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Effect of Temperature on Intra- and Interspecific Crosses of Diploid and Tetraploid Red Clover, *Trifolium pratense* L.*

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Summary. Reciprocal intraspecific crosses of diploid and tetraploid *Trifolium pratense* L. and reciprocal interspecific crosses of 4N *T. pratense* and *T. medium* were attempted in conjunction with a 40 °C temperature treatment on the female parent. The temperature treatment was found to be of little benefit in interspecific and intraspecific hybridization attempts. No cytologically verified interspecific hybrids were found from either heat-treated or non-heat-treated crosses. Intraspecific crosses produced no viable triploid offspring. One seedling was found to possess 17 chromosomes but died before positive verifications could be made. However, several seedlings from male-sterile diploid × tetraploid crosses were found to possess 28 chromosomes. These resulting tetraploids were cross compatible with other tetraploids.

Introduction

Red clover, *T. pratense* L., is a short-lived perennial species and benefit could be derived from hybridization with a long-lived perennial species. Difficulties in obtaining interspecific hybrids in the genus *Trifolium* are widespread and only a few attempts have been successful. The only reported interspecific hybrids with red clover involved the annual species, *T. diffusum* L. (Taylor *et al.*, 1963) and *T. pallidum* L. (Armstrong and Cleveland, 1970). *T. medium* is a species which could provide increased longevity to red clover germ plasm.

Triploid offspring from diploid-tetraploid crosses in red clover could provide the genetic material necessary to develop a trisomic series in red clover. Ploidy level crosses in red clover have been attempted by Laczynska-Hulewicz and Mackiewicz (1963) and Maizonnier (1969). Diploid pollen grains were found to grow faster than haploid pollen grains by Laczynska-Hulewicz and Mackiewicz (1963). Low frequencies of triploid plants were found in diploid-tetraploid crosses by Maizonnier (1969).

Red clover is a self-incompatible species (Williams, 1931). Self-incompatibility has been overcome by using relatively high temperatures in *T. pratense* (Leffel, 1963; Kendall and Taylor, 1969) and *T. hybridum* L. (Townsend, 1968). Dadson (1969) reported temperature treatments decreased interspecific incompatibilities in certain *Trifolium* species.

Newton *et al.* (1970) applied a temperature treatment of 40 °C to female buds to facilitate interspecific hybridization of diploid *T. pratense* with several other *Trifolium* species. Although no hybrids were obtained, some seeds were produced. Whether the *T. pratense* × *T. medium* seeds were of hybrid origin or selfs could not be determined because the seeds would not germinate. They suggested that tetraploid (4N = 28) red clover would be easier to hybridize with *T. medium* (ca. 72 chromosomes) than diploid red clover. The objectives of this research were to determine the effect of high temperature (40 °C) on (i) hybridization of red clover with *T. medium* and (ii) diploid-tetraploid crosses of red clover.

Material and Methods

The diploid red clover used in this study was obtained from clonal stocks at the University of Kentucky. Two male sterile diploid clones were used in diploid-tetraploid crosses. We obtained one male sterile clone (740-3-7) from our backcross breeding program, and the other (66-1) was obtained from R. R. Smith, University of Wisconsin. *T. medium* plants were obtained from an open-pollinated seed source in our field nursery. All tetraploid red clover and *T. medium* plants were transplanted to the field in the late summer and were dug in early February and placed on greenhouse benches along with diploid red clover. Artificial light sources (16 hr. daylength) were used to induce flowering.

Intact stems instead of the excised stem method reported by Kendall and Taylor (1969) were used for temperature treatments during anthesis. Buds showing floret color were inserted into holes drilled in the sides of a seed germinator maintained at 40 ± 1 °C. The remainder of the plant was exposed to normal greenhouse temperature (ca. 25 °C). After 2 or 3 days, the temperature-treated buds were removed and pollinated. Buds not subjected to the temperature treatment were maintained at ca. 25 °C on the greenhouse bench. Seeds were harvested from plants on greenhouse benches after about 30 days.

All seeds were scarified and placed on moistened filter paper in petri dishes at about 5 °C for 3–4 days and then germinated at 25 °C. Root tips for chromosome counts were collected from germinating seeds. The seedlings

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Table 1. *Seed obtained from reciprocal interspecific hybridization attempts between Trifolium pratense L.(4N) and T. medium*

Cross	Buds (No.)	Florets (No.)	Number of Seeds Obtained			Number of Seeds Germinated		
			Normal	Abnormal	Total	Normal	Abnormal	Total
T. medium (HT)[1] *T. pratense*	40	1433	45 (3.14)[2]	5 (.35)	50 (3.49)	38	0	38
T. medium × *T. pratense*	51	1266	13 (1.03)	26 (2.05)	39 (3.08)	11	0	11
T. pratense (HT) × *T. medium*	30	1219	147 (12.06)	17 (1.38)	164 (13.45)	131	5	136
T. pratense × *T. medium*	26	858	31 (3.62)	5 (.59)	36 (4.70)	27	1	28

[1] HT = heat treatment of 40 °C applied to bud of female parent.

[2] Values in parentheses are percentage of total florets pollinated.

were then transplanted to sub-irrigated vermiculite and one-third strength Hoagland's nutrient solution was applied weekly. The seedlings were later transferred to 10 cm clay pots filled with disinfected soil.

Root tips from germinating seeds were pretreated for 4 hrs in 0.003 M 8-hydroxyquinoline, fixed in alcohol-acetic acid (3:1/v:v), hydrolyzed in 1 N HCl for 6 min, stained in Feulgen, placed in 45% glacial acetic acid for 2 hrs and squashed in 1% acetocarmine.

Results

Interspecific Crosses

Seeds were obtained from all classes of crosses of tetraploid red clover with *T. medium* (Table 1). The largest amount of seed was obtained by heat treatment of tetraploids crossed with *T. medium* pollen. The percentage of total seed set did not change when the heat treatment was applied to *T. medium* buds. However, heat treatment of *T. medium* buds produced a larger number of normal seeds. Most of the seeds that germinated from *T. medium* used as the female crossed to tetraploid red clover gave rise to plants that were apparently selfs. Cytological examination of root tips revealed ca. 72 (52—80) chromosomes in each of the plants. The plants obtained were phenotypically similar to *T. medium* and the varying chromosome numbers were probably due to abnormal disjunction. Five abnormal-appearing seedlings from *T. medium* female crosses were obtained which had small, deformed root tips, consequently, no significant cytological conclusions could be reached. These seedlings were distorted and reached a height of about 1 cm after 6 weeks. All five of these seedlings died before any conclusions about their origin could be made. None of the 31 abnormal seeds germinated.

The heat treatment overcame, to a limited extent, the self-incompatibility present in tetraploid red clover as evidenced by the total amount of seed in heat-treated buds as compared with non-heat-treated buds in tetraploid *T. pratense* crosses with *T. medium*. Of the 158 normal seeds that germinated, 42 gave rise to plants which were sufficiently dissimilar in plant type to justify cytological examination. Forty-one of the 42 plants had 28 chromosomes, and the remaining plant had 31 chromosomes. The six abnormal seeds that germinated appeared to be tetraploid red clover. Two of the five plants from heat-treated buds had 28 chromosomes. Two and one of the remaining 3 plants had 31 and 33 chromosomes, respectively. The plant obtained from the abnormal seed from non-heat-treated crosses had 27 chromosomes. Again, as in crosses using *T. medium* as the female, three abnormal seedlings showing very little growth died before any verification could be made.

Intraspecific Crosses

Seeds were obtained from all classes of diploid by tetraploid crosses of *T. pratense* (Table 2). The percentage of florets which set seed was about five times greater in the non-heat-treated flower heads than for heated florets when the treatment was applied to both male sterile and normal diploid florets.

The 19 normal seeds obtained from the male-sterile diploid-tetraploid crosses germinated and all root tips examined showed 28 chromosomes. The resulting tetraploids had normal anthers and were male fertile indicating they were products of fertilization between male-sterile diploids and tetraploids. The tetraploids were cross-compatible with other tetraploids as evidenced by seed production upon crossing. The one abnormal seed gave rise to a weak seedling which later died. The one root tip examined showed 17 chromosomes. The eight seeds obtained from diploid × tetraploid crosses germinated, and all were selfs (2N = 14).

Many more seeds were obtained from heat—treated tetraploid-diploid crosses. Root tips were collected from all germinating seeds and all proved to be selfs. Fifty-four of the 59 normal seeds possessed 28 chromosomes. Five plants exhibited chromosome numbers of 30—32 as evidenced by several root tips. The root tip obtained from 1 abnormal seed that germinated had 27 chromosomes.

Table 2. *Seed obtained from reciprocal intraspecific hybridization attempts between diploid (2N) and tetraploid (4N) Trifolium pratense L.*

Cross	Buds (No.)	Florets (No.)	Number of Seed Obtained			Number of Seed Germinated		
			Normal	Abnormal	Total	Normal	Abnormal	Total
MS[1] diploid (HT)[2] × tetraploid	16	798	1 (.12)[3]	2 (.25)	3 (.38)	0	0	0
MS diploid × tetraploid	73	3911	44 (1.15)	16 (.41)	61 (1.56)	19	1	20
Diploid (HT) × tetraploid	11	409	3 (.73)	9 (2.20)	12 (2.93)	0	0	0
Diploid × tetraploid	56	1937	30 (1.54)	240 (12.38)	270 (13.92)	8	0	8
Tetraploid (HT) × diploid	14	586	75 (12.80)	7 (1.19)	82 (13.99)	43	1	44
Tetraploid × diploid	48	1915	31 (1.62)	19 (.99)	50 (2.61)	16	0	16

[1] MS — male sterile.
[2] HT — heat treatment of 40 °C applied to bud of female parent.
[3] Values in parentheses are percentages of total florets pollinated.

Discussion

All of the cytologically verified seedlings obtained from our interspecific hybridization attempts proved to be selfs. Self-incompatibility of *T. medium* was not affected by the high temperature treatment as evidenced by number of seeds obtained. Newton *et al.* (1970) found similar conditions in using excised pistils in *T. medium.* However, Newton *et al.* (1970) found a significant increase in the number of pollen tubes growing through heat-treated excised *T. medium* styles that had been pollinated with *T. pratense* compared with non-heat-treated excised styles. This indicated that a temperature treatment should facilitate interspecific hybridization. Our data showed no differences in seed amounts on intact plants between heat-treated and non-heat-treated crosses using *T. medium* as the female parent. The apparent discrepency in results between pollen growth and seed yields may reflect problems associated with fertilization or post fertilization seed abortion. Müller (1960) concluded that failure of interspecific hybridization of many *Trifolium* species is mainly dependent upon abnormal pollen tube growth while post fertilization disorders are of secondary importance. Pandey (1969) has suggested that intraspecific incompatibility is a secondary specificity and that interspecific incompatibility is a primary specificity. He suggests that the sites of pollen inhibition in inter- and intraspecific incompatibilities are in the stigma and style, respectively. Chen and Gibson (1972) concluded that failure or ineffectiveness of pollen germination and of pollen tube growth are not primary causes of incompatibility in certain *Trifolium* species. They found fertilization to occur in higher frequencies in some species combinations but did, in fact, find that fertilization occurred in all interspecific crosses attempted. They postulated "that post-fertilization seed abortion must be an additional and perhaps a significant barrier". Our data and earlier work by Newton *et al.* (1970) tend to support the recent findings of Chen and Gibson (1972).

Several selfed seedlings of tetraploid *T. pratense* were found with hypo- or hyperploid chromosome numbers. Similar findings of aneuploid plants have been reported by Ellerström and Sjödin (1966) and Maizonnier and Picard (1970). Low frequencies of abnormal disjunction most likely gave rise to these plants. Several weak seedlings suspected as being interspecific hybrids died before any verification could be made.

In intraspecific crosses, all but 1 of the crossed seedlings showed 28 chromosomes. Unreduced diploid gametes from our male sterile clones most likely gave rise to the tetraploid plants found in the diploid-tetraploid crosses. Maizonnier (1969) found a frequency of 1 triploid/1000 florets and 3/1000 from diploid × tetraploid crosses and reciprocal crosses, respectively. We obtained only 1 seedling showing an abnormal chromosome number (17) and that seedling soon died. It may be that the use of a wider number of genotypes would provide more success in recovering triploid offspring. Male-sterile plants were used to limit the number of selfed seeds; however, we were restricted in our male-sterile sources to two clones and most of the crosses involved only one. Meiotic analyses of our male-sterile sources may reveal higher frequencies of abnormal disjunction patterns than in other diploid clones.

Two other points of interest are denoted by these data. The heat treatment has been found to overcome self-incompatibility in diploid red clover (Leffel, 1963; Kendall and Taylor, 1969). Yet, we obtained less seed in crosses using the heat treatment in diploid × tetraploids. This illustrates that the heat treatment reduced an S-allelic compatible mating. Diploid pollen grains have been found to grow faster than haploid pollen grains by Laczyńska-Hulewicz (1963) and this may have been the condition in our crosses. The same female parent was used in all non-male sterile diploid × tetraploid crosses so no differences attributable to pseudo-self-compatibility in different clones was involved. Secondly, it would be expected that male-sterile diploids × tetraploids and diploids × tetraploids would produce uniform numbers of seeds less selfed seed via pseudo-self-compatibility in fertile diploids. Clone 66-1 was the female parent in most of our male-sterile crosses, and it has shown reduced female fertility when compared with 740-3-7 crossed to compatible diploid testers. Partial female sterility as well as the lack of selfed seed could account for the reduced numbers of seeds in male-sterile diploid × tetraploid crosses. Most of the 240 abnormal seeds from non-heat-treated diploid-tetraploid crosses were probably of triploid origin but did not germinate.

On the basis of these results, it may be concluded that the heat treatment of female buds is of little value in interspecific hybridization of *T. pratense* and *T. medium*. The use of tetraploid male sterile red clover in crosses may facilitate interspecific hybridization because the problem of selfed-seed production would be eliminated. Our results likewise indicate no benefit due to the heat treatment in intraspecific crosses. No doubt the use of a wider number of genotypes in combination with male sterility would provide viable triploid offspring.

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