A Biochemical Investigation into Self-Incompatibility in *Lotus corniculatus*

A Dissertation Presented to the

Faculty of Graduate Studies of

McGill University

in partial fulfillment of the Requirements

for the Degree Master of Science

by

Shirley Dobrofsky

December, 1978

Short title: Self-Incompatibility in *Lotus corniculatus*

ABSTRACT

The influence of self-pollination, cross-pollination, and absence of pollination on ovary protein subunit banding patterns using SDS-polyacrylamide gel electrophoresis was examined in clones of Lotus corniculatus L. cultivar 'Mirabel'. Banding patterns from self-pollinated florets revealed the highest overall protein content and the greatest number of bands of the three treatments, whereas cross-pollinated florets revealed the lowest overall protein content and the least number of bands. Banding patterns differed between treatments within clones, and within treatments between clones. All clones examined produced more seed per pod and longer pods after cross-pollination than after self-pollination, indicating that self-incompatibility did occur in these clones. Ovule position did not appear to affect the probability of fertilization of an ovule. Further research is needed on the genetics of self-incompatibility in this species, to permit establishment of clones with well-defined complements of incompatibility alleles.

RESUME

Nous avons étudié chez divers clones de Lotus corniculatus L. cultivar Mirabel l'effet produit par des autofécondations, des croisements et par l'absence de pollinisation sur les diagrammes électrophorétiques de protéines d'ovaire obtenus au moyen de la technique d'électrophorèse en gel de polyacrylamide. La plus forte concentration de protéines totales ainsi que le plus grand nombre de bandes furent relevés chez les fleurs autofécondées, tandis que l'on notait la plus faible concentration de protéines totales et le moins de bandes chez les fleurs ayant subi un croisement. Les électrophorégrammes ont différé entre les traitements appliqués à un même clone, ainse que pour le même traitement administré aux divers clones. Tous les clones étudiés ont produit plus de graines par gousse et des gousses plus longues après un croisement, mettant ainsi en évidence le phénomène d'autoincompatibilité existant chez ces plants. La probabilité qu'un ovule soit fécondé n'a pas semblé être reliée à la position occupée à l'interieur de l'ovaire. D'autres études sur les mécanismes génétiques gouvernant le phénomène d'autoincompatibilité chez L. corniculatus L. sont nécessaires afin de produire des clones possedant des systèmes d'allèles S bien définis.

ACKNOWLEDGEMENTS

I thank Dr. W. F. Grant, Genetics Laboratory, Department of Plant Science, McGill University, Macdonald Campus, for suggesting the area of research, for his confidence in me, and for providing the pleasant atmosphere in his laboratory that is so conducive to individual research.

I also thank my colleagues, Graduate Students M. C. Therrien for photography and G. Séguin for providing the French translation of the Abstract. Discussions with Graduate Students R. R. Papenburg of the Institute of Parasitology and D. Brener, Department of Microbiology, were invaluable, and I am indebted to them for their help.

I am very grateful to Dr. I. W. Devoe, Department of Microbiology and Immunology, for the use of his centrifuge, and to Dr. C. E. Tanner, Institute of Parasitology, for use of his densitometer. In addition, I thank Dr. J. F. Peterson, Department of Plant Science, McGill University, and Dr. S. J. Peloquin, Department of Horticulture, University of Wisconsin-Madison, for a constructive criticism of the manuscript.

I thank also my parents for their genuine interest in my work.

I am very grateful to the Department of Education of the Province of Québec and to the National Sciences and Engineering Research Council of Canada for providing me with Fellowships for the duration of my studies.

CLAIM TO ORIGINAL RESEARCH

This thesis represents the original work of the author and is her contribution to an understanding of self-incompatibility in Lotus corniculatus cv. 'Mirabel'. Differences in protein concentration and SDS - polyacrylamide electrophoretic banding patterns were demonstrated between unpollinated, self-pollinated, and cross-pollinated ovaries (collected 24 hours after pollination) for the first time in this genus and family. An electrophoresis technique for the analysis of ovary proteins for plants with very small ovaries, containing low protein concentrations, was developed. In addition, an original technique was used to circumstantially determine the ability of the pollen tube to penetrate to the end of the ovary.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

Lotus corniculatus L. (birdsfoot trefoil), a perennial legume and a natural tetraploid ($2n = 24$), originated in the Mediterranean region (Hertsch, 1959) and advanced over most of western Europe (including the British Isles) and eastward to Asia Minor (Giles, 1949). Within this century it has become a major forage crop in temperate regions of the world.

Birdsfoot trefoil is an important species to investigate as it is increasing in usage in southeastern Canada and northeastern United States (Seaney, 1973). It withstands extremes of flooding and drought with less loss than other forage legumes and can thrive in this cool, humid climate. The forage produced is very digestible, even after the plants go to seed. The leaves are high in protein but do not cause bloat in cattle or sheep, and are low in the estrogens that cause breeding problems (Langille and Bubar, 1978). Milk from cows fed trefoil hay contains more carotene, vitamin A, and tocopherol, and exhibit better keeping qualities than milk from cows receiving Ladino clover and timothy hays (Loosli et al., 1950).

Lotus corniculatus is an outcrossing species and plant breeders generally agree that it produces less seed when self-pollinated than when cross-pollinated. Although the morphological and genetical aspects of this reduction in seed set have been studied in great depth, no work has been done on the biochemical basis of this phenomenon. Some workers feel that both self-sterility and self-incompatibility contribute to the reduced seed set upon self-pollination. A study of the biochemistry of the self-incompatibility reaction would be of considerable interest as it may reveal

the presence of a biochemical barrier to fertilization, and so provide a first step towards more detailed experimentation aimed at chemically by-passing the incompatibility reaction.

The ability to control the self-incompatibility reaction is advantageous to the plant geneticist and the plant breeder. A depressed seed set due to self-incompatibility may seriously alter gene frequencies in a population, such that analysis of traits linked, or biochemically related, to self-incompatibility may be grossly impaired. This is especially relevant when one is trying to determine if a trait is tetrasomically or disomically inherited. Even characters that are not linked or somehow related biochemically to self-incompatibility will have altered gene frequencies at the population level due to the high ovule mortality that results from self-incompatibility. If self-incompatibility is no longer present, greater genetic variability may be revealed, homozygosity of certain loci may finally be accomplished, and persistent undesirable traits may be more easily selected against. A survey of the protein differences between self-pollinated and cross-pollinated ovaries may provide a base from which to uncover the mechanism that may some day be exploited to control the self-incompatibility reaction.

Since both tetrasomic and disomic inheritance for different characters have been reported for Lotus corniculatus (Bubar, 1958), tetrasomic inheritance of self-incompatibility has not been ruled out. Consequently, the number of loci controlling the incompatibility reaction is as yet undetermined. It is considered that an investigation into the protein differences between ovaries of self-pollinated and cross-pollinated florets may provide an insight into the number of gene loci involved.

Lotus corniculatus is an interesting species to choose as a subject for research in incompatibility, due to the unusual apparent location of the self-incompatibility reaction. In most plants with this type of incompatibility (gametophytic) the first evidence of incompatibility is in the style, whereas in birdsfoot trefoil, the first morphological indication of incompatibility appears at the level of the ovary. Lotus corniculatus is a favorable plant to study as it is a perennial, is easily propagated vegetatively, and can flower profusely for extended periods given proper conditions. This permits an exhaustive study on a single genotype. Since the species is entomophilous, cross-pollination is easily prevented when plants are maintained in bee-proof enclosures.

The object of this study is to compare protein subunit banding patterns in self-pollinated and cross-pollinated ovaries using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. If consistent differences between self-pollinated and cross-pollinated ovaries of several plants are found, this may suggest that these protein subunits are implicated in, related to, or genetically linked with the self-incompatibility reaction. To insure that the self-incompatibility reaction is occurring in the plants examined, the seed yield and length of the mature pods will be analyzed. To gain insight into the difference between compatible and incompatible pollen-tube lengths, seed position will be examined.

II. LITERATURE REVIEW

A. Terminology

According to Brewbaker (1954), self-incompatibility is "the inability of a plant producing functional male and female gametes to set seed upon self-pollination". Because self-incompatible plants carry factors for incompatibility, such plants may also be involved in cross-incompatibilities. For this reason, many researchers prefer simply to use the term "incompatibility".

The word "self-incompatibility" was proposed by Stout in 1917, but "self-sterility", the term suggested by Darwin in 1876, was still being used by Giles in 1949. "Self-sterility" is now restricted to those forms of sterility which result from chromosomal abnormalities, the production of non-functional gametes, or post-fertilization failure (Williams, 1964).

Hogenboom (1975) suggested the term "incongruity", as opposed to incompatibility, to describe interspecific or intervarietal hybridization failure. He defined incongruity as "nonfunctioning of a relationship resulting from a lack of genetic information in one partner about some relevant character of the other".

Self-incompatibility is not restricted to higher plants and has been recognized in the Ascomyctes (fungi) since 1934 (Drayton in Sclerotinia gladioli and Ames in Pleurage anserina; for a review of self-incompatibility in fungi, see Lewis, 1954). In addition, hermaphroditic animals have been described as self-incompatible, which include Ciona intestinalis (Castle,

1896) and Styela partita (Plough, 1933). In fungi the incompatible interaction occurs between two haploid tissues, whereas in animals it occurs between haploid and diploid tissues.

B. Genetics

Incompatibility in flowering plants has been observed for over two centuries. It was first reported in 1764 by Kolreuter working on Verbascum phoeniceum (East and Park, 1917). There have been several reviews on the genetics of incompatibility in Angiosperms (Darwin, 1876; East and Park, 1917; Stout, 1938; Bateman, 1952; Arasu, 1968) of which the one by de Nettancourt (1977) has been the most recent. Since the various incompatibility systems have been described in detail in these and other reviews, only a general summary will be presented here.

Incompatibility systems are divided into "heteromorphic" and "homomorphic" on the basis of floral morphology. In the heteromorphic system, incompatibility is correlated with differences in stamen and pistil height. Flowers are of two or three types, depending on the species. In species with two types, flowers with long stamens and short pistils will only accept pollen from plants (of the same species) with short stamens and long pistils. A single incompatibility locus, S/s, controls the inhibition reaction. In the homomorphic system, there are no morphological differences to foretell the potential result of a cross.

In 1937, Sears distinguished three classes of homomorphic incompatible plants, based on the region in the pistil in which pollen-tube growth ceases. In Class I, pollen germination is inhibited on the stigma. These

plants tend to exhibit "sporophytic" self-incompatibility, in which case substances produced by the male parent (carried by the pollen) react with the female tissue to block fertilization. The genetics of this system was first described in guayule by Gerstel (1950) and in Crepis foetida by Hughes and Babcock (1950).

In Class II, pollen germination is normal, but pollen-tube growth is inhibited in the style. These plants typically exhibit "gametophytic" self-incompatibility, in which case the male gametophyte (the pollen tube) reacts in some way with the female tissue to prevent ultimate gametic fusion. The genetic basis of the gametophytic system of incompatibility was determined by Prell in 1921 (Arasu, 1968), but it was overlooked in the literature for many years. East and Mangelsdorf (1925), working with Nicotiana hybrids, and Lehman (1927) and Filzer (1927), working with Veronica, independently arrived at the same conclusion about the mode of inheritance of this incompatibility system as did Prell.

In Class III, the self-incompatibility reaction is manifested at some point after the pollen tube has entered the ovary. Narcissus (Frankel and Galun, 1977), Theobroma cacao (Cope, 1958), Rubus arcticus L. (Tammisola and Ryynänen, 1970), and Lotus corniculatus (Giles, 1949) fall into this class. This is a rather heterogeneous group both in terms of the precise point at which the incompatibility reaction occurs and in terms of the genetics involved. Whereas in Theobroma cacao the pollen tube has already liberated its contents into the ovule before compatible and incompatible pollen tubes are distinguished (Cope, 1962), this is not the case in Lotus

corniculatus (Giles, 1949). In Theobroma cacao the genetical control of the reaction is sporophytic, whereas in Lotus corniculatus and Rubus arcticus control is apparently gametophytic. Thus, in the ovary, the incompatibility reaction may be either between the male gametophyte and the female sporophyte (the ovary) or between the male gametophyte and the female gametophyte (in the ovule). This latter type has been reviewed by Tammisola and Ryynänen (1970).

Most of the early work dealt with incompatibility systems that were apparently controlled by a single gene locus, often designated as "S." Later, Owen (1942) discovered that the incompatibility system of Beta vulgaris was controlled by several gene loci. Since then, similar complex systems have been uncovered in many plant species (See Lundqvist (1975) for a review).

C. Biochemistry

Pollination is an event that causes major changes in the metabolism of the flower, the most profound occurring in the pistil (Linskens, 1974).

As a consequence of compatible pollination, respiration is stimulated, growth hormones increase rapidly, starch and free sugars alter, the amount and composition of proteins change (Shivanna et al., 1974; Mascarenhas, 1975; Roggen, 1967; Tupý et al., 1974; Ascher and Drewlow, 1970) and enzyme activity is affected (Paton, 1921; Mascarenhas, 1975; Stanley and Linskens, 1974).

Incompatible pollination can initiate strikingly different developments. The following is a summary from various gametophytically incompatible species and does not apply specifically to any one species.

During progression of the pollen tube through the style, the pollen-tube nuclei in an incompatible pollination may behave abnormally (Linskens, 1974; van den Ende, 1976). As well, carbohydrate metabolism may be disturbed, resulting in a proliferation of callose plugs and a higher density of fibrils in the pollen tube walls (Currier, 1957; Linskens, 1964; de Nettancourt, 1973; Dickenson and Lawson, 1975; van den Ende, 1976).

Self-incompatibility is also characterized by different activities of some enzyme systems (Linskens, 1964; Pandey, 1967; Bredemeijer and Blaas, 1975). Pandey (1973) observed that self- and cross-pollinated Lilium and Nicotiana plants differ in their heat sensitivity of esterase isoenzymes in the style. However, this need not imply that esterase is involved in the self-incompatibility system, since temperature sensitivity of pollen-tube growth is known, at least in Trifolium hybridum L., to be genetically linked to the self-incompatibility system (Townsend, 1970, 1971). This may explain the relationship observed between self-incompatibility, temperature - sensitivity, and esterase isoenzymes. Pistil amino acid patterns differ according to whether the plant has been self- or cross-pollinated (Linskens and Tupý, 1966; Pipkin and Larson, 1973). Self-pollinated plants in some species have been demonstrated to respond differently to RNA synthesis inhibitors (Ascher, 1971; Linskens, 1975). Quantitative and qualitative differences in protein and glycoprotein synthesis have been demonstrated between the two types of matings (van den Ende, 1976; Sarfatti et al., 1974; Ascher and Drewlow, 1970; Deurenberg,

1976; van der Donk, 1974a,b, 1975). Van der Donk (1974a, 1975), using sodium dodecyl sulfate (SDS) gel electrophoresis, found great differences in the protein banding patterns of self- vs. cross-pollinated styles of Petunia hybrida L.

The biochemical timing of the self-incompatibility reaction may anticipate the morphological manifestation of the event. Linskens (1975) and Deurenberg (1976), working with Petunia hybrida, found that pre-activation of RNA and protein synthesis in the ovary occurs following self-pollination, even though the "self"-pollen tubes in this plant will never reach the ovary. In Abelmoschus esculentus, pollination induces dissolution of the nucellar cells at the micropylar end of the ovule, forming a passage for pollen-tube entry even when the pollen tubes are in the stylar region (Chandra and Bhatnagar, 1975). This may provide some insight on how and when the pollen tube "communicates" with the ovary and/or ovules.

Experiments using immunological techniques suggest that the self-incompatibility reaction is associated with specific proteins. Lewis (1952), working with Oenothera organensis, produced antisera to protein from macerated pollen with different incompatibility genotypes. Using the precipitation technique, antigens were found that were specific to the different pollen genotypes. This was confirmed in an experiment he carried out with Burrage and Walls (1967) using leachates from intact pollen grains. Linskens (1960, cited in Mäkinen and Lewis, 1962), working with Petunia hybrida extended the immunological technique to include stylar proteins,

and showed that the self-incompatibility reaction between pollen tube and style is correlated with specific proteins. Using radioactive tracers and paper electrophoresis, Linskens (1958, cited in Mäkinen and Lewis, 1962) showed that a complex was formed between pollen and stylar proteins in Petunia after the incompatibility reaction had occurred.

Staining tests and heat denaturation on pollen extracts and on exudates into agar indicated that the antigenic substance is a protein, but could include a polysaccharide component (Mäkinen and Lewis, 1962).

Lectins, or phytohaemagglutinins, have been proposed as a class of substances that may enable recognition of 'self' and 'non-self' in plants. Lectins are soluble globular proteins and glycoproteins that can bind specific carbohydrates with high affinity (Knox, 1976; Clarke et al., 1975). They have been implicated as a 'gluing' substance in cell wall extension (Kauss and Glaser, 1974), possibly binding different carbohydrates more or less reversibly (Kauss and Bowles, 1976). Jermyn (1975) suggests on the basis of precipitation reactions, due mostly to lectins, between plant tissue extracts, that a wide range of specific interactions between macromolecules of plant origin are possible, and that for plant species there is at least a rudimentary recognition of the differences between substances that are "self" and "non-self". Burnet (1971) has pointed out in relation to the human immune system that possible defence mechanisms have evolved as secondary elaborations in a basic system evolved for self-recognition.

In 1913, Compton proposed that substances are formed in the pistil which stimulate or retard the growth of the pollen tube. He drew an analogy between self-sterility and the growth of fungus hyphae into the host tissue, and compared the mechanism of self-sterility with that of immunity against a pathogen. Later, lectins were found to be involved in fungus-plant reactions, such as that between Lotus corniculatus and Rhizobium (Currier and Strobel, 1977). The possible involvement of lectins in the recognition reaction in pollen-tube growth is suggestive of Compton's original comparison in 1913.

D. Self-Incompatibility in Lotus corniculatus

It is generally agreed that Lotus corniculatus L. produces less seed when self-pollinated than when cross-pollinated. This feature is common to other perennials within the genus, but has not been observed in the annuals examined which seem to be self-pollinators for the most part (Zandstra and Grant, 1968). Self-incompatibility can serve as an effective outcrossing mechanism for a plant species that is likely to produce offspring many times, and can afford to depend upon bees for pollination. Stebbins (1958) indicated that long-lived species tend to be more allogamous than short-lived ones. Oka and Morishima (1967), working with Oryza sativa, where strains exhibit a perennial-annual continuum, found that perennial forms outcrossed to a greater extent than annual forms. In four species of Melilotus in which both annual and biennial forms were present, the annual forms were found to have higher self-fertility than their biennial conspecifics (Sano, 1977).

1. Seed Production

Many researchers have compared seed set in Lotus corniculatus between self- and cross-pollinated plants. As far back as 1876, Darwin observed that insects were crucial to seed production in this species.

In 1931, Silow noted that Lotus corniculatus was practically "self-sterile", but that occasionally plants set a few seeds after self-pollination. Three out of 22 plants were slightly self-fertile voluntarily (8.7 to 30.3 seeds/100 flowers) but 14 out of 22 were self-fertile when deliberately self-pollinated (1.1 to 416.0 seeds/100 flowers).

Then in 1946 MacDonald claimed 100% floret self-fertility when single plants were isolated with bee pollinators. Giles, in 1949, repeated this experiment but was unsuccessful, thus refuting the virtue of bee pollination over the human imitation, and thereby concluded that MacDonald had erred. Possibly, he argued, progeny of the parental plant had inadvertantly been allowed to germinate and ultimately contribute "non-self" pollen; or perhaps the bees had not been rendered sufficiently "pollen-free" between trials.

Giles' (1949) results agreed with those of Tome and Johnson (1945), who found that in 78% of the plants, fruits were produced from only 0 to 5% of the florets self-pollinated. In contrast, 40 to 69.99% of the florets when cross-pollinated produced pods (Giles, 1949). Also, pods resulting from cross-pollinations contained at least three times more seed and were longer than pods from self-pollination. Giles was doubtful that any fruit produced had occurred without manipulation. He observed that

fertile ovules were more frequent in the basal half of the ovary (the region farthest from the style) following cross-pollination than following self-pollination. Brandenburg (1961), like Giles, found that outcrosses produced significantly higher pod set, more seeds per pod, and longer pods. He also noted that outcrossers had significantly more plump seed and matured faster. However, he did not find as great a difference in seed set between pods from self- and cross-pollinated florets as did Giles. He confirmed that the average length of pods resulting from cross pollination was 20 millimeters while the average length of pods resulting from self-pollination was 14 millimeters. Giles (1949) noted that post-fertilization abortion further reduced the amount of seed from a self-pollination, but did not mention post-fertilization abortion in connection with pods resulting from cross-pollinations.

In 1963, Wojciechowska reported 41.2% fertilization in ovaries from cross-pollinated florets (in 1958, Bubar reported 50% or less) as opposed to 6.7% after self-pollination. Post-fertilization abortion was reported to be slightly greater after self-pollination. This author concluded that Lotus corniculatus was partially self-incompatible. In contrast to Giles (1949), Wojciechowska maintained that there was probably no relation between fertility and the location of the fertile ovules.

In a hybridization study involving 360 plants, Seany (1964) reported that only 7% of the plants produced more than an average of 0.5 seeds per self-pollinated flower, whereas 45% of the plants produced no seeds.

In 1977, Schaaf reported that average differences in cross-fertility among 13 plants ranged from 1.5 to 9.8 seeds per flower pollinated, although 17 out of 156 crosses produced greater than 13 seeds per flower pollinated and 14 crosses were sterile. No relationships were apparent between a plants performance as a female and as a male. There was considerable variation in the success of an individual plant as a male or a female, depending upon the plant with which it was cross-pollinated.

It would appear that researchers will most likely continue to obtain widely variable results when comparing self-pollination with cross-pollination in Lotus corniculatus. The literature suggests wide variation in number of seeds per pod between plants in a population. This variation becomes even more apparent when one considers the origin of the germplasm and allows for the discrepancies between different taxonomic collections and local cultivars of Lotus corniculatus.

2. Genetics

The genetic basis of self-incompatibility in Lotus corniculatus has been explored in great depth (Bubar, 1957; Brandenburg, 1959; Miri, 1964) but has not yet been determined with certainty (Seaney and Hensen, 1970). Since Lotus corniculatus is considered a segmental allopolyploid (Stebbins, 1950), the genetic basis for the incompatibility reaction may be more complex than for diploid species. Typically, diploid species tend to reduce their self-incompatibility when their chromosome complement is doubled (Lewis, 1943; Brewbaker, 1954). This has evidently not been the case with Lotus corniculatus. Loci from several original parents may have

combined in this species to contribute to the resulting incompatibility system. It is proposed that this species exhibits gametophytic incompatibility, and that several loci are involved in the control of the incompatibility reaction. Since some seed are produced from self-pollinations, the incompatibility reaction is designated "incomplete" (Wojciechowska, 1963).

3. Morphology

Giles (1949) first observed that a difference in the rate of pollen-tube growth after self- vs. cross-pollination occurred only at the level of the ovary. Spiss (1969) and Spiss and Paolillo (1969) confirmed Giles' observation that no difference in the pollen-tube growth rate occurred at the level of the stigma or style. Brewbaker and Majumder (1961) stated that inhibition occurs in the ovary after completion of pollen-tube mitosis. According to Giles, pollen tubes reach the ovary within 24 hours after pollination under field conditions. By 36 hours some reach the micropyle (Hansen, 1953). Wojciechowska (1963) reported pollen tubes within the ovary 24 to 48 hours after pollination.

Various factors combine to contribute to a lower seed set after self-pollination. According to Bubar (1958), outcrossing Lotus species stagger the readiness of ovules to be fertilized, to prolong the total length of the period of anthesis and increase the probability of being visited by a bee during the period when pollination is still possible. For this reason, rarely more than 50% seed set will result from even the most favourable cross-pollination, assuming a single pollination event (Bubar, 1958). In contrast, self-pollinating congenerics exhibit synchronous ovule development within a single ovary (Bubar, 1958).

Bubar (1959) considered self-incompatibility and self-sterility as two distinct explanations for the reduced seed set. Self-incompatibility is expressed by a slower rate of growth of self-pollen tubes within the ovary, as observed by Giles (1949). The rupture of the stigmatic membrane, which is a prerequisite for pollen penetration into the style, is typically performed by a bee. Therefore, in all probability, cross-pollen (from the bee) will be included in each natural pollination. Since cross-pollen has an advantage over self-pollen at the level of the ovary, cross-fertilization is favoured (Bubar, 1959). Self-sterility is expressed by post-fertilization abortion. Giles (1949) does not consider this a major cause of lowered seed set after self-pollination in Lotus corniculatus, although Bubar (1959) does. In genetical studies, self-incompatibility effects may easily be confounded with self-sterility.

4. Biochemistry

Experiments on the biochemical basis of incompatibility have been attempted in various species but none to date on Lotus corniculatus.

III. MATERIAL AND METHODS

A. Plant Material

Lotus corniculatus L. (cultivar Mirabel) plants used were grown in December 1976 from seed from a single 1976 accession (A. E. Lods Research Station, Macdonald College), and were maintained under controlled environmental conditions prior to the experiments. Plants were grown in a mixture of soil, peatmoss, vermiculite, perlite, and sand (5:3:2:1:1) and were fertilized regularly. Cuttings were propagated when the plants were five months old. Data from cuttings and parental plants were pooled.

In October 1977 six randomly selected clones (designated P, PY, OB, PB, O and Y) were placed in controlled environment chambers with a constant temperature of 20 $\pm$ 1°C, an 18:6 hour day:night light cycle, and a relative humidity of 75 $\pm$ 5%, to optimize flowering conditions. Four of the clones (P, PY, PB and OB), consisting of four plants each, were used for electrophoretic studies from May to July 1978. Mature pods were collected from all six clones from October 1977 to August 1978.

Large morphological differences distinguished the six clones (Table 1), such that the origin of the plant was obvious. After complete floret removal, plants of the same clone would begin to produce florets one or two days apart from each other, but possibly weeks before or after plants of other clones had begun to flower.

B. Plant Treatment

Florets used for electrophoresis were plucked at anthesis (the "erect standard" stage; Giles, 1949), when presumably they would be most attractive to bee pollinators. Ascher and Peloquin (1966) found that the

Table 1. Morphological characteristics distinguishing the clones.

Clone	Floret production	Plant height	Leaf colour	Habit	Keel tip colour
P	high	short	yellow-green	ascending	yellow
PY	high	intermediate	dark green	ascending	yellow
OB	low	tall	green	almost erect	yellow
O	moderate	short	yellow-green	semi-prostrate	brown
Y	low	tall	green	almost erect	brown
PB	moderate	intermediate	yellow-green	ascending	yellow

self-incompatibility reaction was strongest at this time in Lilium longiflorum Thunb. Care was taken to exclude florets that were beginning to show signs of senescence, such as wilting or the onset of floral abscission. Flowers plucked were either unpollinated, or had been self-pollinated, or cross-pollinated, 24 to 26 hours prior to removal. According to Giles (1949), pollen tubes reach the ovary 24 hours after pollination under optimum (field) conditions. Some pollinated florets were not removed until maturity, when parameters described below were analyzed.

Florets were self-pollinated by slitting the carina (keel) with a sterilized dissecting needle (dipped in 95% ethanol and flamed) to expose the stigma, rupturing the stigmatic membrane by rubbing the stigma with fine-grained sandpaper (a new piece was used for each clone), and fresh pollen from the same plant placed on the stigma using a sterilized dissecting needle. Treated umbels were labelled with white Time Tape (Professional Tape, Inc.).

Florets were emasculated prior to cross-pollination. A sterilized dissecting needle was used to slit the carina of a floret at the "pointed bud" stage (Giles, 1949), just prior to anther dehiscence. A 145 mm pasteur pipette fastened to a vacuum pump was used to remove the anthers. The umbel was labelled with red Time Tape after treatment. Emasculation at earlier stages caused more damage to the bud, thus reducing the probability of the floret surviving to anthesis. Some plant clones withstood emasculation much better than others. Large flower size and sturdier stems increased the probability of a clone reacting favourably to emasculation.

Only two of the six initial clones survived emasculation to a degree that permitted electrophoretic analysis of cross-pollination. Coincidentally, both these clones had been cross-pollinated with the same plant of a third clone. Within two to three days after emasculation, the flowers that had survived to reach anthesis were cross-pollinated, such that an entire clone would only be cross-pollinated with one plant of the clone acting as the pollen parent. The same pollination technique was used as for self-pollination, except that in this case, after pollination the umbels were labelled with orange Time Tape rather than with white.

Clones P and PY were cross-pollinated with cutting No. 1 of clone OB, clones OB and PB with the parental plant of clone P, clone Y with cutting No. 1 of clone O, and clone O with cutting No. 1 of clone Y. Sufficient numbers of florets survived emasculation in clones OB, PB, O, and Y to permit some cross-pollination for analysis of the mature pod.

C. Electrophoretic Studies

1. Chemicals and Apparatus

Chemicals used in this study and the sources from which they were obtained are as follows: sodium azide (NaN_3), THAM (Tris (hydroxymethyl) aminomethane), sodium dodecyl sulfate (sodium lauryl sulfate, SDS), glycine, and bromophenol blue were obtained from Fisher Scientific Company; KH_2PO_4 and 2-mercaptoethanol were obtained from J. T. Baker Chemical Company; Na_2HPO_4 was obtained from Merck and Company Limited; N,N',N',N'-Tetramethylethylenediamine (TEMED) and N,N'-methylenebisacrylamide (bis) from Canal Industrial Corporation; acrylamide and the Bio-Rad Protein Assay

kit from Bio-Rad Laboratories; glycerol from Matheson, Coleman and Bell Manufacturing Chemists; Coomassie Brilliant Blue R from Sigma Chemical Company; and the Electrophoresis Calibration Kits for high and low molecular weight protein subunit determination from Pharmacia Fine Chemicals. Other chemicals were obtained from the most convenient commercial sources. All solutions were prepared using double (glass)-distilled water and were filtered with Whatman No. 3 (9 cm) filter paper.

Plant tissue was macerated in a 7 ml Ten Broeck type Pyrex macerator; and centrifuged in Eppendorf micro-test tubes (polypropylene) in an Eppendorf centrifuge Model 5412, both manufactured by Brinkmann Instruments. Supernatant was concentrated in a Minicon Macrosolute Concentrator (B15) manufactured by Amicon Corporation. The electrophoresis unit used was built by Mr. Hagemann of the Instrument Shop, McIntyre Medical Building, McGill University; and was powered by a Beckmann Duostat, Model RD. A Unicam SP 1800 ultraviolet spectrophotometer with a Unicam AR 25 Linear Recorder was used to scan the gels and record band positions and relative dye concentrations.

2. Ovary Weight and Protein Content

Unpollinated Lotus corniculatus ovaries were removed when the florets were at anthesis (the "erect standard" stage - Giles, 1949), and the fresh weight, dry weight, and protein content determined. Ovaries were oven-dried at 100°C to determine dry weight. Samples of 50 ovaries were weighed on a Mettler balance.

The Bio-Rad protein determination assay was used to determine protein concentration, as plant phenolics were known to interfere with the more sensitive Lowry assay (Lowry et al., 1951). For the assay, fresh ovaries

were macerated in 0.1 M prechilled phosphate buffer (0.1 M Na_2HPO_4, 0.005 M KH_2PO_4, 0.01% NaN_3; pH 8.0) in a prechilled hand homogenizer. The homogenate was prepared as described for electrophoresis below to a final volume of 0.1 ml.

3. Sample Preparation

Each sample consisted of 250 ovaries macerated in prechilled phosphate buffer (see above) in a prechilled hand homogenizer. The homogenate was evenly distributed into 1.5 ml micro test tubes and centrifuged at 12,800 X G for five minutes. The supernatant was then removed and spun for five minutes at 12,800 X G. The final supernatant was concentrated to 50 µl in an Amicon Minicon macrosolute concentrator that removes molecules below a molecular weight of approximately 15,000.

To each of the concentrated samples was added 10 µl of the following solution (modified from Laemmli, 1970): 60% (v/v) glycerol, 6% (v/v) 2-mercaptoethanol, 6% (w/v) sodium dodecyl sulfate (SDS), and 0.045% (w/v) bromophenol blue, dissolved in the phosphate macerating buffer. This yielded the following concentrations in the final solution after addition of the protein sample: 10% glycerol, 1% 2-mercaptoethanol, 1% SDS, and 0.0075% bromophenol blue. The modification permitted addition of a smaller volume of the above solution to the protein sample than would be possible according to Laemmli (1970), but maintained the recommended concentrations of glycerol, 2-mercaptoethanol, and SDS. The phosphate buffer was used instead of the recommended buffer (0.5 M Tris-Cl, pH 6.8; Laemmli, 1970) because addition of such a small volume of pH 6.8 buffer to the sample would not have changed the final pH appreciably from the sample pH of 8.0.

4. Electrophoresis

Siliconized glass tubes used for electrophoresis were 125 mm long with an inner diameter of 5 mm. The gels and electrode buffer were prepared according to Laemmli (1970), with a 90 mm separating gel and a 250 µl stacking gel. A 7.5% separating gel was used instead of the 9% separating gel described by Laemmli (1970). Electrophoresis was performed for 3 hours and 20 minutes at a constant current of 2 milliamperes per gel. The bromophenol blue marker was permitted to travel 8 ± 0.5 cm through the separating gel.

Simultaneous with the samples, two standard gels were run, each containing a mixture of proteins for molecular weight determination. Standard protein subunit mixtures were dissolved in phosphate macerating buffer (described above) instead of the recommended buffer. 0.01 ml of the standard protein subunit solution (high or low molecular weight) were used for each standard gel.

After completion of electrophoresis, each gel was stabbed with a syringe needle coated with India Ink to indicate the position of the bromophenol blue marker, and was stained in a solution of 0.1% Coomassie Blue R, 25% 2-propanol, and 10% acetic acid (Fieldes et al., 1977) for 18 hours at 37°C. Gels were then destained for two days in a solution of 10% 2-propanol and 10% acetic acid. The destaining solution was changed frequently. Gels were stored in 7.5% acetic acid and photographed. They were analyzed on a densitometer at 590 nm.

D. Mature Pod Analysis

Pod length, seed number per pod, length from the farthest seed from the style to the distal end of the pod (with respect to the style), and number of seed in the distal vs. the proximal half of the pod were tabulated for mature pods (pods and sepals brown or straw-coloured). Pod length and position of the farthest seed were analyzed for clones P and PY using the Student's t-test (Steel and Torrie, 1960) and the Kolmogorov-Smirinov test (Siegel, 1956). Mean and standard deviation were computed for all clones examined. Seed number for clones P and PY were analyzed using the Kolmogorov-Smirinov test, and for all clones the median and values one quintile above and below the median were computed. Maximum seed set was noted, as well as total number of ovules per pod.

IV. RESULTS

A. Electrophoretic Studies

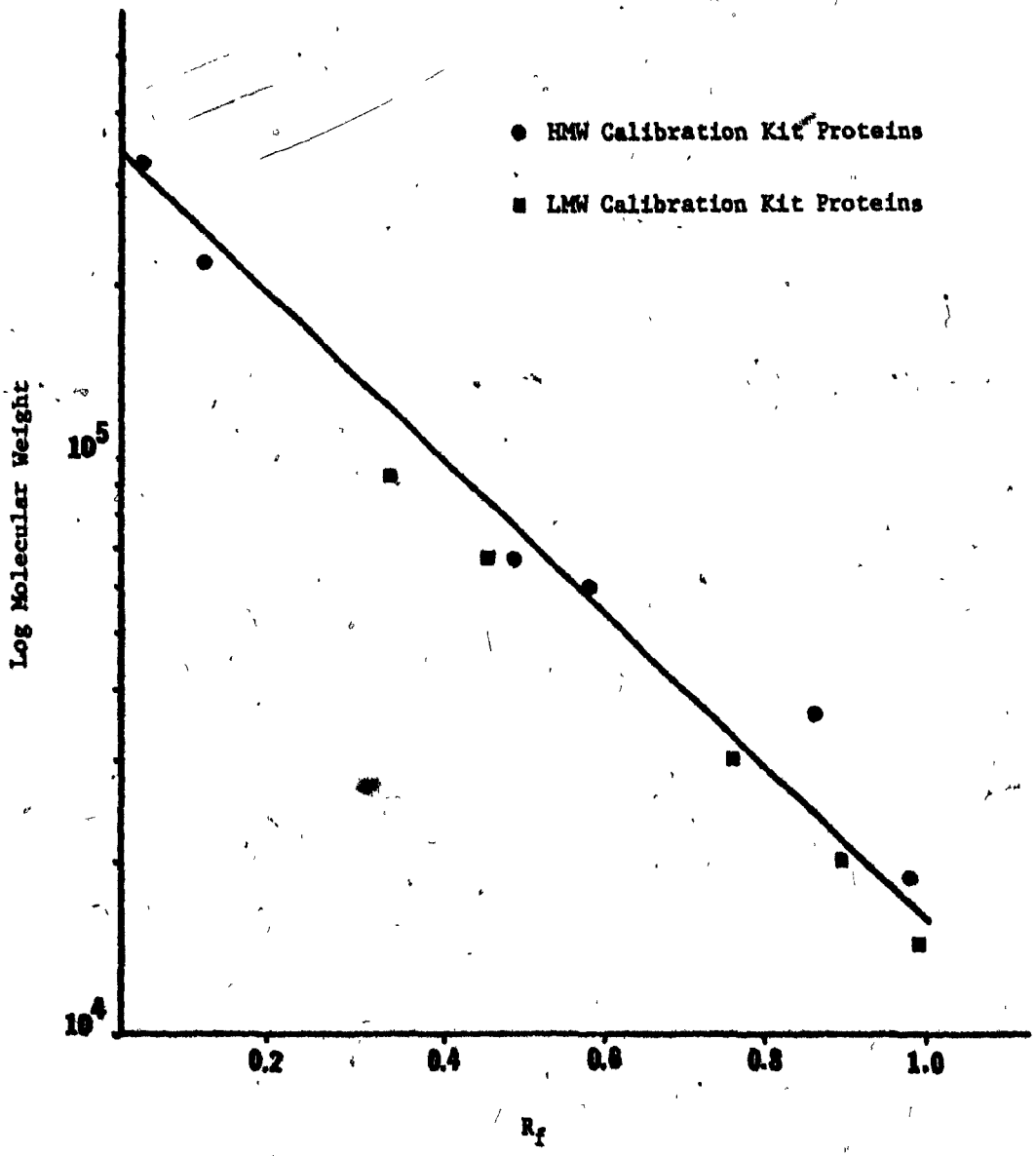
1. Preliminary Analysis

a) Ovary Weight. The fresh weight of 50 unpollinated ovaries at anthesis was determined to be 0.10 ± 0.01 g (mean and standard deviation for 10 samples) and the dry weight for the same number of unpollinated ovaries at anthesis was 0.025 ± 0.001 g (5 samples).

b) Protein Content. Using the Bio-Rad protein assay, 100 unpollinated ovaries plucked at anthesis were found to contain 40 to 60 µg of buffer-soluble protein (three replicates). Since Maizel (1971) recommended using 100 µg of protein per gel for samples whose electrophoretic banding pattern is unknown, 250 ovaries were used per gel in order to attain an approximate protein content of 100 to 150 µg per gel. For consistency, the same number of ovaries were used per gel for the self-pollinated and cross-pollinated ovary samples. Results from electrophoresis would thus indicate relative protein concentrations between unpollinated, self-pollinated, and cross-pollinated ovary samples.

c) Standard Curve for Protein Subunit Molecular Weight Determinations. To estimate protein subunit molecular weight, protein subunits of known molecular weight were run concurrent with the gels containing the samples. A plot of relative mobility (R_f) against log molecular weight was then constructed (Fig. 1) from which to derive approximate molecular weights of the protein subunits in the sample for which only the R_f values were known. The points obtained using the

Figure 1. Standard curve of migration distance vs. log molecular weight for molecular weight determinations.


● HMW Calibration Kit Proteins
■ LMW Calibration Kit Proteins
Log Molecular Weight
10^5
10^4
0.2
0.4
0.6
0.8
1.0
R_f

standard protein subunits were found to approximate a line (Fig. 1) in the region where most of the protein subunit bands appeared in the sample gels, so this technique was considered applicable for molecular weight determination of protein subunits in the samples.

Two mixtures of protein standards were used; one mixture for the determination of molecular weight of protein subunits with relatively high molecular weights and the other mixture for the determination of molecular weights of protein subunits with relatively low molecular weights. The high molecular weight protein subunit calibration kit contained the following: thyroglobin (molecular weight (M.W.) = 33,000), ferritin (M.W. = 18,500; M.W. of the half unit which appeared as well = 220,000), albumin (M.W. = 67,000), catalase (M.W. = 60,000), and lactate dehydrogenase (M.W. = 36,000). The low molecular weight determination kit contained the following: phosphorylase b (M.W. = 94,000), albumin (M.W. = 67,000), ovalbumin (M.W. = 43,000), carbonic anhydrase (M.W. = 30,000), trypsin inhibitor (M.W. = 20,000), and α-lactalbumin (M.W. = 14,400). Densitometer tracings for the two types of standard gels are indicated in Figs. 2 and 3. Apparently the gel system used could accommodate protein subunits of molecular weights extending from 14,400 to over 330,000.

2. Electrophoresis

Samples of unpollinated ovaries at anthesis were analyzed electrophoretically from four plant clones (P, PY, PB and OB). Ovaries from self-pollinated florets were analyzed from clones P, PY and PB.

Figure 2. Densitometer tracing and corresponding gel for low molecular weight protein subunit standard.

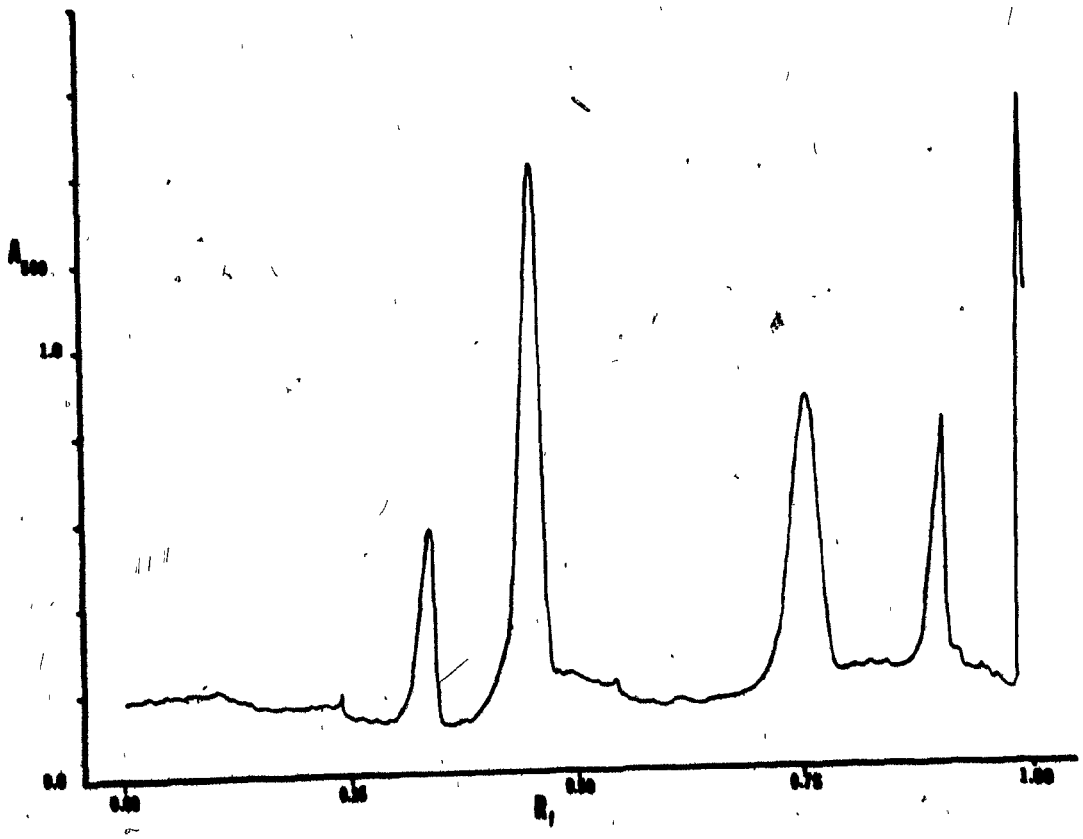
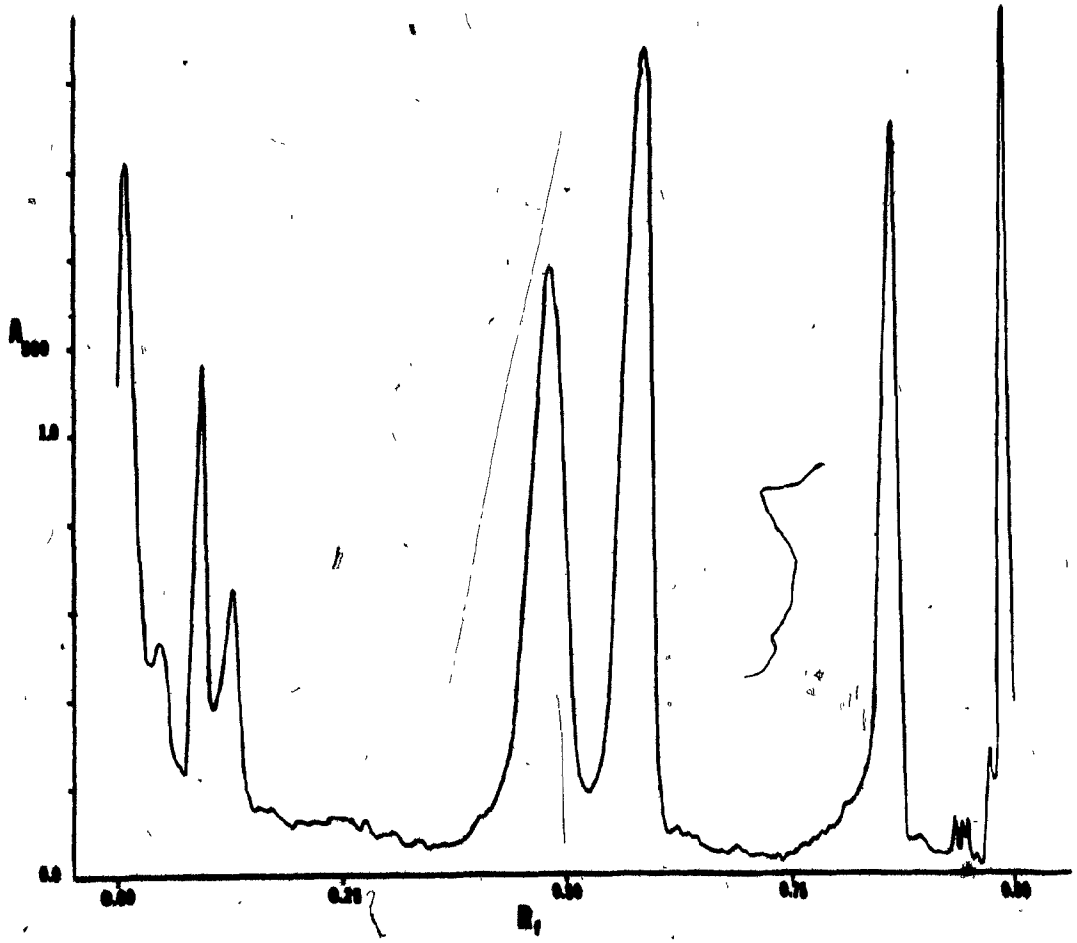

A_{560}
1.0
0.0
0.00
0.25
0.50
0.75
1.00
R_f

Figure 3. Densitometer tracing and corresponding gel for high molecular weight protein subunit standard.


1.0
0.0
0.50
0.75
0.00

Ovaries from cross-pollinated florets were only obtained in quantities sufficient for analysis from clones P and PY. Pollen for cross-pollination was obtained from cutting No. 1 of plant clone OB, and was not examined electrophoretically.

a) Clone P. Figure 4 compares densitometer tracings obtained after electrophoresis of buffer-soluble protein subunits of ovaries of unpollinated, self-pollinated, and cross-pollinated florets of plant clone P. A photograph of the gel obtained is included with each tracing and the correspondence between the densitometer peaks and the observable bands is indicated. Note that although the graphs are drawn to the same scale with respect to R_f (the abscissa), the concentration axes (the ordinate) vary slightly between all three tracings.

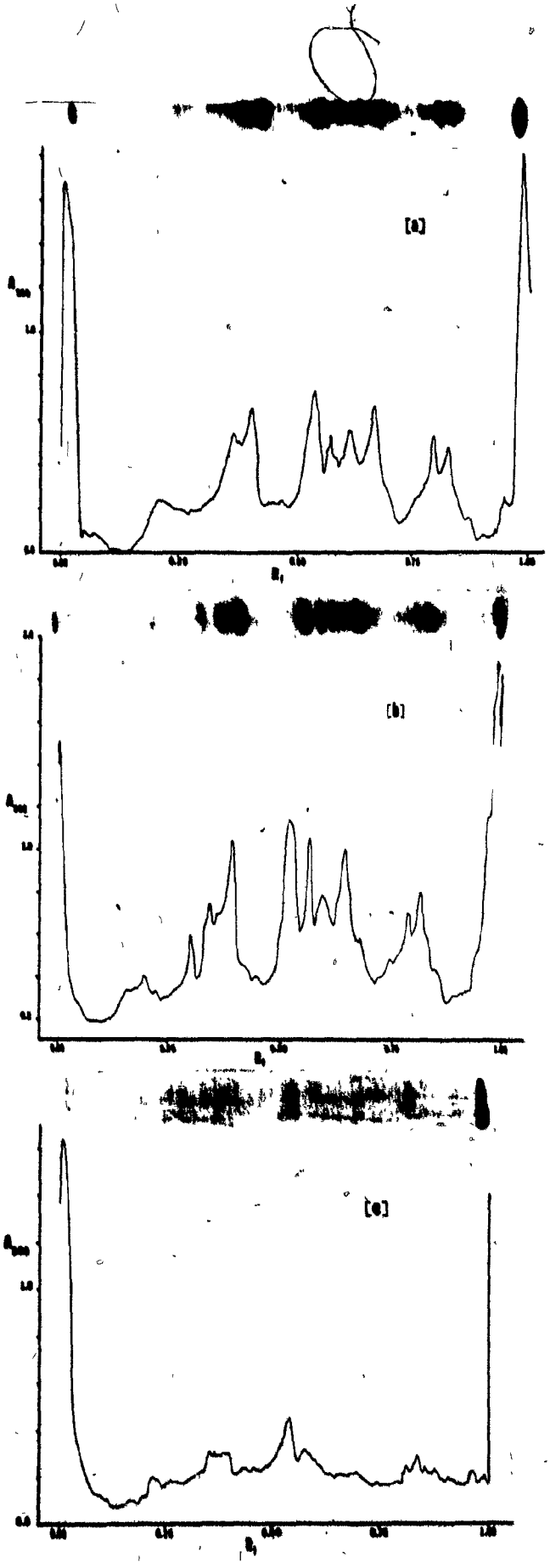
The overall protein content (or possibly, stainability) of the cross-pollinated sample is lower than that of either the unpollinated or self-pollinated samples. The self-pollinated sample has the greatest protein content. The unpollinated and self-pollinated samples have rather similar banding patterns, but an additional major peak (R_f = 0.30) is evident in the self-pollinated sample. Although bands appear in all cases at the gel fronts, at R_f = 0.50 to 0.55, and at the gel origin (except for the cross-pollinated sample), radical differences are evident. Many band position and band number differences exist between the three treatments, and relative and absolute concentrations of the same bands are different.

Figure 4. Densitometer tracings and corresponding gels for clone P.

a. Ovary sample from unpollinated florets.

b. Ovary sample from self-pollinated florets.

c. Ovary sample from cross-pollinated florets.


[a]
[b]
[c]

b) Clone PY. Figure 5 compares densitometer tracings and the corresponding gels of unpollinated, self-pollinated, and cross-pollinated ovary samples for clone PY. Again, the ordinates of the three tracings differ slightly. As in clone P, the self-pollinated sample contains more protein than the unpollinated sample, which contains more protein than the cross-pollinated one. Although the number of peaks appears to differ little between results obtained for unpollinated and self-pollinated ovaries, the relative concentrations have shifted dramatically such that in the gel from self-pollinated ovaries, there are more than the four dominant peaks observed in the gel from the unpollinated ovaries. Replicates of the unpollinated ovary sample (not shown) contained bands in the same positions but which varied in protein subunit concentration. The dominant position of the central peaks of the self-pollinated and unpollinated samples is not observed here. Different peaks are dominant in the three samples, although a central region of high protein concentration is consistent in the three samples. This is also true for the plant clone discussed above.

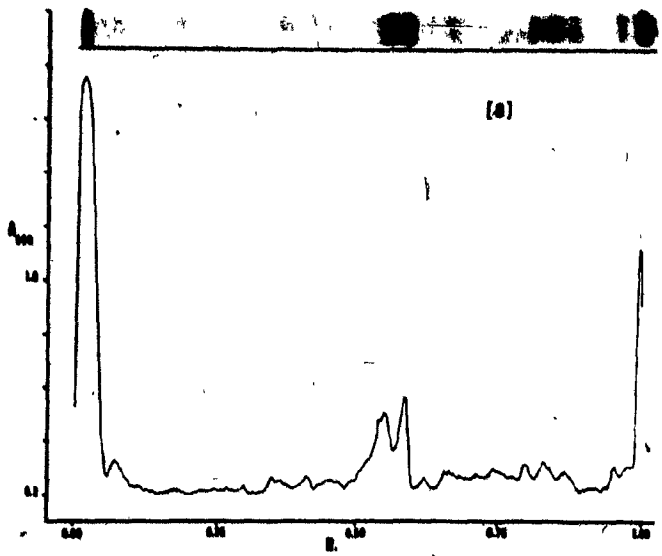
c) Clone PB. Figure 6 compares densitometer tracings and the corresponding gels of unpollinated and self-pollinated ovary samples of plant clone PB. Again, the self-pollinated ovary sample has a larger total amount of protein than the unpollinated sample, and this protein is distributed more evenly over protein subunits of varying molecular weights in the self-pollinated sample as compared to the unpollinated sample. As with clone PY, replicates of the unpollinated ovary sample (not shown) contained bands in the same positions but which varied in protein subunit concentration.

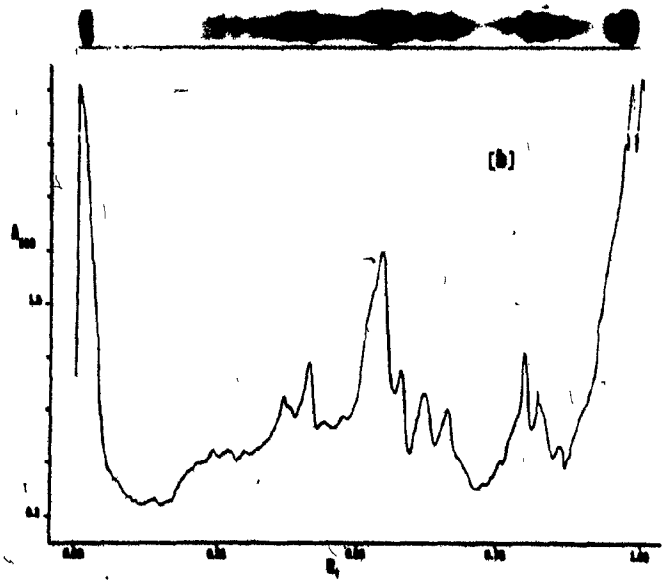
Figure 5. Densitometer tracings and corresponding gels for clone PY.

a. Ovary sample from unpollinated florets.

b. Ovary sample from self-pollinated florets.

c. Ovary sample from cross-pollinated florets.


(a)


(b)

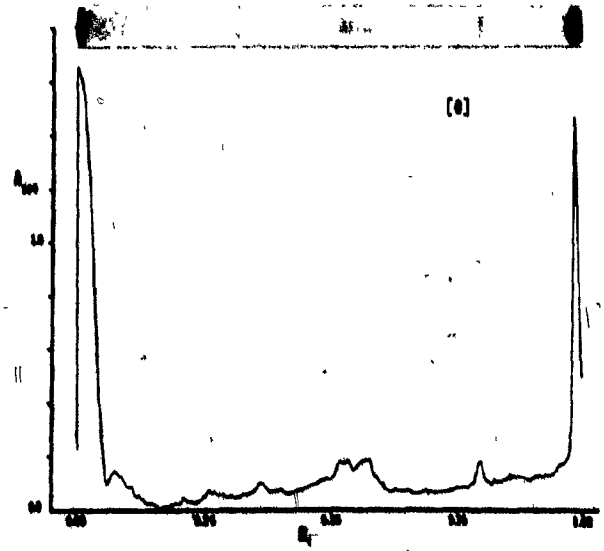
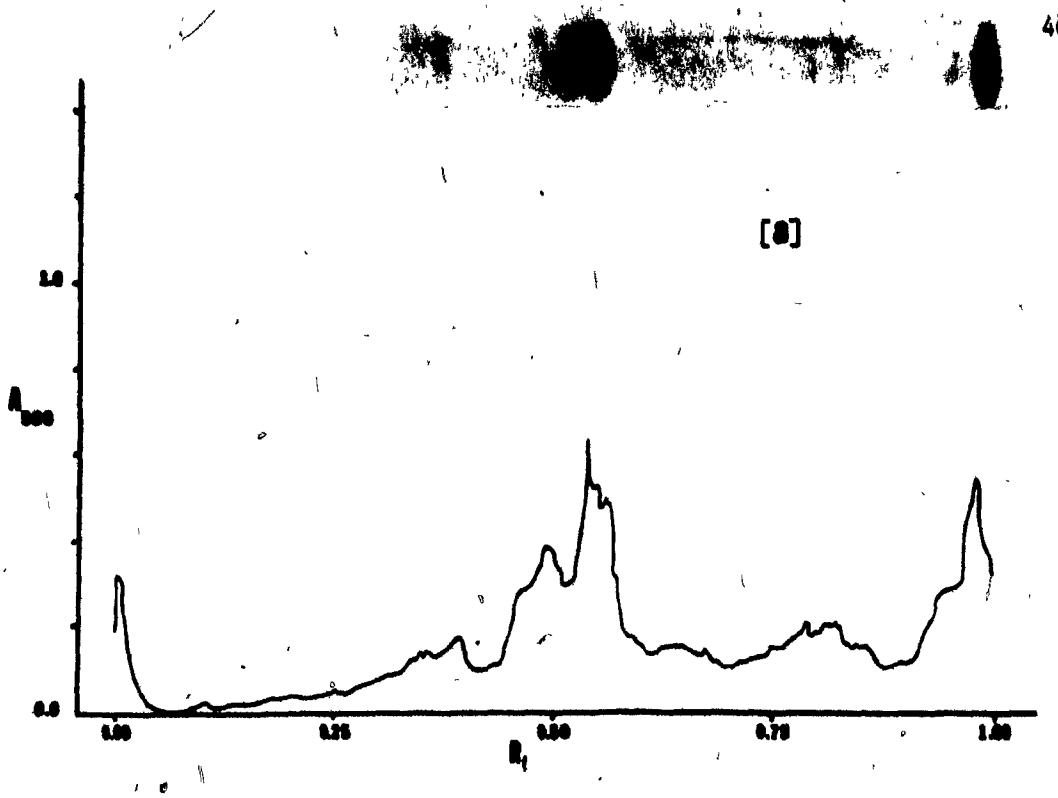
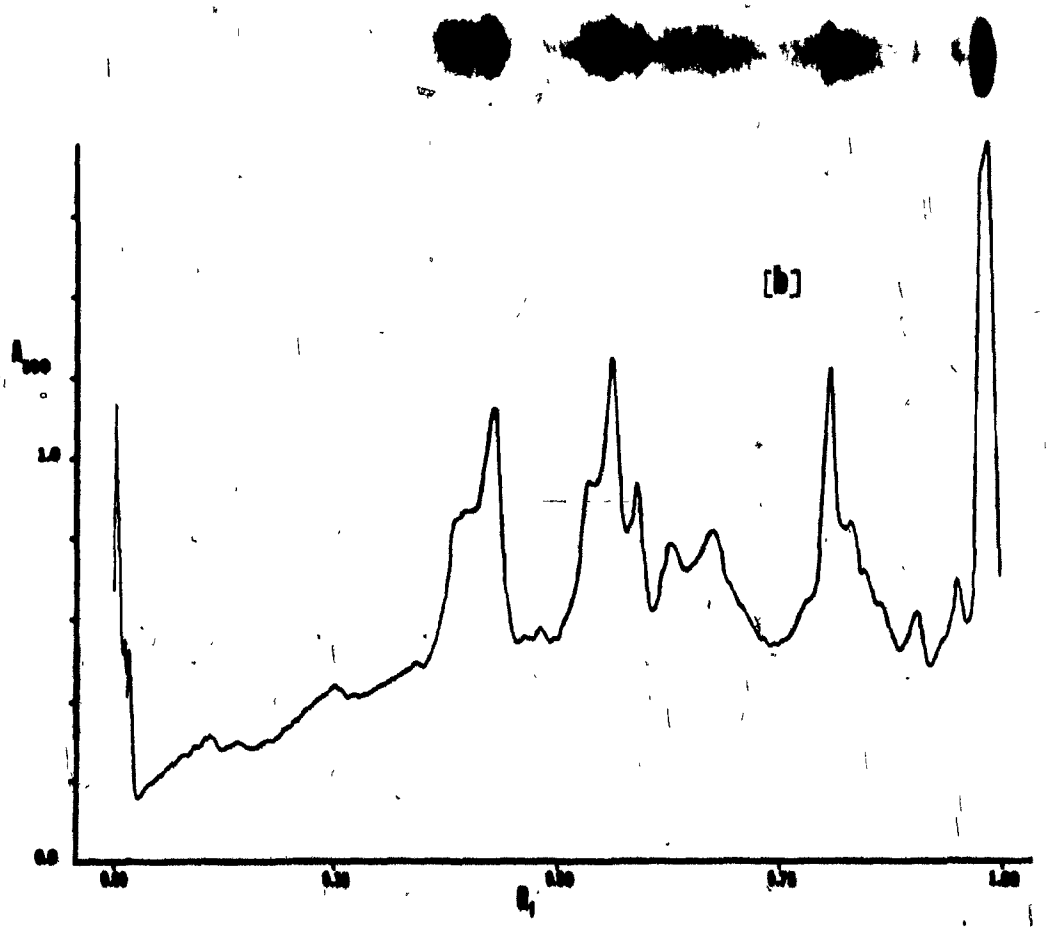

(c)

Figure 6. Densitometer tracings and corresponding gels for clone PB.

a. Ovary sample from unpollinated florets.

b. Ovary sample from self-pollinated florets.


[a]
1.0
0.0
0.00
0.25
0.50
0.75
1.00


[b]
1.0
0.0
0.00
0.25
0.50
0.75
1.00

d) Clone OB. The densitometer tracing and corresponding gel of the unpollinated ovary sample of clone OB are given in Figure 7. This result is much different from the electrophoretic results obtained from unpollinated ovaries in the three plant clones mentioned above, in that there is no dominant region of very high protein concentration. The protein content here is apparently less than that of gels from self-pollinated ovaries from other clones, and greater than that of gels from cross-pollinated ovaries from other clones.

In all gels examined, intensely-stained bands were visible at the extreme ends (except at the origin of the cross-pollinated sample of clone P), indicating that the protein subunits examined extended across the full molecular weight range of the gel system. Gels contained from 20 to 32 bands visible to the naked eye.

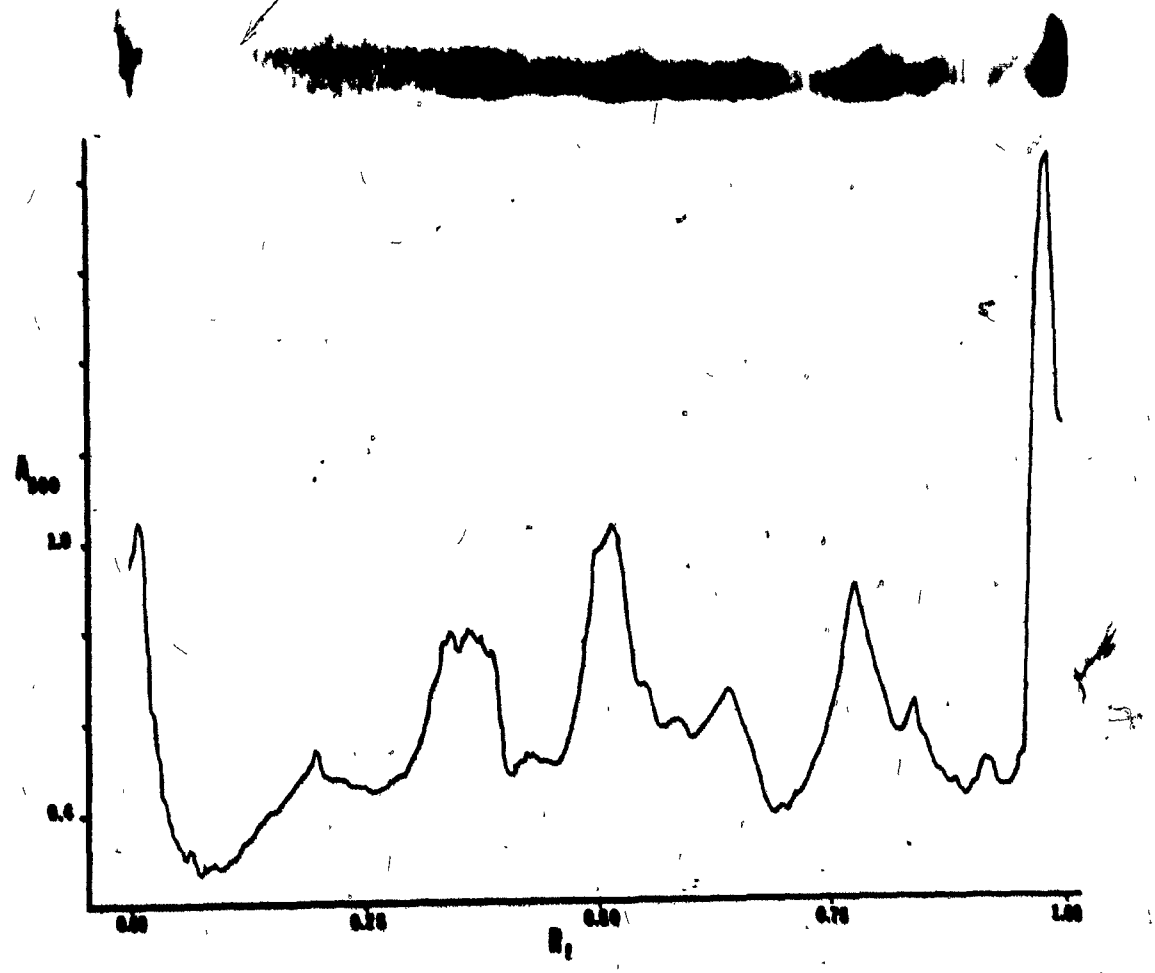
B. Mature Pod Analysis

Mature pods were collected from six clones (P, PY, PB, OB, O and Y). The number of seeds per pod, pod length, and relative position of the seed farthest from the style were compared between pods resulting from self-pollinations and pods resulting from cross-pollinations. Also compared were number of seeds in proximal and distal pod halves, and percent seed germination.

1. Number of Seeds per Pod

One cannot assume that the number of seeds per pod corresponds to a normal distribution, as the values would be discretely rather than continuously distributed. Therefore, the Kolmogorov-Smirinov one-tailed

Figure 7. Densitometer tracing and corresponding gel for clone OB: ovary sample from unpollinated florets.


A_{600}
1.0
0.6
0.00
0.25
0.50
0.75
1.00
R_f

test for unequal, large samples (Siegel, 1956) was applied to compare the distribution of number of seeds per pod in pods resulting from self- and cross-pollinations in clones P and PY, where the sample sizes were large for both types of pollinations. In both instances, number of seeds per pod was found to be significantly different at the 0.0001 level between pods resulting from self-pollinations and pods resulting from cross-pollinations.

The median and the values lying 20% above and below the median are indicated for seed number comparisons in all six clones (Table 2). Also noted are the range of the number of seeds per pod and the number of samples used. In each clone examined, there appears to be great differences in the values between pods resulting from self- and cross-pollinations. In none of the clones do the 20% limits above and below the median overlap. The medians for the self-pollinated samples are almost half, or more than half, those for the cross-pollinated ones. The maximum number of seeds is larger in the cross-pollinated sample than in the self-pollinated sample in all clones examined. Note, however, that there exists considerable variation in the distribution of number of seeds per pod between the six plant clones.

The largest seed set resulting from a cross-pollination was 39 seeds out of 42 ovules, and the largest seed set resulting from a self-pollination was 18 seeds out of 38 ovules. No pods examined contained more than 60 ovules (over 700 pods were examined), and most contained approximately 40 ovules.

Table 2. Comparisons of number of seeds per pod after cross- and self-pollinations in all six clones. Median values are given, plus one quintile above and below the median. Range and sample size are indicated.

	Self-pollination				Cross-pollination			
Clone	Median	±20%	Range	Sample size	Median	±20%	Range	Sample size
P	3	3-5	2-8	24	11	9-14	2-26	50
PY	6	4-7	1-14	94	11	8-16	2-25	58
OB	4	3-6	1-11	45	13	9-13	1-19	7
O	8	6-9	1-18	124	17	12-17	7-28	11
Y	4	3-4	1-7	14	7	5-7	1-11	8
PB	6	5-8	3-10	9	20	17-35	6-39	11

2. Pod Lengths

The Student's t-test (Steel and Torrie, 1960) was used to compare the lengths of pods from self- and cross-pollinations. For both clones P and PY, the difference was significant at the 0.0001 level. This was true whether variances were assumed to be equal or unequal. The F-test was performed on the same material. For clone P, the variances between the two samples were found to be significantly different at the 0.03 level, and for clone PY the variances between the two samples were found to differ significantly at the 0.005 level.

The Kolmogorov-Smirinov test was applied to analyze pod length. To convert the data to correspond to a discrete distribution, the range of pod lengths was divided into 5 mm increments. For both clones P and PY, the difference in pod length between pods resulting from self- and cross-pollinations was significant at the 0.0001 levels.

Table 3 contains the mean pod length and corresponding standard deviation for pods resulting from self- and cross-pollinations from each of the six clones. The number of pods in each sample is indicated. Although there is a wide variation between the clones, a longer average pod length is observed for pods resulting from cross-pollinations.

3. Relative Position of the Seed Farthest from the Style

Giles (1949) suggested that one cause for a reduced seed yield for self-pollinated ovaries may be that the pollen tubes from self-pollen tend not to reach the end of the ovary, whereas pollen tubes from cross-

Table 3. Comparisons of pod lengths after cross- and self-pollination in all six clones. Mean, standard deviation, and sample size are indicated. Mean and standard deviation are in mm.

	Self-pollination			Cross-pollination		
Clone	Mean	Standard deviation	Sample size	Mean	Standard deviation	Sample size
P	18.12	3.1	24	25.9	4.63	50
PY	17.4	3.53	95	22.7	4.87	58
OB	17.3	4.06	45	20.0	6.35	7
O	22.0	7.05	109	27.5	7.03	11
PB	19.1	2.85	11	28.1	5.94	8
Y	13.7	2.57	14	19.4	4.14	8

pollen reach the distal end in much greater numbers. If this were a cause for reduced seed set after self-pollination, one would expect that the seed most distal from the style would be farther from the distal end of the pod after self-pollination than after cross-pollination. This distance, expressed as a percent of the total pod length, was compared between pods resulting from self- and cross-pollinations.

The Student's t-test was used to compare the relative position of the most distal seed in pods from self- and cross-pollinations. Assuming equal variances, the difference was found to be significant at the 0.0001 level for clone P and at the 0.006 level for clone PY. Assuming unequal variances, the difference was found to be significant at the 0.001 level for clone P and at the 0.0018 level for clone PY. The F-test was performed on the same material to test the null hypothesis that the variances of the two samples for each clone were equal. For clone P, the null hypothesis was rejected at the 0.01 level, and for clone PY the null hypothesis was rejected at the 0.0001 level. Thus in both clones, the variances of the self-pollinated and cross-pollinated samples were significantly different.

In Table 4 the mean and standard deviation of the relative position of the most distal seed, expressed as a percent, are indicated for the six clones. Sample size is indicated. Except for clones OB and Y, it appears that more space remains at the distal end of the pod after self-pollination than after cross-pollination. This is most obvious in clones P and PY where the distal seed location values for pods resulting from self-pollination are almost double those resulting after cross-pollination.

Table 4. Comparisons of the distance of the most distal seed, to the distal end of the pod (relative to the style), expressed as a percent of the total length of the pod, after cross- and self-pollination in all six clones. Mean, standard deviation, and sample size are indicated. Measurements in mm.

	Self-pollination			Cross-pollination		
Clone	Mean	Standard deviation	Sample size	Mean	Standard deviation	Sample size
P	22.8	15.5	24	10.2	10.0	46
PY	12.6	13.6	88	6.88	7.71	52
OB	19.6	16.3	44	23.3	11.0	7
O	12.1	12.6	108	10.6	10.0	10
PB	12.0	11.4	10	8.71	13.0	8
Y	17.5	17.1	14	18.6	13.7	6

Although the distal seed-location values follow a continuous distribution, the Kolmogorov-Smirinov test was applied to clones P and PY, by grouping percents (such that class one contained values from 0 to 4.99%, class two contained values from 5 to 9.99%, etc.). No significant difference was found between self-pollinated and cross-pollinated samples in either clone.

4. Number of Seeds in Proximal and Distal Pod Halves

No significant difference was observed in pods from either cross- or self-pollinations, for the number of seeds in proximal and distal pod halves. The numbers tended to be equal in the pods examined.

V. DISCUSSION

Biochemical aspects of self-incompatibility in Lotus corniculatus L. were investigated. Ovary protein subunit banding patterns on polyacrylamide gels were compared between unpollinated, self-pollinated, and cross-pollinated florets. Various parameters of the mature pod were measured (1) to insure that self-incompatibility was occurring to a greater extent than cross-incompatibility (which can occur), and (2) to gain insight into the behaviour of the pollen tube after self- and cross-pollinations.

A. Electrophoretic Studies

1. Preliminary Analysis

Polyacrylamide gel electrophoresis is a powerful analytical tool for the study of proteins. It requires only a small quantity of protein (about 5 µl per band; Maizel, 1971) and gives greater resolution than the ultracentrifuge or other electrophoretic media, such as paper, cellulose acetate, or starch gels (Derbyshire et al., 1976). Polyacrylamide, which is a simple organic substance of non-biological origin, is less likely to be contaminated with biological impurities (Maizel, 1971).

The buffer-soluble protein content of Lotus corniculatus ovaries at anthesis was assayed to determine the number of ovaries required to insure the appearance of bands using polyacrylamide gel electrophoresis. The dry weight of the ovaries was 25% of the fresh weight. Phosphate buffer-soluble proteins with subunit molecular weights greater than 15,000 comprised approximately 0.1% of the total dry weight of unpollinated ovaries. The protein content value is a rough estimate, as the concentrating device used is meant primarily as a qualitative tool.

Most of the literature to date reports on legume protein content in leaves (3 to 5% protein; Derbyshire et al., 1976) or in mature seed from large-seed horticultural crops such as peas and beans (20 to 25% protein; Derbyshire et al., 1976). Little work has been done on the protein content of unfertilized whole pods at anthesis in small-seed forage legumes. Phosphate buffer-soluble protein would comprise a fraction of this value.

The anionic detergent sodium dodecyl sulfate (SDS) denatures and solubilizes proteins into rod-shaped subunits, the lengths of which vary with the molecular weight of the protein moiety for most proteins (Reynolds and Tanford, 1970). Since SDS minimizes native charge differences, a good estimate of molecular weight can be obtained knowing migration distance (Shapiro, Viñuela and Maizel, 1967). Relatively crude extracts may be used, as protein - nucleic acid and protein - lipid interactions are eliminated by SDS treatments as well (Maizel, 1971).

To estimate molecular weight of the sample protein subunits observed, migration distances of protein subunits of known molecular weight were compared with sample migration distances. However, the use of a different solubilising buffer for the standard protein mixtures (0.1 M phosphate buffer at pH 8.0 rather than 0.01 M Tris-HCl buffer containing 0.001 M EDTA (ethylenediamine tetraacetic acid)) resulted in the loss of one band using the low molecular weight mixture of proteins, and the appearance of several anomolous bands using the high molecular weight mixture of proteins. The observed protein subunits were easily distinguished on the basis of concentration differences. In experiments performed prior to those presented

here, standard gels prepared using the gel system of Weber and Osborn (1969) resulted in the expected banding patterns. R_f values derived from standard gels used in this experiment still maintained a linear relation between migration distance and the logarithm of the molecular weight.

2. Electrophoresis

Twenty-four to 26 hours after pollination, Lotus corniculatus pollen tubes have reached the ovary and most have not yet entered the ovule micropyles (Giles, 1949). Therefore, any electrophoretic banding pattern differences observed at this time between self- and cross-pollination would be due to self-incompatibility rather than self-sterility in the form of post-fertilization abortion.

Any incompatibility reaction manifested in the ovary at this time may either be due to the influence of pollen tubes in the style, in the ovary, or a combination of both. Ovarian response to pollen tubes that have not yet reached the ovary has been observed in other plants. In Abelmoschus esculentus, dissolution of nucellar cells at the micropylar end of the ovule to form a passage for pollen tube entry occurs when the pollen tubes are still in the style (Chandra and Bhatnagar, 1975). Linskens (1975) observed that shortly after pollination (and subsequent excision of the pistil) in Petunia hybrida L., the electrical potential in the style changes, suggesting that information may be reaching the ovary or ovules before the incompatibility reaction begins. In self-pollinated pistils of the same plant, proteins are transported to the ovary as the pollen tubes approach, even though the pollen tubes never reach the ovary.

a) Differences in Protein Content. Twenty-four hours after self-pollination, self-pollen tubes in the Lotus corniculatus ovary may be growing at a slower rate than cross-pollen tubes, so it may appear surprising that the protein content of the ovary of a self-pollinated flower contains more proteins than that of a cross-pollinated flower, as was true of the gels examined. However, van der Donk (1974a), working with Petunia hybrida, observed that stylar protein synthesis in vitro from polysomes extracted at various times after pollination was at first higher after self- than after cross-pollination. Later, protein synthesis was higher in styles from cross-pollinated flowers, and by the time the cross-pollen tubes had entered the ovary and self pollen tubes were degrading in the style, protein synthesis was higher in styles of self-pollinated flowers. In the above case, flowers were removed immediately after pollination and compared with unpollinated controls removed simultaneously.

Linskens and Typý (1966) observed that the increase in stylar protein content 18 hours after self-pollination in Petunia hybrida corresponded to cessation of pollen-tube growth (cross-pollen tubes reach the ovary after 36 hours). Deurenberg (1976) working with Petunia hybrida ovaries as opposed to styles, observed the greatest amount of protein synthesis after self-pollination as opposed to cross-pollination or nonpollination (control flowers were unpollinated, but were picked and examined at intervals coincident with the pollinated flowers) when the flowers had just been pollinated. By the time pollen tubes were nearer the ovary, the most protein synthesis was observed in cross-pollinated flowers and the least in unpollinated controls.

Linskens and Tupý (1966) noted that incompatible pollen tubes in this species stopped growth after 18 hours. At this point protein synthesis recommenced, protein content increased, and respiration intensity was reduced after incompatible pollinations; but protein content decreased and respiration intensity showed continuous increase after compatible pollinations. Increased respiration demands may cause protein degradation to amino acids that could provide accessory carbon sources. In Lotus corniculatus, differences between compatible and incompatible pollinations manifest in the ovary (Giles, 1949), as opposed to the style. Therefore, if a similar situation occurs we would expect to observe the reaction to the incompatible pollen tubes in the Lotus corniculatus ovary after 24 hours as is observed in Petunia hybrida styles after 18 hours; increase in protein content upon cessation of pollen-tube growth, due to reduced respiration demands.

There exist other possible explanations for the large difference observed in protein content between incompatible and compatible pollinations. Proteins that may be the most important in enhancing or permitting compatible pollen-tube growth may be synthesized at concentrations too low to be manifested as bands. To effect self-incompatibility, synthesis of large quantities of proteinaceous material may be required to block further pollen-tube growth. Perhaps degradation of the incompatible pollen tube material demands synthesis of large quantities of digestive proteins. An alternate explanation for the apparent reduction of protein content in the ovaries after cross-pollination is that the ovaries have been trauma-

tized by emasculation, such that although the ovules are healthy enough to produce seeds and the pollen tubes are unharmed by the process, the ovarian tissue and other parts of the floret are utilizing any surplus protein for reparations and maintenance. Protein may also be lost through tissue lesions caused by emasculation.

The emasculation operation can cause great damage to a floret. Only clones P and PY produced florets that were consistently capable of surviving emasculation. Umbels of these clones were more frequently subtended by sturdier peduncles, buds were produced at a faster rate, and florets on a single umbel tended to reach anthesis more or less synchronously as compared to those of other clones. Thus a large number of emasculated florets were available for cross-pollination. However, emasculation of florets of clones P and PY for cross-pollination and keel damage to permit self-pollination may have contributed to some differences observed between the three treatments.

b) Differences in Banding Patterns. The banding patterns between clones and for different treatments within clones were extremely divergent. Except for a few consistent features, bands changed greatly in colour intensity, position, and number. Van der Donk (1974b, 1975) compared electrophoretograms containing stylar proteins translated in vitro from RNA extracted from self- and cross-pollinated flowers of Petunia hybrida plucked after pollination, and examined at intervals following pollination. Comparison of self- and cross-pollinations at the same intervals revealed great differences in band number, position, and dye concentration, as was noted above for Lotus corniculatus. He concluded that different protein activity accompanied compatible and incompatible pollen-tube growth.

Some banding pattern disparities between self- and cross-pollinated samples must be attributed to the differences in the background genotype of the types of pollen that were used.

In all cases examined, gels containing protein from self-pollinations revealed a greater number of bands in the densitometer profiles than gels containing protein from unpollinated ovaries which demonstrated a greater number of bands than gels containing protein from cross-pollinated ovaries. This may indicate that self-pollinated ovaries produce a greater variety of protein subunits, or perhaps an overall protein concentration reduction is occurring in the cross-pollinated samples, preventing detection of certain bands. Important proteins, such as regulatory enzymes, may occur in insufficient quantities for detection by these methods.

The same subunits on different gels run at the same time can have different R_f factors under certain circumstances. Impurities in the protein sample can affect migration rates, in spite of the presence of SDS. Unstained sample gels in this study contained greenish and yellowish bands that were possibly caused by plant pigments. These disappeared after the gels were stained. According to Lewis et al. (1967), R_f shifts may occur due to cofactors which are released upon grinding the fresh tissue. When many similar bands appear close together, it is often difficult to ascertain which correspond to the same subunits on different gels when band shifting is not ruled out.

The binding patterns obtained do not permit a general conclusion concerning the possible molecular weight of proteins associated with the self-incompatibility reaction. Differences observed between clones for the same pollination treatment were large, corresponding to the large degree of difference observed between clones (Table 1). As plants within clones were found to be morphologically similar, gels replicated for unpollinated samples were very similar. The differences between gels of different clones are too great, and the similarities between gels for different clones subject to the same treatment are too few, to permit definite assignation of any band(s) to the self-incompatibility reaction.

Similarities that do occur between different clones for the same treatment, and that tend not to be present for the other treatments, may be explained in several ways. These may indicate the presence of proteins or protein subunits that are involved in the self-incompatibility reaction as substrates, catalysts, or products. They may be proteins that are not functionally related to self-incompatibility, but that are genetically linked to some loci involved in self-incompatibility (such as perhaps is temperature sensitivity, Townsend, 1971). Some of these bands may be for proteins that follow in the wake of the self-incompatibility reaction, as has been suggested for some peroxidase isoenzymes (Bredemeijer and Blaas, 1975).

One should not expect to find one band representing one protein or protein subunit solely responsible for controlling the self-incompatibility reaction in L. corniculatus, since several loci most likely contribute to

the incompatibility system in this species. If the genetic basis of the incompatibility system was more fully understood, it would be possible to compare electrophoretograms of plant clones with the same incompatibility alleles but with different genetic backgrounds.

The plants were all grown under identical rigorously controlled conditions so different banding patterns should not be attributable to temperature, humidity, or soil condition differences. However, since the clones are genetically different, they may be differentially sensitive to the environmental conditions imposed, for example, an optimal temperature for flowering in one clone may be suboptimal for another, thus augmenting the differences observed between clones.

I consider that this study has indicated a biochemical difference between pistils subjected to cross-pollination and those subjected to self-pollination.

B. Mature Pod Analysis

To test whether the plant clones examined were possibly self-incompatible, the number of seeds per pod and pod length were examined in the mature pods. To compare the behaviour of the pollen tube after self- and cross-pollination, the distance of the most distal seed (from the style) to the distal end of the pod, and the number of seeds in the proximal as compared to the distal half of the pod were examined in mature pods after self- and cross-pollination.

Giles (1949) found that cross-pollinated florets produced three times more seed than self-pollinated florets. Bubar (1958) found that while less than 50% of the ovules produced seeds after cross-pollination, only an average of 6.7% of the ovules produced seeds after self-pollination. Brandenburg (1961) observed that significantly higher pod set resulted from cross-pollination as compared to self-pollination, but did not observe differences as great between the two types of pollination as was observed by the above two authors. My results agree with those of Brandenburg, in that cross-pollinated florets produce significantly more seeds than self-pollinated florets for all clones examined, but the median number of seeds produced per pod after cross-pollination was approximately double rather than triple the median number of seeds produced per pod after self-pollination (this is true if means are compared as well, but these are not reported here as the mean is a less appropriate statistic for a discrete distribution).

Although Bubar (1958) indicated that 50% pod set was an upper limit for even the most favourable cross-pollination, the best seed set observed in the study presented here was 92.9% (39 seeds out of 42 ovules, resulting from a cross-pollination). The greatest seed set resulting from a self-pollination was 18 seeds out of 38 ovules (47.4%). Wojciechowska (1963) found 41.2% fertilization after cross-pollination. No pods observed in the six clones examined in this study contained more than 60 ovules (over 700 pods were examined), and most contained approximately 40 ovules. Thus the

median seed set after self-pollination would range from 7.5 to 20%, and for a cross-pollination from 17.5 to 50%, depending on the clone considered. It must be noted that the cultivar Mirabel has only been recently developed. Cultivars used in previous studies may have had different seed sets.

Giles (1949) observed that cross-pollinated florets produced longer pods than self-pollinated florets. This was confirmed by Brandenburg in 1961, who indicated that the average pod length after cross-pollination was 20 mm, and the average pod length after self-pollination was 14 mm. In this study, the average pod length after self-pollination ranged between 13.7 $\pm$ 2.57 mm and 22.0 $\pm$ 7.05 mm, and after cross-pollination ranged between 19.4 $\pm$ 4.14 mm and 28.1 $\pm$ 5.94 mm, depending on the clone examined. These values are somewhat greater than those of Brandenburg (1961) but a difference between the two types of pollination is still apparent.

The form of the Kolmogorov-Smirinov test that was used, was for sample sizes greater than forty. This test was used for comparisons on clone P even though the self-pollinated sample contained only 24 values. In drawing conclusions from the results, one should remain aware of this fact.

The large range in seed number and the great variances observed for pod length emphasizes the need for large sample sizes. It is obvious that the self-incompatibility mechanism is incomplete in this species, in that a considerable amount of self seed is produced.

Plant clones varied as to the degree of their self-incompatibility. Since each clone was only cross-pollinated with one plant of another clone, potential variation in seed number and pod size values for cross-pollinations was reduced. One cannot assume that the plants acting as female parents would produce the same seed yield after cross-pollination if other plant clones are used as pollen parents.

Although emasculation took its toll of floret buds, some emasculated buds in each clone did survive to anthesis, and produced pods when cross-pollinated. However, since self-pollinated florets were not emasculated, the two treatments are not exactly equivalent. Since any damage due to emasculation would more likely reduce fitness than enhance it, it is possible that cross-pollinated florets would produce even more seeds per pod if not subject to emasculation. This would enhance the difference between self- and cross-pollination.

Variation in number of seeds per pod and pod length was greatest between clones and between pods taken from the same clone.

Variation between clones can be partially attributed to a genetical difference between the clones. Many loci contribute to the control of self-incompatibility in L. corniculatus, and a wide range of alleles can be present at each of the loci. Thus several different allelic combinations are possible, and great variability in degrees of self- and cross-incompatibility might be expected to occur in a population. Factors such as seed yield and pod length can also be influenced by background genotype,

for example, plants that contribute more energy to pod and seed development in general should have higher values for seed yield and pod length for both types of pollinations than plants that contribute more energy to foliage production.

Differences in number of seeds per pod and pod length varied more within clones than the median values between clones. Bubar (1958) indicated that in L. corniculatus, ovules within an ovary do not develop synchronously so that the ovary as a whole is receptive to fertilization over a longer period. For this reason, one should not expect an equal number of seeds per pod when the same type of pollination is performed on the same plant.

Environmental factors are known to affect seed set. Ascher and Peloquin (1966), working with Lilium longiflorum Thunb. found that floral age influences pollen-tube growth. The difference between a self- and a cross-pollination was found to be greatest during the interval in which pollination occurred in nature. Sirks (1927) noted that pollination success depended on the age of the pistil, the pollen, and the plant. A common phenomenon to students of self-incompatibility is end-season fertility, where self-pollinations produce seed sets comparable to cross-pollinations when the flowering season is drawing to a close. Where floret production is artificially enhanced for long periods (as was true for this experiment), there is the possibility that end-season fertility is occurring in spite of profuse flowering. The physiological basis for this phenomenon is not yet completely understood. Other factors that have been found to influence seed

set in some plants, such as temperature, relative humidity, and illumination (Kendall, 1973), have been kept constant throughout these experiments. However, due to positional differences between florets, microclimatic differences may have caused some observed variation in the observed seed sets and pod lengths.

Measurement of the gap between the most distal seed with respect to the style and the distal end of the pod revealed a significant difference between pods resulting from cross- and self-pollinations. This difference may indicate that the self-pollen tube tends not to travel as far into the ovary as the cross-pollen tube, or that since fewer seeds are present in pods resulting from self-pollination, the distance between seeds are, on average, greater, in spite of the evidence that pods resulting from self-pollinations tend to be shorter than pods resulting from cross-pollinations.

To distinguish between these two possibilities, the number of seeds in the distal and proximal halves of pods was compared for pods resulting from self- and cross-pollinations. No significant difference between seed content of the two pod halves was revealed. This suggests that the difference in the distance from the most distal seed from the distal end of the pod, between self- and cross-pollinations, is indicative of an overall reduced seed set rather than a shorter ultimate pollen-tube length after an incompatible pollination. It is equally likely that incompatible pollen tubes grow as far into the ovary as compatible tubes, but fail to fertilise ovules as efficiently.

C. Suggestions for Further Research

This study initiated biochemical research on self-incompatibility in Lotus corniculatus. It is recommended that in further studies more plants per clone be used. This would permit a sufficient amount of floret buds to reach anthesis after emasculation, allowing comparison of emasculated florets for all treatments, rather than just for cross-pollinated samples. Use of more clones would permit more exact characterization of the proteins involved in the self-incompatibility or cross-compatibility reactions. A variety of pollen parents should be used with each clone, to distinguish banding pattern differences attributable to the incompatibility reaction from those due simply to the disparate background genotypes of the self- vs. cross-pollen.

Protein synthesis (as opposed to protein content), RNA synthesis, and specific enzyme activities should be assayed in an attempt to account for the increase in protein content and number of bands in electrophoretic analyses after self-pollination, and the diminution after cross-pollination. Once specific bands have been designed as corresponding to the self-incompatibility or cross-compatibility reactions, one should determine if these protein subunits are in reality glycoprotein subunits, as glycoproteins have also been postulated to be involved in the recognition of "self". An immunological (in terms of lectin biochemistry) or histochemical approach to this problem would reveal more about the relationship between the biochemical differences and the morphological differences. Because

a biochemical difference between ovaries from self- and cross-pollinated florets has been demonstrated to occur as early as 24 to 26 hours after pollination in this study, continuing research in this area would appear fruitful.

Before forecasting too freely into the future, one must be aware that lack of sufficient information about the self-incompatibility mechanism in L. corniculatus presents a major handicap to the researcher. The production of various clones with the same or completely different incompatibility alleles cannot be assured. However, a biochemical approach to the problem of self-incompatibility may provide insight into the genetic basis of self-incompatibility in L. corniculatus.

VI. SUMMARY

Lotus corniculatus L., an increasingly important forage legume, produces less seed after self-pollination than after cross-pollination. Some workers consider that both self-sterility and self-incompatibility contribute to the reduced seed set upon self-pollination. In *L. corniculatus* many gene loci control the self-incompatibility reaction, which is assumed to be gametophytic. However, no conclusive results have established the number of loci or the number of alleles per locus involved. Morphological studies have determined that by about 24 hours after pollination under optimal conditions, most pollen tubes have reached the ovary where the self-incompatibility response is thought to occur.

Biochemical experiments with other plants exhibiting the gametophytic mode of self-incompatibility (especially *Lilium longiflorum* Thunb. and *Petunia hybrida* L.) suggest that proteins play a fundamental role in the control of the self-incompatibility reaction. Changes in protein synthesis may even anticipate the arrival of the pollen tubes, for example, ovaries often respond to pollination before the pollen tubes have even penetrated the style.

The biochemical nature of the self-incompatibility response in *L. corniculatus* has not been investigated to date. Since proteins are thought to play a fundamental role in the self-incompatibility reaction in other plants, it was attempted in this thesis to begin biochemical research into self-incompatibility in *L. corniculatus* by comparing the protein subunit electrophoresis banding patterns between unpollinated, self-pollinated and cross-pollinated ovaries.

Lotus corniculatus ovaries were collected at anthesis (unpollinated samples), or 24 to 26 hours after self- or cross-pollination of the florets. All cross-pollinated florets were emasculated prior to anthesis. Under optimal conditions, most pollen tubes will have reached the ovary 24 hours after pollination, but will not have affected fertilization. Thus any protein subunit differences between the two pollinated samples of ovaries may be attributable to the self-incompatibility (or cross-compatibility) reaction rather than to post-fertilization effects. Since 100 unpollinated ovaries at anthesis were found to contain 40 to 60 µg of protein, 250 ovaries were used per sample.

Phosphate buffer-soluble ovary protein extracts were treated with 2-mercaptoethanol, and analyzed using SDS-polyacrylamide gel electrophoresis, to distribute protein subunits along the gel according to molecular weight. Molecular weight standards were run coincident with the sample gels.

Gels from the "self-pollinated sample" contained a higher protein concentration and a larger number of bands than the other sample groups. Gels from the "cross-pollinated sample" contained the lowest protein concentration and the fewest number of bands. This was consistent over all clones examined. Different treatments produced different banding patterns. In replicates of some samples containing protein from unpollinated florets, the band positions did not change appreciably, but the resulting concentrations differed. It was deemed premature to assign particular bands to the self-incompatibility reaction.

The plant clones used for this study exhibited partial self-incompatibility, as demonstrated by reduced seed set and mature pod length. These results agree with reports in the literature on other L. corniculatus cultivars (Giles, 1949; Bubar, 1958; Brandenburg, 1961). From results of seed position studies in the mature pod, it was proposed that no seed location is more or less favoured during fertilization after either self- or cross-pollination, thus biochemical analysis of the entire ovary is recommended.

Suggestions were proposed for further research in this area. Emasculation of all florets used would permit distinguishing whether the profound changes observed after cross-pollination are in part attributable to emasculation. A more complete understanding of the genetic basis for self-incompatibility would permit more clear-cut results, as one could compare plants with the same complement of incompatibility alleles in different background genotypes.

VII. LITERATURE CITED

Ames, L. M. 1934. Hermaphroditism involving self-sterility and cross-fertility in the Ascomycete Pleurage anserina. Mycologia, 26: 392-414.

Arasu, N. T. 1968. Self-incompatibility in Angiosperms: A review. Genetica, 39: 1-24.

Ascher, P. D. 1971. The influence of RNA-synthesis inhibitors on in vivo pollen tube growth and the self-incompatibility reaction in Lilium longiflorum Thunb. Theor. Appl. Genet. 40: 75-78.

Ascher, P. D. and L. W. Drewlow. 1970. The effect of cycloheximide and 6-methylpurine on in vivo compatible and incompatible pollen tube growth in Lilium longiflorum. Theor. Appl. Genet. 40: 173-175.

Ascher, P. D. and S. J. Peloquin. 1966. Effect of floral aging on the growth of compatible and incompatible pollen tubes in Lilium longiflorum. Am. J. Bot. 53: 99-102.

Bateman, A. J. 1952. Self-incompatibility systems in angiosperms. Heredity, 6: 285-310.

Brandenburg, N. G. 1959. Self- and cross-fertility in birdsfoot trefoil, Lotus corniculatus L. Ph.D. Thesis, Univ. Maryland.

Brandenburg, N. G. 1961. Fertility and pod development of self-fertile clones of birdsfoot trefoil with selfing and outcrossing. Crop Sci. 1: 373-374.

Brademeijer, G. M. M. and J. Blaas. 1975. A possible role of a stylar peroxidase gradient in the rejection of incompatible growing pollen tubes. Acta Bot. Neerl. 24: 37-48.

Brewbaker, J. L. 1954. Incompatibility in autotetraploid Trifolium repens L. I. Competition and self-compatibility. Genetica, 39: 307-317.

Brewbaker, J. L. and S. K. Majumder. 1961. Cultural studies of the pollen population effect and the self-incompatibility inhibition. Am. J. Bot. 48: 457-464.

Bubar, J. S. 1957. Genetics and cytotaxonomy in birdsfoot trefoil (Lotus corniculatus L.). Ph.D. Thesis, McGill University, Montreal, Québec.

Bubar, J. S. 1958. An association between variability in ovule development within ovaries and self-incompatibility in Lotus (Leguminosae). Can. J. Bot. 36: 65-72.

Bubar, J. S. 1959. Differences between self-incompatibility and self-sterility. Nature, 183: 411-412.

Burnet, F. M. 1971. "Self-recognition" in colonial marine forms and flowering plants in relation to evolution of immunity. Nature, 232: 230-235.

Castle, W. E. 1896. The early embryology of Ciona intestinalis Flemming (L.). Bull. Mus. Comp. Zool. Harv. Univ. 27: 201-280.

Chandra, S. and S. P. Bhatnagar. 1975. Pollination and nucellus in Abelmoschus esculentus. Biol. Plant. 17: 154-155.

Clarke, A. E., R. B. Knox, and M. A. Jermyn. 1975. Localization of lectins in legume cotyledons. J. Cell Sci. 19: 157-167.

Compton, R. H. 1913. Phenomena and problems of self-sterility. New Phytol. 12: 197-206.

Cope, F. W. 1958. Incompatibility in Theobroma cacao. Nature, 181: 279.

Cope, F. W. 1962. The mechanism of pollen incompatibility in Theobroma cacao. Heredity, 17: 157-182.

Currier, H. B. 1957. Callose substance in plant cells. Am. J. Bot. 44: 478-488.

Currier, W. W. and G. A. Strobel. 1977. Chemotaxis of Rhizobium spp. to a glycoprotein produced by birdsfoot trefoil roots. Science, 196: 434-435.

Darwin, C. R. 1876. The effect of cross- and self-fertilization in the vegetable kingdom. (2nd Ed., 1916 reprint). John Murray, London.

Deurenberg, J. J. M. 1976. Activation of protein synthesis in ovaries from Petunia hybrida after compatible and incompatible pollination. Acta Bot. Neerl. 25: 221-226.

Derbyshire, E., D. J. Wright, and D. Boulter. 1976. Legumin and vicilin, storage proteins of legume seeds. Phytochemistry, 15: 3-24.

Dickenson, H. G. and J. Lawson. 1975. Pollen tube growth in the stigma of Oenothera organensis following compatible and incompatible intra-specific pollinations. Proc. R. Soc. Lond. Ser. B. 188: 327-344.

Drayton, F. L. 1934. The sexual mechanism of Sclerotina gladioli. Mycologia, 26: 47-72.

East, E. M. and A. J. Mangelsdorf. 1925. A new interpretation of the hereditary behaviour of self-sterile plants. Proc. Natl. Acad. Sci. U.S.A. 11: 166-171.

East, E. M, and J. B. Park. 1917. Studies on self-fertility. I. The behavior of self-sterile plants. Genetics 2: 505-509.

Fieldes, M. A., C. L. Deal, and H. Tyson. 1977. Preliminary characterization of peroxidase isozymes isolated from two flax genotrophs. Can. J. Bot. 55: 1465-1473.

Filser, P. 1926. Die Selbsterilität von Veronica syriaca. Z. Indukt. Abstamm. Vererbungsl. 41: 137-197.

Frankel, R. and E. Galun. 1977. Pollination mechanisms, reproduction, and plant breeding. Springer-Verlag, Berlin, Heidelberg, New York.

Gerstel, D. U. 1950. Self-incompatibility studies in guayale. II. Inheritance. Genetics, 35: 482-506.

Giles, W. 1949. The morphological aspects of self-sterility in Lotus corniculatus L. Ph.D. Thesis, Univ. of Missouri.

Hansen, H. W. 1953. Developmental morphology of Lotus corniculatus L. Iowa State Coll. Sci. 27: 563-600.

Henny, R. J. and P. D. Ascher. 1975. Kinetin removes self-incompatibility reaction in detached styles of Lilium longiflorum Thunb. HortScience, 10: 25-26.

Hertzsch, W. 1959. Hornklee, Lotus-Arten. Handbuch der Pflanzenzüchtung, 4: 342-345.

Hogenboom, N. G. 1975. Incompatibility and incongruity: Two different mechanisms for the non-functioning of intimate partner relationships. Proc. R. Soc. Lond. Ser. B. 188: 361-375.

Hughes, M. B. and E. B. Babcock. 1950. Self-incompatibility in Crepis foetida L. subsp. rhoeadifolia (Biel) Schinz et Keller. Genetics, 35: 570-588.

Jermyn, M. A. 1975. Precipitation reactions between components of plant tissue extracts. Aust. J. Plant Physiol. 2: 5335-5542.

Kauss, H. and D. J. Bowles. 1976. Some properties of carbohydrate-binding proteins (lectins) solubilised from cell walls of Phaseolus aureus. Planta, 130: 169-174.

Kauss, H. and C. Glaser. 1974. Carbohydrate-binding proteins from plant cell walls and their possible involvement in extension growth. FEBS Lett. 45: 304-307.

Kendall, W. A. 1973. Simplification of the heat treatment technique to enhance pseudo-self-compatibility in Trifolium pratense L. Crop Sci. 13: 559-561.

Knox, R. B. 1976. Cell recognition and pattern formation in plants. In The developmental biology of plants and animals. C. F. Graham and P. I. Wareing (Eds.). W. B. Saunders, Philadelphia, Toronto, pp. 141-168.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.

Langille, J. E. and J. S. Bubar. 1977. Growing birdsfoot trefoil in the Atlantic Provinces. Publication prepared for the Forage Subcommittee of the Atlantic Field Crops Committee. Publ. No. 130 of the Ministers of Agriculture of New Brunswick, Nova Scotia, Prince Edward Island and Newfoundland.

Lehmann, E. 1926. The heredity of self-sterility in Veronica syriaca. Mem. Hortic. Soc. N. Y. 3: 313-320.

Lewis, D. 1943. Physiology of incompatibility in plants. III. Autopolyploids. J. Genet. 45: 171-185.

Lewis, D. 1952. Serological reactions of pollen incompatibility substances. Proc. R. Soc. Lond. Ser. B. 140: 127-135.

Lewis, D. 1954. Comparative incompatibility in angiosperms and fungi. Adv. Genet. 235-285.

Lewis, D., S. Burrage, and D. Walls. 1967. Immunological reactions of single pollen grains, electrophoresis and enzymology of pollen protein exudates. J. Exp. Bot. 18: 371-378.

Linskens, H. F. 1964. Pollen physiology. Annu. Rev. Plant Physiol. 15: 255-270.

Linskens, H. F. 1974. Translocation phenomena in the Petunia flower after cross- and self-pollination. In Fertilisation in higher plants. H. F. Linskens (Ed.). North-Holland Publ., Amsterdam, pp. 285-292.

Linskens, H. F. 1975. Incompatibility in Petunia. Proc. R. Soc. Lond. Ser. B. 188: 299-311.

Linskens, H. F. and J. Tupý. 1966. The amino acids pool in the style of self-incompatible strains of Petunia after self- and cross-pollination. Genet. Breed. Res. 36: 151-158.

Loosli, J. K., V. N. Krukovsky, G. P. Lofgreen, and R. B. Musgrave. 1950. The comparative value of ladino clover, birdsfoot trefoil, timothy, and alfalfa hays for yield and quality of milk. J. Dairy Sci. 33: 228-236.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Lundqvist, A. 1975. Complex self-incompatibility systems in angiosperms. Proc. R. Soc. Lond. Ser. B. 188: 235-245.

MacDonald, H. A. 1946. Birdsfoot trefoil (Lotus corniculatus L.): Its characteristics and potentialities as a forage legume. Cornell Agric. Exp. Sta. Mem. No. 261, pp. 1-184.

Maizel, J. V., Jr. 1971. Polyacrylamide gel electrophoresis of viral proteins. In Virology 5. K. Maramorosch and H. Koprowski (Eds.). Academic Press, New York, pp. 179-246.

Mäkinen, Y. L. A. and D. Lewis. 1962. Immunological analysis of incompatibility (S) proteins and of cross-reacting material in a self-compatible mutant of Oenothera organensis. Genet. Res. 3: 352-363.

Mascarenhas, J. P. 1975. The biochemistry of angiosperm pollen development. Bot. Rev. 41: 259-314.

Miri, R. K. 1964. Genetics of incompatibility and other segregating characters in Lotus corniculatus L. M.Sc. Thesis, McGill Univ., Montreal, Québec.

Nettancourt, D. de. 1973. Ultrastructural aspects of the self-incompatibility mechanism in Lycopersicon peruvianum. J. Cell Sci. 12: 403-419.

Nettancourt, D. de. 1977. Incompatibility in angiosperms. Springer-Verlag, Berlin, Heidelberg, New York.

Oka, H. I. and H. Morishima. 1967. Variations in the breeding systems of a wild rice, Oryza perennis. Evolution, 21: 249-258.

Owen, F. V. 1942. Inheritance of cross- and self-sterility and self-fertility in Beta vulgaris. J. Agric. Res. 64: 679-698.

Pandey, K. K. 1967. Origin of genetic variability: Combinations of peroxidase isozymes determine multiple allelism of the S gene. Nature, 213: 669-672.

Pandey, K. K. 1973. Heat sensitivity of esterase isozymes in the styles of Lilium and Nicotiana. New Phytol. 72: 839-850.

Paton, J. B. 1921. Pollen and pollen enzymes. Am. J. Bot. 8: 471-501.

Pipkin, J. L., Jr. and D. A. Larson. 1973. Changing patterns of nucleic acids, basic and acidic proteins in generative and vegetative nuclei during pollen germination and pollen tube growth in Hippeastrum belladonna. Exp. Cell Res. 79: 28-42.

Plough, H. H. 1933. Selective fertilisation in Styela. Biol. Bull. 65: 365.

Reynolds, J. A. and C. Tanford. 1970. The gross conformation of protein - sodium dodecyl sulfate complexes. J. Biol. Chem. 245: 5161-5165.

Roggen, H. P. J. R. 1967. Changes in enzyme activities during the programe phase in Petunia hybrida. Acta Bot. Neerl. 16: 1-31.

Sano, Y. 1977. The pollination systems of Melilotus species. Oecol. Plant. 12: 383-394.

Sarfatti, G., F. Ciampolini, E. Facini, and M. Cresti. 1974. Effects of actinomycin D on Lycopersicum peruvianum pollen tube growth and self-incompatibility reaction. In Fertilisation in higher plants. H. F. Linskens (Ed.). North-Holland Publ., Amsterdam, pp. 293-300.

Schaaf, H. M. 1977. Cross-fertility in birdsfoot trefoil, Lotus corniculatus L. Agron. Abst. 1977 Annu. Meet., Los Angeles. p. 70.

Seaney, R. R. 1964. Cross and self seed set in birdsfoot trefoil plants selected for self-fertility. Crop Sci. 4: 440-441.

Seaney, R. R. 1973. Birdsfoot trefoil. In Forages: The science of grassland agriculture. M. E. Heath, D. S. Metcalfe, and R. F. Barnes (Eds.). Iowa State Univ. Press, Ames, Iowa. pp. 177-188.

Seaney, R. R. and P. R. Hensen. 1970. Birdsfoot trefoil. Adv. Agron. 22: 120-157.

Sears, E. R. 1937. Cytological phenomena connected with self-sterility in the flowering plants. Genetics, 22: 130-181.

Shapiro, A. L., E. Vinuela, and J. V. Maizel, Jr. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS - polyacrylamide gels. Biochem. Biophys. Res. Commun. 28: 815-820.

Shivanna, K. R., V. S. Jaiswal, and H. Y. Mohan Ram. 1974. Inhibition of gamete formation by cycloheximide in pollen tubes of Impatiens balsamina. Planta, 117: 173-177.

Siegel, S. 1956. Nonparametric statistics for the behavioral sciences. McGraw-Hill, New York.

Silow, R. A. 1931. Self-fertility of Lotus spp. Welsh Plant Breed. Sta. Bull., Series H, No. 12: 234-240.

Sirks, M. J. 1927. The genotypical problems of self and cross-incompatibility. Mem. Hortic. Soc. N. Y. 3: 325-343.

Spiss, L. 1969. Laboratory methods of determining compatibility in birdsfoot trefoil (Lotus corniculatus L.). Genet. Pol. 10: 114-115.

Spiss, L. and D. J. Paolillo, Jr. 1969. Semi-vitro methods in the study of compatibility in birdsfoot trefoil (Lotus corniculatus L.). Crop Sci. 9: 173-176.

Stanley, R. G. and H. F. Linskens. 1974. Pollen: biology, biochemistry, management. Springer-Verlag, Berlin, Heidelberg, New York.

Stebbins, G. L. 1950. Variation and evolution in plants. Columbia Univ. Press, New York.

Stebbins, G. L. 1958. Longevity, habitat, and release of genetic variability in the higher plants. Cold Spring Harbor Symp. Quant. Biol. 23: 365-378.

Steel, R. D. G. and J. H. Torrie. 1960. Principles and procedures of statistics. McGraw-Hill, New York.

Stout, A. B. 1917. Fertility in Chicorium intybus. The sporadic appearance of self-fertile plants among the progeny of self-sterile plants. Am. J. Bot. 4: 375-395.

Stout, A. B. 1938. The genetics of incompatibilities in homomorphic flowering plants. Bot. Rev. 4: 275-369.

Tammisola, J. and A. Ryynänen. 1970. Incompatibility in Rubus arcticus L. Hereditas, 66: 269-278.

Tome, G. A. and I. J. Johnson. 1945. Self- and cross-fertility relationships in Lotus corniculatus L. and Lotus tenuis Wald. et Kit. J. Am. Soc. Agron. 37: 1011-1023.

Townsend, C. E. 1970. Inheritance of a self-compatibility response to temperature and the segregation of S alleles in diploid alsike clover, Trifolium hybridum L. Crop Sci. 10: 558-563.

Townsend, C. E. 1971. Further studies on the inheritance of a self-compatibility response to temperature and the segregation of S alleles in diploid alsike clover. Crop Sci. 11: 860-863.

Tupý, J., E. Hrabětová, and V. Balatková. 1974. The release of RNA from pollen tubes. In Fertilisation in higher plants. H. F. Linskens (Ed.). North-Holland Publ., Amsterdam. pp. 145-149.

van den Ende, H. 1976. Sexual interaction in plants: The role of specific substances in sexual reproduction. Academic Press, New York.

van der Donk, J. A. W. M. 1974a. Synthesis of RNA and protein as a function of time and type of pollentube-style interaction in Petunia hybrida L. Mol. Gen. Genet. 134: 93-98.

van der Donk, J. A. W. M. 1974b. Gene activity and the incompatibility reaction in Petunia. In Fertilisation in higher plants. H. F. Linskens (Ed.). North-Holland Publ., Amsterdam. pp. 279-283.

van der Donk, J. A. W. M. 1975. Recognition and gene expression during the incompatibility reaction in Petunia hybrida L. Mol. Gen. Genet. 141: 305-316.

Weber, K. and M. Osborn. 1969. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4406-4412.

Williams, W. 1964. Genetical principles and plant breeding. Blackwell, Oxford.

Wojciechowska, B. 1963. Embryological studies in the genus Lotus. Part I. Fertilisation and seed development following open- and self-pollination of Lotus corniculatus L. Genet. Pol. 4: 53-63.

Zandstra, I. I. and W. F. Grant. 1968. The biosystematics of the genus Lotus (Leguminosae) in Canada. I. Cytotaxonomy. Can. J. Bot. 46: 557-583.