

# Production of 2n Pollen in Red Clover<sup>1</sup>

W. A. Parrott and R. R. Smith<sup>2</sup>

## ABSTRACT

The success of tetraploid red clover (*Trifolium pratense* L.) in Europe has stimulated interest in developing tetraploid red clover adapted to the United States. The use of 2n gametes to produce tetraploid alfalfa (*Medicago sativa* L.) and potato (*Solanum tuberosum* L.) from diploid germplasm suggests that 2n gametes may be an alternative to chemical treatment. Diploid plants that produce 2n eggs are known to occur in red clover, and plants that produce 2n pollen have been reported. The objectives of this study were to search for red clover plants producing 2n pollen, and to determine the cytological origin of such pollen. Six hundred plants, belonging to six different diploid ( $2n = 2x = 14$ ) cultivars, were screened for 2n pollen production by examining dry pollen samples with the aid of a microscope. Eighteen plants (3%) produced at least 1% 2n pollen and were classified as 2n pollen producers. Individual plants ranged from 1 to 84% in frequency of 2n pollen production. Pollen mother cell analyses revealed that 2n pollen resulted from parallel or tripolar spindles during anaphase II of microsporogenesis. This mechanism is genetically equivalent to a first division restitution (FDR) mode of gamete formation. One 2n pollen producer proved to be a synaptic mutant. Normally, synaptic mutants are male sterile, but fertility is restored by parallel spindles in this plant. Because the lack of homologous pairing limits genetic recombination, this plant can produce gametes with the same genetic constitution as the sporophyte. It is apparent from the results that 2n gametes may be useful in red clover polyploidization programs.

**Additional index words:** Parallel spindles, *Trifolium pratense* L., Synaptic mutant, Tetraploid red clover, 2n gametes.

RED clover (*Trifolium pratense* L.) is a diploid ( $2n = 2x = 14$ ) species used as a forage crop throughout the temperate regions of the world. There is no evidence to date of naturally occurring tetraploid forms of the species; however, chemically induced tetraploids ( $2n = 4x = 28$ ) have been produced. In certain parts of Europe, tetraploid forms are sometimes agronomically superior to the diploid forms in yield, disease resistance, and persistence (Taylor and Smith, 1979). Considering the success of tetraploids in Europe, it may be desirable to develop tetraploid germplasm adapted to United States growing conditions.

The conventional method for obtaining red clover tetraploids has been through the use of colchicine or nitrous oxide. Recent research with 2n gametes in other species suggests that these gametes can be used as an alternative to chemical treatments in a breeding program. Production of 2n pollen has been extensively studied in potato, *Solanum tuberosum* L., (Mok and Peloquin, 1975), alfalfa, *Medicago sativa* L., (Vorsa and Bingham, 1979), and peas, *Pisum sativum* L., (Myers, 1981). First division restitution (FDR) 2n pollen has facilitated the transfer of germplasm from the diploid level to the tetraploid level in both potato

(Peloquin, 1982) and alfalfa (Bingham, 1968). In these species, 2n pollen can be produced by disorientation of the meiotic spindles during anaphase II. The spindles may lie in one plane instead of two, in which case they are either parallel or tripolar. Parallel spindles lead to the formation of two restitution microspores, while tripolar spindles form one restitution microspore and two normally reduced microspores.

The production of 2n pollen via parallel or tripolar spindles is genetically equivalent to the FDR mechanism of 2n gamete formation. All the heterozygous loci from the centromere to the first crossover are preserved in each chromosome arm, 50% from the first crossover to the second, and 75% in each successive crossover. Thus, most of the heterozygosity present in the sporophyte is retained in the gametophyte and subsequently transmitted to the progeny (Mendiburu and Peloquin, 1977). A synaptic mutant, occurring together with parallel spindles during anaphase II, has been described in potato. The combination of these two mutants leads to the formation of pollen with exactly the same genetic constitution and heterozygosity as the sporophyte. This occurs as the lack of normal pairing significantly reduces crossing over, resulting in a corresponding increase in the amount of heterozygosity transmitted by a restitution gamete. When the lack of pairing is complete the genome of the gametophyte is identical to that of the sporophyte (Peloquin, 1983). Maximizing the heterozygosity in autopolyploids is important (Dunbier and Bingham, 1975), and FDR 2n gametes can be an effective breeding tool for autopolyploid crops (Mendiburu et al., 1974; Bingham, 1979).

Red clover plants that produce 2n eggs have been identified (Broda and Smith, 1980; Taylor and Giri, 1982), suggesting that 2n gametes can be used to produce tetraploid red clover germplasm. The only report of functional 2n pollen within the true clovers (*Trifolium* spp.) has been in *T. alpestre* L. (Maizonnier, 1972). Strzyzewska (1974, 1976) reported the formation of 2n pollen by post meiotic fusion in diploid red clover, but pollen function was not tested in 4x-2x crosses. Kazimierski and Kazimierska (1970) reported that a hybrid of *T. repens* L. × *T. nigrescens* Viv. produced dyads and triads. The authors also included a microphotograph of tripolar spindles.

The objectives of this investigation were to search for plants which produced 2n pollen in diploid red clover and to determine cytologically the origin of the 2n pollen.

## MATERIALS AND METHODS

In red clover, x and 2x pollen can be easily distinguished by their distinct morphology when dry. Haploid pollen is oblong and regular in shape, while diploid pollen is triangular, square, or irregular in shape (Taylor et al., 1976). Red clover flowers were tripped on a slide, and the pollen was then examined under low magnification. One thousand grains from each plant were scored for ploidy level. In all, 600 plants, 100 each from six diploid red clover cultivars,

<sup>1</sup> Cooperative investigation of USDA-ARS and the Wisconsin Agric. Exp. Stn., Madison, Wis. This research supported in part by U.S. Competitive Grant no. 8100349. Received 11 July 1983.

<sup>2</sup> Research assistant, Dep. of Agronomy, Univ. of Wisconsin, Madison, and supervisory research geneticist, USDA-ARS and professor of agronomy, Univ. of Wisconsin, Madison, WI 53706.

were examined. The six cultivars were 'Arlington', 'Chesapeake', 'Florex', 'Kenstar', 'Pennscott', and 'Redman'. All plants with at least 1% 2n pollen production were identified as 2n pollen producers.

The three plants with the highest frequency of 2n pollen production were selected for cytological study. Pollen mother cells (PMC) and chromosome numbers in root tip cells were examined using the method described by Giri et al. (1981). The procedure was modified to the extent that Hoyer's mounting medium was used to make the slides permanent. No heat and minimal pressure were used to minimize the probability of creating cytological distortions.

Interploidy crosses (4x-2x) were made in the greenhouse to determine if the 2n pollen was functional. The tetraploids (K4x) used as females were obtained through nitrous oxide doubling, and were made available by Dr. N. L. Taylor, University of Kentucky, Lexington, Ky. Leaf mark (a dominant trait) on the pollen parent was used as a genetic marker to identify hybrid progeny. Red clover has a strong triploid block that acts as a selective screen for 2n gametes in interploidy crosses, as 4x embryos survive while 3x embryos abort. Also, the self-incompatibility system in red clover limits the number of selfed progeny. Thus, only tetraploid progeny are expected from 4x-2x crosses, either as a result of a functional 2n gamete from the male (i.e. a cross), or from a breakdown in the self incompatibility system of the female (i.e. a self).

## RESULTS AND DISCUSSION

### 2n Pollen Producers

Eighteen plants were identified producing at least 1% of 2n pollen (Table 1). One plant each was identified from Chesapeake and Kenstar, two from Redman, four from Arlington, and five each from Florex and Pennscott.

The frequency of 2n pollen produced by individual plants ranged from 1 to 84%, with a mean of 10%. A plant of the cultivar Chesapeake (C-51) produced the highest frequency (84%) of 2n pollen. Chromosome counts in root tip cells of these plants verified their diploid nature.

The stability of 2n pollen production was determined by repeat observations of the 2n pollen producing plants after they were exposed to varying environmental conditions in the greenhouse. Original 2n pollen frequencies were determined in March, 1982, when the average daytime high temperature was  $27 \pm 3$  C, and the average nighttime low temperature was  $11 \pm 2$  C. Repeat observations were made in June of the same year. The average high daytime temperature was  $33 \pm 4$  C, while the average nighttime low temperature was  $15 \pm 2$  C. Temper-

**Table 1. Number of 2n pollen-producing plants and range of percent 2n pollen production in six cultivars of red clover.†**

Cultivar	No. of plants with $\geq 1\%$ 2n pollen	Range in % 2n pollen production
Arlington	4	1-24
Chesapeake	1	84
Florex	5	1-40
Kenstar	1	6
Pennscott	5	1-5
Redman	2	1-2
Total	18	1-84

† 100 plants screened per cultivar.

atures were monitored with a hygrothermograph. A constant 16-h photoperiod was maintained at all times. While the frequency of 2n pollen production is somewhat sensitive to environment, the initial identification of a plant as a producer or nonproducer of 2n pollen was consistent, irrespective of the environment in which the plants were examined (Table 2).

### 4x-2x Crosses

In 4x-2x crosses made in the greenhouse, C-51 functioned as a male to produce viable 4x seed. However, C-51 never functions as a male in 2x-2x crosses, suggesting that only 2n pollen was functional. Since this plant produces both 2n eggs and 2n pollen, it can be used successfully in both 2x-4x and 4x-2x crosses (Table 3).

### Cytology of 2n Pollen Production

Cytological observations of PMC's revealed that meiosis proceeded normally through telophase I. Beginning in metaphase II and during anaphase II, the spindles of some PMC's within an anther were in one plane instead of two and were either parallel or tripolar. Parallel spindles led to the formation of a dyad of microspores (two restitution gametes) and tripolar spindles led to the formation of a triad of microspores (one microspore of which is a restitution gamete) instead of the tetrad that is normally formed in microsporogenesis (Fig. 1-6).

Ramana (1979) has argued that only fused spindles, and not parallel spindles, lead to dyad formation. However, the observation of microspores with two

**Table 2. Percent of 2n pollen production in red clover plants exposed to varying greenhouse environmental conditions.**

Plant†	Percent 2n pollen production	
	Mar. (1982)	June
C-51	84	95
A-74	24	19
F-15	2	17
P-76	2	15
R-34	1	8
P-58	5	3
F-40	0	0
R-53	0	0

† A = Arlington, C = Chesapeake, F = Florex, P = Pennscott, R = Redman.

**Table 3. Ploidy classes of progeny of C-51 from intra- and interploidy crosses in the greenhouse.**

Cross†		Type of cross	Ploidy of F <sub>1</sub>
♀	♂		
C-51	F-49	2x-2x	2x
C-51	K-32	2x-2x	2x
C-51	K4x-5‡	2x-4x	4x
C-51	K4x-11	2x-4x	4x
F-49	C-51	2x-2x	§
K-32	C-51	2x-2x	§
K4x-5	C-51	4x-2x	4x
K4x-11	C-51	4x-2x	4x

† Ploidy level of parent indicated under type of cross.

‡ K4x = Tetraploid germplasm from nitrous oxide treatment.

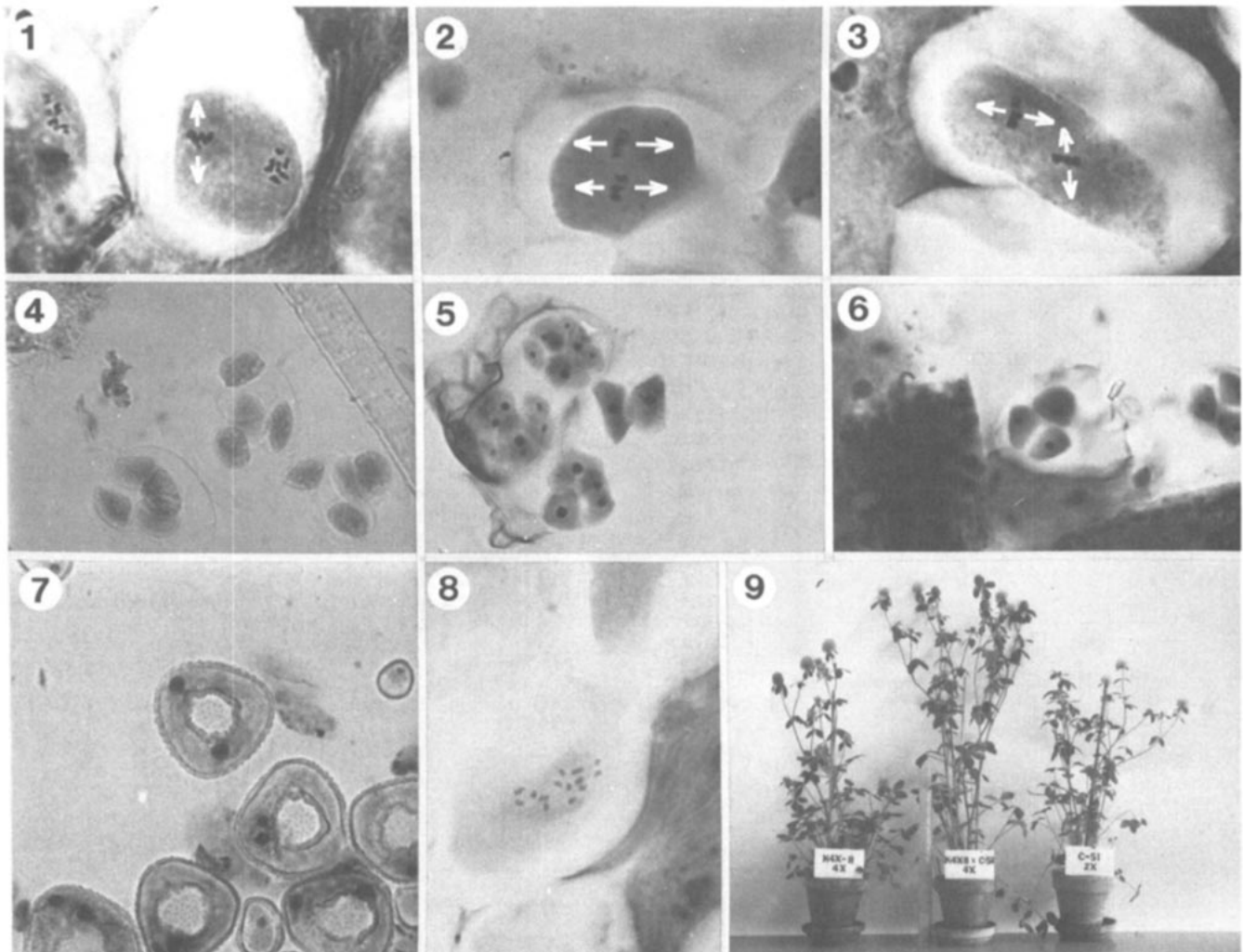
§ Progeny not obtained.

nuclei (Fig. 7) is a strong indication that separate nuclei formed after telophase II in plants with parallel spindles can become enclosed in the same microspore. This also indicates that sometimes nuclear restitution may not occur until after exine deposition has begun. These observations are possible, as the premitotic nucleus in a microspore is morphologically distinct from the vegetative and generative cells of a pollen grain. It may also be possible that sometimes nuclear restitution within the microspores may never occur. This might explain why occasional 2n pollen grains grew two pollen tubes when they were placed on pollen germination medium.

This method of 2n pollen formation is identical to that described in potato (Mok and Peloquin, 1975; Peloquin, 1983), alfalfa (Vorsa and Bingham, 1979) and pea (Myers, 1981). Pollen produced in this manner is especially valuable in autotetraploid breeding

programs because it transmits most of the heterozygosity and epistatic interactions present in the sporophyte.

The plant designated as C-51 proved to be a synaptic mutant. The PMC's had between 0 and 2 bivalents per cell (Fig. 8). This plant also exhibited many of the abnormalities frequently associated with synaptic mutants, including lagging chromosomes at metaphase I, irregular distribution of univalents at anaphase I, and formation of polyads after telophase II (Koduro and Rao, 1981). The irregular distribution of univalents during anaphase I normally leads to male sterility, as it results in the formation of unbalanced gametes that subsequently abort. Similar male sterile plants have been previously reported in red clover (Strzyzewska, 1976; Whittington, 1958). However, formation of parallel spindles during anaphase II in C-51 apparently leads to the formation



**Fig. 1-9.** Cytological observations of pollen mother cells and the consequence of mating 2x C-51 with a tetraploid plant. **Fig. 1.** Metaphase II of a pollen mother cell with normal spindle orientation. **Fig. 2.** Metaphase II of a pollen mother cell with parallel spindle orientation. Arrows depict the spindle axes. **Fig. 3.** Metaphase II of a pollen mother cell with tripolar spindle orientation. Arrows depict the spindle axes. **Fig. 4.** A tetrad, which results from normal spindle orientation. **Fig. 5.** A dyad (center) which results from parallel spindle orientation. Note the polyads (left), which result when PMC's of C-51, a synaptic mutant, lack parallel spindles. **Fig. 6.** A triad, which results from tripolar spindles orientation. **Fig. 7.** Microspores of C-51 with unrestituted nuclei. Note the presence of micropollen, which is formed from polyads. **Fig. 8.** Pollen mother cell of C-51 with 14 univalents. **Fig. 9.** A tetraploid hybrid (center) obtained from a chemically induced tetraploid female (left) and C-51 (right), a diploid synaptic mutant with parallel spindles.

of genetically balanced restitution gametes. This may explain why  $2n$  pollen is the only type of functional pollen that C-51 produces. Peloquin (1983) has described a similar process of fertility restoration by parallel spindles in a synaptic mutant of potato. Strzyewska (1976) also described a synaptic mutant of red clover that produced  $2n$  gametes exclusively. A detailed description of the process by which restitution took place was not given, but it may have been by parallel spindles. The observation of a single metaphase II plate in some PMC's of C-51 suggests that this plant may also produce dyads through fused spindles, or perhaps omission of the first meiotic division. However, evidence at this time would suggest that parallel spindles are the predominant mechanism operating in C-51.

Figure 9 depicts a tetraploid plant, obtained via  $2n$  pollen, and its parents, K4x-11 and C-51. The heterosis shown by the tetraploid hybrid may be due, in part, to the heterozygosity transmitted by  $2n$  pollen of C-51, and may be an indication of the breeding value of such pollen.

With further refinements it should be possible to use  $2n$  gametes for the efficient transfer of additional red clover germplasm to the tetraploid level. A population with a high frequency of  $2n$  pollen production is currently being developed through recurrent selection. Plants from this population could be used directly in  $4x-2x$  crosses (unilateral sexual polyploidization) to obtain tetraploid plants of diverse but adapted background. Appropriate genetic markers would be used to identify hybrid progeny that would then be used to produce subsequent generations. In addition, such a population could be used in conjunction with diploid plants selected for  $2n$  egg production ( $2x-2x$  crosses, bilateral sexual polyploidization) to produce tetraploid cultivars. As in the  $4x-2x$  crosses appropriate genetic markers would be used to identify hybrid progeny.

## REFERENCES

1. Bingham, E.T. 1968. Transfer of diploid *Medicago* spp. germplasm to tetraploid *M. sativa* in  $4x-2x$  crosses. *Crop Sci.* 8:760-762.
2. ———. 1979. Maximizing heterozygosity in autopolyploids. p. 471-489. In W.H. Lewis (ed.) *Polyploidy: Biological relevance*. Plenum Press, New York.
3. Broda, Z., and R.R. Smith. 1980. Production of tetraploids from  $2x-4x$  crosses in red clover. *Agron. Abstr., Am. Soc. of Agron., Madison, Wis.* p. 50.
4. Dunbier, M.W., and E.T. Bingham. 1975. Maximum heterozygosity in alfalfa: results derived using haploid-derived autotetraploids. *Crop Sci.* 15:527-531.
5. Giri, N., N.L. Taylor, and G.B. Collins. 1981. Chromosome numbers in some *Trifolium* species with a karyotype for *T. physodes*. *Can. J. Genet. Cytol.* 23:621-626.
6. Kazimierski, T., and E.M. Kazimierska. 1970. Investigations of the hybrids of the genus *Trifolium* L. III. Morphological traits and cytogenetics of the hybrid *Trifolium repens* L.  $\times$  *Trifolium nigrescens* Viv. *Acta Soc. Bot. Pol.* 64:565-592.
7. Koduro, R.K., and M.K. Rao. 1981. Cytogenetics of synaptic mutants in higher plants. *Theor. Appl. Genet.* 59:197-214.
8. Maizonnier, D. 1972. Obtention d'hybrides entre quatre espèces pérennes du genre *Trifolium*. *Ann. Amélior. Plant.* 22:375-387.
9. Mendiburu, A.O. and S.J. Peloquin. 1977. The significance of  $2n$  gametes in potato breeding. *Theor. Appl. Genet.* 49:53-61.
10. ———, ———, and D.W.S. Mok. 1974. Potato breeding with haploids and  $2n$  gametes. p. 249-258. In K.J. Kasha (ed.) *Haploids in higher plants*. Univ. of Guelph, Guelph, Canada.
11. Mok, D.W.S., and S.J. Peloquin. 1975. Three mechanisms of  $2n$  pollen formation in diploid potatoes. *Can. J. Genet. Cytol.* 17:217-225.
12. Myers, J.R. 1981. Description, inheritance, linkage, and allelism of genetic male sterility in peas (*Pisum sativum* L.). M. Sc. Thesis. Univ of Wisconsin, Madison.
13. Peloquin, S.J. 1982. Meiotic mutants in potato breeding. *Stadler Genet. Symp.* 14:(In press.)
14. ———. 1983. Genetic engineering with meiotic mutants. p. 311-316. In D.L. Mulcahy and E. Ottaviano (ed.) *Pollen: Biology and implications for plant breeding*. Elsevier Science Publishing Co., Inc., New York.
15. Ramana, M.S. 1979. A re-examination of the mechanisms of  $2n$  gamete formation in potato and its implications for breeding. *Euphytica* 28:537-561.
16. Strzyewska, C. 1974. Sib-mating in *Trifolium pratense* L. I. Some morphological traits; properties of euploids, aneuploids and polyploids. *Genet. Pol.* 15:255-293.
17. ———. 1976. Sib-mating in *Trifolium pratense* L. II. Cytogenetics of euploids, aneuploids and polyploids. *Genet. Pol.* 17:497-517.
18. Taylor, N.L., M.K. Anderson, K.H. Quesenberry, and L. Watson. 1976. Doubling the chromosome number of *Trifolium* species using nitrous oxide. *Crop Sci.* 16:516-518.
19. ———, and N. Giri. 1982. Frequency and stability of tetraploids from  $2x-4x$  crosses in red clover. *Agron. Abstr., Am. Soc. of Agron., Madison, Wis.* p. 84-85.
20. ———, and R.R. Smith. 1979. Red clover breeding and genetics. *Adv. Agron.* 31:125-154.
21. Vorsa, N., and E.T. Bingham. 1979. Cytology of  $2n$  pollen formation in diploid alfalfa, *Medicago sativa*. *Can. J. Genet. Cytol.* 21:525-530.
22. Whittington, W.J. 1958. Asynapsis in red clover. *J. Hered.* 49:202.