Recurrent Selection for 2*n* Pollen Formation in Red Clover[1]

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ABSTRACT

The superior quality and yielding ability of tetraploid red clover (*Trifolium pratense* L.) in Europe has led to interest in developing tetraploid red clover adapted to the USA. It is possible to use 2*n* gametes (gametes with the chromosome number of the sporophyte) to develop tetraploid red clover, but a relatively high frequency of 2*n* gamete production appears to be necessary before 2*n* gametes can be used efficiently to obtain tetraploid plants. Three cycles of phenotypic recurrent selection for 2*n* pollen production were conducted on adapted diploid red clover germplasm grown in the greenhouse. Average 2*n* pollen production per plant increased from 0.04% in the original population to 47.38% in the third cycle. In an effort to limit inbreeding, selection was restricted by families such that only the best plant from each family was selected. This appears to have been successful, as mean dry matter production per plant did not decrease during the selection process. Realized heritability for 2*n* pollen formation was about 0.50. It was estimated that two to six genes control the frequency of 2*n* pollen formation within a plant. After one cycle of selection the frequency of 2*n* pollen production was sufficiently high to successfully obtain tetraploid plants, although at a low frequency. After three cycles of selection against 2*n* pollen formation in a control population, we were unable to change the frequency of 2*n* pollen-producing plants from that in the original population.

Additional index words: **Restricted selection, Sexual tetraploidization, 2*n* gametes, *Trifolium pratense* L.**

THE DISCOVERY of 2*n* gametes (gametes with the chromosome number of the sporophyte) in red clover, *Trifolium pratense* L., and the ability to produce polyploids in this species (Parrott and Smith, 1984; Parrott et al. 1985) indicate their possible use to routinely develop tetraploid ($2n = 4x = 28$) red clover. Red clover occurs naturally as a diploid ($2n = 2x = 14$) species, but tetraploid strains developed in Europe frequently out-perform diploid strains (Taylor and Smith, 1979), suggesting it would be desirable to develop tetraploid germplasm adapted to the red clover–growing regions of the USA. A high frequency of 2*n* pollen production is a key to successful sexual tetraploidization in red clover, such that tetraploid seed would be obtained following either $4x-2x$ or $2x-2x$ crosses. Recurrent selection is one method to develop a population with a high frequency of 2*n* pollen formation, and is currently being used to develop such a population of potato (*Solanum* spp.) (McHale, 1983).

Phenotypic recurrent selection has been used successfully in red clover breeding (Taylor and Smith, 1979) to develop persistent and pest-resistant cultivars. One possible limitation of recurrent selection is that inbreeding may result if no effort is made to minimize the incidence of consanguineous matings. Cornelius and Taylor (1981) used phenotypic recurrent selection to increase the intensity of flower petal color in red clover. They successfully met this goal, but a corresponding linear decrease in dry weight over cycles of selection was attributed to inbreeding depression.

A recurrent selection program was initiated in 1981, in an attempt to develop a red clover population with a high frequency of 2*n* pollen formation. This population would then serve as a future source of 2*n* pollen for a sexual tetraploidization program.

MATERIALS AND METHODS

Selection Procedures

Eighteen red clover plants that produced at least 1% 2*n* pollen were identified initially after screening 600 plants from six cultivars (Parrott and Smith, 1984). The cultivars were 'Arlington', 'Chesapeake', 'Florex', 'Kenstar', 'Pennscott', and 'Redman'. Ten of these C_0 plants, each of which produced 1 to 3% 2*n* pollen, were selected to create a Low (L) population and were intercrossed in a field cage using honey bees (*Apis mellifera* L.) to effect pollination. Progenies from these matings were designated as the L C_1 population. Also, five C_0 plants, each producing 4 to 6% 2*n* pollen, were intercrossed to produce the C_1 for a medium (M) population. Finally, six C_0 plants, selected for producing no 2*n* pollen, were intercrossed to produce the C_1 for a zero (Z) population.

Twenty-five C_1 progeny from each selected plant in the L and M C_0 populations were planted in the greenhouse. The first 20 plants to flower in each half-sib progeny group were evaluated for 2*n* pollen production. The presence of 2*n* pollen was determined by examining 250 dry pollen grains from each of two flowers per plant under low magnification of a microscope. Ploidy identification of pollen grains was based on the morphology of individual grains. Haploid grains are oblong in shape, whereas 2*n* grains are tetrahedral in shape. Because the average 2*n* pollen formation in the L population (5.14%) and that of the M population (4.45%) were very similar after one cycle of selection, selected plants from each were combined to create a low-medium (LM) population. Because heterozygosity is of utmost importance in autotetraploids (Mendiburu et al., 1974; Bingham, 1979), and as the population resulting from recurrent selection is to be used to develop an autotetraploid population, it was considered essential to minimize the amount of inbreeding that might result during the selection process. Consequently, selection was largely restricted to families. The best 2*n* pollen producer in each of 11 C_1 half-sib families was selected. Four of the total 15 families were not included due to their extremely low levels of 2*n* pollen production. To compensate, an additional plant was selected from each of four families to make a total of 15 C_1 plants. These were then intercrossed to produce the C_2 generation. Again, C_2 progeny from each C_1 plant were planted in the greenhouse and evaluated as previously described. The two highest 2*n* pollen formers from most families were selected. As two families were dropped from the LM population due to their low levels of 2*n* pollen formation, three plants were selected from four superior families to obtain a total of 30 selected C_2 plants. These were intercrossed to obtain the C_3 generation. For the LM population, selection intensities [(number of plants selected/number of plants evaluated) × 100] were 2.5% from C_0 to C_1, 5.0% from C_1 to C_2, and 10.0% from C_2 to C_3, with respective selection differentials of 2.4, 43.5, and 38.8%.

Five C_1 progeny from each of the six C_0 parents in the Z

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population were evaluated as described previously for the L and M populations, after the first cycle of selection. Plants producing any 2*n* pollen were discarded and the remainder intercrossed. Twenty C_2 plants from each C_1 plant were established in the greenhouse and evaluated. Sixty C_2 plants that produced no 2*n* pollen were selected and intercrossed in a field isolation cage to obtain the C_2 generation. Although a family structure was maintained during selection, different families contributed differing numbers of progeny to the next generation.

Testing Procedures

To measure progress from selection, seed from the original cultivars and from each family in each cycle of selection for the LM and Z populations were planted in the greenhouse. Plants were established in 10.2-cm (4-inch) pots, which were arranged in blocks in replications design, with five blocks in each of two replications, such that each cycle of selection was represented by 60 plants. Plants that flowered within a 2-month period after the onset of flowering were evaluated for 2*n* pollen formation. For analyses of variance, data were transformed using the arcsine square root transformation. Cycle means within blocks were analyzed using the Statistical Analysis System (SAS) General Linear Models procedure (Ray, 1982). Cycles and blocks within replications were tested using the error means square, and replications were tested using the blocks/reps mean square. Cycles were partitioned into single degree of freedom contrasts for linear, quadratic, and cubic effects. These effects were tested using the error mean square. Individual analyses of variance were performed on each cycle of selection, C_1 to C_3 taking into consideration the family structure within each cycle. As the families were half-sib families, additive genetic variance was estimated as being four times the among-family variance component. The number of genes affecting 2*n* pollen expression was estimated using the approximate formula, $n = R^2 / (8\sigma^2_{(f)})$ (Falconer, 1981a, b), where R is the total range of response to selection, in this case being 0 to 1.57, which is the arcsine square root transformation for 0 to 100% 2*n* pollen formation, with 100% being a theoretical maximum. Narrow sense heritability (h^2) was estimated as a ratio of the additive genetic variance ($\sigma^2_{(f)}$) to the phenotypic variance. The phenotypic variance was calculated as the sum of the variance components for family, replication per family, and error. Realized heritability was calculated for each cycle by dividing the realized gain by the selection differential. To help compensate for annual fluctuations in the frequency of 2*n* pollen formation in each cycle, the mean percent 2*n* pollen formation of the selected parents for each cycle was adjusted by the percentage change in 2*n* pollen formation between the year each cycle was first evaluated, and the final year when all cycles were evaluated together.

As 2*n* pollen frequencies in the Z population consisted almost exclusively of null values, it was impossible to use analysis of variance to analyze the data. Consequently, the frequencies of 2*n* pollen-producing plants in the C_1 to C_3 generations were tested by chi-square to determine if they differed from the frequency of 2*n* pollen-producing plants in the C_0.

Dry weight of herbage regrowth was measured on plants to detect possible effects of inbreeding. After all pollen samples had been collected, the herbage of each plant was removed to a height of 2 cm. Herbage of 40-day regrowth of each plant was removed, dried in a forced-air drier, and the weight recorded. Data were analyzed as described previously for the frequency of 2*n* pollen except that no transformation was necessary on the raw data.

Procedure for Testing the Effectiveness of 2*n* Pollen

To test whether the increased frequency of 2*n* pollen formation would help obtain tetraploid plants, the original 2*n* pollen producers (C_0's) were used as males in 4*x*-2*x* crosses. The tetraploid plants used as females were developed by Dr. N.L. Taylor, University of Kentucky, Lexington, KY, using nitrous oxide to double the chromosome number of Kenstar plants. We also attempted to produce tetraploids from 2*x*-2*x* crosses using selected 2*n* pollen-producing C_1 plants as males. These selected C_1 plants were placed in an isolation cage together with nine previously identified 2*n* egg-producing, male-sterile diploid plants as females. Honey bees were used as pollinators. Seed heads were manually harvested from the male-sterile diploid plants. Progeny were evaluated for ploidy using pollen morphology as an initial indicator of tetraploidy. Final verifications were made by chromosome counts in root-tip squash preparations (Parrott et al., 1985).

RESULTS AND DISCUSSION

Average frequency of 2*n* pollen production per plant in the LM population increased from 0.04% in the base cycle (C_0) to 47.38% in the third cycle population (Table 1). There were highly significant differences among cycles, with the greatest increase realized between the later cycles, which was evidenced by a significant quadratic effect. Both the frequency of 2*n* pollen production per plant and the frequency of plants that produced 2*n* pollen in each cycle increased. Whereas only 7% of the plants in the original population (C_0) showed evidence of 2*n* pollen production, 75 and 97% of the plants in the C_1 and C_2, respectively, produced 2*n* pollen to some extent (Table 2). All plants in the C_3 produced at least some 2*n* pollen. While original plants were found to produce 2*n* pollen as a consequence of parallel spindle orientation during the second meiotic division (Parrott and Smith, 1984), it is possible that other mechanisms of 2*n* pollen formation (e.g., first division restitution or premature cytokinesis) may have been selected during the selection process.

In the final test of this study, unselected red clover

Table 1. Mean percent 2*n* pollen production per plant for three cycles of selection in the zero (Z) and the low-medium (LM) populations of red clover.

	Population			
	Z		LM	
Cycle	Mean	Range	Mean	Range
0	0.04	0.0-1.2	0.04	0.0-1.20
1	0.02	0.0-0.2	3.09	0.0-59.5
2	0.02	0.0-0.2	17.86	0.0-88.3
3	0.03	0.0-0.4	47.38	0.8-99.6

Table 2. Chi-squares comparing the frequency of 2*n* pollen producing plants in C_1 to C_3 of the low-medium (LM) population with the frequency of 2*n* pollen producing plants (12.5:1, non-producers/producer) in the C_0.

	Observed values				Expected (12.5:1) values		
Cycle	Non-producer	Pro-ducer	Total	% Pro-ducers	Non-producer	Pro-ducer	χ^2 (1 df)
0	50	4	54	7	–	–	–
1	14	42	56	75	51.52	4.48	341.55**
2	2	44	46	97	42.34	3.68	480.20**
3	0	56	56	100	51.52	4.48	621.22**

** Highly significant differences at the 1% level.

Table 3. Chi-squares comparing the frequency of 2*n* pollen-producing plants in C_1 to C_3 of the zero (Z) population, with the frequency of 2*n* pollen-producing plants (12.5:1, nonproducers/producer) in the C_0.

Cycle	Observed values: Non-producer	Producer	Total	Expected (12.5:1) values: Non-producer	Producer	χ^2 (1 df)
0	50	4	54	--	--	--
1	44	4	48	44.16	3.84	0.007 NS†
2	23	2	25	23.00	2.00	0.000 NS
3	48	7	55	50.60	4.40	1.670 NS

† NS = nonsignificant.

Table 4. Mean plant dry weight (g) in three cycles of selection for 2*n* pollen producers in the zero (Z) and the low-medium (LM) populations of red clvoer.

Cycle	Population: Z	LM
0	4.09	4.09
1	4.72	5.02
2	4.87	4.26
3	4.41	4.71
LSD (0.05)	0.58	0.67

populations were found to have, on the average, one producer of 2*n* pollen for every 12.5 nonproducers of 2*n* pollen (i.e., 7% of the population produced 2*n* pollen). Only 3% of the plants in the original population (consisting of 600 plants) produced 2*n* pollen to some extent (Parrott and Smith, 1984). The higher frequency observed in the final test is probably due to sampling and environmental effects. Only 54 plants of the original population were sampled in the final test. Therefore, the frequency of 2*n* pollen-producing plants in each cycle of the LM and of the Z population were compared to the 7% observed in the unselected populations (Tables 2 and 3). Frequencies in each cycle of the LM population were significantly greater than the frequency of the unselected population, but frequencies of 2*n* pollen-producing plants in any of the cycles of the Z population were not significantly different from that of the unselected population. The cause of this low frequency of 2*n* pollen in the Z population is unknown. It may represent meiotic accidents that are occurring, recessive genes still segregating in the population, or both.

Significant differences were observed between cycles for dry weight in the LM population (Table 4). This population showed a weight increase in the first cycle, followed by a decline in the C_2 and another increase in the C_3 cycle. The initial weight increase probably was due to heterosis that resulted from intercrossing the different cultivars. This effect appears to have persisted in the LM population, as the average weight after the third cycle, while not significant, was still greater than the average weight of the original population.

There were no significant differences for mean plant dry weight of progeny between cycles in the Z population, although the quadratic effect was significant, suggesting an initial heterosis that declined as the cycles advanced. Due to the small size of the initial population (six plants), even restricting selection by families was unable to prevent inbreeding depression.

Table 5. Estimates, based on transformed data, of additive genetic variance, realized heritability, and number of genes affecting 2*n* pollen formation in the C_2 and C_3 cycles of the low-medium (LM) population of red clover.

Cycle	Additive variance	Realized h^2	Number of genes
2	0.0496	0.54	6
3	0.1728	0.49	2

Based on the response to selection, it was expected that heritability of 2*n* pollen formation would be moderate to high. The heritabilities for C_2 and C_3 were moderate and quite similar (0.54 and 0.49, respectively, Table 5).

The presence or absence of 2*n* pollen formation is controlled by one gene in potatoes (Mok and Peloquin, 1975), alfalfa (*Medicago sativa* L.) (McCoy, 1982), and peas (*Pisum sativum* L.) (Myers et al., 1984). Segregations for the presence or absence of 2*n* pollen formation in red clover suggest monofactorial control for this trait (Smith et al., 1985). However, the frequency with which 2*n* pollen is produced in a given plant is probably under the influence of modifying genes upon which recurrent selection is effective. Estimates for the number of genes affecting the frequency of 2*n* pollen formation per plant were six and two for C_2 and C_3, respectively. To the degree with which estimates for gene number are reliable, at least two genes, but probably not more than six, affect the frequency of 2*n* pollen formation. The estimate for σ^2_A used was not obtained from the base population and the base population was not in random mating equilibrium; therefore, linkage may be of importance and gene frequencies may have been other than 0.5. Consequently, the limit of six genes may be too high or too low. However, a low number of genes is consistent with the heritability associated with the frequency of 2*n* pollen formation and the rapid rate of gain (an average rate of gain of 15.8% per cycle) achieved through selection.

Original 2*n* pollen-producing plants (average 2*n* pollen production = 5.5%) and the selected plants from the C_1 generation (average 2*n* pollen production = 45.5%) were tested for their ability to serve as 2*n* pollen parents. The original plants were used in 4*x*-2*x* crosses, while C_1 plants were used in 2*x*-2*x* crosses to nine diploid, male-sterile plants that produced both *n* and 2*n* eggs (average frequency of 2*n* egg production = 3.4%, as determined from previous 2*x*-4*x* crosses). No tetraploid seeds were detected when C_0 plants were used as pollen sources in 4*x*-2*x* crosses. However, two tetraploids were among a sample of 209 progeny evaluated when C_1 plants were used as pollen sources in 2*x*-2*x* crosses. There were no tetraploid plants in the pollination cage, but there were 2*n* pollen and 2*n* egg producers. Bilateral sexual tetraploidization (the union of a 2*n* egg and a 2*n* pollen to produce a tetraploid sporophyte) is the most plausible origin of these tetraploid plants. It appears that increasing the frequency of 2*n* pollen formation was necessary before sexual tetraploidization could occur.

The two tetraploid plants obtained represent a success rate of about 1% in the attempt to obtain tetraploids through sexual polyploidization. This success rate reported here is still lower than that reported for

the use of colchicine or nitrous oxide, but it compares very favorably with the expected success rate of 1.5% [calculated from the frequency of $2n$ eggs present (0.034) $\times$ the frequency of $2n$ pollen present (0.454)]. However, the plants obtained through sexual polyploidization in this and other attempts appear vigorous, highly fertile, and directly usable in a breeding program as compared with plants derived through chemical chromosome doubling. As recurrent selection was very effective in increasing the frequency of $2n$ pollen formation in red clover and when egg parents with higher frequencies of $2n$ egg formation are obtained, it should be possible to greatly increase the efficiency with which the sexual polyploidization of red clover can occur in the future. It appears the LM population will serve as a broad-based, heterozygous population that will lead eventually to the development of a tetraploid red clover population well adapted to the USA.

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