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Pollen Protein Synthesis and Control of Incompatibility in *Brassica**

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Summary. Excised but otherwise intact cabbage *(Brassica oleracea* var. *capitata)* stigmas release a water-soluble substance which selectively inhibits germination of self- but not cross-pollen. The inhibitory effect on self-pollen germination is dependent on both the concentration of stigma extract and on the time of addition. Low concentrations of stigma extract inhibit when present from the start (zero time additions) of pollen imbibition, whereas high concentrations do not. High concentrations inhibit when stigma extracts are added 1 to 2 minutes after the start of pollen imbibition, but germination is increasingly less inhibited when additions are delayed 2 to 4 minutes. Similar inhibition kinetics are also observed with delayed additions of cordycepin and cycloheximide. Stigma extracts selectively inhibit leucine-^{14}C incorporation into proteins of self-pollen. We conclude that germination does not require protein synthesis whereas the regulation of self-incompatibility does.

Introduction

Flowers of many higher plants contain an intraspecific recognition-system operating between pollen and papilla cells located on the stigma surface (Heslop-Harrison 1975). This recognition system controls self-incompatibility, which is widespread in the plant kingdom, and enhances evolutionary change by permitting cross- but not self-fertilization (Arasu 1968). This intraspecific incompatibility system is different from interspecific incompatibility, where responses of pollen to fertilization barriers are phenomenologically similar (Heslop-Harrison 1975).

Differentiation between self- and cross-pollen occurs within about 4 minutes after *Brassica* pollen contacts papillae cells on the stigma surface (Kroh 1967). Following self-recognition, pollen fails to germinate or germinates poorly so that germ tube penetration into the stigma, style and/or ovary is prevented. However, pollen from plants of compatible genotypes (cross-pollen) germinates readily.

In *Brassica*, at least 50 different alleles at the "S" locus control self-incompatibility (Ockendon 1975; Thompson 1968). The two alleles of a heterozygous plant interact with expressions of dominance, co-dominance, partial dominance or mutual weakening (Nasrallah 1974; Okendon 1975; Thompson and Taylor 1966). Interactions between alleles occur in both the stigma and pollen or both; but they may not be the same in both.

Immunological and electrophoretic data indicate that an S allele-specific incompatibility protein is produced in stigmas of *Brassica* flowers (Nasrallah and Wallace 1967; Nasrallah *et al.* 1970; Sedgley 1974). High concentrations of this protein are present on stigmas of open flowers which express strong self-incompatibility. Only low concentrations are present in stigmas prior to anthesis when they accept either self-or cross-pollen (Nasrallah 1974). This coincides with a stage in flower development (the bud stage) when self-incompatibility is either weak or not expressed (el Murabaa 1957). Also, a dominant suppressor or regulator allele at a specific locus, reduces the quantity of this stigma protein in a normally incompatible genotype, and thereby confers compatibility (Nasrallah and Wallace 1968; Nasrallah 1974). In addition to immunologically-detectable, genotype-specific stigma proteins, *Brassica* pollen also possesses biologically-detectable substances [which may or may not be the same molecule(s)]. These produce a rejection response (callose deposition) on papillae of self-, but not cross-stigmas (Heslop-Harrison *et al.* 1974). Further, excised cabbage stigmas *(B. oleracea* var.

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Table 1. Germination media for pollen from different incompatibility genotypes[a]

Incompatibility genotype	Medium	Medium components			
		PEG	Sucrose	H_3BO_4	$CaCl_2$
		% (w/v)	(M)	(mM)	(mM)
$S_{14}S_{14}$[b]	A	20	0.5	0.06	1.0
$S_{15}S_{15}$[b]	B	15	0.5	0.06	1.0
ǂ236[b], ǂ223, ǂ115	C	20	0.6	0.1	0.4
CS_3CS_3[b], $S_{14}CS_3$ $CS_{7a}CS_{7a}$, $CS_{7b}CS_{7b}$	D	30	0.4	0.4	12

[a] Alleles S_{14} and S_{15} are identified by the internationally accepted nomenclature originated by Thompson (1968), while CS_3, CS_{7a} and CS_{7b} have been studied only at Cornell and international nomenclature identity has not been determined. Genotypes ǂ236, ǂ223 and ǂ115 are plants of unknown and unstudied S allele genotypes.
[b] Media components optimized for pollen germination from these genotypes.
[c] Polyethylene glycol (PEG) was purified as described earlier (Ferrari and Wallace 1975).

capitata) release a water-soluble and heat-labile substance that inhibits self- but not cross-pollen from germinating *in vitro* (Ferrari and Wallace 1975).

Because the incompatibility response can be demonstrated *in vitro*, intraspecific incompatibility of *Brassica* is an ideal system for studying physiological, genetic and biochemical mechanisms involved in intercellular recognition. This investigation provides evidence that genotype-specific substances from stigmas rapidly interfere with pollen protein synthesis. We propose that early products of pollen protein synthesis regulate incompatibility.

Materials and Methods

Flowers were harvested from greenhouse-grown plants of *Brassica oleracea* var. *capitata* 1 to 3 days after anthesis. The stigmas were excised, and if not used immediately, air-dried at 20-24° C and kept frozen (-21° C) until used. Fresh pollen was released by stirring intact anthers into germination medium (one anther/0.05 ml) with a thin glass rod. Anthers were removed after pollen was released. Composition of the pollen germination medium was varied to optimize germination for the different genotypes studied (Table 1). All media contained 0.01 M Na phosphate buffer (pH 5.8).

Unless otherwise indicated, germination was measured 20-24 hours after addition of pollen to germination medium. Conditions for pollen germination and methods of measuring tube length and percent germination were as described earlier (Ferrari and Wallace 1975).

"Stigma extract" was prepared by incubating excised stigmas in germinated media (1 stigma/0.01 ml) at room temperature for approximately 15 minutes with occasional shaking. After removing the stigmas with a fine wire loop, aliquots were added to germination media to give a final volume of 0.05 ml and stigma extract concentrations as indicated in tables and figures.

Medium D (Table 1) was used for the two isotope incorporation experiments, variable conditions for which are indicated in Table 2. First stigmas were removed from germination media after approximately 15 min preincubation at room temperature with occasional shaking. For control purposes, isotope incorporation rates were determined in the absence of any stigma extract. To start the experiment, pollen was added by stirring into the media the indicated number of excised anthers (Table 2). At 10 minute

Table 2. Assay conditions for isotope incorporation experiments

Assay condition	Experiment	
	1	2
Vol. (ml) medium	0.8	0.5
uc leu-^{14}C (270 mc/mM)	0.80	0.25
No. self (CS_3CS_3) stigmas	20	10
mg/stigma (dr.wt.)	0.102	0.122
No. cross ($S_{14}S_{14}$) stigmas	20	10
mg/stigma (dr.wt.)	0.104	0.102
Pollen (CS_3CS_3), - no. anthers	12	10

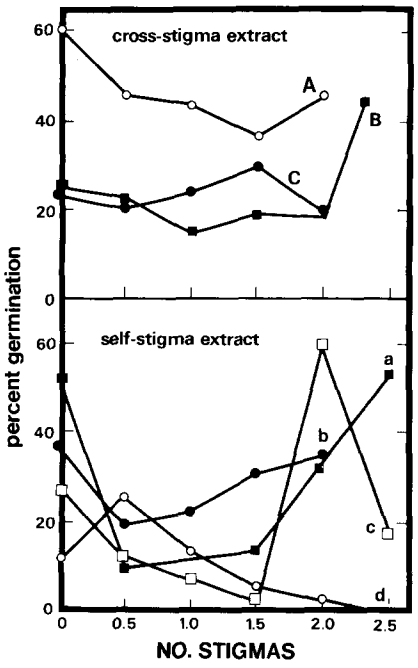

Fig. 1. Pollen germination in the continuous presence of increasing amounts of extracts from self- or cross-stigmas. Extracts were derived from the indicated number of stigmas with $S_{14}S_{14}$, $S_{15}S_{15}$ or CS_3CS_3 S-allele genotypes. Selfs: curve a was with CS_3CS_3 pollen; b, with $S_{15}S_{15}$ pollen; c and d with $S_{14}S_{14}$ pollen. Crosses: curve A was $S_{15}S_{15}$ pollen with CS_3CS_3 stigma extract; B, CS_3CS_3 pollen with $S_{15}S_{15}$ stigma extract; C, $S_{14}S_{14}$ pollen with CS_3CS_3 stigma extract. Each treatment contained pollen released from 1 anther. Air dry weights per stigma were: CS_3CS_3 = 0.098 mg; $S_{15}S_{15}$ = 0.099 mg; $S_{14}S_{14}$ = 0.092 mg

intervals, duplicate aliquots (0.03 ml each) of the medium were added to 10^{-3} M L-leucine-^{12}C in 2 ml of either: (A) methanol: chloroform: water mixture (12:5:3 w/v) to measure protein synthesis, or (B) 2 ml of 0.6 M sucrose to measure isotope uptake (Ferrari and Widholm 1973). (The high sucrose molarity in (B) was used to prevent grain bursting.)

Results

Pollen germination in the continuous presence of stigma extract

The effect on pollen germination of 6 stigma extract concentrations ranging between 0.0 (control) and 2.5 stigmas is shown in Fig. 1. Four experiments were performed with self-stigma extract (curves a-d) and three with cross-stigma extracts (curves A-C). Pollen germination was strongly inhibited in media containing self-stigma extracts derived from 0.5 to 1.5 stigmas, as compared to controls without stigma extract. Germination was much less inhibited by similar concentrations of cross-stigma extracts. At higher

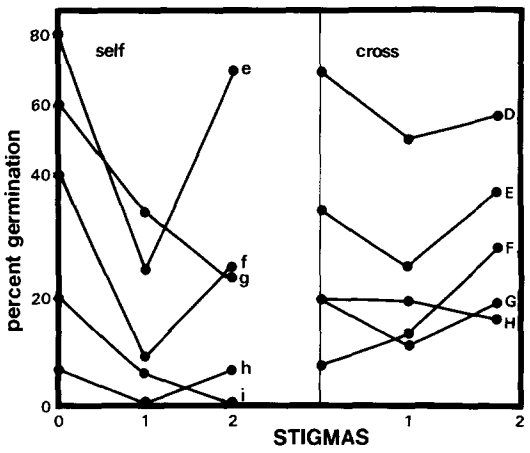

Fig. 2. Pollen germination in the continuous presence of extracts from cross- or self-stigmas. Extracts were derived from the indicated number of $S_{14}S_{14}$ or CS_3CS_3 stigma genotypes. Selfs: curves e-g were with CS_3CS_3 pollen; curve h and i with $S_{14}S_{14}$ pollen. Crosses: curves D, E and G were with $S_{14}S_{14}$ pollen; curves F and H were with CS_3CS_3 pollen. Each treatment contained pollen released from 1 anther. Air dry weights per stigma were: CS_3CS_3 = 0.103 mg; $S_{14}S_{14}$ = 0.118 mg

concentrations of both self- and cross-stigma extracts, beginning sometimes at 1.0 stigmas and most evident at about 2.0 to 2.5 stigmas, inhibition was decreased or not evident and germination often exceeded that of the controls (all curves except d).

Data for ten additional experiments using extracts from 0.0, 1.0 and 2.0 stigmas are presented in Fig. 2. For the five using self-stigma extract (curves e-i), pollen germination was strongly inhibited by extract from 1.0 as compared to 0.0 stigmas (control). For three of these five experiments (curves e, f and h), inhibition of germination disappeared and germination approached control percentages when extract concentrations were increased from 1 to 2 stigmas. For the five experiments using cross-stigmas, extracts from 1 stigma caused little or no inhibition of pollen germination (curves D-H). Higher concentrations of cross-stigma extracts caused stimulation (curve F) or partial to complete recovery of germination (curves D, E and G). Germination percentages exceeding control levels occurred for two of these five experiments with cross-stigma extracts (curves E and F).

Average maximum inhibition of germination as compared to controls was 77% ± 19% for the nine experiments (a-i) with self-stigma extracts, and 30% ± 15% for the eight (A-H) experiments with cross-stigma extracts (Fig. 1 and 2).

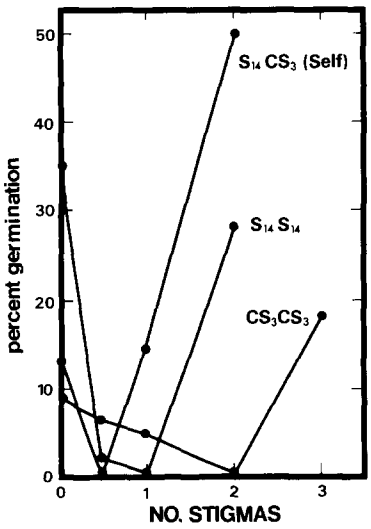

Fig. 3. Effect of stigma extract on germination of pollen from a heterozygous plant. Extracts were derived from the indicated number of stigmas with $S_{14}S_{14}$, CS_3CS_3 or $S_{14}CS_3$ S-allele genotypes. Media for each treatment contained $S_{14}CS_3$ pollen released from one anther. Air dry weights per stigma were: $S_{14}CS_3$ = 0.086 mg; $S_{14}S_{14}$ = 0.114 mg; CS_3CS_3 = 0.117 mg

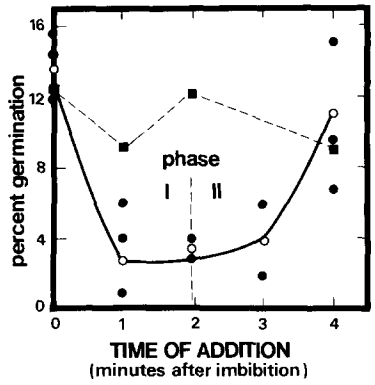

Fig. 4. Pollen germination following delayed additions of stigma extracts. Extracts were obtained from stigmas of plants 236, $S_{14}S_{14}$ or 115. Aliquots equivalent from 2 to 3 stigmas were added to self pollen at the indicated times after the start of imbibition. Open circles represent averages (solid line). Stigma extracts from plant 233 were added to $S_{14}S_{14}$ pollen as a cross pollen control (dashed line). Germination rates for pollen incubated without any stigma extract (control) were as follows: $S_{14}S_{14}$ = 12%; 236 = 12%; 233 = 8%; $S_{14}S_{14}$ = 9%

Considerable variability was observed in control pollen germination percentages, which ranged from about 10 to 80% (Figs. 1 and 2). Nevertheless, in all 9 experiments using self-stigma extract, strong inhibition of pollen germination resulted. In 6 of these 9 experiments (curves a, b, c, e, f, and h), self-stigma extract in excess of concentrations producing maximum inhibition caused germination to approach or exceed control percentages. The "U-shaped" character of these self-stigma inhibition curves was further supported in 3 additional experiments utilizing pollen from a plant heterozygous for incompatibility alleles S_{14} and CS_3. With this pollen, germination was inhibited by low concentrations of self-stigma extract or of extracts from stigmas of either the $S_{14}S_{14}$ or CS_3CS_3 homozygous parent (Fig. 3). *In vivo*, allele S_{14} is more strongly expressed than CS_3 for pollen from $S_{14}CS_3$ plants (unpublished data). This is consistent with the *in vitro* requirement of a higher concentration of CS_3 than S_{14} stigma extract to completely inhibit germination for this heterozygous pollen (Fig. 3).

Pollen germination following delayed additions of stigma extract

The inhibition of pollen germination by relatively low concentrations of self-stigma extracts, as discussed above and shown in Figs. 1-3, occurred when pollen was imbibed and germinated in the continuous presence of these extracts: yet higher concentrations often did not inhibit germination. When these same high concentrations (equivalent to about 2.0 or more stigmas) of self-stigma extract were added to media at increasing time intervals after addition of pollen, 2 response phases were distinguished (Fig. 4). Phase I was characterized by pollen germination becoming increasingly inhibited by self-stigma extract as time of extract addition was delayed about 1 to 2 minutes. Phase II was characterized by pollen germination becoming less and less inhibited as the time of addition of self-stigma extract was delayed from about 2 to 4 minutes. Pollen germination in the presence of cross-stigma extract did not show these inhibition kinetics (Fig. 4).

Pollen germination following delayed additions of cycloheximide and cordycepin

Cycloheximide (2×10^{-4}M) and cordycepin (10^{-3}M, 3-deoxyadenosine) affected pollen germination with the same inhibition kinetics as high concentrations of self-stigma extract (Figs. 5 and 6A). Phase I occurred when inhibitor addition was delayed 1-2 minutes after adding pollen to germination medium. During Phase II inhibition of germination decreased as inhibitor addition was delayed beyond 2 to 4 minutes. With a 5-

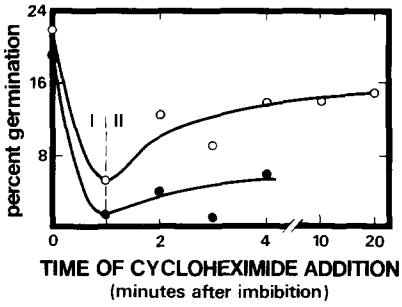

Fig.5. Pollen germination following delayed additions of cycloheximide. Pollen from plants #115 or #236 was added to germination medium (0.05 ml) containing 2×10^{-4} M cycloheximide, or added to medium to which cycloheximide was added at the indicated times after pollen suspension. Germination rates for pollen not treated (control) with inhibitor were 22% for #236 and #115

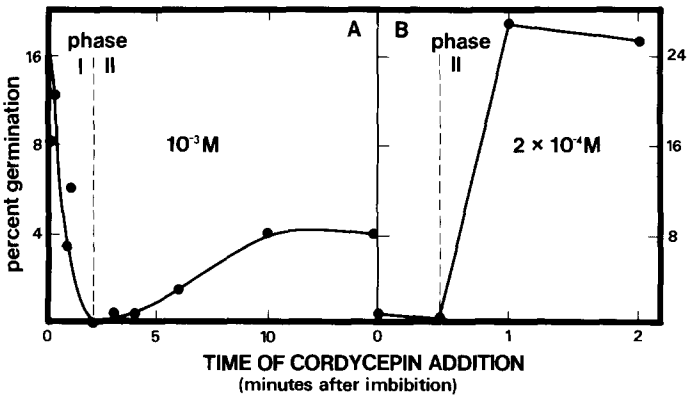

Fig.6. Pollen germination following delayed additions of cordycepin. Pollen from plant #236 was suspended in germination medium (0.05 ml) containing 10^{-3}M or 2×10^{-4}M cordycepin, or in medium to which cordycepin was added at the indicated time after pollen suspension. Germination rates for pollen not treated with inhibitor (controls) where 15% for the experiment with 10^{-3} M cordycepin (panel A) and 21% for that with 2×10^{-4} M (panel B)

fold lower concentration of cordycepin (2×10^{-4}M), stimulation during Phase II was detected, but increasing inhibition during Phase I was not (Fig.6B).

Effect of stigma extract on leucine-^{14}C incorporation into pollen protein

Leucine-^{14}C incorporation into newly synthesized pollen proteins was 70-90% inhibited by self- relative to cross-stigma extract or controls with no stigma extract (Fig.7). The incorporation rate in the presence of self-stigma extract was linear with no lag phase. Cross-stigma extract had little or no effect on the rate of isotope incorporation relative to control incorporation rates. A higher concentration of isotope was used for experiment 2 than 1. Leucine-^{14}C uptake was relatively unaffected by self-stigma extract compared to cross-stigma extract; however, both were inhibited about 50% compared to the control with no stigma extract.

Discussion

Germination of *Brassica oleracea* var. *capitata* pollen was selectively inhibited by relatively low concentrations of soluble substances present in self- but not in cross-stigma extracts (Figs.1-3). However,

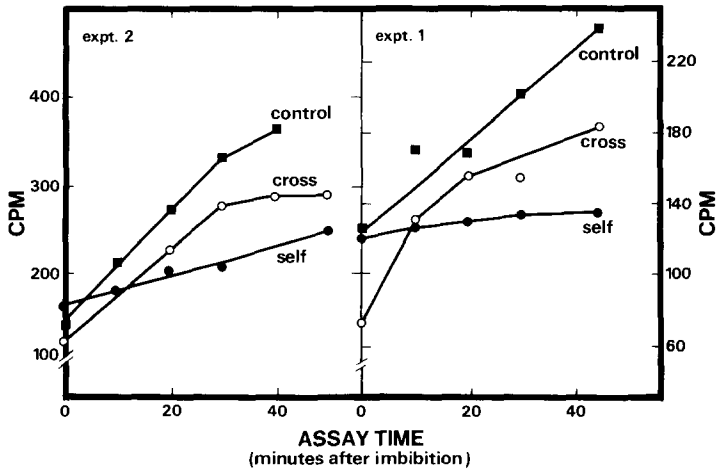

Fig.7. Effect of stigma extracts on incorporation of leucine-^{14}C into pollen protein. Experimental conditions are presented in Methods

at increasingly higher concentrations, inhibition disappeared and pollen germination sometimes exceeded controls with no stigma extract. These concentration dependent effects held only when pollen was imbibed and germinated in the continuous presence of self-stigma extract. When high concentrations of self-stigma extract were added to media 1 to 2 minutes after the start of pollen imbibition, germination was increasingly inhibited (Phase I) (Fig. 4). Delaying addition of stigma extract approximately 2 to 4 minutes caused germination to become increasingly insensitive to inhibition (Phase II). (Time of maximum inhibition of germination by stigma substance varied from experiment to experiment, but usually occurred 1 to 2 minutes after adding pollen to medium.) Similar inhibition kinetics occurred using high concentrations (10^{-3}M to 10^{-4}M) of cordycepin and cycloheximide, inhibitors of RNA and protein synthesis, respectively (Fig. 5 and 6A). The high concentrations of these chemicals apparently had no side effects, because pollen germination in their continuous presence (zero time additions) was not different from germination in their absence (values in legends to Figs. 5 and 6). That high concentrations of cycloheximide and cordycepin failed to block germination when pollen was incubated in their continuous presence, is strong evidence that mature pollen contains in presynthesized form all the enzymes required for tube elongation. Cycloheximide also had no effect on *in vitro* germination of *Lilium* (Ascher and Drewlow 1970), *Impatiens* (Shivanna *et al.* 1974a) and *Tradescantia* pollen (Mascarenhas 1975), and only inhibited *Trigonella* pollen germination by about 30% (Shivanna *et al.* 1974b). However, with *Impatiens* and *Trigonella* pollen, nuclear division was totally inhibited (Shivanna *et al.* 1974a and b).

That pollen germination inhibition kinetics for self-stigma extract (Fig. 4) were similar to those of protein and RNA synthesis inhibitors (Figs. 5 and 6A), suggested that incompatibility substance(s) in self-stigma extracts might also interfere with protein synthesis. Indeed, substances in self-stigma extract did selectively inhibit leucine-^{14}C incorporation into pollen protein (Fig. 7). Inhibition of protein synthesis was linear, had no lag phase and was detected only 10 minutes after adding pollen to germination medium. This was long before emergence of the pollen tube was observed, which *in vitro* usually occurred 3 to 4 hours after pollen imbibition. This implies that at the time inhibition was observed, protein synthesis was concerned more directly with events leading to recognition and control of germination, than with processes related to subsequent tube elongation. *In situ* pollen transfer experiments support our conclusion that recognition of self vs. non self is complete within about 4 minutes of imbibition. Kroh (1967) observed that *Brassica* pollen germinated and penetrated papillae on self-stigmas at rates comparable to cross-pollen if self-pollen was first placed on a cross-stigma for a minimum of 4 minutes. Thus, events that result after failure to recognize self, were irreversibly completed during the 4 minute "preincubation" of pollen on a cross-stigma.

We conclude that germination does not require protein synthesis whereas the regulation of incompatibility does. A hypothesis on the role of protein synthesis and of the genotypespecific recognition system in control of sexual incompatibility in pollen-stigma relationships is in preparation.

Inhibition of pollen germination by self-stigma extract averaged 70% and 30% for cross-stigma extract. Cross-stigma extract did not always inhibit germination, whereas self-stigma extract did. This inconsistency with cross-stigma extract suggests the need for further characterization of inhibitory and stimulatory substances in crude stigma extracts. Sephadex chromatography of stigma extract indicated the presence of a low molecular weight substance(s) which was stimulatory to both cross and self pollen germination (unpublished experiments); this also indicates the need for separating inhibitory from stimulatory substances that are present in crude stigma extracts.

Literature

Arasu, N.T.: Self-incompatibility in angiosperms: a review. Genetica 39, 1-24 (1968)

Ascher, P.D.; Drewlow, L.W.: The effect of cycloheximide and 6-methyl purine on *in vivo* compatible and incompatible pollen tube growth in *Lilium longiflorum*. Theoret. Appl. Genetics 40, 173-175 (1970)

Ferrari, T.E.; Wallace, D.H.: Germination of *Brassica* pollen and expression of incompatibility *in vitro*. Euphytica 24, 757-765 (1975)

Ferrari, T.E.; Widholm, J.M.: A simple, rapid, and sensitive method for estimation of DNA, RNA, and protein synthesis in carrot cell cultures. Analyt. Biochem. 56, 346-352 (1973)

Heslop-Harrison, J.: Incompatibility and the pollen-stigma interaction. Ann. Rev. Plant Physiol. 26, 403-425 (1975)

Heslop-Harrison, J.; Knox, R.B.; Heslop-Harrison, Y.: Pollen-wall proteins: exine-held fractions associated with the incompatibility response in Cruciferae. Theoret. Appl. Genetics 44, 133-137 (1974)

Kroh, M.: Reaction of pollen after transfer from one stigma to another (Contribution to the character of the incompatibility mechanism in *Cruciferae*). Züchter 36, 185-189 (1967)

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

Murabaa, A.I.M.el: Factors affecting seed set in Brussels sprouts, radish and cyclamen. Publ. 168 Lab. voor Tuinbouwplantenteelt Landbouwhogeschool, Wageningen, Nederland (1957)

Nasrallah, M.E.: Genetic control of quantitative variation in self-incompatibility proteins detected by immunodiffusion. Genetics 76, 45-50 (1974)

Nasrallah, M.E.; Barber, J.T.; Wallace, D.H.: Self-incompatibility proteins in plants: detection, genetics, and possible mode of action. Heredity 25, 23-27 (1970)

Nasrallah, M.E.; Wallace, D.H.: Immunogenetics of self-incompatibility in *Brassica oleracea* L. Heredity 22, 519-527 (1967)

Nasrallah, M.E.; Wallace, D.H.: The influence of modifier genes on the intensity and stability of self-incompatibility in cabbage. Euphytica 17, 495-503 (1968)

Ockendon, D.J.: Dominance relationships between S-alleles in the stigmas of Brussels sprouts (*Brassica oleracea* var. *gemmifera*). Euphytica 24, 165-172 (1975)

Sedgley, M.: The concentration of S-protein in stigmas of *Brassica oleracea* plants homozygous and heterozygous for a given S-allele. Heredity 33, 412-416 (1974)

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

Shivanna, K.R.; Jaiswal, V.S.; Mohan Ram, H.Y.: Effect of cycloheximide on cultured pollen grains of *Trigonella foenumgraecum*. Plant Science Letters 3, 335-339 (1974b)

Thompson, K.F.; Taylor, J.P.: Non-linear dominance relationships between S alleles. Heredity 21, 345-362 (1966)

Thompson, K.F.: Classified S alleles for *Brassica* breeders. In: Dixon, G.G. (Ed.), Proceedings of Brassica Meeting of Eucarpia, pp. 25-28. National Vegetable Research Station, Wellesbourne, England (1968)

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