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Growth of *Trifolium pratense* L. Pollen Tubes in Compatible and Incompatible Styles of Excised Pistils

II. Pollen Treatments[1]

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Summary. The excised pistil technique was used to study effects of pollen treatments on self-incompatibility in red clover. Pollen was treated directly with infrared or ultraviolet radiation, and indirectly with chemicals that were applied to the stigmas before pollination. The chemicals (boric acid, calcium nitrate, colchicine, cyclohexamide, gibberellic acid, glycine, and indole butyric acid) were each used at five concentrations. High dosage rates of most treatments inhibited pollen growth in styles that were genetically compatible, but none of the treatments had a specific effect on the self-incompatibility mechanism.

The effects of growth of compatible or incompatible pollen tubes in a style on subsequent growth of both types of pollen tubes in the same style were studied in an experiment with 6 hr between pollinations. The passage of some compatible pollen tubes through the style did not influence either self- or cross-compatibility in the style after the second pollination. The passage of some self-incompatible tubes into the styles caused a slightly higher number of incompatible tubes to penetrate the style after the second pollination. Each pollen tube appeared to traverse the style independently of other tubes.

Introduction

The first papers of this series (Kendall, 1967; Kendall and Taylor, 1969) describe experiments in which heat or chemical treatments were applied to red clover (*Trifolium pratense* L.) plants to influence self-incompatibility. Application of high temperature to flower heads during anthesis inactivated the fraction of the self-incompatibility mechanism located in the styles but did not affect the fraction in the pollen. The chemicals were applied to the styles of excised pistils. Some of the chemicals enhanced growth of pollen in the styles but none were effective in breaking down self-incompatibility. In one of three experiments the application of large amounts of pollen to the stigma rendered the incompatibility mechanism less effective.

The mechanism of self-incompatibility cannot be described in detail; however it may be inferred from some lines of evidence (Lewis et al., 1967; Nasrallah et al., 1970) that it involves protein molecules in an antigen-antibody type of reaction. It has been possible to block some antigen-antibody systems of animal origin with amino carboxylic acids (Atchley and Bhagavan, 1962). Two amino carboxylic acids (gamma aminobutyric acid and glycine) enhanced growth of some cultures of red clover pollen *in vitro* (Kendall et al., in press). Thus these chemicals at low concentrations might serve as a nutrient for pollen tube growth in the styles, while high concentrations might interfere with the incompatibility mechanism.

The apparent high osmotic pressure of red clover pollen (Kendall, 1967) and the failure to influence the pollen fraction of the incompatibility mechanism suggested that the pollen grains were more tolerant than the pistils to heat and chemical treatments. Pollen treatments might serve to supplement (or provide a more convenient alternative) to the anthesis temperature treatment for inbreeding red clover, provided pollen viability and its portion of the self-incompatibility mechanism were independently affected by the treatment. This paper describes treatments which were designed to influence the pollen fraction of the self-incompatibility mechanism. Objectives of the research were to develop a more effective means for breaking self-incompatibility, and to provide more information on the mechanism involved.

Materials and Methods

Flowers were obtained from plants of 'Kenland' red clover (*Trifolium pratense* L.) grown in a greenhouse. The greenhouse was shaded during summer months, and in winter months the photoperiod was extended to 18 hr with tungsten bulbs.

All treatment effects were evaluated with cultures of excised pistils. The method was modified from that used previously (Kendall, 1968) in that the media were partially solidified with 0.5% agar to hold the basal end of the styles in position; and the glass beads previously used for that purpose were omitted. The florets were cut off the

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flower heads at a height just above the ovulary of the pistil, and the keel petals were discarded. Thus, an excised pistil consisted of the stigma and style surrounded by the anthers and corolla tube. With compatible matings pollen germinated on the stigmas and grew through the styles into the agar-nutrient medium.

Chemicals were applied to the stigmas of flowers on excised stems. Flower stems about 10 cm long bearing heads with most of the florets opened were excised daily and maintained during treatment in the laboratory with the basal 5 cm of the stems in jars of water. For each treatment 10 florets of a flower head were tripped and the keel petals pulled off. Usually the stigma and anthers remained intact and in close proximity to the standard petal. The stigma was then submerged in a drop of solution which was held in place by the anthers and standard. The drop of solution was maintained around the stigma for 1 to 2 min and then blotted off with absorbent paper. Pollen of the same (incompatible) or different (compatible) genotypes as the floret was placed on the stigma with a dissecting needle.

The concentrations of chemicals used were determined in preliminary experiments with compatible matings. Solutions that inhibited pollen growth in compatible matings were used in final experiments with both mating types at the concentration that caused 50% inhibition, and one above and three below that level. Solutions which did not inhibit pollen growth were used at five levels up to the maximum solubility of the chemical or within the usual physiological ranges.

Pollen spread on a piece of aluminium foil was irradiated with infrared or ultraviolet light. About 10 to 20 florets were inverted and tripped a few cm over the aluminium foil to accumulate the pollen. Each source of radiation was located 5 cm above the pollen and was operated to provide treatments of 0, 5, 15 or 30 min. The infrared treatment was obtained with a Sylvania* infrared 250 watt bulb. The ultraviolet source provided 1600 microwatts/cm^2 at a wavelength of 254 mμ as indicated by the manufacturer's specifications. Pollen was transferred with a dissecting needle from the aluminium foil to florets that had their keel petals removed. The pollinated florets were excised and set in caps as described above.

To study effects of pollen growth through a style on subsequent growth of other pollen tubes in the same style we first either self or cross-pollinated all of the florets on an excised flower stem. Immediately after pollination 10 florets were excised and established in caps. Six hours after the first pollination the florets that were left on the excised stems were divided into three groups of 10 florets per group, and treated as follows: no treatment, self-pollinated, or cross-pollinated. The repollinated florets, and their controls, were excised and established in the caps.

Results

Chemicals which were applied to the stigmas are listed in Table 1. This table also shows the concentrations of the chemicals which inhibited pollen growth when used with genetically compatible matings. The same chemicals at various concentrations did not enhance growth of pollen tubes through styles of excised pistils with genetically incompatible matings.

* Mention of specific products is for identification only and does not imply endorsement of the product by the U.S. Department of Agriculture.

Table 1. *Chemicals applied to the stigmas of red clover and the concentrations that either prevented pollen growth through 50% of the styles or the maximum concentrations used with their corresponding percent of inhibition when evaluated with genetically compatible matings in excised pistils*

Chemicals	Concentrations	Pollen inhibition (%)
Cycloheximide	28 ppm	50
Boric acid	0.03 M	45
Calcium nitrate	0.08 M	0
Colchicine	500 ppm	50
Gibberellic acid	1750 ppm	0
Glycine	2500 ppm	0
Indole butyric acid	100 ppm	25

None of the radiation treatments with the infrared source affected pollen growth in either the compatible or incompatible mating types of excised pistils. The 5-min treatment with ultraviolet radiation caused a 50% inhibition with compatible matings. The maximum inhibition was 58% at the 30-min treatment. None of the ultraviolet radiation treatments affected pollen growth in matings that were incompatible.

Results of experiments with two pollinations are shown in Table 2. After the first pollination pollen tubes grew through most excised pistils with genetically compatible matings, and a small percentage of pistils with incompatible matings. Pistils that were originally crossed remained compatible to additional cross-pollen and incompatible to self-pollen. Pistils that were originally selfed became slightly less incompatible to additional self-pollen and retained their compatibility with cross-pollen.

Table 2. *Percentage of excised pistils of red clover with pollen tubes protruding from the basal end of the style after single or double pollinations and a time interval of 6 hr between pollinations*

Original mating	Styles pollen traversed (%)			
	First pollination	Second pollination		
		None	Self	Cross
Cross	92 a*	69 c	69 c	84 b
Self	2 c	3 c	12 b	73 a

* Duncan's Multiple Range Test. Means within a row with the same letter do not differ at the 5% level of significance.

Discussion

The chemicals and pollen were placed on the stigma separately in lieu of liquid pollination as described by Binek and Bingham (1969) for alfalfa. Although the liquid pollination technique was designed for treating pollen with chemicals, we found that red clover pollen did not readily separate from the solutions, and that wet pollen grains did not adhere to the stigma.

Two experiments with compatible matings of excised pistils were designed to demonstrate that

chemical applied to the stigma might influence subsequent growth of pollen in the styles. In one experiment stigmas were treated with cycloheximide at various concentrations. Cycloheximide inhibits protein synthesis and therefore was expected to inhibit pollen tube elongation. No pollen tubes grew through styles when the pistils were treated with more than 50 ppm of cycloheximide. At concentrations between 5 and 50 ppm the inhibition was linear which indicated the technique provided good sensitivity to this chemical. In a second experiment a drop of neutral red (1000 ppm) was applied to the stigmas. The portion of the pollen tubes which grew out of the cut ends of the pistils contained traces of neutral red which were visible only under high magnification in a microscope.

Cycloheximide was also applied to pistils that were self-pollinated to evaluate effects of the chemical on the self-incompatibility mechanism. None of the concentrations were effective in breaking self-incompatibility. These results with compatible and incompatible matings for red clover were the same as reported by Ascher and Drewlow (1970) for similar types of experiment with *Lilium longiflorum* Thunb.

The organic and inorganic nutrients that are known to affect red clover pollen tube elongation were supplied in high concentrations to the stigmas in the present experiments and to the basal cut ends of the styles in the former study. None of these treatments enhanced pollen tube elongation sufficiently to overcome self-incompatibility in red clover. The present experiments with calcium nitrate were repeated on three occasions, and each time treatment with 0.04 M at least doubled the number of selfed-pistils with pollen tubes protruding from the cut ends. These differences were not significant by the analyses of variance test. However, they indicate that a technique with less variability might show a positive effect for calcium in red clover as already reported for *Oenothera* (Kwack, 1965).

An abundant supply of plant growth substances applied either to the stigma or styles has also proven to be ineffective in preventing self-incompatibility. The conclusions from these experiments, as well as those with red clover pollen *in vitro* are that the added plant growth substance cannot function to stimulate pollen tube elongation or to interfere directly with the possible protein entities that comprise the incompatibility mechanism. The hormone probably functioned as a general growth stimulator and caused growth of pseudo- self-compatible florets when sprayed on intact red clover plants (Eyster, 1941).

Glycine at various concentrations up to 2500 ppm did not serve to block the self-incompatibility mechanism. Some chemicals that affect surface properties of proteins (i.e., tannin, urea, and caffein) (Goldstein and Swain, 1965) were applied to the styles of excised pistils in a continuation of our earlier study. These chemicals inhibited growth of pollen in compatible matings, but high concentrations did not block self-incompatibility. All of our treatments with chemicals to the stigma and to the styles of red clover pistils have failed to influence the self-incompatibility mechanism.

Changes in self-incompatibility may result from mutagenic activity of the chromosomes (Brown, 1954). Colchicine inhibited pollen-tube elongation in pistils of compatible matings but it did not affect the incompatibility mechanism. Similar results were obtained in preliminary experiments with ethyl methyl sulfonate. Several of the ultraviolet light treatments killed about 50% of the pollen grains but did not affect the self-incompatibility mechanism in the surviving pollen grains.

In experiments with two pollinations the number of styles with pollen tubes that grew out the cut ends in compatible matings was less in pistils excised 6 hr after pollination than in pistils excised immediately after pollination. The decrease probably occurred as tips of the pollen tubes passed the point in the style where the cut was made, and only the tips of the slowest growing tubes were left to pass that point after 6 hr. The passage of some compatible pollen tips through the style did not interfere with either self- or cross-compatibility in the style after the second pollination. The passage of some self-incompatible tubes into the styles did cause a slightly higher number of incompatible tubes to penetrate the style after the second pollination. However, the change in self-compatibility was slight, and the general conclusion is that each pollen tube traverses the style independent of other tubes. The incompatibility mechanism then is uniquely limited to individual pollen tubes.

Two heat treatments were used originally in this study. The infrared lamp as described above was used for one heat source, and the second was an oven at 80 C for various periods of time up to 2 hr. The maximum dosage of both treatments slightly reduced pollen growth with compatible matings. Oven treatments at above 80 C were lethal to the pollen. In early experiments the treatments with the infrared source appeared to be more effective than the oven and the latter were discontinued. Both treatments demonstrated a relatively high degree of heat tolerance for the pollen in comparison with the other parts of the plant. In comparison with Aspen pollen, which was treated in an infrared oven at 30 C for 5 min (Winton and Einspahr, 1968), the red clover pollen ranks higher in heat tolerance.

In our previous study it was shown that heat treatments applied during anthesis caused a change in the portion of the self-incompatibility mechanism located in the styles. The treatments were not effective if applied immediately after pollination. In alsike clover (Townsend, 1965) and in red clover (unpublished data in this laboratory) plants made compatible by heat treatments reverted back to self-

incompatible types within a few hours after the plants were changed from a hot to cool environment. Therefore, it is likely that the heat treatment blocks a process in the synthesis of the stylar portion of the mechanism, and after it is formed it is more heat stable than the pistil. A conclusion from the present data is that the portion of the mechanism located in the pollen is more heat tolerant than the pollen grain, despite the relatively high degree of tolerance of the latter.

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