Bio-Gel P-2 column void volume fraction (~15 ml) was passed through the C-25 column, and the column was then eluted with 20 ml of water. These fractions were combined and passed through the A-25 column, and it was eluted with 20 ml of water. Large amounts of yellow and brown contaminants were retained on the columns. These contaminants had no chemotactic activity.

10. The molecular weight standards used were: bovine immunoglobulin IgM (845,000), bovine IgG (169,000) (Sigma), human IgG (160,000), horse apoferritin (466,900), and bovine serum albumin (68,000) (Schwarz/Mann).
11. A column (1 cm by 5 cm) of concanavalin A–Sepharose (Sigma) was washed with 0.1*M* KCl or 0.1*M* α-methyl-D-mannoside and then rinsed with distilled water. Purified chemotactin was passed through the column, followed by a distilled water rinse. The chemotactin was removed by 0.1*M* KCl or 0.1*M* α-methyl-D-mannoside and dialyzed against water for 2 days.
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13. Chemotaxis was negative (i) if the repellent gave counts that were significantly lower than those of the buffer control in the standard assay or (ii) if more bacteria swam into a pipette free of the repellent than into one that contained repellent, when the compound was present in the bacterial solution (repellent in the pool experiments). Peanut agglutinin and concanavalin A are repellents in both of these tests.
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19. We thank N. Sharon, Weizmann Institute, for his gift of lectins from peanut, soybean, and wheat germ; R. Valentine, University of California, Davis, for the cowpea strain of *Rhizobium* (32H1); and J. Burton, Nitragin Company, for the other five strains of rhizobia (trefoil strain 95C13, sainfoin strain 116A12, *Rhizobium japonicum* strain 61A76, and *R. meliloti* strains 102F51 and 102F66). We also thank M. McNeil in the laboratory of P. Albersheim for sugar analyses and K. Hapner for amino acid analysis. This work was supported in part by grant BM 575-06758 from the National Science Foundation and is paper No. 700 of the Montana Agricultural Experiment Station.

1 June 1976; revised 2 November 1976

## Incompatibility on *Brassica* Stigmas Is Overcome by Treating Pollen with Cycloheximide

Abstract. *Pollen of* Brassica *exhibited strong self-incompatibility. It did not germinate on, adhere to, or extend into the stigmatic tissues of the female parent plant. In contrast, pollen that had been treated with cycloheximide germinated on and penetrated into self-stigmas to the same degree as untreated pollen placed on cross-stigmas (compatible). Germ tubes from cycloheximide-treated pollen did not continue growth through stylar tissues and therefore were unable to effect fertilization.*

The translational inhibitor cycloheximide (*1*) only partially reduces or has no effect in vitro on the germination of pollen from *Lilium* (*2*), *Trigonella* (*3*), *Impatiens* (*4*), *Antirrhinum*, *Amaryllis*, and *Tradescantia* (*5*). In contrast, cycloheximide has blocked germination of *Tradescantia* (*5*) and *Lilium* and *Clivia* (*6*) pollens in vitro. This same contrast was obtained with *Brassica* by adding cycloheximide at different times to media containing germinating pollen (*7*). A high concentration ($2 \times 10^{-4}M$) of cycloheximide in the medium when the pollen was added did not inhibit germination while, paradoxically, strong inhibition resulted when the same high concentration was added to the medium 1 to 2 minutes after the pollen.

The germination in vitro in the presence of a high cycloheximide concentration suggested that new protein synthesis by pollen was not required for germination. The inhibition of germination caused by the delayed addition of the same high concentration of cycloheximide suggested that a protein or proteins synthesized by pollen between the time of placement of pollen in synthetic media and the addition of cycloheximide 1 to 2 minutes later might cause the inhibition of pollen germination.

These apparently protein-regulated variations of pollen germination in vitro might be involved with the regulation of incompatibility in vivo, since extracts of self-incompatible stigmas (that is, stigmas of the same plant that produced the pollen) had the same concentration- and time-dependent effects on pollen germination as cycloheximide (*7*). Extracts of cross-stigmas (compatible) had little or no effect (*7*). The extracts consisted of medium in which a given number (concentration) of stigmas had been incubated for about 10 minutes and then removed. Also, extracts from self- but not cross-stigmas rapidly (within 10 minutes) blocked the incorporation of $^{14}C$-labeled leucine into pollen proteins (*7*). These results suggested that the endogenously synthesized pollen protein or proteins postulated to "turn off" pollen germination in vitro are involved with the regulation of self-incompatibility. Developmentally, the expression of incompatibility in *Brassica* in vivo is failure of the pollen to germinate on and adhere to the papillae of self-stigmas (*8*).

Additional evidence, obtained in situ, is now reported which supports the evidence obtained in vitro that germination of *Brassica* pollen does not require protein synthesis, whereas expression of self-incompatibility does. The prior treatment of *Brassica* pollen with cycloheximide overcame developmental expression of incompatibility: pollen so treated germinated and adhered to papillae of self-stigmas.

Pollen was treated in a "dry" state with cycloheximide as follows. Cycloheximide was dissolved in absolute methanol, and 0.1-ml portions were added to glass (Pyrex or Kimax) tubes (10 mm by 70 mm) to give the quantities indicated in Tables 1 and 2 and Figs. 1 to 3. The methanol was evaporated off by passing a gentle stream of air over the liquid surface while the tube was rotated. Heat applied to the base of the tube in a water bath at 60°C sped evaporation, which took approximately 30 seconds and coated 0.5 to 1.0 cm of the lower part of the tube with cycloheximide. Six fully dehisced anthers from one flower of greenhouse-grown *Brassica oleracea* var. *capitata* were added to each tube, and the pollen was released by tapping the tubes firmly against a wood bench. Emptied anthers were discarded by carefully inverting each tube. Most of the released pollen adhered to the cycloheximide-coated tube wall. From 30 to 60 minutes later, the pollen was

Table 1. Mean scores for relative number of pollen tubes detected in stigmatic and stylar tissues after treatment of either the pollen or the stigmas with cycloheximide (10 μg). The scores are averages of five or six pollinations. A score of 0.0 indicates no pollen tubes and 5.0 indicates 100+ tubes, as defined in more detail in Fig. 1. The untreated pollen represents a solvent control (methanol minus cycloheximide).

| Experimental | Penetration of pollen tubes into stigmas and styles (relative score) | | | |
|---|---|---|---|---|
| | Self-pollinated | | Cross-pollinated | |
| | Stigma | Style | Stigma | Style |
| Untreated pollen (control) | 0 | 0 | 4.3 | 3.8 |
| Cycloheximide-treated stigma | 0.7 | 0.0 | 4.0 | 0.5 |
| Cycloheximide-treated pollen | 4.2 | 0.4 | 3.8 | 0.0 |

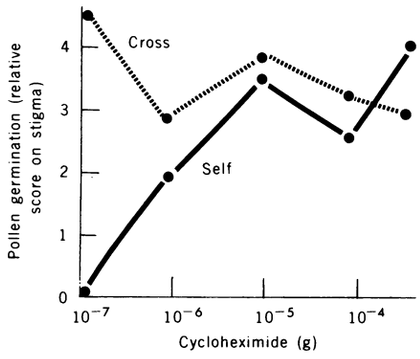


Fig. 1. Effect of cycloheximide on the relative number of germinated pollen grains detected in self- and cross-stigmas. Arbitrary scores were assigned as follows: scores of 0, 1, 2, 3, 4, and 5 represent, respectively, 0, 1 to 3, 4 to 10, 11 to 50, 51 to 100, and more than 100 germ tubes in or on the indicated tissues. Controls (without cycloheximide) were obtained by placing pollen in tubes from which methanol had been evaporated.

picked up with a camel's-hair brush and transferred to self- or cross-stigmas. For a control, pollen was also added to tubes which had contained the methanol but no cycloheximide. As controls for the treated stigmas, pollen was omitted from tubes containing cycloheximide, a brush was wiped against the inside of the tube, and stigmas were then brushed as though pollen were present; untreated pollen was subsequently applied to effect pollination. About 24 hours after pollination, pollen germination on the stigma and tube penetration into stylar tissues were assayed by means of the stigma squash fluoresence technique (*9*), and arbitrary scores were assigned as described in Fig. 1.

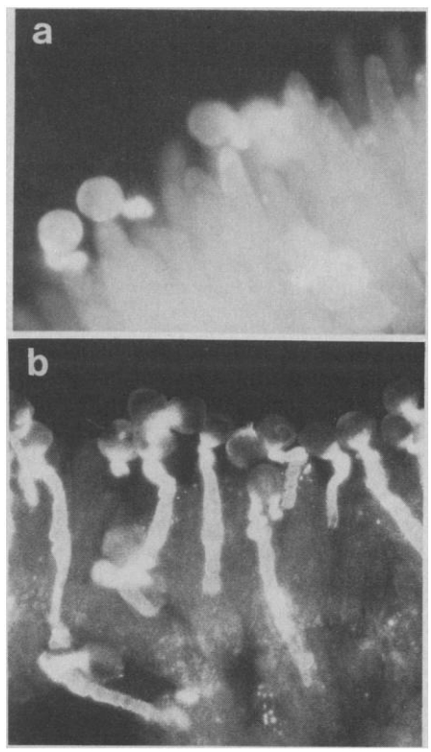


Fig. 2. Fluorescent micrographs of stigmas showing papillae and germinated self-pollen grains following pollination without (a) or with (b) cycloheximide-treated pollen. Tissues were stained with aniline blue (*9*) (×90).

Control pollen with no cycloheximide exhibited strong self-incompatibility as evidenced by its inability to germinate on and adhere to the papillar tissues of self-stigmas; when pollen grains did occasionally germinate, they formed short, twisted, or distorted germ tubes which did not extend through the papillar tissue (Fig. 2a). However, pollen treated with increasing amounts of cycloheximide germinated (Fig. 1) and adhered to papillae of self-stigmas (Fig. 2b); furthermore, the tubes frequently extended into the immediately subjacent stigmatic tissue.

The pollen was treated with cycloheximide in test tubes, and was then picked up on a camel's-hair brush and applied to stigmas. Thus, it was possible that cycloheximide carried to stigmatic papillae on bristles of the brush overcame the inhibition of self-pollen germination by interfering with stigmatic protein synthesis. However, untreated pollen placed on self-stigmas previously brushed with cycloheximide still manifested strong incompatibility. The scores for pollen tube penetration of self-stigmas for untreated pollen, cycloheximide-treated stigmas followed by untreated pollen, and for cycloheximide-treated pollen indicate average germ tube numbers of 0, below 3, and above 50, respectively (Table 1 and Fig. 1). The approximately 20-fold larger effect of treating the pollen with cycloheximide rather than the stigma strongly indicates that cycloheximide overcame the incompatibility by perturbing protein synthesis in the pollen. The slight increase in germination caused by treating the stigma with cycloheximide could have been the result of some pollen rapidly absorbing cycloheximide from the treated stigmas. The experiments in vitro (*7*) as well as those in situ suggest that pollen that absorbs sufficient cycloheximide during imbibition will germinate. It may be impossible to distinguish the small effect on pollen germination caused by treatment of the stigma from the large effect of treating the pollen with cycloheximide, because with these assays in situ there is always the possibility of cycloheximide being transferred from the stigma to the pollen or vice versa.

The inability of pollen to germinate and adhere to tissues of self-stigmas was overcome by cycloheximide, but germ tubes from treated pollen did not penetrate either self- or cross-styles (Fig. 3 and Table 1). Consequently, seed set was also strongly inhibited when cycloheximide-treated pollen was used for self- or cross-pollinations (Table 2). Thus, cycloheximide treatment of pollen overcame the developmental expression of incompatibility, but it also prevented a subsequent stage of pollen tube development: that of penetration into and growth through the style. The data do not distinguish whether germ tube penetration into the style was prevented by a perturbed pollen or stigmatic function, because untreated pollen also did not penetrate the styles of cycloheximide-treated cross-stigmas (Table 1). Thus, either the cycloheximide blocked synthesis by the stigma or pollen (or both) of some essential enzyme or regulator necessary for development of a stylar penetration capacity, or unknown side effects were responsible, for example, a wound to the stigma might prevent tube penetration into the style. Cycloheximide

Table 2. Effect of cycloheximide-treated pollen on the number of seed set after self- or cross-pollination. Pollen was treated with cycloheximide prior to pollination as described in the text. Each seed set value is an average of six to eight separate pollinations.

| Cycloheximide ($\mu$g) | Seeds set (number of seeds per pod) | |
|---|---|---|
| | Self-pollination | Cross-pollination |
| 0 | 0.2 | 18.0 |
| 2 | 0.0 | 3.0 |
| 10 | 0.0 | 0.0 |
| 100 | 0.0 | 0.9 |
| 500 | 0.0 | 0.0 |

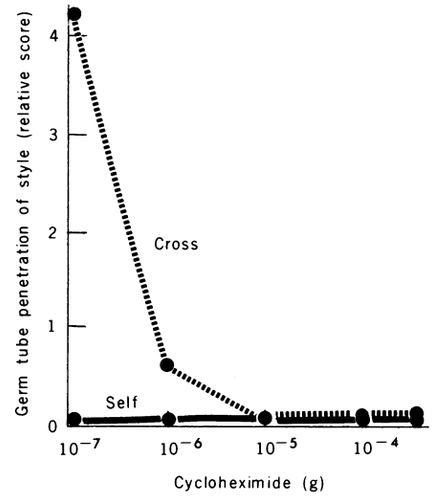


Fig. 3. Effect of cycloheximide on the number of pollen tubes detected in self- or cross-styles. Relative scores and controls are as described in Fig. 1.

caused numerous fluorescent spots to develop on papillae (Fig. 2b) which were similar to, but considerably smaller than, the fluorescent "callose rejection reaction" noted by others (*8*). Cycloheximide has also been shown to block migration of pollen nuclei into the germ tubes of *Trigonella* (*3*), *Impatiens* (*4*), and *Tradescantia* (*5*); and to prevent or retard entry of nuclei into metaphase with pollen from *Ornithogalum* and *Tradescantia* (*5*).

Sarfatti *et al.* (*10*) observed that treating pollen from *Lycopersicon* with actinomycin D overcame self-incompatibility. The concentric array of endoplasmic reticulum found in incompatible pollen tubes of *Lycopersicon* suggested that incompatibility might lead to a general cessation of protein synthesis (*11*).

That cycloheximide failed to block germination in situ of *Brassica* pollen on cross-stigmas (compatible), and facilitated germination of self-stigmas (incompatible), supports the previous results in vitro indicating that pollen contains all the enzymes required for tube elongation (*7*). That the cycloheximide-treated self-pollen germinated and adhered to papillae of tissues of self-stigmas, whereas untreated self-pollen did not, indicates that pollen protein synthesis must be required for expression of incompatibility in vivo. These data for *Brassica* pollen in situ are in agreement with the hypothesis that the control of pollen germination in vivo and in vitro by cycloheximide is indirect and results from the blockage, in pollen, of the synthesis of proteins that regulate the expression of self-incompatibility.

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## In vitro Growth of Imaginal Disks from *Drosophila melanogaster*

Abstract. *Imaginal disks were cultured in a new medium in vitro. Optimal growth occurred when the medium was supplemented with insulin and a juvenile hormone analog and conditioned by larval fat body. The disks grow in vitro by normal cell division and maintain their capacity to differentiate into normal patterns of adult cuticular structures.*

During the embryogenesis of *Drosophila melanogaster* and related insects, some of the cells are programmed (that is, determined) to become imaginal disk cells (*1*). These cells do not immediately differentiate or become polytene as do larval cells. Instead, they proliferate as determined cells throughout larval life.

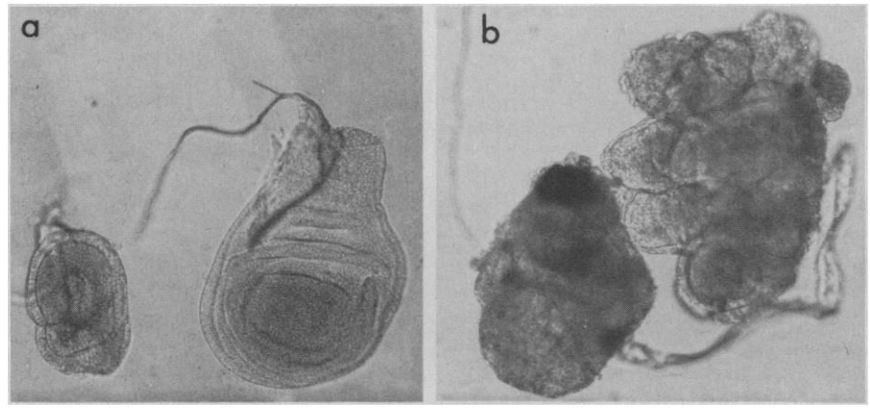


Fig. 1. Wing and leg imaginal disks. (a) Disks dissected from mature third instar larvae. (b) Explanted disks cultured for 2 weeks in medium XCS in vitro.

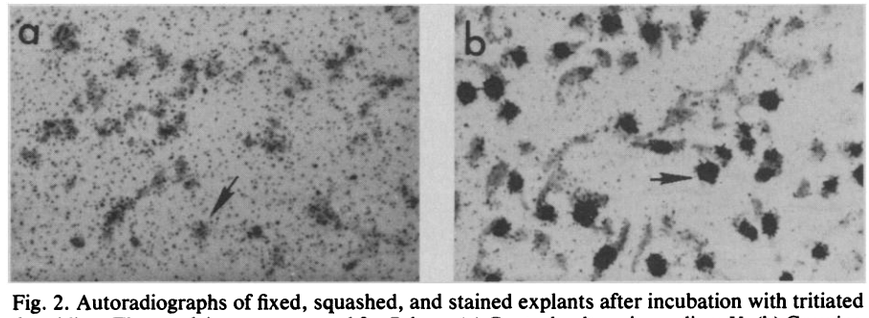


Fig. 2. Autoradiographs of fixed, squashed, and stained explants after incubation with tritiated thymidine. The emulsion was exposed for 7 days. (a) Control culture in medium X. (b) Growing culture in medium XCS. Arrow indicates intense grain density over a nucleus.

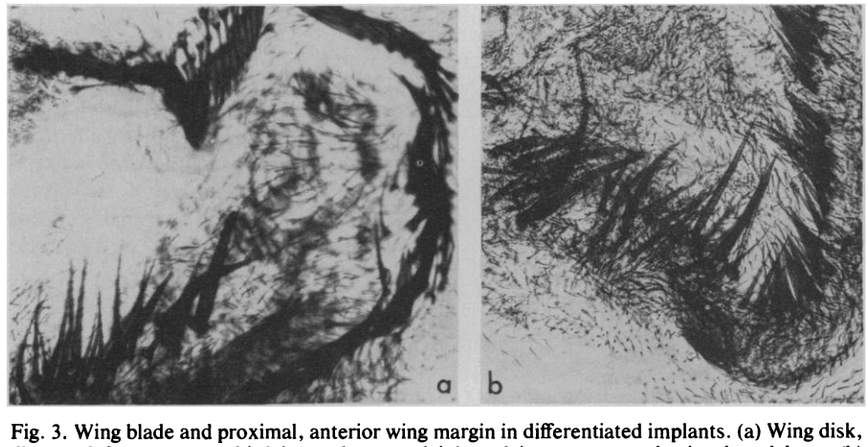


Fig. 3. Wing blade and proximal, anterior wing margin in differentiated implants. (a) Wing disk, dissected from mature third instar larva and injected into metamorphosing larval host. (b) Fragment of an explant cultured in medium XCS for 2 weeks and injected into metamorphosing larval host.