STUDIES ON SELF-INCOMPATIBILITY IN 'BRASSICA NAPUS'

Sumedha Dharmaratne

A Thesis Submitted for the Degree of PhD
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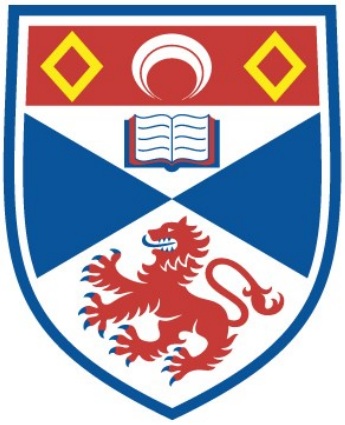
University of St Andrews



1990

STUDIES ON SELF-INCOMPATIBILITY

IN

BRASSICA NAPUS

By

SUMEDHA DHARMARATNE

A thesis submitted for the Degree of Doctor of Philosophy in the University of St Andrews, October 1989. The work was carried out in the Scottish Crop Research Institute, Invergowrie, Dundee, under the supervision of Dr Toby Hodgkin.

DEDICATION

To my parents, my husband Gamini

and son Nuwan

CERTIFICATE

I certify that SUMEDHA CHANDANI DHARMARATNE has fulfilled the conditions of the Ordinance and Regulations (University of St Andrews) and is thus qualified to submit her thesis for the Degree of Doctor of Philosophy.

Signature of Supervisor _____

Date 20 September 1989

DECLARATION

I, Sumedha Chandani Dharmaratne, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other professional qualification.

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CHAPTER 5 - SELF- AND CROSS-COMPATIBILITY OF F_1's PRODUCED FROM *B. NAPUS* SYNTHETIC LINES

ABSTRACT

A detailed investigation was conducted on the self-incompatibility system operating in the amphidiploid species, Brassica napus. Seven synthetic B. napus lines were produced by intercrossing plants of the parental species B. campestris and B. oleracea and doubling the chromosome numbers of the F_1 hybrids, so formed. Each of the parental lines used in the synthesis were homozygous for different S-alleles. B. oleracea parental lines were homozygous for S_{29}, S_{14} and S_2 while the B. campestris line was homozygous for S_a and S_b. Consequently, the synthetic lines of B. napus were homozygous for different alleles at each of two S-loci. Following synthesis, S-allele activity and expression was examined in the synthetics and their F_1's and F_2's, using genetical and biochemical methods. In addition, a study of interspecific incompatibility between B. napus and B. oleracea was conducted.

It was established that the production of synthetic B. napus by ovary culture in B. campestris was more successful than production using embryo culture in B. oleracea. Amphidiploid B. napus plants produced from F_1 hybrids by chromosome doubling were easily identified, exhibiting a typical B. napus morphology, producing fertile buds and reflecting B. campestris and B. oleracea isozyme banding patterns.

Synthetic B. napus plants were generally self-incompatible and the self-incompatibility alleles of both B. oleracea and B. campestris were expressed and showed interlocus epistasis similar to that found in a single locus sporophytic system. Several F_1 lines produced from crosses between B. napus syntheticsexpressed only 3 alleles in any 4

S-allele combination, and this expression occurred only in the pistils. The B. campestris alleles were functional in F_1 stigmas but not in the pollen, showing that allele activity in F_1 pollen would appear to be equivalent to that of a single locus system.

In the F_2 generation, all 4 S-alleles were active in a given genotype and it was established, therefore, that hidden S-loci could persist in a species with a sporophytic self-incompatibility system if alleles were partially or completely recessive.

Test crosses between B. campestris, B. oleracea and B. napus showed that pollen from B. oleracea usually failed to penetrate the stigmatic surface of B. napus, despite all other combinations of interspecific crosses being compatible. It was found that this interspecific incompatibility could be overcome by bud pollinations or by treatment with cycloheximide.

Biochemical studies showed that stigma-specific proteins, resolved by isoelectricfocusing, correlated with some S-allele expression and could be detected in synthetic B. napus. However, stigma-specific proteins, which correlated with the presence of the S_a and S_{14} alleles, were detected in stigmas of F_2 plants even when they were not active, indicating that S-protein expression is not correlated with the full function of self-incompatibility alleles.

CHAPTER 1

LITERATURE REVIEW

1.1. INTRODUCTION

1.1.1. Background to self-incompatibility systems in plants

General reviews of the literature on self-incompatibility systems in plants have been written by Lewis (1954), Arasu (1968), Linskens & Kroh (1967), Townsend (1971) and de Nettencourt (1977). More specialised reviews on specific aspects of the systems are also available; Lundqvist (1965) on genetic aspects, Linskens (1965) on biochemical aspects and Crowe (1955) on evolutionary aspects. In this review I will emphasise the studies which have had particular significance to the development of our understanding of the genetics of sporophytic self-incompatibility systems (particularly those involving Cruciferae) and the mechanism of self-incompatibility.

Kolreuter (1764) was the first to recognise that self-fertilisation is not common among flowering plants. He pointed out that many species possess mechanisms that prevent self-fertilisation such as separation of the pollen production and pistil receptivity in time, so that few seeds produced under natural conditions are products of self-fertilisation. For many years the failure to produce seed on selfing was termed self-sterility. Darwin (1862) suggested that many features of orchid flowers act as devices to reduce or prevent self-fertilisation thus enabling plants to avoid the effect of inbreeding depression. Prevention of inbreeding depression was considered by Darwin as the main reason why plants had evolved mechanisms to prevent

selfing. He pointed out that the different flower forms found in Primula and Oxalis, and the partial self-incompatibility which he detected in Brassica oleracea, were mechanisms that promoted outcrossing and prevented the adverse effects of inbreeding.

Darwin (1876) was first to study self-incompatibility in the Cruciferae in any detail. He found that Brassica oleracea was partially self-incompatible, setting about four times as much seed on outcrossing as on selfing. Raphanus sativus was slightly less self-compatible than B. oleracea while Iberis umbellata was self-fertile. He further showed that, in species where self-sterility was present, self-sterile individuals could be successfully fertilised using pollen from other plants of the same species. Jost (1907) demonstrated that in self-sterile plants, self-pollen tubes showed limited growth whereas cross pollen quickly developed tubes of the necessary length for fertilisation. From his results, Jost proposed that each self-sterile plant contains its own 'individualstoffe' which stimulates the pollen of other plants, but not its own pollen.

Following the establishment that self-sterility was common in plants, Correns (1912) was first to attempt to determine its genetic control using the homomorphic species, Cardamine pratensis. However, his interpretation did not explain all the results obtained from crosses he made between sibs, and he believed that the deviations found were due to other factors. Compton (1913) regarded self-fertility in Reseda odorata to be due to a factor showing simple Mendelian dominance to self-sterility. He agreed with Jost's interpretation of an individual substance in each plant that stimulates the pollen of other plants and drew attention to the

similarity of self-sterility to immunity. East (1915) crossed self-sterile Nicotiana forgetiana and N. alata plants to produce the F_1, F_2, F_3 and F_4 generations, and tested a large number of crosses, the results of which disagreed with the simple Mendelian dominance theory advanced by Correns and Compton. East put forward the hypothesis that a hexose sugar in the style was a pollen stimulator and that pollen contained an enzyme which acted on this sugar and which differed slightly from plant to plant. He suggested that this hypothesis could be linked to Jost's single direct stimulus and the need of 'individualstoffe'.

The first detailed studies on self-incompatibility in Crucifers related to Brassicacea were carried out by Stout (1917) who put forward the word 'incompatibility'. He found (Stout, 1920) that Raphanus sativus was self-incompatible. Stout (1922, 1927) later described three types of sterility in Brassica pekinensis and B. chinensis: flower abortion and arrested development, proliferation with the destruction of the pistil by vegetative growth, and physiological incompatibility. In regard to the latter, he assumed that self-pollen tubes secreted an antigen which stimulated the style to produce antibodies and that these prevented further tube growth.

In 1925 East and Mangelsdorf proposed an oppositional factor hypothesis to explain self-incompatibility within Nicotiana sunderae. This hypothesis proposed that a series of multiple alleles was controlled by a single locus. In the style of a self-incompatible plant two alleles were active and pollen was only stimulated to grow if it contained an allele different from that in the style. An interesting finding by East (1923) was that in self-incompatible Nicotiana, very young buds would set seeds when pollinated with self

pollen. Pearson (1929, 1932) also showed bud self-compatibility to occur in B. oleracea with younger buds producing much higher seed set than mature flowers. Pearson suggested that mature ovules died before the sperm nuclei reached them whilst in buds, fertilisation occurs. In contrast, East (1923) considered that in Nicotiana the compatibility barrier developed only a day or so before buds opened.

A detailed account of incompatibility in B. oleracea was given by Kakizaki (1930). He proposed two series of alleles; one oppositional and the other sympathetic with the oppositional series being epistatic to the sympathetic set. He also suggested that inhibiting substances were produced most abundantly when the pistil was in its 'full vigour'. He found that bud pollinations showed high levels of pseudocompatibility, and assumed that this was due to both insufficient inhibitory action in immature styles and to the longer time available for pollen tube growth as well as shorter distance to be traversed.

Stout (1931) reported that self-incompatibility in B. pekinensis involved powerful inhibition of pollen germination and tube growth on the stigma. In incompatible crosses, he found that there was a lower percentage of germinated grains than in compatible crosses and that grains grew around the stigmatic papillae rather than growing straight into the stigma. He put forward the theory that self-sterility and incompatibility are not to be considered as strictly contrasted characters. He agreed with Kakizaki's interpretation of incompatibility that oppositional factors were involved when the style and pollen were of similar genetic composition.

It was established by Sears (1937) that compatible pollinations in

B. oleracea var. italica occurred if part or all of the stigma was removed. He proposed that two series of oppositional factors of varying inhibitory potency were present in the species and that germination of pollen did not depend on a specific stimulating substance. Rather the stigma possessed or produced substances which actively inhibited pollen. He suggested that one could recognise 3 different types of incompatibility response depending on the site at which male failure occurs: pollen germination does not occur or only short tubes are produced (eg B. oleracea var italica), tube growth stops shortly below the stigma but some tubes grow nearly to the bottom of the style (eg Petunia violacea), incompatible tubes grow as fast as compatible ones and appear to affect fertilisation. Tatebe (1939) repeated some of Sears' work and confirmed that removal of half or all of the stigma resulted in seed set.

A major advance in resolving the genetics of species with sporophytic incompatibility system was made by Hughes & Babcock (1950) who described a novel type of incompatibility in the composite Crepis foetida. They proposed that there was a single incompatibility gene with multiple alleles which acted individually in the styles, as in Nicotiana, but that the behaviour of the pollen was determined by the sporophyte and that the alleles exhibited dominant relationships.

Crowe (1954) found that self-incompatibility in Cosmos bipinnatus closely resembled that described by Hughes & Babcock (ob. cit) and involved a single incompatibility locus with multiple alleles showing sporophytic action in pollen. She also found that the S-alleles showed dominance or individual action in both pollen and style. Bateman (1954) reported that a sporophytic self-incompatibility system also operated in the Crucifer, Iberis amara and that the two alleles

of a heterozygote may act independently or show dominance/recessive relationships. Bateman estimated that the number of 'self-incompatible' alleles in I. amara was probably greater than 22 and that these could be arranged in a linear order of dominance. Sampson (1957) and Thompson (1957) later showed that a sporophytic system of self-incompatibility was also present in Raphanus sativus and B. oleracea respectively. The presence of this form of system in B. oleracea has since been confirmed by numerous workers using a variety of different cultivars. (Odland, 1962; Adamson, 1965; Haruta, 1966; Hoser-krauze, 1971).

1.1.2. The genetics of the self-incompatibility system

Self-incompatibility systems can be divided into heteromorphic or hormomorphic gametophytic and homomorphic sporophytic systems (Lewis, 1954). Heteromorphic systems are those associated with different floral morphologies which act to reinforce cross pollination. Homomorphic incompatibility systems are characterised by the absence of morphological differences between the mating types. In the gametophytic self-incompatibility system the phenotype of the pollen is determined by the individual microspore, while in sporophytic systems it is determined by the genotype of the pollen producing plant. Sporophytic systems are common in the Cruciferae, Compositae and Rubiaceae. A central feature of the sporophytic system is that the expression of the self-incompatible gene in pollen must be premeiotic. Thompson & Taylor (1966) and Van Hal (1968, unpublished) have carried out detailed surveys of S-allele activities in

B. oleracea and the inter-relationships between different S-alleles showing that non-linear dominance relationships are common.

In a sporophytic system the two different S-alleles in a heterozygote may either both be active or one may be dominant over the other. The interaction between two alleles may differ in pollen and stigma. Dominance is more common in the pollen and all possible allele relationships have been described, i.e. dominance of one allele to another in the pollen, independence in the style; dominance in the style, independence in the pollen; independence in both pollen and style; dominance in both pollen and style. Van Hal (1968, unpublished) has found some incomplete dominance situations in which the activity of both alleles in certain combinations is reduced. Thompson & Taylor (1966) and Thompson (1968) classified the known S-alleles into groups in which all the alleles have common characteristics, but these classifications have only been moderately successful. Thompson (1968) analysed some 170 combinations involving 28 different S-alleles in B. oleracea var acephala and found that 23 S-alleles showed activity or co-dominance of both S-alleles in the pollen. However, in the stigma the relationships were different. The pollen recessive alleles were sometimes recessive in the stigma in combination with the other alleles. However, no dominance occurred in a high proportion of combinations and a linear dominance relationship between recessive S-alleles was not found. It was also noted by Thompson that plants homozygous for recessive S-alleles tended to be self-incompatible. Ockendon (1974, 1980) extended the S-allele analysis initiated by Thompson and he found a total of 19 S-alleles in Brussels sprouts, 12 of which had been previously found in kale.

It has been reported by Odland (1962) that the activity of S-

alleles is correlated with dominance in B. oleracea, with alleles high in the dominance series more active than those low in the series. Wallace (1979) has determined S-allele activity in both pollen and stigma using pollen tube penetration and seed set data from reciprocal crosses between S-allele heterozygotes and their corresponding S-allele homozygotes. He showed that within S-allele heterozygotes each pair of S-alleles had a specific interaction in the pollen and the same or another in the stigma and confirmed that pollen tube penetration into the style was highly correlated with seed set. In B. campestris Richards & Thurling (1973) have shown that self-incompatibility is also determined by a single locus multiallelic sporophytic system, and that allele co-dominance is more frequent in the stigma than pollen.

Despite the general acceptance of the presence in diploid Brassica species of a single locus sporophytic self-incompatibility system, anomalous results have been reported on occasion and Zuberi et al. (1981) have considered that a second gene I might also be involved in the control of self-incompatibility in B. campestris. Most recently, Lewis et al. (1988) and Zuberi & Lewis (1988) have suggested that in Raphanus sativus and B. campestris there is an additional gene, G, which is active only in certain limited S-allelic combinations.

1.1.3 Morphology and physiology of the self-incompatibility system

With very few exceptions (see Lawrence, 1975; Knight & Rodgers, 1955), the incompatibility reaction in sporophytic species mostly occurs at the stigma surface whilst in species showing gametophytic self-

incompatibility inhibition of pollen tube growth occurs in the stylar region. As mentioned earlier, many workers have shown that incompatibility in Brassica species is a stigma surface reaction (Stout, 1931; Kakizaki, 1930; Tatebe, 1939; Kroh, 1956).

In Cardamine pratensis it was suggested (Christ, 1959) that the cuticle is the incompatibility barrier and later Kroh (1964) showed that in the Cruciferae, incompatible pollen tubes fail to penetrate the cuticle. Ockendon (1972) has used scanning electron microscopy to confirm that in B. oleracea self pollen tubes are inhibited at the stigmatic surface before they penetrate the papillae. More recently Dickinson & Lewis (1973a, b) showed that in Raphanus tapetally-derived proteins are deposited in the pollen exine (tryphine) and that the compatibility of the pollen is determined by the character of this tryphine. They proposed that an interaction between a protein on the stigmatic surface and the tryphine stimulates callose formation in incompatible pollinations and hence inhibits tube growth. In support, Heslop-Harrison et al. (1974) found that the deposition of callose in the stigma papillae could be induced by agar gels onto which incompatible pollen had been placed for sufficient time to permit the diffusion of exine held materials. They also showed that such a response did not occur when agar was used onto which compatible pollen had been placed. It was suggested by Mattson et al. (1974) that the 'pellicle' (i.e. the external coating overlying the cutinised stigmatic papillae) could be the recognition site of the incompatibility responses, being functionally important in both the capture and hydration of the pollen grains. Subsequently Heslop-Harrison (1975) proposed that the pellicle determines the production of cutinase and the penetration of the pollen tube into the stigma and

that the incompatibility reactions take place at the stigmatic surface between antigens (S-allele related stigmatic proteins) detected by Nasrallah et al. (1970) as an exine bound protein.

As part of the incompatibility reaction, Heslop-Harrison (1979) has recognised that regulation of the passage of water from stigma to pollen is particularly important for successful pollen tube development. Zuberi & Dickinson (1985) have since shown that, under normal field conditions Brassica self-pollen may often undergo a brief period of hydration which causes inhibition of many pollen grains.

1.1.4. Biochemistry of the self-incompatibility system

Lewis (1952) applied serological techniques to identify incompatibility substances in Oenothera organensis and concluded that the different S-alleles produce specific and antigenically distinguishable substances in the pollen. Linskens (1960) raised antisera to both pollen and style extracts of Petunia and reported that both pollen and style have identical antigens. Nasrallah & Wallace (1967a) applied the same technique to B. oleracea and found that S-specific antigens were only present in the stigma. They (1967b) described the segregation of the antigenically active proteins in the F_1 and F_2 and found that it correlated with the segregation of the S-alleles as tested by pollination and seed set. Sedgely (1974a, b) later noted that plants heterozygous at the S-locus contained about half as much of a particular S-antigen as did the S-allele homozygote.

Isoelectricfocusing (IEF) has been used by Nishio & Hinata (1977, 1979, 1980) to show that certain S-specific molecules are

glycoproteins. Roberts et al. (1979) have also used isoelectricfocusing to examine stigmatic extracts of B. oleracea and found that mature stigmas possess large quantities of glycoprotein which is not present at earlier stages of bud development. Nasrallah et al. (1983) have since examined S-specific proteins in Brassica stigmas at different developmental stages using IEF and have also observed increased levels of S-specific protein bands in mature stigmas. Finally, Nasrallah et al. (1985a) have presented preliminary evidence of having cloned the DNA of the S_6 allele of B. oleracea and further showed that the protein encoded by the S-DNA is detected by an antibody to the S_6 glycoprotein produced in intact stigmas. Subsequently, Nasrallah et al. (1988) have demonstrated a cell-type specific expression of self-incompatibility sequences for several S-transcripts in the stigma and shown that these S-transcripts are expressed exclusively in the papillae cells of the stigma and not in other stigma style or ovary tissues.

1.1.5. Self-incompatibility in Brassica napus

Studies of the genetics of self-incompatibility in amphidiploid Brassicas are limited. B. napus is the allotetraploid derived from B. campestris and B. oleracea. Although both parental species have a sporophytic self-incompatibility system, B. napus is usually self-compatible. Olsson (1960a) found some naturally occurring self-incompatible plants of B. napus spp rapifera (swedes) and B. napus ssp oleifera (oilseed rape), and Davey (1958) and Olsson (1960b) have shown that self-incompatible B. napus plants can be obtained by crossing B. oleracea and B. campestris followed by embryo culture.

Functional S-alleles have also been successfully introgressed into forage rape B. napus L. from turnips, B. campestris ssp rapifera (Mackay, 1977a). Mackay (1976) showed that in forage rape self-incompatibility was dominant to self-compatibility, and moreover, B. campestris alleles were active in B. napus. Recently Gowers (1989) and Gemmell et al. (1989) have demonstrated that active S-alleles in B. napus have been inherited either from B. oleracea genome or the B. campestris genome; however, they were not able to determine which parental genome donated the active allele.

Self-incompatibility in B. napus has been of interest to breeders because of the possibility of producing F_1 cultivars. In the diploid Brassica species self-incompatibility is of central importance in the production of cultivars e.g. cabbage, calibrese (B. oleracea), Chinese cabbage and turnip (B. campestris). This reflects the monetary value of these crops, where both uniformity and hybrid vigour (heterosis) are of importance. The need for uniformity in amphidiploid Brassica is not as great; however hybrid vigour has been shown to occur in swede (Gowers, 1973), thus justifying the need for a cheap method of producing hybrid lines.

1.2. Objective of the proposed Research

Successful use of self-incompatibility in the production of F_1 cultivars of B. napus crops would be greatly enhanced by a more complete knowledge of the self-incompatibility system in the species. Because two S-loci are present in B. napus, the amphidiploid provides a unique opportunity to investigate self-incompatibility in a species

known to possess a 2 locus sporophytic self-incompatibility system. In turn, it can be viewed as a model system for studies of recognition in flowering plants, which complements and extends the work done on Brassica species with a single S-locus.

The detection and production of self-incompatible B. napus raises a number of questions concerning the operation of the system in the amphidiploids, viz:

(a) Are both loci donated by the self-incompatible parents expressed in the synthetic B. napus?

(b) If not, then which locus is expressed? Is it always the same locus that is expressed or is it sometimes the oleracea locus and sometimes the campestris locus?

(c) If the alleles at both loci are expressed, do they operate independently or is there an interaction between the two loci?

(d) What interaction, if any, occurs between individual alleles at different loci and to what extent do inter- and intra-locus interactions occur together?

(e) If an interaction occurs what form does it take?

Previous studies have not addressed these questions owing to inadequate information on the incompatibility status of the parents of B. napus.

The objective of the present research was therefore to synthesise B. napus from parents of known S status (Chapter 2) and to investigate the expression of the alleles at each locus in the synthetics (Chapter 3), their F_1 progeny obtained by intercrossing them (Chapter 5), and the F_2 families derived from the F_1's (Chapter 6). Detection in tests between B. napus and B. oleracea of a form of interspecific incompatibility led to an investigation of the nature and extent of

this phenomenon (Chapter 4). Finally, studies were also carried out to detect the stigma protein profiles of the families raised in order to detect if S-correlated protein bands could be identified (Chapter 7).

CHAPTER 2

PRODUCTION OF SELF-INCOMPATIBLE BRASSICA NAPUS

2.1. INTRODUCTION

The first description of a hybrid between two different species of the Cruciferae was that reported by Sageret (1926) who successfully crossed Raphanus sativus with Brassica oleracea. This was followed by the synthesis of the first fertile allopolyploid in the family produced by Kapechenko (1928) who crossed Raphanus sativus with B. oleracea to yield Raphanobrassica. In 1932 Frandsen & Winge described another interspecific allopolyploid hybrid produced from crosses between B. napus and B. campestris and named it Brassica napocampestris. In both cases, fertility in the allopolyploid apparently resulted from spontaneous chromosome doubling in the sterile hybrid.

Based on studies of chromosome pairing, Morinaga (1934) proposed that the high chromosome Brassica species B. napus (2n = 38), B. juncea (2n = 36) and B. carinata (2n = 34) originated as amphidiploid hybrids from interspecific crosses between low chromosome species B. nigra (2n = 16), B. oleracea (2n = 18) and B. campestris (2n = 20). Following this proposal, the first synthesis of B. napus was made by U in 1935 (Fig. 2.1). He crossed diploid B. campestris and B. oleracea using B. campestris as the female parent and obtained four true hybrid B. napus plants from 732 pollinations (0.54 hybrids/100 pollinations). Colchicine was used to double the chromosome number of the hybrid to obtain the fertile allopolyploids. Later, Frandsen

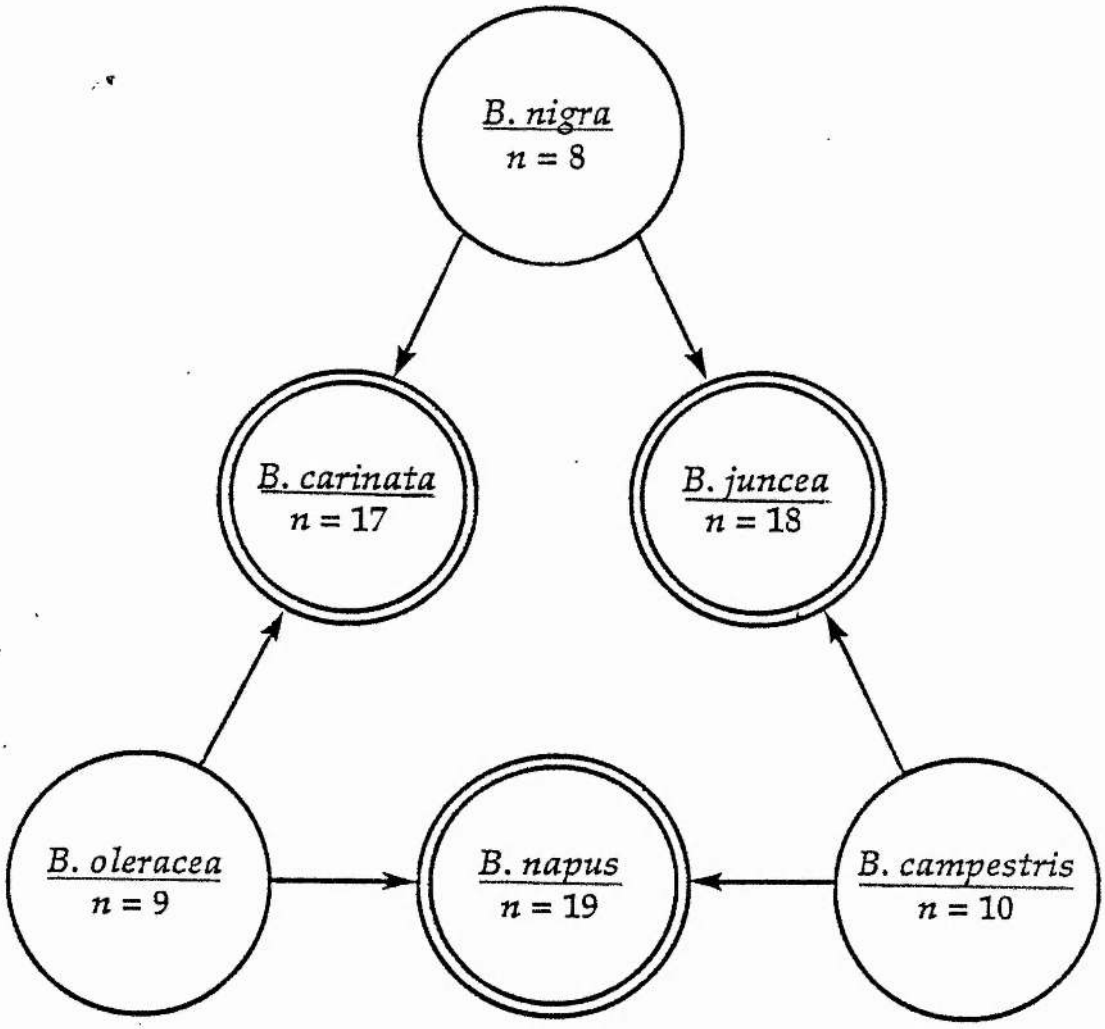


Fig. 2.1 Diagramatic representation of the genomic relationship among species of Brassica (From U, 1934)

(1943, 1947) synthesised B. juncea, B. napus and B. carinata, following intercrossing between the diploid species that Moringa (1934) had identified as parents.

Artificial synthesis of the amphidiploid B. napus by hybridisation between B. campestris and B. oleracea followed by chromosome doubling provides a means of increasing variability in the species (B. napus)

and, in turn, obtaining suitable material for genetic studies. For this reason, artificial B. napus has been produced by a number of breeders in Europe as part of their breeding work over the past 50 years. The frequency of hybrids obtained by the different investigators has varied but, as a rule, only a few hybrid seeds have been produced per 1,000 pollinated flowers. Several methods have been used to synthesise the amphidiploid B. napus. Hoffmann and Peters (1958) intercrossed diploid B. campestris with diploid B. oleracea reciprocally and treated the resulting offspring with colchicine following the methods used by U. (1935). They found that the number of hybrids obtained per cross depended on the species used as the female parent. When B. campestris was used as the female, 48 hybrids were obtained from 20,283 pollinations, (ie 0.24 hybrids/100 pollinations), but with B. oleracea as the female only one hybrid plant was produced from 15,288 pollinations made (0.006 hybrids/100 pollinations). Olsson (1960b) has obtained similar results from his crossing programme, producing 15 hybrids from 7,434 crosses (0.20 hybrids/100 pollinations) when B. campestris was the female parent but only one hybrid from 2,961 crosses when B. oleracea was the female (.034 hybrids/100 pollinations).

The use of tetraploid parents to produce fertile synthetics directly has also been exploited. Olsson (1960b) obtained more hybrids from intercrosses between tetraploid B. campestris and B. oleracea (263 hybrids from 41,758 pollinations i.e. 0.62 hybrids/100 pollinations) than were obtained with diploids as the parents (16 hybrids from 14,469 crosses - 0.11 hybrids/100 pollinations). McNaughton (1963) also showed that the number of pollinations required to produce a hybrid was considerably greater at

the diploid than the tetraploid level, and reported the interesting finding that there was no great advantage as to which species was used as female parent in crosses at the tetraploid level (Table 2.1).

Environment, the physiological state of the mother plant plus its genotype, have all been found to influence the yield of hybrids in crosses. The influence of the genotype of the mother plant on the yield of hybrid seeds was clearly demonstrated by Olsson (1960b) in a series of crosses between tetraploids of B. campestris and B. oleracea. He obtained 133 hybrid plants from 22,884 pollinations (0.58 hybrids/100 pollinations) of which 103 hybrids originated from only 4 mother plants. The remaining 30 hybrids were produced from several mother plants. Of 130 hybrids obtained from the reciprocal combination, 126 were derived from one mother plant and the remaining 4 from 4 different combinations.

A major improvement occurred in the production of hybrids between B. campestris and B. oleracea with the development of an embryo culture technique (Nishi et al., 1959). Initially, Harberd (1971) used a simple liquid culture technique based on a White's medium (see Appendix 1) for raising hybrids from various crosses between Brassica species, while Snell (1977) obtained increased numbers of hybrids (8 hybrids per 100 pollinations) using a modification of Nishis' solid agar culture technique. Subsequently, Inomata (1978a) produced hybrids between B. campestris and B. oleracea comparatively easily by culturing in vitro excised pollinated ovaries. He obtained 15 hybrid plants from 116 ovaries (12.93 hybrids per 100 pollinations) in an initial study and then, following the development of an improved medium (Inomata, 1985), he increased the rate of success to 381 hybrid plants from 568 pollinations using B. campestris as female parent and

B. oleracea as male (67 hybrids per 100 pollinations). He also obtained a very high success rate with interspecific crosses between B. cretica[1] and B. campestris, irrespective of which species was used as the female parent.

Since the breakthrough made by Inomata and others, ovary culture has been developed into a reliable technique at the Scottish Crop Research Institute for the synthesis of B. napus (J. Middlefell-Williams, personal communication) and was the method chosen for this research.

In this Chapter, I describe the methods used to produce self incompatible B. napus of known S allele constitution and the results obtained.

2.2. MATERIALS AND METHODS

2.2.1 Artificial synthesis of Brassica napus of known S-allele genotype

2.2.1.1 Ovary culture

Three inbred lines of B. oleracea var alboglabra obtained as S-allele homozygotes from Dr D J Ockendon (Institute of Horticulture, Wellesbourne, Warwickshire, U.K.) were used as pollen parents in crosses. These were coded thus:

83-456 - DJ 2025 Homozygous for $S_{29}S_{29}$

83-443 - DJ 2020 " " $S_{14}S_{14}$

85-353 - DJ 2023 " " S_2S_2

[1]A species with 2n = 18 chromosomes, closely related to B. oleracea.

B. campestris var. chinensis plants of known S-allele composition were chosen to represent the female parent. These plants were produced by self pollinating an inbred derived from B. chinensis juslen var chinensis; 81X023 yielding:

86-155A - Homozygous for S_aS_a

86-155B - " " S_bS_b

The dominance relationship of the S-alleles in the B. oleracea lines were known from the work by Van Hal (1968 - unpublished) and are as follows:

Stigma	Pollen
$S_{29} = (S_{14})$	$S_{29} = S_{14}$
$S_{29} > (S_2)$	$S_{29} > S_2$
$S_{14} = S_2$	$S_{14} > S_2$

Key to symbols

= both allele active
() partially active or recessive
> One allele dominant to the other

Similarly, the dominance relationship of S-alleles in B. campestris (See Appendix 2) have been identified as:

Stigma	Pollen
$S_a = S_b$	$S_a > S_b$

Growth and maintenance of plant material followed a standard regime throughout this work. Seeds were sown in individual peat compost pots (Jiffy 7's) and placed in a heated glasshouse providing a 20°C day and 15°C night temperature. Plants were transplanted to 8.8 cm pots at the 3-5 leaf stage and then, between four to five weeks from sowing, were transplanted again to 13.2 cm pots in which they were subsequently maintained. Mature plants were treated every 2

weeks with Hoaglands solution (for composition, see Appendix 3).

B. campestris 86-155A and 86-155B plants were bud pollinated by brushing pollen from a freshly dehisced anther of the required B. oleracea pollen parent line on to the stigmatic surfaces of buds from which anthers had been previously removed. Pollinated buds were placed in white-paper-waxed bags to prevent contamination until collection. The number of pollinations made for each cross are given in Table 2.2.

Pistils with approximately 0.2 mm of pedicel were collected nine days after pollination when they were 1.5 - 2.5 cm long. These were surface-sterilised with 10% chloros for 5 minutes, rinsed in three changes of distilled water and cultured on modified Nitsch and Nitsch's medium (See Appendix 4 for composition). Each pistil was planted in 20 ml medium poured as a slope culture (Fig. 2.2.).

All manipulations were conducted under aseptic conditions in a laminar flow cabinet while cultures were maintained at 22 ± 2°C at a radiant flux density of 74 $\mu mol/m^2/s$ continuous illumination by fluorescent lamps. The pistils showed elongation within 10-15 days. After 31 days, some of the ovaries had increased considerably in size and showed swelling at places where embryos had developed. At this stage, they were dissected under a stereoscopic microscope and the size and stage of development of the embryo determined. Culture bottles were surface-sterilised by dipping them quickly into ethyl alcohol and flaming. The embryos were extracted and transferred to basic embryo culture medium (for composition see Appendix 5 - Snell, 1977) where they were incubated at 25 ± 2°C under continuous illumination. Most embryos grew normally on this medium (Figs. 2.3 and 2.4), but some produced many shoots and leaves (Fig. 2.5). These latter types were

divided into three or four masses and sub-cultured for further manipulations in a basic embryo culture media supplemented with 0.1 µg/L Indole Acetic Acid (IAA) and 0.1 µg/L Kinetin.

Some of the sub-cultured masses produced further abnormalities in cotyledon shape and formation. These abnormal shootlets developed into normal plants on transfer to a basic embryo culture media containing 0.2 µg/L Napthalic Acetic Acid (NAA) and 0.1 µg/L 6 Benzyl amino purine (BAP).

Once plantlets with an adequate root system had developed they were transplanted to a mixture of peat and Perlite (1:1 by volume Fig. 2.6.) placed in a mist propagator and gradually transferred to the glasshouse under supplementary light.

2.2.1.2 **Embryo culture**

B. oleracea var alboglabra 83-456, 83-443 and 85-353 were bud pollinated using freshly dehisced anthers of B. campestris 86-155A in the manner described above. The number of pollinations made for each cross are presented in Table 2.3.

Thirty-six days after pollination, pistils were collected, surface-sterilised by dipping them in 10% chloros for 5-7 minutes and rinsed in three changes of distilled water. Embryos were removed aseptically under a steroscopic microscope by lengthwise dissecting of the developed seed pod. Excised embryos were transferred to basic embryo culture media (See Appendix 5 - Snell, 1977) and incubated at 25 ± 2°C under continuous illumination of fluorescence light.

Once the embryos had grown an adequate root system and developed

active growing points, they were planted in a mixture of peat and perlite (1:1 by volume) and transferred to a mist propagator. After one to two weeks, they were transplanted directly in compost and gradually transferred to the glasshouse.

2.2.2. Chromosome doubling

All the hybrids produced were expected to have a dihaploid genomic constitution. A few hybrid plants, however, were fertile, presumably due to spontaneous chromosome doubling. Colchicine (0.1%) was applied on five consecutive days to each axillary bud of the dihaploid plants to induce chromosome doubling. Plants treated in this way were left unwatered for at least five hours.

2.2.3. Chromosome counts

Chromosomes were observed in meristematic tissues of root tips. The tips were pre-treated with iced cold water for four hours at 4°C. They were then treated with bromonapthalene (C-mitotic agent) to arrest mitosis at metaphase and also to contract the chromosomes for one hour at room temperature before fixing in alcohol:acetic acid: chloroform (6:2:1) for 24 hours. After fixing, root tips were washed and placed in 1M HCl for 20 minutes at 60°C to soften the tissues. Following removal from HCl, root tips were washed with water and stained with 2% aceto-orcein for four hours. Chromosomes were counted in cells at the late metaphase or early anaphase stage. Two samples were counted from each hybrid line.

2.2.4. Screening for acid phosphatase genotype

Extracts were made of 1 cm discs cut from fresh leaves of both hybrids and their parents in 100µl of 0.21 M Tris-HCl buffer (pH 8.8) in individual wells of a microtitre plate. Samples were crushed using a steel rod for approximately one minute. The supernatant was loaded into the sample wells of a gel, with 5 ml of tracker dye added to a few sample wells. Discontinuous electrophoresis was carried out at 4°C on an 8% polyacrylamide gel slab using 0.25 M Tris-glycine running buffer (pH 8.3). Current was applied at 100V for 16 hours or until the tracker dye began to run off the gel. The gel was then rinsed in three changes of warm 0.14M acetate stain buffer and stained for acid phosphatase following the technique of Allen, Misch and Morrison (1963).

2.3. RESULTS

2.3.1 S-allele analysis of B. campestris 86-155

From the analysis of family 86-155 it was possible to identify four S_aS_a plants and eight S_bS_b plants for use in the production of synthetic B. napus. A further 14 plants were heterozygous S_aS_b with S_a dominant to S_b in the pollen ($S_a > S_b$) and codominant in the stigma ($S_a = S_b$) (see Appendix 2).

2.3.2. Ovary culture

Not all pistils developed from the pollinations that were made.

However, losses were slight except for the crosses 86-155B x 83-456 (27% failed to develop) and 86-155A x 83-353 (17% failed to develop). All incubated ovaries were examined for embryos. Overall, embryo production ranged from 26 per 100 pollinations to 131 per 100 pollinations (Table 2.4), with a mean of 76. The parent 83-443 ($S_{14}S_{14}$) appeared to be a much poorer pollen source than 83-456 ($S_{29}S_{29}$) or 85-353 (S_2S_2); 83-456 was the best pollen parent, producing over one embryo per pollination.

The number of naked embryos produced per ovary ranged from 0-15 and varied in colour, size and shape. The various embryo developmental stages observed included the globular, torpedo and walking stick types (Fig. 2.7, Table 2.5). The number of abnormal cultures obtained is given in Table 2.5. All the abnormal cultures were normalised after treating with NAA and BAP except for one from which only nine out of ten shoots were obtained. Losses also occurred for crosses 1, 2 and 4 (Table 2.2), so that a final value of 143 synthetics were obtained. Thus, the number of plants produced per 100 pollinations obtained by the techniques employed varied from 21 to 128 with a mean of 63 (Table 2.5). Weaning losses were not associated with parentage, thus differences in final plant yields depended largely on differences in embryo production.

2.3.3. Embryo culture

The results of embryo culture are shown in Table 2.6. The number of embryos produced from the three different crosses ranged from 33 per 100 pollinations to 0 per 100 pollinations. In fact embryos were obtained only from the cross 83-456 x 86-155A.

2.3.4. Chromosome analysis

All the plants obtained following ovary or embryo culture were intermediate between the parents for general morphological characters. Thus all had almost white flowers, a characteristic of the B. oleracea parent which is controlled by a single gene with white dominant to yellow (Figs. 2.8, 2.9). The leaves were lobed as in B. campestris and possessed a layer of surface wax as in B. oleracea. In addition, most of the hybrids showed anthocyanin pigmentation in the leaf axis at the seedling stages, which is normal for a B. oleracea. Cytological analysis showed that the plants tested following colchicine treatment had 38 chromosomes in late metaphase or early anaphase cells.

2.3.5. Isozyme analysis

The electrophoretic phenotype of acid phosphatase in B. oleracea, B. campestris and the synthetics gave characteristic banding patterns that differed from each other (Figs. 2.10, 2.11).

2.3.5.1 B. campestris

Enzyme activity was characterised by three zones and shown in lane 1 (Figs. 2.10, 2.11). The first zone, positioned at Rf x-y, presented no clear banding pattern. The second zone was three banded with the two 'faster' bands always located in the same position, but with the slower band positioned either at Rf x or y. The third zone of activity was equivalent to that denoted as ACP 3 by Wills et al.

(1974, 1979). Within the zone three patterns of banding occurred; a single slow band at Rf a, a single fast band at Rf c or 3 bands of Rf a, b, c. This banding pattern is consistent with that described by Wills et al. (1979) who reported the presence of an intermediate heterodimer band in triple banded heterozygote at the ACP3 locus.

2.3.5.2 B. oleracea

In contrast to what was found in B. campestris, enzyme activity was characterised by two zones in B. oleracea (lane 3 - Figs. 2.10 & 2.11). The first zone of activity was similar to that of B. campestris, i.e. darkly stained between Rf x-y, but no sharp banding pattern. The second zone was four banded, of which the faster pair was always at the same distance from the origin whilst the slower pair was found either at Rf x or y.

2.3.5.3 Synthetic B. napus

The enzyme activity of the synthetics was present in four different zones on a gel (lane 2 - Figs. 2.10 & 2.11). Zone one was equivalent to that found in B. campestris and B. oleracea. Zone two was three banded, with the lower two bands equivalent to those of B. oleracea and B. campestris faster bands and the uppermost with the same as slow B. campestris bands. Zone three which consisted of a single band at Rf z was not presented in either of the parent species while zone four, which represent the ACP 3 zone mentioned above, contained a single band at Rf c which was equivalent to the fast band present in B. campestris.

2.4. DISCUSSION AND CONCLUSIONS

By means of ovary culture, 143 synthetics of B. napus were produced from 228 pollinations (63 hybrids/100 pollinations) as compared with ten from 88 pollinations (11.3 hybrids/100 pollinations) using embryo culture. Production of synthetics by ovary culture in B. campestris was therefore approximately six times more successful than that produced by embryo culture in B. oleracea. The production rate of synthetics using ovary culture varied between pollen parents (Table 2.6). Whether this reflects differences in pollen fertility or other factors is unknown. Table 2.7 summarises the data of different workers who have successfully synthesised B. napus from crosses of B. campestris x B. oleracea using an in vitro culture procedure. The results of the present study compare well with those of Inomata (1985) and confirm the high rate of success obtained using ovary culture. Moreover, despite the lower success rate achieved with embryo culture, the level of success achieved in the present study was an improvement on that of earlier workers (Table 2.7).

Successfully doubled dihaploid plants were easily identified. They exhibited a typical B. napus morphology, producing normal buds and anther development and were usually fully fertile. Haploid plants produced thin small buds with reduced anthers that lacked pollen. Isozyme banding patterns in the hybrids (synthetics) reflected a combination of the distinctive patterns of the parents. Zone activity of synthetics characterised with B. campestris Rf c band and zone three single band (T. Hodgkin, pers. comm.).

Synthetic amphidiploid B. napus is expected to have 2n = 38 chromosomes. Mitotic chromosome counts were carried out on 14 plants

derived from seven different genotypes and all plants had the normal chromosome number of 38. Based on this, it was concluded that seven different synthetic B. napus plants had been produced with the following S-allele genotype. The genotypes subscripted as 1-7 are referred to as line numbers 1-7 in following Chapters.

1. $S_{29}S_{29}$ S_bS_b
2. $S_{14}S_{14}$ S_bS_b
3. S_2S_2 S_bS_b
4. $S_{29}S_{29}$ S_aS_a
5. $S_{14}S_{14}$ S_aS_a
6. S_2S_2 S_aS_a
7. $S_{29}S_{29}$ S_aS_a

Table 2.1 Number of pollinations required by various workers to obtain a hybrid from the cross B. campestris x B. oleracea (from McNaughton, 1963)

Cross combinations female x male	Required number of pollinations per hybrid
2n *B. campestris* x 2n *B. oleracea*	432
4n *B. campestris* x 4n *B. oleracea*	211
2n *B. oleracea* x 2n *B. campestris*	15,288
4n *B. oleracea* x 2n *B. campestris*	192

Table 2.2 Number of bud pollinations made for each cross combination in which ovary culture was employed

	Cross combination			No. of pollinations
	B. campestris female	x	B. oleracea male	
1.	86-155B S_bS_b	x	83-456 $S_{29}S_{29}$	30
2.	86-155B S_bS_b	x	83-443 $S_{14}S_{14}$	47
3.	86-155B S_bS_b	x	85-353 S_2S_2	53
4.	86-155A S_aS_a	x	83-456 $S_{29}S_{29}$	33
5.	86-155A S_aS_a	x	83-443 $S_{14}S_{14}$	30
6.	86-155A S_aS_a	x	85-353 S_2S_2	35

Table 2.3 Number of bud pollinations made for each cross combination in which embryo culture was later employed

	Cross combinations *B. oleracea* x *B. campestris*		No. of bud pollinations
	female	male	
1.	83-456 $S_{29}S_{29}$	86-155A S_aS_a	30
2.	83-443 $S_{14}S_{14}$	86-155A S_aS_a	25
3.	85-353 S_2S_2	86-155A S_aS_a	33

Table 2.4 Results of interspecific hybridisation in Brassica ovary culture

Line Number	Cross combination		Number of pollinations	Number of pistils cultured	Number of losses during pistil development x 100	Number of embryos obtained	Number of embryos per 100 pollinations	Number of hybrids obtained	Number of plants per 100 pollinations
	female	male							
1	86-155B	83-456	30	22	27	33	110	25	83
2	"	83-443	47	44	10	15	31	10	21
3	"	85-353	53	49	8	35	66	35	66
4	86-155A	83-456	33	30	9	37	112	20	60
5	"	83-443	30	27	10	8	26	8	26
6	"	85-353	35	29	17	46	131	45	128
TOTAL			228	201	12	174	76	143	63

Table 2.5 Number of different developmental stages obtained in ovary culture and number of abnormalities observed during culturing

Line Number	Cross combination		Embryo stage			Number of embryos with abnormal shoots	Number of shoots normalised
	female	male	globular	torpedo	walking stick		
1	86-155B	83-456	20	3	10	-	-
2	"	83-443	10	2	3	-	-
3	"	85-353	15	5	15	8	8
4	86-155A	83-456	26	3	8	-	-
5	"	83-443	8	-	-	3	3
6	"	85-353	18	8	20	10	9

Table 2.6 Results of hybridisation between B. campestris and B. oleracea by embryo culture

Cross combination		No. of pollinations	No. of pistils cultured	No. of embryos obtained	No. of plants obtained	No. of plants obtained per 100 pollinated
female	male					
83-456	86-155A	30	10	13	10	33
83-443	"	25	12	0	0	0
85-353	"	33	5	0	0	0

Table 2.7 Past and present - results of the production of the interspecific hybrids between B. campestris and B. oleracea

	Author and Year	No. of flowers pollinated	No. of hybrids obtained	No. of hybrids per 100 pollinations
1.	U (1935)	732	4	0.54
2.	Hoffmann & Peters (1958)	20,283	48	0.24
3.	Olsson (1960b)	7,434	15	0.20
4.	Nishi et al.[1] (1970)	539	17	3.18
5.	Snell (1977)[1]	100	8	8.0
6.	Inomata (1978a)[2]	116	15	12.9
7.	Inomata (1978b)[2]	692	44	6.35
8.	Inomata (1985)[2]	568	381	67.07
9.	Present results[1]	88	10	11.36
10.	Present results[2]	228	143	63.0

[1] Embryo culture

[2] Ovary culture

Fig. 2.2. In vitro cultured pistils of a cross between B. oleracea x B. campestris on modified Nitsch and Nitsch's medium, after 18 days of culture. Arrows indicate the places where embryos have developed.

Fig. 2.3. Growth of an embryo of B. oleracea x B. campestris 10 days after culturing on basic embryo culture media.

Fig. 2.4. Growth of an embryo of *B. oleracea* x *B. campestris* 3 weeks after culturing on basic embryo culture media.

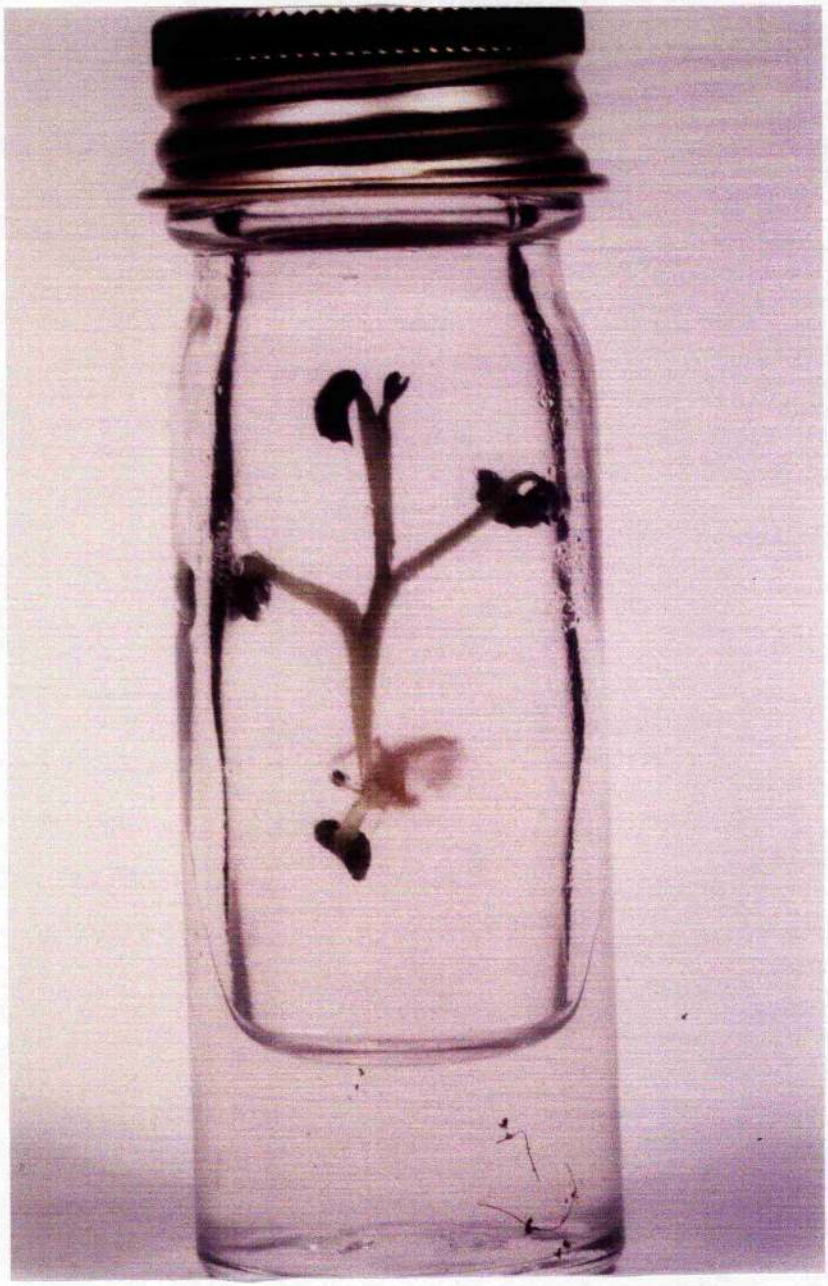


Fig. 2.5. A 10 week old culture of a hybrid embryo showing multiple shoot formation. (Arrow indicates the callusing).

Fig. 2.6. Hybrid B. napus (dihaploid) plants obtained from ovary culture.

Fig. 2.7. Stages of embryo development 1: Heart, 2 and 3: Torpedo, 4: Late torpedo, 5: Walking stick, 6: Mature embryo.

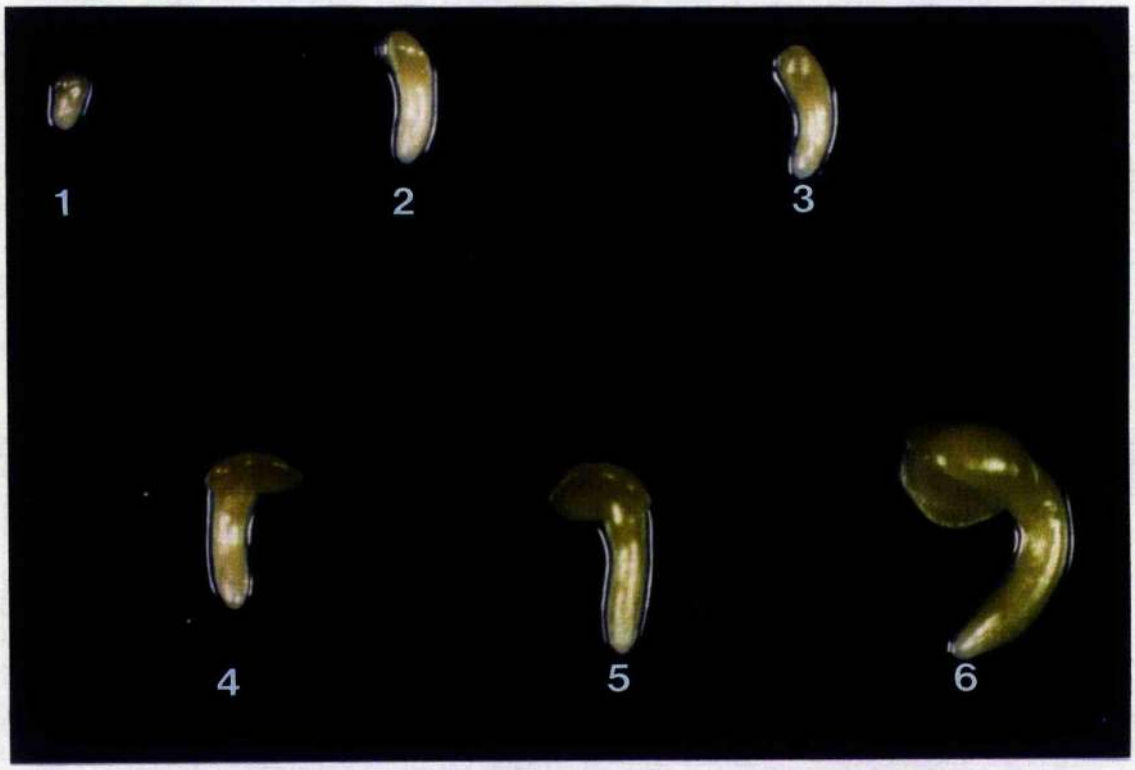


Fig. 2.8. Hybrid B. napus plant derived from ovary culture - showing leaf and flower morphology.

Note: Lobed leaves and almost white flowers.

Fig. 2.9. Leaf and flower morphology of the parents of the hybrid *B. napus*.

1. *B. oleracea* parent.

   Note: White flowers and leaf shape.

2. *B. campestris* parent.

   Note: Yellow flowers and lobed leaves.

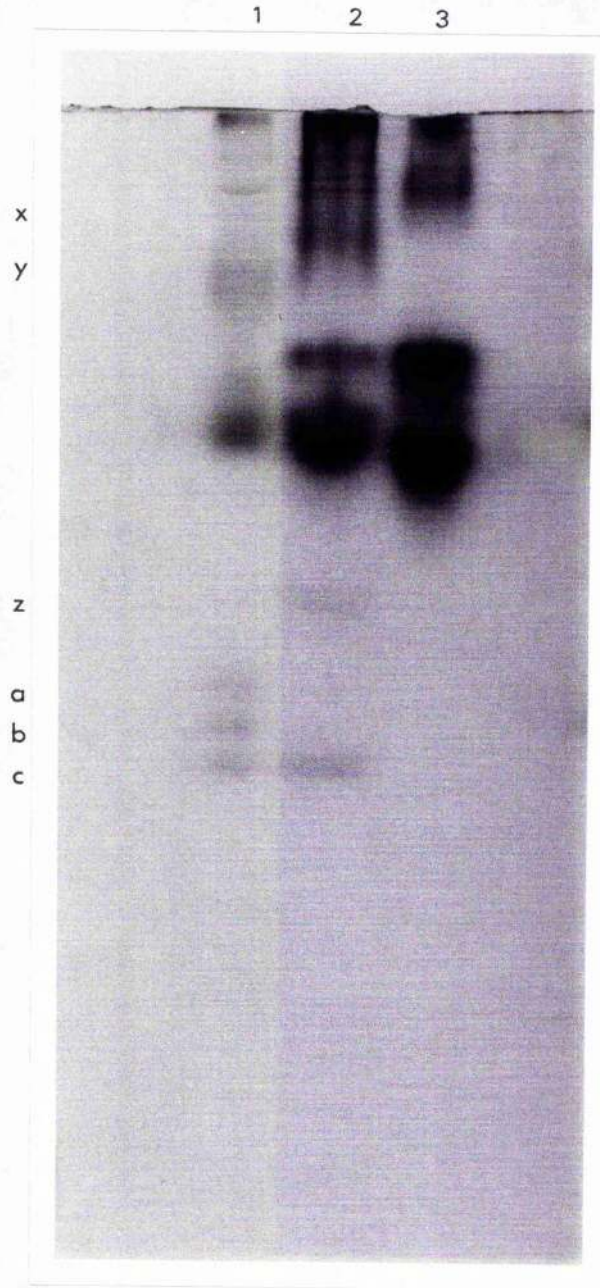


Fig. 2.10. Electrophoretic patterns of extracts of leaf samples from synthetic B. napus and parents B. oleracea and B. campestris.

Lane 1: Acid phosphatase banding of B. campestris (S_b).

Lane 2: Acid phosphatase banding of B. napus line 1 ($S_{29}S_b$).

Lane 3: Acid phosphatase banding of B. oleracea (S_{29}).

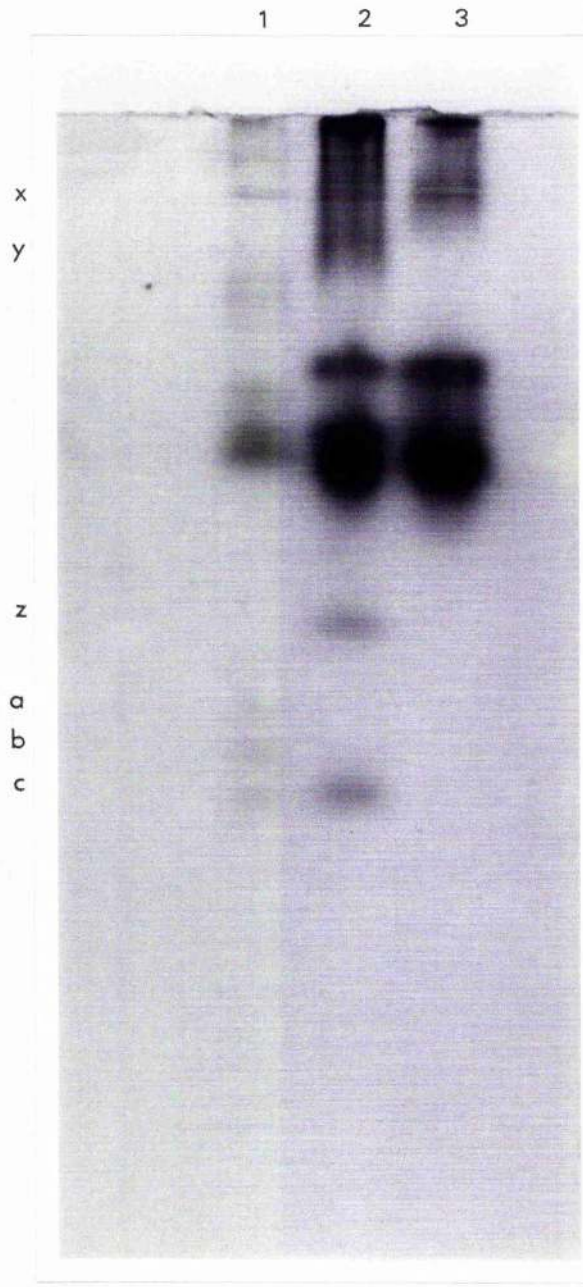


Fig. 2.11.: Electrophoretic patterns of extracts of leaf samples from synthetic B. napus and parents B. oleracea and B. campestris.

Lane 1: Acid phosphatase banding of B. campestris (S_a).

Lane 2: Acid phosphatase banding of B. napus line 4 ($S_{29}S_a$).

Lane 3: Acid phosphatase banding of B. oleracea (S_{29}).

CHAPTER 3

SELF- AND CROSS-INCOMPATIBILITY STATUS OF SYNTHETICS

3.1. INTRODUCTION

As stated in Chapter 1, B. napus is usually self-compatible, despite the fact that both parental species possess a sporophytic self-incompatibility system, controlled by a multiple series of S-alleles at a single locus (Bateman, 1955; Thompson, 1957; Mackay, 1977b). However, Olsson (1960a) found naturally occurring lines of self-incompatible B. napus ssp. oleifera and, moreover, showed that artificially produced B. napus could exhibit self-incompatibility. Gowers (1974) has also reported self-incompatibility in B. napus ssp. rapifera and functional S-alleles have been successfully introgressed into forage rape B. napus ssp. L. from turnips, B. campestris (Mackay, 1976).

In the early part of the present research (described in Chapter 2), seven B. napus lines were produced using parents of known S-allele constitution. The objective of the work reported in this Chapter was to determine the self-incompatibility status of these lines, and the activity and interactions of the parental S-alleles in synthetic B. napus.

The approach chosen was to intercross all seven lines, in all combinations, and, further, to cross them reciprocally with the parental S-allele homozygotes. These crosses were examined to determine the extent to which pollen tubes had penetrated the stigmatic surface utilising the staining procedures of Martin (1959).

3.2. MATERIALS AND METHODS

The origin of the synthetic lines of B. napus used in the present study was described in Chapter 2. The genotypic status of these lines is listed below:-

1. $S_{29}S_{29}S_bS_b$
2. $S_{14}S_{14}S_bS_b$
3. $S_2S_2S_bS_b$
4. $S_{29}S_{29}S_aS_a$
5. $S_{14}S_{14}S_aS_a$
6. $S_2S_2S_aS_a$
7. $S_{29}S_{29}S_aS_a$

Plants of each line were maintained in 13.2 mm pots, watered daily, treated with Hoagland's solution (see Appendix 3) once a week, and maintained at approximately 20°/14°C day/night temperature until they began to flower.

3.2.1. Experiment I

Intercrosses were made between all seven lines. All possible crosses were made between each line, using 3 plants per line. Test pollinations were carried out on five newly opened flowers, and pollen tube penetration assessed, as described later in this Chapter. All test pollinations were repeated using three flowers and all plants were pollinated with pollen from a known compatible cross, so that allowance could be made for female sterility when assessing compatibility levels. In addition to these treatments, each line was self-pollinated to determine its self-incompatibility.

3.2.2. Experiment II

All seven lines were reciprocally crossed with the B. oleracea and B. campestris parental lines that were homozygous for the S_{29}, S_{14}, S_2, S_a and S_b alleles. These test pollinations were carried out on three newly opened flowers and pollen tube penetration assessed as described later in this Chapter. Each pollination was repeated on at least three flowers per cross.

3.2.3. Determination of pollen tube penetration

All the required pollinations were carried out in the laboratory on open flowers by brushing freshly dehisced anthers of the required pollen parent onto the stigmatic surface of a flower from which the anthers had been removed before dehiscence. After 24 hours the entire gynoecium was removed and fixed in approximately 2 ml of chloro-acetic-alcohol prepared as follows:-

Absolute alcohol	60 ml
Chloroform	30 ml
Acetic acid	10 ml

Pistils were left in fixative for 2-24 hours, rinsed in distilled water for about 30 seconds and softened in 1N NaOH for one hour at 60°C. They were then left to stain in a solution of 0.1% water-soluble aniline blue in 0.1N tripotassium phosphate (K_3PO_4). Sufficient staining of the tissue usually occurred after three to four hours. For examination, pistils were mounted in 80% glycerol and gently squashed by touching the cover slip with the forceps. Counts

of pollen tubes were made within a day, after which the slides were discarded. A Zeiss universal microscope with incident illumination was used with barrier and exciter filters, providing a maximum level of light transmission at wave lengths 365 and 404.7 nm.

The fluorescent properties of pollen tubes stained with aniline blue were first fully described by Martin (1959). Aniline blue is a water-soluble acid dye of the triphenylamine series with molecular weight of 738. It stains the callose layer of the pollen tube wall and callose plugs within the pollen tube, such that they fluoresce yellow to green in uv light. Callose is a cell wall polysaccharide composed of beta 1-3 glucan units (Currier, 1957). It is common in phloem sieve tubes and the tapetum of the developing pollen grain, as well as in the pollen tube. Currier reported that callose is rapidly synthesised during differentiation of certain plant tissues, especially following wounding or infection by a pathogen.

In incompatible pollinations callose formation provides a useful phenotypic bioassay, relating to rejection phenomena. Heslop-Harrison et al. (1973) showed the rejection includes the deposition of leticules of callose in the stigma papillae adjacent to the incompatible pollen and within the germinating pollen tubes, as well as the tips of the rejected tubes.

3.3. RESULTS AND ANALYSIS

3.3.1. Compatible and incompatible pollinations

Throughout the project, large numbers of compatible and incompatible pollinations were examined. In this study, a compatible cross was

considered to be one in which the number of pollen tubes per stigma was above 30 and an incompatible one below 20 (see section 3, below, for discussion of this). Accuracy in counting pollen tubes following a compatible pollination was extremely difficult. Great variation in the appearance of both compatible and incompatible pollinations was noted.

Stigmas which have been pollinated with compatible pollen exhibit a mass of brightly stained fluorescent material. With careful squashing, it is often possible to achieve reasonable separation of this fluorescent mass and to distinguish the individual pollen tubes (Fig. 3.1). Pollen grains are observed attached to the papillae as dark circular bodies, with a faintly fluorescent rim and a yellow fluorescent spot at the point of tube emergence. Pollen tubes which have penetrated the stigmatic papillae immediately deposit a relatively large amount of fluorescent callose material. Following penetration, individual pollen tubes grow down the side of the papillae cells usually at a density of no greater than one per papilla. While growth through the papillae may appear uneven and be associated with the deposition of relatively large amounts of callose, tube growth through the rest of the stigma, and in the style, is extremely regular (Fig. 3.2). Tubes appear to be almost straight and smooth-walled with occasional callose plugs occurring throughout their length. In the style, the tubes merge into a cone until they reach the ovary at which point they spread out and appear to wander individually through the ovary tissue. As the tube enters an ovule, it tends to thicken with the deposition of greater amounts of callose. Penetration of an ovule by more than one pollen tube is extremely rare

although the tube that enters an ovule sometimes appears to divide shortly before entry. After entry, it ceases to be visible (Fig. 3.3).

The fate of tubes which do not reach an ovule is not clear. Since over 100 tubes may penetrate the stigma to fertilise 20-30 ovules and only 20 seeds be set, there must be considerable wastage. Counts of tube numbers made at the top of the ovary would seem to indicate that more than half of the penetrating tubes fail to reach this point (see also Ockendon, 1972). Some have been observed to grow directly through the ovary and others, while travelling laterally towards ovules, fail to enter an ovule.

Although the above may be true for the great majority of pistils pollinated with compatible pollen, considerable variation can occur. Often pollen tubes are obscure in the stigma and upper part of the style, and only become distinct as they move down through the style. In some extreme cases, such poor resolution is associated with the absence of any clear plug formation in the tubes which appear to possess, instead, a large number of very small depositions which fluoresce only faintly.

In a fully incompatible pollination no tubes will penetrate through the stigmatic papillae. However, there is frequently considerable variation in the extent of grain development. The most extreme manifestation of incompatibility is one in which no pollen grains are visible on the style as they are presumably washed off during processing, having failed to adhere to the stigmatic surface. In such cases there is little or no fluorescent tissue present, apart from papillae (Figs. 3.4).

In most incompatible pollinations, pollen grains adhere to the stigma and often show a degree of germination. The extent of this

germination, without apparent stigma penetration, can vary and sometimes reaches extreme proportions, with tubes growing to more than twice the diameter of the pollen grain and curling round the papilla. However, even when pollen tubes appear to penetrate the pellicle, cuticle or cellulose pectin layer of the papilla cell, they do not grow out from the base of the papillae and it was this character that was used to distinguish a successful tube from an unsuccessful one in assessment of the degree of incompatibility.

Counts were made in the stigma immediately below the papilla layer wherever possible, and as confirmation, a further count was made at the top of the style. In the style, pollen tubes and phloem sieve tubes may occasionally be confused and the differences in appearance described by Chu Chou & Harberd (1970) proved useful to distinguish them.

3.3.2 Determining the degree of incompatibility

Pollen tube counts penetrating the stigma provided the data used to determine the compatibility or incompatibility of the test pollinations. Other workers have used seed set data and the presence of S-allele specific glycoproteins in stigmas to determine S-allele constitution and the self-incompatibility level in Brassica spp (e.g. Thompson, 1957; Nasrallah & Wallace, 1967b; Nasrallah et al., 1970; Nishio & Hinata, 1977, 1979; and Roberts et al., 1979). Seed set provides a much more indirect measure than pollen tube number of the compatibility of a pollination, since it is subject to fertilisation and post-fertilisation factors that affect the final number of seeds

produced. Although still used (e.g. Gemmell et al., 1989), seed set numbers are less satisfactory estimators of incompatibility than pollen tube penetration. The presence of stigmatic glycoproteins, even when S-specific ones have been identified, provides no data on S-allele activity, and the degree of incompatibility. Indeed, as will be shown in Chapter 7, S-allele correlated proteins may be present even when the allele is inactive.

As noted above, inhibition of pollen tubes on the stigmatic surface is characteristic of the incompatibility response, and Roberts et al. (1984a) have shown that this is associated with a reaction occurring at the surface of the stigmatic papillae. Considerable difficulties were encountered in distinguishing compatible and incompatible pollinations, on the basis of tube numbers. Variation in pollen tube numbers between flowers used in the same test cross was often considerable. In extreme cases, adjacent flowers could appear fully compatible (>75 tubes) or fully incompatible (0 tubes) although this was rare. However, a significant number of pollinations gave tube numbers above 0, but well below the number (30+) that would be expected in a normally functioning fully compatible pollination. As Fig. 3.5 shows, approximately 80% of test results can be clearly distinguished as having less than 10 or more than 50 pollen tubes per stigma. From Fig. 3.5, it can be seen that the smallest class was that containing 21-30 pollen tubes. It was therefore decided to consider a cross which yielded a mean of over 30 pollen tubes per stigma as compatible, while crosses which yielded less than 20 tubes per stigma as incompatible. Intermediates which gave 21-30 pollen tubes were evaluated individually.

3.3.3. Results of Experiment I

Interline pollination results are presented in Table 3.1. Each line, except line 5, was fully self-incompatible with mean tube numbers of 0. Line 5 was self-compatible with a mean tube number of 41. Using the criteria that below 20 tubes per test is incompatible and over 20 is compatible, one can deduce the activity of the S-alleles in all the seven lines.

3.3.3.1. Line 1 - $S_{29}S_{29}S_bS_b$

The reactions of line 1 with the other lines were as follows:

Line no.	Allelic constitution	$S_{29}S_b$ male	$S_{29}S_b$ female
2	$S_{14}S_b$	+	+
3	S_2S_b	+	-
4	$S_{29}S_a$	-	-
5	$S_{14}S_a$	+	+
6	S_2S_a	+	+
7	$S_{29}S_a$	-	-

Line 1 was incompatible in both directions with lines 4 and 7 with never more than 5 tubes produced per stigma. This showed that S_{29} was active in both pollen and stigma of line 1. The line was fully compatible with lines which did not possess any common S-alleles, although pollen from line 6 gave a mean tube number of 24 with a range from 0 to >75, either through poor pollen fertility or because of some

interaction unrelated to S-allele identity. Pollinations with lines 2 and 3 (possessing S_b) as female parents were compatible, providing evidence that either S_{29} was dominant to S_b in pollen of line 1, or alleles S_{14} and S_2 were both dominant to S_b in the stigma of lines 2 and 3 (see below). Pollen from line 3 was incompatible in stigmas of line 1 demonstrating that S_b was active in the stigma. (Thus indicating that in line 2, S_{14} was dominant to S_b in the pollen). Therefore S_{29} and S_b were codominant in stigma ($S_{29} = S_b ♀$).

3.3.3.2. Line 2 - S_{14} S_{14} S_b S_b

The reaction of line 2 with other lines was as follows:

Line No.	Allelic constitution	$S_{14}S_b$ male	$S_{14}S_b$ female
1	$S_{29}S_b$	+	+
3	S_2S_b	+	-
4	$S_{29}S_a$	+	+
5	$S_{14}S_a$	+	-
6	S_2S_a	+	+
7	$S_{29}S_a$	+	+

The results from intercrosses using line 2 as pollen were consistent with the hypothesis that S_{14} was dominant to S_b in pollen ($S_{14} > S_b ♂$) although the cross between line 5 stigma and line 2 pollen gave a compatible result. However, since line 5 was self-compatible, it is impossible to deduce the dominance of the S-alleles from these results. In fact, self-compatibility in line 5 appeared to

result from a stigmatic rather than pollen-related lesion (see below). Pollinations with lines 3 and 5 as pollen parent were incompatible, indicating activity of both S_{14} and S_b in the stigma of line 2 (S_{14} = S_b ♀). These findings were consistent with the hypothesis that S_{29} was dominant to Sb in pollen of line 1 ($S_{29} > S_b$ ♂) as suggested in the previous section.

3.3.3.3. Line 3 - $S_2 S_2 S_b S_b$

The reactions of line 3 with other lines were as follows:

Line No.	Allelic constitution	S_2S_b male	S_2S_b female
1	$S_{29}S_b$	-	+
2	$S_{14}S_b$	-	+
4	$S_{29}S_a$	+	+
5	$S_{14}S_a$	+	+
6	S_2S_a	-	+
7	$S_{29}S_a$	+	+

Pollen of line 3 was incompatible with lines 1 and 2 with no more than 4 tubes produced per stigma. This result shows that S_b was active in the pollen of line 1, and is consistent with the hypothesis that S_b was active in the stigmas of both line 1 and 2. Pollen of line 3 was also fully incompatible with line 6 producing a mean of 3 pollen tubes per stigma. This indicates that S_2 is also active in pollen of line 3 as well as in the stigmas of line 6 (see below). Thus it is concluded that S_b and S_2 were both active in the pollen of

the synthetics (codominant, $S_2 = S_b♂$).

Owing to dominance of S_{14} and S_{29} to S_b in pollen (see above), the activity of S_b in stigmas of line 3 could not be determined. Compatibility with line 6 pollen indicated either that S_b was dominant to S_2 in the stigma or that S_a was dominant to S_2 in the pollen of line 6. Resolution of this relationship could only be determined from the results of the analysis that employed S-allele tester lines (Expt. 2 below).

3.3.3.4. Line 4 - S_{29} S_{29} S_a S_a

The reaction of line 4 with other lines was as follows:

Line No.	Allelic constitution	$S_{29}S_a$ male	$S_{29}S_a$ female
1	$S_{29}S_b$	-	-
2	$S_{14}S_b$	+	+
3	S_2S_b	+	+
5	$S_{14}S_a$	+	+
6	S_2S_a	-	+
7	$S_{29}S_a$	-	-

Line 4 was incompatible in both directions with line 1 with a mean tube number of 3. This indicated that S_{29} was active in both pollen and stigma in line 4. Also it was consistent with the hypothesis that S_{29} was active in stigmas of line 1 (see above). Pollinations with line 6 (possessing S_a) as female parent were incompatible with no more than 9 tubes per stigma; this suggests that S_a was active in the

pollen of line 4 and stigmas of line 6 (see below). Thus, in pollen it appears that S_{29} and S_a were both active (codominant, $S_{29} = S_a$♂). Pollinations with lines 5 and 6 as pollen parents were compatible. However, since family 5 was self-compatible with a mean of 41 tubes, it is impossible to deduce the allelic relationship from this pollination. But pollen from line 6 was compatible on stigmas of line 4, indicating either that S_{29} is dominant to S_a in the stigma of line 4 or S_2 is dominant to S_a in the pollen of line 6 (see below).
Line 4 was also fully compatible with lines which did not possess a common S-allele.

3.3.3.5. Line 5 - S_{14} S_{14} S_a S_a

The reactions of line 5 with the other lines were as follows:

Line No.	Allelic constitution	$S_{14}S_a$ male	$S_{14}S_a$ female
1	$S_{29}S_b$	+	+
2	$S_{14}S_b$	-	+
3	S_2S_b	+	+
4	$S_{29}S_a$	+	+
6	S_2S_a	-	+
7	$S_{29}S_a$	+	+

Pollen of line 5 was incompatible on stigmas of lines 2 and 6 with no more than 5 pollen tubes produced per stigma: this shows that both S_{14} and S_a alleles were active in the pollen of line 5 ($S_{14} = S_a$♂) and S_a and S_{14} were active in stigma of lines 6 and 2 respectively.

This provides evidence that in line 4, S_{29} was dominant to S_a in the stigma ($S_{29} > S_a$ ♀). Stigmas of line 5 were fully compatible with all other lines consistent with the hypothesis (see above) that self-compatibility in line 5 appeared to result from a stigmatic rather than pollen-related lesion, thus preventing the determination of allele activity in stigmas of line 5.

3.3.3.6. Line 6 - $S_2 S_2 S_a S_a$

The reactions of line 6 with the other lines were as follows:

Line No.	allelic constitution	S_2S_a male	S_2S_a female
1	$S_{29}S_b$	+	+
2	$S_{14}S_b$	+	+
3	S_2S_b	+	-
4	$S_{29}S_a$	+	-
5	$S_{14}S_a$	+	-
7	$S_{29}S_a$	+	-

Line 6 was incompatible with pollen from lines 3, 4, 5 and 7 with fewer than 9 tubes per stigma. This shows that S_2 and S_a were both active in the stigmas of line 6, ($S_a = S_2$ ♀) and provides supporting evidence for the hypothesis that S_2 was active in line 3 and S_a was active in line 4 (see above). Line 6 was compatible with all the other lines when used as the female parent, although in crosses with line 1 there was a wide range in the tube numbers per stigma (from 0 to 75). Two further cross pollinations were made between these two

lines and gave a mean of 24 tubes (Table 3.2).

Outcrosses showed that stigmas of line 1 were fully fertile as was line 6 pollen. Since these lines have no S-alleles in common, it appears that some interaction occurred between pollen of line 6 and line 1, preventing pollen tube penetration.

Owing to the dominance of S_{29} to S_a in stigmas of line 4 (see above), activity of S_a in pollen of family 6 could not be determined. Compatibility with line 3 stigmas indicated either that S_a was dominant to S_2 in pollen of line 6 or S_b was dominant to S_2 in the stigma of line 3. This relationship could only be determined from the results with S-allele tester lines.

3.3.3.7. Line 7 - S_{29} S_{29} S_a S_a

The reaction of line 7 with the other lines was as follows:

Line No.	Allelic constitution	$S_{29}S_a$ male	$S_{29}S_a$ female
1	$S_{29}S_b$	-	-
2	$S_{14}S_b$	+	+
3	S_2S_b	+	+
4	$S_{29}S_a$	-	-
5	$S_{14}S_a$	+	+
6	S_2S_a	-	+

Line 7 has the same allelic constitution as line 4 and the reactions with the other lines showed the generally same results (see above). It was confirmed therefore, that S_{29} and S_a were both active

in the pollen (S_{29} = S_a ♂ codominant) and, in the stigmas, S_{29} was dominant to S_a (see above).

3.3.4. Results of Experiment II

The results of the test crosses of the synthetics with the parental stocks i.e. B. campestris var chinensis 86-155A and 86-155B which were homozygous for S-alleles S_a and S_b respectively; and B. oleracea var alboglabra lines which were homozygous for S_{29}, S_{14} and S_2, are summarised in Tables 3.3 and 3.4.

3.3.4.1. **The reaction of the synthetics with B. campestris**

Crosses between the synthetic lines, (acting as the male parent) and the B. campestris stocks (S_a and S_b - as female parents) gave results in agreement with those from intraline pollinations (see above - Experiment I) and are presented in Table 3.3.

Synthetic lines 1 and 2 were compatible with both B. campestris S_a and S_b as female parent and, therefore, in agreement with the interline results, which showed that S_{29} and S_{14} were dominant to S_b in the pollen (see above - Experiment I). Pollen of line 3 was incompatible with the B. campestris S_bS_b parent producing 0-20 tubes per stigma; thus S_b was active in the pollen of line 3. Lines 4, 5 and 6 were incompatible with the S_aS_a homozygote suggesting that S_a was active in the pollen, a finding which was consistent with that deduced from the intraline results (see above - Experiment I) and one which shows that the S_a allele was active in line 6 as well as in lines 4 and 5. All the crosses between B. campestris and the 7 lines

which did not involve a common S-allele, were fully compatible.

For the reciprocal crosses, it was established that lines 1 and 2 were compatible with S_a pollen and incompatible with S_b pollen with never more than 1 pollen tube produced per stigma. This was consistent with the hypothesis that S_{29} and S_{14} were both codominant with S_b ($S_{29} = S_b$, $S_{14} = S_b$ ♀) in the stigma (see above - Experiment I). Lines 4 and 7 treated with either S_a and S_b pollen gave compatible pollinations with a mean of 30 tubes, a finding which was again consistent with the intraline results ($S_{29} > S_a$ ♂). Line 6 as female parent was incompatible with S_a and compatible with S_b pollen showing the activity of the Sa allele (see above - Experiment I). Line 3 was incompatible with S_b pollen but compatible with S_a pollen, suggesting that S_b was active in the stigma of line 3.

3.3.4.2. **The reaction of synthetics with B. oleracea**

Crosses between the B. oleracea parental lines as female parent and the synthetic lines as male parent gave the expected results except for the cross between $S_{29}S_{29}$ and pollen from line 6 (Table 3.4). B. oleracea plants homozygous for the S_2 allele were incompatible with pollen from line 3, confirming that S_2 was active in the pollen. All other crosses with this tester were compatible except that the number of tubes in the cross with line 5 pollen was low. The results from Experiment I showed that line 6 pollen was compatible on line 3 stigmas, indicating that either S_a was dominant to S_2 in pollen of line 6 or S_b was dominant to S_2 in the stigma of line 3 or both, and, as noted above, this could not be determined. The tests in

Experiment II showed that S_2 was not active in pollen of line 6. As expected, the B. oleracea parent homozygous for the S_{29} allele was incompatible with pollen from lines 1, 4 and 7 which confirms the hypothesis that $S_{29}>S_b$ and S_{29} and S_a were codominant in pollen (see above Experiment I). However, as noted above, the S_{29} homozygote was also incompatible with pollen from line 6 which was an unexpected result. Two further test crosses (3 stigmas/test) made between the B. oleracea homozygote and line 6, gave means of less than 1 pollen tube per stigma. Fertility of the $S_{29}S_{29}$ tester and line 6 were confirmed by outcrosses. It is possible that the apparent incompatibility between lines known not to possess S-alleles in common, is similar to that observed between line 1 (which possess S_{29}) and line 6, however its cause cannot be determined from the existing data. Tests with S_{14} stigmas confirmed the activity of S_{14} in line 2 pollen. However, S_{14} stigmas were compatible with pollen from all the other lines, even with line 5 for which results from Experiment I had suggested that S_{14} was active in the pollen. Again, without further data it is not possible to resolve this contradiction.

Interspecific crosses using B. oleracea pollen produced results that did not fit any simple model of S-allele activity. S_2 pollen was incompatible with all 7 lines, S_{29} was only compatible with line 4, and S_{14} only with lines 1, 3, 4 and 5. Thus each tester gave incompatible results with lines known not to contain the S-allele of the tester. Since all testers and lines were fully fertile it seems that some other aspect of pollen stigma recognition may be responsible. The extent and nature of incompatibility between B. napus stigmas and B. oleracea pollen is discussed more fully in Chapter 4.

3.4. DISCUSSION AND CONCLUSIONS

The major finding to emerge from Experiments I and II was that *B. napus* plants synthesised from self-incompatible *B. campestris* and *B. oleracea* were usually self-incompatible and that the self-incompatibility alleles of both *B. oleracea* and *B. campestris* were expressed in the synthesised lines of *B. napus* (Table 3.5). In general the expression and activity of alleles could be determined from crosses between synthetics. Results of crosses between the synthetics and the parental species, *B. campestris* and *B. oleracea* confirmed this and provided useful additional information. Thus the dominance of S_2 to S_a in pollen of line 6 and codominance of S_2 and S_b in stigma of line 3 could not be detected from the intraline test crosses alone. Test crosses with these S-allele tester lines were generally consistent, but there were some anomalies. In particular, the results of the test cross between *B. oleracea* S_{29} stigma with the line 6 pollen contradicted those from Experiment I, and also *B. oleracea* as pollen did not give any consistent evidence for allele relationship.

Codominance in the stigma was detected in stigmas between alleles $S_{29}S_b$; $S_{14}S_b$; S_2S_a; and in the pollen between alleles S_2S_b; $S_{29}S_a$ and $S_{14}S_a$. Except for the combinations S_2S_b and $S_{14}S_a$, one allele (either from *B. campestris* or *B. oleracea*) was dominant to the other either in the pollen or the pistil showing that interlocus epistasis was common in these synthetic lines of *B. napus* (Table 3.5).

The relationship between S-alleles in line 5 stigma was not determined because the line was self-compatible. This self-compatibility could be due to some allele interaction between the allele derived from *B. oleracea* and *B. campestris* or to an interaction

unrelated to S-allele identity.

In conclusion, these results showed that the self-incompatibility system in the synthetic B. napus lines were generally functional, and that both B. campestris and B. oleracea alleles were active and could show interactions similar to those found in single locus sporophytic systems between S-alleles in S-heterozygotes.

B. oleracea as female parent and B. campestris as both female and male parent with synthetics could be used for the detection of active S-alleles, but B. oleracea as pollen was not informative.

Table 3.1 Mean numbers of pollen tubes penetrating the stigma following crosses between 7 synthetic B. napus lines of known S-allele constitution

Stigma Line Number	Allelic constitution	Pollen line number 1 $S_{29}S_b$	2 $S_{14}S_b$	3 S_2S_b	4 $S_{29}S_a$	5 $S_{14}S_a$	6 S_2S_a	7 $S_{29}S_a$
1	$S_{29}S_b$	0	>75	0	1	59	24	0
2	$S_{14}S_b$	>75	0	1	>75	0	>75	>75
3	S_2S_b	66	37	0	70	47	60	36
4	$S_{29}S_a$	0	64	>75	0	>75	56	0
5	$S_{14}S_a$	63	>75	>75	67	41	>75	63
6	S_2S_a	50	53	2	0	10	0	1
7	$S_{29}S_a$	0	>75	41	0	35	43	0

Table 3.2 Pollen tube per stigma in cross between synthetic B. napus line 1 ($S_{29}S_b$) x line 6 (S_2S_a)

Test	Pollen tube numbers					Mean
1	20	30	0	10	24	17
2	75	12	0			29
3	75	1	0			25
4	75	0	0			25
Total						24

Table 3.3 Mean numbers of pollen tubes penetrating the stigma following crosses between 7 synthetic lines and B. campestris S-allele tester lines

Stigma line number	Allelic constitution	Pollen line number 1	2	3	4	5	6	7	B. campestris	
		$S_{29}S_b$	$S_{14}S_b$	S_2S_b	$S_{29}S_a$	$S_{14}S_a$	S_2S_a	$S_{29}S_a$	S_a	S_b
1	$S_{29}S_b$								>75	0
2	$S_{14}S_b$								>75	0
3	S_2S_b								60	0
4	$S_{29}S_a$								55	>75
5	$S_{14}S_a$								n.t.	>75
6	S_2S_a								0	50
7	$S_{29}S_a$								0-75	>75
B. campestris	S_a	>75	>75	>75	0-25	0-21	0-25	n.t.	0	>75
	S_b	33	50	0-20	>75	>75	>75	>75	>75	0

n.t. - not tested

Table 3.4 Mean numbers of pollen tubes penetrating the stigma following crosses between 7 synthetic lines and B. oleracea S-allele tester lines

Stigma Line Number	Allelic constitution	Pollen line number 1 $S_{29}S_b$	2 $S_{14}S_b$	3 S_2S_b	4 $S_{29}S_a$	5 $S_{14}S_a$	6 S_2S_a	7 $S_{29}S_a$	B. oleracea S_2	S_{29}	S_{14}
1	$S_{29}S_b$								1	0	47
2	$S_{14}S_b$								0	20	0
3	S_2S_b								0	1	32
4	$S_{29}S_a$								0	43	30
5	$S_{14}S_a$								6	7	>75
6	S_2S_a								7	11	3
7	$S_{29}S_a$								0	0	0
B. oleracea	S_2	>75	38	0	67	25	32	>75	4	>75	>75
	S_{29}	0	61	45	2	29	0	0	>75	0	>75
	S_{14}	>75	0	>75	>75	>75	>75	>75	>75	>75	1

Table 3.5 S-allele relationship of synthetic B. napus lines as deduced from Experiment I and II

Stigma	Pollen
$^{1}S_{29} = S_b$	$^{1}S_{29} > S_b$
$^{1}S_{14} = S_b$	$^{1}S_{14} > S_b$
$^{2}S_2 = S_b$ or $S_b > S_2$	$^{1}S_2 = S_b$
$^{1}S_{29} > S_a$	$^{1}S_{29} = S_a$
$^{3}S_{14}$? S_a	$^{1}S_{14} = S_a$
$^{1}S_2 = S_a$	$^{2}S_a > S_2$

1 allele activity deduced from Expt. I
2 allele activity deduced from Expt. II
3 allele activity is not detected, possibly alleles non-functional.

Fig. 3.1. Compatible pollination of a synthetic B. napus pistil (x 350).

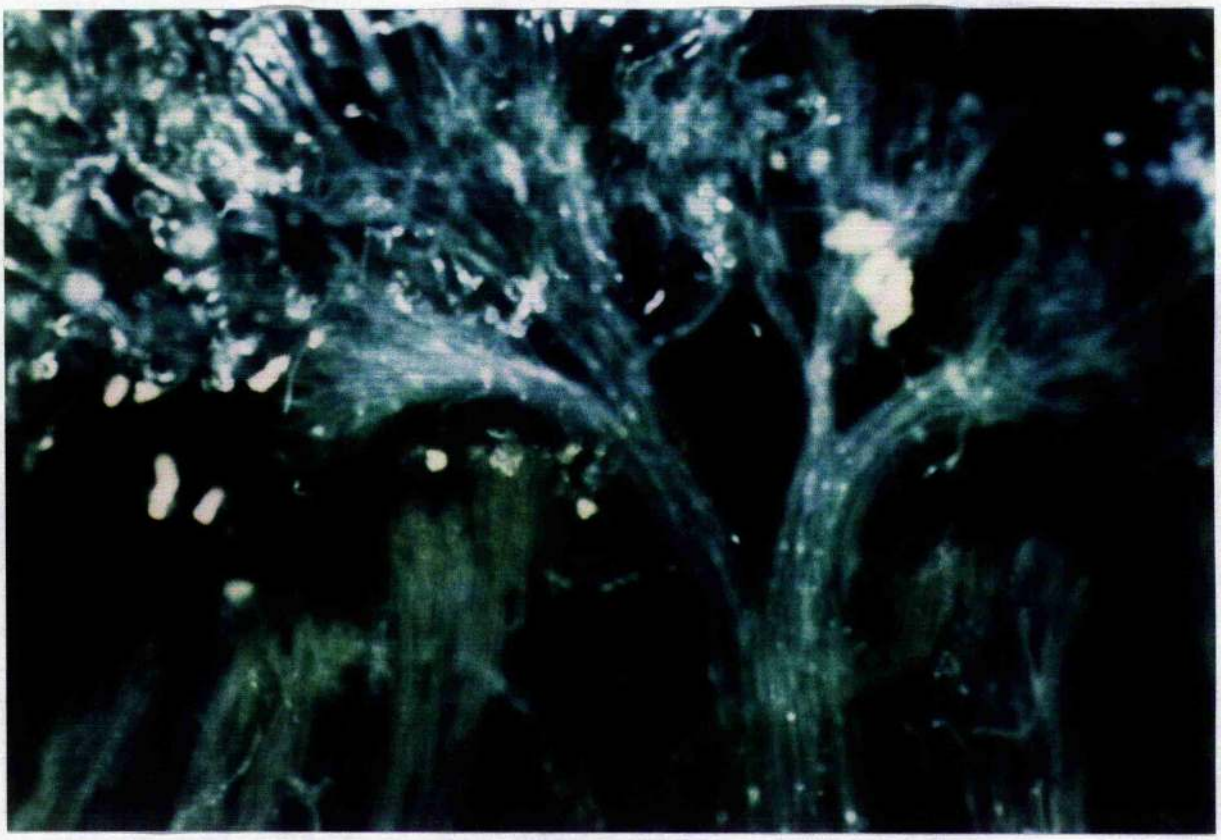


Fig. 3.2. Pollen tubes in the style after a compatible pollination of a synthetic B. napus pistil (x 350).

Fig. 3.3. Pollen tube entering an ovule (x 400).

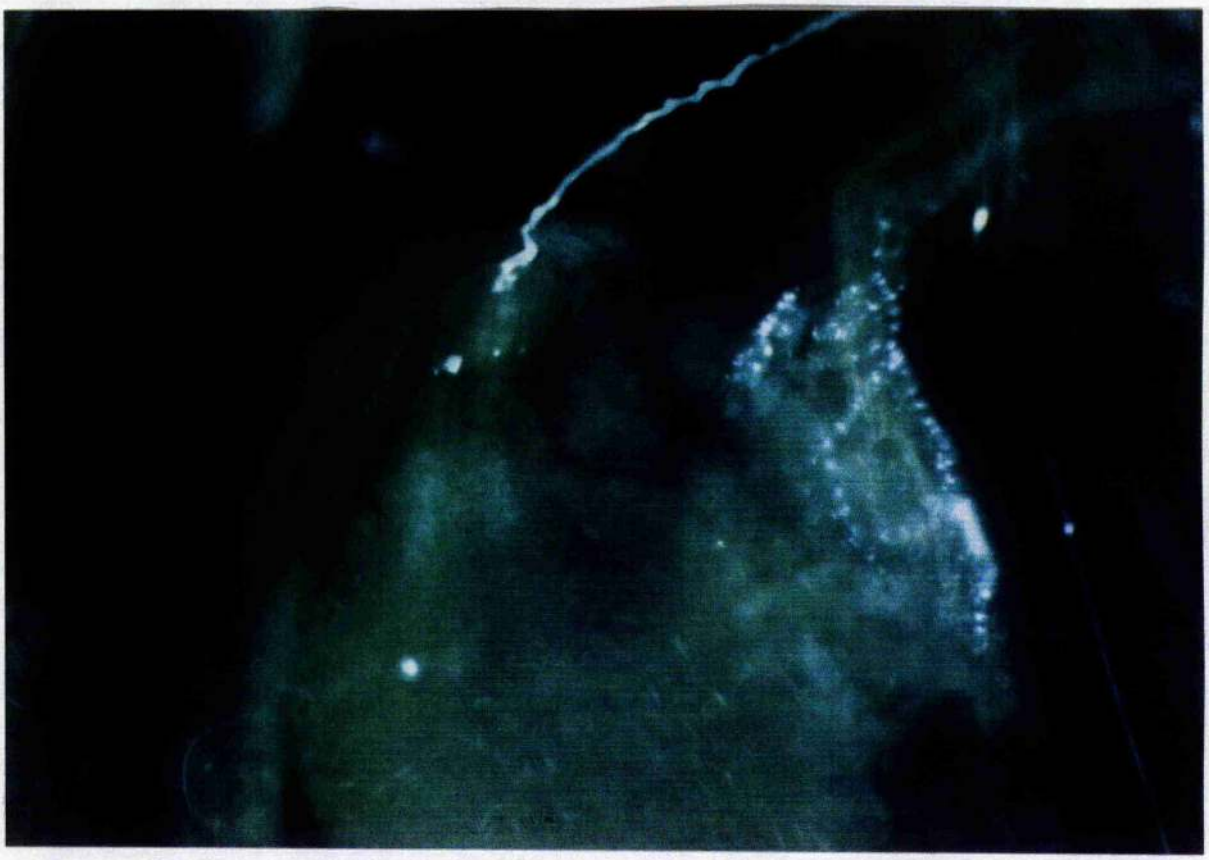


Fig. 3.4. An incompatible self-pollination of a B. napus pistil with no extensive pollen germination and some callose fluorescence in the stigmatic papillae and early germination of pollen grain (x 350).

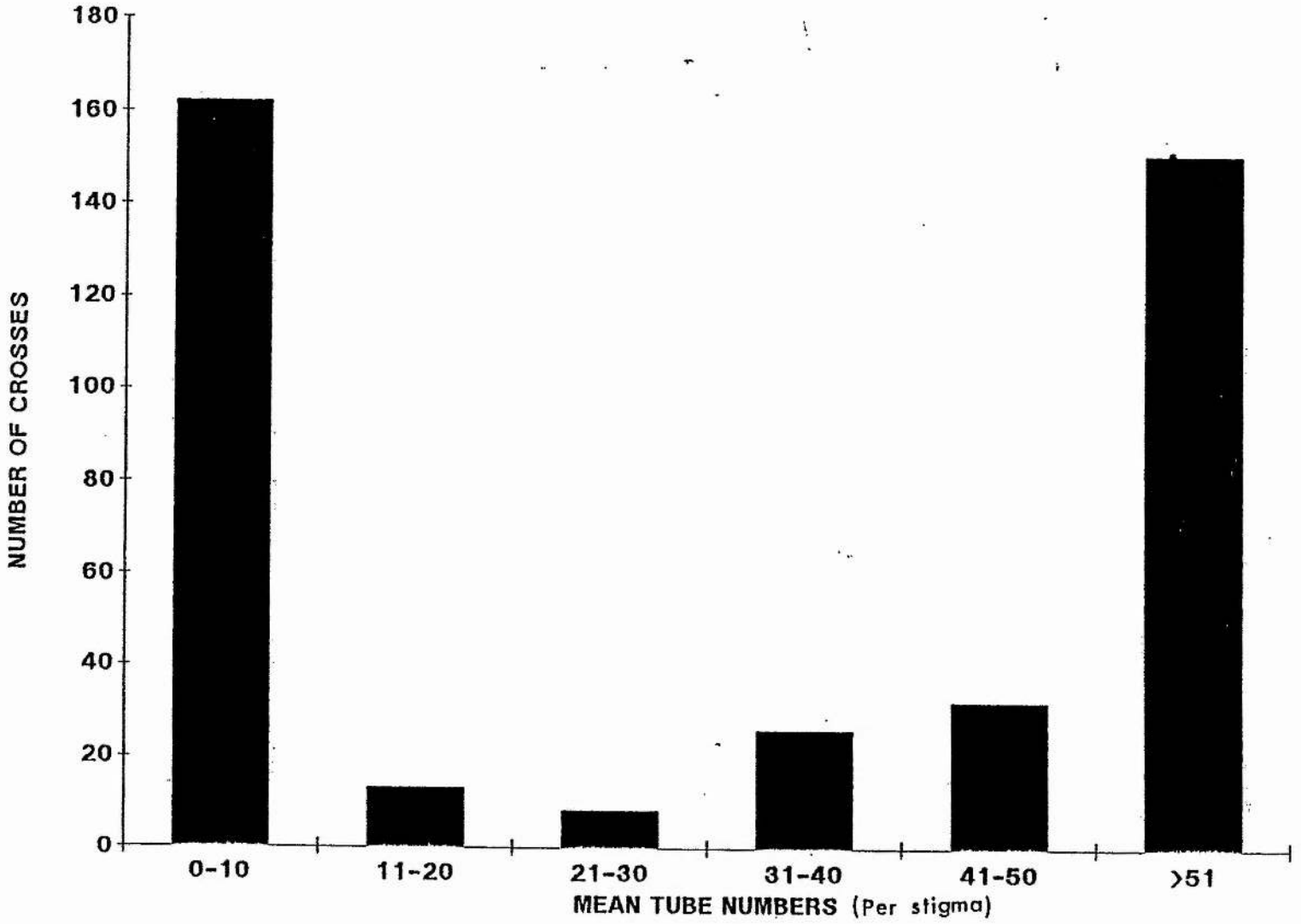


Fig. 3.5. Distribution of mean pollen tube numbers per stigma from crosses between synthetic B. napus lines.

CHAPTER 4

INTERSPECIFIC-INCOMPATIBILITY IN BRASSICAS

4.1. INTRODUCTION

4.1.1. Interspecific-incompatibility

Interspecific-incompatibility may be defined as any post-pollination process preventing cross pollen germination and fertilisation, occurring between two different closely related fertile species within families where there are clear barriers involving some kind of recognition reaction. Used in this sense, interspecific-incompatibility excludes reactions between species with gross physiological differences such as intercrosses between Petunia hybrida and B. oleracea and is restricted to those species where one might otherwise expect to see some kind of compatibility with respect to pollen tube growth and penetration, even if fertilisation was always unsuccessful.

Lewis & Crowe (1958) showed that within the Cruciferae, Onagraceae and Solanaceae, pollen tubes of self-incompatible (SI) species grew successfully through the styles of closely related self-compatible (SC) species, but that pollen tubes from the self-compatible species were inhibited on the stigma or in the styles of SI ones. They also found exceptional SC species in which pollen was not inhibited in SI styles and suggested that these species were recently derived from the self-incompatible ancestors. They considered that the S-locus possessed two functions; one controlling interspecific-incompatibility

and the other controlling self-incompatibility, and that each step in the evolutionary sequence corresponds to a specific change in the interspecific-incompatibility controlling part of the gene.

Hogenboom (1972a), working with self-incompatible Lycopersicon peruvianum and self-compatible L. esculentum, attempted to break the breeding barrier between the 2 species. By selfing L. peruvianum, he first produced progeny that varied in ability to cross with L. esculentum pollen. He also found (Hogenboom, 1972b) a single recessive gene which controlled the loss of interspecific incompatibility caused by high temperature (40°C). Hogenboom (1975) later presented evidence to suggest that dominant genes expressed in the pistil, controlled the acceptance or rejection of foreign pollen. This led him to propose that interspecific-incompatibility should be considered a separate phenomenon from self-incompatibility and instead should be termed incongruity (Hogenboom, 1972a, 1972c, 1973, 1975). Incongruity, he believed, involved processes distinct from self-incompatibility and was controlled at other loci than the S-locus. He concluded that whereas incompatibility was due to pollen-pistil interactions that involved a highly developed recognition reaction at the intraspecific level, incongruity resulted from incomplete matching of the pollen and pistil components.

From similar work conducted on the two tomato species, de Nettancourt et al. (1974) reported that the capacity of Lycopersicum peruvianum to recognise pollen of L. esculentum was governed by an allele at the S-locus or by a gametophytic gene closely linked to it. It was therefore considered that the acceptance of L. esculentum pollen by L. peruvianum pistils depended both on the presence or absence of dominant stylar genes and on the S-locus

itself.

While detailed genetic studies have been largely restricted to species where control of self-incompatibility is gametophytic, interspecific-incompatibility is also known in species with sporophytic self-incompatibility systems. Thus Lewis & Crowe (1958) have described the crossing relationship between several species of Cruciferae (Draba verna, Erysimum suffrutuosum, Sisymbrium officinale, Iberis contracta, Aktssyn argentenum) while Harberd (1976) has carried out an extensive survey of interspecific-incompatibility in the sub-tribe Brassicinae and related sub-tribes. From Harberd's work details of cross-compatibility have been established for the following species:

Erucastrum abyssinicum

Brassica oxyrrhina

Eruca spp.

Brassica tournefortii

Snapodendron spp.

Enarthrocarpus spp.

Diplotaxis erucoides

Diplotaxis tenuifolia

Brassica barrelieri

Diplotaxis muralis

Diplotaxis harra

Erucastrum virgatum

Brassica carinata

Diplotaxis catholica

Brassica napus

Erucastrum canariense

Brassica oleracea

Sinapis alba

Erucastrum gallicum

Brassica campestris

Hirschfeldia incana

Brassica nigra

Sinapis arvensis

Raphanus spp.

Trachystoma spp.

Sinapis pubescens

Brassica juncea

B. cossoneana

B. fruticulosa

Harberd (1976) found that stigmas of species such as B. oxyrrhina, B. tournefortii, Erucastrum abyssinicum, Erucca spp and Sinapodendron spp were very unselective and were compatible with nearly all the other species listed above. In contrast, the pollen of these species were incompatible with most of the others. It was also evident that pollen from species at the bottom of the list such as B. juncea, B. cossoneana, B. fruticulosa and Sinapis pubescens were compatible with the other species but as female parents the same species were very highly selective. Thus, pollen of few other species were compatible with them. More recently, Hodgkin (1986) found that an incompatibility barrier existed between a synthetic B. napus line and B. oleracea. This operated unilaterally, so that the synthetic line tested was incompatible with B. oleracea pollen but not if used as a pollen donor on B. oleracea pistils. Synthetic B. napus was also incompatible with other B. napus plants when used as female.

4.1.2. Breakdown of incompatibility

The conditions under which self-incompatibility breaks down in different species can be considered characteristic of the reaction. In Brassicas, the self-incompatible reaction is only fully operational in mature flowers and self-compatibility of bud stigmas is a characteristic of the species (Pearson, 1929). Methods of breaking down SI have been utilised both to investigate its mechanism and to develop methods of producing inbred seed in the production of F_1 hybrids. Treatments have been applied to Brassica plants both before and after pollination and a number of these have been shown to induce normal tube penetration. Sears (1937) removed stigmas using a razor blade prior to pollination in B. oleracea and found increased numbers of penetrating pollen tubes. Ockendon (1973) showed that high temperature increased self-pollen tube penetration in Brassicas, while Carter & McNeilly (1975) found that increased humidity or the application of hexane to stigmas prior to pollination, had a similar effect in B. oleracea.

The extent to which these treatments provide a significant functional insight into the nature of the self-incompatible mechanism is not yet clear (Hodgkin et al., 1988). In this regard, Ferrari & Wallace (1977) have shown that pretreatment of pollen with cycloheximide increases self-incompatibility in B. oleracea while Roberts et al. (1984b) have reported that self-incompatible stigmas exposed to cycloheximide for 2 hours prior to pollination promotes the germination and growth of both cross- and self-pollen of B. oleracea. It can be deduced from these findings that a continuous synthesis of stigmatic proteins is essential for the operation of the SI system, in

that inhibition of protein synthesis by cycloheximide (translation inhibitor) greatly increases pollen tube penetration. Nakanishi et al. (1969) also showed that elevated concentrations of CO_2 gas (0.1 to 7.5%) increases self-pollen tube penetration in B. oleracea and B. campestris without affecting cross-pollen samples and later demonstrated (Nakanishi & Hinata, 1973) that treatment with CO_2 also increases seed set from 0.2/flower to 10/flower. More recently it has been reported that treatment with either 3% NaCl solution or 4% CO_2 gas decreases self-incompatibility levels in B. campestris (Guohua & Rui, 1986) and that 3% NaCl solution acts by increasing pollen adhesion and pollen germination and reducing callose formation on the papillae (Monteiro et al., 1988).

Much less research has been conducted on the breakdown of interspecific-incompatibility. In Lycopersicum, Hogenboom (1972b) has shown that high temperature increases tube penetration while Knox et al. (1972a) found that pollen wall protein(s) act as recognition substances in incompatibility between Populus deltoides and P. alba.

With regard to Brassica, it was reported in Chapter 3 that pollen from B. oleracea frequently failed to penetrate the stigmas of newly synthesised B. napus, even when the plants used, had no S-alleles in common. This confirmed previous findings by Hodgkin (1986) and suggests that the production of the synthetic results in the creation of barriers absent in the parents.

The objective of the work reported in this Chapter was to investigate more fully the occurrence of interspecific-incompatibility between B. napus and B. oleracea. In particular by using both self-incompatible and self-compatible lines of B. napus it was intended to

determine whether self-incompatibility in B. napus is required for interspecific-incompatibility to be expressed.

Two lines of investigation were conducted:

(a) Interspecific-incompatibility was investigated between a range of self-incompatible lines using material obtained from backcrossing synthetics with naturally-derived swedes and B. oleracea and B. campestris inbred lines. Recently produced synthetic B. napus lines were also crossed with B. campestris and B. oleracea related and unrelated S-homozygotes to give additional data on interspecific-incompatibility.

(b) Tests were carried out to establish the breakdown of interspecific-incompatibility and compared with that of self-incompatibility.

4.2. MATERIALS AND METHODS

4.2.1. Part 1 - The occurrence of interspecific-incompatibility in Brassica species

4.2.1.1. Experiment I

Plants of B. napus, B. campestris and B. oleracea were raised to the flowering stage. The lines chosen from each species for crossing were as follows:

B. napus - Three lines designated 86X024, 86X025 and 86X026. 86X024 was the F_1 between a self-incompatible swede line from cultivar Gullacker and a self-incompatible forage rape inbred obtained by introgressing a B. campestris S-allele into the forage rape cultivar

Bishop.

86X025 was the F_1 between a self-incompatible swede line from cultivar Gullacker and a self-incompatible oilseed rape inbred line obtained from introgressing a B. campestris S-allele into the oilseed rape cultivar Norde.

86X026 was the F_1 between a plant obtained from the cultivar Panter (which originated as a synthetic produced at the tetraploid level) and a self-incompatible oilseed rape inbred obtained from introgressing a B. campestris S-allele into the oilseed rape cultivar Norde.

B. oleracea - Three inbred lines of B. oleracea var acephala homozygous for different S-alleles.

82-103 homozygous for S_{12}

83-457 " " S_{29}

49-85 homozygous for S_2

B. campestris - Two inbred lines of B. chinensis juslin var chinensis.

86-155 - either homozygous for S_a or heterozygous for S_aS_b. (exact genotype unknown).

86-153 - homozygous for S_c.

These 8 lines were intercrossed in a full diallel using 10 flowers per cross taken at random from 3 B. napus plants or 2 B. oleracea and B. campestris plants per line. Crosses and pollen tube counts were made using the procedures as detailed in Chapter 3.

4.2.1.2. **Experiment II**

Four plants of the synthetic B. napus line (86-168) which had been synthesised at SCRI using known B. oleracea and B. campestris homozygotes were crossed reciprocally with the following: the

respective parents; two unrelated B. oleracea and B. campestris lines; self-compatible B. napus and self-incompatible synthetic B. napus line.

Details of the material used in these crosses are as follows. The respective parents were a B. oleracea var. alboglabra line denoted as 82-039 and homozygous for S_{16}, and a B. campestris juslen var. chinensis line denoted as 86-155 and homozygous for either S_a or S_b. The unrelated B. oleracea and B. campestris lines were respectively B. oleracea var. alboglabra 85-353 (S_2S_2) and 83-456 ($S_{29}S_{29}$) and B. campestris cv. purple top inbred line, 84-239 and the F_1 between B. pekinensis var. pekinensis and B. narinosa 87X013.

The self-compatible B. napus lines were 86X084 (B. napus oilseed rape Jet-neuf) and 86X085 (B. napus oilseed rape Mikado) while the chosen synthetic lines were 86-168 (produced at SCRI by crossing B. campestris 82-592 with B. oleracea 82-44) and 44-85 (a product of the cross between B. oleracea 2P-147 with B. campestris cultivar grannat 24X009).

Each cross involved up to 3 flowers which were pollinated in the standard way. Pollen tube numbers were counted using the procedure outlined in Chapter 3.

4.2.2. Part 2 - Methods for overcoming self- and cross-incompatibility

4.2.2.1. **Experiment I - Effect of cycloheximide and bud pollination in intra-and interspecific crosses**

Plants of a synthetic B. napus line 86-483 synthesised at SCRI,

B. napus oilseed rape cultivars 86X086 and Bienvenu 86X090 were raised to flower in a heated glasshouse. Self-pollen and pollen from the B. oleracea cultivar Bittern were used in the experiment.

Each test cross involved 5 flowers and the experiment was carried out on 2 separate occasions to give two replicates for analysis. Excised stigmas from newly opened flowers were placed on a filter paper soaked with 10 mM cycloheximide solution for 2 hours and then brushed with the required pollen from a freshly dehisced anther. For comparison, bud and control stigmas from newly opened flowers were also placed on filter paper soaked in distilled water and pollinated. After 4 hours stigmas were fixed in chloroform : absolute alcohol : acetic acid (6:3:1 v/v), stained and squashed in decolorised aniline blue (as described in Chapter 3) and the number of pollen tubes were counted.

4.2.2.2. **Experiment II - Effect of CO_2 gas**

Plants from synthetic B. napus and B. oleracea were raised to flower. The lines chosen from each species were as follows.

Synthetic B. napus 86-483 - Artificially produced at SCRI by crossing B. campestris 82-592 with B. oleracea 82-44.

Synthetic B. napus 87X048 (line 6) - Artificially produced by crossing B. campestris 86-155A with B. oleracea 85-353 (Chapter 2 in this thesis).

B. oleracea - Two inbred lines of B. oleracea var alboglabra

85-362 - homozygous for S_{14}

85-338 - " " S_2

The B. napus line, 86-483, was pollinated with 87X048, 85-362, 85-338 and self-pollen. All pollinations were carried out in the laboratory, using pollen from freshly dehisced anthers, on open flowers from which the anthers had been removed before dehiscence. Immediately after pollination, flowers were placed in a glass chamber and treated with a 4% CO_2 gas stream for 4 hours. Control stigmas received identical treatments but were placed in a glasschamber flushed with air. After 5 hours the flowers were fixed, stained with aniline blue and the number of pollen tubes counted as described in Chapter 3. Each pollination treatment involved 5 flowers. Eight replicate pollinations were carried out per treatment.

4.3. RESULTS AND ANALYSIS

4.3.1. Incompatible pollinations between species.

Incompatible pollinations between species appear different in several respects from incompatible pollinations within species. In most interspecies crosses that are incompatible, pollen grains adhere to the stigma and often show a degree of germination and some appearance of stigma penetration. Sometimes, tubes grow more than 2-3 times the diameter of the pollen grain. Masses of brightly stained fluorescent material can be seen in these stigmas, suggesting a greatly increased deposition of stigma callose. The tubes appear to coil round the stigma and contain relatively large amounts of fluorescent callose material. Sometimes a few individual pollen tubes can be seen growing down the style. These tubes are extremely clear, smooth and straight, but in the ovary region they cease to be visible.

4.3.2. Results of Part 1

4.3.2.1. Experiment I

Within-species pollinations

All B. napus self-pollinations were incompatible (Table 4.1). Crosses between the lines 86X024 and 86X026 were reciprocally compatible with a mean of 32 or 40 pollen tubes but all other crosses led to no pollen tube penetration.

Self-pollinations of B. oleracea lines were incompatible but crosses between lines were compatible except for the cross 82-103 x 83-457, which yielded no pollen tubes. This cross failed despite the fact that both parents appeared to be fully fertile and were known to be homozygous for different S-alleles.

The B. campestris line 86-155 appeared to be partially self-compatible with a mean of 20 pollen tubes per stigma while 86-153 was self-incompatible. The two lines were fully cross-compatible confirming that they possessed different B. campestris S-alleles.

Between-species pollinations

When B. napus was used as a pollen parent it was compatible with all three B. oleracea lines. Most of the crosses showed high tube counts with little plant-to-plant or flower-to-flower variation. In contrast, B. napus pollen was incompatible with B. campestris 86-155 though compatible with 86-153. Used as the female parent, B. napus was incompatible with all the three B. oleracea lines. In addition pollen from both B. campestris lines was effectively incompatible with B. napus 86X024 line but proved compatible with the 86X025 and 86X026

lines yielding 36-75 tubes per cross. Tube penetration and growth was normal and could not be distinguished from intraspecific compatible pollinations.

In crosses between B. oleracea and B. campestris, B. oleracea pollen was compatible with the B. campestris 86-153 line, but not compatible or only marginally so with B. campestris line 86-155. In contrast, pollen from both B. campestris lines was compatible with the B. oleracea lines tested (49-85 and 83-457).

4.3.2.2. **Experiment II**

The results of crosses using the B. napus synthetic-1 are summarised in Table 4.2. The synthetic B. napus was reciprocally incompatible with its B. oleracea parent (82-039) but compatible with the non-parental B. oleracea lines 85-353 and 83-456 when used as the male rather than female parent.

The synthetic B. napus line was fully reciprocally compatible with the B. campestris non-parental lines (84-239, 87X013), but crosses with other B. napus lines gave more complex results. Although the synthetic produced pollen that was fully compatible with both self-compatible and self-incompatible B. napus testers, as the female it was incompatible with pollen from 44-85 (the other synthetic).

4.3.3. Results of Part 2

4.3.3.1. **Experiment I**

For each treatment a total of 30 measurements and 3 crosses were

available for analysis and comparison. Mean tube numbers for each treatment and each cross are summarised in Table 4.3.

Analysis of variance of tube numbers transformed into square roots (Table 4.4 and 4.5) showed that differences between the means of treatments and crosses were highly significant ($P<0.001$) and, in addition, there was no significant interaction between these two effects. Cycloheximide-treated stigmas contained significantly more pollen tubes than bud stigmas (4.5a), while control stigmas contained the least number of all. It is concluded that both bud pollination and cyclohexamide treatment will overcome interspecific incompatibility as effectively as they overcome intraspecific incompatibility, though cyclohexamide treatment is the more effective.

4.3.3.2. **Experiment II**

Following treatment with CO_2 gas crosses between 86-483 x 85-362 and 86-483 x 85-338, and 86-483 selfing gave fully incompatible pollinations with no more than 2 tubes per stigma. In contrast, B. napus 86-483 crossed with 87X048 pollen gave a mean of 35 pollen tubes per treated stigma; however this was no greater than that recorded for untreated stigmas.

4.4. DISCUSSION AND CONCLUSIONS

The major finding to emerge from Experiments I and II (Part 1) reported in this Chapter was that interspecific incompatibility is a real phenomenon between B. oleracea pollen and B. napus stigmas. In

contrast, the results of crosses between B. napus and B. campestris suggest that no interspecific incompatibility occurs between these two Brassica species. Irrespective of whether self-incompatible or self-compatible lines were used, crosses that involved pollinating B. napus with B. oleracea pollen always proved to be incompatible.

Of equal importance, was the finding (Experiments I and II; Part 2) that the interspecific incompatibility recorded, functioned in much the same way as self-incompatibility with respect to its expression. Thus it was evident only in mature flowers and depended on continuing protein synthesis whereas bud pollination and cycloheximide treatment broke down interspecific incompatibility, little effect followed treatment with CO_2 gas.

In Experiment I (Part 1), the three related B. napus lines presumably shared one or more S-alleles at either the B. campestris or B. oleracea loci. All three of the lines turned out to be incompatible when crossed with 86-155 (B. campestris) pistils (Table 4.1) but two of them (86X025, 86X026) were reciprocally compatible with 86-153 and the 86X024 line was compatible with 86-153 (B. campestris) stigmas. It was concluded that 86-155 was an S-heterozygote with both alleles active in the female and these alleles were present in 86X024, 86X025 and 86X026.

In the interspecific crosses with B. oleracea three different B. oleracea S-allele homozygotes were used, and since no one B. napus line can contain more than two B. oleracea alleles, at least one B. oleracea line should have been compatible with each B. napus line, i.e. if interspecific incompatibility was due entirely to the expression of S-alleles. In fact pollen of the B. napus line was always compatible in B. oleracea stigmas suggesting that none of the

alleles present in B. oleracea were present in the B. napus lines.

In Experiment II (Part 1), synthetics were chosen to ensure compatibility, in S-allele terms, for certain crosses between B. oleracea x B. napus and B. campestris x B. napus. Nevertheless the synthetics were always fully incompatible with pollen of the unrelated B. oleracea S_2 and S_{29} lines although the reciprocal crosses were compatible. Only in crosses between the synthetic and the B. oleracea line homozygous for S_{16} (82-039), was incompatibility apparent in both directions due to the sharing of an active S-allele.

In regard to crosses with B. campestris, synthetic B. napus was always compatible with unrelated B. campestris in both directions, providing further evidence that interspecific incompatibility does not occur between B. campestris and B. napus.

The interspecific incompatibility which was observed to occur between B. napus and B. oleracea is different from that previously discussed by Lewis & Crowe (1958) for the Cruciferae. First, it occurs in a synthetic species and it is the synthesis which appears to be the cause of its occurrence. Secondly, neither species needs to be self-compatible. Thirdly, when it does occur between self-compatible B. napus and self-incompatible B. oleracea (Chapter 3 SC x SI) it is the self-incompatible pollen that is inhibited on the B. napus stigma. In contrast, Lewis & Crowe (1958) normally found incompatibility to result from the reciprocal cross i.e. stigmas of the self-incompatible species pollinated by pollen of the self-compatible species resulted in incompatibility (SI x SC).

Table 4.1. Mean numbers of pollen tubes penetrating the stigma following crosses between Brassica spp. in Experiment I (Part 1)

	Pollen source							
	B. napus			B. oleracea			B. campestris	
Stigma source	86X024	86X025	86X026	82-103	49-85	83-457	86-155	86-153
B. napus								
86X024	0	0	40	2	1	0	3	0
86X025	0	0	0	1	2	0	43	39
86X026	32	0	0	2	1	0	75	36
B. oleracea								
82-103	40	20	32	0	51	0	n.t.	n.t.
49-85	69	61	65	75	0	56	n.t.	75
83-457	67	59	59	75	75	2	25	40
B. campestris								
86-155	3	0	2	2	15	21	20	39
86-153	75	62	75	51	60	60	45	0

n.t. = not tested

Table 4.2. Mean numbers of pollen tubes penetrating the stigma following crosses between synthetic B. napus 86-168 and related and unrelated B. campestris and B. oleracea S-allele homozygotes. (Experiment II - Part 1)

Synthetic B. napus Line Number	Plant Number	B. oleracea 82-039		B. campestris 86-155	S_a/S_b	B. oleracea 85-353		B. oleracea 83-456		B campestris 84-239		B campestris 87X013		B. napus 86X084		B. napus 86X085		Synthetic B. napus 44-85	
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
86-168	1	0	0	n.t.	40	>75	0	>75	3	25	50	45	>75	25	50	30	35	40	2
86-168	2	0	0	n.t.	8	>75	0	>75	0	20	40	50	n.t.	45	n.t.	40	16	>75	n.t.
86-168	3	0	0	n.t.	35	n.t.	0	>75	15	30	n.t.	20	>75	42	>75	>75	40	>75	0
86-168	4	0	0	n.t.	6	>75	0	40	12	20	35	21	>75	20	65	n.t.	30	n.t.	n.t.

n.t. = not tested

Table 4.3. Mean pollen tube numbers in intra and interspecific pollinations following pollination of buds or treatment with cycloheximide. (Experiment I - Part 2)

Cross	Bud stigmas	Stigmas treated with cycloheximide	Untreated stigmas	Total Mean
	Treatment 1	Treatment 2	Treatment 3	
86-483 x 86-483	17.7	47	1.2	22
86X086 x Bittern	51.1	60	7.3	39.5
86X090 x 86X090	5.0	16.2	1.5	7.6
Total mean	24.6	41.1	3.3	23

N.B. 86-483 : *B. napus* synthetic
86X086 : *B. napus* cultivar
86X090 : *B. napus* cultivar (Bienvenu)
Bittern: *B. oleracea* cultivar

Table 4.4. Analysis of variance of square root transformation of pollen tube numbers in Experiment I (Part 2)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio
Day	1	0.554	0.554	
Treatment	2	365.293	182.647	40.835***
Cross	2	195.7	97.85	21.877***
Treatment x Cross	4	40.98	10.245	2.291 n.s.
Residual	8	35.782	4.473	

*** P<0.001
n.s. not significant

Table 4.5 Mean pollen tube numbers (square root transformation) of Experiment I (Part 2)

Table 4.5.a

Treatment	Bud stigmas	Stigmas treated with cycloheximide	Untreated stigmas
Mean	4.18	6.02	1.14
SED	0.546		
DF	8		

Table 4.5.b

Cross	86-483 x 86-483	86-483 x Bittern	86X090 x 86X090
Mean	3.69	4.63	2.02
SED	0.546		
DF	8		

Table 4.6. Results of Experiment II (Part 2)

Mean pollen tube numbers per stigma in crosses treated with or without CO_2 gas

Cross combination			Mean pollen tube number	
Stigma	x	Pollen	CO_2 treated stigmas	control stigmas
86-483		86-483	2	0
86-483		87X048	35	35
86-483		85-362	0	0
86-483		85-338	0	0

CHAPTER 5

SELF AND CROSS-COMPATIBILITY OF F_1s PRODUCED FROM B. NAPUS SYNTHETIC LINES

5.1 INTRODUCTION

In Chapter 3, the operation of the self-incompatibility system and activity of S-alleles was determined in seven synthetic B. napus lines of known S-locus genotype. It was established that both S-loci (one inherited from B. oleracea, the other from B. napus) were functional and that there were interactions between them with respect to S-allele expression. Two locus sporophytic incompatibility systems have not previously been clearly established in the literature with the exception of those reported by Riley (1932, 1936) and Correns (1912) for Capsella bursa-pastoris and Cardamine pratensis respectively.

Riley (1932, 1936) proposed that Capsella bursa-pastoris possessed a self-incompatible system involving two incompatibility loci with one gene epistatic to the other and with sporophytic control of the pollen reaction. However, de Nettancourt (1972) later showed that most of the segregation data accumulated by Riley could equally be explained by a single genetic locus with a polyallelic series. In Cardamine pratensis, Bateman (1954, 1955) followed up earlier work conducted by Correns (1912), and proposed that the species exhibited sporophytic control of both pollen and style incompatibility due to the action of two independent loci at each of which was present a dominant and recessive allele.

In view of the uniqueness of the two locus sporophytic multi-

allelic system created in synthetic lines of B. napus, it was recognised that a major objective of the present research should be to describe the operation and segregation of the particular S-alleles in these lines. To this end the study reported in this Chapter was conducted to assess the incompatibility reactions among F_1's that were produced by intercrossing the seven synthetic lines of B. napus of known S-locus genotype. This allowed an assessment of incompatibility reactions when the S-loci were in the heterozygous rather than homozygous state as was the case in the previous analysis of self- and cross-incompatibility of synthetics reported in Chapter 3.

5.2 MATERIALS AND METHODS

In December 1987 fourteen plants, two from each of the seven lines of synthetic B. napus (Chapter 2), were intercrossed to produce 14 F_1 lines (Table 5.1). Because of poor seed set from crosses between synthetic lines 4 and 2, only 13 F_1's were sown for further analysis. Each of these lines contained a four S-allele combination that was heterozygous at both S-loci.

Eleven of the F_1's were made by crossing parents originally produced by ovary culture (with B. campestris as female). The 2 remaining F_1's came from crosses where the female parent had been produced by embryo culture. A total of three different four S-allele combinations were produced. These heterozygous genotypes plus the parent lines which were crossed to produce them, are presented in Table 5.2.

Ten seeds of each F_1 line were sown at the beginning of April 1988 and plants were raised to maturity in a heated glasshouse maintained

at 20°C day and 14°C temperature at night, under natural light. Using these plants, two experiments were conducted.

5.2.1. Experiment 1

A diallel set of crosses between the 13 F_1 lines was carried out together with self-pollinations to determine the self-incompatibility status of plants. For each cross, five flowers taken randomly from the plants of each family, were pollinated and the degree of incompatibility was determined as described in Chapter 3. Each cross was later repeated using 3 newly opened flowers to confirm results.

5.2.2. Experiment 2

All possible crosses were made between each F_1 line and the *B. campestris* and *B. oleracea* S-allele homozygotes (S_a, S_b, S_{29}, S_{14}, S_2), using randomly taken flowers. Test pollinations were carried out on 3 newly opened flowers, and pollen tube penetration assessed as described in Chapter 3. All test pollinations were repeated using 3 flowers.

In addition to the main crossing programme, each F_1 line was also pollinated with pollen from a known compatible line (82-213), so that an estimate of female sterility could be made. Male sterility was also examined by visual observation of anthers and pollen grains.

In Chapter 3, it was shown that *B. oleracea* pollen could not be used to test S-allele relationships. Therefore, in this experiment, the crosses using *B. oleracea* as male were made to investigate the

continuing occurrence of interspecific incompatibility barriers rather than to study the genetics of the self-incompatibility system itself.

5.3 RESULTS

5.3.1 - Results of Experiment 1

The results of self-pollinations and interline pollinations between F_1s are presented in Table 5.3. Self-incompatibility levels of individual lines and other results are dealt with under the appropriate headings for S-allele constitution. However, in general, plants of allelic constitution $S_{14}S_2S_aS_b$ were partially or fully self-compatible, while F_1 lines possessing the other two allelic combinations were self-incompatible.

5.3.1.1 - Reactions of F_1 lines containing $S_{29}S_{14}S_aS_b$ (Group I) (lines 1.5, 2.4, 5.1 and 7.2)

Lines containing the alleles $S_{29}S_{14}S_aS_b$ were, in the main, incompatible when crossed with each other. However, pollen of the F_1 lines 1.5 and 5.1 produced over 30 pollen tubes per stigma when crossed with line 7.2 - (an F_1 which contained B. oleracea cytoplasm). In reciprocal crosses between lines containing $S_{29}S_{14}S_aS_b$ and the F_1's of $S_{29}S_2S_aS_b$ genotype, pollen always produced less than 13 tubes per stigma. In contrast, when $S_{14}S_2S_aS_b$ lines were used as the pollen parents in crosses with $S_{29}S_{14}S_aS_b$ there was generally considerable tube penetration (>50 tubes).

5.3.1.2 - Reaction of F_1 lines containing $S_{29}S_2S_aS_b$ (Group II) (lines 1.6, 3.4, 4.3, 6.1 and 7.3)

Crosses between F_1 lines containing the alleles $S_{29}S_2S_aS_b$ were always incompatible with no more than 9 pollen tubes produced per intragroup cross. It was also evident that pollen from lines of $S_{29}S_{14}S_aS_b$ genotype was incompatible on stigmas of F_1's containing the $S_{29}S_2S_aS_b$ alleles, producing no more than 8 tubes per stigma in any cross. In contrast, when the $S_{29}S_2S_aS_b$ F_1's were pollinated by $S_{14}S_2S_aS_b$ lines, it was general for more than 20 tubes to be produced per stigma. However, only in the cross between line 6.1 and 2.6 was measure of true compatibility obtained, with a mean of over 50 tubes produced per stigma.

5.3.1.3 - Reaction of F_1 lines containing $S_{14}S_2S_aS_b$ (Group III) (lines 2.6, 3.5, 5.3 and 6.2)

As well as being partially self-compatible, F_1 lines containing the $S_{14}S_2S_aS_b$ allelic combination were moderately compatible with each other based on interline crosses. Only crosses in which line 5.3 was pollinated by lines 2.6 or 3.5 yielded no pollen tubes per stigma. Considerable tube penetration was evident when the $S_{14}S_2S_aS_b$ lines were pollinated by lines from the other two groups (Group I and II). In these crosses tube numbers tended to vary from 20 to over 75, although certain crosses produced fewer tubes.

5.3.2 - Results of Experiment 2

The results of test pollinations between the 13 F_1 lines and *B. campestris* S_a and S_b homozygotes and *B. oleracea* S_{29}, S_{14} and S_2 homozygotes are summarised in Table 5.4.

5.3.2.1 - **Reaction of F_1s with *B. campestris***

$S_{29}S_{14}S_aS_b$ pollen appeared to be effectively compatible with both S_a and S_b *B. campestris*, with pollen tubes per stigma ranging from 28 to 75. Pollen from the $S_{29}S_2S_aS_b$ and $S_{14}S_2S_aS_b$ F_1 lines were also compatible with S_a and S_b. However, tube numbers were low (15 and 17 tubes respectively) in crosses in which lines 4.3 and 5.3 were used to pollinate the S_a *B. campestris* line.

When crosses were made using *B. campestris* testers as the pollen parent the S_b line produced no pollen tubes when crossed with F_1's of $S_{29}S_{14}S_aS_b$ genotype. In contrast, the *B. campestris* S_a line produced over 75 tubes when crossed with line 2.4 but only 8 tubes with line 5.1. Lines of $S_{29}S_2S_aS_b$ genotype were always incompatible with S_b pollen but in general showed good compatibility with S_a pollen lines except, that is, when line 1.6 was the pollen recipient. Rather surprisingly, lines containing the $S_{14}S_2S_aS_b$ alleles appeared to be incompatible with both S_a and S_b pollen, with only 0 to 15 pollen tubes produced per stigma.

5.3.2.2 - **Reactions of F_1s with *B. oleracea***

Using the *B. oleracea* S-allele homozygotes as female parents,

pollen from lines containing $S_{14}S_{29}S_aS_b$ alleles produced less than 3 tubes per stigma on plants of S_{29} or S_{14} constitution, but was compatible on S_2 stigmas.

Pollen from lines containing $S_{29}S_2S_aS_b$ gave no pollen tubes in crosses with S_{29} but, in general, showed good compatibility on S_2 and S_{14} stigmas. Finally, pollen of the $S_{14}S_2S_bS_a$ lines varied in compatibility with B. oleracea homozygote. Over 75 pollen tubes were produced in crosses between line 5.3 pollen and the three B. oleracea S-allele homozygotes while line 6.2 pollen produced only 0 and 7 pollen tubes on S_2 and S_{14} stigmas but 40 pollen tubes on S_{29} stigmas.

There was again evidence of interspecific incompatibility in crosses between B. oleracea pollen and the F_1 lines, although $S_{29}S_{14}S_aS_b$ lines pollinated with S_{14} pollen yielded 60 to >75 pollen tubes per stigma, and intermediate numbers of pollen tubes (30-60) were obtained when the F_1's 6.1, 2.6, 3.5 and 5.3 were pollinated by B. oleracea S_{14} and S_{29} homozygotes. In nearly all other crosses pollen tube numbers were close to 0.

5.4 ANALYSES

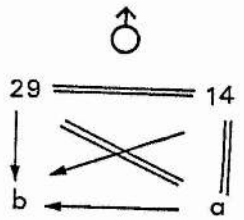
In Chapter 3 the S-allele status of synthetics was described and it was shown that

(a) Where 2 alleles were present, allelic activity could be determined by intraspecific pollinations.

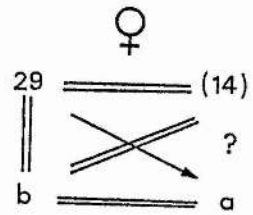
(b) Crosses with the synthetics involving parental B. oleracea lines as female, and B. campestris lines as both female and male, gave results which confirmed the findings of the intraspecific data.

From the data obtained in Chapter 3 and a knowledge of the dominance relationships of the original S-alleles we could expect the following allele activities in the three different 4 S-allele combinations investigated here.

(a) In pollen of F_1s containing the $S_{29}S_{14}S_aS_b$ alleles, S_b is expected to be recessive to S_a, S_{29} and S_{14}; while S_a would be co-dominant to both S_{29} and S_{14} (Chapter 3). [Note: S_{14} and S_{29} are expected to be co-dominant (Van Hal, 1968 - unpublished)]. In stigmas, S_{29} should be dominant to S_a and S_b co-dominant to S_{29}, S_{14} and S_a. The expected relationship between S_{29} and S_{14} is less certain as both co-dominance and partial dominance of S_{29} to S_{14} have been reported by Van Hal (1968 - unpublished). The expected allele activities in pollen and stigmas of the four S-alleles can therefore be summarised as follows:



$S_{29}S_{14}S_a$



$S_{29}S_{14}S_b$

= co-dominant

1 → 2 allele 1 dominant to 2

( ) partially recessive (sometimes)

? allele relationship not determined

(b) In pollen from plants of $S_{29}S_2S_aS_b$ genotype, S_b and S_2 are expected to be recessive to S_{29} and S_a (Chapter 3 and Van Hal). S_a would be expected to be co-dominant to S_{29}, as would S_b with S_2.

In the stigma, S_a and S_2 should be recessive to S_{29} and S_b co-

dominant to S_{29}. The relation between S_b and S_2 would be less certain (Chapter 3) with S_b being either co-dominant or dominant to S_2. Finally S_a is expected to be co-dominant to both S_b and S_2.

Thus, taken overall, the following pattern of allele activity should occur in pollen and stigmas.

$S_{29}S_a$ $S_{29}S_b$

(c) In pollen of F_1's containing the $S_{14}S_2S_aS_b$ alleles, S_b and S_2 are expected to be recessive to S_{14} and S_a; while S_{14} and S_a, and S_b and S_2 should show co-dominance.

In the stigma, S_b and S_2 should be co-dominant to S_{14} and S_a, but the relation between S_b and S_2 would be uncertain (see above), as would that between S_{14} and S_a. Thus, the expected pattern of allele activities in pollen and stigmas should be as follows:

$S_{14}S_a$ $S_{14}S_2S_aS_b$

From the allele activities indicated above for the three different 4 S-allele combinations we can deduce the expected intraline and interspecies compatibilities and compare them to the observed data.

For the comparison, first a summary of the results of crosses between the 13 F_1 lines of B. napus, and between these lines and the B. campestris and B. oleracea genotypes (presented in terms of whether a cross was compatible or not), is given in Tables 5.5 and 5.6 respectively. This is followed by a Table that compares the expected and observed findings (Table 5.7). Crosses between B. napus F_1's involving the $S_{29}S_{14}S_aS_b$ and $S_{29}S_2S_aS_b$ genotypes were mainly incompatible whether used as pollen or stigma. Consistent with the expected results and evidence that the common allele (S_{29}) was active in both pollen and stigma (Table 5.7). However, as noted earlier (Table 5.3), stigmas of line 7.2 appeared to be compatible with pollen of lines 1.5 and 5.1 (Table 5.5). The cause of this is unknown but could be related to the cytoplasm of line 7.2 being derived from B. oleracea while other lines contained the cytoplasm of B. campestris. The results of reciprocal crosses between lines containing the alleles $S_{14}S_2S_aS_b$ and lines containing $S_{29}S_{14}S_aS_b$ or $S_{29}S_2S_aS_b$ did not entirely fit expectations as they appeared to be generally compatible. When used as the female parent, lines containing $S_{14}S_2S_aS_b$ were compatible with all the lines including self-pollen. This was contradictory to expectations. As S_{29} is sometimes dominant to S_{14} (Van Hal, 1968 - unpublished), it would not be unexpected for pollen from lines $S_{14}S_2S_aS_b$ to be compatible on stigmas of lines containing $S_{29}S_{14}S_aS_b$ and this was what was found. It is evident therefore that S_{29} was dominant to S_{14} in stigma and S_2 was inactive in $S_{14}S_2S_aS_b$ pollen and/or the $S_{29}S_2S_aS_b$ stigma.

The results of interspecific crosses between the B. napus F_1's and B. campestris used as the pollen parent (Table 5.6 and 5.7) were consistent with the expected results and showed that, in the F_1 lines

of $S_{29}S_{14}S_aS_b$ and $S_{29}S_2S_aS_b$ genotype, S_b was active while S_a was inactive in the female. In contrast, in the other group of F_1's ($S_{14}S_2S_aS_b$), both S_a and S_b were active. Using B. campestris as the female parent, S_b in the stigma gave compatible pollinations with all F_1 lines which was consistent with the expected results, and evidence that S_b is inactive in the pollen of all three groups of F_1's. However, the results obtained with S_a in stigmas of B. campestris were not as expected. The S_a homozygote of B. campestris was compatible with the pollen of all F_1 lines, evidence that in all three groups of F_1's S_a was inactive in pollen.

Results of crosses between B. oleracea as female parent and the F_1 lines were consistent with those expected, except that lines containing $S_{14}S_2S_aS_b$ gave a range of 0 - 75 tubes with stigmas of S_{14} genotype. This unexpected result could be an effect of the self-compatibility in $S_{14}S_2S_aS_b$ lines.

When B. oleracea was used as the pollen parent very little useful evidence was obtained although lines containing $S_{14}S_2S_aS_b$ were compatible with S_{29} pollen and incompatible with S_2 pollen as expected, evidence that S_2 is active in this group.

From the interspecific crosses, it appeared that S_b was active in all three groups of F_1's but that S_a was active only in Group III ($S_{14}S_2S_bS_a$) stigmas. B. campestris as the female parent gave no evidence for S-allele activity in the pollen of the F_1's, but tests with B. oleracea as the female parent gave evidence for activity of S_{29} and S_{14} in the pollen of the F_1 group of $S_{14}S_{29}S_aS_b$ genotype and of S_{29} in the F_1 group containing $S_{29}S_2S_aS_b$ alleles.

Apart from evidence of activity of S_2 and S_{14} in 6.2, lines

containing $S_{14}S_2S_aS_b$ showed no B. oleracea S-allele activity in the pollen.

5.5 DISCUSSION

5.5.1. Distinguishing between compatible and incompatible crosses

As described in Chapter 3, a great variation in degree of both compatible and incompatible pollinations was noted throughout this research. Figs. 5.1 and 5.2 illustrate the distribution of mean pollen tube numbers per stigma in interspecific and intraspecific pollinations involving the F_1's. The distribution of the pollen tube number classes in the interspecific crosses was similar to the distributions noted in Chapter 3. Most crosses (75%) gave less than 10 or more than 50 tubes and very few crosses yielded between 21-30 pollen tubes per stigma (Fig. 5.1). In crosses between F_1's (Fig. 5.2), however, the class of crosses with the smallest number of pollinations was 41-50. The greater number of crosses which produced tube numbers falling within the 11-20, 21-30 and 31-40 classes may have been due either to partial self-compatibility or partial expression of a particular S-allele within a large number of test crosses. Taking account of the distribution, it was decided that a mean of over 30 pollen tubes would be regarded as a compatible one and below 20 as an incompatible one. Intermediates which gave 21-30 pollen tubes were evaluated individually.

5.5.2 Intraspecific test crosses

The results showed that crosses between the F_1 lines of B. napus in Group I ($S_{29}S_{14}S_aS_b$) and Group II ($S_{29}S_2S_aS_b$) were self-incompatible while those between lines in Group III ($S_{14}S_2S_aS_b$) were either partially self-compatible or fully self-compatible.

Results of crosses between and within groups were largely consistent with those expected, i.e. crosses between lines of Group I and II were reciprocally incompatible (Table 5.3), while lines of both groups appeared more or less compatible with Group III pollen. However, when lines of Group III were used as the female parent, crosses between these lines and those of the other groups were also compatible which did not accord with expectations.

There were a number of crosses in which the individual results were different from the general response for a particular inter- or intra-group cross. These were of two kinds; a compatible response recorded where an incompatible one was expected, and an incompatible one detected where a compatible was expected. The former could be due to the failure of incompatible mechanisms while the latter could be due either to fertility or some form of 'false incompatibility'. Anomalous compatible responses were only recorded in the crosses between lines 5.1 and 1.5 pollen, with line 7.2 in Group I. There is no obvious explanation for this, although it may result from the interaction of the particular genotype (1.5 and 5.1 were reciprocals) with the B. oleracea cytoplasm present in line 7.1. Anomalous incompatible crosses were recorded more often. Group III lines, 2.6 and 6.2 pollen, gave 15 pollen tubes per stigma or less in line 1.5 stigmas, despite the generally high levels of compatibility found in

other Group I and III intercrosses. Line 4.3 (Group II) pistils were incompatible with the pollen from Group III lines and in general, pollen tube numbers in crosses between Group II and III lines were lower than might be expected. Full compatibility was expected between these two groups from the basis of allele dominance relations. Pollen of Group III was clearly fully functional (Table 5.3 Group I and II and Table 5.8) but Group II stigmas were less fertile (see the interspecific results - Table 5.8).

The majority of anomalous incompatible results occurred using Group III stigmas (e.g. 4 crosses with 20 or fewer tubes in Groups III x I, and 7 in crosses between Groups III x II). In fact, the presence of significant levels of self-compatibility in this group makes any interpretation extremely difficult. In addition, there was no particular pattern to the occurrence of incompatibility e.g. it was not more common in lines with lower self-compatibility. Indeed, in crosses involved line 2.6 as the female, only two crosses yielded less than 20 pollen tubes per stigma while, of the crosses involving line 5.3, which was fully self-compatible, seven led to the production of less than 20 tubes per stigma.

Results of crosses between B. napus F_1 pollen and B. campestris (outcross 82-213), an interspecific cross that was normally highly compatible, yielded, as expected, a high number of pollen tubes per stigma (Table 5.8). Thus incompatible crosses between F_1 lines involving 7.3, 2.6 and 3.5 are not apparently due to reduced fertility of the pollen of F_1's.

Amongst the Group III F_1's, the most self-compatible lines, 3.5 and 5.3, had a common parent, line 5 (Chapter 3) which was itself

self-compatible. This self-compatibility appeared to be due to failure of incompatible activity in the stigma, although pollen activity appeared normal. However, not all F_1's produced from line 5 were self-compatible; indeed, lines 1.5 and 5.1 (Group I) were fully self-incompatible. These contrasting results make it difficult to determine allelic activities.

5.5.3 Interspecific test crosses

Results of interspecific crosses between the B. napus F_1 lines and B. campestris using the S_b homozygote as female parent were consistent with expectations. Table 5.4 shows that pollen of all three F_1 groups were compatible on B. campestris S_b stigmas. More surprising was the fact that B. campestris S_a stigmas were also compatible with pollen of all three F_1 groups, contradicting the expected reactions. The latter could be due to the failure of the incompatibility mechanism due to the interspecific nature of the cross.

When the B. campestris S_a and S_b lines were used as the male parent, the results of crosses were once again largely consistent with those expected. S_b pollen was incompatible with lines from all three F_1 groups, indicating that S_b was active while S_a pollen was mainly compatible in crosses with F_1 Groups I and II but incompatible with those in Group III. The latter indicates that S_a is active only when crossed with Group III lines.

There were no individual anomalous compatible results as had been obtained with interspecific crosses but some anomalous incompatible crosses were recorded. Line 5.1 in Group I and line 1.6 in Group II, when pollinated by B. campestris S_a pollen yielded less than 9 pollen

tubes per stigma. Similarly pollen of line 4.3 produced only 15 pollen tubes per stigma on stigmas of the S_a homozygote. Interspecific outcross results (Table 5.8) and inter-group (Table 5.3) results showed that Group II stigmas were less fertile. Therefore the incompatibility that results from using line 1.6 (Group II) may result from reduced female fertility, rather than a true incompatibility reaction. However, the unexpected incompatibility resulting from crosses between line 5.1 and S_a pollen, and line 4.3 pollen and S_a stigmas have no such simple explanation.

Taken overall, the expression of B. campestris alleles can be summarised as follows:

B. napus F_1 Group	Stigma	Pollen
I	S_b	-
II	S_b	-
III	S_aS_b	-

Thus no B. campestris allele expression occurred in the pollen but both S_a and S_b were expressed in stigmas of Group III. The latter finding was particularly surprising in view of the self-compatible nature of Group III plants. Turning to the results of interspecific crosses between the B. napus F_1's and B. oleracea homozygotes, it was known (Chapter 3) that B. oleracea pollen could not be used for the detection of S-allele activity due to interspecific-incompatibility. In the event, B. oleracea pollen gave variable results with F_1's of all the three groups, consistent with previous findings.

The results of crosses using B. oleracea as the female were in the main, consistent with those expected. Table 5.4 shows that Group I lines were incompatible with S_{29} and S_{14} but compatible with line S_2.

Group II appeared mainly compatible with B. oleracea S_2 and S_{14} and incompatible with S_{29}. Finally, Group III lines were more or less compatible with B. oleracea lines S_2, S_{14} and S_{29}.

There were a number of crosses in which the incompatible results were different from the general response for a particular group but again there were no anomalous compatible results. Thus line 6.1 in Group II (Table 5.4) produced seven or less pollen tubes per stigma in crosses with B. oleracea, despite the general high levels of compatibility obtained when crosses were made between other Group II F_1's and homozygotes for the S_2 and S_{14} alleles. These anomalous results could be due to reduced fertility or false incompatibility in B. oleracea. However, a crossing programme between the B. oleracea homozygous lines (Table 5.9) showed that stigmas were fully functional in these lines.

Taken overall, the general pattern of compatibility and incompatibility found in the various crosses suggest that the expression of the B. oleracea S-alleles in the F_1 napus lines is as follows:

B. napus F_1 Group	Stigma	Pollen
I	not detected	$S_{29}S_{14}$
II	"	S_{29}
III	"	-

5.6 CONCLUSIONS

The main conclusion to be drawn for the two crossing programmes reported in this Chapter are as follows. In F_1's of synthetic B. napus which contain a 4 S-allele combination (i.e. the two S-loci

are heterozygous for different S-alleles), the self-incompatibility system functioned provided that the S_{29} allele was present. Among the F_1's which lacked the S_{29} allele (Group III lines), the most self-compatible lines were 3.5 and 5.3 which were reciprocal progeny obtained from crossing B. napus synthetic line 5 ($S_{14}S_a$) which was itself self-compatible (Chapter 3) with line 3 (S_2S_b). Lines 1.5 and 5.1 (Group I) were also obtained from crosses involving line 5, but with line 1 ($S_{29}S_b$). In contrast these F_1's exhibited complete self-incompatibility. This difference suggests that the self-compatibility detected in line 5 was only expressed in the absence of the dominant allele (S_{29}), a situation also reported by Thompson (1968) in B. oleracea.

Table 5.10 summarises the expression of S-alleles in the three groups of F_1's. In Group I and II, S_{29} seems to be expressed in both pollen and stigma while S_b seems to be active in the stigmas of all 3 groups. S_{14} is expressed only in the pollen of Group I, while S_2 and S_a are expressed only in stigmas of Group III.

Finally, data from crosses between the F_1's and B. campestris suggest that B. campestris alleles are functional in the female but not in the pollen. This implies that in this 2 locus system, allele activity in pollen would appear to be equivalent to that in a single locus system, although as Chapter 3 showed, the expressed alleles may be from either locus.

Table 5.1. Intercrosses between synthetic lines of B. napus to produce four allele combinations (i.e. genotypes heterozygous at both S-loci)

		Pollen source						
Line Number		1	2	3	4	5	6	7
	Stigma source	$S_{29}S_b$	$S_{14}S_b$	S_2S_b	$S_{29}S_a$	$S_{14}S_a$	S_2S_a	$S_{29}S_a$
1	$S_{29}S_b$					x (1.5)	x (1.6)	
2	$S_{14}S_b$				x (2.4)		x (2.6)	x n.p. (2.7)
3	S_2S_b				x (3.4)	x (3.5)		x n.p. (3.7)
4	$S_{29}S_a$		x c.f. (4.2)	x (4.3)				
5	$S_{14}S_a$	x (5.1)		x (5.3)				
6	S_2S_a	x (6.1)	x (6.2)					
7	$S_{29}S_a$		x (7.2)	x (7.3)				

c.f. - cross failed

n.p. - not produced

N.B. Numbers in brackets indicate the two parent lines involved in a cross. Hereafter, these combinations are used to identify a particular F_1.

Table 5.2. The three different 4 S-allele combinations produced by intercrossing seven B. napus lines. Parent lines crossed to produce each 4 S-allele combination are also listed.

S alleles combination	F_1 lines
$S_{14}S_{29}S_aS_b$	1.5, 5.1, 2.4, 7.2
$S_{29}S_2S_aS_b$	1.6, 6.1, 3.4, 4.3, 7.3
$S_{14}S_2S_aS_b$	2.6, 6.2, 3.5, 5.3

Table 5.3. Mean numbers of pollen tubes penetrating the stigma following crosses between F_1s of synthetic *B. napus* lines

Line nos.		Pollen source: 1.5	2.4	5.1	7.2	1.6	3.4	4.3	6.1	7.3	2.6	3.5	5.3	6.2
	Stigma source	$S_{29}S_{14}$ S_bS_a	$S_{14}S_{29}$ S_bS_a	$S_{14}S_{29}$ S_aS_b	$S_{29}S_{14}$ S_aS_b	$S_{29}S_2$ S_bS_a	S_2S_{29} S_bS_a	$S_{29}S_2$ S_aS_b	S_2S_{29} S_aS_b	$S_{29}S_2$ S_aS_b	$S_{14}S_2$ S_bS_a	S_2S_{14} S_bS_a	$S_{14}S_2$ S_aS_b	S_2S_{14} S_aS_b
1.5	$S_{29}S_{14}S_bS_a$	0	0	0	0	1	2	0	0	n.t.	10	>75	>75	15
2.4	$S_{14}S_{29}S_bS_a$	0	3	0	0	0	0	0	0	0	58	33	57	30
5.1	$S_{14}S_{29}S_aS_b$	0	2	0	10	7	2	13	3	0	>75	>75	58	>75
7.2	$S_{29}S_{14}S_aS_b$	30	0	>75	0	5	0	12	0	n.t.	>75	53	55	>75
1.6	$S_{29}S_2S_bS_a$	0	0	0	0	2	2	0	0	0	36	21	37	20
3.4	$S_2S_{29}S_bS_a$	0	2	7	0	0	0	0	0	0	39	26	21	35
4.3	$S_{29}S_2S_aS_b$	0	3	0	0	1	0	1	9	0	8	6	7	16
6.1	$S_2S_{29}S_aS_b$	0	0	8	8	0	0	0	6	1	52	44	39	44
7.3	$S_{29}S_2S_aS_b$	n.t.	n.t.	0	n.t.	7	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
2.6	$S_{14}S_2S_bS_a$	33	48	35	35	13	47	39	55	13	28	28	61	23
3.5	$S_2S_{14}S_bS_a$	10	59	60	58	52	43	60	30	12	69	65	57	32
5.3	$S_{14}S_2S_aS_b$	4	67	5	>75	33	28	2	18	0	0	0	>75	35
6.2	$S_2S_{14}S_aS_b$	32	20	21	53	36	23	38	50	15	18	19	24	12

>20 - compatible pollination; < 20 - incompatible pollination; n.t. - not tested

Table 5.4. Mean numbers of pollen tubes penetrating the stigma following crosses between F_1s and their parents homozygous for S-alleles

Line number	Allelic constitution	B. campestris as female (♀)		B. campestris as male (♂)		B. oleracea as female (♀)			B. oleracea as male (♂)		
		S_b	S_a	S_b	S_a	S_{29}	S_2	S_{14}	S_{29}	S_2	S_{14}
1.5	$S_{29}S_{14}S_bS_a$	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
2.4	$S_{14}S_{29}S_bS_a$	>75	>75	0	>75	0	36	3	0	0	60
5.1	$S_{14}S_{29}S_aS_b$	28	>75	0	8	0	31	0	0	0	>75
7.2	$S_{29}S_{14}S_aS_b$	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
1.6	$S_{29}S_2S_bS_a$	>75	>75	11	09	0	>75	>75	0	0	01
3.4	$S_2S_{29}S_bS_a$	n.t.	>75	0	42	0	>75	>75	0-75	09	07
4.3	$S_{29}S_2S_aS_b$	36	15	3	25	0	62	>75	03	0	13
6.1	$S_2S_{29}S_aS_b$	>75	>75	0	>75	0	07	0	0-75	0	62
7.3	$S_{29}S_2S_aS_b$	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
2.6	$S_{14}S_2S_bS_a$	>75	>75	01	01	16	30	35	42	0	30
3.5	$S_2S_{14}S_bS_a$	>75	>75	0	03	62	28	45	45	0	60
5.3	$S_{14}S_2S_aS_b$	n.t.	17	0	0	>75	>75	>75	50	15	35
6.2	$S_2S_{14}S_aS_b$	>75	>75	01	15	40	07	0	2	0	0

>20 - compatible pollinations; <20 - incompatible pollinations; n.t. - not tested

Table 5.5. Interpretation of the results of intraspecific crosses between 13 F_1's of synthetic B. napus

Line number	stigma source	Pollen source: $S_{29}S_{14}$ S_bS_a	$S_{14}S_{29}$ S_bS_a	$S_{14}S_{29}$ S_aS_b	S_aS_b $S_{29}S_{14}$	$S_{29}S_2$ S_bS_a	S_2S_{29} S_bS_a	$S_{29}S_2$ S_aS_b	S_2S_{29} S_aS_b	S_aS_b $S_{29}S_2$	$S_{14}S_2$ S_bS_a	S_2S_{14} S_bS_a	$S_{14}S_2$ S_aS_b	S_2S_{14} S_aS_b
1.5	$S_{29}S_{14}S_bS_a$	-	-	-	-	-	-	-	-	n.t.	-	+	+	-
2.4	$S_{14}S_{29}S_bS_a$	-	-	-	-	-	-	-	-	-	+	+	+	+
5.1	$S_{14}S_{29}S_aS_b$	-	-	-	-	-	-	-	-	-	+	+	+	+
7.2	$S_aS_bS_{29}S_{14}$	+	-	+	-	-	-	-	-	n.t.	+	+	+	+
1.6	$S_{29}S_2S_bS_a$	-	-	-	-	-	-	-	-	-	+	+	+	+
3.4	$S_2S_{29}S_bS_a$	-	-	-	-	-	-	-	-	-	+	+	+	+
4.3	$S_{29}S_2S_aS_b$	-	-	-	-	-	-	-	-	-	-	-	-	-
6.1	$S_2S_{29}S_aS_b$	-	-	-	+	-	-	-	-	-	+	+	+	+
7.3	$S_aS_bS_{29}S_2$	n.t.	n.t.	-	n.t.	-	n.t.	n.t.	n.t.	-	n.t.	n.t.	n.t.	n.t.
2.6	$S_{14}S_2S_bS_a$	+	+	+	+	-	+	+	+	-	+	+	+	+
3.5	$S_2S_{14}S_bS_a$	-	+	+	+	+	+	+	+	-	+	+	+	+
5.3	$S_{14}S_2S_aS_b$	-	+	-	+	+	+	-	+	-	-	-	+	+
6.2	$S_2S_{14}S_aS_b$	+	+	+	+	+	+	+	+	-	+	+	+	-

n.t. - not tested; - incompatible pollination; + compatible pollinations

Table 5.6. Interpretation of interspecific crosses between the F_1's of synthetic B. napus with B. campestris and B. oleracea

Line number	Allelic constitution	B. campestris as female		B. campestris as male		B. oleracea as female			B. oleracea as male		
		S_b	S_a	S_b	S_a	S_{29}	S_2	S_{14}	S_{29}	S_2	S_{14}
1.5	$S_{29}S_{14}S_bS_a$	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
2.4	$S_{14}S_{29}S_bS_a$	+	+	-	+	-	+	-	-	-	+
5.1	$S_{14}S_{29}S_aS_b$	+	+	-	-	-	+	-	-	-	+
7.2	$S_{29}S_{14}S_aS_b$	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
1.6	$S_{29}S_2S_bS_a$	+	+	-	-	-	+	+	-	-	-
3.4	$S_2S_{29}S_bS_a$	n.t.	+	-	+	-	+	+	±	-	-
4.3	$S_{29}S_2S_aS_b$	+	-	-	+	-	+	+	-	-	-
6.1	$S_2S_{29}S_aS_b$	+	+	-	+	-	-	-	±	-	+
7.3	$S_{29}S_2S_aS_b$	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
2.6	$S_{14}S_2S_bS_a$	+	+	-	-	-	+	+	+	-	+
3.5	$S_2S_{14}S_bS_a$	+	+	-	-	+	+	+	+	-	+
5.3	$S_{14}S_2S_aS_b$	n.t.	-	-	-	+	+	+	+	-	+
6.2	$S_2S_{14}S_aS_b$	+	+	-	-	+	-	-	-	-	-

n.t. - not tested; - incompatible pollinations; + compatible pollinations

Table 5.7. A summary of expected and observed results of intra- and interspecific crosses

	Line numbers	Allelic constitution	1.5, 2.4 5.1, 7.2 $S_{29}S_{14}$ S_a S_b	1.6, 3.4 4.3, 6.1 as male $S_{29}S_2$ S_a S_b	2.6, 3.5 5.3, 6.2 $S_{14}S_2$ S_a S_b	B. campestris as female (♀) S_b	B. campestris as female (♀) S_a	B. campestris as male (♂) S_b	B. campestris as male (♂) S_a	B. oleracea as female (♀) S_{29}	B. oleracea as female (♀) S_2	B. oleracea as female (♀) S_{14}
expected results	1.5, 2.4 5.1, 7.2	$S_{29}S_{14}S_aS_b$	-	-	±	+	-	-	+	-	+	-
	1.6, 3.4 4.3, 6.1 7.3	$S_{29}S_2S_aS_b$	-	-	+	+	-	-	+	-	+	+
	2.6, 3.5 5.3, 6.2	$S_{14}S_2S_aS_b$	-	-	-	+	-	-	-	+	+	-
obtained results	1.5, 2.4 5.1, 7.2	$S_{29}S_{14}S_aS_b$	-	-	+	+	+	-	+	-	+	-
	1.6, 3.4 4.3, 6.1 7.3	$S_{29}S_2S_aS_b$	-	-	±	+	+	-	+	-	+	+
	2.6, 3.5 5.3, 6.2	$S_{14}S_2S_aS_b$	±	±	±	+	+	-	-	+	+	+

- incompatible pollinations; + compatible pollinations

Table 5.8 Number of pollen tubes penetrating a stigma following crosses between synthetic B. napus F_1s and B. campestris 82-213

Stigma source	Pollen source: Synthetic B. napus F_1 Group I	Group II	Group III	B. campestris 82-213
Group I	n.t.	n.t.	n.t.	20, 66, 65
Group II	n.t.	n.t.	n.t.	20, 25, 3
Group III	n.t.	n.t.	n.t.	40, >75, >75
B. campestris 82-213	>75	>75	>75	n.t.

n.t. - not tested

Table 5.9 Compatible and incompatible crosses between B. oleracea homozygous lines.

Stigma source	Pollen source: B. oleracea $S_{29}S_{29}$	$S_{14}S_{14}$	S_2S_2
$S_{29}S_{29}$	-	+	+
$S_{14}S_{14}$	+	-	+
S_2S_2	+	+	-

\- incompatible pollinations
\+ compatible pollinations

Table 5.10 Active alleles in F_1s as expected and detected

Group	line numbers	allelic constitution	active alleles			
			expected		detected	
			stigma	pollen	stigma	pollen
I	1.5, 2.4 5.1, 7.2	$S_{29}S_{14}S_aS_b$	$S_{29}S_{14}S_b$	$S_{29}S_{14}S_a$	$S_{29}S_b$	$S_{29}S_{14}$
II	1.6, 3.4, 4.3 6.1, 7.3	$S_2S_{29}S_aS_b$	$S_{29}S_b$	$S_{29}S_a$	$S_{29}S_b$	S_{29}
III	2.6, 3.5 5.3, 6.2	$S_{14}S_2S_aS_b$	$S_{14}S_2S_a$ S_b	$S_{14}S_a$	$S_2S_aS_b$	

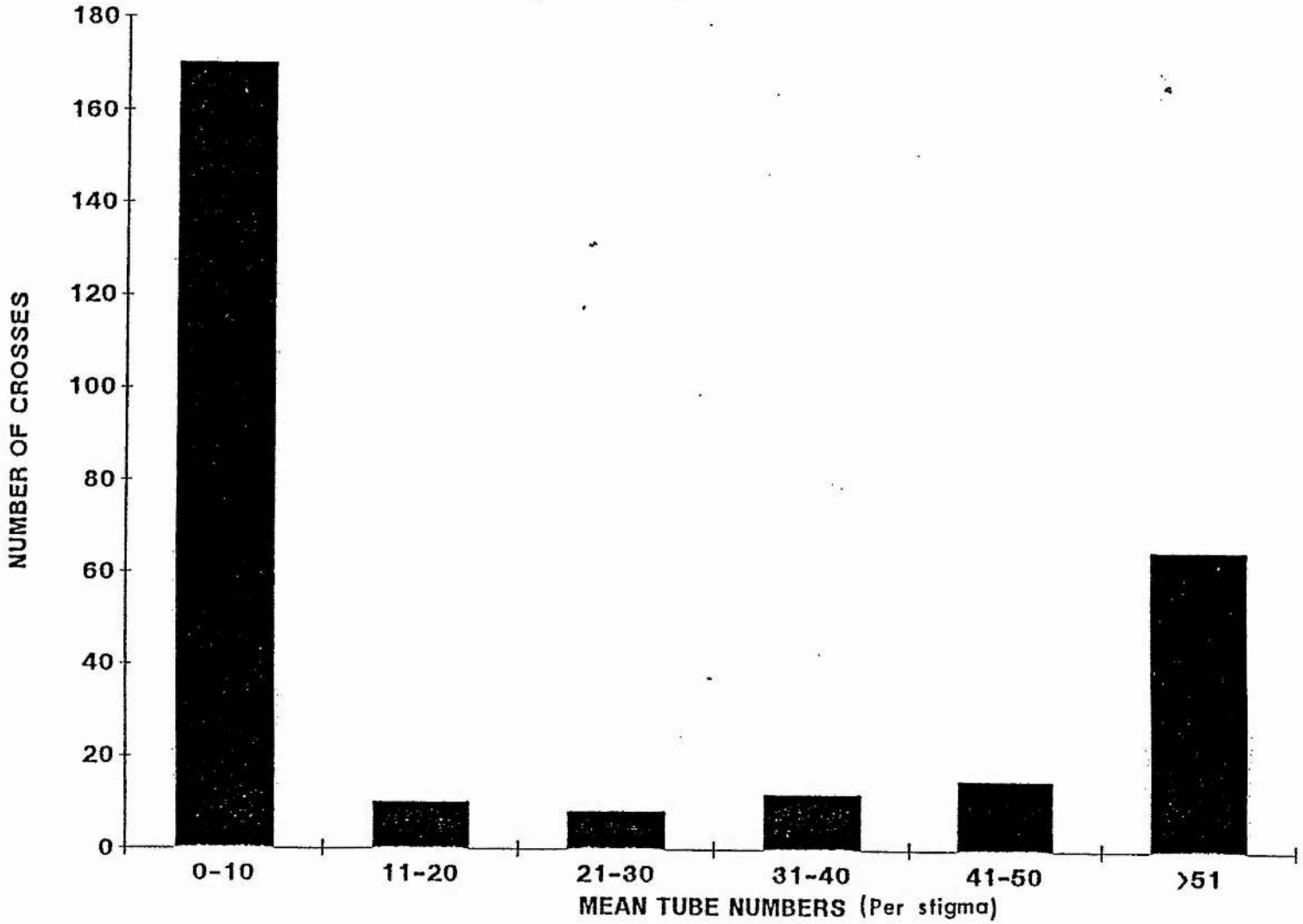


Fig. 5.1. Distribution of mean pollen tube numbers per stigma in crosses between synthetic B. napus F_1's x B. oleracea (Interspecific).

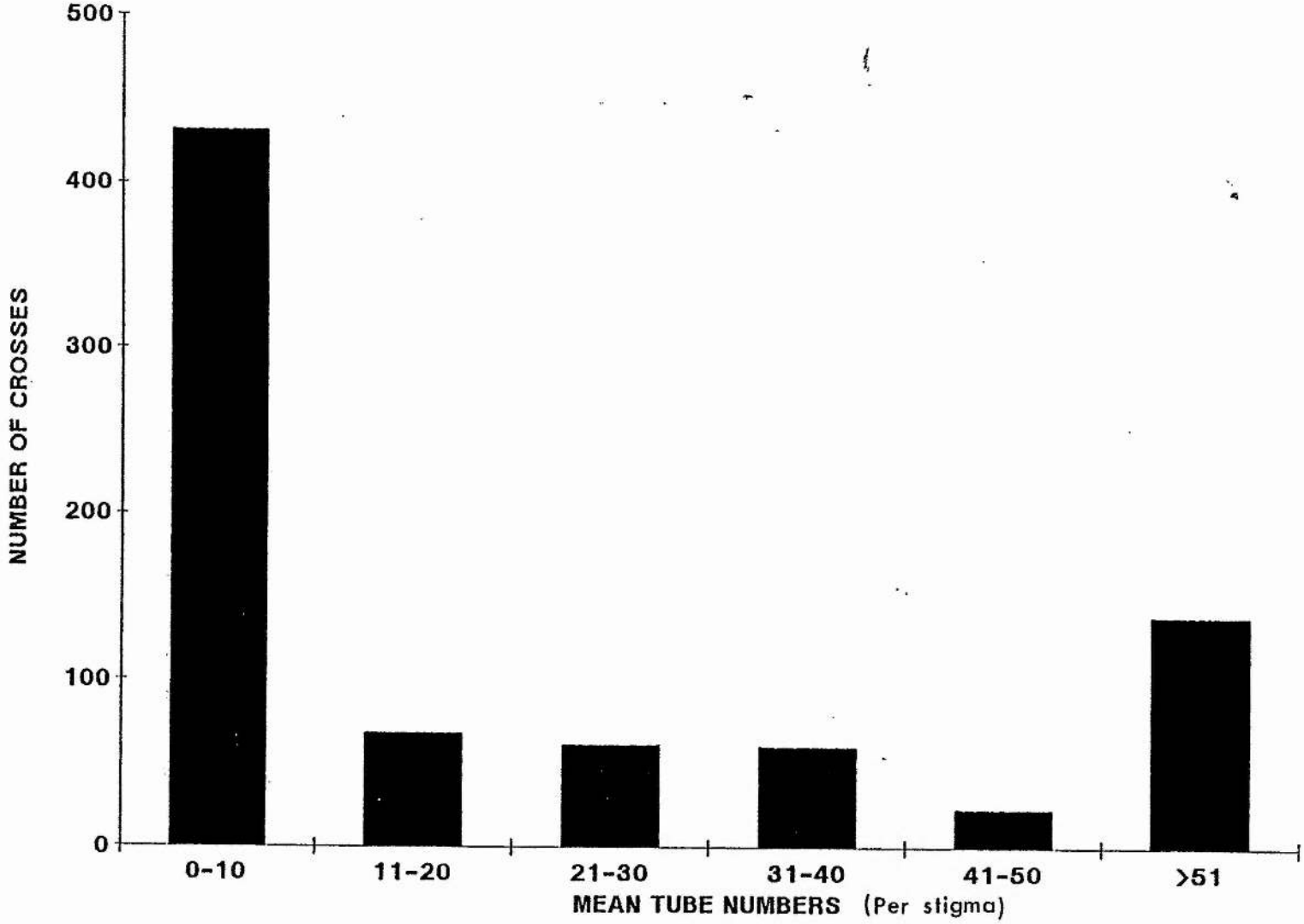


Fig. 5.2. Distribution of mean pollen tube numbers per stigma in crosses between synthetic B. napus F_1's (Intraspecific).

CHAPTER 6

GENETICAL STUDIES OF INCOMPATIBILITY IN SYNTHETIC *B. NAPUS* F_2 LINES

6.1 INTRODUCTION

In Chapters 3 and 5, the operation of the self-incompatibility system and activity of S-alleles at the *B. campestris* and *B. oleracea* S loci were determined in several synthetic *B. napus* lines that were homozygous at each S-locus, and also, in several *B. napus* F_1s that were heterozygous for different alleles at each of the two S-loci. It was established that both S-loci could be expressed in the synthetics, and that there was an interaction between loci such that either *B. campestris* or *B. oleracea* alleles could be dominant in the pollen and/or stigma. In the heterozygous F_1's it was shown that *B. oleracea* S-alleles were expressed in both pollen and stigma, whereas *B. campestris* alleles were expressed only in the stigma. Thus, in the pollen, only 2 alleles were expressed while in the stigma 3 were expressed, even when the expectation from the parents was that the effects of all 4 alleles would be detected (Chapter 5, Table 5.10). An interesting feature to emerge from the results was the presence of self-compatibility in the synthetic line of $S_{14}S_a$ genotype (Chapter 3) and also in F_1's containing the $S_{14}S_2S_aS_b$ alleles. The evidence suggested that this self-compatibility involved the combination of S_{14} and S_a in the absence of the S_{29} dominant allele. Such self-compatibility could be due to allelic interaction between the allele derived from *B. oleracea* and that from *B. campestris* or to gene interaction not associated with the S-alleles.

Following on from the previous analysis conducted, the objective

of the work reported in this Chapter was to investigate the activity and segregation of S-alleles in F_2 families of synthetic B. napus obtained from selfing two F_1 lines analysed in Chapter 5. It was intended that this investigation would also add to the development of the allele identification which could be used in F_1 hybrid production.

6.2 MATERIALS AND METHODS

In July 1988 15 plants, 5 from each of the three different 4 S-allele F_1 types studied in Chapter 5 were self-pollinated to produce F_2 families. Because of limitations of time only 2 families were sown (80 seeds from each) at the beginning of December 1988, and plants were raised to maturity in a heated glasshouse maintained at a temperature of 20°C day and 14°C night under natural light. From February 1989, plants were treated with Hoagland solution (for composition see Appendix 3) every two weeks.

6.2.1 Experiment I - The F_2 obtained from self-pollinating $S_{29}S_{14}S_aS_b$ (line 88-184)

(a) Seventy-one F_2 plants were used in the study. One plant was chosen (88-184-03) and test pollinated reciprocally with the remaining 70 plants. All plants were also self-pollinated to determine their self-compatibility status.

(b) All F_2 plants (71) were crossed reciprocally with B. campestris plants homozygous for the allele S_a or S_b, and also used as the pollen parent in crosses with B. oleracea plants homozygous for either the

S_{29} or S_{14} alleles.

(c) To determine if any F_2 plants were male sterile they were also used as the male parent in crosses with an unrelated B. campestris line (82-339) possessing a different S-allele constitution to that of the B. campestris lines used in (b) above.

For each test cross (in a, b and c) five flowers taken randomly were pollinated per plant. The degree of incompatibility was determined as described in Chapter 3.

6.2.2. Experiment II - The F_2 obtained from self-pollinating $S_{14}S_2S_aS_b$

(a) Thirty-two plants of the F_2 family were crossed reciprocally with B. campestris S_a and S_b homozygotes, and also used as the pollen parent in crosses with B. oleracea S_2 and S_{14} homozygotes. Self-pollinations to determine the self-compatibility status of F_2 plants were also made as well as crosses with an unrelated B. campestris line as female parent to determine male sterility. For each test cross, three flowers taken randomly from the required plant were pollinated and the degree of incompatibility was determined as described in Chapter 3.

6.3 RESULTS AND ANALYSIS

6.3.1 Determining the number of progeny to be tested in an F_2 family and distinguishing compatible from incompatible pollinations.

Self-pollination of an $S_{29}S_{14}S_aS_b$ heterozygote may be expected to

yield 9 genotypes thus: $S_{29}S_{14}S_aS_b$; $S_{29}S_{14}S_aS_a$; $S_{29}S_{14}S_bS_b$; $S_{29}S_{29}S_aS_b$; $S_{14}S_{14}S_aS_b$; $S_{14}S_{14}S_aS_a$; $S_{14}S_{14}S_bS_b$; $S_{29}S_{29}S_aS_a$ and $S_{29}S_{29}S_bS_b$. Where 2 loci are segregating, the number of plants required to detect all genotypes can be calculated from the formula $(15/16)^n = 1/100$ at a 0.01% probability of detecting all possible genotypes, where n is the number of plants required. A family size of 71 is therefore required. (Mather, 1951).

Compatible and incompatible pollinations were detected as described in Chapters 3 and 5. Again they were distinguished on the basis of tube numbers. As Fig. 6.1 shows, although 70% of the test results can be clearly distinguished as having less than 10 or more than 50 pollen tubes, 30% were intermediates. As with the earlier data, the smallest class were those with 21-30 pollen tubes and it was decided to consider pollinations with a mean of over 30 pollen tubes as compatible and those with under 20 as an incompatible. However the intermediates (3% pollinations) had to be evaluated individually.

6.3.2 Results of Experiment Ia

Self and intraline pollination results are summarised in Table 6.1. All the F_2 plants were effectively self-incompatible. Of the F_2 plants for which results are available, only 4 plants (88-184-11, 12, 28, 62) appeared to be more or less reciprocally compatible with the tester (88-184- 03). Of the remaining plants, 4 gave a compatible result in one direction and a significant number of tubes in the other direction (Plants 88-184-22, 25, 50, 57); one plant (55) was fully compatible as pollen but not as female; and 10 plants gave varying

numbers of tubes from 1-32 in one or both directions. The tester (88-184- 03) therefore identified a group of 8 plants with which it showed either full compatibility (Plants 88-184-11, 12, 28, 62) or almost full compatibility (88-184-22, 25, 50, 57).

6.3.3 Results of Experiment Ib

The results of crosses between F_2 plants and B. campestris, and B. oleracea are presented in Table 6.2. Using the criteria that below 20 tubes per test is incompatible and over 20 is compatible, one can deduce the activity of S-alleles. F_2 plants which gave identical results in the crosses were considered as a single group, and in this way, 14 different groups were identified. Also presented in the tables below is the compatibility shown by F_2 plants used as the male parent in crosses with B. campestris (82-339). All such crosses were compatible, indicating that none of the F_2 plants were male sterile.

Reaction of Group 1

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	$S_{29}S_{29}$	female
24	+	-	-	-	-	-	+

\+ compatible pollinations - incompatible pollinations

Pollen from F_2 plants in this group was compatible in B. campestris S_b stigmas whilst all other crosses were incompatible. This suggests that all four S-alleles ($S_{29}S_{14}S_aS_b$) were present in plants of Group 1, and that S_b was inactive while S_a, S_{14} and S_{29} were

active in the pollen. The conclusion should be regarded as provisional for plants 51, 55 and 59 in view of the significant tube penetration when B. oleracea S_{29} is used as the female (Table 6.2). It may be that these particular plants are more appropriately assigned to Group 10 (see below). Despite these anomalies, plants in Group 1 appear to possess alleles S_{29}, S_{14}, S_a and S_b, with S_b recessive in the pollen.

Reaction of Group 2

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	$S_{29}S_{29}$	female
8	+	-	+	-	-	-	+

Pollen of F_2 plants placed in Group 2 was compatible with both B. campestris S_a and S_b testers, suggesting that both S_a and S_b alleles were inactive in Group 2 pollen. Pollinations with B. oleracea S_{14} and S_{29} as female parent were incompatible, evidence that S_{14} and S_{29} alleles were both active in pollen. The plants were also fully incompatible when B. campestris S_a and S_b testers were used as the pollen parent, showing that both S_a and S_b were active in the stigmas of Group 2. Thus there was evidence that all 4 S-alleles were present in this group, although the allele relationships differed from those of Group 1 plants with both S_a and S_b being recessive in the pollen.

Reaction of Group 3

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	$S_{29}S_{29}$	female
5	+	-	-	+	-	-	+

Pollen of F_2 plants placed in Group 3 was compatible in B. campestris S_b stigmas but incompatible in S_a, B. oleracea S_{29} and S_{14} stigmas, indicating that S_{14}, S_{29} and S_a were active and S_b was inactive in Group 3 pollen. These results are identical to those obtained for Group 1 plants. Rather surprisingly, B. campestris S_a pollen was compatible in the stigmas of Group 3 plants indicating that S_a was inactive in F_2 plants when used as the female parent. Thus there was evidence that all four alleles were present in this group, with S_b being recessive in pollen and S_a recessive in the stigma.

Reaction of Group 4

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	$S_{29}S_{29}$	female
10	+	-	+	+	-	-	+

F_2 plants in Group 4 were reciprocally compatible with the B. campestris S_a line showing that S_a was inactive in both the pollen and stigmas of these plants. This group of plants was also fully compatible when B. campestris S_b was used as the female parent. However, crosses between the F_2's and the B. oleracea S_{14} and S_{29} lines used as female parents were incompatible, showing that the S_{14}

and S_{29} alleles were present and active in the pollen of this group. Plants of the group were also incompatible with S_b pollen, indicating that S_b was active in the stigmas of F_2's. Thus in plants of Group 4, only S_b, S_{14} and S_{29} were expressed; S_a appeared to be absent and S_b was recessive in the pollen.

Reaction of Group 5

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	$S_{29}S_{29}$	female
3	+	+	-	-	-	-	+

Group 5 plants were compatible in both directions with the B. campestris S_b line showing that S_b was inactive in both pollen and stigmas of Group 5. In contrast, the group was incompatible in both directions with the B. campestris line S_a indicating that S_a was active in both pollen and stigma of Group 5 plants. The group was also fully incompatible with the B. oleracea S_{29} and S_{14} homozygotes used as female parents, showing that S_{29} and S_{14} were active. Thus plants assigned to Group 5 appeared to possess the S_a, S_{14} and S_{29} alleles, with S_a showing independent action in the pollen and stigmas.

Reaction of Group 6

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	$S_{29}S_{29}$	female
1	+	+	+	-	-	-	+

The single F_2 allotted to Group 6 was compatible in both directions with the B. campestris line S_b and also with S_a used as the female parent, implying that S_b was inactive in both pollen and stigmas while S_a was inactive in just the pollen of this F_2 plant. Crosses with the B. oleracea lines S_{14} and S_{29} (used as female parent) were incompatible indicating that S_{14} and S_{29} were both present in the F_2 plant. The plant was also incompatible with S_a pollen indicating activity of S_a in the stigma. Thus, the F_2 plant placed in Group 6 possessed the S_a, S_{14} and S_{29} alleles but, in contrast to plants of Group 5, S_a appeared to be recessive in the pollen.

Reaction of Group 7

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	$S_{29}S_{29}$	female
2	+	-	-	+	+	-	+

Group 7 plants were compatible with the B. campestris S_b and S_{14} homozygotes used as the female parent showing that S_b and S_{14} were inactive in the pollen of this group of F_2 plants. The plants of the group were also compatible with S_a pollen, indicating that S_a was inactive in their stigmas. In contrast, Group 7 plants were incompatible with S_b pollen and S_{29} and S_a stigmas. It is concluded that Group 7 plants possessed the S_a, S_b and S_{29} alleles with S_b being recessive in the pollen and S_a recessive in the stigmas.

Reaction of Group 8

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	$S_{29}S_{29}$	female
5	+	-	-	-	+	-	+

Group 8 plants were compatible with S_b and S_{14} as the female parent showing that both S_b and S_{14} were inactive in the pollen. The F_2's were, however incompatible in both directions with the B. campestris S_a line, indicating that S_a was active in both pollen and stigmas. Plants of the group were also fully incompatible with S_b pollen and S_{29} stigmas. It was, therefore, concluded that plants placed in Group 8 possessed the S_{29}, S_a and S_b alleles but S_b was recessive in the pollen.

Reaction of Group 9

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	$S_{29}S_{29}$	female
1	+	-	+	-	-	+	+

The F_2 plant placed in Group 9 was compatible when used as the male parent in crosses with S_b, S_a and S_{29} indicating that S_b, S_a and S_{29} were inactive in its pollen. The same plant, however, was incompatible when used as the female in crosses with S_a and S_b pollen showing that S_a and S_b were active in its stigmas. When crossed with the B. oleracea S_{14} homozygote (female), the cross also proved incompatible. Thus, plants in Group 9 contained S_{14}, S_a and S_b with

S_a and S_b recessive in the pollen.

Reaction of Group 10

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	$S_{29}S_{29}$	female
5	+	-	-	-	-	+	+

Group 10 F_2's were incompatible in both directions with the B. campestris S_a tester showing that S_a was active in both pollen and stigma of this group. Plants of the group were also incompatible with S_b pollen and S_{14} stigmas indicating that S_b was active in the stigma while S_{14} was active in the pollen. Crosses with the testers S_b and S_{29} used as the female parent, were compatible, showing that S_b and S_{29} were inactive in the pollen of the F_2's. It is concluded that plants in Group 10 possessed the S_{14}, S_a and S_b alleles but S_b was recessive in the pollen.

Reaction of Group 11

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	$S_{29}S_{29}$	female
2	+	+	-	-	-	+	+

The two plants placed in this group were compatible in both directions with the B. campestris S_b line and were also compatible with B. oleracea S_{29} used as the female. This shows that S_{29} and S_b were inactive in the pollen and S_b was inactive in the stigmas of both

F_2 plants. The two plants were incompatible, reciprocally with the B. campestris S_a tester, and with B. oleracea S_{14} used as the female. This was evidence that S_{14} was active in the pollen while S_b was active in both pollen and stigmas of Group 11 plants. It is concluded that plants in Group 11 possess only the S_a and S_{14} alleles, with independent action of S_a in pollen and stigma. (Interestingly, neither of the two plants in Group 11 was self-compatible - see Table 6.1).

Reaction of Group 12

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	$S_{29}S_{29}$	female
1	+	-	+	+	-	+	+

Most crosses involving the plant in Group 12 were compatible apart, that is, from those using S_b pollen or S_{14} stigmas. The latter means that S_b was active in the stigma while S_{14} was active in the pollen. It is concluded that the plant placed in Group 12 possessed the $S_{14}S_{14}S_bS_b$ genotype with S_{14} showing dominance to S_b in pollen.

Reaction of Group 13

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	$S_{29}S_{29}$	female
2	+	+	-	+	+	-	+

Crosses involving Group 13 plants were compatible except when stigmas were used of tester stocks containing the S_a or S_{29} alleles. This means that S_{29} and S_a were active in the pollen of Group 13

plants. It is concluded that plants of this group possess only the S_{29} and S_a alleles and S_a is recessive in the stigmas.

Reaction of Group 14

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	$S_{29}S_{29}$	female
2	+	-	+	+	+	-	+

The two F_2's in Group 14 showed cross-compatibility with testers except those producing S_b pollen or S_{29} stigmas. Thus S_b was active in the stigma while S_{29} was active in the pollen of Group 14 plants. It is concluded that the plants in Group 14 possessed the $S_{29}S_{29}S_bS_b$ genotype with S_{29} showing dominance to S_b in the stigma.

6.3.4 Results of Experiment II

The results of crosses between plants of the F_2 family (derived by selfing $S_{14}S_2S_aS_b$) with B. campestris and B. oleracea tester lines, are presented in Table 6.3. Also included are the mean number of pollen tubes produced per stigma when each of the F_2 plants were selfed. From the results of the self-pollinations, it was established that six plants were self-compatible with over 38 pollen tubes produced per stigma, while 16 were clearly self-incompatible (10 plants were not tested for self-compatibility). Among 32 F_2 plants tested, those which gave identical results in interspecific crosses were considered as a single group and in this way 10 groups were identified.

Reaction of Group 1

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	S_2S_2	female
3	+	-	+	-	-	-	+

Of the 3 F_2 plants placed in Group 1, one was self-compatible. However, all plants in the group were incompatible with both B. campestris S_a and S_b pollen, showing that the S_a and S_b alleles were active in the stigmas of these F_2's. Plants of the group also produced pollen that was incompatible in B. oleracea S_{14} and S_2 stigmas, indicating that the S_{14} and S_2 alleles were both active in the pollen of the F_2's. Cross-pollinations using the S_a and S_b homozygotes as female parents were compatible, providing evidence that S_a and S_b were both inactive in the pollen. The evidence suggests, therefore, that Group 1 plants possess all four alleles - S_{14}, S_2, S_a and S_b but S_a and S_b were recessive in the pollen.

Reaction of Group 2

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	S_2S_2	female
2	-	+	-	-	-	-	+

Of the 2 plants assigned to Group 2 only one was tested for self-compatibility and was found to be partially self-compatible with a mean of 18 pollen tubes per stigma. However, both plants were

reciprocally incompatible with the B. campestris S_a tester line showing that S_a was active in both pollen and stigma. Pollen of the two F_2 plants was also incompatible with the testers homozygous for S_b, S_{14} and S_2 indicating that S_b, S_{14} and S_2 were active in the pollen of F_2's. Finally crosses using the S_b homozygote as the male were compatible, providing evidence that S_b was inactive in the stigma. It is concluded that F_2 plants in Group 2 also possess the S_{14}, S_2, S_a and S_b alleles but S_b is recessive in the stigma.

Reaction of Group 3

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	S_2S_2	female
5	+	-	+	-	-	+	+

Of the 5 plants placed in Group 3 only two were tested for self-compatibility. One (plant 36) was fully compatible with over 75 pollen tubes per stigma and another one was partially self-compatible. Plants of the group used as the male parent were cross-compatible with the S_a, S_b and S_2 tester lines indicating that S_a, S_b and S_2 were inactive in the pollen. The plants, however, when used as the female, were incompatible with S_a and S_b pollen, showing that these two alleles were active in their stigmas. The evidence suggests, therefore, that plants placed in Group 3 contain the S_{14}, S_a and S_b alleles, with S_a and S_b being recessive in the pollen.

Reaction of Group 4

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	S_2S_2	female
3	+	-	-	-	-	+	+

Of the 3 plants allotted to Group 4, 2 were self-compatible with over 61 pollen tubes per stigma while the other was self-incompatible with no pollen tube penetration. Plants of the group were reciprocally incompatible with the S_a tester line showing that S_a was active in both pollen and stigma. They were also incompatible when involved in crosses with S_b pollen or S_{14} stigmas indicating that S_b was active in their stigmas and S_{14} in their pollen. When used to pollinate the S_b and S_2 stigmas, the crosses were compatible, showing that S_b and S_2 were both inactive in the pollen of F_2's. It was concluded that plants allotted to Group 4 possessed the S_a, S_b and S_{14} alleles with S_b being recessive in the pollen.

Reaction of Group 5

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	S_2S_2	female
2	-	-	+	-	-	+	+

Of the 2 plants placed in Group 5, one was tested for self-compatibility and shown to produce 47 pollen tubes per stigma. Plants of this group were incompatible with all testers except those with S_a and S_2 stigmas. It is concluded that plants allotted to Group 5

possessed the genotype $S_{14}S_{14}S_aS_b$, with S_a being recessive in the pollen.

Reaction of Group 6

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	S_2S_2	female
4	-	-	-	-	+	-	+

In Group 6 one plant was found to be fully self-compatible producing 38 pollen tubes per stigma while 2 plants were fully self-incompatible. Plants of the group were incompatible with all testers except the B. oleracea line with S_{14} stigmas. Thus Group 6 plants appear to possess the S_a, S_b and S_2 alleles, with S_a and S_b showing independent action in both pollen and stigma.

Reaction of Group 7

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	S_2S_2	female
2	+	+	-	-	-	+	+

The single plant tested for self-compatibility in Group 7 was fully self-incompatible. Plants of the group were reciprocally incompatible with the B. campestris S_a tester showing that S_a was active in both the pollen and stigmas of these plants. Their pollen was also incompatible in S_{14} stigmas indicating that S_{14} was active in the pollen. Crosses involving the S_b tester as male and female and

the S_2 line as female were compatible indicating that S_2 was inactive in the pollen while S_b also inactive in the pollen & stigma. The evidence suggests, therefore, that plants in Group 7 possess only the S_{14} and S_a alleles, with the S_a allele showing independent action in pollen and stigma.

Reaction of Group 8

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	S_2S_2	female
6	+	-	+	+	-	+	+

Of 4 plants tested for the self-compatibility in Group 8 all proved to be self-incompatible producing between 0-6 pollen tubes per stigma. Plants of the group were reciprocally compatible with the B. campestris S_a tester line indicating that the S_a allele was inactive in both pollen and stigmas of this group of plants. Pollen produced by the plants was also compatible in the stigmas of the S_b and S_2 testers indicating a lack of S_b and S_2 activity in the pollen. Finally, the incompatible crosses with B. oleracea S_{14} stigmas and B. campestris S_b pollen showed that in the F_2 plants the S_{14} allele was active in the pollen while S_b was active in the stigma. From this it is concluded that plants allotted to Group 8 possess the S_{14} and S_b alleles, with S_b being recessive in the pollen.

Reaction of Group 9

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	S_2S_2	female
3	-	-	+	+	-	+	+

All plants in Group 9 produced less than 2 pollen tubes per stigma when selfed. Plants of the group were reciprocally incompatible with the B. campestris S_b tester and incompatible with stigmas of the S_{14} tester. The plants were also found to be reciprocally compatible with the S_a tester and compatible with stigmas of the S_2 line. It was concluded, therefore, that plants of this group possessed the S_b and S_{14} alleles with S_b showing independent action in the pollen and stigma.

Reaction of Group 10

Number of plants	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
	female	male	female	male	$S_{14}S_{14}$	S_2S_2	female
2	-	-	+	+	+	-	+

Both plants allotted to Group 10 were fully self-incompatible with no more than 1 pollen tube produced per stigma. Plants of the group were reciprocally incompatible with the S_b tester line showing that S_b was active in both pollen and stigma. Pollen of Group 10 plants was also incompatible in S_2 stigmas suggesting S_2 was active in the pollen. Crosses with B. oleracea S_{14} as the female, and B. campestris S_a in both directions, were compatible. Consequently, the plants in

Group 10 were considered to possess just the S_2 and S_b alleles with S_b showing independent action in the pollen and stigma. Plants chosen at random from each phenotypic group were tested with B. oleracea and B. campestris tester lines to confirm the results and these results were consistent with those of the first set of the tests.

6.4 DISCUSSION

6.4.1 The F_2 obtained from self-pollinating $S_{14}S_{29}S_aS_b$

All 71 plants of this F_2 family were effectively self-incompatible indicating that the self-incompatibility response was fully functional, even in plants of genotype $S_{14}S_{14}S_aS_a$. From the results of crosses made between the F_2 plants and the B. campestris and B. oleracea tester lines (each homozygous for particular S-alleles), the 9 possible S-locus genotypes that could be present in an F_2 were identified (Table 6.4). Plants of the same genotype frequently differed with respect to the expression of particular S-alleles. Thus based on their S-allele constitution (genotype) plus the activity of alleles in pollen and stigmas the 71 plants of the F_2 naturally fell into a total of 14 different groups.

Plants in Group 1 contained all 4 S-alleles (S_{14}, S_{29}, S_a, S_b), but in the pollen S_b was recessive. Group 2 plants also contained all 4 S-alleles but both S_a and S_b were recessive in the pollen. Finally plants assigned to Group 3 again contained the 4 different S-alleles but S_a was recessive in the stigma and S_b in the pollen. Thus plants in Groups 1, 2 and 3 all appeared to possess the genotype $S_{29}S_{14}S_aS_b$

but showed different S-allele activity in stigma or pollen. In similar fashion the S-locus genotypes and activity of alleles have been established for plants in the other 11 groups (Table 6.4).

An interesting feature to emerge from Table 6.4 is that the difference between phenotypic and genotypic groups is entirely due to the variation in expression of allele S_a. Thus activity of S_a in this F_2 could be affected either by an environmental factor or by genetic factors unrelated to the S-locus. It is difficult to identify an environmental factor that might be responsible for variation of S_a expression. No obvious flower to flower basis or plant to plant factor could be detected. The possibility of it being due to another single dominant gene unlinked to the S-allele would reflect a similar situation to one described by Verma et al. (1977) in Eruca sativa.

By means of the interspecific crosses the genotype of the F_2 plant (88-184-03) used as the tester in crosses to other F_2 plants (Experiment Ia) was established to be $S_{29}S_{29}S_aS_b$ with S_a being recessive in the stigma and S_b in the pollen. The results obtained from crosses between 88-184-03 and remaining 70 plants are given in Table 6.5, ordered according to the groups identified in interspecific tests, but 4 test crosses (plants 51, 55, 22 and 57) gave anomalous compatible results which did not fit the general pattern of the genotypic groups. In two of these crosses (involving plants 88-184-55 and 57) full compatibility occurred with over 75 pollen tubes per stigma. In fact the results obtained for plants in Group 1 (Table 6.2) showed that plants 51 and 55 might more properly be assigned to Group 10, because of the significant tube penetration that occurred in B. oleracea S_{29} stigmas. It is possible that the anomalous results obtained for plants 51 and 55 were the result of partial inactivity of

the S_{29} allele. However anomalous results obtained for plants 22 and 57 could not be accounted for. Anomalous incompatible results were obtained in 3 test crosses (plants 88-184-19, 35 and 50). However these crosses gave a significant number of pollen tube penetrations and may have been false negatives.

6.4.2 F_2 obtained from self-pollinating $S_{14}S_2S_aS_b$

As noted earlier self-compatible plants occurred in all the phenotypic groups detected in this family, but detection of allele activity was still possible. In Experiment II, 10 different phenotypes were identified and these are shown in the Table 6.6 together with their presumed S-allele constitution and allele activity. All four alleles were detected in plants assigned to Groups 1 and 2 but in the former S_a and S_b were recessive in pollen whilst in the latter all 4 alleles were expressed in pollen but S_b was recessive in the stigmas. Thus it was concluded that plants in Groups 1 and 2 each possess the genotype $S_{14}S_2S_aS_b$. As in Experiment I, it was possible to deduce the genotypes of the other 8 groups in the same way (Table 6.6). In contrast to the F_1's all four alleles could be active in the pollen of heterozygous plants. As in Experiment I, activity of B. campestris S-alleles showed between plant variation. In this family activity of both S_a and S_b varied and this variation could occur in groups possessing only 2 S-alleles (e.g. Groups 8 and 9). Only 32 plants were tested in this family and only 6 genotypes were identified. Furthermore, the number of plants in the different genotypes appeared to deviate considerably from expectations for this family. The Chi

square test showed that the ratio of S_aS_b:S_aS_a:S_bS_b plants was not significantly different from the expected 2:1:1 ratio at 0.05%, but that the ratio of $S_{14}S_2$:S_2S_2:$S_{14}S_{14}$ did differ significantly from expectation. The difference between phenotypic and genotypic groups was entirely the result of variation in expression of B. campestris S_a and S_b alleles. In the pollen of those plants which contained both S_a and S_b alleles (19 plants), S_a and S_b were both active in 6 plants, S_a was active in 3 plants and S_b in 2 plants. In 8 plants none of the B. campestris alleles were active.

6.4.3 Consequences of the results obtained from analysing F_2 progenies

Inherited form of self-compatibility was present in the synthetic material. However, neither genetic control of this self-compatibility nor its precise character were resolved. The analysis of both F_2 progenies showed that it was not linked or associated with the $S_{14}S_{14}S_aS_a$ genotype in which it has first been detected. The occurrence of self-compatibility in family 88-188 and its absence in 88-184 suggests that it may only be expressed in the absence of dominant S-alleles.

Analysis using a single plant tester from within the family as in 88-184, was uninformative. This was not surprising, given the activities of the S-alleles found in the synthetics and F_1's. As Table 6.7 shows, only 3 groups would be distinguished from such tests indicating that successful allele identification in such complex situations requires homozygous testers. In fact, 'hidden' S-loci could persist in a species with sporophytic self-incompatibility if

the alleles at the locus were partially or completely recessive. Verma et al. (1977) reported that Eruca sativa possessed a sporophytic system with 2-3 loci. They assumed interlocus interaction of the type found in grasses, such that the 2 alleles at S and Z loci, together produced a particular specificity. In the 2 loci system described in B. napus the alleles at each locus retain individual specificities i.e. S_a remains S_a, its expression being dependent on the inter and intra locus dominance relations with other loci. The 9 genotypes were therefore expected to produce up to 9 phenotypes depending on dominance relationships of the alleles present. In fact, more than 9 genotypes were found depending on the expression of S_a in the family containing alleles $S_{29}S_{14}S_aS_b$ (88-184) and S_a and S_b in family containing alleles $S_{14}S_2S_aS_b$ (88-188). Table 6.8 gives the results that would be expected from a complete diallel set of crosses for family 88-184. It should be noted that there are more than 9 detectable phenotypes and that if the incompatibility status of the material had been unknown and tester lines unavailable, 3 locus systems would have been required to account for the results. It may be of value for those working with other complex self-incompatibility systems to re-evaluate their results with this data in mind. Finally, the activity of B. oleracea alleles in stigma remains undetermined and could only be deduced in some cases. The production of testers homozygous for single B. oleracea alleles is required for these tests.

Table 6.1 Mean number of pollen tubes penetrating the stigma following self and intraline crosses between synthetic B. napus F_2 plants containing alleles $S_{29}S_{14}S_aS_b$ (88-184)

Plant No. (88-184)	Self pollinations	88-184-03 female	88-184-03 male
1	0	0	0
2	0	0	0
3	0	0	0
4	0	n.t.	0
5	0	0	1
6	0	0	0
7	0	0	0
8	0	7	0
9	3	0	0
10	0	0	0
11	9	21	29
12	0	23	>75
13	0	0	17
14	0	0	0
15	0	0	0
16	0	0	0
17	0	11	15
18	4	0	0
19	2	23	10
20	0	0	0
21	0	0	0

/...

Plant No. (88-184)	Self pollinations	88-184-03 female	male
22	0	17	25
23	0	0	0
24	0	0	0
25	2	20	31
26	0	0	0
27	0	0	0
28	13	>75	>75
29	0	0	0
30	0	0	0
31	0	0	0
32	0	0	0
33	0	0	0
34	0	0	0
35	6	10	26
36	0	0	0
37	0	0	0
38	0	0	0
39	0	0	0
40	0	0	0
41	0	0	0
42	0	0	0
43	0	0	0
44	0	0	0
45	0	0	0
46	0	0	0

/...

Plant No. (88-184)	Self pollinations	88-184-03 female	male
47	0	7	0
48	0	0	0
49	0	0	0
50	7	43	18
51	18	8	32
52	0	2	20
53	0	0.	n.t.
54	0	0	0
55	0	>75	0
56	0	5	n.t.
57	0	20	>75
58	0	0	0
59	0	0	n.t.
60	0	0	0
61	0	0	0
62	0	60	34
63	0	0	0
64	0	0	0
65	0	0	0
66	0	0	0
67	0	0	0
68	0	0	0
69	0	0	0
70	0	0	0
71	0	0	0

Table 6.2 Mean numbers of pollen tubes penetrating the stigma following crosses between line 88-184 ($S_{29}S_{14}S_aS_b$) with B. campestris and B. oleracea tester lines

Group No.	Plant No.	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
		female	male	female	male	$S_{14}S_{14}$	$S_{29}S_{29}$	female
1	1	23	3	6	7	0	1	6
	4	23	0	8	6	0	0	61
	5	35	7	8	6	0	0	>75
	6	25	0	4	11	0	0	>75
	8	29	0	1	0	0	0	>75
	15	61	0	14	0	0	0	>75
	21	37	0	11	0	0	0	40
	23	31	0	0	0	5	5	>75
	30	40	0	4	0	0	0	32
	33	>75	0	17	3	0	0	44
	36	>75	0	7	0	0	0	n.t.
	37	>75	0	2	8	0	0	38
	38	>75	0	7	8	0	0	25
	41	43	0	13	0	0	0	24
	43	20	0	0	0	0	0	8
	44	56	0	18	11	0	0	48
	47	50	0	0	0	0	0	37
	48	>75	0	8	10	4	0	43
	51	>75	0	1	0	0	12	36
	54	63	0	6	10	0	0	50
	55	>75	0	3	1	0	17	30 /...

Group No.	Plant No.	B. campestris S_bS_b female	B. campestris S_bS_b male	B. campestris S_aS_a female	B. campestris S_aS_a male	B. oleracea female $S_{14}S_{14}$	B. oleracea female $S_{29}S_{29}$	B. campestris 82-339 female
1	58	20	0	0	13	0	0	23
	59	25	0	0	0	9	16	40
	63	43	0	0	0	0	0	>75
Total		48	0	6	4	1	2	43
2	7	20	0	25	6	0	0	>75
	13	44	0	33	6	0	0	>75
	18	>75	7	20	4	0	0	>75
	24	49	0	28	9	0	0	60
	26	>75	0	52	0	0	0	n.t.
	32	45	0	20	2	0	0	>75
	64	20	0	>75	19	0	0	>75
	65	60	0	35	0	11	0	>75
Total		49	1	36	6	2	0	64
3	14	>75	0	10	>75	0	0	47
	31	>75	0	4	54	0	0	n.t.
	42	20	0	12	>75	0	11	>75
	60	27	0	0	22	5	0	47
	68	52	16	6	28	0	0	39
Total		50	3	6	51	1	2	42

/...

Group No.	Plant No.	B. campestris S_bS_b female	B. campestris S_bS_b male	B. campestris S_aS_a female	B. campestris S_aS_a male	B. oleracea female $S_{14}S_{14}$	B. oleracea female $S_{29}S_{29}$	B. campestris 82-339 female
4	2	68	0	>75	>75	0	0	32
	10	>75	0	40	27	0	0	65
	16	48	0	54	22	0	0	>75
	17	46	0	63	>75	0	0	>75
	22	>75	0	29	36	0	0	>75
	29	>75	0	47	40	0	0	>75
	45	>75	16	28	40	0	0	>75
	52	61	0	20	58	16	0	>75
	57	>75	0	49	27	0	0	>75
	66	>75	0	20	49	0	0	25
Total		67	2	43	45	1	0	65
5	40	>75	42	0	0	0	0	>75
	53	69	69	0	0	0	0	>75
	69	57	58	17	0	0	0	29
Total		59	56	7	0	0	0	55
6	71	>75	>75	>75	0	0	0	>75
7	3	>75	0	2	>75	56	0	35
	70	62	0	10	>75	62	0	25
Total		69	0	6	>75	59	0	30

/...

Group No.	Plant No.	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
		female	male	female	male	$S_{14}S_{14}$	$S_{29}S_{29}$	female
8	20	36	0	0	0	>75	0	33
	27	>75	0	4	0	>75	0	54
	34	>75	0	8	0	>75	0	25
	49	20	12	0	0	24	0	20
	61	36	0	0	12	>75	0	36
Total		48	4	2	4	70	0	36
9	11	53	0	40	0	0	32	>75
10	19	63	0	13	0	0	46	65
	28	>75	0	9	3	0	60	30
	35	>75	0	0	0	0	51	>75
	50	39	0	4	0	2	27	21
	62	23	0	0	0	11	38	>75
Total		50	0	6	0	2	40	52
11	25	24	21	7	0	0	20	47
	56	50	41	4	0	0	25	61
Total		37	31	6	0	0	23	54
12	12	>75	0	>75	22	0	29	>75

/...

Group No.	Plant No.	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339
		female	male	female	male	$S_{14}S_{14}$	$S_{29}S_{29}$	female
13	39	>75	>75	4	35	>75	0	>75
	46	54	25	7	27	56	0	>75
Total		65	50	5	31	66	0	>75
14	9	>75	0	26	31	>75	0	>75
	67	65	0	>75	40	60	0	>75
Total		70	0	51	36	68	0	>75

Table 6.3 Mean numbers of pollen tubes penetrating the stigma following crosses between line 88-188 ($S_{14}S_2S_aS_b$) with B. campestris and B. oleracea tester lines.

Group No.	Plant No. (88-188)	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339	Self pollination
		female	male	female	male	$S_{14}S_{14}$	S_2S_2	female	
1	10	50	0	31	0	0	4	>75	49
	12	33	0	31	0	12	5	>75	10
	30	>75	0	>75	0	0	0	>75	n.t.
Total		53	0	46	0	4	3	>75	
2	18	16	>75	7	0	0	3	>75	18
	34	0	40	0	0	13	0	>75	n.t.
Total		8	58	4	0	7	2	>75	
3	2	>75	0	35	13	0	>75	>75	18
	4	20	0	59	2	0	61	>75	n.t.
	25	40	0	40	18	0	>75	>75	n.t.
	29	35	0	63	12	0	>75	45	n.t.
	36	>75	0	20	6	0	>75	>75	>75
Total		49	0	43	10	0	73	69	

/...

Group No.	Plant No.	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339	Self pollination
		female	male	female	male	$S_{14}S_{14}$	S_2S_2	female	
4	6	26	0	2	8	0	>75	n.t.	65
	20	58	0	15	0	12	61	>75	0
	28	>75	3	0	8	0	20	>75	61
Total		53	1	6	5	4	52	>75	
5	15	13	17	45	0	10	32	>75	47
	24	15	11	63	0	0	28	>75	n.t.
Total		14	14	54	0	5	30	>75	
6	1	10	0	0	0	33	9	>75	0
	7	0	11	12	0	25	12	>75	0
	14	0	0	4	1	>75	0	2	38
	38	19	0	7	0	>75	15	>75	n.t.
Total		7	3	8	0	52	9	57	

/...

Group No.	Plant Number	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339	Self pollination
		female	male	female	male	$S_{14}S_{14}$	S_2S_2	number	
7	19	>75	57	12	5	0	>75	>75	n.t.
	31	55	47	18	0	0	>75	>75	0
Total		65	52	15	3	0	>75	>75	
8	3	36	0	52	47	0	>75	n.t.	0
	22	35	0	55	28	0	23	>75	n.t.
	26	>75	0	>75	55	0	33	>75	5
	33	25	0	37	>75	0	>75	25	6
	39	>75	0	>75	60	0	>75	>75	0
	40	>75	0	>75	61	0	>75	>75	n.t.
Total		54	0	62	54	0	59	65	

/...

Group No.	Plant Number	B. campestris S_bS_b		B. campestris S_aS_a		B. oleracea female		B. campestris 82-339	Self pollination
		female	male	female	male	$S_{14}S_{14}$	S_2S_2	number	
9	5	14	0	>75	63	0	>75	>75	0
	21	10	0	38	>75	0	>75	>75	0
	23	0	0	39	21	5	>75	>75	2
Total		8	0	51	53	2	>75	>75	
10	32	0	0	>75	>75	>75	2	n.t.	1
	11	0	0	65	>75	40	6	>75	0
Total		0	0	70	>75	58	1	>75	

Table 6.4 Phenotypic and genotypic groups deduced in F_2 family containing alleles $S_{29}S_{14}S_aS_b$ together with their allele activity

Group	Expressed alleles	Active alleles (Phenotypes) female	Active alleles (Phenotypes) male	No. of plants	Genotypes	No. of plants
1	$S_{29}S_{14}S_aS_b$	$S_{29}S_{14}S_aS_b$	$S_{29}S_{14}S_a$	24		
2	"	"	$S_{29}S_{14}$	8	$S_{29}S_{14}S_aS_b$	37
3	"	$S_{29}S_{14}S_b$	$S_{29}S_{14}S_a$	5		
4	$S_{29}S_{14}S_b$	$S_{29}S_{14}S_b$	$S_{29}S_{14}$	10	$S_{29}S_{14}S_b$	10
5	$S_{29}S_{14}S_a$	$S_{29}S_{14}S_a$	$S_{29}S_{14}S_a$	3	$S_{29}S_{14}S_a$	4
6	"	"	$S_{29}S_{14}$	1		
7	$S_{29}S_aS_b$	$S_{29}S_b$	$S_{29}S_a$	2	$S_{29}S_aS_b$	7
8	"	$S_{29}S_aS_b$	"	5		
9	$S_{14}S_aS_b$	$S_{14}S_aS_b$	S_{14}	1	$S_{14}S_aS_b$	6
10	"	$S_{14}S_aS_b$	$S_{14}S_a$	5		
11	$S_{14}S_a$	$S_{14}S_a$	$S_{14}S_a$	2	$S_{14}S_a$	2
12	$S_{14}S_b$	$S_{14}S_b$	S_{14}	1	$S_{14}S_b$	1
13	$S_{29}S_a$	S_{29}	$S_{29}S_a$	2	$S_{29}S_a$	2
14	$S_{29}S_b$	$S_{29}S_b$	S_{29}	2	$S_{29}S_b$	2
Total				71		71

Table 6.5 Interpretation of the results obtained from intraline crosses containing alleles $S_{29}S_{14}S_aS_b$

Group	Genotype	Plant No.	Mean pollen tube no. 88-184-03 female	male
1	$S_{29}S_{14}S_aS_b$	1	0	0
		4	n.t.	0
		5	0	1
		6	0	0
		8	7	0
		15	0	0
		21	0	0
		23	0	0
		30	0	0
		33	0	0
		36	0	0
		37	0	0
		38	0	0
		41	0	0
		43	0	0
		44	0	0
		47	7	0
		48	0	0
		51	8	32
		54	0	0
		55	>75	0

/...

Group	Genotype	Plant No.	Mean pollen tube no. 88-184-03	
			female	male
1	$S_{29}S_{14}S_aS_b$	58	0	0
		59	0	n.t.
		63	0	0
2	"	7	0	0
		13	0	17
		18	0	0
		24	0	0
		26	0	0
		32	0	0
		64	0	0
		65	0	0
3	"	14	0	0
		31	0	0
		42	0	0
		60	0	0
		68	0	0
Total			4	3

/...

Group	Genotype	Plant No.	Mean pollen tube no. 88-184-03	
			female	male
4	$S_{14}S_{29}S_bS_b$	2	0	0
		10	0	0
		16	0	0
		17	11	15
		22	17	25
		29	0	0
		45	0	0
		52	2	20
		57	20	>75
		66	0	0
Total			5	14
5	$S_{14}S_{29}S_a$	40	0	0
		53	0	n.t.
		69	0	0
6	"	71	0	0
Total			0	0

/...

Group	Genotype	Plant No.	Mean pollen tube no. 88-184-03	
			female	male
7	$S_{29}S_{29}S_aS_b$	3	0	0
		70	0	0
8	"	20	0	0
		27	0	0
		34	0	0
		49	0	0
		61	0	0
Total			0	0
9	$S_{14}S_{14}S_aS_b$	11	21	29
10	"	19	23	10
		28	>75	>75
		35	10	26
		50	43	18
		62	60	34
Total			39	37
11	$S_{14}S_{14}S_aS_a$	25	20	31
		56	25	n.t.
Total			23	31
12	$S_{14}S_{14}S_bS_b$	12	23	>75

/...

Group	Genotype	Plant No.	Mean pollen tube no. 88-184-03 female	male
13	$S_{29}S_{29}S_aS_a$	39	0	0
		46	0	0
Total			0	0
14	$S_{29}S_{29}S_bS_b$	9	0	0
		67	0	0
Total			0	0

Table 6.6 Phenotypic and genotypic groups deduced in F_2 family containing alleles $S_{14}S_2S_aS_b$ together with their allele activity

Group	Expressed alleles	Active alleles in (phenotypes) female	male	No. of plants in each group	Genotype	No. of plants in each genotype
1	$S_{14}S_2S_aS_b$	$S_{14}S_2S_aS_b$	$S_{14}S_2$	3	$S_{14}S_2S_aS_b$	5
2	"	$S_{14}S_2S_a$	$S_{14}S_2S_aS_b$	2		
3	$S_{14}S_aS_b$	$S_{14}S_aS_b$	S_{14}	5		
4	$S_{14}S_aS_b$	$S_{14}S_aS_b$	$S_{14}S_a$	3	$S_{14}S_{14}S_aS_b$	10
5	$S_{14}S_aS_b$	$S_{14}S_aS_b$	$S_{14}S_b$	2		
6	$S_2S_aS_b$	$S_2S_aS_b$	$S_2S_aS_b$	4	$S_2S_2S_aS_b$	4
7	$S_{14}S_a$	$S_{14}S_a$	$S_{14}S_a$	2	$S_{14}S_{14}S_aS_a$	2
8	$S_{14}S_b$	$S_{14}S_b$	S_{14}	6	$S_{14}S_{14}S_bS_b$	9
9	$S_{14}S_b$	$S_{14}S_b$	$S_{14}S_b$	3		
10	S_2S_b	S_2S_b	S_2S_b	2	$S_2S_2S_bS_b$	2
Total				32		32

Table 6.7 Possible results could be obtained from intercrossing B. napus F_2 line containing alleles $S_{29}S_{14}S_aS_b$ according to the allele activity detected in F_1's. $\overline{S}$ indicate the active alleles as detected in F_1's

		Pollen source								
Group	Stigma source	$\overline{S}_{29}\overline{S}_{29}S_aS_a$	$\overline{S}_{29}\overline{S}_{29}S_aS_b$	$\overline{S}_{29}\overline{S}_{29}S_bS_b$	$\overline{S}_{29}\overline{S}_{14}S_aS_a$	$\overline{S}_{29}\overline{S}_{14}S_aS_b$	$\overline{S}_{29}\overline{S}_{14}S_bS_b$	$\overline{S}_{14}\overline{S}_{14}S_aS_a$	$\overline{S}_{14}\overline{S}_{14}S_aS_b$	$\overline{S}_{14}\overline{S}_{14}S_bS_b$
I	$\overline{S}_{29}\overline{S}_{29}S_aS_a$	-	-	-	-	-	-	+	+	+
	$\overline{S}_{29}\overline{S}_{29}S_a\overline{S}_b$	-	-	-	-	-	-	+	+	+
	$\overline{S}_{29}\overline{S}_{29}\overline{S}_b\overline{S}_b$	-	-	-	-	-	-	+	+	+
II	$\overline{S}_{29}\overline{S}_{14}S_aS_a$	-	-	-	-	-	-	-	-	-
	$\overline{S}_{29}\overline{S}_{14}S_a\overline{S}_b$	-	-	-	-	-	-	-	-	-
	$\overline{S}_{29}\overline{S}_{14}\overline{S}_b\overline{S}_b$	-	-	-	-	-	-	-	-	-
III	$\overline{S}_{14}\overline{S}_{14}S_aS_a$	+	+	+	N	N	N	N	N	N
	$\overline{S}_{14}\overline{S}_{14}S_a\overline{S}_b$	+	+	+	-	-	-	-	-	-
	$\overline{S}_{14}\overline{S}_{14}\overline{S}_b\overline{S}_b$	+	+	+	-	-	-	-	-	-

\+ compatible pollinations
\- incompatible pollinations
N could not be detected due to the self-compatibility of $S_{14}S_{14}S_aS_a$ genotype.

Table 6.8 Possible results could be obtained from intercrossing B. napus F_2 line 88-184 ($S_{29}S_{14}S_aS_b$) according to the detected phenotypes

Stigma source \ Pollen source	$S_{29}S_{14}S_a$	$S_{29}S_{14}$	$S_{29}S_{14}S_a$	$S_{29}S_{14}$	$S_{29}S_{14}S_a$	$S_{29}S_{14}$	$S_{29}S_a$	$S_{29}S_a$	$S_{14}S_a$	$S_{14}S_a$	$S_{14}S_a$	S_{14}	$S_{29}S_a$	S_{29}
$S_{29}S_{14}S_aS_b$	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$S_{29}S_{14}S_aS_b$	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$S_{29}S_{14}S_b$	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$S_{29}S_{14}S_b$	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$S_{29}S_{14}S_a$	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$S_{29}S_{14}S_a$	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$S_{29}S_b$	-	-	-	-	-	-	-	-	+	+	+	+	-	-
$S_{29}S_aS_b$	-	-	-	-	-	-	-	-	-	-	-	+	-	-
$S_{14}S_b$	-	-	-	-	-	-	+	+	-	-	-	-	+	+
$S_{14}S_aS_b$	-	-	-	-	-	-	-	-	-	-	-	-	-	+
$S_{14}S_a$	-	-	-	-	-	-	-	-	-	-	-	-	-	+
$S_{14}S_b$	-	-	-	-	-	-	+	+	-	-	-	-	+	+
S_{29}	-	-	-	-	-	-	-	-	+	+	+	+	-	-
$S_{29}S_b$	-	-	-	-	-	-	-	-	+	+	+	+	-	-

+ compatible pollinations - incompatible pollinations

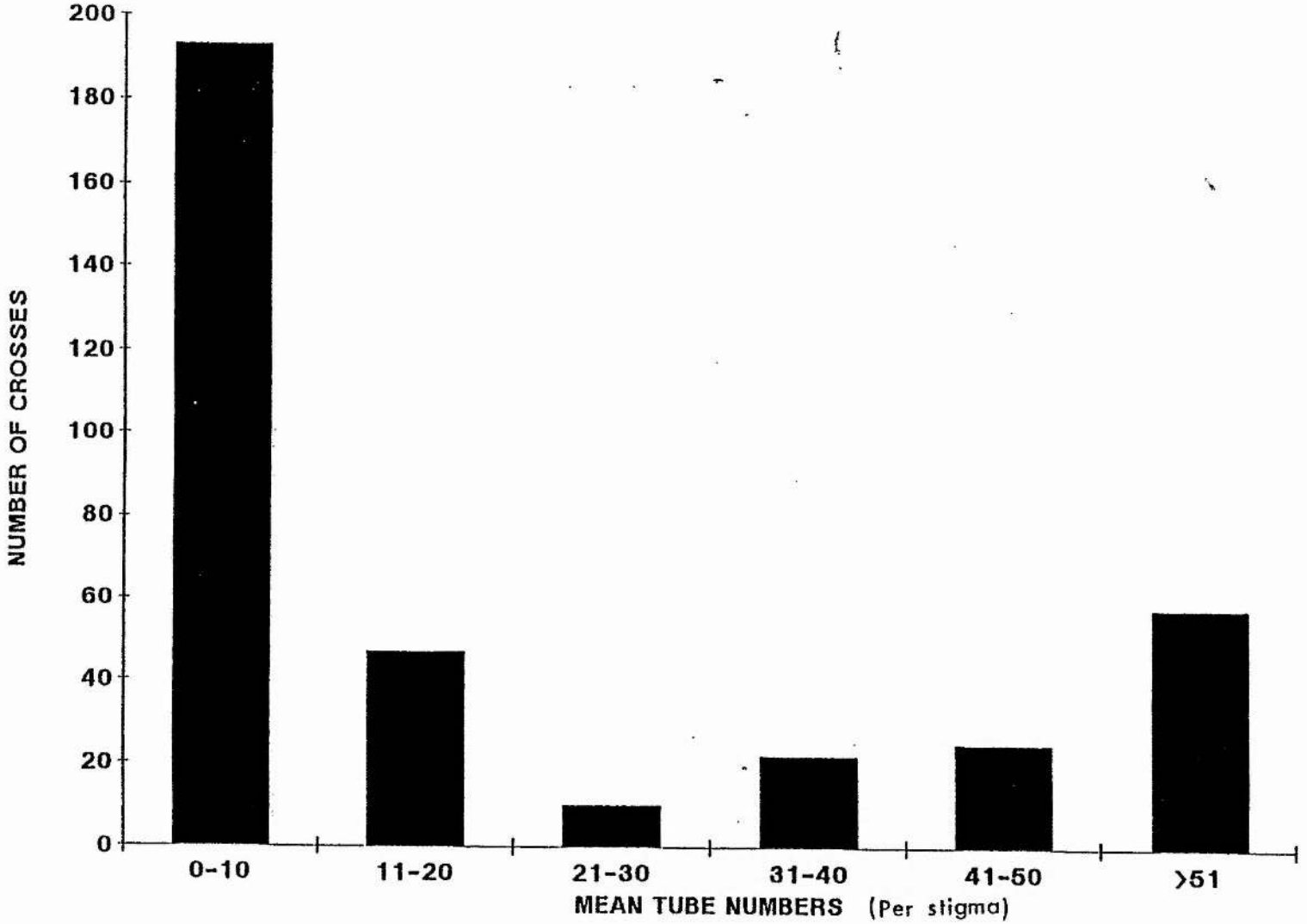


Fig. 6.1. Distribution of mean pollen tube numbers per stigma crosses between synthetic *B. napus* F_2's x *B. campestris*.

CHAPTER 7

BIOCHEMICAL STUDIES OF STIGMATIC PROTEINS IN BRASSICAS

7.1 INTRODUCTION

Lewis (1952) used serological techniques to identify incompatibility substances and proposed that different S-alleles produced specific and antigenically distinguishable substances in Oenothera organensis pollen. Following this approach, Nasrallah & Wallace (1967a) employed immunodiffusion techniques to assay the antigens present in stigmas of different incompatibility genotypes of cabbage. They showed that genotype specific antigens could be detected in self-incompatible stigmas of different B. oleracea S-allele homozygotes and also in the stigmas of their F_1 hybrids. Later, using acrylamide gel electrophoresis, Nasrallah et al. (1970) detected differences in protein patterns from stigmatic homogenates of several S-allele genotypes of B. oleracea with homogenates from heterozygotes exhibiting the S-allele specific bands of both parents. Further studies conducted on developing stigmas (Nasrallah, 1974) demonstrated a correlation between self-incompatibility and 'S' protein concentration in stigmas. A sudden increase in the level of self-incompatibility protein was detected during the development of the stigma; this suggested that a threshold quantity of protein was necessary for the complete manifestation of the self-incompatibility reaction. Of further significance was the finding by Sedgley (1974b) which showed that B. oleracea stigmas homozygous for S_{16} and S_{23}, contained twice as much antigenically detectable S-protein as those heterozygous for the same alleles.

Nishio & Hinata (1977) were first to use polyacrylamide

isoelectricfocusing (IEF) to show that in B. oleracea, S-allele specificity could be ascribed to a combination of the protein bands detected. They reported that the parental bands were transmitted to their progeny and were specific to stigmatic tissue. They (Nishio & Hinata, 1978, 1980) further established that certain S-specific molecules were glycoproteins, and following a study of different S-allele lines of B. oleracea and B. campestris (Hinata & Nishio, 1978) were able to correlate 7 S-alleles with distinct glycoproteins. Subsequently they reported (Nishio & Hinata, 1980) that among the S_2, S_7, S_{22}, S_{39} and S_{45} alleles, the recessive allele S_2 did not produce clear bands when subjected to acrylamide gel or cellulose acetate electrophoresis; however, the dominant alleles S_{39}, S_{22}, S_7 and S_{45} gave clear bands. Nishio & Hinata (1982) next purified S-correlated glycoproteins in B. oleracea using gel electrophoresis and recorded distinct differences between alleles in amino acid composition.

An investigation by Nasrallah & Nasrallah (1984) of nine S-homozygotes of B. oleracea revealed that 7 genotypes produced single bands after IEF, while two produced a complex of isoforms. Continuing this work, Nasrallah et al. (1985a) produced a complementary DNA clone which encoded two S-locus-specific-glycoproteins. Nasrallah et al. (1987) later published a complete amino-acid sequence of the cDNA clones and suggested this represented part of the base sequence of the S-gene in Brassica.

More recent studies on cDNA clones of the putative S-gene in Brassica have revealed several complexities. Trick & Flavell (1989) have isolated 2 different cDNA clones for transcripts which are equally abundant in stigmas competent for self-incompatibility and

each of which is homologous to previously reported S-locus-specific-glycoprotein sequences. In addition, Lalonde et al. (1989) have reported that the Brassica genome contains multiple S-locus-specific-glycoprotein related sequences.

Taken overall, the evidence to date suggests that in Brassicas, a glycoprotein specific to the S-allele is produced in the stigma during the later stages of bud maturation. This glycoprotein appears to have a relatively high pI, (Isoelectric point) amino-acid constitution, molecular weight and in some cases different isoforms. Difficulties have been experienced in identifying S-glycoproteins specific to recessive S-alleles and the significance of difficult isoforms in some but not all S-glycoproteins are unknown. Most importantly no progress appears to have been made in identifying the S-specific pollen product.

Studies aimed at determining whether a protein present in the stigma or pollen is a component of the self-incompatible interaction, and specific to a particular S-allele, need to demonstrate that the protein: is present in the flower, but absent in the bud; exhibits S-allele specific characteristics; and has some functional significance.

This Chapter describes the use of isoelectricfocusing to investigate proteins that are specific to the stigmas of synthetic B. napus lines and their progeny. The investigations reported were also aimed at determining whether the occurrence of any stigma specific bands could be correlated with the presence of specific S-alleles.

7.2. MATERIALS AND METHODS

7.2.1. Materials

During the course of the experiments, stigma, style and leaf tissue was obtained from the synthetic B. napus lines 1, 2, 3, 4, 5 and 6 (described in Chapter 2) and their B. campestris parents homozygous for S_a and S_b and B. oleracea parents homozygous for S_{29}, S_{14} and S_2. To investigate the segregation of protein bands that differed between synthetics, F_2 plants obtained by selfing F_1's were also surveyed.

7.2.2. Determination of protein concentration in tissue samples using microassay procedure

Four dilutions of standard Bio-Rad protein (Bio-Rad-Laboratories - U.K.) containing from 1 to 25 µg/ml were prepared to provide the standard curve for comparison with plant tissue. Sample extract was prepared and the Bio-Rad protein dye reagent added. The optical density was then recorded and compared with that from the protein standard.

7.2.3. Analytical Isoelectricfocusing

(a) Preparation of gels for ultrathin polyacrylamide gel-isoelectric-focusing.

Ultrathin polyacrylamide gels were prepared as follows. The following ingredients were combined to produce a solution that was subsequently degassed.

Biorad Acrylamide/bis 37.5 : 1 (dissolve 5 g in 7.5 ml H_2O)

Glycerol (16% v/v)

Ampholytes (pH 3.5 - 9.5 ampholines)

Two plates - a top glass plate (10 mm thick x 25 cm x 13 cm with a strip of Dymotape stuck along each long edge) and a bottom glass plate (2 mm thick x 25 cm x 13 cm) - were cleaned with 70% ethanol. The top plate was then wiped with repel silane (2% v/v dimethyldichlorosilane in 1, 1, 1, trichloroethane) and the bottom one with bind silane (A-174 silane from Pharmacea U.K., dissolved in 0.2% v/v in chloroform). After wiping the bottom plate was placed on the gel pouring apparatus. Ammonium persulphate (10 mg/ml) was added to the solution which was gently swirled and poured on to the plate to give a bubble-free layer that was left to set for 45 minutes.

Alternatively pre-poured pH 3.5-9.5, 1.5 mm thick polyacrylamide gel plates obtained from Pharmacia LKB Biotechnology, were used.

(b) Electrophoresis procedure

Gels were layed over a film of water on a cooling plate. Electrode strips, previously soaked in electrolyte solution were placed along each long edge of the gel about 0.5 - 1 mm from the edge. Electrolyte solutions of 1M H_3PO_3 (anode) and 1M NaOH (cathode) were used in all experiments. Gels were prefocused for 0.5 hour at a constant power (25 mA). Samples (20 µl) were then loaded in paper wicks at appropriate positions on the gel, allowed to run into the gel for 0.5 hour, and the paper wicks were then removed. Samples were subsequently fully focused for 2000 volt/hours (v/h).

7.2.4 Detection methods

7.2.4.1. Staining procedures

Ultrathin gels were fixed for 25 minutes in a solution of 35 g sulphosalicylic acid, 130 g trichloroacetic acid and 350 ml acetic acid dissolved in 1 litre of distilled water. Gels were then washed to remove the ampholines for 15 minutes in 'destaining' solution containing 35% ethanol and 10% acetic acid in distilled water. Proteins were visualised by staining in a solution containing 1.5 g Coomassie Brilliant Blue R250 (Sigma - U.K.), 35% C_2H_5OH and 10% CH_3COOH in distilled water for 15 minutes. Gels were then returned to fresh destaining solution for 15 minutes or until the background was clear. The gel surface was wiped with a piece of cotton wool soaked in destaining solution to remove any precipitated stain.

Gels purchased from Pharmacia LKB Biotechnology were treated differently. These were fixed for 30 minutes, immediately after focusing, in a solution of 34.6 g sulphosalicylic acid and 115 g trichloracetic acid made up in 1 litre distilled water. They were then washed for 15 minutes in destaining solution containing 25% C_2H_5OH and 8% acetic acid in distilled water. Proteins were visualised by staining in solution containing 0.45 g Coomassie Brilliant Blue R250 (Sigma - U.K.) in 400 ml destaining solution (heated to 60°C before use) for 30 minutes. Gels were subsequently destained in fresh destaining solution for 15 minutes or until the background was clear.

7.2.4.2. Densitometry of focused bands

Stained gels were scanned using a US-9000 'Shimadzu' dual wavelength flying-stop scanner. Each band was scanned by a laser beam (0.05 x 2.0 µm) at a single wavelength of 565 nm, resulting in a trace which plotted the absorbance value against the distance along the electrophogram, with the area under the trace proportional to the total protein content. The minimum area, minimum width and drift line used for the peak detection was 300, 0 and 0.1.

7.3 EXPERIMENTS

7.3.1. Determination of appropriate extraction procedure and sample loading position for isoelectricfocusing

Protein was extracted from stigmas using two methods

(a) From open flowers from which anthers were removed before dehiscence, 0.02 g of stigmas (approximately 50 stigmas) of the synthetic B. napus lines 5 ($S_{14}S_a$) and 3 (S_2S_b) (Chapter 2) were collected and homogenised in 80 µl 0.1 M phosphate-buffered saline (0.01 M phosphate buffer pH 7.1 plus 8.5 gm/l of NaCl - Nishio & Hinata, 1977). Each homogenate was centrifuged for 20 minutes at 6,000 g.

(b) Approximately fifty stigmas were collected as above and homogenised in 80 µl distilled water and centrifuged as before.

For electrophoresis, approximately 20 µl of the supernatant from B. napus lines 3 and 5 were loaded in a different position in a pH

3.5 - 9.5, 1.5 mm thick polyacrylamide gel plate and positioned 0.5 cm from anodal and/or 0.5 cm from cathodal end. Isoelectricfocusing was carried out as described in section 7.2.

7.3.2. Measurements of protein

The synthetic B. napus line 1 was used for preliminary measurement of protein in Brassica stigmas. Four dilutions of standard Bio-Rad protein (Bio-Rad Laboratory, U.K.) containing from 1 to 25 µg/ml were prepared to provide a standard. Stigmas or styles taken from clean flowers (0.01 g) were homogenised in distilled water (40 µl/sample) in an eppendorf tube and centrifuged for 2,000 g for 20 minutes. Distilled water was added to the supernatant to give 0.8 ml of extract to which 0.2 ml Bio-Rad protein dye reagent was added to the sample tubes. After 20 minutes the OD_{595} was measured with a reagent blank as control.

7.3.3. Identification of stigma-specific proteins in synthetic and their parents

Synthetic B. napus lines 3, 4, 5, and the B. oleracea and B. campestris parental lines homozygous for S_{29}, S_2, S_{14}, S_a and S_b were subjected to analysis. Stigmas were collected (25/sample), from open flowers from which anthers had been removed before dehiscence, and homogenised in 40 µl of distilled water. Each homogenate was centrifuged for 20 minutes at 6,000 g. The same technique was used for preparation of extracts from leaves and styles of the lines S_2,

S_{29}, S_{14}, $S_{14}S_a$.

Each sample was loaded on to the anodal end of an ultrathin pH 3.5 - 9.5 polyacrylamide gel plate and isoelectricfocusing was carried out as described in section 7.2. Gels were stained and scanned using US-9000 laser densitometer at 565 nm.

7.3.4. Detection of interline differences in protein band patterns of synthetic B. napus lines and their parents.

Synthetic B. napus lines 1, 3, 4, 5 and 6, heterozygous respectively for $S_{29}S_b$, S_2S_b, $S_{29}S_a$, $S_{14}S_a$ and S_2S_a and their B. campestris and B. oleracea parents homozygous for S_a, S_b, S_{29}, S_{14} and S_2 were surveyed. Stigmas (25/sample) were collected and homogenised in 40 µl distilled water as before and loaded onto the anodal end on a pH 3.5 - 9.5 which was then run using standard conditions.

7.3.5. Detecting S-specific proteins in synthetic B. napus F_2's (88 - 184)

Seven different genotypes which self-incompatibility tests had shown possessed the S-allele genotypes, $S_{14}S_{29}S_aS_b$, $S_{29}S_{29}S_aS_b$, $S_{14}S_{14}S_aS_b$, $S_{29}S_{14}S_aS_a$, $S_{29}S_{14}S_bS_b$, $S_{29}S_{29}S_aS_a$ and $S_{29}S_{29}S_bS_b$ were used, together with the parental controls. Stigmas (25/sample) were collected from respective plants. Collection of samples, electrophoresis and analysis was carried out as previously described.

7.4. RESULTS

7.4.1. Determination of appropriate extraction procedure and sample loading position for isoelectricfocusing.

Fig. 7.1 shows the protein bands which were separated in experiment 7.3.1. No clear bands were detected in phosphate buffer extracts (lanes 1, 2, 6, 7) but clear bands appeared in some water extracted samples (lanes 8, 9, 10), though not all (lanes 4 and 5).

In fact, loading the samples at the cathodal end resulted in no bands appearing (lanes 1, 2, 3, 4, 5) whereas loading at the anodal end, showed clear protein bands (lane 8, 9, 10).

These results suggested that distilled water exhibited more efficient extraction for the separation of stigmatic proteins by Isoelectricfocusing, and that the anodal position was best for loading samples.

7.4.2. Measurements of proteins

Bio-Rad protein assay is a dye-binding assay based on the differential colour change of a dye in response to differing concentrations of protein. Fig. 7.2 shows the standard plot for the Bio-Rad protein assay obtained with a spectrophotometer. Stigma and style samples (0.01 g) showed an absorbance of 0.8652 and 0.8531, and thus the concentration of protein was respectively 9.1 µg and 9.0 µg per sample. It was concluded that 0.01 g (25 stigmas) of stigma and style material contained the same amount of soluble protein extractable in

distilled water; consequently 25 stigmas were subsequently used per sample for IEF.

7.4.3. Identification of stigma specific protein in synthetic B. napus and their homozygotes parents.

Isoelectric bands exhibited in stigma, style and leaf extractions are shown in Fig. 7.3. Lanes 1-8 represent protein bands of stigmas, 9-12 protein bands of styles and 13-16 protein bands of leaf samples. Lane 1, 2 and 3 contain samples from B. oleracea homozygous for S_{29}, S_2 and S_{14} respectively. In lanes 1 and 2 very faint bands appeared. In lane 3, however, two bands (d and f) unique to the S_{14} genotype were detected together with bands e, g, h, i, j, k and l which were present in other lanes. Lanes 7 and 8 were loaded with samples from the B. campestris S_b and S_a homozygotes. In lane 8 a single IEF band-c characteristic of the S_a genotype was detected. Bands nearest the cathode, which were common to all lanes, appeared very faintly.

Lanes 4, 5 and 6 show the patterns of the $S_{14}S_a$ (line 5) S_2S_b (line 3) and $S_{29}S_a$ (line 4) stigma samples. It was noted that lane 4 exhibited the single IEF band characteristic of the S_a homozygous genotype (band c) and two bands characteristic of the S_{14} genotype (d and f) plus bands (e, g, h, i, j, k and l) common to B. oleracea or B. campestris lines. (Note - The S_{14} and S_a genotype specific bands c, d and f, from heterozygous stigmas, occur at a slightly lower intensity). Lane 5 did not exhibit any characteristic bands but contained the bands produced by S_2 and S_b samples. Lane 6 exhibited the band characteristic of S_a stigmas (band c) as well as the common

bands exhibited by B. campestris and B. oleracea samples. Finally, none of the samples in lanes 9-12 representing style samples of S_{29}, S_2, S_{14}, $S_{14}S_a$, or lanes 13-16 contained the leaf samples of the same genotypes produced any clear band patterns except in arrears of very low pI. This was evidence that the bands detected in stigma samples were stigma specific.

Densitometry Results

Densitometry plots of Coomassie-blue-stained IEF bands of the above stigma and style extracts are presented in Fig. 7.4-7.8. Fig. 7.4 represents the scans for S_{14}, S_a and $S_{14}S_a$ (lanes 3, 8, 4) stigmas. The results show that peaks d and f were specific to S_{14} samples and peak c to S_a samples. [Extraction of stigmas from the heterozygous $S_{14}S_a$ genotype (Fig. 7.4c) produced both S_{14} and S_a area peaks but at a lower density]. Interestingly, several peaks (m, n, o) were detected in the $S_{14}S_a$ samples but were not present in either S_{14} or S_a, suggesting that new stigmatic specific proteins were produced in the synthetic.

Fig. 7.5 shows the densitometer results for the $S_{29}S_{29}$ and $S_{29}S_a$ genotypes (lanes 1 and 6). The S_{29} homozygote produced many low density peaks (<0.2) while $S_{29}S_a$ exhibited the peaks c, c', e, g, h, i, j, k and l, that were also evident in the S_aS_a sample (Fig. 7.4b). These results suggest that genotype-specific stigmatic proteins could not be identified in S_{29} samples.

The densitometer results from the S_2, S_b and S_2S_b genotypes (lanes 2, 7 and 5) are illustrated in Fig. 7.6. Results for S_2 (Fig. 7.6a) reveal many low density peaks although peaks at e, h, i, j, k and l were also detected. S_bS_b samples (Fig. 7.6b) produced bands e and g

in the acidic portion of the gel and h, i, j, k and l in the basic portion. Finally the pattern of peaks produced by S_2S_b (Fig. 7.6c) was very similar to that of S_b homozygous but at a slightly lower density.

Scans for lanes 9, 10, 11 and 12 (S_{29}, S_2, S_{14} and $S_{14}S_a$ styles) are illustrated in Figs. 7.7 and 7.8. The scans in general show very low density gradients, except, that is, for style extracts of the S_{29} genotype, which produced a peak with a density between 0.450 - 0.650 in the basic portion of the gel.

Taken overall the densitometer scans confirm that the protein bands which were resolved in IEF gels were correlated with the stigmas of particular S-locus genotypes.

7.4.4. Isoelectricfocusing band patterns of synthetic B. napus lines and their parents.

Coomassie blue stained IEF protein bands of synthetic B. napus lines 1, 3, 4, 5, 6 ($S_{29}S_b$, S_2S_b, $S_{29}S_a$, $S_{14}S_a$, S_2S_a) and B. oleracea and B. campestris lines homozygous for S_{29}, S_2, S_{14}, S_a and S_b are illustrated in Fig. 7.9. It is noted that the samples from the heterozygous synthetics which contained the S_{14} and S_a alleles show the c, d and f bands that are characteristic of the S_a and S_{14} parental homozygotes.

7.4.5. Detecting S-specific proteins in synthetic B. napus F_2's

Coomassie blue stained protein bands of the plants surveyed are shown in Figs. 7.10 and 7.11. In Fig. 7.10, lanes 1, 18, 19 and 21 showed

extracts from the B. campestris line homozygous for S_a. These extracts exhibit the single c band in the acidic portion of the gel as well as bands e and g present in other samples. Similarly, contained within lanes 3 and 4 are the stigmatic extract of B. oleracea S_{14} and S_{29} homozygotes respectively. Lane 3 shows the characteristic d and f bands of the S_{14}.

With regard to the F_2 plants surveyed: Lanes 5, 7, 9 and 13 contain samples from plants with the genotype $S_{14}S_{29}S_aS_b$ for which bands c and d were resolved as well as bands e and g; Lanes 6 and 8 contain extracts from $S_{29}S_{14}S_a$ genotype and stained clearly for band c (characteristic of the S_a homozygote) and band d (characteristic of the S_{14} genotype); Lanes 10, 14 and 16 containing samples from the genotype $S_{29}S_aS_b$ possessed band c, while Lanes 11 and 12 contain samples from genotype $S_{14}S_aS_b$ which stained for band c and d. Finally lane 15 contains a sample from genotype $S_{29}S_b$ and exhibits only bands e and g (common to all tested plants).

In Fig. 7.11 lanes 17 and 19 contain samples of the B. campestris line homozygous for S_b while lane 18 shows the pattern of banding for the B. oleracea line homozygous for S_{29}. With regard to the F_2 plants surveyed, lane 1 contains extracts from the $S_{29}S_b$ genotype and exhibits bands e and g which occur in other lanes; lanes 3, 4, 6, 9, 10 and 14 contain samples from the $S_{14}S_{29}S_aS_b$ genotype which produce bands c and d; lanes 2 and 15 contain extracts of genotype $S_{14}S_aS_b$ and show the characteristic S_a (band c) and S_{14} (band d) bands; lane 5 contains samples of genotype $S_{29}S_{14}S_a$ with bands c and d again present; lanes 7 and 16 contain extracts from the genotype $S_{29}S_aS_b$ with the characteristic band c; and lane 8 contains a sample from the $S_{29}S_a$ genotype again showing the S_a band (band c). Finally lanes 11,

12 and 13 contain extracts of genotype $S_{29}S_{14}S_b$ and show the band characteristic to the S_{14} genotype, band d.

7.5 DISCUSSION AND CONCLUSIONS

Measurement of protein contents (Experiment 7.3.2) showed that Brassica stigmas and styles contain approximately equal quantities of water soluble proteins. However, when subjected to IEF, many more protein bands were detected from stigma samples in the pH 3.5 - 9.5 region of gels. Resolution of bands depended on extraction being made with distilled water and loading extracts at the anodal position on a gel.

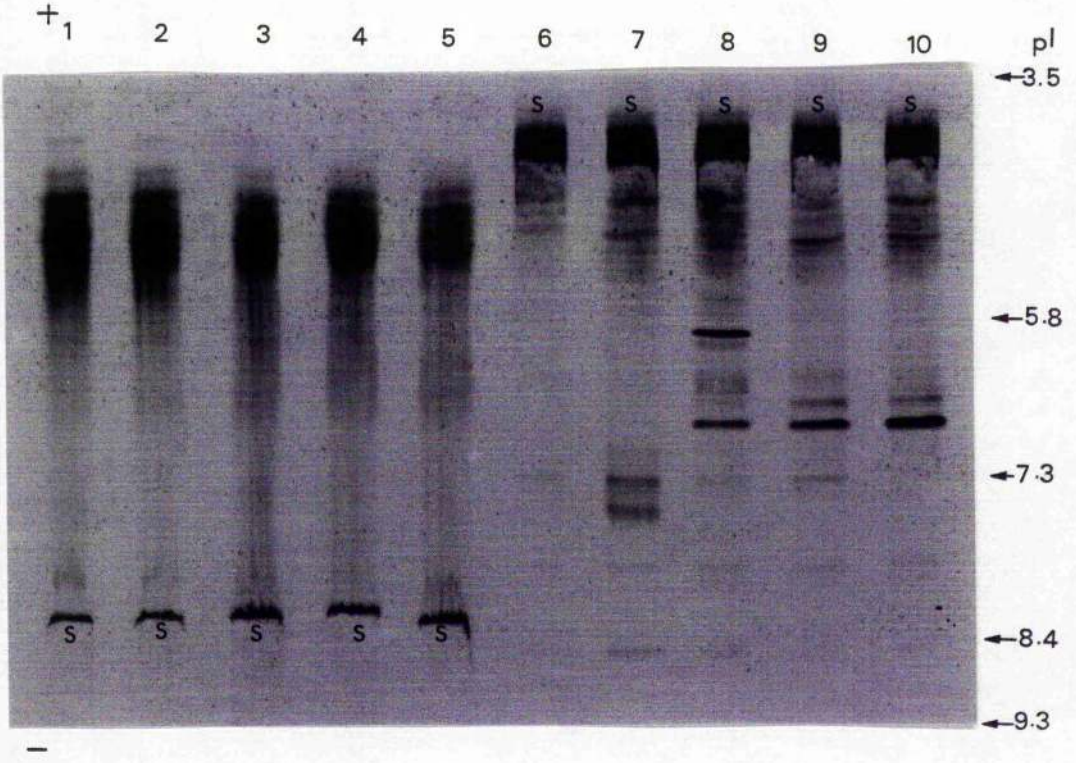
Differences in stigmatic protein band patterns were resolved between synthetic B. napus lines and the B. campestris and B. oleracea parents. In this way, Coomassie blue stained protein bands of synthetic lines 1 ($S_{29}S_b$), 3 (S_2S_b), 4 ($S_{29}S_a$), 5 ($S_{14}S_a$) and 6 (S_2S_a) and the B. campestris and B. oleracea parents homozygous for S_a, S_b, S_{29}, S_{14} and S_2 were distinguished (Fig. 7.9). It was established that the B. campestris S_a genotype possessed a single stigmatic protein band in the acidic portion of the gel (band c), as well as bands e, g, h, i, j, k and l in the basic region. B. campestris S_b genotype also possessed bands e, g, h, i, j, k and l but lacked band c suggesting the possibility that band c was characteristic of the allele S_a. Similarly, B. oleracea lines homozygous for S_{14} possessed two stigmatic protein bands in the acidic portion of the gel (bands d and f), whereas B. oleracea lines homozygous for S_{29} or S_2 could not be distinguished from each other. Among the synthetic B. napus lines,

line 5 (heterozygous for $S_{14}S_a$) possessed bands c, d and f showing that they were inherited from the respective parents. In contrast, the synthetic lines of $S_{29}S_b$ and S_2S_b genotype did not possess any differential bands.

From an analysis of banding patterns in the F_2 generation, it was evident that bands unique to S_a and S_{14} genotypes were fully correlated with these alleles (Figs. 7.10 and 7.11). Thus these protein bands were unique to a specific parental S-homozygote, were transmitted to the F_1's and segregated in the F_2 with the appropriate S-allele that had been identified from pollen tube penetration test (Chapter 6). A point of interest was that the banding pattern obtained for the B. oleracea S_{14} genotype was consistent with that reported by Nasrallah et al. (1983, 1985b).

It is concluded from the work reported in this Chapter that stigma specific bands correlated with S_{14} and S_a allele expression can be detected in synthetic B. napus, and that these co-segregate with the appropriate S-alleles. In contrast, no S-specific protein bands were identified in S_2, S_{29} or S_b genotypes. It was not possible to test whether the proteins identified were glycoproteins, since large numbers of stigmas are needed for glycoprotein staining (T. Hodgkin - pers. comm.). The research indicated that S-protein expression is not correlated with the full function of self-incompatibility. Thus, genotype $S_{14}S_a$ was self-compatible with no loss of stigmatic function although S-correlated bands were still detected. Similarly in the F_2 the S_a band was detected in stigma extracts of plants in which S_a was not detected in compatibility tests.

Fig. 7.1. Coomassie blue stained Isoelectricfocusing patterns of Brassica stigma homogenate in water or phosphate buffer. Figures on the right hand side indicate the position of pI standards and 'S' indicate the position of the sample.



Lanes 1, 2, 6 and 7: S_2S_b stigmas homogenate in phosphate buffer.

Lanes 3, 4, 5, 8, 9 and 10: Stigmas homogenate in water - 3 : $S_{14}S_a$, 4 : S_2S_b, 5 : S_2S_b, 8 : $S_{14}S_a$, 9 : S_2S_b, 10 : S_2S_b.

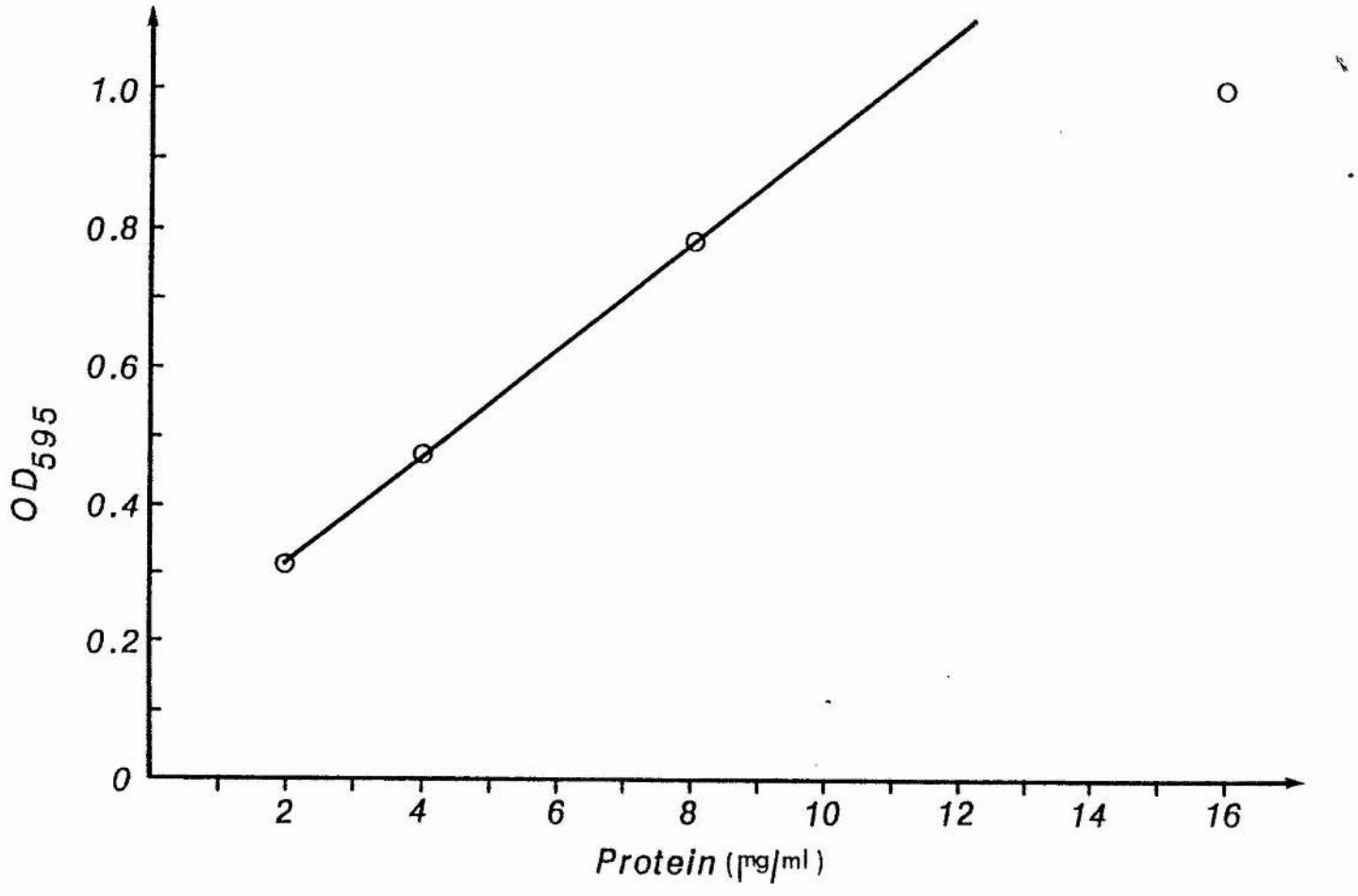
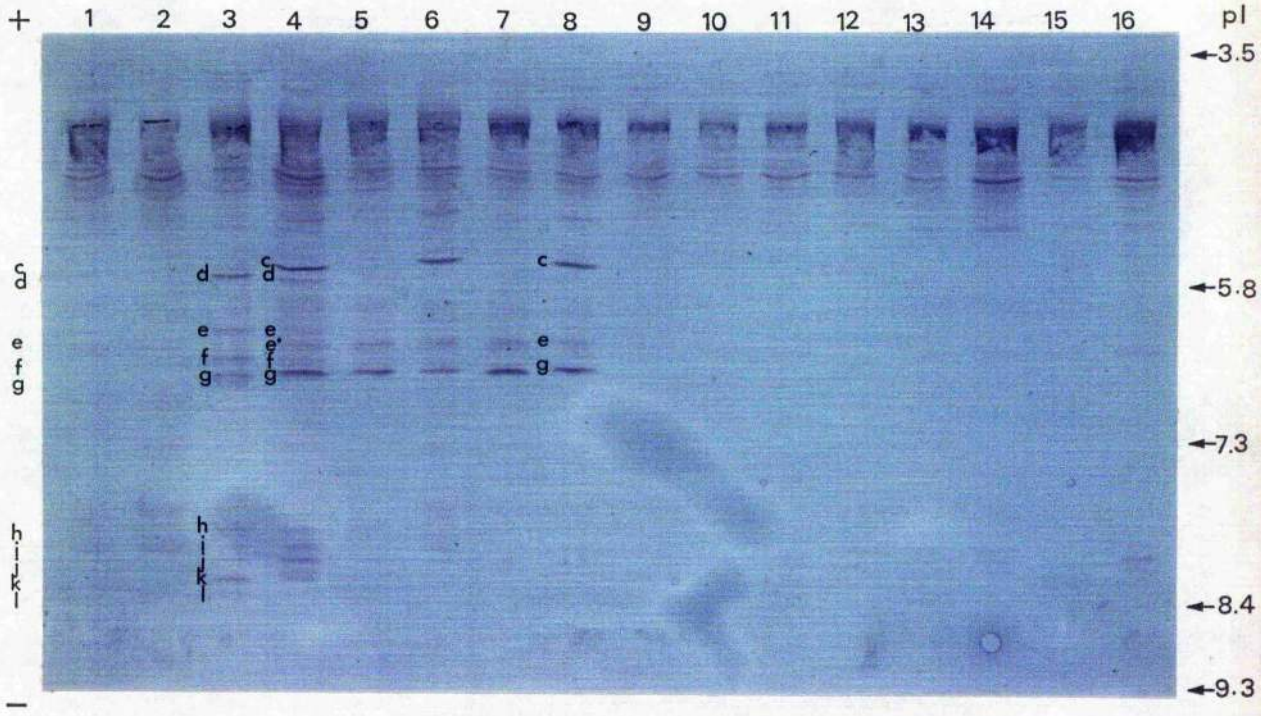


Fig. 7.2. Standard curve for the Bio-Rad protein assay (1-20 µg) using the Bio-Rad protein standard.

Fig. 7.3. Coomassie blue stained IEF patterns of Brassica stigma, style and leaf homogenates. Figures on the right indicate the pI standards, and figures marked as c - l indicate proteins characteristic to stigmas.



Lanes 1-8 : stigmatic proteins.

1 : S_{29}, 2 : S_2, 3 : S_{14}, 4 : $S_{14}S_a$,

5 : S_2S_b, 6 : $S_{29}S_a$, 7 : S_b, 8: S_a.

Lanes 9 - 12 : stylar proteins.

9 : S_{29}, 10 : S_2, 11 : S_{14}, 12 : $S_{14}S_a$.

Lanes 13 - 16 : leaf proteins.

13 : S_{29}, 14 : S_2, 15 : S_{14}, 16 : $S_{14}S_a$.

Fig. 7.4. Densitometer tracings of proteins from Brassica stigmas resolved by IEF. Figures c - o indicate the density peaks.

7.4a: Patterns for B. oleracea homozygous for S_{14} stigmas.

7.4b: Patterns for B. campestris homozygous for S_a stigmas.

7.4c: Patterns for synthetic B. napus line 5 heterozygous for $S_{14}S_a$ stigmas.

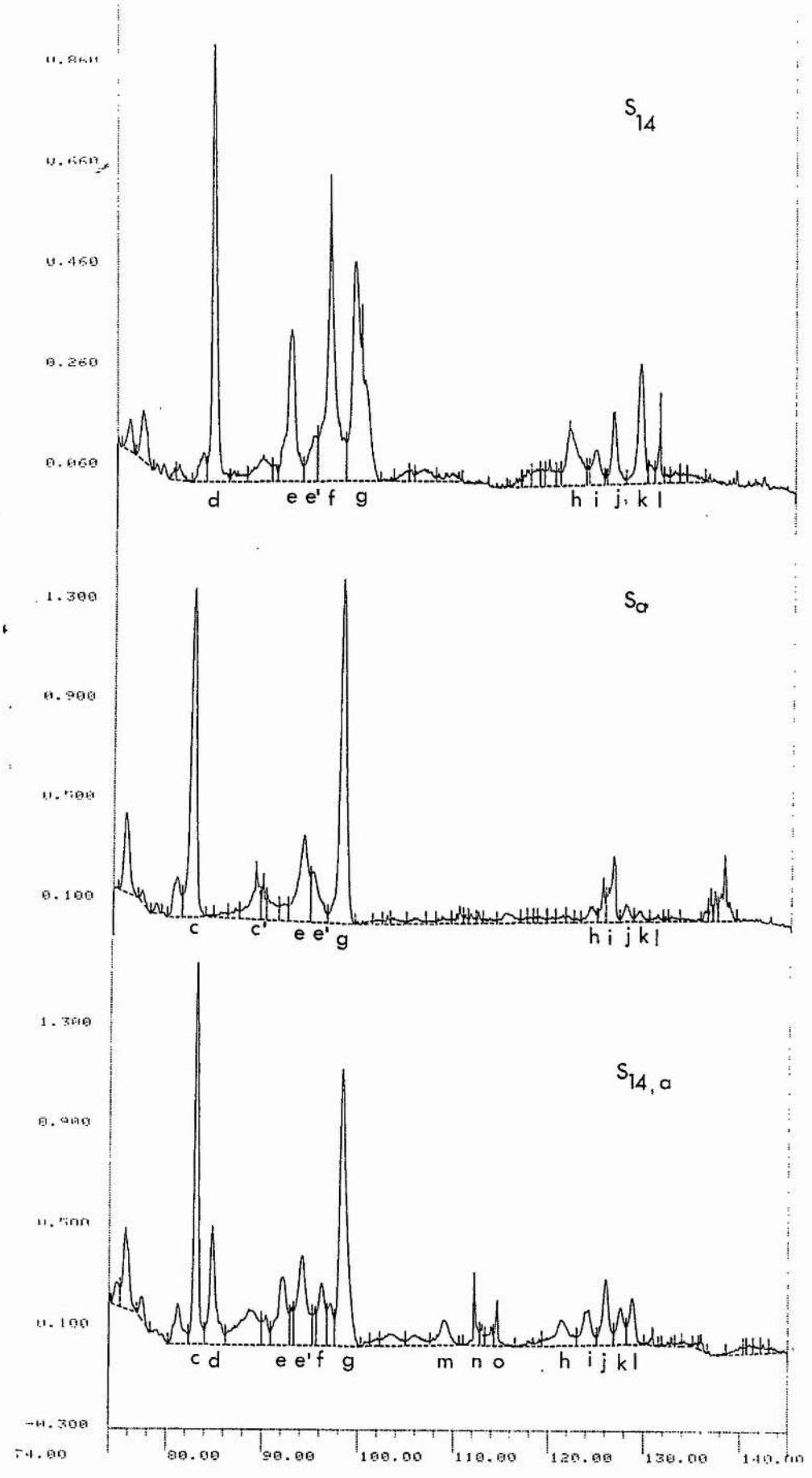

S14
7.4a
d
e e' f g
h i j k l
Sα
7.4b
c
c'
e e' g
h i j k l
S14,α
7.4c
c d
e e' f g
m n o
h i j k l
80.00
90.00
100.00
110.00
120.00
130.00
140.00

Fig.7.4

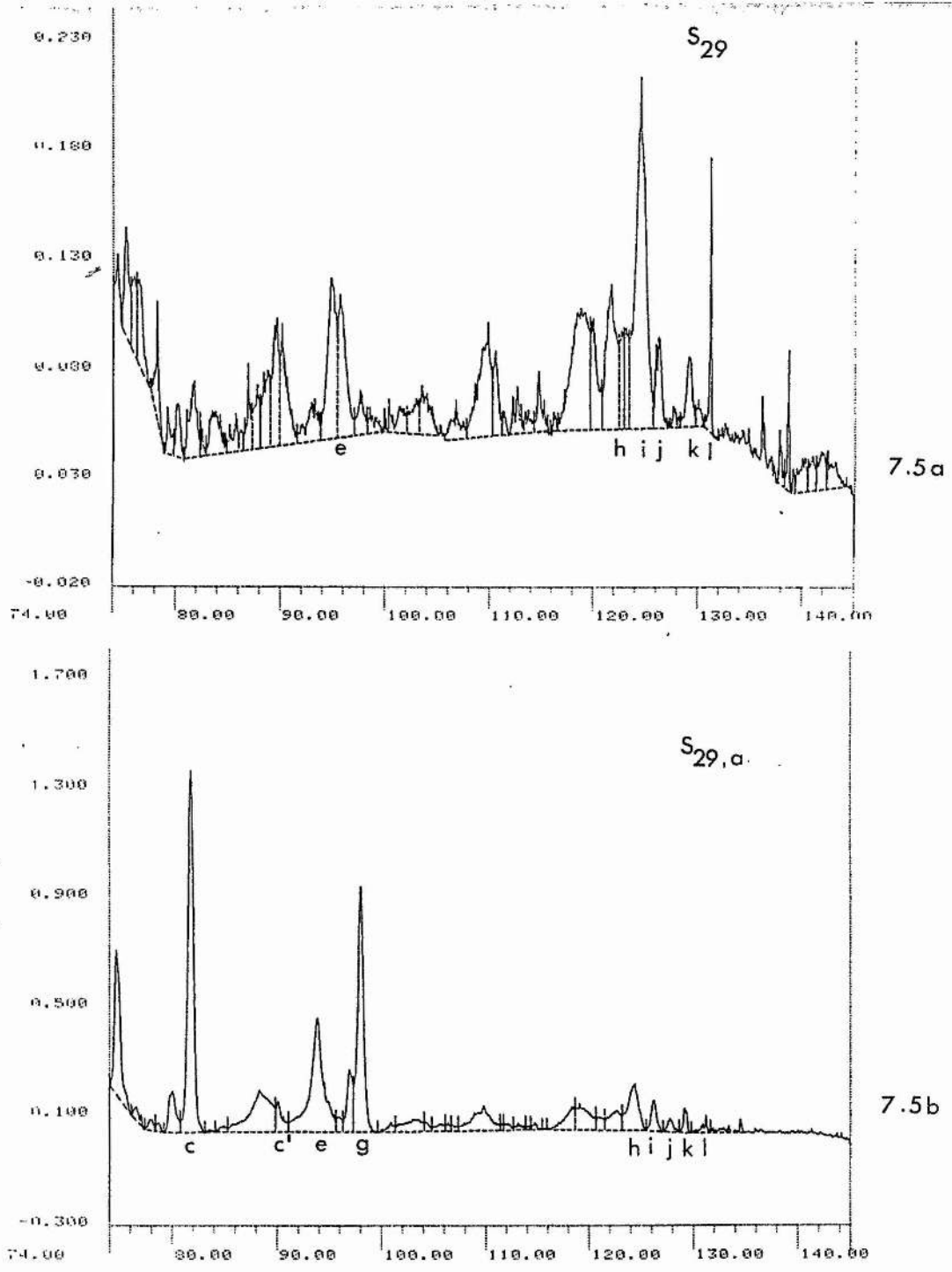


Fig. 7.5. Densetometer tracing of proteins from Brassica stigmas resolved by IEF. Figures c - l indicate the density peaks.

7.5a: Patterns for B. oleracea homozygous for S_{29} stigmas.

7.5b: Patterns for synthetic B. napus line 4 heterozygous for $S_{29}S_a$ stigmas.

Fig. 7.6. Densitometer tracings of proteins from Brassica stigmas resolved by IEF. Figures e - l indicate the density peaks.

7.6a: Patterns for B. oleracea homozygous for S_2 stigmas.

7.6b: Patterns for B. campestris homozygous for S_b stigmas.

7.6c: Patterns for synthetic B. napus line 3 heterozygous for S_2S_b stigmas.

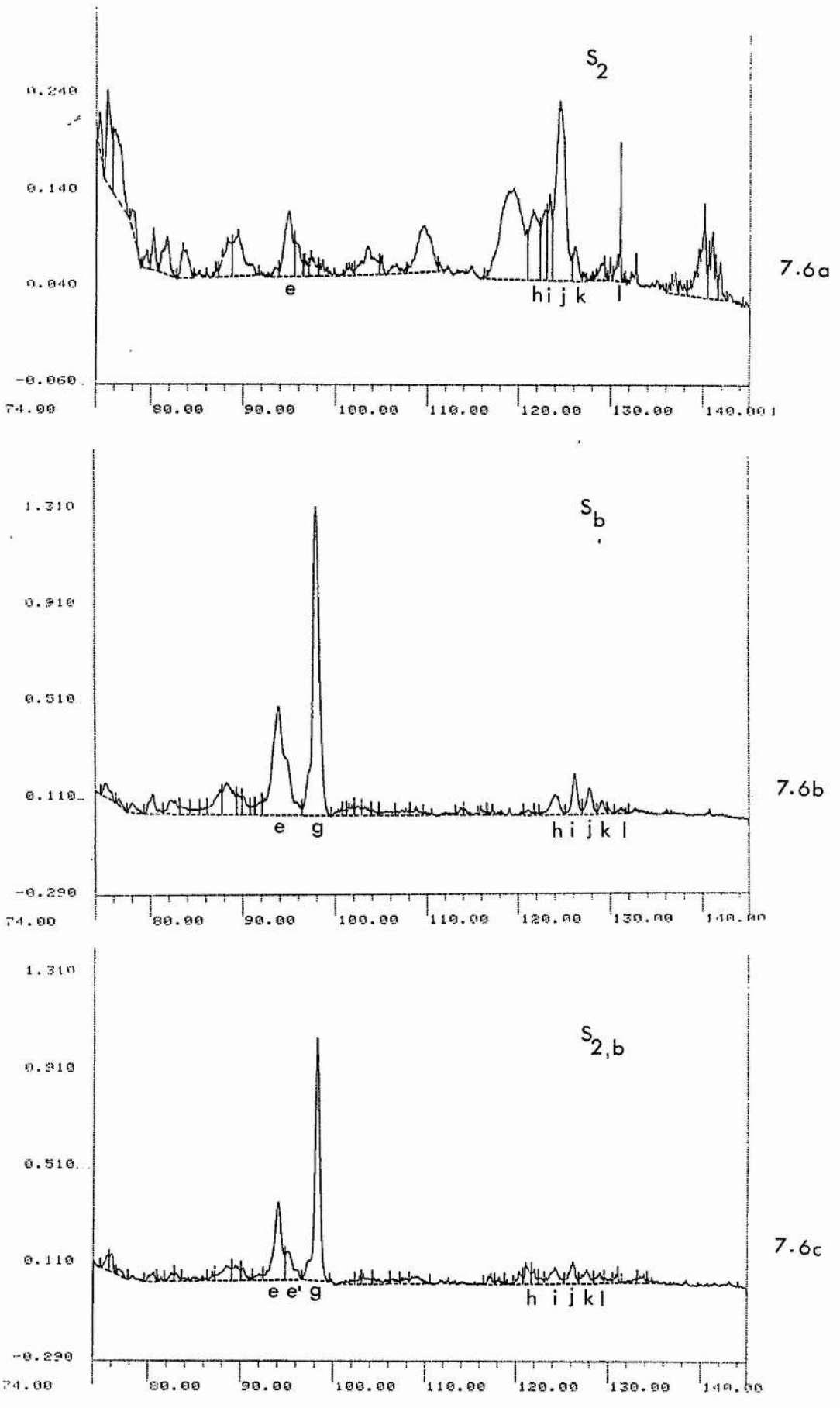

S2
0.240
0.140
0.040
-0.060
74.00
80.00
90.00
100.00
110.00
120.00
130.00
140.00
e
h i j k l
7.6a
Sb
1.310
0.910
0.510
0.110
-0.290
74.00
80.00
90.00
100.00
110.00
120.00
130.00
140.00
e g
h i j k l
7.6b
S2,b
1.310
0.910
0.510
0.110
-0.290
74.00
80.00
90.00
100.00
110.00
120.00
130.00
140.00
e e' g
h i j k l
7.6c

Fig. 7.6

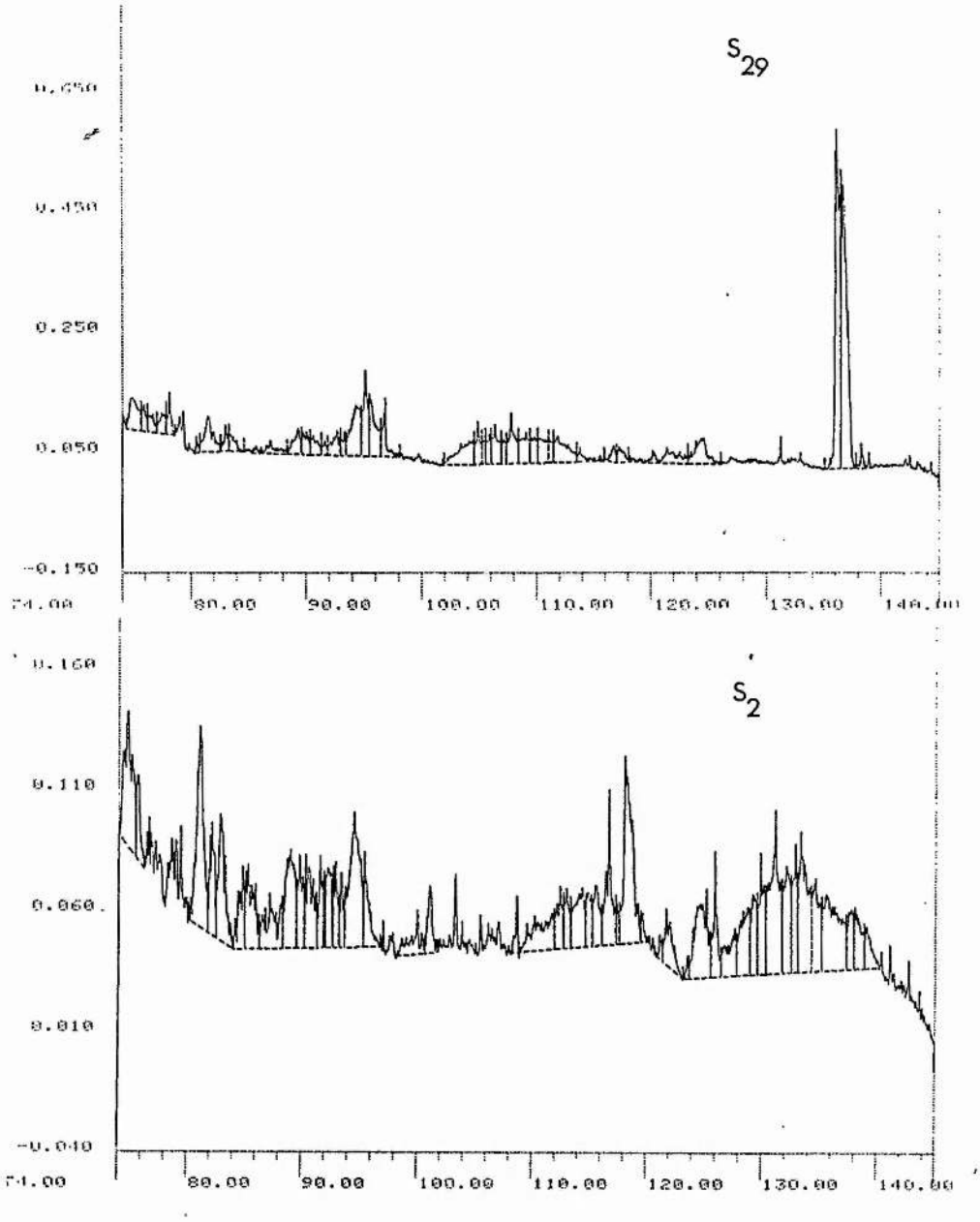


Fig. 7.7. Densitometer tracings of proteins from Brassica oleracea styles resolved by IEF.

7.7a: Patterns for B. oleracea homozygous for S_{29} styles.

7.7b: Patterns for B. oleracea homozygous for S_2 styles.

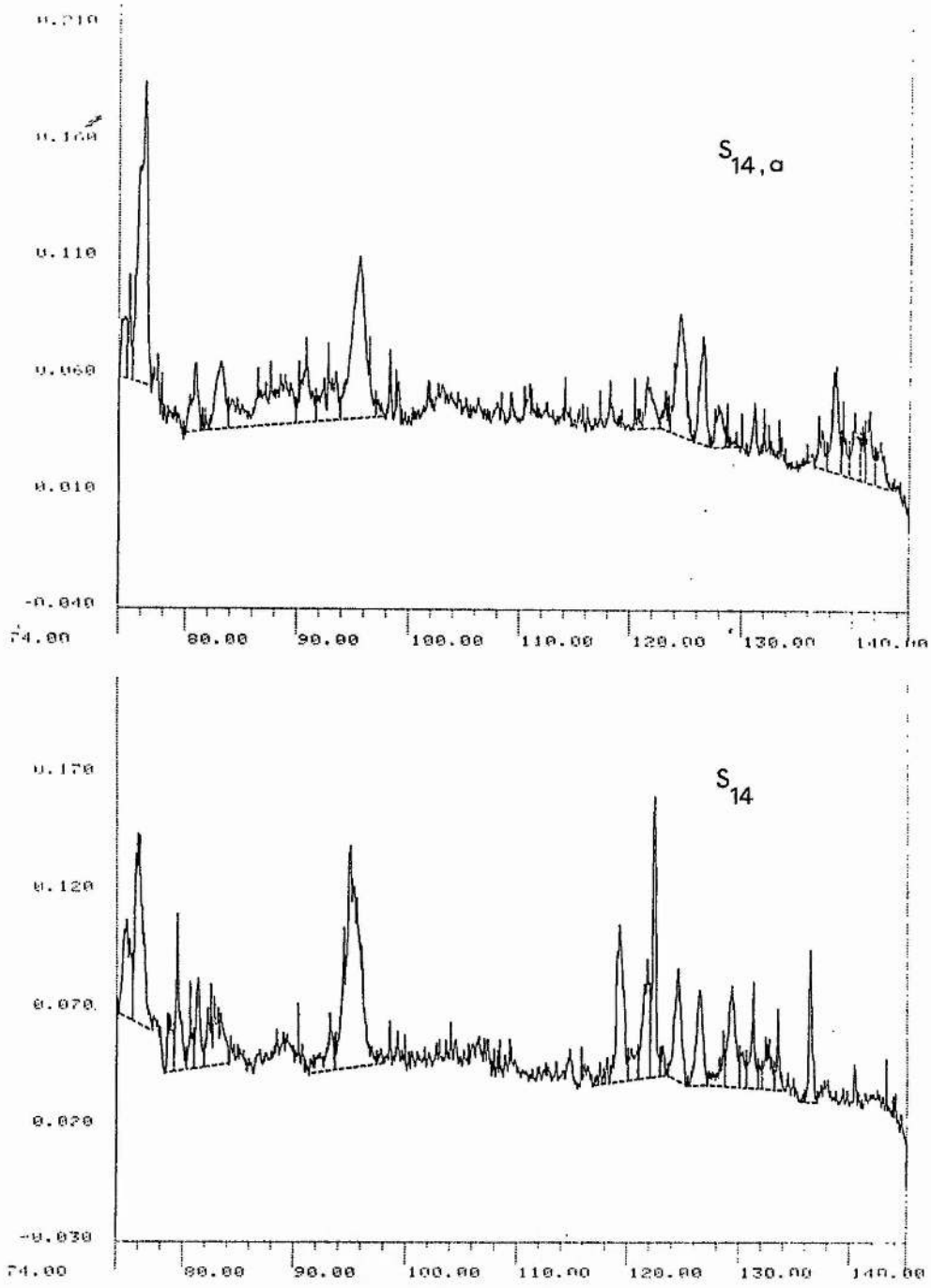
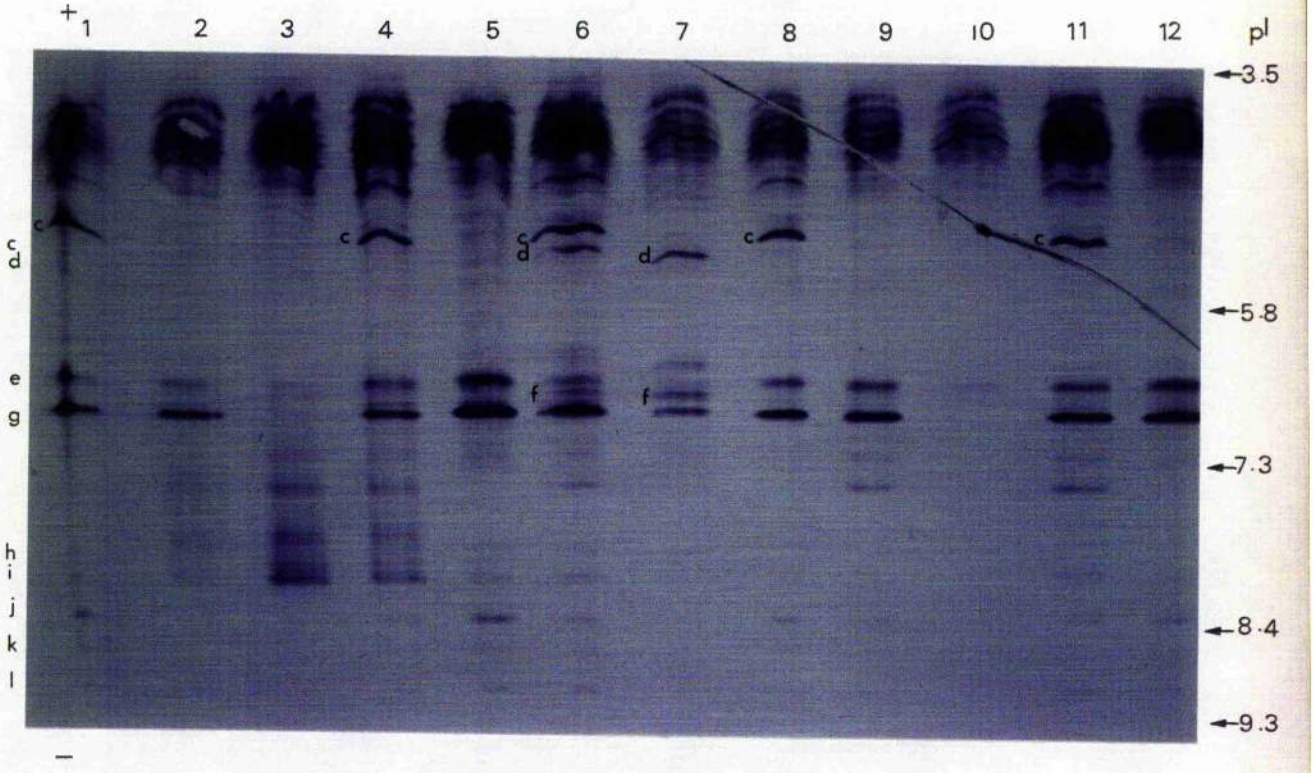


Fig. 7.8. Densetometer tracings of proteins from Brassica styles resolved by IEF.

7.8a: Patterns for B. oleracea homozygous for $S_{14}S_a$ styles.

7.8b: Patterns for B. oleracea heterozygous for S_{14} styles.

Fig. 7.9. Coomassie blue stained IEF patterns of Brassica stigma homogenates of different S-genotypes. Figures on the bottom indicate the S-allele constitute of each sample and on the right indicate the position of the pI standard. Figures c - l denote the proteins characteristic of stigmas.



Lanes 1 and 8: B. campestris homozygous for S_a stigmas.

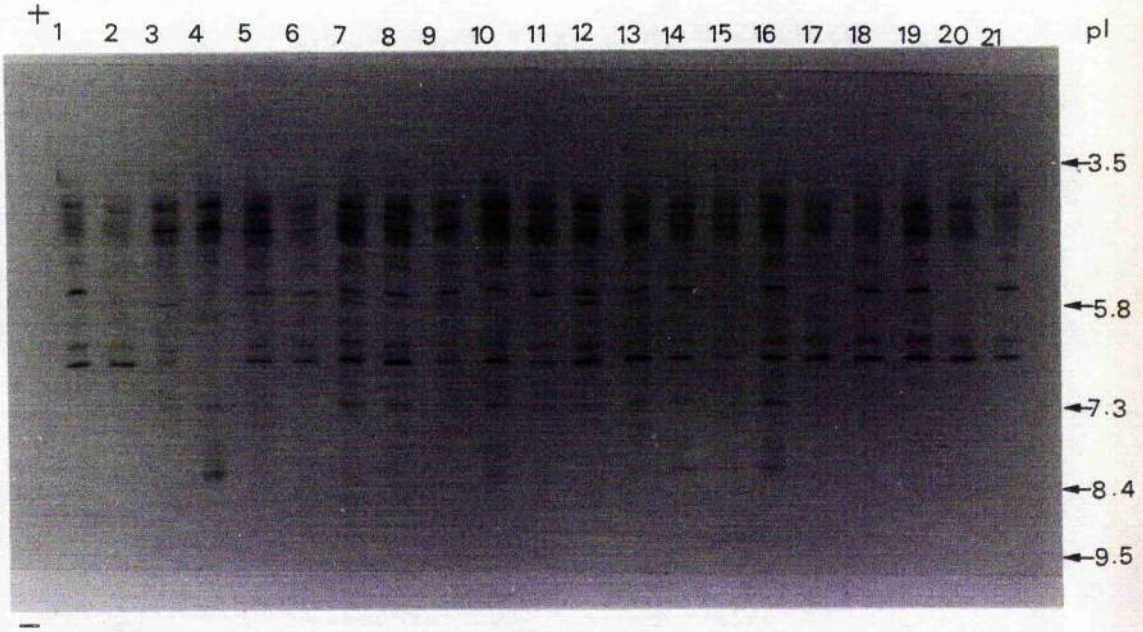
Lanes 5 and 12: B. campestris homozygous for S_b stigmas.

Lanes 3, 7 and 10: B. oleracea homozygous for S_{29}, S_{14} and S_2.

Lanes 2, 4, 6, 9 and 11: synthetic B. napus lines.

2 : $S_{29}S_b$, 4 : $S_{29}S_a$, 6 : $S_{14}S_a$, 9 : S_2S_b, 11 : S_2S_a.

Fig. 7.10. Coomassie blue stained IEF patterns of synthetic B. napus F_2 genotypes and their original parents B. oleracea and B. campestris S-homozygote, stigma homogenates. Figures on the bottom indicate the S-genotype of the sample and on the right indicate the position of the pI standard. Figures c - g denote the S-specific protein bands.



Lanes 1, 18, 19 and 21 : B. campestris,. S_a.

Lanes 2, 17 and 20 : B. campestris, S_b.

Lanes 3 and 4 : B. oleracea, S_{14} and S_{29}.

Lanes 5, 7, 9, 13 : B. napus F_2, S-genotype $S_{14}S_{29}S_aS_b$.

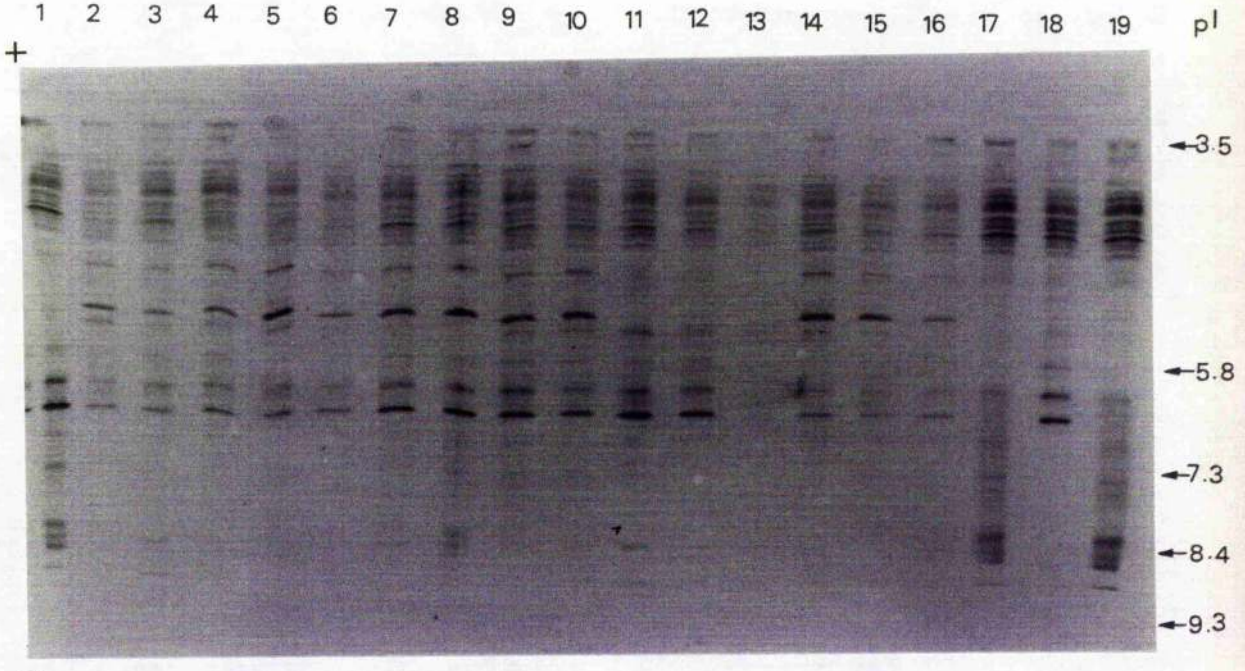
Lanes 6, 8 : B. napus F_2, S-genotype $S_{29}S_{14}S_a$.

Lanes 10, 14, 16 : B. napus F_2, S-genotype $S_{29}S_aS_b$.

Lanes 11, 12 : B. napus F_2, S-genotype $S_{14}S_aS_b$.

Lane 15 : B. napus F_2, S-genotype $S_{29}S_b$.

Fig. 7.11. Coomassie blue stained IEF of synthetic B. napus F_2 genotypes and B. oleracea and B. campestris homozygous for S_{29} and S_b genotype, stigma homogenates. Figures on the bottom indicate the S-genotype of the sample and on the right indicate the position of the pI standard. Figures c-g denote the S-specific bands.



Lanes 17 and 19: B. campestris S_b

Lane 18: B. oleracea S_{29}

Lane 1: B. napus F_2 S-genotype $S_{29}S_b$

Lanes 3, 4, 6, 9, 10, 14: B. napus F_2 S-genotype $S_{29}S_{14}S_aS_b$

Lanes 2, 15: B. napus F_2 S-genotype $S_{14}S_aS_b$

Lane 5: B. napus F_2 S-genotype $S_{29}S_{14}S_a$

Lanes 7, 16: B. napus F_2 S-genotype $S_{29}S_aS_b$

Lane 8: B. napus F_2 S-genotype $S_{29}S_a$

Lanes 11, 12, 13: B. napus F_2 S-genotype $S_{29}S_{14}S_a$

CHAPTER 8

CONCLUSIONS

8.1. INTRODUCTION

The amphidiploid B. napus is normally self-compatible, although self-incompatible plants have been found in some B. napus subspecies rapifera and oleifera. Olsson (1960a), Davey (1958) and Olsson (1960b) showed that self-incompatible B. napus plants could be produced by doubling the chromosome numbers of F_1 hybrid plants obtained by crossing B. oleracea and B. campestris followed by embryo culture. Moreover, functional S-alleles have been successfully introgressed into forage rape, B. napus L., from turnips, B. campestris (Mackay, 1977a).

Self-incompatibility in B. napus is of value to breeders of forage crops and, if suitable methods of inducing self-compatibility were available, would be of equal importance in the breeding of oilseed rape. As B. napus has 2 self-incompatibility loci (one inherited from B. oleracea, the other from B. campestris) significant questions are raised about the operation of such a sporophytic system. The present study used artificial synthesis of B. napus as a way of producing plants of known S-allele status to investigate the operation of a two locus sporophytic self-incompatibility system. The first aim of the investigation was to answer the following questions:-

(a) Are both loci of the self-incompatible parents expressed in synthetic B. napus?

(b) If not, then which one is expressed? Is it always the same locus or sometimes that from B. oleracea and other times that from

B. campestris?

(c) If alleles at both loci are expressed, do they operate independently or are there interactions between the S-alleles at the two, i.e. in terms of dominance and epistasis?

(d) If interactions occur between S-alleles at the two loci, what form does this take?

8.2. PRODUCTION OF SYNTHETICS

For this research seven synthetic B. napus lines of known S-locus genotype were produced, 6 by ovary culture, using B. campestris as the female parent and 1 by embryo culture, using B. oleracea as the female parent. Rates of production were favourable compared with previous attempts to synthesise B. napus (Inomata, 1985). By means of ovary culture 63 hybrids were produced per 100 pollinations, as compared with 11.3 hybrids per 100 pollinations using embryo culture. The production rate of synthetics using ovary culture tended to vary between pollen parents. One of the lines of B. oleracea used as a parent 83-443 (S_{14}), appeared to be a much poorer pollen source than either of the other two B. oleracea lines 83-456 ($S_{29}S_{29}$) or 85-353 (S_2S_2).

Amphidiploid plants were identified from the acid phosphatase isozyme profiles following acrylamide gel electrophoresis. Isozymes that were specific to B. campestris and B. oleracea were combined in the amphidiploid. In addition, isozymes of synthetics were characterised by a novel single band found in zone 3 on gels. Synthesis of the amphidiploid was confirmed by chromosome counts.

8.3. INCOMPATIBILITY IN SYNTHETICS WITH 2 S-ALLELES

Each of the seven synthesised lines of B. napus was homozygous for different S-alleles at the two S-loci. One of the lines was found to be self-compatible, whilst the remaining lines were fully self-incompatible. A crossing programme involving a full diallel between lines, showed that both S-loci functioned and that interactions occurred between loci similar to those found between S-alleles in S-heterozygotes in single locus sporophytic systems. In the stigma, codominance was detected between the alleles $S_{29}S_b$; $S_{14}S_b$; S_2S_a; and in the pollen, occurred between alleles S_2S_b; $S_{29}S_a$ and $S_{14}S_a$. However, except for the combinations S_2S_b and $S_{14}S_a$, one S-allele inherited either from B. campestris or B. oleracea was dominant to the other, either in the pollen or the pistil, showing that interlocus epistasis was common in these synthetic lines of B. napus.

In general, the expression and activity of alleles was determined from the diallel cross between synthetics and was later confirmed by additional information obtained from interspecific crosses, i.e. between the synthetic lines and the parental species. An interesting point to emerge from the latter was that crosses between B. napus and B. oleracea were incompatible when B. oleracea was used as the pollen parent. Further studies showed (Chapter 4) that B. oleracea pollen was incompatible in B. napus stigmas even when they possessed no S-alleles in common. In such situations the reciprocal cross was always compatible.

8.4. F_1 PROGENIES WITH 4 S-ALLELES

Having investigated the functioning of the two S-loci in synthetic lines of B. napus that were homozygous for S-alleles at each locus (i.e. in 2 S-allele genotypes), the next aim of the research was to study the expression of alleles in genotypes that were heterozygous for different alleles at each S-locus (i.e. in 4 S-allele genotypes). To this end, crosses were made between the seven B. napus synthetics to produce several F_1 lines of known heterozygous genotype at each S-locus. These F_1's were reciprocally intercrossed to determine the degree of compatibility/incompatibility between them. The results showed that the self-incompatibility mechanisms were functioning in two sets of F_1's whilst the remaining set was either fully self-compatible or partially self-compatible. The self-compatibility detected was only expressed in genotypes containing the S_{14} and S_a alleles and in which the S_{29} allele, which showed dominance, was absent. Thompson (1968) has reported similar self-compatibility in B. oleracea.

Among the F_1's only 3 alleles were expressed in any four allele combinations. In the F_1 groups of genotype $S_{29}S_{14}S_aS_b$ and $S_{29}S_2S_aS_b$, S_{29} seemed to be expressed in both pollen and stigma, S_b was active only in the stigma and S_{14} was active only in the pollen. However, in the $S_{14}S_2S_aS_b$ F_1 group S_2, S_a and S_b were active in the stigma but allelic activity in pollen could not be detected. This implies that in this 2 locus system, allele activity in pollen would appear to be equivalent to that in a single locus system, and that the alleles from either S-locus may be expressed. It is concluded that the sporophytic self-incompatibility system still functions and breeders may use

double cross methods with agronomically desirable types.

Two kinds of anomalous results were found when crosses were made between F_1 groups. Compatible pollinations were recorded where incompatible pollinations were expected and incompatible pollinations were recorded where compatible pollinations were expected. Further research on progeny production and cytogenetic studies would be useful to clarify these anomalous results. Compatible pollinations were most commonly found where incompatible results were expected in the group containing $S_{14}S_2S_aS_b$ alleles, and it is possible that the presence of S_{14} and S_a in the absence of S_{29} could be a cause of the breakdown of the self-incompatibility mechanism.

8.5. SEGREGATION OF S-ALLELES IN F_2 PROGENIES

Analysis of S-allele expression in F_2 families produced from certain F_1's presented an even more complex picture. Only the data for the F_2 family produced from the F_1, $S_{29}S_{14}S_aS_b$, were sufficient to consider in depth, although data from the analysis of an F_2 of $S_2S_{14}S_aS_b$ provided useful supporting evidence. All plants in the F_2 of $S_{29}S_{14}S_aS_b$ tended to be self-incompatible, including plants with the genotype $S_{14}S_{14}S_aS_a$, showing that self-compatibility was not correlated with $S_{14}S_a$. In contrast to what had been found from the analysis of F_1's, 4 S-alleles were expressed in some F_2 plants. The most significant finding to emerge was that the activity of the S_a allele varied over plants. In 37 plants which contained the S_a allele as one of 3 or 4 different S-alleles in the genotype, it was established that S_a was active in both pollen and stigma. However in

7 such plants S_a was active only in the pollen while in another 10 such plants S_a was active only in the stigma (Chapter 6 - Table 6.4). The origin of this variation is unknown and its investigation would require the production of further generations of progeny and a study of the role of environmental factors.

The findings from the F_2 analysis clearly demonstrated a number of problems involved in the analysis of complex incompatibility systems. From the results of the F_1 analysis, it was expected that the intrafamily test would give only 3 groups (Table 8.1), a result identical with that which would have been obtained if only a single locus were present. S_a and S_b would remain undetected. In fact, varying expression of S_a led to a more complex picture and an intrafamily diallel would have indicated more than 2 loci (rather like Eruca sativa - Verma et al., 1977). Table 8.2 and 8.3 summarises the results that would be obtained from a complete diallel set of crosses of this family. The results show that 7 different incompatibility groups would be identified. Without the precise knowledge of the S constitution of the parents and the available S homozygous testers, any interpretation of such a pattern would have been extremely difficult. It might well have been necessary to produce a system involving more than 2 loci, or some sympathetic allele system to complement the oppositional one.

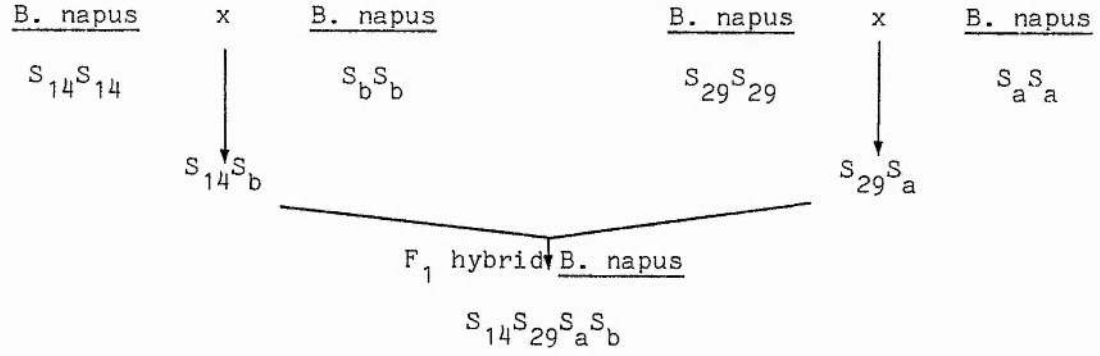
8.6. STIGMATIC PROTEINS

Isoelectricfocusing surveys of stigmatic proteins in B. napus material showed that stigma specific bands could be identified which co-segregated with the S_{14} and S_a alleles in the F_2. However, no S-

specific bands were identified in S_2, S_{29} or S_b genotypes. Rather surprisingly, protein expression was not correlated with function of self-incompatibility or with allele expression. Thus, genotype $S_{14}S_a$ was self-compatible with loss of stigmatic function although S-correlated bands were still detected. Moreover, the S_a band was detected in stigma extracts of F_2 plants in which S_a was not active.

8.7. FUTURE APPLICATIONS

From the studies of self-incompatibility in B. napus that have been reported here, it is evident that synthetic lines possess a functioning self-incompatibility system. Thus it is clear that the production of F_1 hybrids in forage Brassicas on a commercial scale basis would present no problems. For example inbred lines obtained from introgression or synthesis and back crossing to agronomically desirable types could be used to produce double cross hybrids. Thus:



However, the complexities of allele interaction described in this thesis indicate that studies on a larger number of alleles would be desirable as part of such breeding programmes. Oilseed rape is a seed crop in which sufficient cross compatibility is difficult to obtain except in an extremely complex hybrid production programme where it

could reach 50% (T. Hodgkin, pers. comm.). Alternatively the hybrid cultivar would need to be self-compatible. The results obtained for the $S_{14}S_a$ synthetic and $S_{14}S_2S_aS_b$ F_1 progeny suggest that it may be possible to develop self-incompatible inbred lines which when crossed gave self-compatible progeny, i.e.

$$S_{14}S_b \quad \times \quad S_2S_a$$

$$\downarrow$$

$$S_{14}S_2S_aS_b$$

This thesis has clarified S-allele expression in B. napus and has shown that both S-loci were expressed either independently or with interlocus interaction which took the form of dominance of the allele at one of the loci. New artificial B. napus created using B. campestris and B. oleracea of known S status provided an essential starting point for analysis of the action of S-alleles in the amphidiploids. Such well characterised material also permitted the analysis of complex results from F_1 and F_2 progenies.

Much remains to be discovered about the genetics of self-incompatibility and interspecific incompatibility in Brassica as well as the physiology and morphology of the incompatibility mechanisms. This thesis provides a foundation for future work on B. napus.

Table 8.1 Results expected from intercrossing B. napus F_2 plants containing alleles $S_{29}S_{14}S_aS_b$ according to the allele activity detected in F_1's. $\overline{S}$ indicate the active alleles detected in F_1's

Group	Stigma source	Pollen source: $\overline{S}_{29}\overline{S}_{29}S_aS_a$	$\overline{S}_{29}\overline{S}_{29}S_aS_b$	$\overline{S}_{29}\overline{S}_{29}S_bS_b$	$\overline{S}_{29}\overline{S}_{14}S_aS_a$	$\overline{S}_{29}\overline{S}_{14}S_aS_b$	$\overline{S}_{29}\overline{S}_{14}S_bS_b$	$\overline{S}_{14}\overline{S}_{14}S_aS_a$	$\overline{S}_{14}\overline{S}_{14}S_aS_b$	$\overline{S}_{14}\overline{S}_{14}S_bS_b$
I	$\overline{S}_{29}\overline{S}_{29}S_aS_a$	-	-	-	-	-	-	+	+	+
	$\overline{S}_{29}\overline{S}_{29}S_a\overline{S}_b$	-	-	-	-	-	-	+	+	+
	$\overline{S}_{29}\overline{S}_{29}\overline{S}_b\overline{S}_b$	-	-	-	-	-	-	+	+	+
II	$\overline{S}_{29}\overline{S}_{14}S_aS_a$	-	-	-	-	-	-	-	-	-
	$\overline{S}_{29}\overline{S}_{14}S_a\overline{S}_b$	-	-	-	-	-	-	-	-	-
	$\overline{S}_{29}\overline{S}_{14}\overline{S}_b\overline{S}_b$	-	-	-	-	-	-	-	-	-
III	$\overline{S}_{14}\overline{S}_{14}S_aS_a$	+	+	+	N	N	N	N	N	N
	$\overline{S}_{14}\overline{S}_{14}S_a\overline{S}_b$	+	+	+	-	-	-	-	-	-
	$\overline{S}_{14}\overline{S}_{14}\overline{S}_b\overline{S}_b$	+	+	+	-	-	-	-	-	-

\+ compatible pollinations
\- incompatible pollinations
N results obtained would depend on expression of self-compatibility in the $S_{14}S_{14}S_aS_a$ genotype.

Table 8.2 Results expected from an interfamily diallel test of family 88-184 ($S_{29}S_{14}S_aS_b$) synthetic B. napus F_2 progeny

Group	1						2		3		4	5	6	7
active alleles in pollen / active alleles in stigma	29,14,a	29,14	29,14,a	29,14	29,14,a	29,14	29,a	29,a	14,a	14,a	29,a	14,a	14	29
29,14,a,b	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29,14,a,b	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29,14,b	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29,14,b	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29,14,a	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29,14,a	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29,b	-	-	-	-	-	-	-	-	+	+	-	+	+	-
29	-	-	-	-	-	-	-	-	+	+	-	+	+	-
14,a,b	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14,a	-	-	-	-	-	-	-	-	-	-	-	-	-	+
29,a,b	-	-	-	-	-	-	-	-	-	-	-	-	+	-
14,b	-	-	-	-	-	-	+	+	-	-	+	-	-	+
14,b	-	-	-	-	-	-	+	+	-	-	+	-	-	+
29,b	-	-	-	-	-	-	-	-	+	+	-	+	+	-

+ compatible pollination

- incompatible pollination

Table 8.3 Summary of pattern obtained from interfamily diallel of family 88-184

Group as detected in Table 8	1	2	3	4	5	6	7
1	-	-	-	-	-	-	-
2	-	-	+	-	+	+	-
3	-	-	-	-	-	-	+
4	-	-	-	-	-	+	-
5	-	+	-	+	-	-	+
6	-	+	-	+	-	-	+
7	-	-	+	-	+	+	-

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APPENDIX 1

Modified Whites Medium

Containts	mg/l
KCl	65
KNO_3	80
$Ca(NO_3)_2$	300
$Fe_2(SO_4)_3$	.2.5
NaH_2PO_4	16.5
Na_2SO_4	200,
$MgSO_4\ 7H_2O$	360
$MnSO_4\ 4H_2O$	7
H_3BO_3	1.5
$ZnSO_4\ 7H_2O$	3.0
KI	0.75
FeNaEDTA	4.59
Nicotinic acid	2.5
Pyridoxine HCl	0.5
Thiamine HCl	0.5
Casein hydrolysate	300
Glycine	15
Sucrose	50g
Agar	8g
PH 5.8	

APPENDIX 2

Detection of S homozygotes in B. campestris

Introduction

B. campestris homozygotes for S alleles S_a, S_b and heterozygote S_aS_b were categorised using the method described by Thompson & Howard (1959).

Materials and Methods

In March 1987 30 seeds of B. campestris family 85-155 were sown and raised to flower. All plants were test crossed as described in Thompson & Howard (1959). All pollinations were done in the laboratory as described in Chapter 3. On each occasion up to three flowers were pollinated and the number of pollen tubes penetrating the stigma counted.

Results and Discussion

A summary of the results for the test cross is shown in Table 1. In Table 2 plants showing a similar breeding response are grouped together. Plants 3, 13, 26 and 27 form one group, plants 2, 8, 11, 12, 15, 16, 18 and 22 form another group, and plants 1, 4, 5, 6, 7, 9, 10, 13, 14. 17, 19, 20, 21, 23, 24, and 25 form a third group.

According to a classification of breeding responses (Thompson & Howard, 1959) these plants fall into type 3 dominance relationship, where as S_a dominant to S_b in pollen and codominant in stigma.

Table 1. Results of an intra family diallel cross of B. campestris 86-155.

Stigma source	Pollen source 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	+	-						-		+																	
3	-	+						+		-																	
4	-	-						-		-																	
5	-	-						-		-																	
6	-	-						-		-																	
7	-	-						-		-																	
8	+	-						-		+																	
9	-	-						-		-																	
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	+	-	+	+	+	+	+	-	+	+	-	-	+	+	-	-	+	-	+	+	+	-	+	+	+	+	+
12	+	-	+	+	+	+	+	-	+	+	-	-	+	+	-	-	+	-	+	+	+	-	+	+	+	+	+
13	-	+						+		-																	
14	-	-						-		-																	
15	+	-						-		+																	
16	+	-						-		+																	
17	-	-						-		-																	
18	+	-						-		+																	
19	-	-						-		-																	
20	-	-						-		-																	
21	-	-						-		-																	
22	+	-						-		+																	
23	-	-						-		-																	
24	-	-						-		-																	
25	-	-						-		-																	
26	-	+						+		-																	
27	-	+						+		-																	

+ compatible pollinations

- incompatible pollinations

Table 2. Intrafamily diallele cross of B. campestris line 86-155 - Interpretation

Stigma source		Pollen source S_aS_a				Pollen source S_aS_b												Pollen source S_bS_b							
		3	13	26	27	1	4	5	6	7	9	10	14	17	19	20	21	23	24	25	2	8	11	12	15	16	18	22
S_aS_a	3																											
	13	-											-											+				
	26																											
	27																											
S_aS_b	1																											
	4																											
	5																											
	6																											
	7																											
	9																											
	10	-											-											-				
	14																											
	17																											
	19																											
	20																											
	21																											
	23																											
	24																											
	25																											
S_bS_b	2																											
	8																											
	11																											
	12	+											+											-				
	15																											
	16																											
	18																											
	22																											

+ compatible pollinations

- incompatible pollinations

APPENDIX 3

Hoagland solution

Containts	g/l
KNO_3	5.1
$MgSO_4$	2.46
$NH_4H_2PO_4$	1.15
$Ca(NO_3)_2$	2.46
FeEDTA	0.12
H_3BO_3	14 mg
$MnCl_2$	9 mg
$Cu(SO_4)_2$	0.4 mg
$ZnSO_4$	1 mg
Molybdic acid	0.45 mg

APPENDIX 4

Revised Nitsch & Nitsch's medium - Inomata (1985)

Containts	g/l
Nitsch's medium - H	2.27
Casin acid hydrolysate	0.3
Sucrose	50.0
Agar	7.0

APPENDIX 5

Basic embryo culture medium - (Snell 1977)

Containts	mg/l
KNO_3	800
KCl	850
$MgSO_4\ 7H_2O$	370
$Ca(NO_2)_4\ 4H_2O$	290
$CaSo_4\ 5H_2O$	0.025
$NaMoO_4\ 2H_2O$	0.032
KI	0.75
H_3BO_3	1.5
KH_2PO_4	75
$MnSO_4\ 7H_2O$	07
Na_2SO_4	100
$NaH_2PO_4\ 2H_2O$	18.65
NH_4NO_3	750
FeEDTA	30
Meso - Inositol	100
Glycine	02
Nicotinic acid	0.5
Thiamine HCl	0.1
Pyridoxzine HCl	0.1
Sucrose	30 g
Agar	07 g