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

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Summary. The stigma and style portions of red clover pistils were cultured *in vitro* after cross- (genetically compatible) or self- (incompatible) pollination. Pollen tubes grew through styles in which they were compatible and, in some treatments, through styles which were incompatible.

Boric acid, calcium nitrate and a carbohydrate in the medium, and moderate-to-cool temperatures generally enhanced pollen tube growth of compatible and incompatible matings. Several plant hormones at high concentrations inhibited pollen growth with compatible matings and did not retard the incompatibility mechanism. Application of high temperatures to the flower heads during the period of anthesis retarded the incompatibility mechanism. This temperature treatment affected the styles and not the pollen. In one of three experiments the application of relatively large amounts of pollen to the stigma rendered the incompatibility mechanism less effective.

Introduction

Self- and some interspecific-incompatibility in red clover (*Trifolium pratense* L.) is caused by a failure of pollen tubes to grow through the styles (SILOW, 1931; MÜLLER, 1960). This led us to initiate studies concerning the growth of red clover pollen. We have previously reported that to obtain appreciable pollen tube elongation *in vitro* the medium must contain 35% (w/v) sucrose, and boric acid, yeast extract and calcium nitrate at 100, 200 and 2000 ppm, respectively (KENDALL, 1967). Although these constituents of the medium are recommended for pollen of many species of plants (JOHRI, 1961), the concentrations required for red clover are exceptionally high. This paper reports the results of studies concerning the effectiveness of the *in vitro* medium and variable temperatures on pollen tube growth in the styles. The semi- vitro or excised pistil technique (described most recently by KWACK, 1965) which involves culturing only the stigma and style of the pistil on nutrient medium appeared to be especially suited for this study. The styles of excised pistils are easily accessible for treatments, and treatment effects on pollen tube elongation cannot be confounded with difficulties associated with fertilization.

Methods and Materials

Flowers were generally obtained from plants of Kenland red clover grown in controlled environment rooms with a light intensity of 200 ft-c from tungsten bulbs plus 1000ft-c from fluorescent bulbs during a 16-hour photoperiod and a temperature of 25 and 16 ± 3 °C during the light and dark period, respectively. Some flowers were obtained from plants in a glass house, except during summer months when the glass house temperature greatly exceeded temperatures in the controlled environment rooms.

Excised pistils were obtained by separating a floret from a flower head and then dissecting away the petals and a sufficient amount of the base of the floret to remove the ovulary. This left the stigma and style of the pistil which was held loosely in the stamen tube. Such a unit will be referred to hereafter as an excised pistil. Stripping off the petals actuated the tripping mechanism which self-pollinated all pistils while they were being prepared for the treatments. Additional incompatible or compatible pollen was applied by rubbing the stigma, plus the surrounding anthers, through an accumulation of pollen on a microscope slide.

The excised pistils were cultured in plastic containers which were originally made to serve as caps for vials. The caps were 23 mm in diameter and 5 mm tall. The bottom of the cap was covered with a single layer of glass beads (3 mm diameter), and then sufficient nutrient medium was added to cover the glass beads to a depth of about 1 mm. The excised pistils were then laid on the caps with their stigmas protruding over the outside edge and the cut end of the styles resting on the glass beads. The purpose of the beads was to make an uneven surface on which to lay the cut end of the excised pistils and thus prevent them from sloshing around in the medium. The medium entered the stamen tube and was in contact with at least the cut end and lower 5 mm of the style. Unless stated otherwise, the medium contained boric acid and Tween 20 each at 50 ppm and 0.14 M raffinose. Each cap with excised pistils was held in a separate tin sample box (50 mm diameter by 20 mm deep) which was tightly covered and incubated at 15 °C (except when temperature was an experimental variable) for 24 hours. The temperatures were controlled to ± 1 °C in Biochemical Oxygen Demand type incubators.

After the incubation period the excised pistils were transferred to a microscope slide which contained a few drops of a dilute solution of methyl blue. Pollen tubes which grew through the styles and extruded from the cut ends absorbed methyl blue and could be easily seen under the dissecting microscope at a magnification of 10 X. The number of excised pistils with extruded pollen tubes were counted, and this value was used for estimating treatment effects.

Each cap generally contained two excised pistils from each of five flower heads. From 10 to 20 caps were used for each treatment, and each treatment was repeated at least once with plant materials collected at a different season of the year. The results expressed as the number of styles with pollen tubes protruding from the cut ends were transformed to

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$\sqrt{x+1}$ and these data were analysed by the standard methods for the analyses of variance (SNEDECOR, 1956). In the tabular data reported, all means with the same letters within a column do not differ at the 5% significance level according to Duncan's Multiple Range Test.

Variable temperature treatments were applied during the period of flower anthesis or during growth of the pollen in the excised pistils. For the former treatment, stems about 14 cm long, each bearing one flower bud that was just beginning to show some petal color were cut off the plants. The excised stems were placed in glass bottles which contained 2.5% sucrose and held for a period of 3 days in incubators at 25 or 40 °C. To retard contamination of the sucrose solution and stems at 40 °C, it was necessary to place a water bath maintained at 25 °C inside the incubator held at 40 °C. The culture bottles were partly submerged in the water bath which then held the sucrose medium and submerged portion of the excised stems at 25 °C while the flower buds opened in an air temperature of 40 °C.

Results

In a series of experiments with sucrose at concentrations of 0, 0.07, 0.14 and 0.28 M and boric acid at 50 ppm, the pollen tubes grew through the maximum number of styles at a concentration of 0.14 M sucrose. This concentration was then used to test the utility of the carbohydrates listed in Table 1. Mannose was the only carbohydrate which inhibited growth (as compared with no carbohydrate source), and in general the di- and trisaccharides provided a better medium than the monosaccharides. The relative order of utility of the carbohydrates for compatible and incompatible matings were similar, with the possible exception of fructose and galactose.

Effects of various concentrations of boric acid with 0.14 M raffinose are shown in Table 2. Boric acid enhanced growth of pollen tubes in the styles, with the lowest concentration for a maximum response at 10 and 100 ppm for compatible and incompatible matings, respectively.

Calcium nitrate was evaluated at various concentrations up to 2,000 ppm with flower parts harvested during October, December, and January. Results at the three testing dates were statistically similar; i.e., calcium enhanced growth of pollen tubes in the compatible but not in the incompatible matings. In the incompatible matings calcium caused a slight inhibition in all tests. Data from the test made in January are shown in Table 3. Calcium was also tested with yeast extract at 10 and 200 ppm. The yeast extract when used alone or with calcium did not enhance pollen tube growth in excised styles.

Several plant hormones were added to the nutrient medium at concentrations of 0, 1, 10, and 100 ppm. At the highest hormone concentration pollen tube growth in styles in which they were compatible was inhibited to the extent indicated in the following data: gibberellic acid, 25%; a-naphthalene acetamide, 29%; traumatic acid, 64% and 3-indolebutyric acid 81%. None of these chemicals enhanced growth of pollen tubes in styles in which they were compatible or incompatible.

Effects of various temperatures on the growth of pollen tubes through excised pistils is shown in Table 4. These data were obtained with plants which flowered in January and February. Optimum growth of pollen tubes occurred at relatively cool temperatures after self- and cross-pollination. These treatments were repeated with plant material collected during September. Results of the second test were similar to those of the first for compatible matings, but differences between temperatures were very slight for incompatible matings.

The mean number of styles with pollen tubes protruding from the cut ends after self-pollination in experiments with flowers which had opened at either 25 or 40 °C is shown in Table 5. Pollen tubes grew through more of the styles from the 40 °C treatment than did styles from 25 °C. This pre-pollination effect of high temperature in lessening the degree of self- incompatibility was limited to the styles and did not affect the pollen.

Several variations of the usual method of pollination were employed to study effects of the quantity

Table 1. *The mean number of styles (10 per treatment) with pollen tubes protruding from the dissected basal end after self- or cross-pollination and incubated with various carbohydrates*

Carbohydrate (0.14 M)	No. styles with extruded pollen tubes	
	Compatible	Incompatible
Raffinose	8.6 a*	2.6 a
Lactose	8.6 a	1.9 b
Maltose	8.4 ab	1.8 b
Trehalose	8.0 b	1.8 b
Sucrose	8.0 b	1.6 c
Fructose	7.3 c	1.0 e
Arabinose	7.0 cd	1.4 c
Rhamnose	6.6 de	1.1 de
Dextrose	6.4 ef	1.2 d
Galactose	6.2 f	1.6 c
None	4.7 g	0.8 f
Mannose	0.6 h	0.0 g

* Means within a column followed by the same letter do not differ significantly at the 0.05 level of probability.

Table 2. *Effect of various concentrations of boric acid on the growth of pollen tubes through excised styles after self- or cross-pollination*

H_3BO_3 (ppm)	No. styles with extruded pollen tubes	
	Compatible	Incompatible
0	2.6 b*	0.2 c
1	3.3 b	0.3 c
10	8.2 a	0.6 b
50	8.0 a	1.3 ab
100	8.4 a	1.7 a
200	8.3 a	0.8 b

* Means within a column followed by the same letter do not differ significantly at the 0.05 level of probability.

Table 3. *Effect of various concentrations of $Ca(NO_3)_2 \cdot 4\,H_2O$ on growth of pollen tubes through excised pistils after cross- or self-pollination*

Calcium (ppm)	No. styles with extruded pollen tubes	
	Compatible	Incompatible
0	6.2 a*	1.0 a
250	7.9 bc	0.5 a
500	6.9 ab	0.8 a
1000	8.9 c	0.8 a
2000	9.0 c	0.5 a

* Means within a column followed by the same letter do not differ significantly at the 0.05 level of probability.

Table 4. *Effect of various temperatures on the growth of pollen tubes through excised styles after self- or cross-pollination*

Temperature (°C)	No. styles with extruded pollen tubes	
	Compatible	Incompatible
10	6.4 a*	1.9 a
15	8.5 a	2.8 a
20	8.3 a	2.2 a
25	6.8 a	1.7 ab
30	4.0 b	0.8 b
35	2.7 b	1.0 b

* Means within a column followed by the same letter do not differ significantly at the 0.05 level of probability.

Table 5. *The mean number of styles (10 styles per treatment with 20 replications) with pollen tubes protruding from the cut end with flower parts which had opened at either 25 or 40 °C and subsequently self-pollinated*

Anthesis temp. (°C)		No. styles penetrated
Pistil	Pollen	
40	25	4.4 a*
40	40	4.4 a
25	25	0.4 b
25	40	0.2 b

* Means within a column followed by the same letter do not differ significantly at the 0.05 level of probability.

of pollen and the number of pollinations on the degree of self-incompatibility. In one experiment (conducted in May when the plants were growing vigorously) a comparison was made between pollination by tripping the florets vs. tripping the florets and immediately adding to the stigma a mixture of pollen accumulated from other florets from the same head or from other plants of the same clone. The mean number of styles with pollen tubes protruding from the cut ends was 3.5 for pistils pollinated only by tripping and 4.0 for pistils with pollen from other florets. This difference was not significant at P = 5%.

In a second pollination experiment, 20 florets on a flower head were pollinated by tripping the florets and adding additional self-pollen. Immediately after pollination 10 of these florets were set up as excised pistils. The remaining florets were left intact on the head for either 1, 2, 3, 4, 5, or 6 hours and then more pollen was added as they were set up as excised pistils. In this test there were no differences between the control treatment (self-pollinated once) and treatments involving re-pollination at various times after the initial pollination.

In the third pollination experiment, 10 florets per flower head were pollinated by tripping, and the florets were left intact on the flower head for 2 hours at 25 °C. Next, pollen was added to the stigmas of the 10 florets previously tripped, plus an additional 10 florets; and then each group of 10 florets were set up as excised pistils. The mean number of styles with pollen tubes extending from the cut ends was 0.6 for styles with pollen added to the stigmas immediately after tripping, and 1.0 for styles that were re-pollinated 2 hours after tripping. The differences between these treatments were significant at P = 5%.

Discussion

The optimum concentration of carbohydrate for growth of red clover pollen through excised pistils was much lower than the values previously reported (KENDALL, 1967) to be optimum for pollen tube growth *in vitro*. The osmotic pressure of the *in vitro* medium is such that it may dehydrate the style tissue. This raises the question as to how the pollen tubes can function in either the relatively high osmotic pressure of the *in vitro* medium or the low and more usual value of the style tissue. This problem is not encountered by the pollen grains which germinate in a high osmotic environment *in vitro* or on the stigmatic surface.

It is not likely that either the style tissue or pollen tubes had an enzyme system capable of deriving energy from all of the carbohydrates tested. Probably the major effect of all the carbohydrates was to provide, first, a more suitable osmotic pressure for maintenance of style tissue, and, second, they may have been utilized in metabolic processes. In the secondary capacity, difference between carbohydrates would be related to rates of absorption and whether they were utilized or inhibited by the metabolic processes. It is obvious, and the most significant point of this phase of the study, that the carbohydrates were utilized equally well in cultures that were either self- or cross-pollinated. That is, none of the carbohydrates were effective specifically against the incompatibility mechanism.

The percentage of pollinated florets which set seed on red clover plants usually ranges from about 50 to 95 following cross-pollination and at values of less than 1% for florets which were self-pollinated (WILLIAMS, 1931). I obtained no seed following self-pollination of 1000 florets (10 florets on each of 10 flower heads from plants of 10 clones) that had been grown in the controlled environment room, which indicates a very low degree of self-compatibility for our plants also. From the data in Table 2 it can be seen that without boric acid in the medium for excised pistils the pollen tube growth through styles in which they were compatible was much less than expected for intact pistils. With boric acid in the medium pollen tubes grew through about the expected percentage of pistils after cross-pollination but exceeded the expected percentage after self-pollination. Therefore, it was not possible to define a single medium with one concentration of boric acid which would make possible the growth of pollen tubes through excised pistils to the same degree as the one that is expected to occur in the case of intact flowers following self- and cross-pollination.

The optimum concentration of boric acid for incompatible matings was 10 times greater than the optimum for compatible matings. This observation, plus the fact that the highest concentration inhibited incompatible matings but not compatible combinations, indicates that boric acid may influence the incompatibility mechanism as well as the elongation of pollen tubes.

KWACK (1965) reported that calcium in combination with boric acid, potassium, magnesium and sucrose was effective in retarding the degree of self-incompatibility in stylar cultures of Oenothera. In my studies calcium enhanced pollen tube growth for compatible but not for incompatible matings.

Potassium, magnesium and yeast extract did not contribute to the effectiveness of calcium when tested with incompatible matings.

EYSTER (1941) reported that self-incompatibility in red clover did not occur if the plants were treated with a-naphthalene acetamide. Results presented here and in earlier experiments (KENDALL and TAYLOR 1965) indicated that applying hormones directly to the styles did not retard incompatibility.

Red clover plants grown at high temperatures produced more seed after self-pollination than plants grown in cool environments (LEFFEL 1963). I previously reported (KENDALL, 1967) that high temperatures were not optimum for pollen tube elongation *in vitro*. The present study has shown (Table 1) that the optimum temperature for pollen tube elongation in styles with both compatible and incompatible matings is at medium or cool temperatures. The technique used with excised pistils had an inherent limitation for temperature studies because the cultures were held in tin sample boxes which were tightly covered. The tin container caused some undetermined delay in providing the desired temperature for the plant material. Therefore, it may be concluded that the data provide a tentative estimate of the optimum temperature range for pollen growth in styles, and that there is no indication that pollen tube growth in either compatible or incompatible matings is enhanced by high temperature. PANDEY (1956) studied pollen tube elongation after self-pollination at temperatures of 15, 20, and 25 °C. His data indicated that incompatibility was not influenced differentially at the temperatures studied.

Our experiments with flowers that had opened at 25 vs. 40 °C showed that incompatibility was greatly reduced in styles which had developed at the higher temperature (Table 4). Similar effects have been shown for Oenothera (KWACK, 1965), except that the styles were exposed to a higher temperature but for a brief interval of time.

It may be inferred from our two experiments with temperature that, to obtain maximum seed production after self-pollination, anthesis should occur at high temperatures; but a cool environment should be provided for pollen tube growth.

LACZYNSKA-HULEWICZ and MACKIEWICZ (1963) and KWACK (1965) working with red clover and Oenothera respectively, showed that pollen tubes grew further into styles in which they were incompatible if relatively large amounts of pollen were placed on the stigma at the time of the initial pollination, or if additional self-pollen was added at various times after the first pollination. In one of our three experiments repollination did render the incompatibility mechanism less effective. All three experiments might have produced the same positive effect if we had measured the length of the pollen tubes in the styles where they were not protruding from the cut end. However, it must be concluded that any slight difference in the lengths of the pollen tubes confined to the styles that was influenced by repollination would be of little interest to the plant breeder concerned with the production of seed following selfpollination.

We repeated all treatments with plant materials collected at different times from either the glass house or the controlled environment rooms. The results of all treatments at each different sampling date were similar in trends, but some were not statistically different at each date. This variability can be most conveniently ascribed to the pollen involved, a factor which is generally recognized to be easily influenced by the environment (JOHRI, 1961). For example, we observed that plants not growing vigorously produced a small quantity of pollen, and when this pollen was used to make compatible matings only a small portion of the tubes grew through the styles. Thus repollination with self-incompatible matings might be beneficial if the flowers were taken from slowly growing plants, and show no effect at all with flowers from vigorously growing plants. The response of the pollen to temperature and chemical treatments probably reflects the variable environment in which it was produced.

Zusammenfassung

Narben und Griffel von Rotklee-Stempeln wurden nach Fremd- (genetisch kompatibel) oder Selbstbestäubung (inkompatibel) in vitro kultiviert. Die Pollenschläuche wuchsen durch die Griffel, mit denen sie kompatibel waren, und nach bestimmter Behandlung auch durch Griffel, mit denen sie inkompatibel waren. Ein Zusatz von Borsäure, Kalziumnitrat und einem Kohlenhydrat zum Nährmedium sowie mäßige bis kühle Temperaturen förderten im allgemeinen das Pollenschlauchwachstum der kompatiblen und inkompatiblen Kombinationen. Verschiedene Wuchsstoffe, die dem Kulturmedium zugesetzt wurden, hemmten bei höherer Konzentration das Pollenschlauchwachstum kompatibler Kombinationen, verzögerten auf der anderen Seite aber nicht den Inkompatibilitätsmechanismus. Letzterer Effekt trat ein, wenn die Blütenköpfe während der Anthesis hohen Temperaturen ausgesetzt wurden. Diese Temperaturbehandlung wirkte auf die Griffel, nicht auf den Pollen. In einem der drei Experimente führte eine Bestäubung der Narbe mit großen Pollenmengen dazu, den Inkompatibilitätsmechanismus weniger wirksam werden zu lassen.

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