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Hybridization of Some *Trifolium* Species through Stylar Temperature Treatments*

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Summary. Plants of seven perennial *Trifolium* species were interspecifically pollinated. The female flowers opened at 40 °C, and the male flowers opened in the greenhouse or field without a temperature treatment. *T. pratense* was pollinated with *T. ambiguum*, *T. hirtum*, *T. ochroleucum*, *T. rubens*, *T. sarosiense*, and *T. medium*. *T. medium* was pollinated with *T. pratense*. Seeds were obtained from all pollinations; however, 86 of the progeny were morphologically and cytologically similar to their female parent, and probably resulted from self-fertilization. Many seeds of possible hybrid origin were small and/or did not germinate. Some seeds produced weak or albino seedlings which died at an early age. Excised pistils of *T. medium* from flowers that opened at 40 °C were more compatible with pollen of *T. pratense* than pistils of *T. medium* that developed in the field, indicating that the elevated temperature caused greater hybrid seed production than was observed in our earlier studies. The high temperature technique may prove useful in obtaining hybrids in *Trifolium*.

Introduction

Plants in the genus *Trifolium* are generally self- and interspecific incompatible (Williams, 1931; Atwood, 1940; Townsend, 1968; Taylor, et al., 1963). One cause of these incompatibilities is the failure of pollen tubes to grow through the styles (Silow, 1931; Müller, 1960). Considerable success in overcoming self-incompatibility by relatively high temperatures has been reported for *T. pratense* L. (Leffel, 1963; Kendall, 1969) and for *T. hybridum* L. (Townsend, 1968). Dadson (1969) reported that heat treatments increased the degree of interspecific compatibility among certain species of *Trifolium*. This paper reports the use of temperature treatments during anthesis on hybridization of seven *Trifolium* species. Some experiments with excised pistils of *T. medium* L. are included to demonstrate effects of the heat treatment on growth of pollen tubes independent from effects of the heat on fertilization and seed maturation.

Materials and Methods

The species of *Trifolium* used, and the 2n number of chromosomes reported for them, were: *T. ambiguum* M. Bieb., 48 (Britten, 1963a); *T. hirtum* All., 10 (Britten, 1963b); *T. medium* L., ca. 48, 80, 126 (Darlington and Janaki Ammal, 1945; Cincura, 1965); *T. ochroleucum* L., 16 (Keim, 1953); *T. pratense* L. 14 (Britten, 1963a); *T. rubens* L., 16 (Senn, 1938); and *T. sarosiense* Haszl., 48 (Cincura, 1965).

We obtained plants from a collection of *Trifolium* species maintained at the Kentucky Agricultural Experiment Station, Lexington, Ky. Specimens of these plants are filed in the Kentucky Agricultural Experiment Station herbarium. The plants grew in a controlled environment chamber, greenhouse or field.

A slight modification of the excised pistil technique (Kendall, 1968) was used to evaluate effects of heat treatments during anthesis on subsequent growth of pollen tubes through the styles of *T. medium*. Florets that had opened on excised stems at 40 °C were pollinated with pollen of *T. pratense* or were self-pollinated. After pollination the upper part of each floret was cut off the flower head at a height immediately above the ovulary. The upper part of the floret was laid in a plastic cap, 23 mm in diameter × 5 mm tall, which contained glass beads (3 mm in diameter) and a nutrient solution. The cut ends of the florets were submerged in the nutrient solution near the center of the cap. The distal end rested on the rim and protruded outside the cap. The nutrient solution contained boric acid and Tween 20 (a wetting agent) each at 50 ppm, and 0.14 M raffinose. The cultures were incubated at 20 °C for about 20 hr. On each flower head 10 florets were crossed with pollen from *T. pratense* and 10 florets were self-pollinated. Each group of florets with the same pollen was placed in one plastic cap. In matings that were compatible, pollen germinated on the stigmas, grew through the styles and into the media. The cut ends of the styles were examined on a microscope slide at a magnification of 10 × for the appearance of pollen tubes. We estimated treatment effects by the number of styles with pollen tubes protruding from the cut ends that were observed in each cap. Five flower heads that had opened on excised stems at 40 °C and five flower heads that had opened in the field (control) were used each day. The experiment was repeated on each of 8 days (providing a total of 40 flower heads or 400 florets for each treatment).

The excised stem method (Kendall and Taylor, 1969) was used for temperature treatments during anthesis and seed production. Stems about 15 cm long, bearing flower buds, were excised when petal color first became visible in the bud. We washed the stems in tap water; crimped the cut ends with a pair of pliers, and then placed the stems in glass bottles containing an aqueous solution of 2.5% sucrose and 25 ppm boric acid. These bottles were set in a water bath at 25 °C which in turn was maintained in an incubator at 40 °C. This treatment maintained the submerged portion of the stems at 25 °C while the flowers

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were at 40 °C. Under these conditions anthesis occurred within 2 or 3 days.

About 20 to 30 florets of flower heads which opened at 40 °C were crossed with pollen from flowers which opened without the temperature treatment. After pollination an incubation temperature of 20 °C was maintained during pollen tube growth and seed development. Compatible crosses produced mature seed in about 14 days. We changed the nutrient solution and washed the end of the excised stems at about 4-day intervals to limit contamination from microorganisms.

Seeds (suspected F_1 hybrids) were germinated in 100mm sterile petri dishes on one thickness of stationary blotter paper, and kept moist with tap water. Incubation was at 25 °C. Seedlings were transferred to 2-inch clay flower pots containing a 1:1 mixture of vermiculite and sand until the first trifoliolate leaf appeared. Then they were changed to 4-inch flower pots containing autoclaved soil

Chromosomes from root tips of the progeny were counted by methods similar to those of Brooks, Bradley and Anderson (1963) except that cells were squashed in acetocarmine rather than 45% acetic acid. A sheath-like layer of cells was removed from the root tips after the pretreatment to facilitate squashing. Root tips were collected as the seedlings were transferred from the vermiculite-sand mixture to soil.

Results

Some pollen tubes grew through styles of excised pistils of *T. medium* in each treatment (Table 1). Styles of *T. medium* were more compatible with pollen of *T. pratense* than with self-pollen. Heat treatment of *T. medium* styles increased compatibility with pollen of *T. pratense* but not with self-pollen.

Table 1. *Percentage of excised styles of T. medium with pollen tubes protruding from the cut end after pollination with self or T. pratense pollen following anthesis in the field or at 40 °C on excised stems*

Anthesis Treatment	Compatible (%)	
	Self-pollinated	Crossed with *T. pratense*
Field	11 b*	30 b
40 °C	28 b	62 a

* Means followed by the same letter do not differ at the 5% level of significance based on Duncan's Multiple Range Test.

No seeds were obtained from approximately 2000 florets of *T. medium* on excised stems that were self-pollinated after anthesis at 40 °C. This test was made with stems that were collected at several dates from plants growing in the field.

Seeds were obtained from all interspecific pollinations made at 40 °C (Table 2) although the number of flower heads was small, particularly of *T. ambiguum*. Most of the seeds from each cross were small and shriveled. Seed matured in 5 to 14% of the florets that were pollinated for each cross except for *T. pratense* × *T. sarosiense* which was 43%.

The number of seeds that germinated, and the number of seedlings that appeared to be normal, albino, and deformed are listed in Table 2. A large number of the small and shriveled seeds, particularly seeds from *T. pratense* × *T. medium*, *T. medium* × *T. pratense*, and *T. pratense* × *T. sarosiense*, did not germinate. The small seeds obtained from the cross of *T. medium* × *T. pratense* had small and disfigured embryos.

Many of the seeds produced plants with an abnormal appearance, which died in the seedling stage of growth. The most common abnormality was albino plants. The roots of several seedlings were twisted and curled in a corkscrew manner and failed to grow more than 5 mm long.

A total of 86 normal plants were obtained from all crosses with *T. pratense* as the female parent. All of these plants were morphologically indistinguishable from the female parent. Root tips of the female parent and of each of the putative hybrids had 14 chromosomes. It was concluded that all of these plants were selfs of the female parent, *T. pratense*.

The number of chromosomes in root tips of the parent plants was the same as generally reported in the literature except for *T. medium* which had about 76 chromosomes (Figure 1).

Discussion and Conclusion

A small percentage of self-pollen tubes grew through styles of excised pistils of *T. medium*. Self-compatibility of *T. medium* was not affected by high temperature during anthesis as has been shown for *T.*

Table 2. *The number of flower heads treated at 40 °C, florets pollinated, seed obtained, and seeds germinated and appearance of seedlings from interspecific crosses in Trifolium*

Cross	No. Pollinated		No. of Seeds			No. of Seedlings		
	Heads	Florets	Small	Normal	Germinated	Normal[1]	Albino	Deformed
T. pratense × *T. ambiguum*	7	170	14	4	2	2	0	0
T. pratense × *T. hirtum*	21	475	46	19	19	6	9	4
T. pratense × *T. ochroleucum*	22	549	35	6	47	39	5	3
T. pratense × *T. rubens*	14	360	12	7	15	12	1	2
T. pratense × *T. sarosiense*	18	565	225	39	4	3	0	1
T. pratense × *T. medium*	77	2316	138	40	41	24	9	8
T. medium × *T. pratense*	99	2650	156	30	0	0	0	0

[1] Proved to be selfs.

pratense (Kendall and Taylor, 1969). Possibly a modification of temperature might cause significant changes in self-compatibility of *T. medium.*

Pollen of *T. pratense* was more compatible with excised pistils of *T. medium* flowers that opened at 40 °C than with pistils that developed in the field at a lower temperature. Heat treatments affect both self- and interspecific-incompatibilities suggesting that a general mechanism is involved. One possibility is the peroxidase isozymes which have been shown by DeJong et al. (1968) to be different in warm- than in cool-grown cultures and also have been implicated by Pandey (1967) as determinants of multiple allelism of the S gene.

No seeds were obtained after self-pollinating flowers on excised stems of *T. medium* that opened at 40 °C, although comparable selfs of excised pistils indicated that pollen tubes grew through up to 28% of the pistils that were pollinated. Therefore, lack of seed production indicates that failure during fertilization or seed development, is a further cause of self-incompatibility in this species.

The number of crosses of *T. pratense* particularly with *T. rubens* and *T. ambiguum* was limited by the number of flowers produced. Several plants of each species used in the hybridization study were maintained in the greenhouse for over one year, and several treatments; e.g., spraying with gibberellic acid and various temperature and photoperiod cycles, did not enhance flowering.

Flower buds of *T. pratense* did not open at 40 °C on excised stems when the buds were picked before the color of the petals became visible. We delayed picking the flowering stems of *T. medium* until the most mature florets (the lowest row of florets on the flower head) had opened completely. The mature florets were discarded when the stems were picked and transferred to the incubator. The flower head of *T. medium* is much longer than the round head of *T. pratense,* and allowing the lower florets to mature was probably necessary to bring the buds at the upper end of the flower head to a critical stage of development. Flower buds of *T. medium* from plants growing in the field did not open unless they were washed in tap water before the heat treatment. The washing was not necessary for buds from plants in the greenhouse. The buds from the field were apparently infested with thrips.

The percentage of pollinated florets which set seed was from 5 to 14% for all interspecific crosses except *T. pratense* × *T. sarosiense* which was 43%. Kendall and Taylor (1969) reported that 10 clones of *T. pratense* differed from 2 to 20% for seed production after self-pollination of florets that opened at 40 °C. The number of seeds produced per floret crossed with pollen from various species was within the range expected for self-pollination for all crosses except *T. pratense* × *T. sarosiense.* The unusually large number of seeds and the large proportion of seeds that were small and nonviable indicate that cross-fertilization occurred relatively frequently in this cross.

Fig. 1. Photograph of chromosomes in root tips of *T. medium*

The cross of *T. medium* with *T. pratense* produced 186 seeds. Although about 30 of these seeds appeared normal in size and shape for *T. medium,* none of the seeds germinated. Inasmuch as no seeds were produced when *T. medium* was self-pollinated, the 186 seeds must have represented various hybrid genotypes that died during the time of seed maturation.

The morphological appearance and chromosome counts of the 86 plants obtained from crosses of *T. pratense* with six *Trifolium* species were all the same as the mother parent. This indicates that these plants resulted from self-pollination. Failure of many seeds to germinate, and of many seedlings to survive past the seedling stage, are evidence that some hybrids were formed. However, their identity could not be proven, and they were of no value to the plant breeding program.

The number of chromosomes in the *T. medium* plants we used was about $2n = 76$. This figure is intermediate in the range of numbers that have been reported for this species. It is likely that plants of *T. medium* exist at lower levels of polyploidy which might be more compatible with *T. pratense.*

Heat treatments during anthesis allowed the formation of a large number of seeds from self-fertilization. This difficulty could be avoided by the use of male-sterile female plants or an efficient technique for emasculation. Wit (1960) reported that varying degrees of male sterility could be induced in *T. pratense* by spraying the plants with 2, 3-dichloroisobutyrate. We used 2,3-dichloroisobutyrate, coumarin, and maleic hydrazide, each at various concentrations in the medium during anthesis for excised stems. We also sprayed these chemicals on intact plants at prebloom stage to induce male sterility. High concentrations of the chemicals blocked development of all parts of the florets. At low concentrations viable pollen was formed.

Although no hybrids were produced in this study, treatment of heads with 40 °C temperature resulted in many more seeds albeit nonviable than previous techniques employed (Taylor et al., 1963). The previous study showed that when shriveled or small seeds were produced, more intensive work using a large number of different genotypes sometimes produced hybrids. For example, the cross *T. pratense* × *T. difusum* produced large amounts of shrivel seed and finally a hybrid was obtained.

T. pratense used in the present studies was at the diploid level. Hybridization might be effected by using the tetraploid in conjunction with embryo culture techniques. In particular, the large numbers of small nonviable seed obtained from *T. pratense* × *T. medium*, *T. pratense* × *T. sarosiense*, and *T. medium* × *T. pratense* indicates that further investigations with these species using the high temperature technique is warranted.

Zusammenfassung

In der Gattung *Trifolium* ist sowohl nach Selbstung als auch nach Artkreuzungen häufig Inkompatibilität zu beobachten. Es wird über Untersuchungen berichtet, durch Wärmebehandlung während der Blüte die Inkompatibilität zu überwinden.

Zu diesen Zweck wurden die Blüten der mütterlichen Eltern einer Temperatur von 40 °C ausgesetzt und danach mit Pollen von unbehandelten Pflanzen bestäubt, die auf dem Feld oder im Gewächshaus aufblühten: *Trifolium pratense* wurde mit *T. ambiguum*, *T. hirtum*, *T. ochroleucum*, *T. rubens*, *T. sarosiense* und *T. medium* bestäubt, *T. medium* mit *T. pratense*. Aus allen Bestäubungen wurden Samen erhalten. 86% der Nachkommen entsprachen jedoch morphologisch und cytologisch ihrem mütterlichen Elter und waren vermutlich das Ergebnis einer Selbstbefruchtung. Viele Samen, die möglicherweise aus Kreuzungen stammten, waren klein und/oder keimten nicht. Aus einigen Samen erwuchsen schwache oder albino-Sämlinge, die bald abstarben. Griffel von *T. medium*, die aus Blüten herausgelöst wurden, die sich bei 40 °C geöffnet hatten, waren für Pollen von *T. pratense* verträglicher als Griffel, die sich auf dem Felde entwickelten. Dieser Befund läßt den Schluß zu, daß die höhere Temperatur einen besseren Hybridsamenansatz bewirkt hat, als er in unseren früheren Untersuchungen beobachtet wurde. Eine Temperaturbehandlung kann sich demnach bei *Trifolium* für eine Erzeugung von Hybriden als günstig erweisen.

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Literature cited

1. Atwood, S. S.: The genetics of cross incompatibility among self-incompatible plants of *Trifolium repens* L. Agron. Jour. **32**, 955–968 (1940). — 2. Britten, E. J.: Chromosome numbers in the genus *Trifolium*. Cytologia **28** (4), 428–449 (1963a). — 3. Britten, E. J.: Chromosome number of rose clover, *Trifolium hirtum*. Sci. **142**, 401–402 (1963b). — 4. Brooks, R. M., Bradley, M., Anderson, Th.: Plant microtechnique manual. Dept. Pomol. Univ. of Calif. 70 pp. (1963). — 5. Cincura, F.: Cytotaxonomic analysis of *Trifolium sarosiense*. Biologica **20** (4): 300–305 (1965). (As reviewed in Biol. abst. **46**, 1965). — 6. Darlington, C. D., Janaki Ammal, E. K.: Chromosome Atlas of Cultivated Plants. London: George Allen and Unwin Ltd. 1945. — 7. Dadson, R. D.: Interspecific hybridization between some perennial *Trifolium* species and *T. pratense* L. Unpublished Doctoral Thesis, McGill University, Montreal, Canada, 1969. — 8. DeJong, D. W., Olsen, A. C., Hawker, K. M., Jansen, E. F.: Effect of cultivation temperature on peroxidase isozymes of plant cells grown in suspension. Plant Physiol. **43**, 841–844 (1968). — 9. Keim, W. F.: Interspecific hybridization in *Trifolium* utilizing embryo culture techniques. Agron. Jour. **45**, 601–606 (1953). — 10. Kendall, W. A.: Growth of *Trifolium pratense* L. pollen tubes in compatible and incompatible styles of excised pistils. Theoretical and Applied Genetics **38**, 351–354 (1968). — 11. Kendall, W. A., Taylor, N. L.: Effect of temperatures on Pseudo-self-compatibility in *Trifolium pratense* L. Theoretical and Applied Genetics **39**, 123–126 (1969). — 12. Leffel, R. C.: Pseudo-self-compatibility and segregation of gametophytic self-incompatibility alleles in red clover, *Trifolium pratense* L. Crop Sci. **3**, 377–380 (1963). — 13. Müller, Gertrud: Untersuchungen über das Pollenschlauchwachstum bei verschiedenen *Trifolium*-Artkreuzungen. Züchter **33**, 11–17 (1960). — 14. Pandey, K. K.: Origin of genetic variability: combinations of peroxidase isozymes determine multiple allelism of the S gene. Nature **18**, 669–672 (1967). — 15. Senn, H. A.: Chromosome numbers of *Leguminosae*. Bibliog. Genet. **12**, 175–336 (1938). — 16. Silow, R. A.: A preliminary report on pollen tube growth in red clover, *T. pratense* L. Welsh Plt. Breeding Sta. Bul., Series S **12**, 228–240 (1931). — 17. Taylor, N. L., Stroube, W. H., Collins, G. B., Kendall, W. A.: Interspecific hybridization of red clover (*Trifolium pratense* L.). Crop Sci. **3**, 549–552 (1963). — 18. Townsend, C. E.: Self-compatibility studies with diploid alsike clover, *Trifolium hybridum* L. III. Response to temperature. Crop Sci. **8**, 269–272 (1968). — 19. Williams, R. D.: Self- and cross-sterility in red clover. Welsh Plt. Breeding Sta. Bul., Series H. **12**, 181–208 (1931). — 20. Wit, F.: Chemically induced male sterility — a new tool in plant breeding? Euphytica **9**, 1–9 (1960).

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