

# METHODS OF OVERCOMING INTERSPECIFIC BARRIERS IN *TRIFOLIUM*<sup>1</sup>

N. L. TAYLOR, R. F. QUARLES and M. K. ANDERSON<sup>2</sup>

Department of Agronomy, University of Kentucky, Lexington, Kentucky 40506, USA

Received 28 February 1979

## INDEX WORDS

*Trifolium*, interspecific barriers, interspecific crossing.

## SUMMARY

Barriers to interspecific hybridization in *Trifolium* were investigated by manipulation of mentor pollen treatments, ploidy levels, and compatibility and male sterility systems. Crosses involving the addition of mentor pollen produced fewer seeds and hybrids than crosses involving normal pollination. Lower seed set with mentor pollen was deduced to result from the use of less viable pollen, approximately half the pollen having been killed by alcohol. Pollinations at the diploid level resulted in more hybrids than at the tetraploid level, perhaps because genes for male sterility produced higher female sterility in the tetraploids. The self-compatible stock produced more seeds, mostly selfs, than the self-incompatible stock, but produced more hybrids only in one cross, *T. pratense* L. × *T. diffusum* EHRH. The use of male-sterile female parents reduced selfing but produced fewer hybrids than male-fertile female parents. Techniques of this study were designed to affect prefertilization barriers, but the lack of effect may indicate that postfertilization barriers in *Trifolium* are of greater importance.

## INTRODUCTION

Attempts to hybridize red clover (*Trifolium pratense* L.) with other *Trifolium* species have met with minimal success. To date only three species have been crossed with red clover and only one of the hybrids obtained was fertile (ARMSTRONG & CLEVELAND, 1970; SCHWER & CLEVELAND, 1972; TAYLOR et al., 1963). All hybrids obtained were annuals which did not fulfill the objective of breeding programs to obtain greater longevity.

Some of recognized techniques for breaking interspecific barriers have been attempted in *Trifolium* (ANDERSON & TAYLOR, 1974; CHEN & GIBSON, 1972; EVANS, 1962a, 1962b; MAIZONNIER, 1972; NEWTON et al., 1970; TAYLOR et al., 1963). Two techniques for overcoming sterility barriers which have not been attempted include use of a self-compatible species as the female parent, as reported by GRUN & RADLOW (1961) and STEBBINS (1957) with *Solanum*, and the use of mentor pollen as described by STETTLER (1968) and KNOX et al. (1972) with *Populus*. One difficulty with using a self-compatible species as the female parent is the large amount of self seed that is obtained unless the female is male sterile (SCHWER & CLEVELAND, 1972).

<sup>1</sup> Journal Article No. 98-3-208 of Kentucky Agricultural Experiment Station. Published with approval of the Director.

<sup>2</sup> Present address of M. K. Anderson: North American Plant Breeders, Berthoud, Colorado 80513.

The objective of the present research was to examine techniques for overcoming barriers to interspecific hybridization of red clover. These techniques included manipulation of ploidy levels, compatibility and male-sterility systems in addition to the application of mentor pollen. It was also possible to examine the interactions of different interspecific crosses at different ploidy levels, of ploidy levels with different compatibility and male-sterility systems, and the overall interaction of these levels and systems with the mentor pollen technique. Further objectives were to describe a male-sterile self-compatible genotype produced at the tetraploid level, and the inheritance of male sterility at the tetraploid level.

#### MATERIALS AND METHODS

The four species of *Trifolium* used in this study consisted of 12 genetic stocks (Table 1). To test the effect of male sterility at the diploid level, male sterile (MS) and male fertile (MF) sources of *T. pratense* (SMITH, 1971; TAYLOR et al., 1978) were crossed with *T. diffusum* EHRH. The hybrid of the two species was obtained previously by TAYLOR et al. (1963).

To test the effect of ploidy levels, and different compatibility systems, two male-sterile lines of *T. pratense* were developed for matings with *T. diffusum*; one with a self-compatible genotype ( $S_f S_x$ ) and one with a self-incompatible genotype ( $S_x S_y$ ).

*Self-incompatible line:* An MS, self-incompatible (SI) diploid plant was mated with a diploid SI clone to produce an  $F_1$  heterozygous ( $Ms_1 ms_1$ ) at a male-sterility locus. The  $F_1$  plants were intercrossed and subjected to nitrous oxide (TAYLOR et al., 1976) to induce tetraploidy. After the resulting seeds were germinated and plants grown to flowering, tetraploids were selected from the diploids based on stomate size. The MS plants were separated from the male-fertile (MF) plants based on the absence of pollen.

*Self-compatible line:* MS, SI, and MF, SC diploid clones were mated to produce an  $F_1$  heterozygous ( $Ms_2 ms_2$ ) for a male-sterility locus (TAYLOR et al., 1978). All  $F_1$  plants were self-fertile. The MF  $F_1$  plants were selfed and subjected to nitrous oxide. All progeny were examined and tetraploid, MS plants were selected on the bases of stomate size and absence of pollen.

To further test the effect of compatibility systems at the tetraploid level, MS, SI, and SC *T. pratense* stocks were crossed as females with SC, MF stocks of *T. medium* L. and *T. sarosiense* HAZSL.

Two other crosses involved MS *T. medium* with tetraploid MF, SI *T. pratense* and with MF, SI *T. sarosiense*. Finally, the effect of mentor pollen was superimposed on all crosses.

For each cross, approximately 2000 florets were pollinated with and without the addition of mentor pollen. In all crosses where mentor pollen was not applied, pollen was obtained from the male parent by tripping the floret by hand to deposit the pollen onto a small section of fine emery paper glued to a toothpick. The pollen was then transferred to the stigma of the female parent to effect pollination. For mentor pollinations, female compatible pollen was collected from a compatible plant of the same species as the female used in the mating. Flowers were tripped manually and pollen was collected on a microspatula. Methyl alcohol (70%) was added to the pollen in a watchglass in an amount sufficient to evaporate in one hour. This time of methanol

INTERSPECIFIC CROSSES IN TRIFOLIUM

Table 1. Identification, ploidy level (x), chromosome number, accession no., designation, fertility level, compatibility system and origin of *Trifolium* species used in interspecific pollinations.

Species	Ploidy level	Number of chromosomes	Accession No.	Designation	Fertility system <sup>1</sup>	Compatibility system <sup>2</sup>	Origin
<i>T. pratense</i>	2x	14	29-L38-96	Clones	MF	SI	Kentucky
	2x	14	12-L38-740-3-7	740-3-7	MS	SI	Kentucky
	2x	14	19-L38-1546	66-1	MS	SI	Wisconsin
	2x	14	01-L38-1547	F-47-3	MF	SC	Kentucky
	4x	28	29-L38-1522	NK	MF	SI	Northrup-King
	4x	28	32-L38-1548		MS	SI	Kentucky
<i>T. diffusum</i>	4x	28	32-L38-1549		MS	SC	Kentucky
	2x	16	P.I. 204517	S-46	MF	SC	Turkey
	4x	32	P.I. 204517-T	S-46	MF	SC	Kentucky
<i>T. medium</i>	8x	64	P.I. 186574	S-25	MF	SI	Canada
	8x	72	P.I. 186574	2-75-4	MS	SI	Canada
<i>T. sarosense</i>	6x	48	P.I. 292827	S-122	MF	SI	Sweden

<sup>1</sup> Male-fertile (MF) of male-sterile (MS).

<sup>2</sup> Self-incompatible (SI) or self-compatible (SC).

saturation was found to give 100% 'kill' of the pollen as shown by test crosses. Pollinations were made with a visually estimated mixture of 50% 'killed' and 50% viable pollen.

Seeds were allowed to mature for approximately 20 to 30 days prior to threshing by rubbing the heads across a section of corrugated rubber. Seeds were scarified with emery cloth, and germinated. Seedlings were transplanted to plastic trays containing a 1:1 mixture of vermiculite and peat moss. The trays were placed under continuous light in a growth chamber maintained at 22°C. When the first trifoliolate leaf emerged, seedlings were transplanted to pots in a greenhouse maintained at a photoperiod of approximately 12 hours at 24°C.

For cytological examination, actively growing root tips were removed from six-to-eight-week old plants and placed in vials of 10 to 15 ml 0.003N hydroxyquinoline with 1 to 2 drops of maltose for a period of four hours at room temperature. Root tips were then transferred in a solution of 6 parts 95% ethanol: 3 parts chloroform: 1 part acetic acid (v:v:v) at room temperature for 48 to 72 hours. Root tips were hydrolyzed for 15 minutes in 5N HCL, stained in leuco-basic fuchsin for 20 minutes, washed in water and then refrigerated in 45% acetic acid for 30 minutes. They were then mounted on a glass slide, stained with acetocarmine, squashed and covered with a cover glass for microscopic examination. Based on the cytological results and phenotype, each plant was identified as either hybrid or self.

RESULTS

*Production of diploid and tetraploid self-compatible and self-incompatible male fertiles and steriles of T. pratense.* From a total of 323 plants produced after N<sub>2</sub>O treatment, 61% were classified as tetraploids based on size of stomates. Fifteen abnormal appearing plants, probably octoploids, were discarded. Seventy-seven of the 323 plants were the result of selfs of SC F<sub>1</sub>'s (S<sub>f</sub> gene) which carried a gene for MS in the heterozygous condition (Ms<sub>2</sub>ms<sub>2</sub>) and theoretically should have segregated three MS to one MF (Table 2). However, the actual number obtained was 22 MF to 25 MS, obviously a significant deviation from the expected ratio. The reason for this deviation is unknown. Fifteen of 25 MS's were tetraploids.

Two hundred seventy-six of the total of 323 plants were the result of intercrosses of SI F<sub>1</sub>'s (no S<sub>f</sub> gene) but heterozygous for a MS gene (Ms<sub>1</sub>ms<sub>1</sub>) (Table 2). These F<sub>2</sub> populations also deviated from a 3:1 MF to MS ratio. Thirty of 50 MS plants were tetraploids.

Table 2. Segregation of F<sub>2</sub> plants for male-sterility in self-compatible (F<sub>1</sub>'s selfed) and self-incompatible (F<sub>1</sub>'s intercrossed) populations based on presence or absence of pollen.

Compatibility System	Observed		Expected		χ <sup>2</sup>	Prob.
	MF : MS (No.) (No.)	MF : MS (No.) (No.)	MF : MS (No.) (No.)	χ <sup>2</sup>		
Self-compatible	22 : 25	3 : 1	19.92	0.005		
Self-incompatible	226 : 50	3 : 1	6.975	0.005-0.01		

INTERSPECIFIC CROSSES IN TRIFOLIUM

Table 3. Flowers, seeds, selfs and hybrids from interspecific pollinations of *Trifolium* species with or without the addition of mentor pollen.

Cross	Cross identification	Mentor				Non-mentor			
		flowers (No.)	seeds (No.)	selfs (No.)	hybrids (No.)	flowers (No.)	seeds (No.)	selfs (No.)	hybrids (No.)
1.	<i>T. pratense</i> (2x, MS, SI) × <i>T. diffusum</i> (2x, MF, SC)	1014	16	4	4	1072	25	9	3
2.	<i>T. pratense</i> (2x, MF, SI) × <i>T. diffusum</i> (2x, MF, SC)	1007	37	11	6	1029	132	30	7
3.	<i>T. pratense</i> (4x, MS, SI) × <i>T. diffusum</i> (4x, MF, SC)	1102	0	0	0	1126	20	0	1
4.	<i>T. pratense</i> (4x, MS, SC) × <i>T. diffusum</i> (4x, MF, SC)	1039	12	3	2	991	21	9	5
5.	<i>T. pratense</i> (4x, MS, SI) × <i>T. medium</i> (8x, * MF, SI)	1123	0	0	0	1208	0	0	0
6.	<i>T. pratense</i> (4x, MS, SC) × <i>T. medium</i> (8x, MF, SI)	1036	3	2	0	1101	4	1	0
7.	<i>T. pratense</i> (4x, MS, SI) × <i>T. sarosense</i> (6x, MF, SI)	1095	0	0	0	1099	0	0	0
8.	<i>T. pratense</i> (4x, MS, SC) × <i>T. sarosense</i> (6x, MF, SI)	1180	1	0	0	1077	7	2	0
9.	<i>T. medium</i> (8x, MS, SI) × <i>T. pratense</i> (4x, MF, SI)	1034	0	0	0	1195	1	1	0
10.	<i>T. medium</i> (8x, MS, SI) × <i>T. sarosense</i> (6x, MF, SI)	1041	28	0	13	1055	110	0	47
Total		10671	97	20	25	10953	320	52	63

\*Chromosome number varies between 8 and 10x.

Thus 10 diploid and 15 tetraploid SC plants and 20 diploid and 30 tetraploid SI plants were available for the study of the effect of compatibility systems on interspecific hybridization, and its interaction with mentor pollen. The specific plants used in crosses were examined cytologically and diploids were found to possess 14 and tetraploids 28 root-tip chromosomes.

*Use of male steriles to prevent selfing during hybridization.* MS *T. pratense* (2x, SI) crossed with *T. diffusum* (2x, MF, SC), produced 41 seeds of which seven were hybrids, 13 were selfs and the remainder failed to germinate (Table 3, cross 1, pooled across mentor treatments). The corresponding cross (No. 2) with an MF parent produced 169 seeds from which 13 hybrids were grown. Although the use of the MF resulted in more selfs, it also resulted in more hybrids than did the MS female parent. Apparently, this MS *T. pratense* stock is also slightly female sterile, and slightly male fertile. Mentor pollinations produced fewer seeds and selfs, but the same number of hybrids as non-mentor pollinations. Male steriles and fertiles produced similar results with both types of pollination.

*Effect of compatibility system.* Crosses involving SC, MS consistently gave higher seed production than the SI, MS strain (crosses 4, 6, 8 vs. crosses 3, 5, 7, Table 3). A total of 6753 SI crosses produced 20 seeds, no selfs, and one hybrid, and 6424 SC crosses produced 48 seeds, 17 selfs, and 7 hybrids. As in other treatments, normal pollinations resulted in more seeds, selfs and hybrids than did mentor pollinations. The SC strain produced more seeds, selfs, and hybrids than did the SI strain in both types of mentor treatments. Only cross 4 (*T. pratense* 4x, MS, SC) produced more hybrids (6 additional plants) than the same cross (No. 3) using the SI source. From crosses 5 and 6, *T. pratense* (4x, MS, SI + SC) × *T. medium* (8x, MF, SI) and 7 and 8, *T. pratense* (4x, MS SI+SC) × *T. sarosiense* (6x, MF, SI), all plants produced were morphologically identical to the female parent and were probably selfs. Therefore, in these crosses no differences between the two strains existed in the number of hybrids produced.

*Effect of ploidy level.* Crosses of MS *T. pratense* with *T. diffusum* at the diploid level produced 41 seeds, from which 13 selfs and 7 hybrids were obtained, and at the tetraploid level, 20 seeds, no selfs, and one hybrid (crosses 1 and 3 summed across mentor treatments, Table 4). Mentor pollination produced fewer seeds, selfs and hybrids than did normal pollinations. Diploid crosses produced more seed, selfs and hybrids than tetraploid in both types of pollinations. It is probable that the MS stock at the tetraploid level was more female sterile than at the diploid level but no crosses were made for direct comparison.

*Effect of mentor pollen.* Intraspecific test crosses of *T. pratense* using 'killed' pollen were compared with viable and viable plus 'killed'. Fewer seeds were produced from mentor pollinations than from normal pollinations (Table 4).

In total 97 seeds were produced from mentor and 320 from normal interspecific pollinations (Table 4). Likewise, more selfs and hybrids resulted from normal than mentor pollinations. Very likely the same factors that reduced seed set in the intraspecific test crosses were operational in the interspecific crosses.

INTERSPECIFIC CROSSES IN TRIFOLIUM

Table 4. Heads, flowers, seeds and seeds per head from intraspecific pollinations of *T. pratense* (2x) using 'killed' or viable pollen or a 1 : 1 mixture of the two.

Pollen	Heads (No.)	Flowers (No.)	Seeds (No.)	Seeds/flower (No.)
'Killed' only	6	226	0	0
Viable only	8	298	225	0.755
'Killed' + viable	9	442	241	0.545

In two crosses not involved in other treatments, *T. medium* × *T. sarosiense* (No. 10) produced more seeds and hybrids than *T. medium* × *T. pratense* (No. 9) and reciprocals (Nos. 5 to 8) in both the mentor and normal pollinations. Normal crosses produced more seeds and hybrids than mentor crosses.

*Hybrid classification and cytology.* Five hybrid plants from the crosses of *T. pratense* (2x, MS and MF, SC) with *T. diffusum* (2x, MF, SC) contained 15 root-tip chromosomes (Table 5). The hybrids produced 9.7 pollen grains per flower of which 7.6% were stainable with acetocarmine. The hybrids were intermediate in morphology between the parents.

Four hybrid plants from crosses of *T. pratense* (4x, MS, SC and SI) with *T. diffusum* (4x, MF, SC) contained 30 root-tip chromosomes (Table 5). Hybrids produced pollen of which 78.3% was stainable with acetocarmine.

Two hybrid plants from the cross of *T. medium* (8x, MS, SI) with *T. sarosiense* (6x, MF, SI) contained 60 root-tip chromosomes (Table 5). Again the hybrid plants were intermediate in morphology between the parent species. Seeds were produced when the hybrid was pollinated by both parents and also when the hybrid was used to pollinate both of its parents. When selfed, no seeds were produced indicating self-incompatibility. When the hybrid was pollinated with *T. pratense* (4x) one seed was obtained, apparently a self.

Table 5. Chromosome number and percent pollen stainability of hybrid *Trifolium* plants from interspecific crosses.

Hybrid	Plants examined (No.)	Cells counted (avg. No.)	Chromo- somes per cell (No.)	Total flowers (No.)	Total pollen grains		
					stained (No.)	unstained (No.)	stainable (%)
<i>T. pratense</i> (2x) × <i>T. diffusum</i> (2x)	5	6.2	15	54	40	485	7.6
<i>T. pratense</i> (4x) × <i>T. diffusum</i> (4x)	4	5	30	12	2310	641	78.3
<i>T. medium</i> × <i>T. sarosiense</i> *	2	10	60	6	1645	355	82.3

\*Determined by QUESENBERRY & TAYLOR (1977).

## DISCUSSION

This investigation encountered many of the same difficulties as earlier work in that barriers to interspecific hybridization were not circumvented by any of the techniques employed. Apparently, the type of interspecific barriers that occurs in *Trifolium* is different from that which occurs in other species (GRUN & RADLOW, 1961; KNOX et al., 1972; STETTLER, 1968).

Mentor pollen as used in this study definitely was detrimental for interspecific hybridization. Fewer hybrids and seeds were obtained from mentor than from normal pollination. Perhaps different methods of obtaining 'killed' pollen other than by methanol application would have given different results. However, methanol was one of the agents used by KNOX et al. (1972). The primary factor involved in lowered seed set with mentor pollen is thought to be the reduced amount of viable pollen applied to the stigma.

Fewer hybrids were produced between *T. pratense* × *T. diffusum* at both the diploid and tetraploid levels than in earlier work (TAYLOR et al., 1963). Also, in contrast to earlier work, the diploid crosses produced more hybrids than the tetraploid level crosses. The tetraploid *T. pratense* plants used as females in the present study were male-sterile and possibly may have been somewhat female-sterile.

The use of SC as contrasted to SI females in *T. pratense* was of little benefit for interspecific hybridization as compared with similar usage in other species (GRANT, 1956; GRUN & RADLOW, 1961). More seeds were produced with the SC than the SI genotype but much of this was the result of selfing. Also, the use of different male steriles in generating the two compatibility genotypes may have contributed to the difference in seed set. The male sterile ( $ms_1ms_1$ ) for the SI genotype was derived from male sterile 66, which possibly is less female-fertile than the SC genotype ( $ms_2ms_2$ ) derived from 740.

Use of male steriles also resulted in lowered female fertility. Although the male-sterile genes eliminated selfing, the fewer hybrid seeds produced is a serious disadvantage. Selection for higher female fertility in the male-sterile stocks is necessary if they are to be used routinely for interspecific hybridization.

This work tends to follow the conclusion drawn by earlier workers in that manipulation of factors affecting pollen growth and development in the stigma and style probably will not affect interspecific hybridization (ANDERSON & TAYLOR, 1974; EVANS, 1962a; NEWTON et al., 1970). With the possible exception of ploidy level treatments, all techniques used in this study operate primarily at the level of the style and stigma and do not influence post-fertilization barriers. Combination of treatments each of which overcome individual interspecific hybridization barriers will have to be utilized for maximum success.

*T. pratense* × *T. diffusum* hybrids were obtained at a somewhat lower frequency than those obtained by TAYLOR et al. (1963) and SCHWER & CLEVELAND (1972). *T. medium* × *T. sarosiense* hybrids had also been obtained by QUESENBERRY & TAYLOR (1977). However, in the first-listed hybrid, pollen stainability was greater than that obtained earlier.



REFERENCES

- ANDERSON, M. K. & N. L. TAYLOR, 1974. Effect of temperature on intra- and interspecific crosses of diploid and tetraploid red clover, *Trifolium pratense* L. *Theoret. Appl. Genet.* 44: 73-76.
- ARMSTRONG, K. C. & R. W. CLEVELAND, 1970. Hybrids of *Trifolium pratense* × *Trifolium pallidum*. *Crop Sci.* 10: 354-357.
- CHEN, CI-CHANG & P. B. GIBSON, 1972. Barriers to hybridization of *Trifolium repens* with related species. *Can. J. Genet. Cytol.* 14: 381-389.
- EVANS, ALICE, 1962a. Species hybridization in *Trifolium*. I. Methods of overcoming species incompatibility. *Euphytica* 11: 165-176.
- EVANS, ALICE, 1962b. Species hybridization in *Trifolium*. II. Investigating the pre-fertilization barriers to compatibility. *Euphytica* 11: 256-262.
- GRANT, VERNE, 1956. The influence of breeding habit on the outcome of natural hybridization in plants. *Amer. Nat.* 90: 319-322.
- GRUN, P. & A. RADLOW, 1961. Evolution of barriers to crossing of self-incompatible with self-compatible species of *Solanum*. *Heredity*: 16: 137-143.
- KNOX, R. B., R. R. WILLING & L. D. PRYOR, 1972. Interspecific hybridization in poplars using recognition pollen. *Sil. Genet.* 21: 65-69.
- MAIZONNIER, D., 1972. Obtention d'hybrides entre quatre especes perennes du genre *Trifolium*. *Ann. Amélior. Plantes.* 22: 375-387.
- NEWTON, D. L., W. A. KENDALL & N. L. TAYLOR, 1970. Hybridization of some *Trifolium* species through stylar temperature treatments. *Theoret. Appl. Genet.* 40: 59-62.
- QUESENBERRY, K. H. & N. L. TAYLOR, 1977. Interspecific hybridization in *Trifolium* L. Sect. *Trifolium* Zoh. II. Fertile polyploid hybrids between *T. medium* L. and *T. sarosiense* Hazsl. *Crop Sci.* 17: 141-145.
- SCHWER, J. F. & R. W. CLEVELAND, 1972. Tetraploid and triploid interspecific hybrids of *Trifolium pratense* L., *T. diffusum* EHRH., and some related species. *Crop Sci.* 12: 419-422.
- SMITH, R. R., 1971. Inheritance of a male-sterile character in red clover (*Trifolium pratense* L.). *Crop Sci.* 11: 326-327.
- STEBBINS, G. L., 1957. Self-fertilization and population variability in the higher plants. *Amer. Nat.* 91: 337-354.
- STETTLER, R. F., 1968. Irradiated mentor pollen: its use in remote hybridization of black cottonwood. *Nature* 219: 746-747.
- TAYLOR, N. L., M. K. ANDERSON, K. H. QUESENBERRY & LINDA WATSON, 1976. Doubling the chromosome number of *Trifolium* species using nitrous oxide. *Crop Sci.* 16: 516-518.
- TAYLOR, N. L., C. KITBAMROONG & M. K. ANDERSON, 1978. Genetic male sterility in red clover. *Crop Sci.* 18: 1033-1036.
- TAYLOR, N. L., W. H. STROUBE, G. B. COLLINS & W. A. KENDALL, 1963. Interspecific hybridization of red clover (*Trifolium pratense* L.). *Crop Sci.* 3: 549-552.