

POLLEN-PISTIL INTERACTIONS

FOUR ALDER SPECIES
AFTER SELFING AND CROSSING

A Senior Thesis
under the
College of Forest Resources
Honors Program

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INTRODUCTION

In sexually reproducing plant species, the prevailing breeding system is of enormous importance in determining the patterns of group variation and subsequent race and species formation. The breeding system regulates the limits of the breeding population and the extent to which hybridization occurs. The breeding system in a species is governed by certain reproductive isolating mechanisms, which may be effective at many levels.

One level at which isolation is operative is the interaction of pistil and pollen. In this case, pollen may be preferentially inhibited from germinating or growing in the pistil of a particular type. This phenomenon has long been studied in the widely occurring case of self-incompatibility.

The genetics of self-incompatibility has been thoroughly investigated for many species (reviews: Aarssen, 1968; Crowe, 1964; Linskens, 1965). The incompatibility reaction is controlled by a multi-allelic (S_1 - S_n) system composed of one to two loci. Pollen tubes having a particular S-allele grow slowly (or do not germinate) in styles that carry the same allele, and grow rapidly in those that do not (East and Mangelsdorf, 1925; Lewis, 1954, 1955, 1965; Pandey, 1967; Linskens, 1968). Two major types of incompatibility have been documented for homomorphic¹ systems -- gametophytic and sporophytic (Brewbaker, 1957; Pandey, 1960). In the gametophytic system, the pollen behavior is determined by the S-allele in each pollen grain; the inhibition occurring at the level of the pollen tube. By contrast, in the sporophytic system, the inhibition is conditioned by the maternal genotype (S-allele in the sporophyte of the pollen donor) and inhibition occurs

1. Homomorphic systems are characterized by non-varying floral morphology in contrast to heteromorphic systems, wherein incompatibility is correlated with certain differences in floral morphology.

at the stigmatic surface. Gametophytic incompatibility has been associated with binucleate pollen grains, simultaneous pmc division, and time of S-allele action after cytokinesis (Pandey, 1960; Crowe, 1964), whereas sporophytic incompatibility is associated with trinucleate pollen grains, successive pmc division, and time of S-allele action after Anaphase II. The gametophytic system is the most widespread, having been recorded in more than sixty Angiosperm families (Crowe, 1964), while the sporophytic system occurs in lower frequency and appears to be a derived system.

Evolutionarily, the negative effects of inbreeding exert positive selective pressures for genes that inhibit self-fertilization. Consequently, it is not surprising that self-incompatibility of one type or the other has arisen independently in many families of flowering plants.

Pistil-pollen barriers also exist in outcrossing conditions. Although this phenomenon has been widely observed, the genetics and physiology of it are poorly understood. In many instances, authors have suggested that the mechanisms that control self-incompatibility are similar or identical to the interspecific crossing barriers at this level. Indeed, many refer to all types of pistil-pollen interactions as incompatible or compatible.

Only recently has attention been drawn to the fact that interspecific or inter-populational barriers may operate quite differently from the mechanisms of self-incompatibility. Hogenboom (1975) presented comparative models for incompatibility and what he has termed incongruity. The former refers to a specific, positively selected genic system such as the S-loci system of self-incompatibility. Within such a system, matching genic components in pistil and pollen have resulted from co-evolution. Pistil and pollen operate in a one-to-one relationship which is rendered non-functional only if both of the components (pistil and pollen) are identical.

In contrast incongruity, according to Hogenboom, results in a non-functional pistil-pollen relationship due to an incompleteness in the matching of the genic

systems of the two components. This occurs most frequently in interspecific crosses, although it may also operate in limiting inter-population crosses. When two genotypes are incongruent, one of the partners lacks sufficient genetic information about some relevant character in the other and the process is inhibited. Whereas incompatibility may have been actively selected as the evolutionary solution to inbreeding, incongruity is, according to Hogenboom, a by-product of evolutionary divergence. Hence, although the two systems both affect the functioning of the pistil-pollen relationship, their execution is widely disparate and should be considered separately.

Although incompatibility and the phenomenon of incongruity have been studied mostly in herbaceous flowering plants, forest-tree breeders and geneticists have become increasingly interested in the breeding systems of forest trees. Since the success of plant improvement by breeding depends on the constraints and the flexibility of the prevailing breeding system, no attempt at breeding should be made without some knowledge of the reproductive system in the plant under consideration.

Self-incompatibility has been observed in many angiospermous tree species (reviewed by Hagman, 1975), whereas the gymnosperm trees studied have displayed low fertility after selfing as the result of inviability of the embryo (Stebbins, 1958; Hagman, 1975). Cases of incongruity in forest trees have been observed (review Hagman, 1975), but these have been investigated mostly at the level of seed set and not at the level of pistil-pollen interaction.

The purpose of this study was to investigate self-incompatibility and incongruity in four species of *Alnus*. Due to its recently acknowledged qualities of nitrogen fixation, fast growth, and value as a pulpwood, *Alnus* has ascended from a relative position of neglect to one of higher priority for forest tree breeders and managers. Although research is underway on the biology and utilization of *Alnus*, relatively little is known as yet about its reproductive behavior. The remainder of this introduction will review the relevant literature on *Alnus*,

and outline the research objectives of this study.

A. Literature Review

From a breeding standpoint, the taxonomy of a genus often offers indications about the crossability of the species. Murai (1964) has extensively studied the world taxonomy and phylogeny of the genus *Alnus*. He divided the genus into two subgenera and seven sections, as indicated in Figure 1.

Figure 1. Taxonomy of the Genus *Alnus* according to Murai (1964)

GENUS <i>ALNUS</i>	29 species
SUBGENUS <i>ALNASTER</i>	5 species
SECTION <i>BIFURCATUS</i>	3 species
SECTION <i>ALNOBETULA</i>	2 species
SUBGENUS <i>GYMNOTHYRSUS</i>	24 species
SECTION <i>CREMASTOGYNE</i>	2 species
SECTION <i>CLETHROPSIS</i>	3 species
SECTION <i>JAPONICAE</i>	6 species
SECTION <i>FAURIAE</i>	2 species
SECTION <i>GLUTINOSAE</i>	11 species

Murai based his treatment of the genus on morphological comparison of structures of the aments. More recently Furlow (1974) made an extensive investigation into the taxonomy of the American species of *Alnus*. He utilized techniques from numerical taxonomy and studied many morphological, physiological, biochemical, and ecological characteristics. His studies indicate some major differences from Murai in grouping of the taxa. Because he utilized such a wide variety of techniques and observed many characters, his treatment is probably more thorough than Murai. However, until a further documentation of the world species of *Alnus* is presented, I have chosen to use Murai's system.

A survey of the natural and artificial hybrids formed indicates the types of potential intrageneric crosses, and the degree to which taxonomic divisions demonstrate genetic and morphological distance. Figure 2 lists the known interspecific hybrids in *Alnus* compiled from Murai (1964). From this evidence, ten intra-sectional crosses have been documented in the section *Glutinosa*, one in *Japonica* and two in *Bifurcata*. Inter-sectional crosses have been most successfully recorded between *Glutinosa* and *Japonica*, whereas only one case of an

Figure 2. Interspecific Hybrids of *Alnus*, after Murai (1964).

Hybrid Name	Hybrid Origin	Taxonomic Level of Hybrid
X A. Hanedae Sugimoto	A. firma X sieboldiana	Bifurcatus X Bifurcatus
X A. peculiaris Hiyama	A. firma X pendula	Bifurcatus X Bifurcatus
X A. spaethii Callier	A. japonica X subcordata	Japonicae X Japonicae
X A. hybrida Braun	A. glutinosa X incana	
	A. incana X glutinosa	
X A. Svaleovens m.	A. glutinosa X hirsuta, hirsuta	Glutinosae X Glutinosae
var. japonica m.	A. glutinosa X hirsuta, tinctoria	Glutinosae X Glutinosae
X A. takisawaensis	A. glutinosa X inokumae	Glutinosae X Glutinosae
	A. inokumae X glutinosa	Glutinosae X Glutinosae
X A. Ljungeri m.	A. glutinosa X rubra	
	A. rubra X glutinosa	Glutinosae X Glutinosae
X A. silesiaca Fick	A. glutinosa X rugosa	
	A. rugosa X glutinosa	Glutinosae X Glutinosae
X A. Aschersoniana Callier	A. rugosa X incana	
	A. incana X rugosa	Glutinosae X Glutinosae
X A. (no name)	A. incana X hirsuta, hirsuta	Glutinosae X Glutinosae
X A. Purpusi Callier	A. rugosa X tenuifolia	Glutinosae X Glutinosae
X A. Fosoi Mizushima	A. pendula X crispa ssp Maximowiczii	Glutinosae X Glutinosae
X A. (no name)	A. rubra X japonica	Bifurcatus X Alnobetula
X A. moriokaensis m.	A. sieboldiana X hirsuta, tinctoria	Glutinosae X Japonicae
X A. Mayrii Callier		Bifurcatus X Glutinosae
var. Mayrii Callier	A. hirsuta, hirsuta X japonica arguta	Glutinosae X Japonicae
var. glabrescens Nakai	A. hirsuta, tinctoria X japonica, japonica	Glutinosae X Japonicae
var. takisawaensis	A. japonica, japonica X hirsuta, hirsuta	Japonicae X Glutinosae
	A. inokumae X Mayrii, glabrescens	Glutinosae X Japonicae
X A. (no name)	A. inokumae X japonica, japonica	Glutinosae X Japonicae
X A. (no name)	A. cordata X glutinosa	
X a. elliptica Requier	A. glutinosa X cordata	Japonicae X Glutinosae
	A. glutinosa X japonica, japonica	
X A. tabuchii m.	A. japonica, japonica X glutinosa	Glutinosae X Japonicae

Figure 2. Continued

Hybrid Name	Hybrid Origin	Taxonomic Level of Hybrid
X A. Koehnei Callier	A. incana X subcordata	Glutinosae X Japonicae
X A. vacillans m.	A. subcordata X incana	Glutinosae X Japonicae
X A. spectabilis Callier	A. incana X orientalis	Glutinosae X Japonicae
	A. incana X japonica	Glutinosae X Japonicae
	A. japonica X incana	Glutinosae X Japonicae

SUMMARY OF HYBRIDS

~~♀ parent~~ ♂ parent	Bifurcatus	Japonicae	Glutinosae	Total
Alnobetula	1			1
Bifurcatus	2			2
Japonicae		1	7	8
Glutinosae	1	5	11	17
Total	4	6	18	28

inter-subgeneric cross between *Glutinosae* and *Bifurcatus* is known. Whether crosses within and between the other sections are unsuccessful or have merely not been investigated is unknown.

Many of the hybrids listed in Figure 2 have shown good growth and hybrid vigor (Ljunger, 1959). In addition, several crosses have shown unilateral fertility, notably *A. glutinosa* X *rubra* (Ljunger, 1959; Hagman, 1970) and *A. incana* X *glutinosa* (Gram, Muhle Larsen, Syrach Larsen, Westergaard, 1941; Ljunger, 1959), which are reportedly fertile only in the direction indicated.

Studies on the filled seed yield resulting after self-pollination have also been undertaken for some species of *Alnus*. Johnsson (1951) reported self-sterility in *Alnus*, and Ehrenberg, Gustafsson, Forshell, and Simak (1955) concluded that selfing leads to low seed set in *A. glutinosa* and *A. incana*. To the contrary, Heitmüller (1957) found these two species to be self-fertile.

While a survey on interspecific hybrids and seed set after selfing may give information on taxonomic distance in a genus, it aids little in resolving the level at which reproductive isolating mechanisms are operative. The fact that a particular cross does not yield viable offspring suggests a whole spectrum of isolating mechanisms from incongruity to embryo inviability, acting singly or in concert. To date, relatively little work has been done on the level of pistil-pollen relationship in *Alnus*.

The earliest study to this effect in *Alnus* was undertaken by McVean (1955) in which he attempted to measure pollen-tube growth after selfing *A. glutinosa*. However, he encountered problems in staining for the pollen tubes and eventually resorted to evaluating the success of controlled pollinations strictly on the percentage of embryos formed the following Autumn. In this manner, McVean reported self-sterility in *A. glutinosa*, but could not resolve the exact level of the barrier.

Subsequently, most of the work on pistil-pollen relationships has been done by Hagman (1969, 1970). Hagman observed pollen-tube development and fertility of seed in self-pollination and cross-pollination experiments on *A. incana*, *A. glutinosa*,

and A. incana X glutinosa. His results from selfing these species indicate that germination of pollen is good, but that with occasional exceptions, pollen-tube growth is retarded in the style. He found the length of selfed A. glutinosa tubes to be equal to those of selfed A. incana. Furthermore, he found considerable variation between different years. Pollen tube development after interspecific pollination was intermediate between selfing and intra-specific crosses.

Hagman's analysis of filled seed was in agreement with his results on pollen tube development. He did, however, find that some of his unpollinated bags yielded filled seeds, apparently the result of apomixis. Apomixis has been reported earlier (Woodworth, 1929) and its occurrence may cause unreliable results when studying seed set after control pollinations.

On the basis of these studies, Hagman suggested that the self-incompatibility mechanism in *Alnus* was of the gametophytic type.

Investigation into pollen-tube growth in other genera of the Fagales indicates that they are also controlled by a gametophytic self-incompatibility system. Hagman (1966) documented retardation of selfed pollen tubes in Betula verrucosa and B. pubescens. Also, when the two species were crossed, the inhibition was of the same type. Furthermore, Hagman found that intraspecific crossing barriers existed in *Betula* to a certain extent. Clausen (1966, 1970) studied filled seed set after selfing and interspecific crossing in twelve species of *Betula*. His results indicated that self-incompatibility was predominant in the birches studied, although annual variation was high. Interspecific crosses were mostly incongruent, although crosses involving different ploidy levels (high ploidy female by low ploidy male) often gave increased seed set. He suggested that high temperatures and pollination at later stages of flowering may render ineffective existing barriers to selfing and interspecific crossing. Finally, his results on a certain amount of intraspecific incongruity agree with Hagman.

Self-incompatibility in other Fagales was attributed to inhibition of pollen-tube growth in the case of *Corylus* (Johansson, 1935), *Quercus* (Pjatnitsky, 1947),

and *Castanea* (Jaynes, 1964). Self-incompatibility was also found in *Fagus* (Blinkenberg, 1958) although individual trees were found to be self-fertile.

B. Objectives of Study

The current study involved investigation of the pistil-pollen relationship after 1) selfing, and 2) crossing *A. rubra*, *A. glutinosa*, *A. cordata*, and *A. sinuata*, in an effort to elucidate incompatibility and incongruity mechanisms. It was hoped that the results would shed light on the taxonomic distance and hybridization potential among the four species.

The species studied (Figure 3) were chosen both for their taxonomic position and their economic importance. Three of the four species (*A. rubra*, *A. glutinosa*, *A. cordata*) are in the Subgenus *Gymnothyrsus* and represent two different sections; the fourth species is in the Subgenus *Alnaster*. With this distribution, it was possible to assess incongruity at the intra-, intersectional, and inter-subgeneric levels.

Figure 3. Taxonomy of the Four Species Studied

SUBGENUS ALNASTER
SECTION ALNOBETULA
A. sinuata
SUBGENUS GYMNOTHYRSUS
SECTION JAPONICAE
A. cordata
SECTION GLUTINOSAE
A. glutinosa
A. rubra

The major emphasis in this study was put on *A. rubra*. Due to many commercially attractive facets of its biology and utilization potential, basic research on *A. rubra* is currently in demand in the Pacific Northwest. In order to facilitate breeding efforts, information on its reproductive behavior and crossability with other commercial alders is needed. *A. glutinosa* is an important forest tree in Northern Europe. As such, it is a likely candidate for any hybridization trials with *A. rubra*. Furthermore, information is available as to its commercial use.

A. cordata is a medium-sized tree of the Mediterranean region with possible potential for breeding. Only A. sinuata is a shrub species which grows in disturbed sites at high elevations in the Pacific Northwest. It was chosen as representative of the subgenus Alnaster.

A further criterion for selecting these species was their time of flowering. Whereas some of the Asiatic alders flower in early winter, the four study species flower in late winter and early spring. This allowed for short term pollen storage with higher likelihood of maintaining pollen viability.

A subordinate objective of this study was to record major phenological events in the reproductive cycle of Alnus, and to provide basic descriptive information which may be helpful in future breeding or genetic research. In addition, I have documented the occurrence of bisexual strobili in three of the four species. This information is presented in two appendices.

MATERIALS AND METHODS

A. Study Trees

Figure 4 tabulates the individual trees used in this study. All trees are growing in the University of Washington Arboretum. Those which are numbered have been used in previous *Alnus* studies. For purposes of this study, FG 36 is referred to as *A. rubra-s* (location near slough), and FG 35 is referred to as *A. rubra-b* (location near Broadmoor).

Figure 4. Location of Individual Study Trees

<u>Species</u>	<u>FG No.</u>	<u>Arboretum Map Location</u>
<u>A. rubra-s</u>	36	E-6; along slough
<u>A. rubra-b</u>	35	F-5; near lagoon & Broadmoor entrance
<u>A. glutinosa</u>	32	N-7; northernmost of the two <u>A. glutinosa</u> trees
<u>A. cordata-1</u>		N-7; NE from FG 32, middle tree of 3 <u>A. cordata</u> trees
<u>A. cordata-2</u>		N-7; southernmost of the three <u>A. cordata</u> trees
<u>A. sinuata</u>		F-5; alongside Foster's Island Road

B. Pollen Collection and Handling

Pollen was obtained in the lab by "forcing" branches carrying staminate catkins. Four-foot branches were collected in late winter after the staminate catkins had started to elongate and the bracts had separated, but before the anthers had dehisced. The branches were placed in large cans of water in the lab, or in the cooler, lit stairwells until anthers dehisced. The location of the branches during forcing did not seem to affect pollen shedding except that higher temperatures hastened catkin maturation. If the branches were collected immediately prior to the natural pollination time, anther dehiscence occurred very rapidly -- in one day or less. However, I found that branches could be forced as much as one month

ahead of natural anthesis without apparent decrease in pollen viability. If such branches were forced, the length of time to anther dehiscence was much longer (7-10 days) than when nearly mature catkins were collected. Furthermore, due to the shrivelling of aments, the amount of pollen produced from these branches was considerably smaller than from the branches with nearly ripe catkins.

Once the anthers began to dehisce, the catkins were cut off from the branches into small cardboard boxes. These catkins were allowed to dry at room temperature for one day before being sifted over four layers of cheesecloth into clean cardboard boxes. Only a shallow layer (3-5mm) of pollen was collected per box to facilitate even drying. This sifted pollen was air dried for an additional two days before being stored in rubber-capped 6cc vials at 4°C. *Alnus* pollen stored in this manner remained viable for at least one month, but when used a year later, had almost completely lost viability.

Relative pollen viability was estimated for *A. rubra* and *A. cordata* by using outcrossed pollinations (eg., *A. rubra*-s X *rubra*-b) as standards. No pollen viability stains or germination tests were run due to their lack of reliability.

Before using pollen for pollinations, it was allowed to stand at room temperature for one to two hours. All uncapped pollen was discarded if not used.

C. Pollinations

All trees were bagged in early February (Figure 5) prior to any external signs of maturation in male or female inflorescences. In all cases, synthetic sausage casing (Teepak Co., Chicago) was cut into sections 12" to 20" long, folded over twice at one end, and stapled in a double row. These bags were placed over branches carrying female flowers from which the staminate catkins had been removed. The bags are stiff enough without wire enforcement so as not to crush the developing inflorescences. They were secured tightly at the base around a piece of non-absorbent cotton and fastened with a wire tie (Figure 6).

Bags were checked every week until development was visibly underway. Then



Figure 5.

Bagging of A. cordata in early February. University of Washington Arboretum.



Figure 6. Synthetic sausage casing bags were secured tightly at the bases around a piece of non-absorbent cotton and fastened with a wire tie.

bags were checked every one to two days until the female strobili appeared receptive. Receptivity may be judged roughly by anthesis in the same tree, although protandry occurs to varying extents in the different species. A better indication of receptivity is the protrusion of succulent pistils beyond the bracts of the strobili (Figure 7). In A. cordata and A. sinuata, this may be as much as 3mm, whereas in A. rubra, pistils extend approximately 2mm, and in A. glutinosa, they extend even less. The period of receptivity appears to last four to five days, after which the pistils wither and turn dark.

A word should be said here about floral morphology in *Alnus*. Figure 8 and Appendix I illustrate and describe the pistils of *Alnus*. The ovary is located at the base of the pistil with two appendages extending from one side. Each of these appendages is an effective stylar /stigmatic unit, the style on the interior of the appendages, the stigma on the surface. For the purposes of this study, the term "pistil" will be used to indicate what is actually half of the complete pistil. This convention has been adopted because the half-pistil units usually disjoin from one another and appear to act as independent, functional units.

Furthermore, although technically the male and female inflorescences in *Alnus* are catkins or aments, I refer for clarity to the staminate inflorescences as catkins or aments, and the female inflorescences as strobili.

When bagged flowers were judged to be receptive (generally a few days before unbagged flowers), pollinations were made with the aid of a syringe using air as a carrier. The injector holes in the bags were then covered with plastic tape to prevent contamination. The identity of the cross was marked with color-coded chicken rings. The isolation bags were left in place until strobilus sampling was completed.

In one case, I used pollination vials provided by Weyerhauser (Figure 9). These were constructed of screw-capped test tubes into which small paint brushes were inserted. When the pollen was placed in the base of the test tube, the brush could be dipped into the pollen and used to "paint" the female strobili.



Figure 7. Maximum receptivity of *A. cordata* strobilus as indicated by the extrusion of succulent, red pistils.

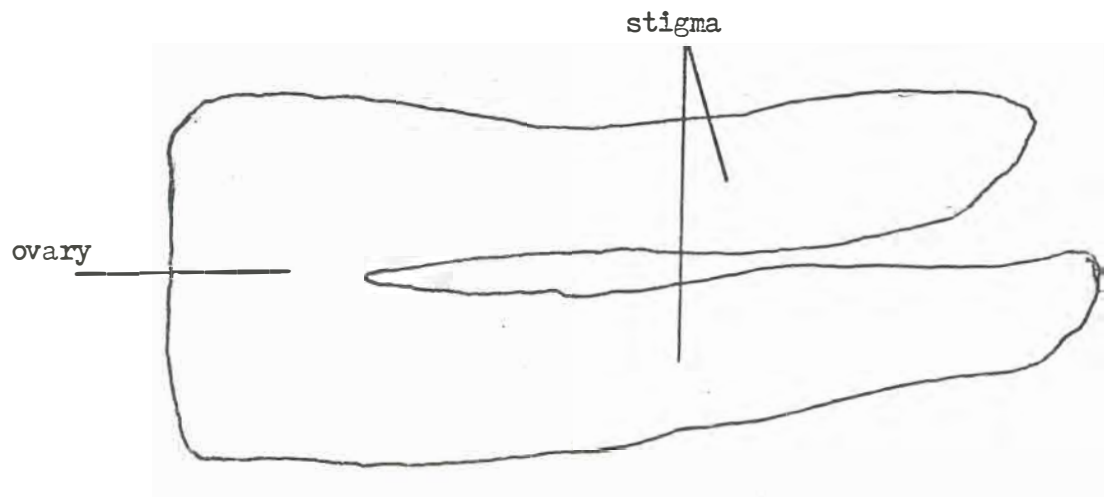


Figure 8. Schematic drawing of an *Alnus* pistil showing double-lobed stigma. X 120.

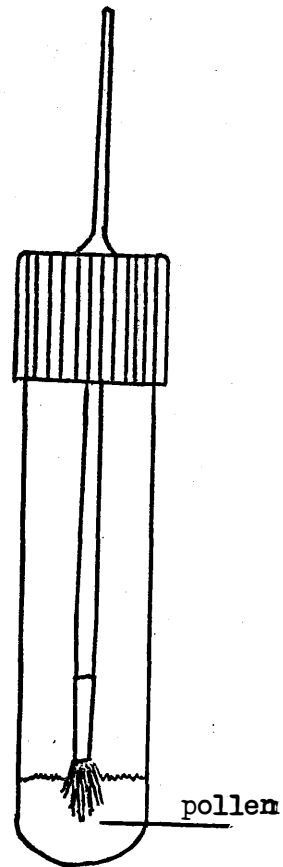


Figure 9. Screw-capped pollination vial designed at Weyerhaeuser.

The advantages of this method are that pollen is placed directly on the stigmatic surface, and that there is no problem with clogged hypodermic syringes. The disadvantages are that with sausage casing bags, it is difficult to apply the pollen-dipped brush without causing large tears in the bags, and that each time the vial is opened, the pollen is subject to ambient moisture and contaminating pollen.

D. Pistil Sampling

In 1976, female strobili were sampled from each control pollination experiment at 4, 12, 24, 36, 48 hours after pollination. Since the pollination and subsequent sampling took approximately the same amount of time, the samples were collected in the same chronological sequence as the pollinations, thus assuring that incubation periods were equal in all cases. In 1977, strobili were sampled only 48 hours after pollination. Strobili were fixed in FAA for at least two days, and stored in 70% etoh at 4°C. Strobili may be stored in this manner indefinitely.

E. Analysis of Pollen Germination and Pollen-Tube Growth

Martin's (1959) aniline-blue technique of fluorescence microscopy was modified for use with the small and opaque pistils of *Alnus*. The following schedule was developed:

Strobili stored in 70% etoh --

1. rinse in several water soaks for at least one hour
2. clear and soften in 8N NaOH for 8-48 hours
3. rinse in several water soaks for two hours
4. dissect pistils from strobili
5. bleach 5 minutes in commercial chlorox (5% NaOCl)
6. rinse in several water soaks for 20 minutes
7. stain pistils in 0.1% water soluble aniline blue in 0.1% K_3PO_4
8. mount pistils in depression slides in stain

Pistils mounted in this manner were observed using fluorescence microscopy. The ultra-violet source was an OSRAM HBO 200 mercury arc lamp in a Zeiss illuminator equipped with a Zeiss UG 5 excitation filter. Observations were made using a

Zeiss Standard GFL microscope equipped with Zeiss 47 and -65 barrier filters.

Pollen grains and tubes fluoresced yellow green against a blue background.

Measurements of pollen-tube length were made using an ocular micrometer at 80X.

At this magnification, 10 micrometer units correspond to 0.15mm.

For each treatment, three strobili were dissected in entirety, and the pistils were allowed to mix in the staining vials. In the first fifty pistils observed, the five longest pollen tubes were measured. Pistils with no tubes were recorded as zero units. Pistils were selected sequentially on the microscope slide. Since there were approximately sixty pistils per slide, most of the slide was examined in the process. There was no attempt made to discriminate among pistils, although any obviously injured or fungally infected pistils were disregarded.

Due to the nature of the aniline blue fluorescence microscopy wherein strobili are subject to repeated washing and handling, many ungerminated pollen grains become detached from the pistils. Hence, it is impossible to obtain reliable estimates of pollen germination. The best alternative appeared to be a count of the number of grains adhering to the pistils. Pollen grains seem to stick to the pistil only if they have some (however slight) indication of a tube, and as such would offer estimates of pollen germination.

The counts of pollen grains were made on the same pistils as the tube measurements. Since only one side of the cylindrical pistils may be viewed, the counts reflect only a portion of the total number of grains per pistil. Since this factor was constant, I used these counts for relative comparison between crosses.

Descriptive observations were also noted at the time of pollen-tube measurement.

F. Statistical Analysis

Three types of statistical tests were made on the pollen-tube length and the number of pollen grain per pistil data. They were: 1) t-tests comparing self pollinations vs. intra-specific pollinations, 2) t-tests comparing reciprocal

crosses, and 3) Analysis of Variance (ANOVA) comparing interspecific crosses. In all cases involving the numbers of pollen grains per pistil, the variances were stabilized by common logarithm transformations. Some other combinations also required log transformations due to heterogeneity of variance.

All statistical tests were performed with the SPSS statistical package of the University of Washington Computer Center. For the purpose of statistical analysis, only the longest tube on each of the fifty pistils per treatment was used. In this manner, routine t-tests comparing independent sample means, and oneway ANOVA were performed. The 1976 data indicated that pollen-tube length and number of pollen grains per pistil were best represented at 48 hours after pollination since, 1) pollen tubes did not grow significantly until approximately 20 hours after pollination, and by 48 hours differences between treatments were discernible, and 2) the longest tubes had not yet penetrated the micropyle.

In those cases where ANOVA indicated that significant differences existed, Duncan's multiple comparison test was used to compare treatment means. This test was chosen as having considerable power when treatment sizes are equal.

All tests were recorded for significance at the 95% probability level.

G. Photographic Documentation

Photographs of catkins were made using a Canon Ftb body with a Canon macro-lens mounted on a copy stand. Kodacolor II daylight print film ASA 80 was used. Illumination was provided by two blue 3800°K 250 Watt bulbs. At this light intensity, exposure times can be read through the lens meter.

Photographs of bisexual aments were made with the same camera body mounted on a Wild Stereoscope. Using the same film as above, at a photometer reading 140, the best exposures were for one minute.

Attempts at documenting pollen-tube growth failed. Neither Ektachrome 200 (Daylight) processed at ASA 200 or ASA 400, nor Ektachrome 160 (Tungsten) were successful in recording fluorescence. The fluorescence of pollen tubes was

typically weak and faded after exposure to uv illumination. Nevertheless, none of these films were used to successfully record visible fluorescence which did not fade. Technical advice from personnel at Medical Photography and Zeiss advisors did not prove useful. Lacking photographs, I have made line drawings to illustrate some of the important features of pollen-tube growth.

RESULTS

In presenting the results of this study, the following sequence will be followed: 1) survey of selfing barriers, 2) survey of interspecific crossing barriers. In the latter section, results will be presented first for pollen-tube growth and pollen germination as a function of time, then for each of the two A. rubra trees used as female parents, and finally for reciprocals. Pollen germination data are handled in each section after pollen-tube growth since they represent only estimated figures. A final section will cover miscellaneous observations.

The ANOVA and t-test tables are tabulated in Appendix III.

A. Survey of Selfing Barriers

Figure 10 presents pollen-tube growth and number of grains per pistil in selfed pistils versus outcrossed (where made) 48 hours after pollination. The important trend to note is that outcrossed tubes and pollen germination performed consistently superior to selfed treatments. No statistical tests were performed for A. rubra, A. glutinosa, or A. sinuata selfed treatments because the comparisons to outcrossed pollinations were so obviously significant. A. cordata was the only case where selfed pollen showed an indication of germination and pollen-tube growth. A t-test to compare selfed A. cordata with outcrossed A. cordata showed that even in this case there was a significant difference.

The standard deviations listed in Figure 10 give an indication of the differences in variance between treatments. For A. rubra and A. glutinosa, the variances were smaller in the selfed treatments than in the outcrossed. The variance in A. sinuata was also small. Only in A. cordata was the variance in selfed treatments higher than in outcrossed treatments.

Apart from differences in tube length and its variance, there was a consistent difference between species relative to the orientation of tubes. In

Figure 10. Mean Pollen Tube Length and Number of Grains
per Pistil 48 Hours After Pollination

Female Parent	Male Parent	Mean Tube Length $\pm$ 1 S.D.	Mean Grain No. $\pm$ 1 S.D.	Male Parent	Mean Tube Length $\pm$ 1 S.D.	Mean Grain No. $\pm$ 1 S.D.
<u>A. rubra-b</u>	<u>rubra-b</u>	1976: 0.00 $\pm$ 0.00* 1977: 6.20 $\pm$ 5.3	0.00 $\pm$ 0.00 0.52 $\pm$ 3.33	<u>rubra-s</u>	1976: 41.02 $\pm$ 10.74 1977: 37.40 $\pm$ 12.24	31.12 $\pm$ 9.87 21.52 $\pm$ 5.33
<u>A. rubra-s</u>	<u>rubra-s</u>	1976: 0.00 $\pm$ 0.00 1977: 0.56 $\pm$ 4.32	0.00 $\pm$ 0.00 0.06 $\pm$ 4.44	<u>rubra-b</u>	1976: 42.62 $\pm$ 6.34 1977: 39.06 $\pm$ 7.27	46.94 $\pm$ 10.55 42.18 $\pm$ 9.87
<u>A. glutinosa</u>	<u>glutinosa</u>	1976: 0.00 $\pm$ 0.00 1977: 0.00 $\pm$ 0.00	0.00 $\pm$ 0.00 0.00 $\pm$ 0.00			
<u>A. cordata-l</u>	<u>cordata-l</u>	1976: 12.62 $\pm$ 18.43 1977: 10.53 $\pm$ 13.67	6.54 $\pm$ 12.22 7.11 $\pm$ 10.53	<u>cordata-2</u>	1976: 1977: 27.20 $\pm$ 7.33	 90.84 $\pm$ 8.23
<u>A. sinuata</u>	<u>sinuata</u>	1977: 0.36 $\pm$ 4.22	1.54 $\pm$ 4.32			

* 1 pollen-tube unit = 15 microns

A. rubra-s, A. rubra-b (1976), A. glutinosa, and A. sinuata, the tubes generally did not grow into the style, but remained convoluted at the surface of the stigma. The exception was A. cordata, wherein all of the tubes measured grew in the style in a similar manner as the outcross tubes. In 1977, a few tubes in selfed A. rubra-b penetrated the style, although most were similar to other A. rubra tubes and did not penetrate the style.

Data on the number of grains per pistil are indirect estimates of pollen germination. Because many grains are washed off the pistils in the preparatory process, a direct measure of pollen germination cannot be made. However, since pollen grains seemed to adhere only if there was some indication of a tube, these data may be used as estimates.

The data on the number of pollen grains per pistil (Figure 10) more or less parallel those on tube growth. Selfed pistils in A. rubra, A. glutinosa and A. sinuata had much fewer grains than outcrossed. Hence, not only were the tubes shorter in the selfed treatments, but many fewer grains germinated. This discrepancy was less pronounced in A. cordata.

Finally, it should be noted from Figure 10 that variation exists not only from year to year, but between different individuals of the same species.

In sum, the data on selfing suggest the inferior performance of selfing compared to outcrossing. The relative ranking order of species based on their selfing ability is: A. cordata > A. sinuata > A. rubra > A. glutinosa. Pollen grains grow into the style of A. cordata and marginally if at all in the other species.

B. Survey of Interspecific Crossing Barriers

1. Pollen-Tube Growth and Pollen Germination as a Function of Time

Part of the purpose of the 1976 work was to give information about the rate of pollen-tube growth. This was accomplished by sampling pistils at five times after pollination. Figures 11-14 show the plotted mean pollen-tube lengths at

4, 12, 24, 36, and 48 hours after pollination for A. rubra-s, A. rubra-b, A. glutinosa, and A. cordata, respectively. (Due to extenuating circumstances, A. sinuata was not included in this aspect of the study.) What is most evident from these graphs is that pollen-tube growth is negligible before 20 hours, but picks up rapidly after 24 hours. This is especially obvious in Figures 11 and 12, in the crosses A. rubra-s X rubra-b, and their reciprocals. To a lesser extent, growth in the other treatments also accelerated around 24 hours after pollination.

A second trend that is evident from these graphs is the much slower tube growth in the interspecific crosses than in the intraspecific crosses. Only in A. cordata (Figure 14) does the rate of growth and the length of pollen tubes compare with some of the interspecific pollinations. (The comparison of the mean pollen-tube lengths will follow in a later section.)

In the cases of A. glutinosa and A. rubra (Figures 11-13), the selfed treatments exist in the lowest category, their rate of growth barely evident.

Figures 15-18 show the plotted means for the number of pollen grains per pistil. The low figures on the graphs (Figures 15, 16) for A. rubra-s X rubra-b and its reciprocal at four hours suggest that the absence of pollen grains is probably a reasonable estimation of germination potential since the number of grains per pistil increased rapidly after four hours. It is conceivable that although the grains may germinate early, growth may not accelerate until beyond 24 hours as indicated by results from pollen-tube lengths.

Referring to the other crosses in Figures 15 and 16, the germination of pollen was low in most cases except A. rubra-s X cordata which demonstrated a high retention of grains even quite soon after pollination. In other cases A. rubra-b X glutinosa and A. rubra-s X glutinosa -- germination appeared to be delayed as much as 35 or 48 hours. Since no further samplings were made beyond 48 hours, nothing can be said about pollen-tube growth potential beyond this point. It is possible (compare Figure 11) that the inter-specific crosses

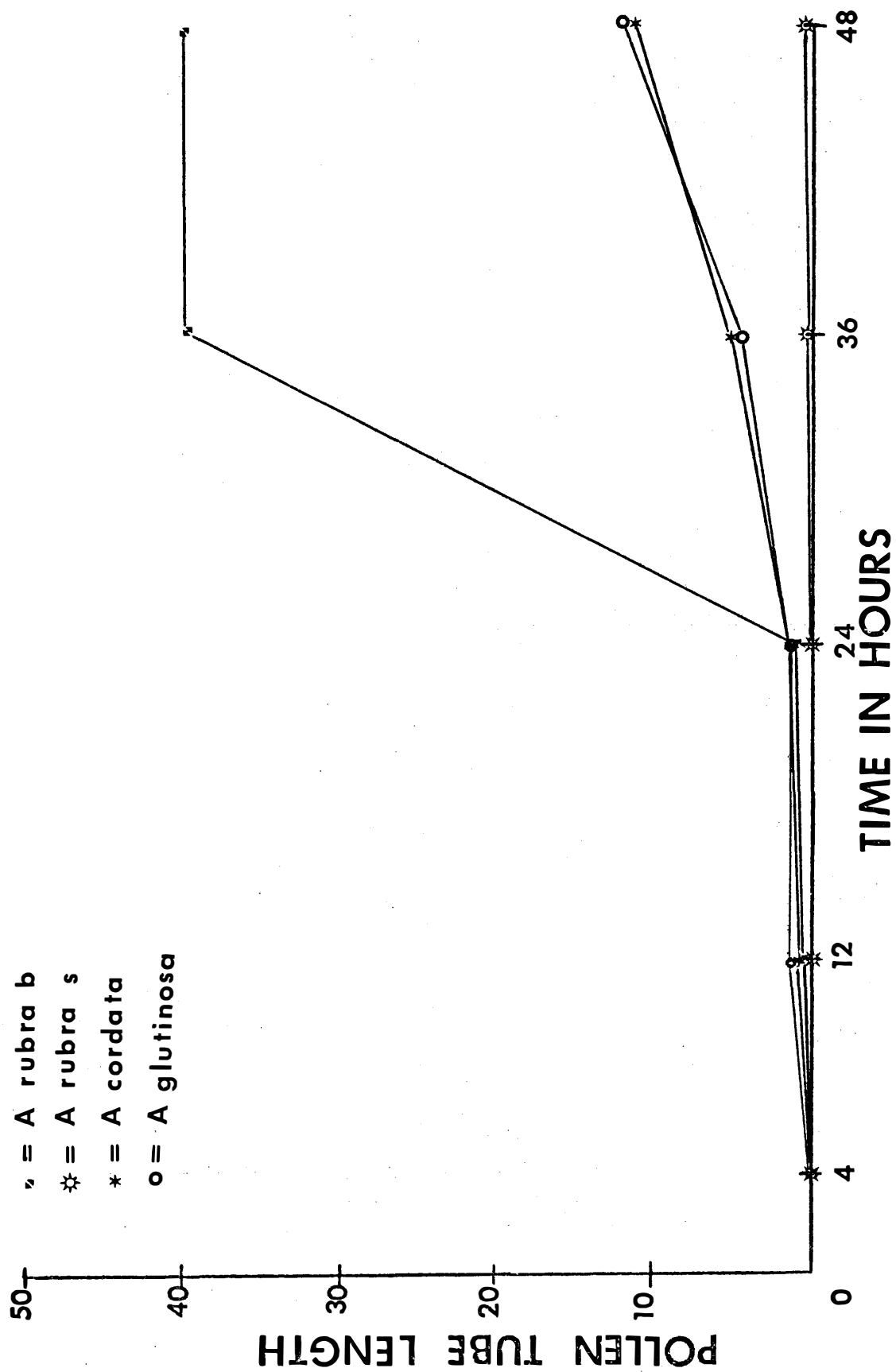


Figure 11. Pollen-tube growth following pollination of *A. rubra-s* flowers (length measured in micrometer units, 50 units (= .75 mm)).

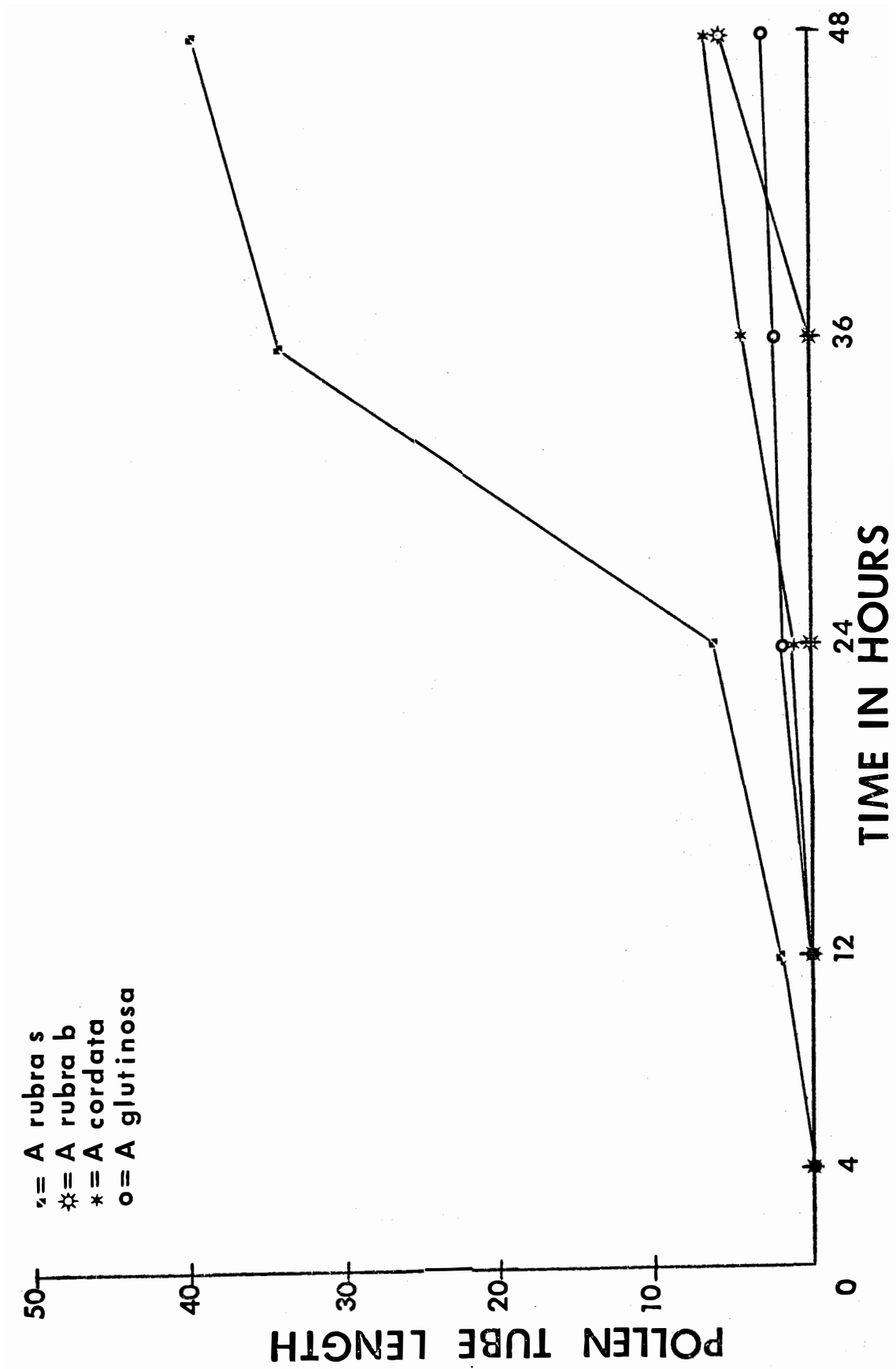


Figure 12. Pollen-tube growth following pollination of *A. rubra-b* flowers (length measured in micrometer units, 50 units = .75 mm).

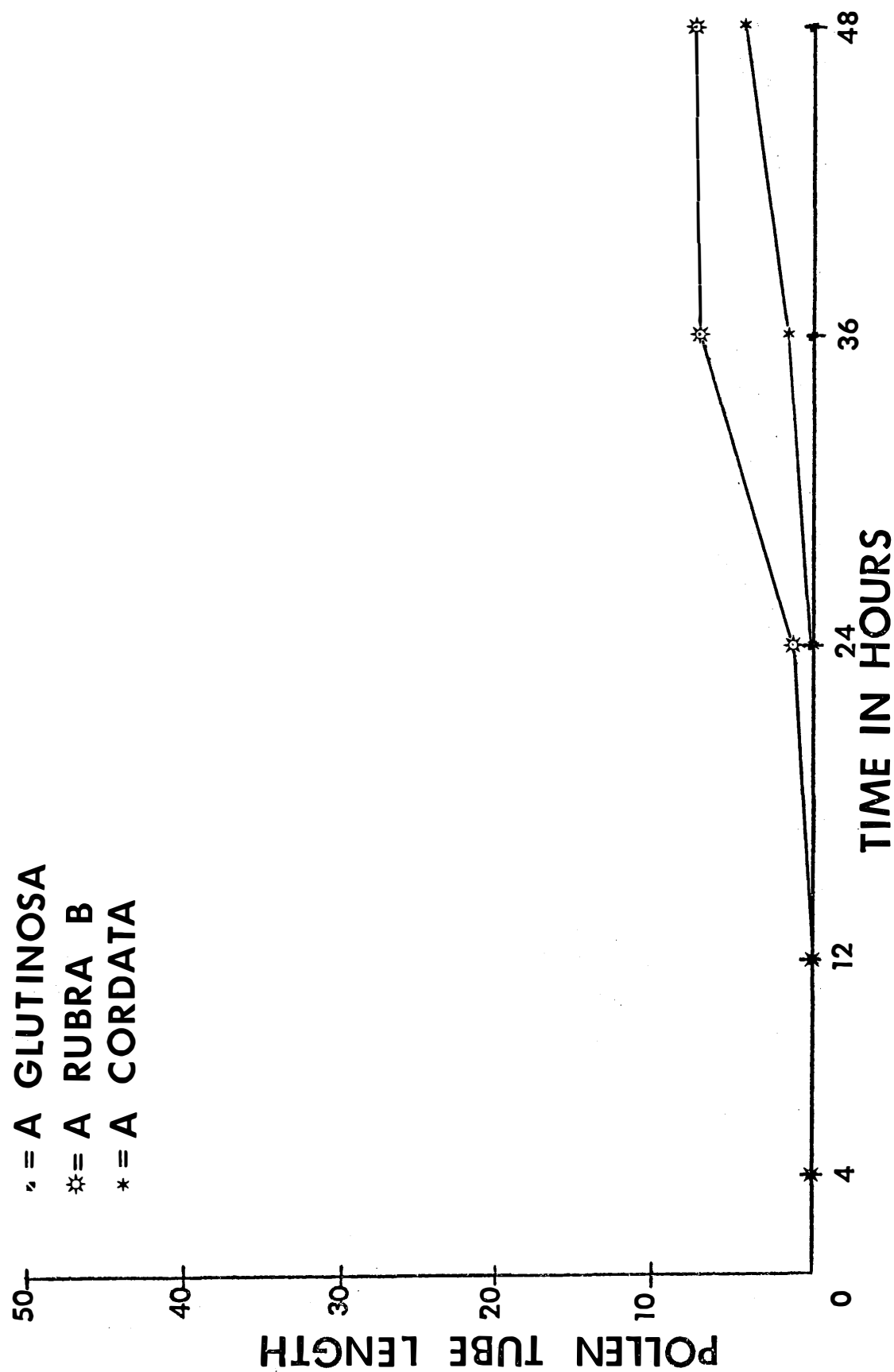


Figure 13. Pollen-tube growth following pollination of *A. glutinosa* flowers (length measured in micrometer units, 50 units = .75 mm).

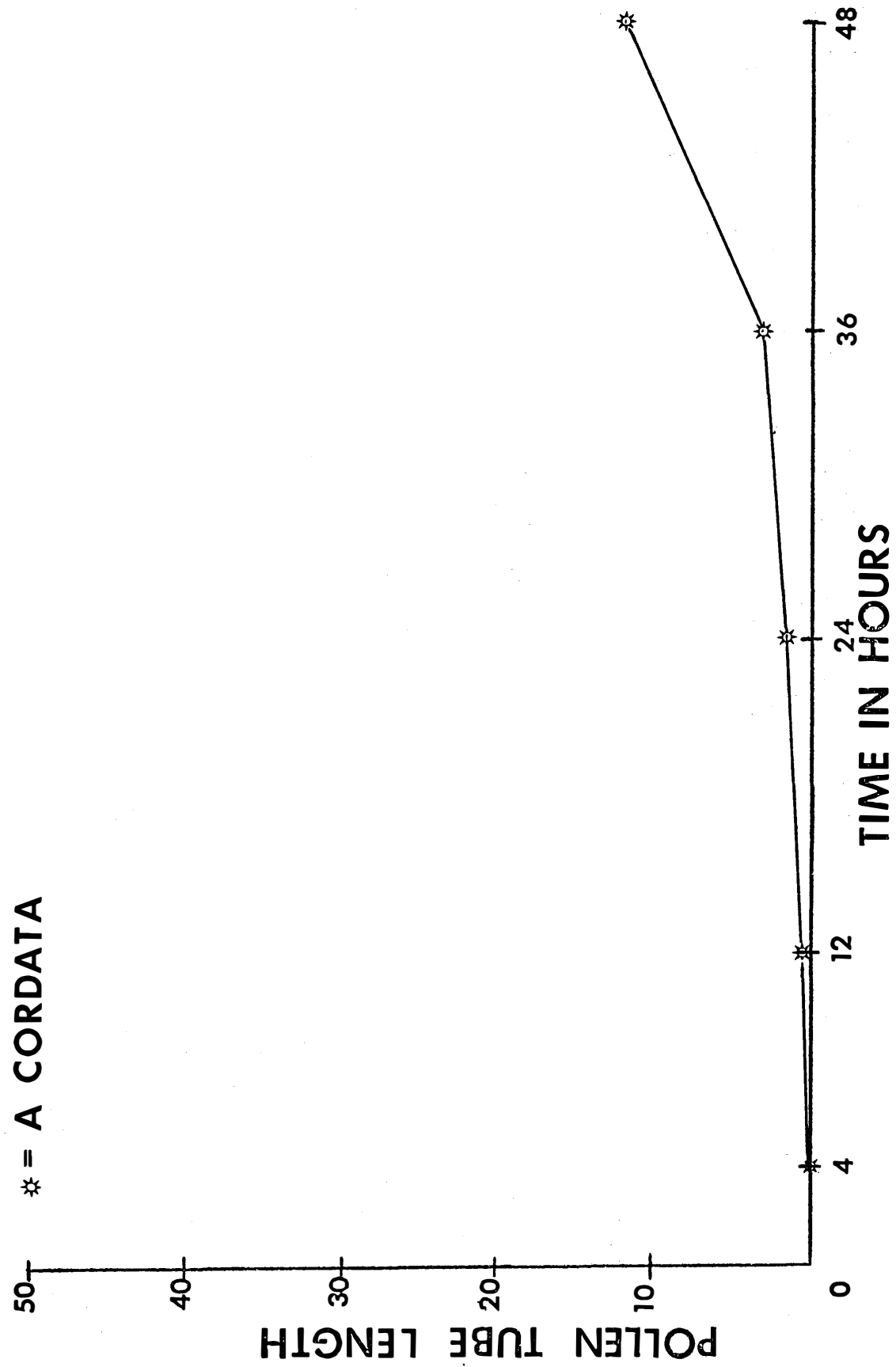


Figure 14. Pollen-tube growth following pollination of *A. cordata* flowers (length measured in micrometer units, 50 units(= .75 mm)).

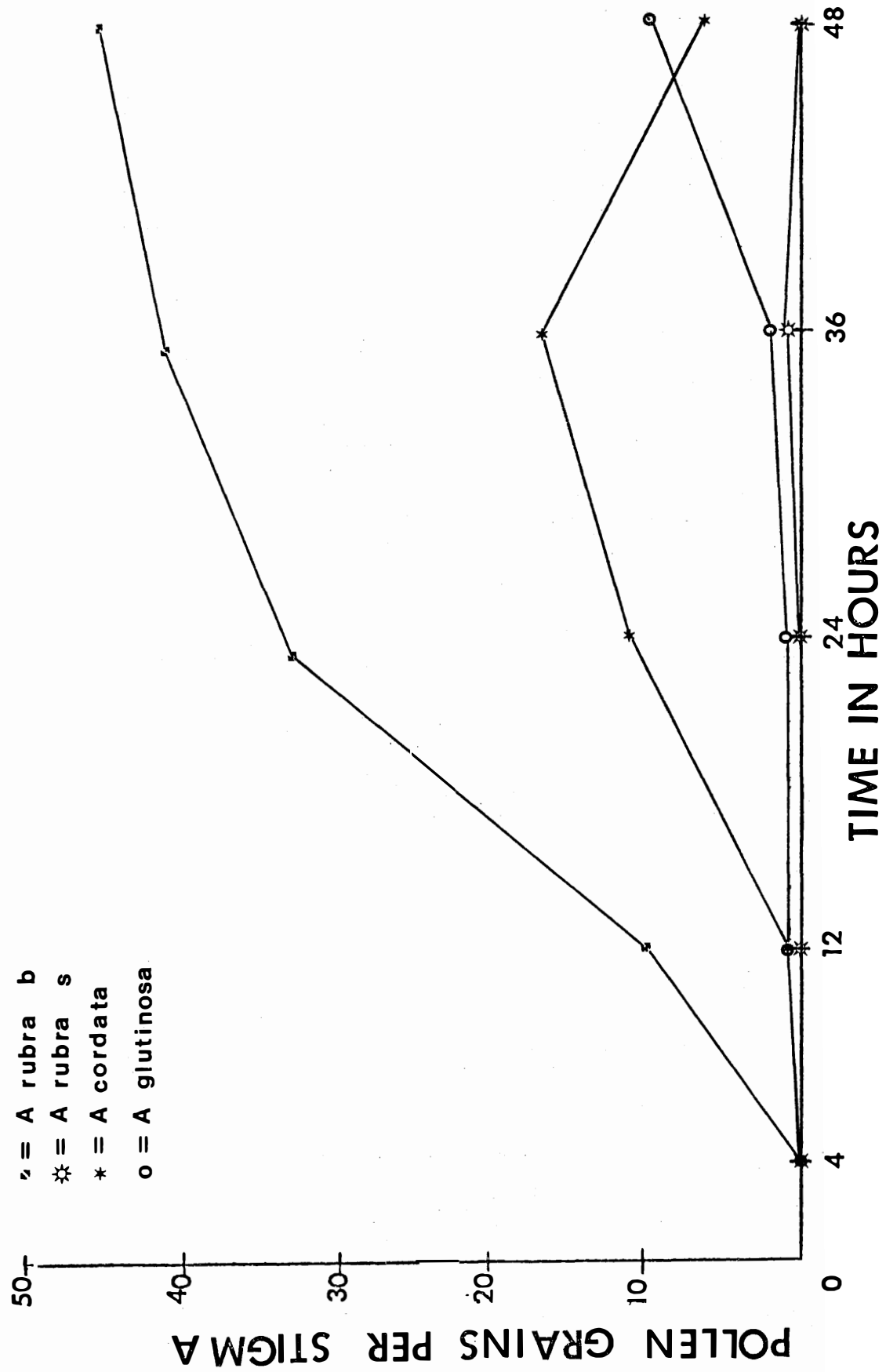


Figure 15. Number of pollen grains per stigma following pollination of *A. rubra-s* flowers.

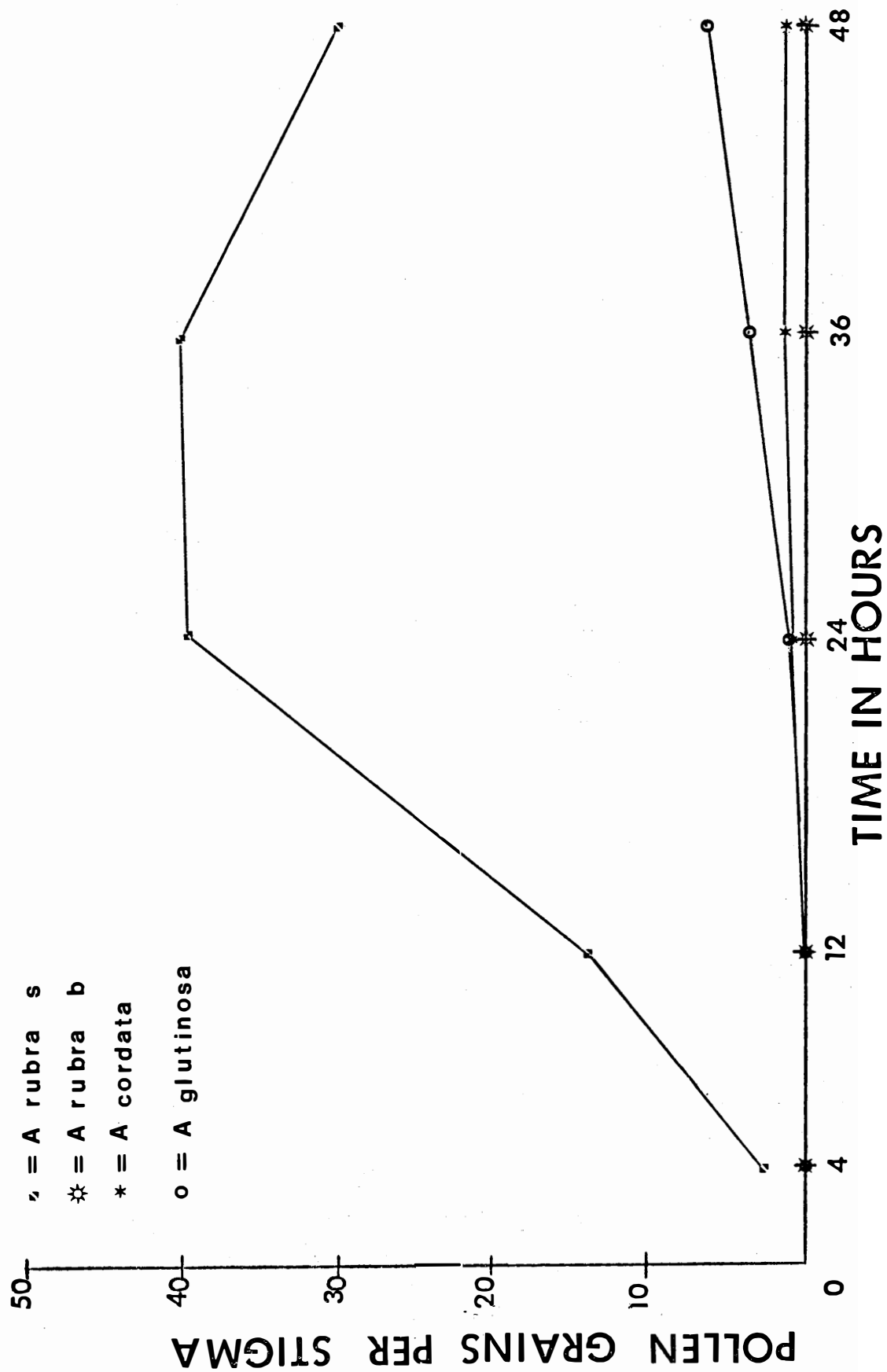


Figure 16. Number of pollen grains per stigma following pollination of *A. rubra-b* flowers.

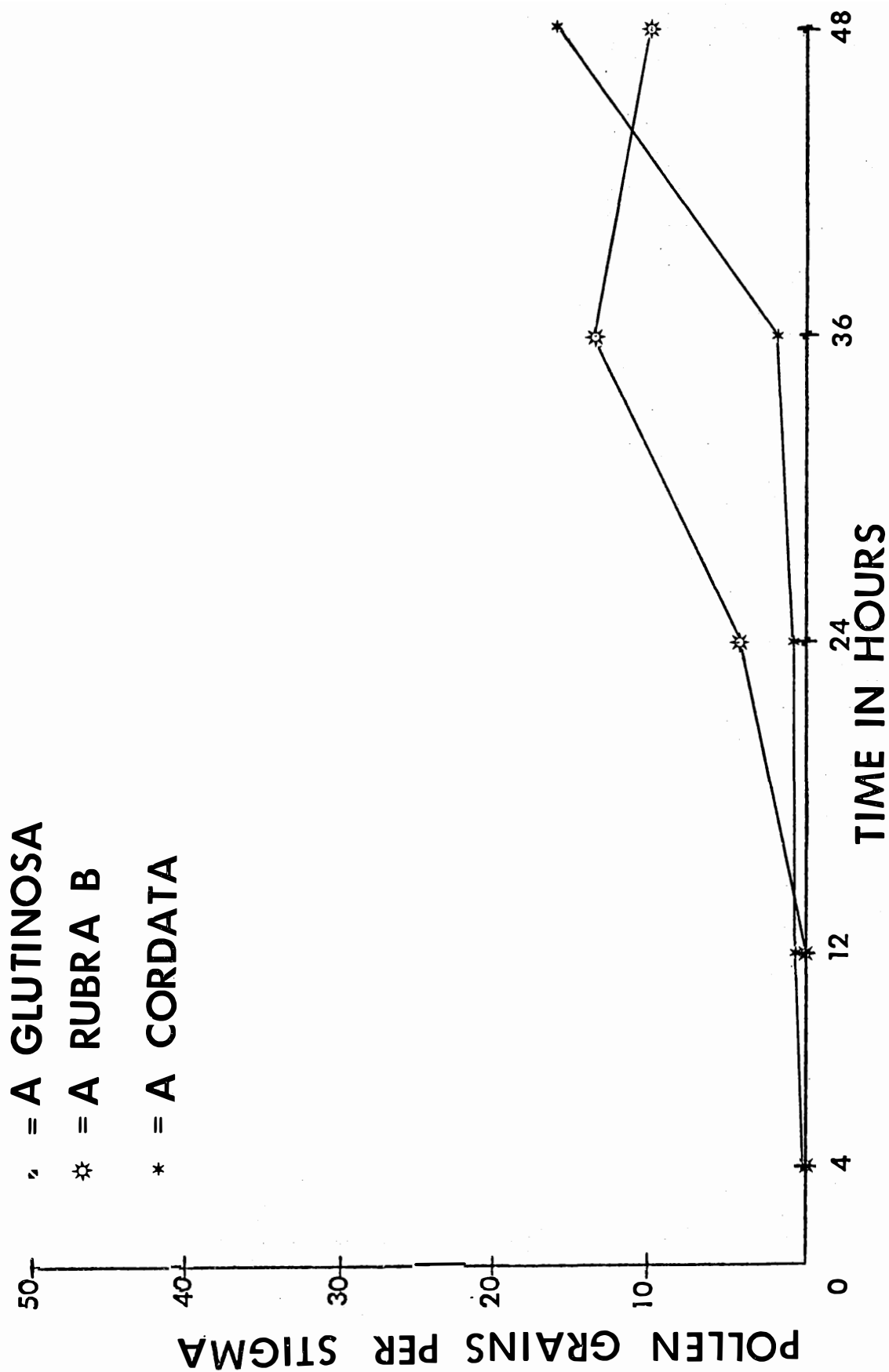


Figure 17. Number of pollen grains per stigma following pollination of A. glutinosa flowers.

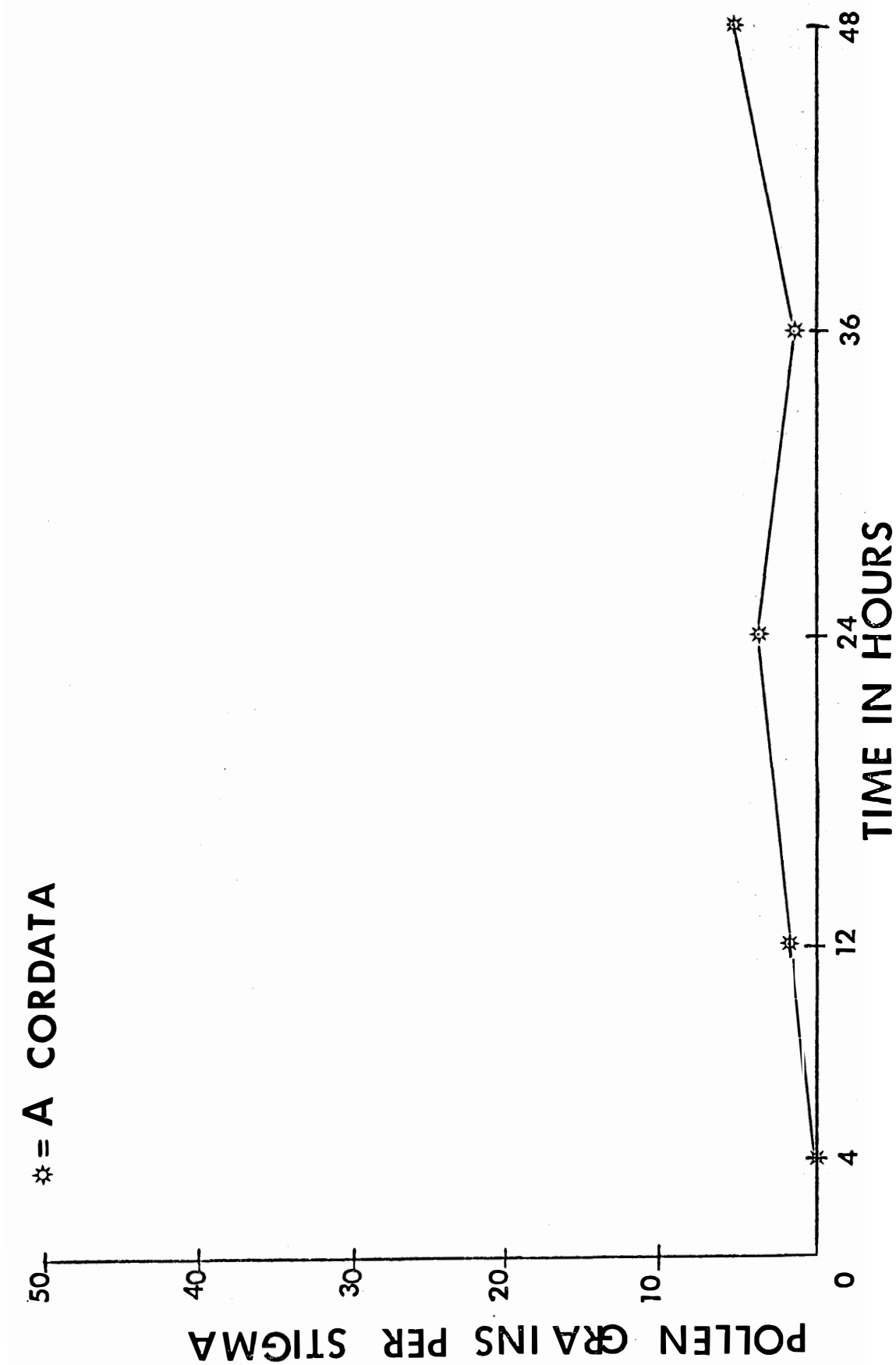


Figure 18. Number of pollen grains per stigma following pollination of *A. cordata* flowers.

need more time for pollen germination, after which pollen-tube growth may occur. In the cases of A. rubra X self, germination was very low and changed little over time.

In Figure 17, a similar situation may be observed. In the A. glutinosa X cordata cross, germination appeared to be delayed and did not change significantly until after 48 hours. A comparison with Figure 13 shows that pollen-tube growth increased somewhat at this point. Similarly, in A. glutinosa X rubra-b, germination increased at 24 hours, and pollen-tube growth increased in like fashion. Selfed A. glutinosa showed no germination over time.

Figure 18 demonstrates that pollen germination had occurred in a small amount as early as 12 hours after self-pollination of A. cordata and thereafter did not increase significantly. Comparison with pollen-tube growth (Figure 14) shows that despite germination of the grains, pollen-tube growth did not accelerate until after 24 hours -- similar to the intra-specific crosses in A. rubra.

These results on pollen-tube growth and pollen germination as a function of time formed the basis for the decision to concentrate on sampling at 48 hours after pollination for the comparative studies.

2. Comparison at 48 Hours: A. rubra-b as Female Parent

Analysis of Variance for pollen-tube length in A. rubra-b pistils at 48 hours after pollination showed that significant differences existed between the pollen parents. Figure 19 summarizes the results from multiple comparison tests.

Figure 19. Duncan test for mean pollen-tube length 48 hours after pollination. Means underscored are not significantly different at .05 level.

<u>rubra-b</u> X <u>rubra-s</u>	<u>rubra-b</u> X <u>cord.</u>	<u>rubra-b</u> X <u>glut.</u>	<u>rubra-b</u> X <u>sin.</u>
41.14*	6.88	3.58	3.24

*1 unit = 15 microns

The most important point to note from Figure 19 is that the closest (intra-specific) and most distant (inter-subgeneric) crosses are at opposite ends for mean

pollen-tube growth. This conforms with expected results where the closest cross should be the most congruent and the most distant cross should be the least congruent.

Secondly, the closest cross (intraspecific) is an order of magnitude larger than the others. In this case, the interspecific crosses are clustered together, despite statistically significant differences, at relatively low mean values.

Finally, the order for inter- and intra-sectional crosses is reversed from that expected on the basis of taxonomic distance. Expected results would place A. rubra-b X cordata and A. rubra-b X glutinosa in opposite positions. Nevertheless, one need not attribute too much weight to this, despite the significant difference, since the mean values are of the same order of magnitude.

Analysis of Variance for pollen grains per pistil also showed that significant differences existed between pollen parents. Duncan multiple comparison test results are tabulated in Figure 20.

Figure 20. Duncan test for mean number of pollen grains per pistil at 48 hours after pollination. Data transformed to common logarithms. Means underscored are not significantly different at .05 level.

<u>rubra-b X rubra-s</u>	<u>rubra-b X glut.</u>	<u>rubra-b X sin.</u>	<u>rubra-b X cord.</u>
1.44	0.51	0.10	0.07

As was the case for pollen-tube lengths, the closest and the most distant crosses are at opposite ends for number of grains per pistil, agreeing with expected results. The intraspecific cross also is significantly higher than all of the other crosses. In this case, however, the intrasectional cross A. rubra-b X glutinosa showed higher performance than the intersectional cross A. rubra-b X cordata. This agrees with results expected on the basis of taxonomic distance.

A comparison with pollen-tube lengths for these crosses show that there is some correlation between germination and pollen-tube length. Generally, the intraspecific and the inter-subgeneric crosses appear correlated, and the intraspecific cross performed an order of magnitude better in both cases. The absolute ranking of the other interspecific crosses was less consistent.

3. Comparison at 48 Hours: A. rubra-s as Female Parent

Similar ANOVA tests were performed for pollen-tube length and number of pollen grains per pistil after interspecific pollination of A. rubra-s flowers. Analysis of variance showed significant differences between mean pollen-tube lengths at 48 hours after pollination. Figure 21 illustrates the results of multiple comparison tests.

Figure 21. Duncan test for mean pollen-tube length at 48 hours after pollination. Means underscored are not significantly different at .05 level.

<u>rubra-s X rubra-b</u>	<u>rubra-s X glut.</u>	<u>rubra-s X cord.</u>	<u>rubra-s X sin.</u>
42.49*	13.20	12.49	5.38

*1 unit = 15 microns

Most importantly, the results in Figure 21 emphasize the close correlation with expected results on the basis of taxonomic distance. The closest cross (intraspecific) had the longest mean tube length, whereas the most distant cross (inter-subgeneric) had the shortest mean length. Secondly, the mean tube length for the intraspecific cross was considerably larger than all the others. Finally, the ranking of the intrasectional and intersectional crosses was exactly as expected.

A comparison of these results to those when A. rubra-b was used as the female parent shows some similarities and differences. In terms of absolute pollen-tube lengths, the intraspecific crosses A. rubra-b X rubra-s and its reciprocal produced very similar pollen-tube lengths. By contrast, the interspecific crosses had quite different pollen-tube lengths. On the whole, pollen tubes growing in A. rubra-s pistils grew faster than those growing in A. rubra-b pistils. In regard to expected taxonomic ranking of the crosses, results from A. rubra-s crosses agreed perfectly with the expected results, whereas A. rubra-b showed reverse order in the intra- and intersectional crosses.

Analysis of Variance for number of pollen grains per pistil showed that significant differences existed between pollen parents on A. rubra-s pistils. Results from Duncan multiple comparison tests are listed in Figure 22.

Figure 22. Duncan test for mean number of pollen grains per pistil at 48 hours after pollination. Data transformed to common logarithms. Means underscored are not significantly different at .05 level.

<u>rubra-s X rubra-b</u>	<u>rubra-s X glut.</u>	<u>rubra-s X cord.</u>	<u>rubra-s X sin.</u>
1.69	0.97	0.75	0.05

These results also compare favorably with those expected. There is perfect agreement on the ranking of crosses on the basis of taxonomic distance. The intra-specific cross had a greater mean number of pollen grains per pistil by one order of magnitude than the intra- and intersectional crosses, and by two orders of magnitude than the inter-subgeneric cross.

Differences in absolute figures may be better illustrated from non-transformed data. Figure 23 shows differences in mean numbers of pollen grains per pistil between the two A. rubra females.

Figure 23. Comparison of the mean number of pollen grains per pistil between two A. rubra trees used as female parents. Number of grains recorded 48 hours after pollination.

<u>rubra-b X rubra-s</u>	<u>rubra-b X glut.</u>	<u>rubra-b X cord.</u>	<u>rubra-b X sin.</u>
31.12	4.24	0.69	1.18
<u>rubra-s X rubra-b</u>	<u>rubra-s X glut.</u>	<u>rubra-s X cord.</u>	<u>rubra-s X sin.</u>
52.83	11.29	6.79	1.40

The consistent superiority of A. rubra-s Stigmata as a substrate for pollen germination relative to those of A. rubra-b is again evident from these data, thus reinforcing the findings on pollen-tube lengths.

4. Reciprocal Tests

Statistical tests were performed to determine if significant differences existed between reciprocal crosses. The results are shown in Figure 24.

Figure 24. Results from t-tests for differences between mean pollen-tube lengths of reciprocal interspecific crosses. Probability = 95%.

<u>Cross</u>	<u>Reciprocal</u>	<u>Differences</u>
<u>rubra-s X glut.</u> 12.82*	<u>glut. X rubra-s</u> 7.24	significant
<u>rubra-b X glut.</u> 3.58	<u>glut. X rubra-b</u> 8.38	significant
<u>rubra-s X cord.</u> 12.94	<u>cord. X rubra-s</u> 9.98	significant
<u>rubra-b X cord.</u> 7.02	<u>cord. X rubra-b</u> 3.38	significant
<u>rubra-b X sin.</u> 6.02	<u>sin. X rubra-b</u> 3.25	not significant

*1 unit = 1 micron

These results differ from what might be expected for the performance of reciprocal crosses, since all reciprocals but one performed significantly different from one another. This again suggests variability between individual trees. The only set of reciprocal crosses which was not significantly different was A. rubra-b X sinuata and its reciprocal.

A further observation which reinforces previous results is the superior performance of A. rubra-s as female parent over A. rubra-b. This is shown in the crosses with A. glutinosa as well as with A. cordata. It appears that A. rubra-s also performs better than A. rubra-b as a male parent, as indicated in the cross with A. cordata.

A final point is the reversal in order of the A. glutinosa X rubra and reciprocal between the two A. rubra parents. For A. rubra-s, the cross was superior when A. rubra was the maternal parent; for A. rubra-b, the cross was superior when A. glutinosa was the maternal parent. This may be linked with the superiority of A. rubra-s stigmata as substrate for pollen over A. rubra-b.

Figure 25 lists t-test results comparing mean pollen grains per pistil between reciprocals.

Figure 25. Results from t-tests for differences between mean number of pollen grains per pistil of reciprocal interspecific crosses. Probability = 95%. Data transformed to common logarithms.

<u>Cross</u>	<u>Cross</u>	<u>Difference</u>
<u>rubra-s X glut.</u> 0.97	<u>glut. X rubra-s</u> 0.96	not significant
<u>rubra-b X glut.</u> 0.50	<u>glut X rubra-b</u> 0.94	significant
<u>rubra-s X cord.</u> 0.75	<u>cord. X rubra-s</u> 1.31	significant
<u>rubra-b X cord.</u> 0.07	<u>cord. X rubra-b</u> 0.03	not significant
<u>rubra-b X sin.</u> 0.03	<u>sin. X rubra-b</u> 0.09	significant

These data reinforce the results from pollen-tube growth in that the dominant trend is one of significant differences between reciprocals. Only in the cases of A. rubra-s X glutinosa and reciprocal and A. rubra-b X cordata and reciprocal were reciprocal crosses not significantly different. These data again indicate the superior performance of A. rubra-s over A. rubra-b both as female and male parent. Finally, the order of A. rubra X glutinosa and reciprocal crosses in the two A. rubra parents is reversed as it was in the pollen tube data.

C. Miscellaneous Observations

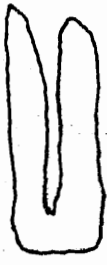
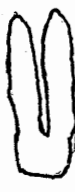
This section presents some of the descriptive observations made during the course of pollen-tube measurement. The descriptions are gathered under descriptive headings for reference. Other descriptive and phenological information is contained in Appendix I.

1. Physical Size of Pistils

The size and shape of pistils differs considerably from species to species. Figure 26 presents comparative information on some physical properties of the pistils of the four species studied.

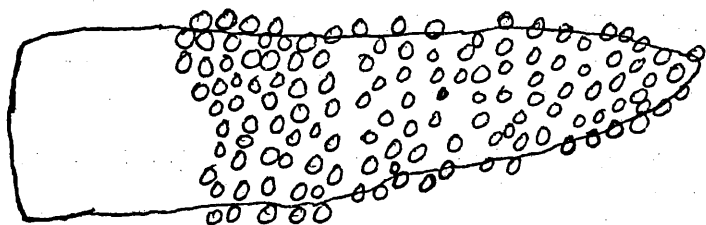
The stigmatic surface of Alnus pistils is extensive, and covers the area from the tip of the pistil nearly to the base. Figure 27 illustrates the extent

Figure 26. Comparative information on some physical properties of the pistils in four *Alnus* species.

Species	Size Length X Width -mm	X-Section	Surface Cells	Extrusion of Pistils at Receptivity-mm	Shape
<u>A. cordata</u>	1.65-2.00 X 0.4-0.5	flat	jacket of rough cells; 75% of time these cells extended over the tip of the stigmata	0.6-0.8	
<u>A. sinuata</u>	1.35-1.70 X 0.2-0.25	flat	irregular but not jacket-like; often warty	0.5-0.7	
<u>A. rubra</u>	1.05-1.22 X 0.15-0.2	round	smooth	0.3-0.5	
<u>A. glutinosa</u>	0.9-1.05 X .15-0.2	round	smooth or warty	0.2-0.4	

of the stigmatic surface as defined by the adhesion of pollen grains which may occur in an intraspecific cross in A. rubra.

Figure 27. Stigmatic surface area of Alnus pistil illustrated by pollen grains adhering in an intraspecific cross in A. rubra.



X90

2. Fluorescence Specks and Callose Deposits

In many cases of interspecific and selfed pollinations, surface specks or often collars of fluorescence and deposits of callose at tips of pollen tubes were observed on styelar cells. These initially appeared similar to short pollen tubes without grains, but with a little experience, tubes could be readily discerned from such vascular fluorescence. This type of fluorescence was not observed in intraspecific crosses, nor was it the result of primary styelar fluorescence (based on observations of unstained pistils). Surface fluorescence and collars of fluorescence were observed in the following matings: A. rubra-s -- self, A. glutinosa -- self, A. cordata -- self, A. rubra-b X cordata, A. rubra-b X glutinosa, and A. glutinosa X rubra-b.

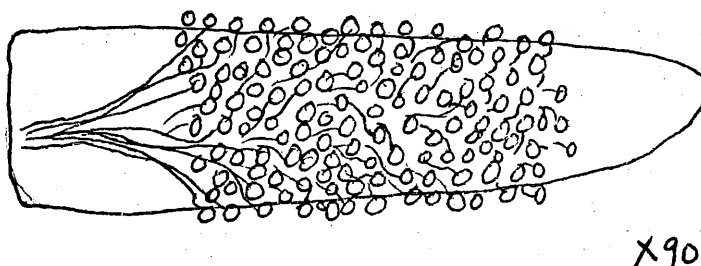
Callose deposits appeared as spots of fluorescence within the style at the terminus of a pollen tube. They were observed in the following matings: A. rubra-s X sinuata, A. rubra-b X sinuata, A. rubra-b -- self, A. rubra-b X cordata, A. rubra-s X glutinosa, A. cordata X rubra-b, and A. glutinosa X rubra-b.

3. Positioning and Density of Pollen Grains on the Style Relative to Growth

In all cases of intraspecific outcrosses, and in two of the interspecific crosses (A. rubra-s X cordata, and A. glutinosa X rubra-b) where grain number per pistil was high, the pollen grains seemed to cluster preferentially away from the tip of the pistil in about 50% of the cases (Figure 28). The length of the unoccupied area was approximately equal to the amount of extrusion of the pistil beyond the bract of the catkin.

In the intraspecific crosses only, regardless of whether the tip was covered with grains or not, the tubes which had reached furthest to the ovaries were those coming from the most proximal grains. These were generally the longest tubes, and there would be five to eight of approximately equal length. The rest of the grains more distally located had very short seemingly aborted tubes (Figure 28).

Figure 28. Positioning and density effects on intraspecific crosses and two interspecific crosses



In the treatments where the density of pollen grains was low (the remaining interspecific crosses and nearly all self-pollinations), there appeared to be neither this limitation of five to eight long tubes, nor did position of the grain on the pistil seem to affect relative growth (Figure 29)a However, in only two cases (A. cordata X self, and 1977 A. rubra-b X self) were there ever tubes as long as the longest intraspecific tubes.

4. Penetration of Style by Pollen Tubes

In almost all interspecific crosses most pollen tubes penetrated the styles.

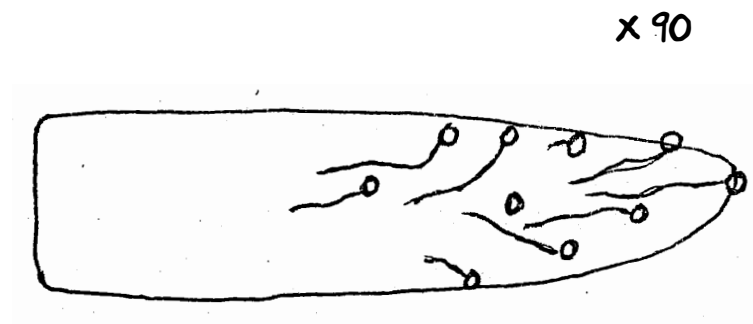


Figure 29. Positioning and density effects on the majority of self- and interspecific pollinations.

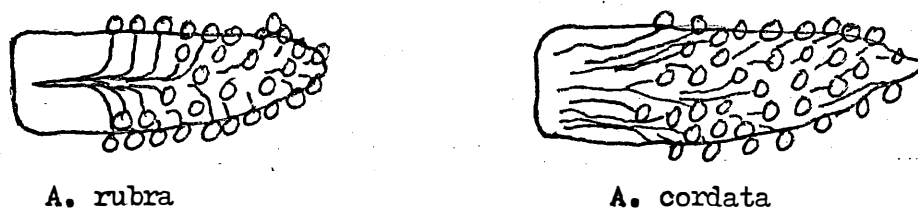
The only exceptions were the crosses A. rubra-b X glutinosa, A. rubra-s X glutinosa (sampled before 48 hours) and A. glutinosa X rubra-b (1977 only). As pointed out in an earlier section, pollen from selfing behaved quite differently and generally did not penetrate the style.

5. Channelling of Pollen Tubes

There appeared to be differences between species as to the preferred location for pollen to grow in the style. In A. rubra, A. glutinosa, and A. sinuata, the pollen tubes grew initially straight into the styles (perpendicular to the long axis) for approximately 4 units, then made an abrupt turn and subsequently grew down the center of the long axis of the style. This was particularly obvious in the intraspecific crosses, A. rubra-s X rubra-b and its reciprocal, where the tubes were long and the density of pollen high (Figure 30).

By contrast, tubes growing in A. cordata styles do not seem to be limited to the central portion of the style, nor do they make a turn as they enter the style. Rather, the growth is more directly toward the base of the style, and the entire styler area appears to be receptive to pollen tube growth (Figure 30).

Figure 30. Channelling of Pollen Tubes in A. rubra and A. cordata Pistils.

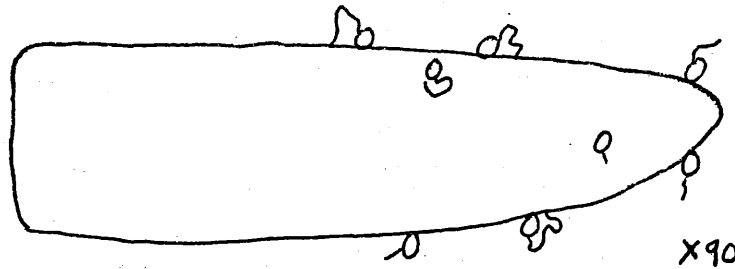


6. Convolution of Tubes at Stigmatic Surface

In most of the selfed and some of the interspecific treatments the lack of styler penetration by the pollen tubes was accompanied by pollen-tube growth outside the pistil. In these instances, the pollen tube would be contorted beside the grain without penetrating the style (Figure 31). This is in contrast to the initial short, straight tubes of crosses wherein pollen tubes grew into

the styles. This convolution occurred in conjunction with short tubes which came off the backs of some grains and headed away from the style. Conditions of this sort were observed in A. rubra-s ~~self~~, A. sinuata-s ~~self~~, A. glutinosa-s ~~self~~, A. rubra-s X cordata, and A. rubra-s X glutinosa. In the cross A. rubra-s-s ~~self~~, loose pollen grains with short pollen tubes were found floating in the staining medium away from the pistil. In one case of A. rubra-s X sinuata, several pollen tubes were observed growing backwards in the style (relative to the ovary).

Figure 31. Convolution of Pollen Tubes at Stigmatic Surfaces



DISCUSSION

A. Survey of Selfing Barriers

The results on pollen germination and tube growth in selfed pistils indicate that a high degree of self-incompatibility exists in A. rubra, A. glutinosa, and A. sinuata, and in A. cordata to a lesser degree. This conforms with the observations made by Hagman (1969, 1970) on self-incompatibility in A. glutinosa. However, Hagman found that the incompatibility reaction operated at the level of retarded pollen-tube growth, whereas the present results indicate that in A. glutinosa selfed pollen grains on the whole were unable to adhere to or germinate on the stigma. This suggests that some incompatibility reaction takes place on the surface -- a phenomenon commonly associated with sporophytic control.

A similar situation was found in A. rubra and in A. sinuata. In 1976, neither of the A. rubra parents studied showed evidence of pollen adhesion, germination or pollen tube growth after self-pollination. The same treatment in 1977 showed some pollen grain adhesion and some pollen tube growth. However, in these cases, and in the case of A. sinuata where a small number of grains adhered to the stigmata and germinated, the majority of the tubes did not penetrate the style, but remained convoluted on the surface of the stigma.

These results contradict the findings of Harry (1974) on seed viability after self-pollination of A. rubra. Harry found that of 800 seeds collected from the control cross A. rubra-b X rubra-s 246 seeds were viable (= 30.8% of 800) and of 1800 seeds collected from the A. rubra-b self mating, 229 seeds (= 12.7% of 1800) were viable. His results may be explained in two ways: 1) Selfed A. rubra-b performs differently depending on the year. My findings support this possibility since a considerable number of self tubes in A. rubra-b were observed in 1977, whereas none were found in 1976. 2) The frequency of apomixis may be increased due to stimulation from pollination. Although Harry's results showed that unpollinated

treatments yielded only a low amount of apomixis, no investigations have been made on the occurrence of apomixis following pollination. Hence, his high results for seed set after selfing A. rubra-b may be distorted by the presence of apomixis.

Nevertheless, as Hagman stated, and as confirmed by the present study, the self-incompatibility reaction seems to be variable rather than absolute, varying from tree to tree and from year to year. Perhaps also the condition of flower maturity and the ambient weather conditions during the period of growth affect results. Accordingly, it is conceivable that conditions arise where a self tube could penetrate the ovary in any of the species studied.

Only A. cordata showed a somewhat higher degree of self-compatibility. The average length of selfed pollen tubes was nearly half that of outcrossed tubes, individual tubes being equally long. Thus, self-incompatibility in A. cordata appears to manifest itself in the slower tube growth in the style. The fact that a number of pollen tubes grew as rapidly as outcross tubes suggests that there are cases in which -- in the absence of further barriers -- self-fertilization may occur. Nonetheless, pollen germination success after selfing was only 6.6% that of outcrossing. Altogether then, barriers to selfing in A. cordata seem to exist at both the stigmatic level and the stylar level. But at neither of the two levels are they absolute.

B. Survey of Interspecific Crossing Barriers

I would like to preface this discussion with a few remarks on the reliability of pollen germination data. As I have stated elsewhere, indirect pollen germination measurements are based on the number of grains adhering to the pistil 48 hours after pollination. Results on number of grains per pistil as a function of time indicate that this measurement is a fairly reliable estimate of pollen germination. However, it is possible that germination may have occurred in tubes in many cases, but that tubes did not penetrate the surface of the stigmata and could not, therefore, anchor the grains.

Estimates of the rate of pollen germination based on the number of pollen grains per pistil demonstrated that germination does not occur until after four hours. Beyond this time, germination occurred rapidly in intraspecific crosses, but slowly in interspecific crosses. In some cases, since germination did not occur until after 36 hours, pollen tubes may grow considerably after 48 hours. When germination occurred early, pollen tubes in most interspecific crosses were still inhibited either at the level of stylar penetration or through retardation of pollen-tube growth in the style. In the majority of interspecific crosses, germination increased only slightly over time, and there appeared to be no striking difference between the taxonomic types of crosses.

Pollen-tube growth, by comparison, was negligible for the first 24 hours, after which growth accelerated rapidly. This was particularly true for intraspecific crosses and to a lesser extent for interspecific crosses. Hagman must have had the same experience since he based his pollen-tube studies on samples collected 36 hours after pollination. I felt that 36 hours was not sufficient time for pollen-tube growth in some of the interspecific crosses, several of which did not show appreciable growth until 48 hours. It is possible that if later samples had been collected, some of the interspecific crosses which showed little or no growth by 48 hours would have shown delayed growth.

2. Comparison at 48 hours.

ANOVA results document the significant amount of incongruity which exists in interspecific crosses in *Alnus*. The barriers to crossing follow expected taxonomic lines in most cases. In all cases, the inter-subgeneric cross yielded the slowest pollen-tube growth of all matings, and the intraspecific crosses yielded the fastest. The intrasectional and intersectional crosses fell in between, their absolute ranking varying with the female parent.

In discussing the results from interspecific crosses, it is noteworthy that all of these crosses are clustered together having very short pollen tubes.

compared with the intraspecific crosses. The variances of pollen-tube length in these crosses were overlapping and, considering the apparent variability which existed from tree to tree and from year to year, absolute ranking cannot be made on the basis of such small differences. Hence the most confident conclusion to be drawn is that significant incongruity exists in interspecific crosses in *Alnus*, even when the crosses are intrasectional. To specify exact rankings of interspecific crossability, a much larger number of trees is needed, and experiments should be conducted over several years.

What can be discussed relative to the present data is the level at which the incongruity barriers exist. The data on pollen tube growth indicate that nearly all those grains that germinate in interspecific-crosses are capable of penetrating the style. In only a few instances (*A. rubra*-b X *glutinosa*, *A. rubra*-s X *glutinosa*, and *A. glutinosa* X *rubra*-b) appeared there to be inhibition of the tube at the stigmatic level. These results varied from year to year. For the majority of interspecific crosses made, the barriers appeared to be in the retardation of pollen-tube growth. Nevertheless, in all the crosses there were some tubes that approached the length of congruent tubes. Hence the distribution (variance) of pollen tubes in interspecific crosses was large -- with some tubes being very short, and others growing long. With such a pronounced variance, it would appear that the genotype of the individual pollen grain was important in determining the extent to which the incongruent reaction was expressed.

Despite the fact that most tubes appeared to penetrate the style and that some grew as long as congruent tubes, the results on the number of pollen grains per pistil indicated that the great majority of pollen grains that landed on the pistils were incapable of adhering and germinating. Hence, the stigmatic surface must act as a strong selective agent in incongruent crosses. The majority of those that are capable of germinating are also capable of penetrating the stigma, but may well be further selected in the style. Only a tiny fraction of the initial grains available grew tubes which even approached the length of those in congruent crosses.

3. Intraspecific Barriers

A brief word may be said about intraspecific crosses in A. rubra. Despite the fact that only two trees were used, the results from pollen-tube lengths and number of grains per pistil indicated that this was a congruent cross. The reciprocals were nearly equal for pollen tube length, although more grains adhered to A. rubra-s pistils than to A. rubra-b pistils. Judging by the comparative results for interspecific crosses, pollen tubes growing in A. rubra-s pistils grew faster and adhered better than those in A. rubra-b pistils. Perhaps A. rubra-s offers a more generally acceptable substrate for pollen-tube growth than A. rubra-b.

4. Reciprocal Tests

Statistical analysis of reciprocal crosses confirms the general finding that much variability exists in crossing barriers among *Alnus* species. Such difference in performance between reciprocal crosses may be expected if incongruity barriers probably operate dissimilarly in the different species. In all but one case, A. rubra used as female allowed greater pollen-tube growth than reciprocals where foreign species were used as female parents. This may be attributed to the fact that A. rubra was the only species tested in its native habitat. A. sinuata, although native to the Pacific Northwest, grows naturally in high elevations, and hence is out of its normal conditions in the Arboretum.

Ljunger (1959) and Hagman (1970) reported unilateral fertility in the cross A. glutinosa X rubra, the cross being fertile only when A. rubra was used as female. My results, however, showed higher fertility when A. glutinosa was used as female, but with pollen-tube growth in the opposite direction. Obviously, a larger sample is needed, with studies spanning several years.

These results on interspecific crossability in the four *Alnus* species agree with current thought about the mechanisms controlling incongruity barriers

(eg., Hogenboom, 1975). Through speciation and evolutionary divergence, the events from pollination to pollen-tube growth and micropylar penetration no longer are uniquely paired as they are in the pistil-pollen relationship of intraspecific crosses. With mismatching of these events, barriers come into existence at various levels, some of which have been elucidated for *Alnus* in this study. Based on this hypothesis, it makes sense that certain genotypes would have a better chance than others to overcome the barriers, and that a great deal of variability should exist from tree to tree, and possibly from year to year. We may well conclude that *A. rubra* has diverged considerably in reproductive attributes relative to *A. glutinosa*, *A. cordata*, and *A. sinuata*.

C. Miscellaneous Observations

1. Physical Size of Pistils

The larger size of *A. cordata* pistils both in width, length, and amount of extrusion over the other species provides greater available stigmatic area on which pollen grains may land. Not surprisingly then, nearly two times as many grains were observed in the intraspecific *A. cordata* cross as in that of *A. rubra*. This should be taken into account when comparing pollen germination results for the different species.

The large surface area of the *A. cordata* pistils, combined with the larger number of pistils per catkin, compensates for the fact that only one pistillate catkin develops per inflorescence, whereas in *A. glutinosa*, *A. rubra*, and *A. sinuata*, there are three to five catkins per inflorescence.

The greater length of *A. cordata* styles requires longer pollen-tube growth before the ovary is reached than in the other species. Hence a pollen tube that may be long enough to penetrate the micropyle in *A. rubra* or *A. glutinosa* may be too short to effect fertilization in *A. cordata*. Since pollen tubes of other foreign species grew no longer in *A. cordata* styles than in *A. rubra* styles, the hypothesis that *A. cordata* may provide a better substrate for pollen-tube growth

than A. rubra can be discredited in my study.

In all *Alnus* species observed, the amount of pollen caught on pistils is maximized by the fact that the effective stigmatic surface extends nearly to the base of the style.

2. Fluorescence Specks and Callose Deposits

Stigmatic cells that fluoresce when pollinated with foreign pollen appear to indicate surface reactions between pistil and pollen. This type of fluorescence was observed most frequently in crosses that were inhibited at the level of pollen germination and of pollen tube penetration into the style (ie., mostly the selfed treatments). This would be expected if the incongruity reaction occurs on the stigmatic surface.

Callose deposits, on the other hand, were observed at the tips of some pollen tubes which were growing in the styles. In these cases, the crucial interaction was obviously at the stylar level. Observations of callose deposits have been made for incongruent crosses in other genera (Martin, 1959; Guries, 1975), although these investigators report callose deposits to be a surface phenomenon. Since callose has been shown to be produced as a wound response (Courier, 1957), it is not unlikely that callose plugs may be a symptom of incongruent pollen-tube inhibition.

3. Positioning and Density of Pollen Grains on the Style Relative to Growth

The observation in certain crosses that pollen grains adhere to the lower 60% of the pistil and not to the tip may be explainable by several reasons:

1) The naked section of the pistil, being relatively equivalent to the extended portion during the period of receptivity, may be more vulnerable to the effects of handling or disturbance than the protected areas between the bracts, 2) because it is exposed, the extruded portion of the pistil may become dehydrated, hence hydration of pollen grains (necessary for germination) on this portion of the

stigma may be impeded, or 3) maturation of the effective stigmatic surface may proceed in basipetal fashion. In the latter case, pollen grains would adhere only to those portions of the stigma which were at maximum receptivity.

The position and density of the grains on the pistil were definitely correlated to growth in the intraspecific crosses. Several explanations may account for the fact that the grains most proximally located on the pistil were always the ones producing the longest tubes: 1) Again, stigmatic maturation may play a role -- wherein the most receptive portion would be furthest down the style. This is unlikely since all grains germinated, but were differentiated at the level of pollen-tube growth. Stigmatic receptivity would discriminate between germination potential; 2) There may exist a gradient in the style relative to promotion of pollen-tube growth. If this were the case, the proximal portions of the pistil must provide more favorable conditions for growth than the distal portions; 3) Since density appears to be a factor (i.e., where density is low as in interspecific crosses, pollen tubes from the tip do not appear to be inhibited), competition may exist between pollen tubes. This is highly speculative and would benefit from further investigation.

4. Channelling of Pollen Tubes

From observations in A. glutinosa, A. cordata, and A. rubra that pollen tubes are restricted to the central portion of the style, it appears that there are morphological or biochemical conditions that distinguish the style from non-receptive pistil tissue. Just from the standpoint of physical space, this could account for pollen-tube competition and inhibition of certain tubes. A. cordata's wider pistils do not appear to have this restriction and hence may be more receptive to various types of pollen.

5. Convolution of Tubes at Stigmatic Surface

The occurrence of convoluted tubes on the surface of pistils has been

observed in other incongruent crosses in certain poplars (Guries, 1975; Stettler, personal comm.). This phenomenon is attributed to a surface interaction between stigma and pollen and may involve inhibition or lack of stimulation of pollen-tube growth.

CONCLUSIONS

This study has shown that in four species of alder, barriers to fertilization operate at the level of pistil-pollen relationships. The specific level of the barrier varies with the species and the direction of the cross.

Self-incompatibility was found in A. rubra, A. glutinosa, A. sinuata, and A. cordata. Barriers to selfing were very strong in the former three species and weakest in A. cordata. In all the species studied, some incompatibility reaction appeared to occur at the stigmatic surface in that pollen grains were unable to adhere to the surface. In cases where some grains germinated, the pollen tubes were almost always inhibited from penetrating the style. This suggests a different mechanism from that which Hagman observed where self pollen-tube growth was retarded in the style. Yearly differences, however, were quite pronounced, and I did observe instances where selfed pollen grew long tubes that penetrated the style, especially in the case of A. cordata.

Incongruity was observed in varying degrees in interspecific crosses between the four species. The barriers appear to exist at several levels including inhibition of pollen grains to adhere to the stigma, inhibition of pollen grains to germinate, inhibition of pollen tubes to penetrate the style, and finally, retardation of pollen-tube growth in the style. In most interspecific crosses, these barriers seemed to reinforce each other so that in one pistil a reduction in the number of grains was evident, some grains persisted ungerminated, some had short tubes outside the stigma, whereas a few actually penetrated the style. It appears that each of these levels acts as a selective sieve of the genotypes represented in the pollen population wherein only a few of the many potential grains are able to actually produce tubes that grow.

The selective strength of the combined barriers more or less followed taxonomic lines. The inter-subgeneric cross (*Gymnothrysus* X *Alnaster*) always

produced the shortest tube lengths, the intraspecific crosses the longest. All interspecific crosses performed somewhere in between although much more poorly than the intraspecific cross. Hence, because of this clustering and because of the variability observed, it is safest to generalize and say that all interspecific crosses performed poorly. To assign an absolute order to crossability, a much wider selection of parent trees would have to be studied, and this for several years.

A tremendous amount of variability was found in performance between trees, species, direction of cross, and year. Most of the variability was quantitative rather than qualitative and hence it is likely that hybrids could be produced and selfings successfully accomplished. What barriers lie beyond the level of the pistil-pollen relationship are obviously crucial factors in the production of seed. Due to the apparent occurrence of apomixis in *Alnus*, however, studies of seed yield are not reliable tests of the success of particular crosses.

Despite the variability observed, this study showed that certain individuals performed consistently better than others of the same species.

Further studies on incompatibility and incongruity in *Alnus* would do well to consider the following points:

- 1) Use a greater number of parents from each species; trees growing in their native habitats would give more reliable results.
- 2) Conduct studies over several years to analyze the annual variation.
- 3) Sample pistils longer than 48 hours past pollination (eg., 60-72 hours) to allow for delayed germination in the interspecific crosses.
- 4) Study levels beyond pollen germination and tube growth (eg., micro-pylar penetration, embryo development) to determine what other barriers exist to crossing.
- 5) Use a method that is less disturbing to the pollen grain in situ. Stettler (personal comm.) has used SEM techniques successfully to achieve this objective in the study of stigmatic surface interactions in *Populus*.

APPENDIX I
PHENOLOGY AND DESCRIPTIVE INFORMATION
ON
ALNUS REPRODUCTIVE BEHAVIOR

This appendix presents a brief and obviously incomplete compilation of phenological and descriptive information on the reproductive behavior in A. rubra, A. glutinosa, A. cordata, and A. sinuata gathered during the course of this study. It is hoped that this information may aid in further breeding studies in Alnus. Figure 32 summarizes the important dates both in flowering and the events of breeding experiments.

Description of Phenological Events

1. Pre-Flowering Period

Figure 33 illustrates the comparative winter forms of flowering branches in A. rubra, A. glutinosa, A. cordata, and A. sinuata as they would be found through late January. All species of Alnus are monoecious bearing male and female flowers on separate aments. Staminate and pistillate aments appear in clusters, with the inflorescence types borne on separate peduncles. Often in the lower portion of the crown, branches carry staminate but no pistillate aments. All staminate catkins are preformed, and in A. rubra, A. glutinosa, and A. cordata the pistillate strobili are preformed and overwinter out of the bud. Only A. sinuata has pistillate strobili that are retained inside the bud until the period of flowering. All of these species have a high retention rate of woody strobili from the previous fall. Figure 34 compares the size and number of woody strobili between species. A. rubra, A. glutinosa, and A. sinuata have oblong-cylindrical strobili which occur in clusters of three to four. The bracts are thin and the pedicels weak, the latter especially true in A. sinuata. By contrast, strobili in A. cordata usually are borne singly or in pairs. They are comparatively larger and more massive than the strobili of the other species, are more spherical in shape, and are supported by firm, thick pedicels.

Figures 35-38 illustrate at higher magnification the flowering structures on each of the species as they would appear in mid-January. In A. rubra (Figure 35), the staminate catkins are borne in clusters of four to five, and

Figure 32. Important dates in the reproductive cycle of *Alnus* with events of breeding experiments.

1976 Date	1977 Date	<u>A. rubra.</u>	<u>A. glutinosa</u>	<u>A. cordata</u>	<u>A. sinuata</u>
Feb. 12	Feb. 5	all catkins tight.	bag flowers, collect branches for forcing; all catkins tight.	bag flowers, collect branches for forcing; male catkins beginning to elongate, no free pollen; female pistils just visible	male catkins tight.
Feb. 13	Feb. 10		forced catkins shed pollen.	forced catkins shed pollen.	
Feb. 17	Feb. 22		male catkins shed pollen.	male catkins shed pollen.	
Feb. 19	Feb. 24			female strobili receptive; control pollination.	
Mar. 4	Feb. 18	collect branches for forcing.			collect branches for forcing.
Feb. 28	Febr 24		female strobili receptive; control pollination.		
Mar. 4	Mar. 5	bag flowers.			
Mar. 7	Mar. 15	forced branches shed pollen.			
Mar. 23	Mar. 15	male catkins shed pollen.			
Mar. 27	Mar. 17.	female strobili receptive; control pollination.			

Figure 32. Continued

1976 Date	1977 Date	<u>A. rubra</u>	<u>A. glutinosa</u>	<u>A. cordata</u>	<u>A. sinuata</u>
	Mar. 31				bag flowers
	April 2				forced branches shed pollen.
	April 14				bud burst.
	April 18				male catkins shed pollen.
	April 20				female strobili receptive; control pollination.
	early July			male catkins for the next year become visible.	



Figure 33. Wintering forms of flowering branches in A. rubra, A. glutinosa, A. cordata, and A. sinuata.



Figure 34. Woody strobili of A. rubra, A. glutinosa, A. cordata, and A. sinuata.



Figure 35. Winter form of flowering branches in A. rubra.



Figure 36. Winter form of flowering branches in A. glutinosa.



Figure 37. Winter form of flowering branches in A. cordata.



Figure 38. Winter form of flowering branches in A. sinuata.

vary from two to five centimeters long in winter form. The preformed female inflorescence can be seen subtending the cluster of male aments. These aments overwinter tightly appressed to one another in clusters of three to five. Occasionally they are partially surrounded by bud scales. The pointed terminal (= lateral) vegetative bud is seen at the base of the reproductive stalk.

A. glutinosa (Figure 36) is similar to A. rubra. The three to four staminate aments are generally shorter than those in A. rubra, and vary from two to three centimeters. The pistillate strobili are about half the length of A. rubra pistillate aments. They are not tightly appressed, nor do they remain partially in a bud, but rather are borne spread out on their elongated pedicels. The length of the pedicels in the female inflorescence does not change significantly as flowering proceeds, whereas in A. rubra, the strobili separate from one another and the pedicels elongate until they are spread out as in the winter form of A. glutinosa. The vegetative bud of A. glutinosa is shorter and more rounded than that of A. rubra.

The organization of flowering branches in A. cordata is quite different (Figure 37). The male catkins are borne in clusters of three to six, and are longer than either A. rubra or A. glutinosa, varying from four to seven centimeters. The female strobili are borne on unbranched peduncles, and occur singly or in pairs. They are the largest in length and width of the four species observed. They overwinter in fully exposed form. The vegetative bud (not shown) is similar to A. rubra.

In contrast, A. sinuata has the smallest overwintering reproductive structures of the four species (Figure 38). The male catkins, in clusters of two to three are only two to three centimeters long, are borne on short pedicels, and stay appressed to one another throughout winter and early spring. They appear to be covered with a protective, resinous substance. The pistillate strobili remain protected over the winter. They are retained in the vegetative bud until about five days before flowering. As far as I could discern, not all vegetative buds

contained pistillate aments, but all buds bearing pistillate aments also bore leaves. I could not distinguish externally between the buds that carried pistillate aments and those that did not.

2. Flowering Period

The dates indicated in Figure 32 list the major events in the flowering of the four species. The order of flowering is: A. cordata, A. glutinosa, A. rubra, and A. sinuata (in the University of Washington Arboretum). An important fact worthy of note is the occurrence of protandry in all four species, as follows:

A. rubra -- 2-3 days
A. cordata -- 1-3 days

A. glutinosa -- 7-10 days
A. sinuata -- 2-3 days

Male flowering and female flowering are not completely separated events within a tree, and there is a certain degree of overlap. The above figures denote what I arbitrarily defined to be maximum maturation of many staminate catkins vs. maximum receptivity of pistillate aments. Only in the A. glutinosa tree used did the two events appear distinctly separate, maximum female receptivity not occurring until all pollen had been shed. The period of pollen shedding lasts about three to four days, depending on temperature and wind. Warmer temperatures and wind hasten the shedding of pollen. Maximum receptivity of pistillate strobili, signified by the extrusion of succulent, red (except in the case of white in A. sinuata) pistils, lasts three to four days also. Beyond this time, the pistils wither and dry up. Protogyny occurred only on bisexual aments as described in Appendix II. McVean (1955) observed some protogyny in A. glutinosa aments, and it is possible that this condition is variable from year to year.

Figures 39 and 40 illustrate the forms of inflorescences at the flowering period in A. cordata. The staminate catkins elongate to 15 centimeters, the bracts become fully separated and the anthers (in Figure 39 visible as brown tissue) are reflexed back on their filaments. Anthesis occurs in a basipetal manner on each catkin, the tip of the catkin maturing and shedding pollen first.

At the time this photo was taken, all pollen had been shed. By contrast, the female strobili (Figure 40) mature more synchronously over the entire strobilus, so that at maximum receptivity, the red pistils appear to cover the exterior of the strobilus. When differential maturation occurs in pistillate strobili, it is in the reverse direction as in staminate catkins (i.e., acropetal). These conditions in A. cordata are similar to those in the other three species, varying only in the absolute length of extended staminate catkins, and of pistils.

It may be recalled that one pistil is actually composed of two styler, stigmatic units, each one of which I have referred to as a pistil. For each double lobed pistil, there is associated one ovary. Two such double-lobed pistils are subtended by one bract in a strobilus, so that there is the potential for two seeds to form per bract.

The pistils of A. cordata are the largest of the four species in length and width. A. sinuata are next in size, but the color of the pistils is a clearer, pale white in contrast to the red of the other species. A. rubra pistils are smaller still, while A. glutinosa has the smallest pistils of the four species. This is important when determining pistillate receptivity because A. glutinosa pistils will never extrude as much as A. cordata.

A word should be said about conditions in A. sinuata. Since the pistillate flowers are borne in some vegetative buds, it would be convenient, for purposes of bagging (when performing crossing experiments) to determine if a rule existed which could be used to determine which buds contain flowers and which do not. I could not establish such a rule, and hence the best alternative may be to overbag.

Finally, a count of the number of pistils per catkin gives an idea of the size of each species' ament.

<u>A. rubra</u>	200-250
<u>A. glutinosa</u>	70-150

<u>A. cordata</u>	280-300
<u>A. sinuata</u>	260-280



Figure 39. Maximum receptivity of A. cordata strobilus as indicated by the extrusion of succulent, red pistils.



Figure 40. Staminate aments of A. cordata soon after anthesis.

3. Post-Flowering Period

Immediately following the flowering period, the pistils wither and eventually dry up. Within a few weeks, the staminate catkins abscise from the branch. Further development of the seed-strobili occurs over spring and summer. The strobili turn green and begin to elongate and swell. By early to mid-July, the initials for the next spring's catkins and strobili become visible on the branch. The seed-strobili fully mature and release their seed by late October through early November. They then turn brown and woody, and are retained on the branch often through the next flowering season.

Female flower-strobili left unpollinated in isolation bags continued to develop into mature strobili for a period of 6 weeks. Hence, parthenocarpy must occur in *Alnus*, and the retention of the strobili is not dependent on fertilization. Finally, other sources (McVean, 1955) have noted that empty seeds also form in the absence of pollination.

APPENDIX II

BISEXUALITY IN THREE ALNUS SPECIES

During the winter of 1977, bisexual aments were found occurring in regular frequencies on A. rubra, A. glutinosa, and A. cordata. Sexual polymorphism has been described frequently in herbaceous flowering plants and in several genera of forest trees (Klaehn, 1958), especially in the dioecious genus *Populus* (Lester, 1963; Melchoir, 1967). However, little direct documentation exists for bisexuality in *Alnus*. This appendix describes the bisexual aments found in three *Alnus* species.

In *Alnus*, clusters of staminate aments and pistillate strobili (solitary in A. cordata) typically occur on separate peduncles in close proximity on the same branch. In the three species, both pistillate and staminate inflorescences are preformed and overwinter entirely (A. cordata and A. glutinosa) or partially (A. rubra) out of the bud. Flowering usually occurs in February and March (in the University of Washington Arboretum). All three species exhibited varying degrees of protandry in the trees used. (See Appendix I for more complete description.)

At the time of flower maturation in winter of 1977, abnormal pistillate strobili were observed in the three species. These aments were characterized by the presence of solitary or four-lobed anthers ingrown behind a bract-like structure at the base of an otherwise normal appearing strobilus (Figure 41). These anthers were afilamentous, being easily dislodged from the strobilus with a dissecting needle. Otherwise, the anthers appeared morphologically similar to those on normal staminate aments. The anthers contained abundant pollen which was morphologically similar to that found in immature normal anthers. The pollen sacks of abnormal anthers during the period of normal flower maturation contained sticky masses of pollen similar to conditions reported in bisexual *Populus* aments (Lester, 1963).

The maturation of abnormal anthers appeared to be about three to four weeks behind that of normal anthers. It should be noted that this is a case of



Figure 41. Abnormal bisexual strobilus in A. cordata.
Anther may be seen ingrown behind bracts
at the base of an otherwise normally
appearing pistillate strobilus.

protogyny in trees that were normally protandrous. Lester (1963) noted this condition in bisexual *Populus* trees. Furthermore, development of anthers on bisexual aments seemed to be inhibited, the anthers deteriorating after the pistils withered

In addition, when bisexuality was observed in *A. rubra* as described above, the distance between abnormal pistillate aments and normal staminate aments was less than that between normal pistillate and staminate clusters. Similar abnormalities have been observed in *Populus* (Lester, 1963).

Another form of sexual deviation was observed in *A. rubra* wherein solitary pistillate strobili grew from the peduncles of staminate aments (Figure 42). Both staminate and pistillate aments were unisexual and appeared normal. Flowers on these aments developed synchronously with normally located pistillate aments and appeared to reach maturation, as evidenced by the extension of swollen pistils beyond the bracts.

On the species examined, bisexuality did not occur on staminate aments.

The frequency of sexual and non-sexual polymorphism was determined by casual observation based on the following sampling: Ten *A. rubra* individuals from the Puget Lowland (Figure 43) were chosen. On each of these trees, 200 male and female inflorescences were checked for abnormalities. Three *A. cordata* and two *A. glutinosa* trees growing in the University of Washington Arboretum were similarly observed. Frequency data are listed in Figure 43.

It is noteworthy that every tree sampled contained at least one bisexual ament in 200. Although bisexuality occurred with similar frequency in Arboretum specimens of *A. cordata* and *A. glutinosa*, nothing is known about their bisexuality in native ranges.

Because the physiology of bisexuality was not studied, and the occurrence of these abnormalities was observed only in one year, no conclusive statements should be made as to their occurrence. Nevertheless, some speculations may guide

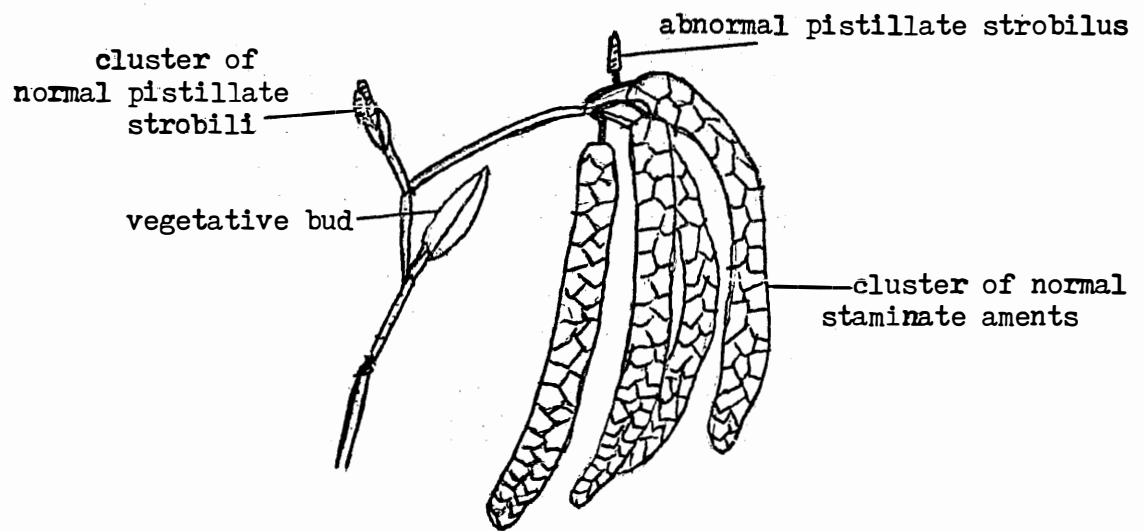


Figure 42. A second form of sexual deviation observed in A. rubra wherein solitary pistillate strobili grow from the peduncles of staminate aments.

Figure 43. Location and frequency of trees sampled for bisexual strobili in A. rubra, A. glutinosa, and A. cordata.

Species	Location	Bisexual strobili/ 200 strobili	% Bisexual strobili
<u>A. rubra</u>			22/2000=1.1%
1 = FG 36	Arb., E-6	4	
2 = FG 35	Arb., F-5	1	
3	Arb., F-7	1	
4	Arb., F-7	1	
5	Arb., F-7	3	
6	Arb., F-7	2	
7	Burke Gilman Trail, UW Campus, W. of overpass to Health Science	2	
8	"	3	
9	Lk. Samamish Picnic	4	
10	Grounds		
	"	1	
<u>A. glutinosa</u>			9/600 = 1.5%
1 = FG32	Arb., N-7	2	
2	"	4	
3	"	3	
<u>A. cordata</u>			10/600 = 1.6%
1	Arb., N-7	4	
2	"	3	
3	"	3	

further investigations.

Since abnormalities were readily apparent only in 1977 (and have not been recorded in the *Alnus* literature), it is conceivable that it is not a regular phenomenon in the genus, and thus may have been triggered by some environmental conditions. Environmental effects on sex expression have been observed in *Populus* (Stettler, 1971; Klaehn, 1958), and in cucumber (Heslop-Harrison, 1959). It is believed that sexual differentiation and floral maturation are influenced by phytohormones and the ratio of minerals and nutrients in reproductive primordia (Galen, Jung, and Lang, 1962; Lang, 1965; Heslop-Harrison, 1959). Studies in growth substances and morpho-genesis indicate that high levels of auxins and nitrogen and low light intensity favor female tendencies, whereas high levels of giberellin, low nitrogen concentration and high light intensity promote male development (Lang, 1959). Late summer and fall of 1976, and winter of 1977 were extremely dry seasons in western Washington. Since catkin initials are formed in early July (*A. glutinosa*, McVean, 1955), it is conceivable that the rare environmental conditions in 1976-1977 altered the ratios and transportation of nutrients and hormones. It would be interesting to observe whether the severe drought of 1976 and 1977 in Europe had any effect in this regard on *A. glutinosa* and *A. cordata* in their native ranges.

Plant growth substances have been known to have an inhibitory effect on the elongation of the flower stalk. In *Fritillaria*, abnormally occurring anthers on a pistillate flower inhibited the growth of the peduncles (Lang, 1959). Conceivably, the presence of staminate flowers on the normally pistillate aments in *Alnus* had a similar effect on the second type of abnormality observed in *A. rubra*.

While the phenomena described may be rare, they may have implications in regard to floral evolution in *Alnus*.

APPENDIX III
ANALYSIS OF VARIANCE AND t-TEST TABLES
FOR POLLEN-TUBE GROWTH AND
NUMBER OF GRAINS PER PISTIL.

Figure 44. Analysis of variance table for pollen-tube length after pollination of A. rubra-s flowers.

Source	df	SS	MS	F
Between groups	4	46828.5906	11707.1476	156.503
Within groups	244	18252.3090	74.8045	
Total	248	65080.8996		

Figure 45. Analysis of variance table for number of pollen grains per pistil after pollination of A. rubra-s flowers. Data transformed to common logarithms.

Source	df	SS	MS	F
Between groups	4	68.4726	17.1182	155.839
Within groups	244	26.7930	.1098	
Total	248	95.2656		

Figure 46. Analysis of variance table for pollen-tube length after pollination of A. rubra-b flowers.

Source	df	SS	MS	F
Between groups	4	53486.3500	13371.5875	324.252
Within groups	246	10144.6141	41.2383	
Total	250	63630.9641		

Figure 47. Analysis of variance table for number of pollen grains per pistil after pollination of A. rubra-b flowers. Data transformed to common logarithms.

Source	df	SS	MS	F
Between groups	4	70.7937	17.6984	174.816
Within groups	246	24.9051	.1012	
Total	250	95.6988		

Figure 48. Table for t-tests using pollen-tube length and number of grains per pistil to compare selfed versus out-crossed pollinations of *A. cordata*. Data for number of grains per pistil transformed to common logarithms.

Variable	Mean	df	t-value
Pollen tube			
Selfed	12.62	98	-7.76
Outcrossed	27.20		
Grain Number			
Selfed	.6904	98	-20.91
Outcrossed	1.9186		

Figure 49. Table for t-test using pollen-tube length and number of grains per pistil to compare reciprocal crosses A. rubra-s and A. glutinosa. Data for number of grains per pistil transformed to common logarithms.

Variable	Mean	df	t-value
Pollen tube			
<u>glut.</u> X <u>rubra-s</u>	17.24	98	-3.94
<u>rubra-s</u> X <u>glut.</u>	12.82		
Grain Number			
<u>glut.</u> X <u>rubra-s</u>	.9587	98	-3.03
<u>rubra-s</u> X <u>glut.</u>	.9738		

Figure 50. Table for t-test using pollen-tube length and number of grains per pistil to compare reciprocal crosses in A. rubra-b and A. glutinosa. Data for number of grains per pistil transformed to common logarithms.

Variable	Mean	df	t-value
Pollen tube			
<u>glut. X rubra-b</u>	8.38	98	6.75
<u>rubra-b X glut.</u>	3.58		
Grain Number			
<u>glut. X rubra-b</u>	.9434	98	6.01
<u>rubra-b X glut.</u>	.5015		

Figure 51. Table for t-test using pollen-tube length and number of grains per pistil to compare reciprocal crosses in A. rubra-s and A. cordata. Data for number of grains per pistil transformed to common logarithms.

Variable	Mean	df	t-value
Pollen tube			
<u>cord.</u> X <u>rubra-s</u>	9.98	98	-2.06
<u>rubra-s</u> X <u>cord.</u>	12.94		
Grain Number			
<u>cord.</u> X <u>rubra-s</u>	1.3129	98	6.42
<u>rubra-s</u> X <u>cord.</u>	.7506		

Figure 52. Table for t-test using pollen-tube length and number of grains per pistil to compare reciprocal crosses in A. rubra-b and A. cordata. Data for number of grains per pistil transformed to common logarithms.

Variable	Mean	df	t-value
Pollen tube			
<u>cord.</u> X <u>rubra-b</u>	3.38	98	-1.76
<u>rubra-b</u> X <u>cord.</u>	7.02		
Grain Number			
<u>cord.</u> X <u>rubra-b</u>	.0320	98	-1.56
<u>rubra-b</u> X <u>cord.</u>	.0768		

Figure 53. Table for t-tests using pollen-tube length and number of grains per pistil to compare reciprocal crosses in A. rubra-b and A. sinuata. Data for number of grains per pistil transformed to common logarithms.

Variable	Mean	df	t-value
Pollen tube			
<u>rubra-b</u> X <u>sin.</u>	6.02	98	1.57
<u>sin.</u> X <u>rubra-b</u>	3.26		
Grain Number			
<u>rubra-b</u> X <u>sin.</u>		98	-2.28
<u>sin.</u> X <u>rubra-b</u>	.0999		

Figure 54. Table for t-tests using pollen-tube length and number of grains per pistil to compare the crosses A. glutinosa X rubra-b with A. glutinosa X cordata. Data for number of grains per pistil transformed to common logarithms.

Variable	Mean	df	t-value
Pollen tube			
<u>glut. X rubra-b</u>	5.72	98	-3.34
<u>glut. X cord.</u>	8.20		
Grain Number			
<u>glut. X rubra-b</u>	1.1334	98	-2.10
<u>glut. X cord.</u>	.9161		

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