; Nishiyama & Yabuno, 1979). Reduced embryo sacs occurred in combinations which had a low percentage of fertilised ovules.

6.3.4 Starting time and rate of development of hybrid embryos.

It was shown in Chapter 5 that the different cultivars did not all start embryo development at the same time. The delay period between pollination and embryo development first being detected could be considerable, as for example the 18 to 20 days in B.oleracea and R.sativus cv Slobolt. Of the interspecific combinations which produced mature embryos, just over 50% had parents with the same starting time of development. The rate of development of the hybrid embryos was compared to the rate of embryo development in both parents. Rates differed in many of the successful combinations, thus a similar rate of embryo development in the two parents was of lesser importance than a similar starting time for successful embryo development. Of the 13 crosses which produced mature embryos, there were 5 pairs of reciprocal crosses, the parents obviously showing good specific combining ability.

In those crosses which have early abortion of the embryo, the parents had widely different development starting times and rates of development. Again many of such combinations were reciprocal crosses. However, two exceptions to this fairly general rule were found. The parent bud selfs of Slobolt x Maris Kestrel (4x) and Crail x Taronda have the same starting time of development, but continue development at different rates. The interspecific combinations however show early abortion and the reciprocal crosses of the former cross showed no embryo development at all.

When embryo development is present the rate of development (ie. the time taken from beginning development until abortion occurs) of the resulting hybrid varies between the different cultivars. There does not appear to be an obvious connection between the percentage of developing ovules or the stage at which development is aborted.

It has been suggested that the failure of many hybrids to develop may be a result of disparity between the mitotic cycle times of the parents (Bennett, Finch & Barclay, 1976); Bennett & Kaltsikes, 1973 and Gustafson & Bennett, 1976). Forster & Dale (1983) concluded that the development of hybrid embryos between barley and rye depends on the early stages of endosperm development, and that mitotic rates in parental endosperms are more important than in embryos. Information on mitotic cycle times in not available for Brassica but the DNA content of the different species is (Verma & Rees, 1974 and Bennett, 1976). Ramsay & Pickersgill (1985) observed that the ranking of the embryo development after crosses with three Vicia species, coincided with the ranking of nucleic DNA content. They did stress however that the difference in maximum embryo development was minimal.

The DNA content of a species may have an influence on the cell cycle time (Bennett, 1972). If this is so, then hybrids between parents of similar DNA contents may be more stable (Cubero, 1982). The 2C DNA contents of the species used in this investigation are: B.campestris 1.64pg, B.oleracea 1.81pg, B.napus 3.21pg (Verma & Rees, 1974) and R.sativus 5.00pg (Bennett & Smith, 1976). B.campestris hybridises well with B.napus but not with B.oleracea or R.sativus. B.campestris and B.oleracea have very similar DNA contents, but do not hybridise readily. Therefore other incompatibility factors must be operating.

When looking at a cultivar overall as a male and female parent, the rate at which the hybrid combinations develop can be divided into four

categories. They can (i) all develop at the same rate, (ii) develop at the same rate as, or faster than, the parents, (iii) develop at the same rate as, or slower than, the parents, or (iv) be a mixture of slower, faster and the same as the two parents. The behaviour is different according to whether a cultivar is used as a male or a female parent. As female parents, category (i) contains Lair, (ii) contains Ruta Otofte and Slobolt (iii) contains Maris Kestrel (2x) and Crail, while category (iv) contains Ponda, Taronda and Maris Kestrel (2x). As male parents category (i) contains Crail, (ii) includes Ponda, Ruta Otofte and Slobolt, (iii) contains Maris Kestrel (4x) while category (iv) includes Taronda, Maris Kestrel (2x) and Lair. This type of information would be important for anticipating the correct time for embryo excision if embryos are to be cultured.

In order to make quantitative comparisons of performance of hybrid embryo development with bud self pollinations, the length of the developing embryos were measured at a number of time intervals after pollination. The results of these measurements are given and discussed in Chapter 7.

6.4 Conclusions

The first observation to be made when making interspecific crosses is whether or not the pollen grains germinate on the style and penetrate the stigma. Most of the combinations examined showed pollen tube penetration of the stigma, but most of the pollen tubes were stopped in the style. Excessive loading does not therefore lead to an increased percentage of fertilisation. In order to increase the percentage of developing ovules, it may be necessary to excise and culture the ovules, applying pollen directly onto them. It was originally thought that the different lengths of gynoecium found in

the different species may affect the percentage of fertilised ovules by showing a bias to either the top or bottom half of the silique. Although overall the bottom half of the silique was shown to contain significantly more developing ovules, on inspection the effect was evident in only four of the crosses.

Examination of the percentage of developing ovules and the percentage of unfertilised ovules showed significant differences between the four species, examined, both as maternal and paternal parents. Species which were found to be productive when used as a female parent were not necessarily productive as a male parent. R.sativus proved to be a good maternal parent and B.oleraçea to be a bad maternal parent. As pollen parents, B.napus was found to produce the best results while R.sativus the worst of the species examined. It was found that increasing the fertilisation percentage did not necessarily increase hybrid production. Increased fertilisation percentage resulted in an increase in abortion. The successful cross combinations of an interspecific hybrid depends on the specific combining ability of the chosen parents as well as the general combining ability of both parents.

The distribution of aborted ovules could be categorised into four sections.

(i) In the early stages all the fertilised ovules develop normally, but with time a small percentage abort while the remainder develop to maturity

(ii) Abortion occurs fairly early in development but with time there was an increase in the percentage abortion, while only a few develop to maturity.

(iii) Sporadic abortion while most of the fertilised ovules continue

to develop.

(iv) No recorded abortion where all the ovules which are fertilised, develop to maturity.

The first category was found to be most common.

All the parents showed a range of the stage of embryo abortion, depending on the cross. The cultivars of a species often showed the same result, producing a block effect on the grid or general combining ability. B.napus was shown to be successful both as a male and female parent. The amphidiploid, itself a successful interspecific combination, has the genomes of both B.campestris and B.oleracea. This common feature was probably responsible for its success. The buffering effect of increased ploidy was perhaps also involved in the limited success of B.napus and R.sativus combinations. With respect to intergeneric crosses, R.sativus was most successful as a female parent. It however showed poor performance as a pollen parent.

"Good" combinations of parents produced mature hybrid embryos and endosperm. Incompatible combinations often showed occlusion of the embryo sac, leaving no room for embryo development; if embryo development is begun, early abortion soon follows. Synchrony of commencement of embryo development may be important in improving the number of successful hybrid combinations. The rate of embryo development would appear to be of lesser importance.

Evidence collected in this chapter shows that both pre-zygotic and post-zygotic barriers to hybridisation operate in these crosses.

CHAPTER 7

OBSERVATIONS ON THE FINAL STAGES OF EMBRYO DEVELOPMENT AFTER INTERSPECIFIC POLLINATION OF *BRASSICA CAMPESTRIS*, *B. OLERACEA*, *B. NAPUS* AND *R. SATIVUS*.

7.1 Introduction.

The development of ovules after interspecific hybridisation and their distribution along the silique was examined in detail in the previous chapter. In order to make quantitative comparisons of the developing embryos, the embryo length for the final stage of development reached in each cross combination (detailed in Chapter 6) was measured, and from this, the mean length was then estimated. This was then compared to the mean embryo length for the same stage of development after bud self pollination of both male and female parents. In those crosses where mature embryos were found, the mean ovule length of the hybrid and parents was also compared. Table 7.1 summarises the results found in Chapter 5 and Chapter 6.

7.2 Results.

The natural logarithm of the mean embryo length for the final stage of embryo development for each of the interspecific cross combinations is shown in Table 7.2. This table also presents the mean embryo length of the comparable stage of development after bud self pollination of the female parent. Figure 7.1 graphically presents the hybrid embryo length plotted against the length of the female parent at the final stage of development reached by the hybrid. The correlation between the embryo length of the female parent and that of the hybrid was highly significant ($r=0.95$, $p<0.001$) however, four distinct groups were found. On examination these groups correspond to the four stages of development - (i) globular, (ii) pre-heart/heart, (iii) torpedo and (iv) mature. This effect is consistent with the results found in Chapter 6, where it was suggested that much of the embryo abortion is a failure to proceed through all the stages of development. The results found here would indicate that these four stages are the most

Table 7.1 A summary of ~~when~~ embryo development showing the number of days after pollination when embryos were first detected, the number of days after pollination, when abortion occurred (or maturity was reached) and the rate of development of any resulting hybrid, as greater than (>>>), less than (<<<) or equal to (---) the parents. The cells are coded according to the stage of development when abortion occured. ● = soon after development began, △ = late globular to pre-heart stage, □ = heart stage and later and ◇ = mature embryos.

	Ponda	Taronda	MK.(2x)	MK.(4x)	Lair	Ru.Ot	Slobolt	Crail
Ponda		12:16 □ ---	14:18 ● <<<	14:14 ● <<<	12:40 ◇ <<<	10:28 ◇ >>>	12:14 ● ---	0:0
Taronda	12:18 △ ---		10:10 ● ---	14:20 ● <<<	10:36 ◇ ---	10:14 □ >>>	10:10 ● ---	0:0
M.Kestrel (2x)	20:36 □ ---	24:28 ● <<<		20:36 ◇ ---	20:22 ● ---	20:24 ● ---	20:20 △ ---	0:0
M.Kestrel (4x)	20:28 △ ---	24:24 ● <<<	14:32 ◇ >>>		19:19 ● ---	16:24 □ >>>	0:0	0:0
Lair	10:30 ◇ ---	10:20 □ ---	10:10 ● ---	10:10 ● ---		6:36 ◇ ---	12:16 △ ---	0:0
Ruta Otofte	14:32 ◇ >>>	14:28 □ ---	0:0	16:20 ● ---	10:24 ◇ >>>		16:20 □ >>>	16:16 ● >>>
Slobolt	14:20 ● ---	14:24 ● >>>	20:32 ◇ ---	14:18 ● ---	18:30 □ ---	12:16 △ >>>		10:16 □ >>>
Crail	*	16:18 ● <<<	12:16 ● ---	12:16 ● ---	14:28 □ <<<	14:28 △ ---	10:35 ◇ >>>	

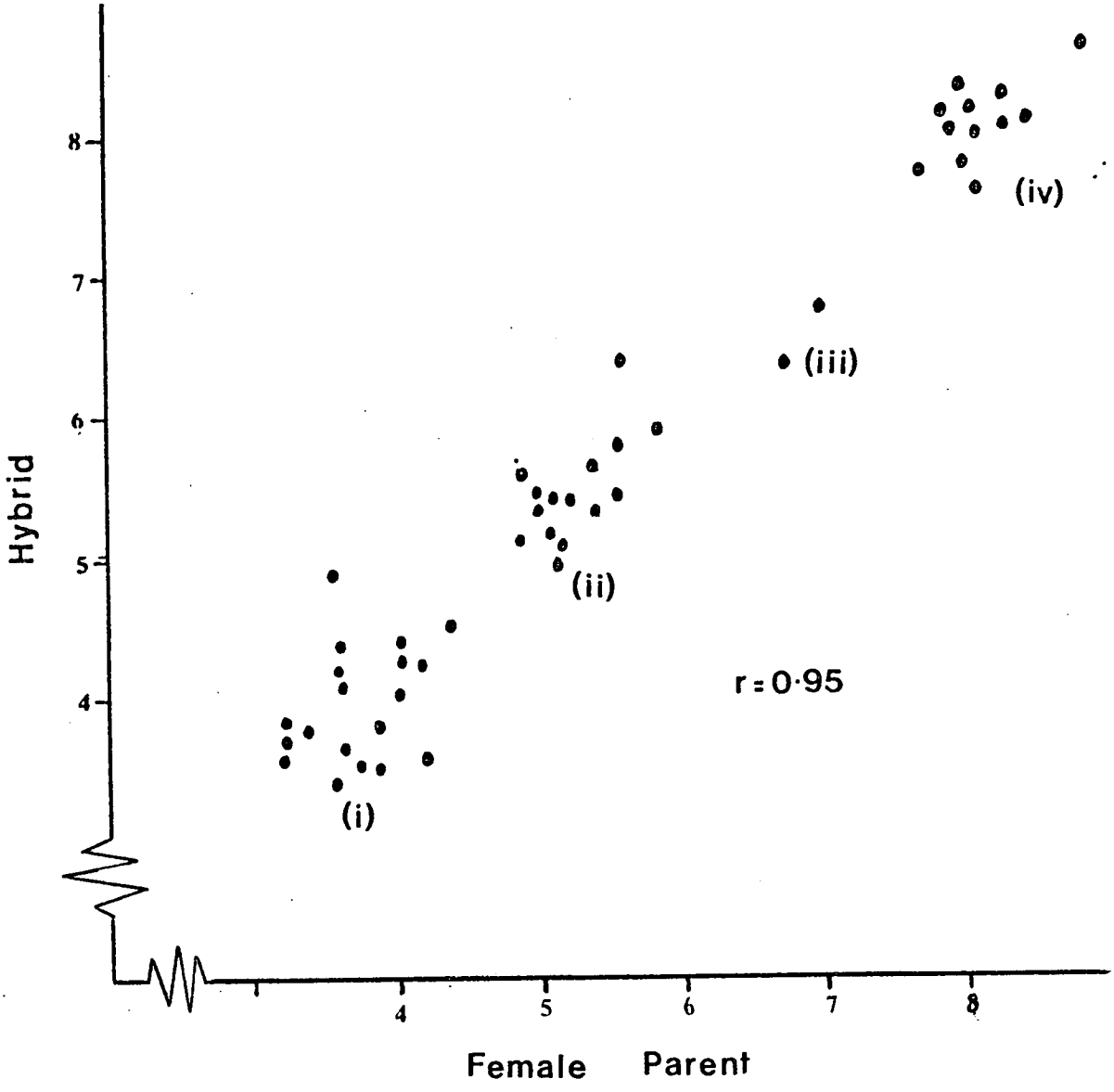
* indicates missing data.

Table 7.2 The natural logarithm of the mean embryo length for the final stage of embryo development recorded after interspecific pollination (H), compared to the embryo length at the equivalent stage of development after bud self pollination of the female parent (F). Combinations which had no embryo development are shown by an asterisk.

		Ponda	Taronda	MK.(2x)	MK.(4x)	Lair	Ru.Ot	Slobolt	Crail
Ponda	H	-	4.96	3.66	4.12	8.08	8.33	4.38	*
	F	-	5.17	3.65	3.65	7.92	8.00	3.65	*
Taronda	H	5.09	-	3.57	4.40	8.16	6.36	4.02	*
	F	4.91	-	3.32	4.04	8.04	6.74	4.04	*
M.Kestrel (2x)	H	5.75	4.23	-	7.60	3.53	3.75	5.14	*
	F	5.58	4.24	-	8.09	4.24	3.40	5.11	*
M.Kestrel (4x)	H	5.40	3.56	7.79	-	*	6.75	*	*
	F	5.23	3.74	8.02	-	*	7.00	*	*
Lair	H	8.07	5.35	3.86	3.73	-	8.21	5.36	*
	F	8.30	5.38	3.27	3.27	-	8.30	5.02	*
Ruta Otofte	H	8.01	5.38	*	4.10	8.10	-	5.34	7.65
	F	8.10	<5.05	*	3.66	7.90	-	<5.05	7.69
Slobolt	H	4.31	3.44	8.09	4.97	6.39	4.91	-	5.43
	F	4.07	3.60	8.43	3.60	>5.57	5.57	-	5.57
Crail	H	*	4.48	3.53	3.85	5.87	5.12	8.63	-
	F	*	4.38	3.92	3.92	5.86	5.18	8.89	-

If comparable stages of development is not available then the length is presented as greater (>) or less (<) than the nearest stage.

Figure 7.1 The natural logarithm of the mean embryo length at the final stage of embryo development recorded after interspecific pollination, plotted against the equivalent stage of development after bud self pollination of the female parent.



critical, although the torpedo stage seems to be of lesser importance. The distribution of the points within each cluster is fairly even. Overall however there were 27 hybrids which produced embryos that were longer than the maternal parent, four hybrids gave embryos of the same length as the female parent and 17 hybrids were shorter than the maternal parent. Similar results were found when the length of the hybrid embryo was plotted against the length of the male parent at the equivelant stage of development. Table 7.3 presents the final stage of embryo development, and whether the length of the embryo at that stage is the same, longer or shorter than either or both of the parents.

There does not appear to be a consistent effect associated with any of the cultivars. The length of the hybrid embryo may be longer, shorter or approximately the same length as the parent cultivar, depending on the interspecific cross combination that is considered. The difference between the length of the hybrid embryo, compared with its parents, does not appear to affect successful hybrid development. Mature embryos have been found which are longer (Ponda x Ruta Otofte) or shorter (Slobolt x Maris Kestrel (2x)) than both parents. Reciprocal crosses of cultivars within a species may produce different results, for example, B.campestris, or they may show the same result, for example, B.oleracea.

The mean length of developing ovules which contained mature embryos was also calculated and compared to the ovule length of both parents (Table 7.4). The ovule length of the hybrid may be longer than either parent (Ponda x Ruta Otofte), shorter than both parents (Maris Kestrel (2x) x Maris Kestrel (4x)), or lie somewhere between the lengths of the two parents (Slobolt x Maris Kestrel (2x)). The ovule length achieved after reciprocal crosses may be similar (between Ponda and

Table 7.3 The final stage of embryo development recorded after each interspecific pollination, and if the resulting embryo length is the same (----), longer (>>>>) or shorter (<<<<) than either parent in the cross. If the hybrid is longer than the female parent but shorter than the male parent >>>< will appear in the table, if the hybrid embryo is the same length as the male parent but shorter than the female parent <<-- will appear in the table, etc..

	Ponda	Taronda	MK.(2x)	MK.(4x)	Lair	Ru.Ot	Slobolt	Crail
Ponda		H <<<<	EG ----	EG >>>>	MII >>><	MIII >>>>	GE >>>>	* .
Taronda	PH >>>>		EG >>><	G >>>>	MIII >>><	T <<<<	G ----	*
M.Kestrel (2x)	H >>>>	G >>><		MIII <<<<	G >>>>	EG >>>>	PH ----	*
M.Kestrel (4x)	PH <<>>	EG >>><	MIII <<<<		*	T <<<<	*	*
Lair	MIII >>><	H ----	EG >>><	EG >>><		MIII ----	PH >>><	*
Ruta Otofte	MIII >>><	H ----	*	EG <<<<	MII >>><		H <<>>	MI <<>>
Slobolt	G >>><	EG <<<<	MI <<<<	EG >>>>	H >>>>	PH <<>>		H <<<<
Crail	*	G >>>>	EG >>><	EG >>><	H <<>>	PH <<>>	MIII <<<<	

Table 7.4 The mean length of ovules (mm) in the hybrid cross combinations which produced mature embryos and the mean embryo lengths of the two parents used in the hybrid cross.

Parent 1	Length	Parent 2	Length	Hybrid 1x2	Hybrid 2x1
s.e.					
Ponda	2.02	R.Otofte	2.15	2.68	2.02
0.167					
Ponda	2.02	Lair	2.50	2.29	2.25.
0.175					
Lair	2.50	R.Otofte	2.15	2.57	2.33
0.142					
MK(2x)	2.87	MK(4x)	2.60	2.02	2.19
0.447					
Taronda	2.28	Lair	2.50	1.95	-
0.130					
Slobolt	3.93	MK(2x)	2.87	3.38	-
0.284					
Crail	4.93	Slobolt	3.93	3.56	-
0.185					

Lair), or they may show variation (between Ponda and Ruta Otofte). All of the cross combinations which produced mature embryos did not necessarily cross reciprocally.

7.3 Discussion.

Crosses between some of the species show a predominant block effect, where all the cultivars abort at approximately the same stage of development, for example, B.campestris x B.oleracea and B.napus x B.oleracea. The length of the embryo does not show this effect. In the cross B.campestris x B.oleracea, the embryos abort at the early globular and globular stages of development. The embryo length achieved may be longer or the same as both parents, or longer than the female parent (Table 7.3). This indiscriminate pattern is apparent over all the cross combinations. It is therefore difficult to assert a pattern of inheritance of embryo length since it appears to depend on the specific combining ability, rather than general combining ability of the parent cultivars.

When Table 7.3 and Table 7.4 are compared a general pattern emerges. The length of the mature hybrid embryos in most cases corresponds to the length of the mature ovules, before desiccation begins. Those mature hybrid embryos which are longer than their parents coincide with mature ovules which are also longer that the parent cultivars. The converse also appears to happen, the smaller hybrid embryos are associated with smaller ovules. The exception to this generalisation is the cross, Taronda x Lair. In this example, the hybrid ovule is smaller than either parent, but the hybrid embryo is larger than the female parent although smaller than the male parent.

The difference in length of hybrid embryos may be due to differences in cell size or cell number, or both. Differences in ovule length may

also be due to proliferation of integument tissue. The length of embryos from some combinations has been measured in the four and eight cell stages of development. These were compared to the parents, to test the theory of different cell sizes.

Hybrid embryos from the cross Maris Kestrel (2x) x Maris Kestrel (4x) measured 22μm long at the four cell stage. The comparable stage of Maris Kestrel (2x) measured 33μm. The mature embryos and ovules from this hybrid combination were both shorter than the female parent. The four celled embryo after bud self pollination of Lair measured 34μm. The hybrid embryo from the cross Lair x Ruta Otofte measured 31μm. In this cross the final length of the mature embryo and ovules of the hybrids were approximately the same as the parents. After bud self pollination of Ruta Otofte the eight celled embryo measured 44μm. The cross Ruta Otofte x Lair measured 59μm at the eight celled stage. Both the mature embryos and ovules were longer in the hybrid than in the bud self of the female parent. The cross combination Ruta Otofte x Ponda gave embryos of 35μm in length at the eight celled stage. The mature embryo was also shorter in length than the female parent at the same stage of development. These examples all suggest that many of the differences in embryo length may be due, at least in part, to a difference in cell size.

However, differences in cell number cannot be overlooked. The four cell embryo stage of Taronda, bud self pollinated, measured 26μm. Embryos from the interspecific cross combination of Taronda x Lair also measured 26μm at the four cell stage. The final length of the mature hybrid embryo was longer than the female parent and the ovule length was shorter than either parent. This would suggest that a difference in cell number of the hybrid embryos and ovules compared to the parent cultivars is possible. It is clear that the final stage of

development and embryo length is showing a degree of specific combining ability and hence it is determined by the specific combination of parents. The cultivars should be considered as both maternal and paternal parents, since different results are often found, with no obvious pattern as to whether a cross or its reciprocal, will yield the better results. In some of the cross combinations, overdominance is evident, where the mature hybrid embryo is larger than either bud selfed parent.

Parents of different genotypes pass on their genes unequally to the next generation (Falconer, 1969). Selection acts to determine the fitness of the individual, which may be shown in the phenotype. Overdominance as shown here with some of the resulting hybrids producing embryos larger than either of the parents. Incomplete dominance is exhibited where the embryos are smaller than either parent. The size of the embryos is not a function of either the male or female parent but it is a result of the combined genetic material.

The critical stages of hybrid embryo development were highlighted in Figure 7.1. Failure of the developing embryo to proceed from globular to the heart stage of development may be related to endosperm development. By the heart stage of development the endosperm has normally become cellular. Many biochemical changes are also taking place at this time (detailed in Chapter 1). These included an increase in the storage protein, starch, RNA and DNA. By the time the embryo has reached the pre-heart/heart stage of development it is green in colour. Perhaps some of the failure is a result of post-zygotic incompatibility at the subcellular level. The green colour of the embryos is directly related to the presence and function of the chloroplasts. But transmission of chloroplasts involves nuclear gene recombination, therefore incompatibility of the

chloroplast genomes may be involved in failure at this crucial step. Also mitochondrial DNA may show some recombination where the resulting mt DNA may be similar but not identical to either parent (Galum, 1982).

To proceed from the heart stage to the torpedo or mature stage involves much differentiation and expansion of the cells. Utilisation of products stored in the earlier part of development occurs during normal development. Failure to complete any of these changes and functions appears to be lethal.

Chapters 6 and 7 have been discussed in terms of the reproductive biology of the different species and their products after hybridisation. The following chapter attempts to explain these results from a genomic view.

7.4 Conclusions

The size of the embryo resulting from a cross pollination is not genetically predetermined by the embryo size produced in the parents after bud self pollination. Differences in cell size, cell number or a combination of size and number, may be contributing factors to differences between the lengths of the hybrid and parental ovules and embryos. Overdominance may be present, resulting in embryos which are larger than either parent.

The two most critical stages of embryo development were highlighted. Transition from globular to heart stages may be associated with failure to accumulate many of the products required for complete embryogenesis, or perhaps related to incompatibility of the chloroplast genomes. Failure to complete development from the heart stage may be related to failure of further differentiation and

expansion of cells in the embryo, or the utilisation of the products stored in the earlier stages of development.

Some combinations may produce mature seed, but developing F_1 may be sterile. The fertility of those embryos which developed to maturity was not examined.

CHAPTER 8

THE INFLUENCE OF GENOME RATIO, NUMBER AND COMPOSITION ON DEVELOPMENT OF EMBRYO AND ENDOSPERM AFTER CROSS-POLLINATION BETWEEN *BRASSICA CAMPESTRIS*, *B.OLERACEA*, *B.NAPUS* AND *RAPHANUS SATIVUS*

8.1 Introduction.

All the possible hybrid combinations of two cultivars from each of the four species B.campestris, B.oleracea, B.napus and R.sativus were made and the results for percentage of fertilised ovules, developing and aborted, their distribution within the silique and a measure of embryo length have been examined in the previous two chapters. This chapter aims to examine endosperm failure and embryo abortion and cytological abnormalities found in the hybrids in relation to the genomic combination.

In all of these cross combinations there are only three genomes - A, C and R. It is therefore possible to examine the effect of hybridity, genome number, ratio and composition and the tissue types of the embryo and endosperm in relation to their success in hybrid production. For example, the products from a AA x CC and a AAAA x CCCC cross combination will have the same genomic ratio and similar cytoplasm, but the ploidy level will be different in both the endosperm and the embryo. Comparisons of the combination AA x CC and the reciprocal cross CC x AA, has the same nuclear type, but different cytoplasmic backgrounds in the embryo. The endosperms differ in genomic ratio. Hybridisation of AACC x AA and AACC x CC, would produce embryos and endosperm which have the same cytoplasm and genome representation, but differ in genome ratio in both the embryo and endosperm.

No report has been found in the literature of an investigation of embryo and endosperm development after interspecific hybridisation in which possible genomic factors are separated in this way.

8.2 Discussion.

8.2.1 Examination of the genomic combination of hybrids.

Table 8.1 presents the expected embryo and endosperm genotype for each of the cross combinations. The table is colour coded to show the stage of embryo development reached before any embryo abortion was observed. Of the 65 hybrid combinations, 23% produced mature embryos, 12.5% showed no embryo development at all, while 62.5% showed some development, although not developing to the mature stages. From the latter group, 57% of the combinations ceased development soon after beginning, and 43% reached the late globular to heart stage of development, and could have perhaps been rescued by embryo culture techniques.

There was no evidence of a reciprocal effect (ie. both reciprocal combinations of a cross gave similar results) in the groups which produced either mature embryos or ceased development soon after it began. Thus 10 out of the 13 combinations producing mature seed belonged to five reciprocal pairs yielding similar results. They all contain the A and C genomes. The remaining three non-reciprocal combinations contained the R genome.

The group of 20 combinations which ceased development soon after it began, included six reciprocal pairs, ie. 12 of the combinations). Only one other reciprocal pair, (showing the same stage of development), was observed outwith these two groups. That was Taronda x Ruta Otofte, which developed to a heart shaped embryo before abortion occurred, which ever direction that the cross was made. The remaining sections, ie. development to the late globular to pre-heart stage, or no development at all, did not show consistent results for reciprocal crosses within a section. This result indicates that some of the cultivars combine better in one direction than in the other.

Table 8.1 The genotype of the embryo and endosperm of all possible combinations between *Brassica campestris*, *B.oleracea*, *B.napus* and *Raphanus sativus*.

	B.campestris		*B.oleracea*		*B.napus*		*R.sativus*	
	Ponda	Taronda	MK.(2x)	MK.(4x)	Lair	Ru.Ot	Slobolt	Crail
Ponda	AA	AAA	AC	ACC	AAC	AAC	AR	ARR
	aaa	aaaa	aac	aacc	aaac	aaac	aar	aarr
Taronda	AAA	AAAA	AAC	AACC	AAAC	AAAC	AAR	AARR
	aaaaa	aaaaaa	aaaac	aaaacc	aaaaac	aaaaac	aaaar	aaaarr
M.Kestrel (2x)	AC	AAC	CC	CCC	ACC	ACC	RC	RRC
	acc	aacc	ccc	cccc	accc	accc	rcc	rrcc
M.Kestrel (4x)	ACC	AACC	CCC	CCCC	ACCC	ACCC	RCC	RRCC
	acccc	aaccccc	ccccc	cccccc	acccccc	acccccc	rcccc	rrcccc
Lair	AAC	AAAC	ACC	ACCC	AACC	AACC	ARC	ARRC
	aaacc	aaaacc	aaccc	aacccc	aaaccc	aaaccc	aarcc	aarrcc
Ruta Otofte	AAC	AAAC	ACC	ACCC	AACC	AACC	ARC	ARRC
	aaacc	aaaacc	aaccc	aacccc	aaaccc	aaaccc	aarcc	aarrcc
Slobolt	AR	AAR	RC	RCC	ARC	ARC	RR	RRR
	arr	aarr	rrc	rrcc	arrc	arrc	rrr	rrrr
Crail	ARR	AARR	RRC	RRCC	ARRC	ARRC	RRR	RRRR
	arrrr	aarrrr	rrrrc	rrrrcc	arrrrc	arrrrc	rrrrr	rrrrrr

8.2.1.1 Combinations forming diploid embryos.

Various combinations of the A, C and R genomes were examined. The simplest of these, the diploid embryos, are considered first. The AC embryo was more successful, in that it achieved the heart stage of development, in an acc endosperm (cell 3.1), than in an aac endosperm (cell 1.3). The embryo is most successful in the maternal 'c' cytoplasm rather than the maternal 'a' cytoplasm. The AR hybrid combination showed early abortion of the embryo in both reciprocal crosses. The RC embryo continued development to produce mature embryos in an rrc endosperm (cell 7.3) and to the late globular to pre-heart stage in an rcc endosperm (cell 3.7). The successful combination was produced with 'r' cytoplasm rather than 'c' cytoplasm. Already the hybridity effect of the embryo is evident, also the direction of the cross, resulting in different genome ratios in the endosperm, and different cytoplasm may be important in supporting the developing embryo.

In general, those embryos which are developing with an 'r' egg cytoplasm do better than those with a 'c' cytoplasm. Those diploid embryos with an 'a' cytoplasm have the poorest development. There also appears to be an endosperm effect. The triploid endosperms which contain two 'r' genomes sustain hybrid embryo development better than those with two 'c' genomes, which in turn, is better than those with two 'a' genomes. Thus the endosperm and cytoplasmic effects are the same with regard to ranking the genomes according to success: the 'r' genome is best, the 'c' genome intermediate and the 'a' genome is poorest.

8.2.1.2 Combinations forming triploid embryos.

The triploid non-hybrid embryos, AAA, CCC and RRR, showed either late abortion or development to produce mature embryos supported in a tetraploid or pentaploid endosperm. Embryos with the A genome sustained development longest in a tetraploid endosperm, while the R genome were best in a pentaploid endosperm. Embryos with the C genome developed to maturity regardless of the endosperm ploidy.

Amongst the triploid hybrid embryos, the AAC hybrid produced mature embryos if the endosperm was tetraploid composed of an aaac in the 'a' cytoplasm (cells 1.5 and 1.6) or pentaploid an aaacc with an 'a' cytoplasm (cells 5.1 and 6.1). However, early abortion of the embryo was observed where the tetraploid endosperm consisted of aacc genomes (cell 3.2) or the pentaploid endosperm had aaaac genome (cell 2.3). The maternal cytoplasmic background was 'c' and 'ac' respectively. The ACC combination showed early abortion, or no development of embryos, regardless of the ploidy in the endosperm. The cytoplasmic background of 'a', 'c' and 'ac' were represented. The exception to this was in cell 4.1 where the embryo developed to the late globular to pre-heart stage in the pentaploid endosperm, acccc and 'cc' cytoplasm. The embryo combination of AAR showed early abortion regardless of endosperm ploidy and cytoplasmic background. Likewise, ARR showed no embryo development (cell 1.8), or it may have shown early abortion (cell 8.1) if development had began. The ARC hybrid combination produced varied results. A genetic effect between the two cultivars of B.napus was noted. The cultivars produced the same genomic ratios and cytoplasmic backgrounds but different results were observed. Embryo development to the late globular to pre-heart stage was observed in the cross shown in cell 5.7 and 7.6. Development to the heart stage was found in the cells 6.7 and 7.5.

Triploid hybrid embryos show a clear genomic effect. Those

combinations producing embryos with two A genomes are likely to show most development, especially if the tetraploid or pentaploid endosperm contains three 'a' genomes in an 'a' or 'ac' cytoplasm. Apart from one exception, all other cross combinations with two C genomes in the embryo showed no development or early abortion. The exception was cell 4.1, whose combination has three 'c' genomes in the endosperm and a 'cc' cytoplasm. All cross combinations with the R genome showed early abortion. With the triploid embryos, AA is better than A. A number of mature embryos were produced with the former in 'a' cytoplasm with three a's in tetraploid endosperm or in cytoplasm with three 'a' genomes in a pentaploid endosperm.

Hybrid embryo effect : A > C > R

Hybrid endosperm effect : 3 a's > 2 a's or 4 a's > c > r

Hybrid cytoplasmic effect : a ≥ ac > c ≥ ac > r

8.2.1.3 Combinations forming tetraploid embryos.

The various hybrid combinations producing tetraploid embryos were also compared. AACC showed early abortion with the hexaploid endosperms aaaacc (cell 2.4) or aacccc (cell 4.2) and 'aa' and 'cc' cytoplasm. Mature embryos were found with the balanced aaaccc endosperm and 'ac' egg cytoplasm (cells 5.6 and 6.5), The AAAC hybrid produced mature embryos in the endosperms aaaaac and egg cytoplasm 'aa' (cell 2.5) and aaaacc and 'ac' cytoplasm (cell 5.2). These embryos developed at the same rate as their parents. The crosses 2.6 and 6.2 produced embryos to the heart stage and later, but not mature embryos, in the endosperms aaaaac and aaaacc and cytoplasms 'aa' and 'ac' respectively. The embryos produced in cell 2.6 developed at a faster rate than 6.2, but development was not sustained for as long. The two pairs of reciprocal crosses, 2.5, 5.2, and 2.6, 6.2, have the same

genomic ratios, which would suggest that the differences observed between them is genetic, and a character of the parent cultivars. The ACCC combination shows the same genetic effect where 4.5 and 4.6 have the same endosperm ratio and 'cc' cytoplasm, but 4.5 aborts sooner than 4.6. The cross combinations 5.4 and 6.4 both abort early, each having the same genomic ratios of embryo and endosperm and 'ac' cytoplasm. The combinations with the faster rate of development (cell 4.6) showed a later stage of abortion. This was the opposite effect as found with the embryo AAAC.

The combination AARR showed early abortion with a Raphanus maternal parent cytoplasmic background 'rr' (cell 8.2) and no development in the reciprocal cross with the cytoplasmic background 'aa' (cell 2.8). Similarly, with the combination RRCC, early abortion when Raphanus was the female parent (cell 8.4) and no development when it was the male parent with 'c' cytoplasmic background (cell 4.8). The reciprocal combinations producing the hybrid ARRC again showed the genetic effect of B.napus. The embryo and endosperms showed the same ratios, but different results were obtained (cells 8.5, 8.6 and cells 5.8, 6.8).

Two patterns emerge from examination of the tetraploid embryos. There is a definite cytoplasmic effect where the 'r' genome is better than the 'a' genome, which in turn, is better than the 'c' genome. The cytoplasmic effect of the different genomes varied depending on the ploidy of the embryo. The two plant types of B.napus showed some genetic control, since hybrid embryo, endosperm and egg cytoplasm are the same, but the results are different.

8.2.1.4 Effect of genome number and ratio.

Examination of effects of differences in genome number, when the composition and the ratio are the same, showed that polyploids gave

results similar to those of the basic genome constitution. The AC unit showed early abortion (cell 1.3) of development of some embryos to the heart stage (cell 3.1). When produced artificially by the combination of B.oleracea and B.campestris, AACC embryos also showed early abortion (cell 2.4 and 4.2), although they obviously developed to maturity when associated with B.napus. The AR unit showed early abortion of embryos (cell 7.1 and 1.7). When doubled up, the AARR combination showed either early abortion (cell 8.2) or no effects of differences in embryo development at all (cell 2.8).

When the effect of differences in genomic ratio was examined, ie. when the genomic composition and ploidy of the embryo was the same, the results were similarly varied. There was a definite species effect rather than development being related to the genome. Similar effects were noted when genome composition, ie. when the genomic number is the same, was examined. For example, the AAC embryos produced by combining B.napus and B.campestris developed to maturity, regardless of endosperm, ploidy or composition. On the other hand, when AAC or ACC were produced using B.oleracea as a parent and early abortion of embryos was evident regardless of the endosperm composition (cells 3.2, 2.3 and 1.4). Similar conclusions were made when the AACC, AAAC and ACCC combinations were examined.

Combinations with the Raphanus genome, AAR, ARR, RRC and RCC often showed no embryo development at all when the R genome was introduced by the male parent and early abortion when Raphanus was the maternal parent. The ARC combination was slightly more successful with embryo development containing up to the late globular to pre-heart stage (cell 5.7 and 7.6) or some embryos developed to the heart stage (cells 6.7 and 7.5). All these combinations were with B.napus as one of the parents. Perhaps their limited success is due to the selected A and C

genomes already successfully combined in the AC hybrid delaying any adverse effect on the introduction of the R genome. Increasing the ploidy of the embryo did not increase the success of the cross. Similar results to those found for the triploid embryos were observed when the AARR, RRCC and ARRC combinations were examined. The latter combination, ARRC, included the AC genomes from B.napus and exhibited slightly improved success.

By comparing the same combinations in embryo and endosperm it was hoped to highlight any effect of endosperm on embryo environment or development. Embryos which had different genomes and endosperms with the same genome were compared. Two sets of comparisons were made, cells 1.4 with 3.2 and cells 4.2 with 5.4 and 6.4. All embryos showed early abortion. When the same embryo genomes were compared with different endosperms, variation was found in the pairs. Cell 3.1 was slightly more productive than cell 1.3 and cell 4.1 was slightly more productive than cell 1.4. Cells 3.5, 3.6, 5.3 and 6.3 all showed early abortion. Combinations of B.campestris and B.napus (cells 1.5, 1.6, 5.1 and 6.1) and between the B.napus cultivars (cells 5.6 and 6.5) produced mature seed. When embryos and endosperm with the same genomic composition were examined, some combinations functioned better as an embryo, while others were better as an endosperm. The combination ACC was more successful as an endosperm (cell 3.1) than an embryo (cell 1.4). The combinations of AAC all showed early abortion of endosperm or embryos (cells 1.3, 2.3 and 3.2).

8.2.1.5 General conclusions from the genomic factors.

Table 8.2 summarises the embryo, endosperm and egg cytoplasm effects on the development of diploid, triploid and tetraploid embryos with regard to different genomes. It is clear that the influence exerted by particular genomes depends on the ploidy of the embryo.

Table 8.2 Summary of the embryo, endosperm and egg cytoplasm effects on the development of diploid, triploid and tetraploid embryos with regard to the different genomes. The symbol X > Y denotes that X showed better development than Y. 'H' = hybrid embryos, 'NH' = non-hybrid (intraspecific) embryos.

Embryo ploidy	Embryo effect	Endosperm effect	Cytoplasmic effect
Diploid, 'H'	-	rr > cc > aa	r > c > a
Triploid, 'NH'	C > R > A	Pent.r > tetr.a	-
Triploid, 'H'	A > C > R	3a>2a or 4a>c>r	a>ac>c>ac>r
Tetraploid, 'H'			
AACC	-	3a+3c > 4a or 4c	ac > aa or cc
AAAC *	-	-	-
ACCC *	-	-	-
AR and RC	-	-	r > a = c
ARC *	-	-	r > a = c

Embryos with genomic formula marked with asterisk, (*), show similar development in reciprocal crosses. B.napus has a positive effect on the development of those hybrids.

Combinations with the two plant types of B.napus produced different results, where tetraploid embryos would have been formed, even though the endosperm and cytoplasm were the same.

There does not appear to be any advantageous effect of altering the genomic number. Similarly, examination of the effects of differences in genomic ratios revealed that any effect was more related to a species rather than to the genome. Some genomic combinations function better as an embryo while others functioned better as endosperms.

The same genomic ratios and number can be produced from various combinations. AC combinations produced from B.campestris and B.oleracea, and B.napus with B.oleracea show poor hybrid development. This poor crossability was also found by Chiang, Chiang & Grant (1977); Hakansson, (1956); Matsuzawa, 1983 and Sarashima, (1973). Combinations between B.campestris and B.napus are more successful, the diploid cultivar of B.campestris being better than the tetraploid, and often producing mature seed. The relative ease by which B.napus and B.campestris combine was also found by Chaing et.al., (1977) and McNaughton (1973). Nwakanti (1970) found that more seed was produced when B.napus was the female parent. These results would suggest that B.oleracea has some genetic control which prevents embryo development when crossed with species which have similar genomes, such as B.napus, or with species which did combine in the past, (ie B.campestris) to produce the amphidiploid B.napus.

R.sativus was a consistently poor pollen parent, especially as the tetraploid cultivar. The R genome is more successfully introduced when present in the female parent. In general, R.sativus as a female parent was the most successful of the species used, in that all combinations produced embryos, although many showed early abortion. The relativly poor crossability of Raphanus with B.campestris,

B.oleracea and B.napus with regard to the production of mature embryos is documented in the literature by Dolstra & Zuidgeest (1979a and 1979b); McNaughton et.al., (1978); McNaughton, (1973) and Subramanyam, (1954) and McNaughton & Ross (1978) respectively.

8.2.2 Observations of cytological abnormalities after interspecific hybridisation.

The cytological abnormalities which were associated with the collapse of the hybrid endosperm were similar to those recorded by other researchers who have studied these hybrids. In the coenocytic endosperm there was an increase in the size of the nuclei, and nucleoli. The large nuclei contained one or more nucleoli. Micronuclei were also present. At mitosis, various widths of metaphase plates were present, perhaps an indication of different ploidy levels present in the endosperm. Lagging chromosomes, anaphase bridges and dumb-bell nuclei were frequently observed, as were split spindles. Most of these abnormalities are shown in Figures 8.1 to 8.4. When the entire embryo sac had been successfully removed from the ovule, holes in the sac like appearance were sometimes observed, showing that development of the endosperm around the inside of the ovule was not complete. In some cross combinations the suspensor as well as the endosperm, often showed signs of degeneration (Figure 8.5 and Figure 8.6). Twin embryos were observed on a number of occasions (Figure 8.7). The occurence of twin embryos has often been explained by the presence of supernumerary egg cells, where egg cells are present instead of synergids. However, in angiosperms the reduced megagametophytes have highly specialised synergid and antipodal cells which makes this explanation appear doubtful (Nogle, 1984). An alternative explanation is where the new sporophyte arises directly from a somatic cell of the ovule usually from the nucellus.

Figure 8.1 Dumb-bell nuclei found in hybrid endosperm resulting from the cross R.sativus cv Crail x B.napus cv Lair, 14 days after pollination. Scale bar 50µm.

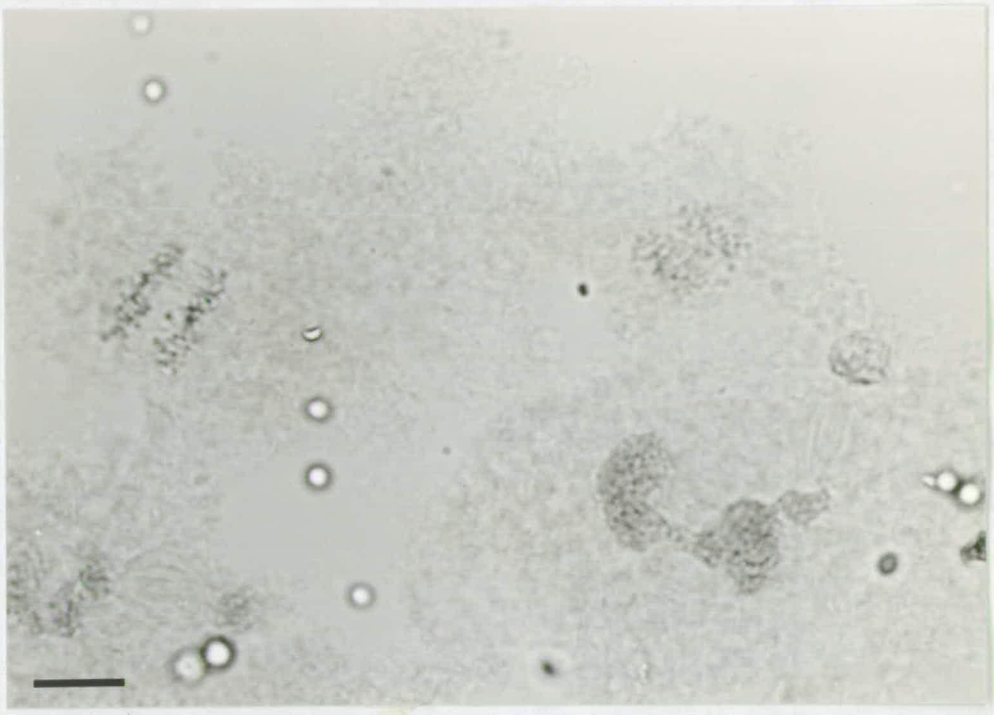


Figure 8.2 Anaphase bridges, lagging chromosomes and different widths of metaphase plates found in hybrid endosperm resulting from the cross R.sativus cv Crail x B.napus cv Lair, 14 days after pollination. Scale bar 25µm.

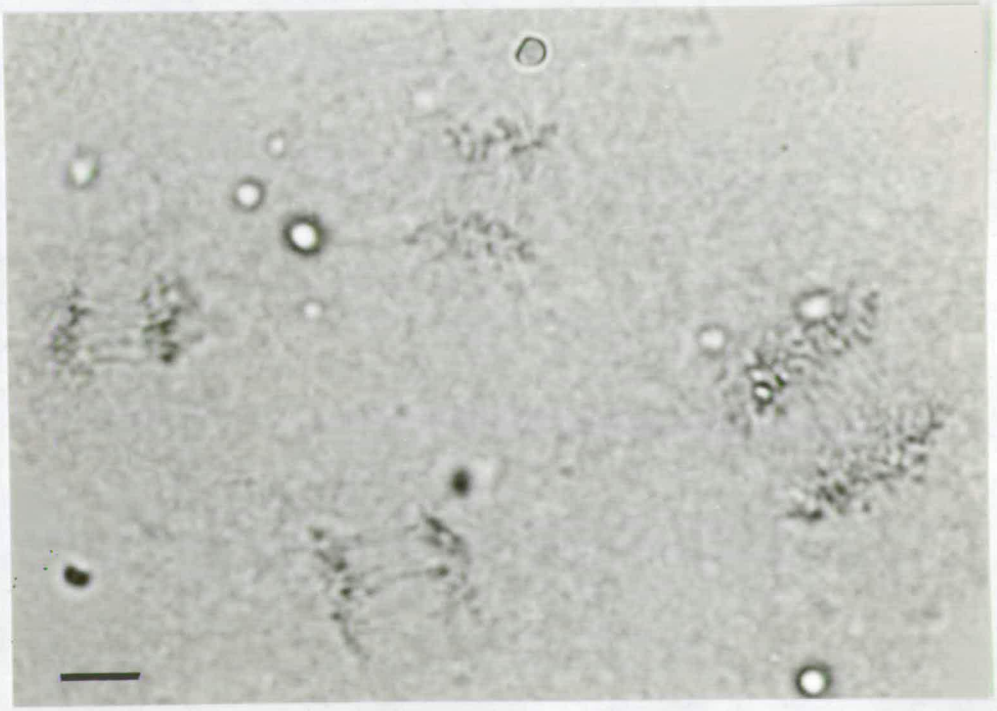


Figure 8.3 Very early abortion of the hybrid embryo formed from the cross B.oleracea cv Maris Kestrel (2x) x B.campestris cv Ponda, 20 days after pollination. Scale bar 100µm.

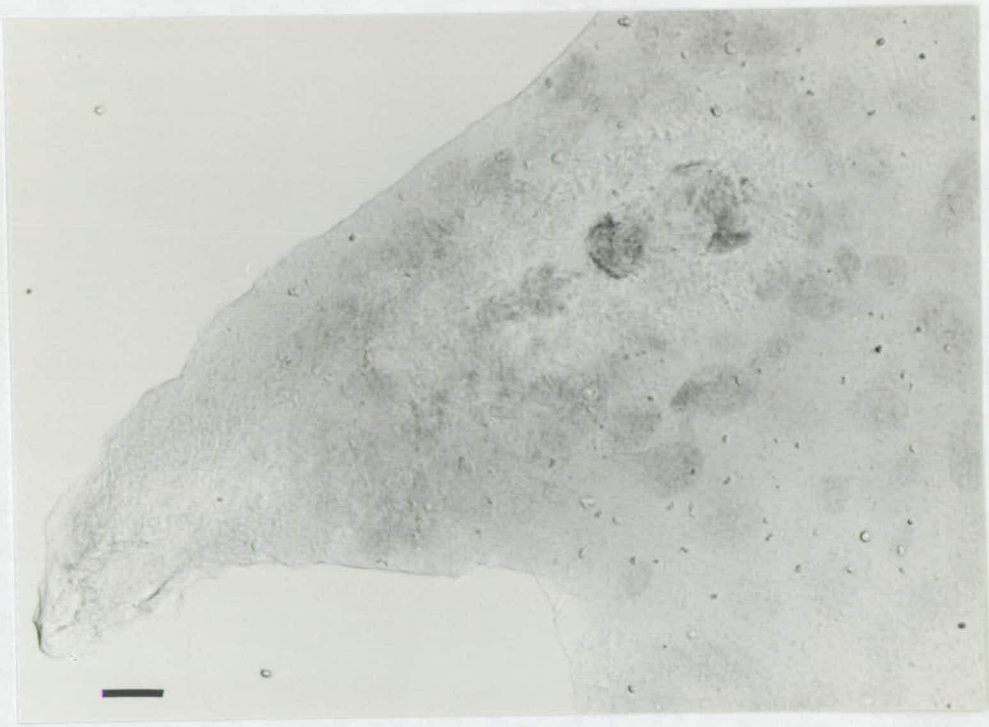


Figure 8.4 Split spindles found at late anaphase in the endosperm resulting from the cross B.oleracea cv Maris Kestrel (2x) x B.campestris cv Ponda, 32 days after pollination. Scale bar 50µm.

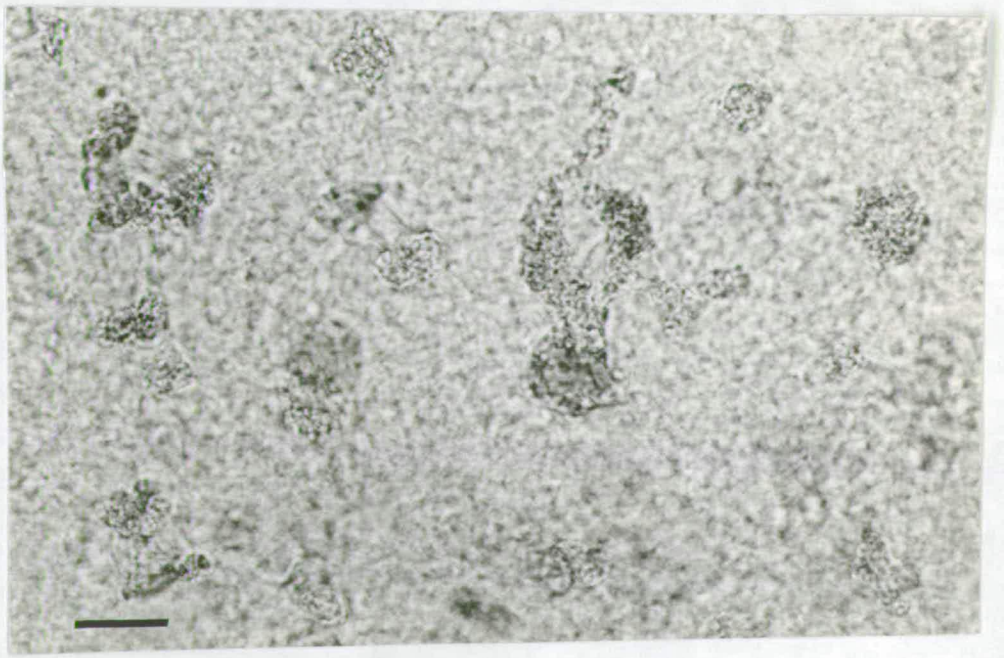


Figure 8.5 Degeneration of suspensor cells after the cross R.sativus cv Slobolt x B.campestris cv Ponda, 16 days after pollination. Scale bar 50µm.

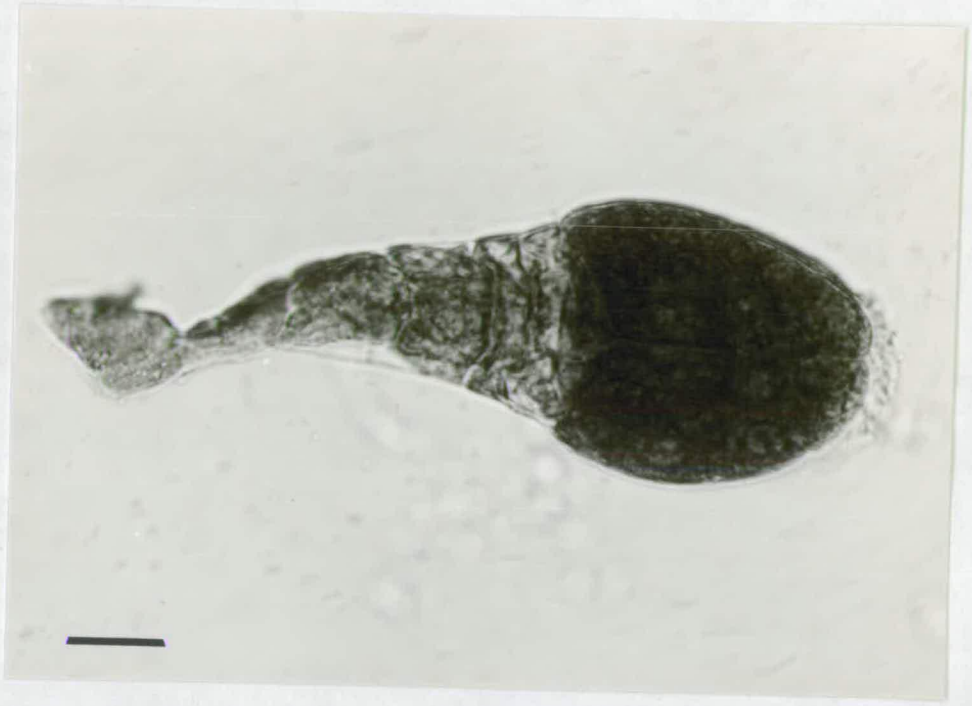


Figure 8.6 Excessive division of the hypophysis cell resulting after the intraspecific cross B.campestris cv Taronda x B.campestris cv Ponda, 12 days after pollination. Scale bar 50µm.

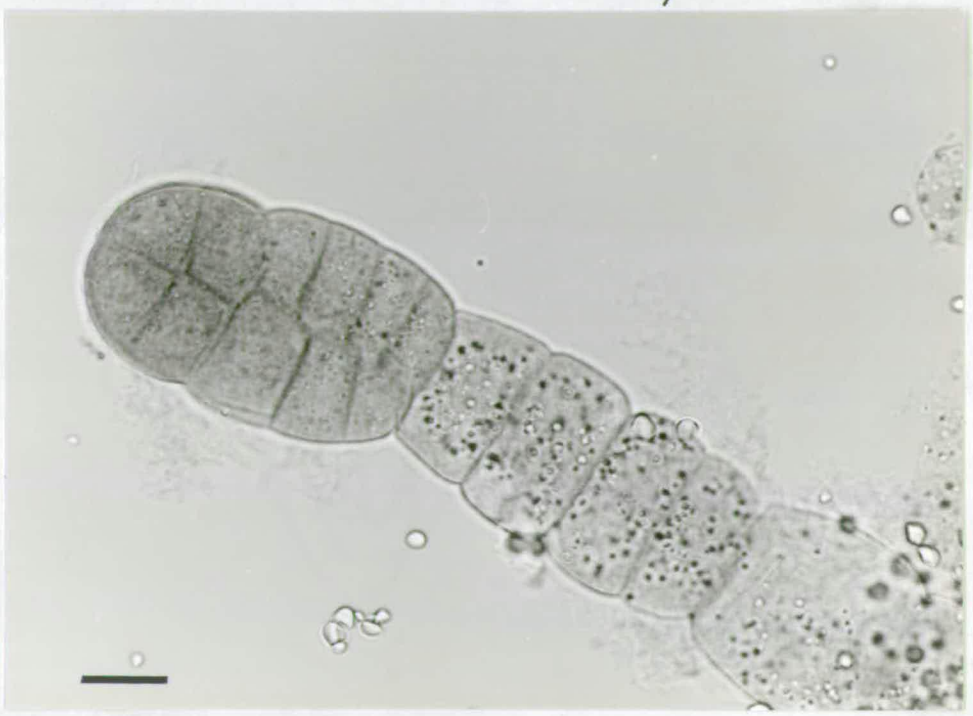
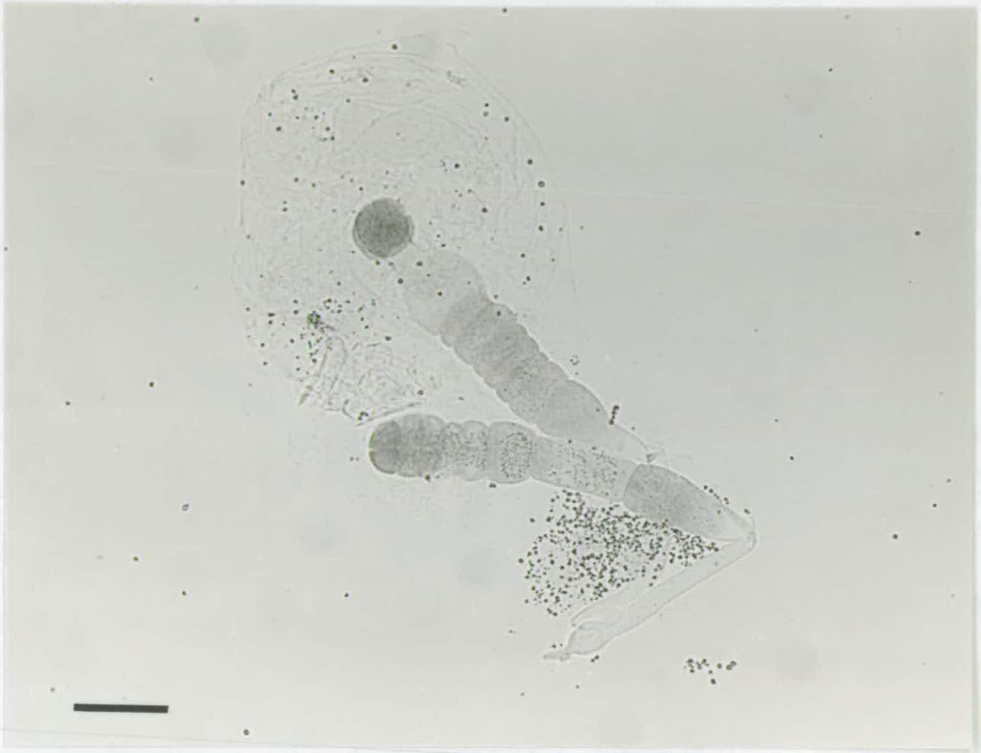


Figure 8.7 Twin embryos resulting from the interspecific cross B.campestris cv Taronda x B.oleracea cv Maris Kestrel (4x), 16 days after pollination. Scale bar 250µm.



Matromorphic progeny (plants with maternal characteristics) have also been observed in the progeny of the cross combination of B.oleracea with B.campestris. They may develop from egg cells which are diploid as a result of different deviations in cell or nuclear divisions. The formation of diploid egg cells may be caused by an absence of meiosis, or the failure of the first or second meiotic division. Diploid embryos may also develop as a result of pre-meiotic endomitosis or a doubling of chromosomes during or after gametogenesis (Eenink, 1974a and 1974b; Mackay, 1972 and Tokumasu, 1970).

Most of the mitotic abnormalities observed result from spindle/centromere defects. Chromosomes can fail to move to the metaphase plate and give rise to lagging chromosomes which then may be eliminated as a micronuclei or included in one of the two daughter nuclei. If a split spindle is present, one to several chromosomes may be eliminated in the form of a micronucleus. Dumb-bell nuclei arise from incomplete separation at anaphase. An increase in the ploidy of a cell can result from reduplication of chromosomes without a nuclear division. This would happen if spindle fibres did not form. Examples of these mitotic faults can be found in the literature. For example, Cooper (1978) and Thomas & Pickering (1983) have reported lagging chromosomes at anaphase. Thomas & Pickering (1983) also reported split spindles.

An increase in the number of micronuclei dumb-bell nuclei and giant nuclei has been recorded for Paris x Trillium hybrids (Rutishauser & Kaltsikes, 1956). An increase in nuclear size in Brassica hybrids was also noted by Hakansson (1956). Cooper (1978) observed that nucleoli may become predominant prior to endosperm degeneration in Hordeum hybrids. Spindle abnormalities leading to polyploidy and aneuploidy and an eventual accumulation of aberrations resulted in the

degeneration of the endosperm of hyacinths (Brock, 1955). Sometimes the frequency of the aberrations is only marginally higher than those found in normally developing endosperms. Rutishauser et.al. (1956) therefore suggest that the aberrations found prior to endosperm degeneration should be considered as being associated with developmental disturbances, but were not wholly to blame. McNaughton (1973) and Ellerstorm & Zagorcheve (1977) are partly in agreement in that they suggest it is the genomic incompatibility in the hybrid, and not wholly the cytological abnormalities which are the cause of sterility of Raphanobrassica. This suggests that the cytological aberrations found are a symptom of post-zygotic incompatibility and not a cause of hybrid failure.

8.3 Conclusion

After an examination of the embryos, endosperm and the egg cytoplasm effects on the development of the embryo, with regard to its genomic combination, it is clear that the ploidy level of the embryo is important. The three genomes can be ranked as to which has best and worst embryo development.

For the diploid embryo, the endosperm and egg cytoplasm have the same ranking. The R genome is better than the C genome, which in turn, is better than the A genome. The non-hybrid triploid embryos show a different ranking to the hybrid triploid embryos. In the non-hybrid embryos the C genome was best, the R genome was intermediate and the A genome the worst. With the C genome the endosperm can maintain embryo development to maturity at the tetraploid or pentaploid level. However, the R genome is best at the pentaploid level and the A genome was best at the tetraploid level. With the triploid hybrid embryos, the A genome was best and the R genome was worst, the C genome being

intermediate. The endosperm also produces an effect. The same ranking of A better than C, better than R applies. However, with the A genome, three A's present in the endosperm are better than two or four A's. The egg cytoplasm effect has the same ranking order as the embryo and endosperm. However, the AC egg cytoplasm does equally well with the A and C genomes, but not the R genome. With the tetraploid hybrid embryos, in particular AACC, are best with a balanced endosperm of three A's and three C's, although the presence of four A's or four C's can maintain embryo growth. The AC egg cytoplasmic background is much better than A or C. When the R genome is introduced at the tetraploid level, the R genome is better than A or C genomes with regard to the egg cytoplasm effect.

B.napus has a positive effect on the development of hybrid embryos at the tetraploid level. This is obvious when the results from the two plant types of B.napus are observed. The genomic constitution of the embryo, endosperm and egg cytoplasm is the same, therefore there must be some other genetic control over the hybrid combinations produced.

After an examination of all the different hybrid combinations the cytological abnormalities observed in the endosperm prior to its collapse are similar to those found in other hybrids. They may, or may not be sufficient to be the solitary cause of abortion, but should be considered to contribute to, or be a stage in, the physiological disturbance of the embryo and endosperm development. It would appear that hybrid embryo development is not determined by chromosomal abnormalities, but by some genetic factor.

The post-zygotic incompatibility after hybridisation may lie at the sub-cellular level. The transmission of chloroplasts involves nuclear gene recombination. Mitochondrial DNA may show some recombination where the resulting mt DNA may be similar, but not identical to either

parent (Galun, 1982). The transmission and reproduction of no cellular organelle is fully understood. For example, abortion at the late globular stage may be related to incompatibility of chloroplast genomes: globular embryos are white, heart shaped embryos are green. Alternatively, it may be related to starch production.

So the post-zygotic incompatibility may be at the subcellular or cellular level. The ploidy level of the hybrid to be produced and the egg cytoplasm, both appear to exert an influence on the outcome of the hybridisation.

So from the results found in this investigation, delimitation of the different species by post-zygotic mechanisms does occur at different levels. In most cases embryo and/or endosperm abortion occurs in the early stages of development. A smaller number of hybrid combinations develop to the later stages of development but are blocked at some stage and fail to complete all the stages to maturity. Some of the F_1 hybrids may be vigorous but sterile, at the diploid level. By intervention fertility may be restored by the use of colchicine, doubling up the chromosome number (eg. Raphanobrassica). A small number of hybridisations, in particular where R.sativus cv Crail was used as a pollen parent, exhibited pre-zygotic mechanism of speciation, where fertilisation failed to take place.

CHAPTER 9

FUTURE DEVELOPMENTS IN INTERSPECIFIC HYBRIDISATION TECHNIQUES FOR *BRASSICA* AND *RAPHANUS*

The previous chapters have indicated the main features of hybrid embryo and endosperm development and failure in interspecific crosses of British crop species of Brassica and Raphanus. Some of the important factors in affecting or determining success with hybridisation techniques have been identified.

9.1 Summary of observations on factors affecting embryo and endosperm formation after interspecific hybridisation.

The success of hybridisation depends to a large extent on the choice of parents. From the combinations examined in chapters 6, 7 and 8 it is evident that the choice of a particular cultivar, different ploidy levels or different genotypes, and the direction of the cross all affect the number of developing seeds. B.campestris with B.oleracea, and B.napus with B.oleracea showed poor combining ability in the Brassica species examined. As a pollen parent, B.oleracea performed badly in that early abortion occurred in nearly all the cross combinations with either the diploid or tetraploid cultivars. As a female parent however, two cross combinations progressed as far as the heart stage embryos, but again most combinations resulted in early embryo abortion. When B.oleracea is combined with B.nigra, early abortion is also observed (Davies & Wall, 1960). B.campestris and B.napus show a better combining ability, often producing mature seed in either direction of the cross. The diploid variety of B.campestris was more productive than its respective tetraploid. R.sativus was successful as a female parent, in that all cross combinations produced developing embryos. However, many of these embryos aborted early in their development. As a pollen parent R.sativus, and in particular the tetraploid variety, performed badly; many of the cross combinations showed no developing embryos. Hybridisation of B.juncea

with B.napus (Roy, 1978) and R.sativus with B.japoncia (Tokumasu, 1970) have also shown limited success.

Most interspecific cross combinations yielded some developing embryos, which ceased development before reaching maturity. This suggests that most genetically determined interspecific incompatibility is operating post-zygotically in these ovules. The percentage of unfertilised ovules varied depending on the species. In five of the eight cultivars examined fewer ovules were fertilised after interspecific pollination compared to those found after bud self pollination. The low percentage of fertilisation may therefore be related to the pollination technique with these cultivars as well as pre-zygotic incompatibility barriers. In some cases, no fertilisation occurred at all; this effect is due only to pre-zygotic incompatibility. Hybrid embryos usually develop at the same rate as the maternal parent, although some cross combinations showed development that was faster or slower than that of the maternal parent.

Among the hybrid combinations, the highest percentage of developing ovules per ovary were often associated with the greater extent of embryo development. Embryo failure at an early stage was often associated with the larger percentages of aborted ovules. In other words, where abortions were few, they usually occurred late in development. Hence, many cross combinations often gave either a relatively high percentage of developing ovules of which most reached a late stage of development, or few developing ovules, most of which aborted early.

9.2 Improving growing conditions and pollination technique for maximum seed set.

All hybridisation begins with the flowers, and it is important that

these are from healthy, disease and pest free plants. It was found that plant material, vernalised under natural conditions, in a cold frame, and transferred to the glasshouse prior to the commencement of flowering produced two good flowering flushes. The plants were regularly fed throughout the flowering period and the period of embryo development. It was found to be possible to induce flowering out of season, but the success of hybridisation was greatest at the natural flowering time.

It has also been shown that the pollen of the four species examined can be stored for at least one year and still remain viable. This possiblity reduces the necessity for synchronous flowering of the male and female parents. The viability in storage of the pollen was found to be species, and in some cases cultivar, dependant. B.campestris cv Civasto showed no storage potential.

Hybrid production has been shown to be sensitive to temperature (Ellerstorm & Zagorcheve, 1977; Chiang, Chiang & Grant, 1977; Pickering & Morgan, 1985; Ramsay & Pickersgill, 1985). A reduction in the frequency of fertilisation was evident when crosses were maintained after pollination at lower temperatures. The glasshouse temperature averaged 19°C during the day for the hybridisation work in this project. Chiang et.al. (1977) found temperatures averaging 18°C in the glasshouse. Increasing or decreasing the temperature has also been reported to affect the self-incompatibility of some Brassica varieties (Visser, 1977).

An investigation of the normal crossing procedure was undertaken. The method of producing maximum seed set varied with the type of cross made. The normal method for bud cross pollination, as for bud self, is emasculation, followed directly by pollination. The results from this examination revealed that, with respect to B.oleracea var

acephala, maximum seed set was obtained when the plants were left unemasculated for bud self and intra-specific pollinations. For interspecific pollinations the conventional method of emasculation prior to pollination produced the most fertilised seeds. The main contribution to the number of seeds set came from the newest flowers and the four oldest buds.

The 'normal' bud pollination technique does not realise 100% fertilisation. Most of the cultivars had a mean percentage fertilised which was lower after interspecific pollination in comparison to that obtained after bud self pollination. So, in addition to considering the type of cross to be made, cultivars should be chosen with a higher seed set after bud self pollination and thereby reduce any effects associated with the bud pollination technique.

9.3 Possible ways of improving success rate of hybrid formation.

Having determined the first necessary steps to maximise the number of fertilised ovules and obtain the maximum possible development, different approaches now need to be examined to further improve success. Those cross combinations with a high percentage of fertilised ovules require to be tackled differently from those with a lower percentage.

9.3.1 Cross combinations with a high percentage of fertilised ovules.

Where there is a high percentage of fertilised ovules, and subsequently embryos approaching the later stages of development, there is a better chance of rescuing and culturing the embryos successfully. There are several examples in the literature where Brassica embryos have been cultured and hybrids produced (Harberd, 1969 and 1971; Nishi, Kawata & Toda, 1959; Snell, 1977).

From observations on the hybrid endosperms formed here, in 96% of the cases examined the endosperm failed to become cellular. Where the exact stage of endosperm collapse is not known, and the embryos abort in the earlier stages of development, then ovule culture is an alternative to embryo culture. In this way the natural endosperm can sustain the initial development of the hybrid embryo, but when it collapses, the nutrient would already be diffusing from the synthetic growth medium to maintain growth. Ovule culture has already been used successfully in the production of Brassica hybrids (Inomata, 1977, 1978a, 1978b and 1979; Kameya & Hinata, 1970; Takeshita, Kato & Tokumasu, 1980). By this technique the ovules and placentae are cultured on a suitable media, and the pollen is applied directly into the ovule. This is also a means to overcome stigma/style barriers to hybridisation.

9.3.2 Cross combinations with a low percentage of fertilised ovules.

From those cross combinations where there is a low percentage of fertilised ovules, often due to poor pollen penetration of the stigma and style, a different approach is required. As mentioned above, fertilisation in vitro with ovule culture may be a possible way of overcoming any stigma/style barriers or chemical attraction to the ovules, since the pollen is applied directly onto the ovules. The pollen tubes only have to find their way to the micropyle.

Pseudo-compatibility, the partial break down of the incompatibility system, in older flowers, or in a second flush of flowering, is a fairly common occurrence in Brassica (Mackay, 1972). If the interspecific incompatibility mechanism operating in the stigma is similar to that of self incompatibility, then this may also break down with time. Protection of stigmas from uncontrolled pollination until

they reach a stage equal to older flowers before controlled pollination, may be a means to improve the number of ovules fertilised.

Low temperature storage of seeds is often associated with chromosome damage and mutations can be seen in the growing plants. Perhaps the storage of pollen at low temperatures has an advantageous effect in helping overcome any interspecific barriers. Due to time limitations on the practical work in this investigation this theory was never tested.

Various other techniques have been experimented with in the hope of increasing the percentage of seeds set after bud self or interspecific pollination. These include the use of CO_2 gas treatment (Nakanishi & Hinata, 1975); the use of N-m-tolypthalamic acid, a growth regulator, applied to the pedicel at the time of pollination (Homma & Heeckt, 1962), and gamma radiation of the female parent (Davies & Wall, 1960). An increase in productivity was achieved when scions of B.oleracea were grafted onto a B.campestris variety before pollination (Hosoda, 1961). Hybrids between B.campestris and B.oleracea have also been produced by cutting the style of the B.campestris maternal parents (Namai & Hosoda, 1965). With the increase in research on protoplast fusion (Shepard, Bideny & Shahin, 1980) and the generation of embryos from somatic tissues (Pareek & Chandra, 1978 and Keller & Armstrong, 1979), it would seem only a matter of time before reports of hybrid production after fusion of somatic tissues of Brassica appear in the literature.

In summary, healthy parental plant material is necessary to maximise hybrid production. When plants are bud pollinated, whether they are best emasculated or not depends whether bud self, intraspecific or interspecific pollinations are to be carried out. B.campestris with

B.oleracea, and B.oleracea with B.napus do not show good combining ability. B.campestris with B.napus combine readily in either direction. R.sativus is much better as a female parent. It was consistently a poor pollen parent, especially the tetraploid cultivar Crail. Most of the cross combinations examined yielded developing embryos of various stages in development. This indicates that most of the incompatability is post zygotic. The different species used for hybridisation were found to develop at different rates. The hybrid embryos usually developed at the same rate as the maternal parent, but some cross combinations were found to show development at a faster or slower rate. Hybrid embryos showing advanced embryo development may be rescued by embryo culture. Embryos which show early abortion, due to collapse of the endosperm, may be able to develop to the later stages if the technique of ovule culture is applied.

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