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Effect of Temperature on Pseudo-Self-Compatibility in *Trifolium pratense* L.*

W. A. KENDALL and N. L. TAYLOR

Department of Agronomy, University of Kentucky

Summary. A relatively high temperature treatment, applied during anthesis, was shown to enhance self-seed production through pseudo-self-compatibility in normally self-incompatible red clover (*Trifolium pratense* L.). The self-seeds were produced in cultures of excised stems held in 2.5 percent sucrose. The stems were excised when petal color was beginning to appear in the buds. During anthesis the cultures were incubated with the flower heads at 40° and the stems at 25 °C. When most of the florets per head had opened the cultures were transferred to 20 °C and held at that temperature during the period of pollen growth through the styles and also during seed development. The addition of calcium nitrate and boric acid to the culture medium did not enhance anthesis, seed weight, or the number of seeds produced.

Plant genotype and the environment provided before anthesis were the primary factors influencing the number of self-seed produced. Although not all attempts to produce self-seed have been successful, with repeated trials all clones we tested produced some seed.

Introduction

The regularly occurring self- and the infrequent cross-incompatibility in red clover (*Trifolium pratense L.*) has been attributed genetically to a number of incompatibility alleles (WILLIAMS, 1931). Generally, pollen tubes cannot grow through styles which carry the same gene for incompatibility (SILOW, 1931; KENDALL, 1968). Occasionally plants with the incompatibility alleles produce a small amount of self-seed in a process which has had several names, the most recent being Pseudo-Self-Compatibility (PSC) (LEFFEL, 1963).

BRANDON and LEFFEL (1968), THOMAS (1955) and many earlier workers cited in their papers, have shown that PSC in red clover is an inherited characteristic i.e., the amount of seed produced is a function of the genotype involved. Usually under field conditions, less than 1 percent of the florets pollinated have set seed, but we estimated from the data by THOMAS (1955) that about 1 percent of the plants he tested produced as much as 5 percent seed per floret pollinated. Sufficient seeds for studies of the inheritance of PSC (BRANDON and LEFFEL, 1968; JOHNSTON, TAYLOR, and KENDALL, 1968) and the effects of inbreeding on growth of progeny (THOMAS, 1955) have been obtained by pollinating large numbers of flower heads produced on vigorously growing single plants or clones of plants. A very small percentage of red clover plants carry a gene for self-compatibility in place of the gene for incompatibility, and these plants produce seed readily after self-pollination (RINKE and JOHNSON, 1941).

The amount of self-seed obtained from flower heads on the same plant has been variable, and it has been suspected for many years that PSC was influenced by the environment. LEFFEL (1963) showed that plants grown at relatively high temperatures produced more seed after self-pollination than plants of the same clones at cool temperatures. KENDALL (1968) reported that the effect of high temperatures on PSC occurred during anthesis and caused an inactivation of a pollen tube inhibitor (s) in the style.

TOWNSEND (1968) found that exposure to 32 °C for 1 or 2 days changed clones of alsike clover (*Trifolium hybridum L.*) from self-incompatible to self-compatible. The site of the change in the incompatibility reaction was in the styles for plants of one clone but could not be identified for plants of a second clone.

At Kentucky we produced self-seed of red clover to isolate inbred lines for the production of double-cross hybrids (JOHNSTON et al., 1968). The self-seeds were obtained from many plants which were isolated under separate cages for each clone. That procedure proved undesirable because of the large labor requirement, the small quantities of seed produced, and the continual occurrence of outcrosses. The present research originated from the desire to overcome these disadvantages and to provide information concerning the mechanism of PSC action.

Methods and Materials

Plants used in this study were grown in either a glass house or a field, or in controlled environment chambers at Lexington, Kentucky. The controlled environment chambers maintained a light intensity of 200 ft-c from

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tungsten bulbs plus 1000 ft-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. The glass house was shaded, but not mechanically cooled, during summer months.

All treatments involved flower heads on excised flower stems, a procedure basically similar to that described by BATTLE (1949) for red clover. We excised the stems for some experiments when petal color first became visible in the flower bud, and for other tests we cut the stems when most florets were suitable for pollination. The stems were cut at a length of about 14 cm, and usually 5 to 10 stems were placed in glass bottles that were a few centimeters shorter than the stems and contained a nutrient solution. The nutrient solution contained 2.5 percent sucrose and 25 ppm boric acid, except where noted otherwise. The lower one cm of stem tissue was squashed with a pair of pliers to facilitate entry of nutrients into the stem.

During the period of anthesis the culture bottles were partially submerged in a water bath at 25 °C which was held in an incubator at 40 °C. Accordingly, the media and submerged part of the stems were at 25° while the aerial part of the stems and flowers were at 40 °C. Under these conditions anthesis was completed in 2 or 3 days. After pollination the media and plant organs were held at 20 °C during the time of pollen growth through the styles and also during seed development, which generally was completed in about 14 days. The temperatures were obtained in Bio-chemical Oxygen Demand incubators, with an accuracy of ± 1 °C, and a fan for constant circulation of air within the chamber. No illumination was provided during the incubation period.

The bottles used to hold the cultures were autoclaved between each use, but the nutrient solutions were not sterilized. When the medium became cloudy, presumably because of the development of microorganisms, it was discarded and the stems were washed under running tap water before they were replaced in a fresh solution. Usually the media had to be changed after anthesis and once or twice during the period of seed development.

All of the pollinations were made on an individual floret basis to provide an accurate estimate of the number of florets involved. Florets were self-pollinated by activating the tripping mechanism with a dissecting needle. Pollen from several flower heads of different genotypes was mixed together on a glass microscope slide and transferred to the florets in a small metal loop for compatible matings. About 15 florets per head were cross-pollinated, and about 30 florets per head were self-pollinated. At least 10 flower heads were used for each treatment.

Results

Compatible matings: Studies with compatible matings were made with plants from the controlled environment and glass house, to ascertain the optimum temperatures and media for growth of pollen tubes and for seed maturation. In these studies stems were excised when most florets in the head were freshly-opened, and the florets were pollinated with compatible pollen immediately after setting the stems in the media.

Several carbohydrates and combinations of carbohydrates were evaluated as a source of nutrients for seed development. Sucrose was used at concentrations of 0, 2.5, 5.0, and 10 percent which produced 14, 71, 56, and 3 percent seed, respectively. Seeds produced on stems in the 2.5 percent solution weighed from 1.0 to 1.5 mg per seed and appeared similar to seed obtained from plants in the field. Chemicals which were shown to influence growth of red clover pollen tubes in styles of excised pistils (KENDALL, 1968), i.e., raffinose, boric acid and calcium nitrate, did not influence seed development when added to media for excised stems.

Effects of various temperatures on seed development are shown in Table 1. In this study, all flowers were held at 20 °C for 25 hours after pollination to insure equal fertilization at all treatments, and then the temperature treatments were applied for a period of 2 weeks. The mean number of seeds produced were similar for all treatments. However, seeds produced at 20 and 25 °C were larger than seeds grown at extreme temperatures.

To ascertain the optimum temperature for pollen tube growth, some excised stem cultures were held at either 15, 20, 25, 30, or 35 °C for 24 hours after pollination; and then all cultures were held at 25 °C for seed development. The numbers of seeds set were used to estimate treatment effects on pollen tube growth. No significant differences between temperature treatments were obtained in this test. We concluded that the technique was not suitable for estimating temperature effects on pollen tube growth, and consequently, we arbitrarily used the temperature found optimum for seed development, i.e. 20 °C.

Table 1. *Mean (10 replications) number and weight of seeds produced on excised stems of red clover at various temperatures from 24 hours after cross-pollination until the seed was mature (two weeks)*

Temperature	Seed	
C°	Number	mg/seed
15	19a*	0.81b
20	18a	1.30a
25	17a	1.23a
30	16a	0.75b
35	12a	0.61b

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

Table 2. *Average percentage of seed produced on stems excised either with opened florets and cross-pollinated, or with flower buds that were held at 40 °C for anthesis and self-pollinated*

Clone	Seed set (percent)	
Number	Cross-pollinated	Self-pollinated
700	51	12
581	57	20
749	66	5
829	69	17
11	71	12
713	77	11
54	78	3
525	79	3
1173	79	2
740	85	5

Incompatible matings: Several studies were made to provide a nutrient solution during anthesis that might enhance the opening of buds or retard development of the self-incompatibility mechanism. Generally most of the florets opened, in a solution of 2.5 percent sucrose, and appeared similar in size but of paler color than florets on intact plants. No florets opened on stems in either 0 or 10 percent sucrose. Various concentrations of calcium nitrate and boric acid generally failed to promote anthesis of flowers or to influence growth of pollen tubes through styles that were incompatible. In some tests the number of seeds obtained was increased by boric acid at 25 ppm.

The maximum temperature in which anthesis would occur was 40 °C. However, the submerged portions of the stems became injured at 40 °C, and they deteriorated before the seed matured. Therefore, it was necessary to provide a means for keeping the media at a cool temperature at the same time that high temperatures were provided for the flower heads.

The florets remained in a condition suitable for pollination for a relatively short time at 40 °C. Usually the maximum number of florets per head were ready for pollination after 2 or 3 days at 40 °C. The average percentage of pollinated florets which set seed after 1, 2, 3, or 4 days at 40 °C were 7, 11, 14 and 10 percent respectively. In one direct comparison of temperature treatments during anthesis, with flower heads on excised stems, we obtained no seed and 25 percent seed from flower heads that had opened at 25 and 40 °C, respectively.

To ascertain the optimum temperature for pollen tube growth in styles that were incompatible, some excised stem cultures were held at either 15, 20, 25, 30 or 35 °C for 24 to 48 hours after pollination, and then all cultures were held at 25 °C for seed development. This experiment was run twice with flower heads that had opened on plants in the glass house, and twice with flower heads from plants in the controlled environment rooms. Practically no seed was obtained in this test. Since pollen tube growth was not strongly influenced by temperature and 20 °C was optimum for seed maturation (see Table 1), we chose to use that temperature for the period of pollen tube growth and seed development for the incompatible matings.

The medium that was found to be optimum for seed development following compatible matings was arbitrarily selected for PSC studies. Seeds produced in this way were generally small and had hard seed coats. The approximate weight of seeds produced on excised stems by the various methods described above were as follows: stems picked with flowers opened and cross-pollinated, 1.3 mg; stems picked with flowers in the bud stage and later cross-pollinated, 0.8 mg; and stems picked with flowers in the bud stage and self-pollinated, 0.8 mg per seed.

The usefulness of the technique may be illustrated by some data with plants of 10 clones of red clover. In this study, 20 florets were pollinated on each of 10 flower heads for each of the 10 clones.

The degree of self-incompatibility of these plants under conditions nearly optimal for growth was established by allowing one set of flower heads to mature on the plants after self-pollination. The plants were maintained in the glass house or controlled environment chamber for this experiment. No seed was obtained from the 2,000 florets pollinated. It was inferred that under normal conditions the flowers on plants of these clones were very self-incompatible.

The percentage of florets that set seed after cross-pollinating flowers that were freshly-opened at the time the stems were excised is shown in Table 2.

The percentage of florets that set seed after self-pollinating florets which had opened with the flower heads at 40 °C and the stems at 25 °C is shown also in Table 2. No seeds were produced in some of the flower heads, but some seed was obtained for all of the clones tested.

Discussion

BATTLE (1949) arbitrarily selected a media with 2 percent sucrose and room temperature for his studies of seed production on excised stems. He excised stems when most of the florets were freshly-opened and all florets were cross-pollinated. He noted that florets which opened on the excised stems produced a relatively small number of small seeds.

Our experiments with compatible matings showed that the medium and temperature selected by BATTLE (1949) were nearly optimum, and the addition of several nutrients to the medium did not enhance seed development from florets which had opened on excised stems. We obtained a small quantity of seed from florets that were cross-pollinated when the florets became dry to the extent that they were brittle a few days after pollination. The excessively dry condition was prevented by maintaining an open pan of water, or a large number of cultures, in the incubators.

The length of time for anthesis to occur on excised stems was generally 2 or 3 days, and it was probably dependent upon the stage of development of the bud at the time the stems were cut. On some occasions the buds did not open. Generally, the inhibition of anthesis was associated with one or more of the following factors: (1) stems cut from plants that were not growing vigorously, (2) the appearance of disease lesions on the stems; and (3) the appearance of an exceptionally large amount of contamination in the stem media. We define a nonvigorous plant as one which, after having grown from a seed or cutting, under a light regime of low intensity and long duration, results in a small plant with only one or two small flower heads.

Disease lesions have developed mainly on stems taken from plants growing in the field. Consequently, we routinely wash the stems and buds taken from plants growing in the field in a large volume of water to reduce the amount of soil, microorganisms and insects on the cultures. The development of a slightly cloudy appearance (presumably due to microorganisms) in the stem medium has generally not prevented anthesis. The contamination could not be controlled with streptomycin sulfate at 62, 250, or 500 ppm, or with sulfanilamide at 25, 500, 1000, or 2000 ppm.

In previous studies of growth of pollen tubes through styles that were genetically incompatible it was shown (KENDALL, 1968) that pollen growth was enhanced by certain carbohydrates, boric acid and calcium nitrate. We added these chemicals, at several concentrations, to the nutrient media for excised stems during anthesis. From the numbers of seed set it was inferred that none of these chemicals consistently influenced growth of pollen tubes through styles that were incompatible. In some tests the number of seeds obtained were increased by boric acid at 25 ppm. At that concentration boric acid was never inhibitory, and therefore, it was regularly included in the media.

Studies on the effects of temperature on the growth of pollen tubes in excised pistils (KENDALL, 1968) showed that the incompatibility mechanism was partially inactivated by high temperatures during anthesis and that high temperatures applied after pollination had only inhibitory effects on pollen growth. In our study, where the effects of temperature were evaluated on the basis of seed production, conclusions concerning the incompatibility mechanism are the same

The technique described here has been used in our laboratory on many occasions in the past 2 years, and independently by Dr. J. THOMAS (personal communication). On some occasions at both laboratories the amount of seed obtained has been unsatisfactory. However, by repeating the procedure on several occasions we have produced self-seed for all of the clones we have tested, and the technique has been useful in the breeding program.

Plant genotype and season of the year influenced effects of high temperature on PSC in alsike clover (TOWNSEND, 1968). Variable stability seems to be a general characteristic of the incompatibility mechanism. Eventually the variability may prove helpful in that, as we identify factors responsible for the fluctuations, we shall come closer to defining the mechanism itself.

Zusammenfassung

Durch eine Behandlung normalerweise selbstunverträglichen Rotklees (*Trifolium pratense* L.) mit verhältnismäßig hohen Temperaturen während der Anthesis ergab sich eine Erhöhung des Samenertrages durch Pseudo-Selbstverträglichkeit. Die Samen aus der Selbstbefruchtung wurden an Kulturen abgeschnittener Stengel erzielt, die in 2.5% Sucrose gehalten wurden. Die Stengel waren zu dem Zeitpunkt, an dem die Petalenfarbe in den Knospen sichtbar wurde, abgeschnitten worden. In einem Brutraum wurden die Blütenköpfe während der Anthesis bei 40 °C und die Stengel durch Eintauchen der Kulturgläser in ein Wasserbad bei 25 °C gehalten. Sobald sich die Mehrzahl der Blütchen geöffnet hatte, wurden die Kulturen in 20 °C übergeführt und in dieser Temperatur während des Pollenwachstums durch die Griffel und auch während der Samenentwicklung belassen. Eine Beigabe von Kalziumnitrat und Borsäure zum Kulturmedium steigerte weder die Anthesis noch das Samengewicht und die Anzahl der erzeugten Samen. Der Genotyp der Pflanze und die Umwelt vor der Anthesis waren die Primärfaktoren, die die Anzahl der Samen aus Selbstbefruchtung beeinflußten. Obwohl nicht alle Versuche, Samen aus Selbstbefruchtung zu erzielen, erfolgreich waren, erzeugten in wiederholten Versuchen doch alle untersuchten Klone etwas Samen.

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W. A. KENDALL, Plant Physiologist,
and N. L. TAYLOR, Professor of Agronomy,
University of Kentucky
Agronomy Department
Agricultural Science Center
Lexington, Ky. 40506 (USA)