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# Interspecific Hybridization of Red Clover (*Trifolium pratense* L.) with *T. sarosiense* Hazsl. Using in Vitro Embryo Rescue*

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**Summary.** Interspecific hybrid clover plants from the cross *Trifolium sarosiense* Hazsl. × *T. pratense* L. were obtained in the present investigation. Immature hybrid embryos were excised aseptically from the pistillate parent, *T. sarosiense* (2 n = 48), and cultured in vitro prior to in situ abortion. Agar-solidified nutrient media modified from that developed previously for tissue and cell cultures of red clover (2 n = 14) were used for embryo rescue.

The heart shaped embryos obtained were cultured for 8 to 14 days on a medium containing a high level of sucrose, a moderate level of auxin, and low cytokinin activity. Viable embryos were then transferred to a standard medium with low auxin and moderate cytokinin levels for the direct germination of shoots. Some embryos produced only callus. Plants were regenerated from callus using an alternate culture scheme. Hybrid shoot numbers were increased on a low auxin, high cytokinin medium and subsequently rooted before transfer to soil in the greenhouse.

About 10% of the hybrid embryos were rescued using the optimal culture sequence. Five full-sib families of the $F_1$ hybrid were successfully grown to maturity. Root-tip cells of hybrid plants possessed the expected somatic chromosome number of 31. The genetically determined leaf-mark trait carried by the staminate parent and the rhizomatous root habit of the pistillate parent were expressed in hybrid plants.

**Key words:** Interspecific hybridization – *Trifolium pratense* L. (red clover) – Forage legume – in vitro – Embryo culture

## Introduction

Red clover (*Trifolium pratense* L.) is an important forage legume in the southcentral United States and other temperate regions of the world. One characteristic desirable in an ideal forage legume but lacking in red clover is long-term persistence (Taylor et al. 1963). Genetic variation available within the species has been used to extend field persistence to only three to five years (Taylor and Anderson 1973). Introgression of genes for longevity from long-lived perennial species may be important for the development of new red clover cultivars.

Red clover has been hybridized successfully only with two annual species in the same taxonomic subsection, Genus *Trifolium* L. Sect. *Trifolium* Zoh. Subsect. *Trifolium* Zoh. Conventional sexual procedures were used to produce the hybrids of red clover with *T. pallidum* Waldst. & Kit. (2 n = 16) (Armstrong and Cleveland 1970; Schwer and Cleveland 1972 b; Taylor et al. 1963) and with *T. diffusum* Ehrh. (2 n = 16) (Schwer and Cleveland 1972 a, b). Numerous unsuccessful attempts have been made to cross red clover with other annual and perennial species (Taylor 1980; Taylor and Smith 1979). Some of the perennial species of Genus *Trifolium* Sect. *Trifolium* do intercross but only with difficulty (Maizonnier 1972; Quesenberry and Taylor 1976, 1977, 1978).

Post-zygotic mechanisms appear to be the primary cause of the reproductive isolation of red clover from related perennials (Kazimierska 1978; Quesenberry and Taylor 1976, 1977; Taylor and Smith 1979). Principal among these mechanisms is endosperm failure followed by embryo abortion. The method most likely to overcome post-zygotic barriers to hybridization is the in vitro culture of immature hybrid embryos prior to abortion (Raghavan 1976). Hannig (1904) was the first worker to investigate the culture of isolated plant

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embryos. Laibach (1925) was the first to apply the method for the growth of interspecific hybrid embryos. Important cultural factors for immature embryo growth include the use of a high level of sugar (Hannig 1904), a complete, sometimes complex, salts and vitamins formulation (Monnier 1978), and growth regulators (Raghavan 1976).

Previous attempts at in vitro rescue of interspecific red clover hybrid embryos were unsuccessful in producing new red clover hybrids, but only simple media were evaluated (Evans 1962; Keim 1953). Investigations conducted in this laboratory included the development of a basal medium, designated L 2, which was experimentally optimized for red clover tissue growth in vitro (Phillips and Collins 1979a). This basal nutrient medium served as the basis for the development of a number of specialty media in which growth regulator specificities were identified for red clover manipulation (Collins and Phillips 1981). Normal diploid red clover plants were regenerated from callus and cell suspension cultures (Phillips and Collins 1979a, 1980). Meristem-tip cultures of red clover were amenable to clonal propagation and virus elimination from plants (Phillips and Collins 1979b). Shoot cultures were rooted and established in soil (Phillips and Collins 1979b).

The major objective of the current study was to hybridize red clover with the perennial species *T. sarosiense* Hazsl., 2 n = 48 (Cincura 1965), by in vitro embryo rescue using improved cultural methods developed for red clover.

## Materials and Methods

Nine clones of *T. pratense* cv. 'Kenstar', 2 n = 2 x = 14, and 18 clones of *T. sarosiense* P.I. 292827, Sweden, 2 n = 6 x = 48, were identified for use in the hybridization study as described previously (Taylor et al. 1981). Vegetative stem cuttings were propagated, plants were grown, and hand pollinations were performed using standard procedures (Taylor 1980; Taylor et al. 1981). Emasculations were not performed since both species possess self-incompatibility systems (Fergus and Hollowell 1960; Quesenberry and Taylor 1977; Taylor and Smith 1979). *T. sarosiense* was used as the pistillate parent and *T. pratense* was used as the staminate parent in the interspecific cross. Non-pollinated florets, self-pollinations, compatible intraspecific crosses, and mature (undisturbed) interspecific crosses were used as controls.

Florets were collected 11 to 23 days after pollination. Surface sterilization followed an earlier routine (Collins and Phillips 1981). Embryos were dissected aseptically from each floret under magnification. Isolated embryos were cultured on solidified variations of the L2 medium (Phillips and Collins 1979a). Media varied in source and level of growth regulators and in concentration of sucrose. The standard medium contained 2.5% sucrose (w/v). The optimal cultural sequence was determined for the survival of immature embryos and direct germination of shoots.

Embryos which produced only callus were transferred to modified L2 medium containing 45 nM 2,4-dichlorophenoxyacetic acid (2,4-D) and 15 μM adenine (LSE medium), identified previously for red clover plant regeneration via somatic embryogenesis (Phillips and Collins 1980). The ML8 medium was used for increasing the shoot numbers of induced regenerates and from directly germinated embryos. The ML8 medium is modified from L2 and includes 12 nM picloram and 2.2 μM N6-benzyladenine (BA) (Collins and Phillips 1981; Phillips and Collins 1980). Several modifications of the red clover rooting medium (Phillips and Collins 1979b) were used for rooting of embryo-derived shoots. In vitro cultures were maintained at 25 °C under continuous light (100 μE $m^{-2}$ $s^{-1}$).

Some shoot cultures were transferred to ML8 medium containing 0.05% colchicine (w/v) for two days and then returned to standard ML8 medium. Plants rescued in vitro were transplanted into vermiculite or soil and kept for two weeks under a plastic tent to provide conditions of high humidity. Plants were grown to maturity in the greenhouse. $F_1$ plants were used as the pistillate parent in attempted backcrosses with diploid red clover. Some stem cuttings of $F_1$ plants were treated with 0.2% colchicine as described previously (Taylor 1980). Plants treated with colchicine were also backcrossed with red clover.

Hybrid plants were evaluated cytologically and compared to parental clones following the methods of Sharma and Sharma (1972). Root-tips were analyzed for somatic chromosome number using 8-hydroxyquinoline and maltose pretreatment, 3:1 (v:v) 95% ethanol:glacial acetic acid fixative, HCl hydrolysis, Feulgen reagent stain, and aceto-carmine squash procedures. Meiosis was analyzed in pollen mother cells following fixation and hydrolysis of immature floral buds. Pollen was tested for viability by aceto-carmine stainability.

Root, stem, leaf, and floral comparisons were made among $F_1$ plants and parental clones. The rhizomatous root habit of the perennial species parent is inherited by dominance in interspecific crosses (Quesenberry and Taylor 1976, 1977). Red clover possesses a tap-root system (Fergus and Hollowell 1960). Red clover has a basal stem crown system (Fergus and Hollowell 1960) while *T. sarosiense* exhibits the zigzag stem branching pattern (Quesenberry and Taylor 1977). The central, V-shaped, light-colored leaf-mark exhibited by red clover is conditioned by a dominant gene (Wexelsen 1932). Leaf-mark is lacking in *T. sarosiense* (Zohary 1972). Red clover generally possesses crimson or pink colored corollas while those of *T. sarosiense* are dark red (Zohary 1972). The calyx of red clover has 10 veins while that of *T. sarosiense* has 12–20 veins (Zohary 1972).

## Results

Hybrid zygote formation and optimal periods of in situ development prior to excision for in vitro culture were first determined. Embryos were present 11 to 23 days after pollination and the embryos were more ontogenetically advanced with time. A lower proportion of embryos was viable with time after pollination. By 23 days after pollination, very few if any viable embryos could be found in the interspecific cross due to premature degeneration. Compatible intraspecific crosses produced fully mature seeds by 30 to 35 days after pollination. At 11 days after pollination, generally

**Table 1.** Summary of interspecific *T. sarosiense* × *T. pratense* $F_1$ and backcross generations, with control pollinations and number of embryos excised from these crosses indicated

| Cross | Number of pollinations | Number of embryos found 14 to 19 days after pollination | Percent embryo formation | Number of embryos rescued in vitro |
|---|---|---|---|---|
| *T. sarosiense* × *T. pratense* | 1921 | 555 | 28.9 | 18 |
| *T. sarosiense* × *T. pratense* | 142 | undisturbed | no hybrid seed | – |
| *T. sarosiense*, non-pollinated | 95 florets | 0 | 0 | – |
| *T. sarosiense*, selfed | 78 | 2[a] | 2.6 | – |
| *T. sarosiense*, compatible cross | 44 | 32[a] | 72.7 | – |
| *T. pratense*, compatible cross | 43 | 39[a] | 90.7 | – |
| (*T. sarosiense* × *T. pratense*) × *T. pratense* | 742 | 6 | 0.8 | 0 |
| (*T. sarosiense* × *T. pratense*) × *T. pratense* | 700 | undisturbed | no seed | – |

[a] Mature embryos were found in these crosses. Only immature embryos were found in other crosses

globular stage embryos were found in the interspecific cross while compatible intraspecific crosses produced nearly mature embryos. Only globular, heart, and early torpedo stages of embryos were found in the interspecific cross. The best compromise between the maximum number of hybrid embryos and the maximum degree of development was observed 14 to 19 days after pollination. Predominantly heart stage embryos were obtained from the interspecific cross during this collection period. Retardation and abortion of hybrid embryos were generally associated with incomplete or degenerated endosperms.

A total of 51 interspecific $F_1$ crossing combinations were attempted. Two $F_1$ combinations failed to produce embryos discernible at 14 to 19 days after pollination (time of dissection). Other $F_1$ combinations produced embryos in frequencies as high as 55%. The total numbers of embryos dissected from interspecific and control pollinations are summarized in Table 1. The frequency of embryo production was 29% for all interspecific pollinations examined. No viable seeds were obtained from undisturbed interspecific pollinations, confirming that in situ abortion was the cause of reproductive isolation. Compatible intraspecific crosses of *T. sarosiense* and of *T. pratense* produced embryos in 73 and 91% frequencies, respectively. Self-pollinations of *T. sarosiense* resulted in less than 3% frequency of embryo formation. In self-pollinations and compatible intraspecific crosses the embryos found were mature with fully developed cotyledons. These are normally associated with viable seeds in situ.

Hybrid embryos were cultured on several media varying in the levels of sucrose, cytokinin, and auxin. The heart stage embryos survived well and did not experience turgor-related problems (precocious germination) on an L2 basal medium including 12.5% sucrose. Ten and 15% sucrose levels were suboptimal for survival. The use of BA as the cytokinin promoted the loss of organization at 0.44 μM or failed to support good survival at 88 nM. The use of 15 μM adenine was satisfactory for the growth of immature hybrid embryos. A moderate level of picloram (25 nM) used as auxin was required for embryo survival. A higher level of auxin (40 nM) promoted the loss of organization, and a lower level (12 nM) did not support survival. These observations led to the identification of the LIH medium for the initial culture of immature hybrid embryos. The LIH medium consists of the basal salts and vitamins composition of the L2 medium with 12.5% sucrose, 15 μM adenine, and 25 nM picloram. The optimal embryo survival rate during this experiment was 19%.

The initial culture medium was used for 8 to 14 days after which embryos turned yellow or white and ceased to grow. The LIH medium was not suitable for shoot germination from immature embryos although young cotyledon differentiation was often observed. Prior to degeneration on LIH medium, embryos were transferred to L2 basal media including the standard level of 2.5% sucrose and adjustments in the growth regulators. The best shoot germination response with minimal callusing occurred on a medium including 4 nM picloram and 0.66 μM BA, designated the LSP2 medium. A higher level of auxin (8 nM picloram) promoted the loss of organization as did a higher level of cytokinin (0.88 μM BA). A lower level of either growth regulator failed to support embryo survival and germination. Adenine was not a suitable cytokinin for promoting shoot germination. About two-thirds of the total number of hybrid embryos used in the hybridization study were utilized in these two initial culture experiments and two $F_1$ lines were rescued.

The subsequent experiment was designed to validate the response of hybrid embryos to the cultural

**Table 2.** Efficiency of in vitro procedures for the rescue of interspecific embryos from the *T. sarosiense* × *T. pratense* cross

| Culture medium[a]: type of response | | Number of embryos in culture | Number responding | Response rate in percent |
|---|---|---|---|---|
| LIH: | Immature embryo survival and growth | 157 | 33 | 21.0 |
| LSP2: | Shoot germination | 33 | 15 | 45.5 |
| | loss of organization | | 13 | 39.3 |
| LSE: | Plant regeneration from callus | 13 | 1 | 7.7 |
| ML8: | Shoot number increase | 16 | 16 | 100 |

[a] The compositions of these modifications of the L2 basal medium (Phillips and Collins 1979a) are described in the text

sequence identified above, summarized in Table 2. Immature embryos were cultured with 21% survival on LIH medium. After 8 to 14 days these embryos were transferred to LSP2 medium. Within 2 to 4 weeks 45% of the embryos germinated shoots directly. Another 39% survived the culture treatment but suffered a loss of organization. After 4 weeks culture on LSP2, embryos which produced only callus were transferred to LSE medium for induction of plant regeneration. Only one (or 8%) of the callus lines regenerated plants but it did not represent a new $F_1$ family. The somatic embryos recovered by monthly subculture on LSE medium sometimes germinated shoots on the LSP2 medium if not on LSE. Whether germinated directly from embryos or indirectly from callus by somatic embryogenesis, the 16 shoots obtained in this experiment were transferred to ML8 medium which was suitable for clonal shoot number increase. Monthly subculture of shoots on ML8 medium yielded a 4 to 10 fold increase/passage in shoot number.

These three experiments produced a total of 18 $F_1$ shoot lines, representing 11 full-sib $F_1$ families. Shoots rooted spontaneously in about 15% of the cultures on ML8 medium. Non-rooted shoots were transferred to rooting media. About 50% developed root systems in 4 to 6 weeks. This response was about the same as using only water for rooting. A well-developed root system facilitated the transfer of hybrid plants from in vitro to in vivo conditions. But only 5 of the 11 $F_1$ families rescued in vitro were successfully grown to maturity in vivo. There was no apparent correlation between in vitro rooting efficiency and survival of these $F_1$ lines to maturity. About 100 plants of each $F_1$ line were generated, but unlimited numbers of shoots were available by serial culture on ML8 medium prior to rooting. Nearly 10% of the available hybrid embryos were rescued on the optimal in vitro culture sequence. Of the 51 $F_1$ crossing combinations attempted, 5 $F_1$ families were grown to maturity.

Rescued plants were analyzed cytologically for somatic chromosome number. All $F_1$ lines possessed the expected chromosome number in root-tip cells of 31, 24 from *T. sarosiense* and 7 from *T. pratense,* as illustrated in Fig. 1.

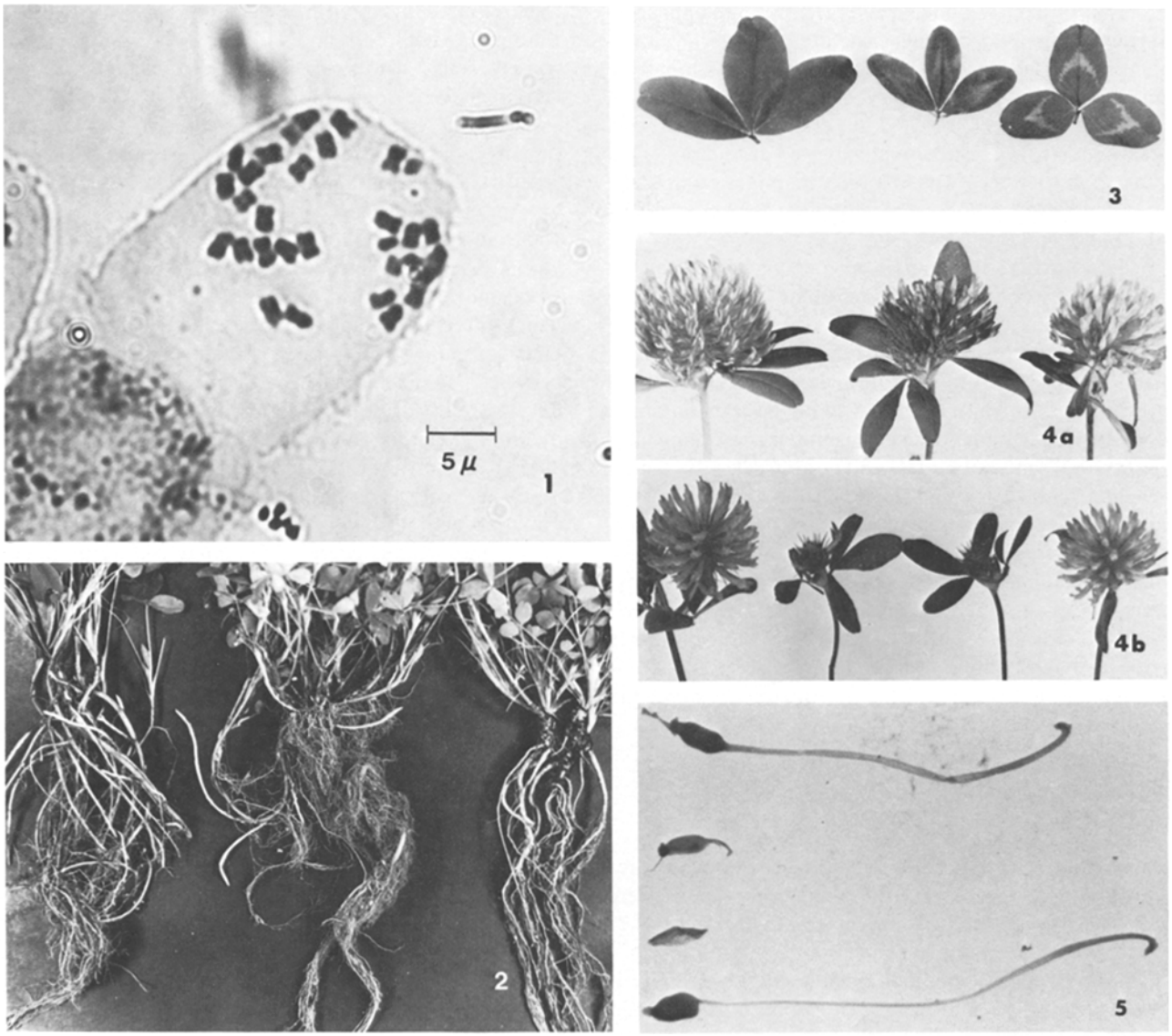
Meiosis was evaluated in rescued plants. Pollen mother cells were observed in the hybrid only rarely. This indicated that meiosis was arrested very early during reproduction and caused sterility. Red clover pollen mother cells exhibited regular pairing of chromosomes during meiosis. *T. sarosiense* pollen mother cells generally exhibited regular chromosome pairing, but irregular pairing and chromosome bridges at anaphase were observed in one parental clone.

Mature flowers of rescued hybrid plants were examined for pollen viability, but pollen grains were observed rarely and never darkly stained. This indicated that there was probably no male fertility in the hybrid. *T. sarosiense* flowers exhibited 75% pollen stainability and flowers of red clover showed 95% pollen stainability.

Rescued hybrid plants were compared to both parental species morphologically. The hybrid was less vigorous than either parent. The hybrid possessed rhizomes although fewer in number than the pistillate parent, as illustrated in Fig. 2. The rhizomatous parent was nodulated at moderate density in the greenhouse soil used, the nodules being small and club-shaped. The tap-root system of red clover was more densely inoculated with larger nodules. The hybrid was not nodulated at the time of examination and is currently being tested in field soils.

The hybrid lacked the basal stem crown of the staminate parent but did exhibit the stem branching pattern of the pistillate parent. The stems of the hybrid were woody, similar to the perennial parent and unlike the softer stems of the commercial parent. The dominant gene leaf-mark trait carried by the staminate parent was expressed in the interspecific hybrid as illustrated in Fig. 3. The expression of leaf-mark was diluted in the hybrid and was developmentally variable.

Flower heads of the parents and hybrid are represented in Fig. 4. Only one $F_1$ family consistently




**Figs. 1–5.** Root-tip cell of the *T. sarosiense* × *T. pratense* hybrid with the expected $F_1$ somatic chromosome number of 31. (Scale on Fig.); **2** Root habits of *T. sarosiense* (left), *T. pratense* (right), and the *T. sarosiense* × *T. pratense* interspecific hybrid (middle) showing the inheritance of rhizomes. (X 0.26); **3** Fully expanded leaves of *T. sarosiense* (left), *T. pratense* (right), and their interspecific $F_1$ hybrid (middle) showing the inheritance of leaf-mark. (X 0.54); **4a and b** Flowering heads of *T. sarosiense* (left), *T. pratense* (right), and the *T. sarosiense* × *T. pratense* hybrid (middle). **a** Normal appearing flower of one $F_1$ family. (X 0.59); **b** Abnormal flowers of the other $F_1$ families. (X 0.50); **5** Pistils excised from red clover and from the *T. sarosiense* × *T. pratense* interspecific hybrid. Left to right: From normal appearing floret of the $F_1$ family which produces normal appearing flowers; from rare normal appearing floret of a $F_1$ family which produces abnormal flowers; from abnormal floret typical of the hybrid; from a normal red clover floret. (X 7.15)

produced normal appearing but sterile flowers as shown in Fig. 4a. The pigmentation of corolla and calyx in the hybrid was intermediate to those of the parental clones. All other $F_1$ families produced abnormal and sterile flowers as shown in Fig. 4b. These $F_1$ plants produced heads and calyces but corollas were rarely produced (2 to 3/head). Hybrid calyces possessed 10 veins as did those of the staminate parent. Calyces of the pistillate parent possessed 12 veins.

Pistil comparisons were striking, as shown in Fig. 5. Compared to a red clover pistil, the pistils of the $F_1$ family which produced normal appearing flowers were slightly reduced in development. Anthers of this $F_1$ family (not shown) were white and immature, perhaps due to the absence of viable pollen. Pistils of all other $F_1$ families were completely aborted or greatly reduced in development. Anthers were not produced or were very rudimentary in these hybrid florets.

The $F_1$ family which produced normal appearing flowers was used as the pistillate parent in an attempted backcross with red clover (Table 1). Only 0.8% embryo formation was observed in the backcross. None of the embryos were rescued in vitro but only one heart stage embryo was successfully placed in culture. One other heart embryo was lost to contamination and the other embryos found were globular. Backcross seeds aborted in situ.

Some hybrid materials were exposed to colchicine in order to double the chromosome number to the amphidiploid level. Hybrid survival after colchicine treatment was low. Only 3 of the 5 $F_1$ families which had been treated were returned successfully to the greenhouse. In some cases the stems became enlarged and stem crown growth or exaggerated stem branching occurred at leaf axil nodes. Only one treated $F_1$ family reached maturity. Some flower heads appeared to be larger in size and possessed a greater density of florets after plant treatment with colchicine but the frequency of corolla tube formation was not altered appreciably. Fertility was not increased in these plants. Root-tip cell analysis indicated that the number of chromosomes had not been doubled, suggesting that stem and floral alterations following colchicine treatment were the result of mutation.

## Discussion

These studies produced the first successful wide hybridization of red clover with a perennial species, by the in vitro culture of immature *T. sarosiense* × *T. pratense* embryos prior to abortion.

Embryo rescue methods were used successfully in this hybridization study where previous efforts with red clover were unsuccessful. One reason for this success was that a basal medium was utilized which had been nutritionally optimized for in vitro tissue culture of red clover. This should have encouraged maximal embryo nutrition and growth with minimal toxicity reaction (Monnier 1978). A second reason was that recent work had identified specific growth regulator source requirements for red clover manipulation in vitro. The present work involved the adjustment of the levels of growth regulators for optimal growth and development of hybrid embryos. Thirdly, the concentration of sucrose was adjusted experimentally for the optimal physical effect on immature embryos.

Heart stage hybrid embryos required a level of 12.5% sucrose, a moderate level of auxin, and low cytokinin activity (the LIH medium). Picloram was used as auxin and is a highly active growth regulator (Collins et al. 1978). The use of picloram at 25 nM for hybrid embryo growth was at a level 50% greater than that required by shoot-tips of red clover (Collins and Phillips 1981; Phillips and Collins 1979 b). Adenine is a weakly active cytokinin whereas BA is highly active (Hecht 1979; Skoog and Miller 1957). Adenine was superior to BA for immature embryo growth and organization, suggesting that immature embryos were sensitive to cytokinin inhibition.

Apparently the needs for auxin and turgor control occurred early in embryo differentiation. Interspecific embryos and/or fertilized ovules may have lacked sufficient endogenous production of these materials which were then supplied in the culture media. The embryo maturation process involves cell elongation growth (Raghavan 1976). Continued growth and development of hybrid embryos occurred using a medium including the standard level of sucrose, less auxin, and more cytokinin (using BA instead of adenine) at levels appropriate for maximizing shoot germination without loss of organization (the LSP2 medium). Once shoots were germinated, shoot numbers were increased using a standard red clover medium (ML8) which contained a low level of auxin and a high level of cytokinin. The initiation of shoot development involves cell division (Raghavan 1976). Since survival and response rates improved as differentiation progressed, genetic imbalances apparently did not disturb cell division and shoot growth as severely as cell elongation and maturation stages of immature hybrid embryo development.

The rescue of 10% of the hybrid embryos cultured on the optimal in vitro sequence offers a reasonably efficient and useful system for producing hybrid plants previously unavailable. However, two-thirds of the materials were used in experiments which determined the optimal in vitro sequence. Using only the optimal sequence a greater number of $F_1$ lines and a greater proportion of attempted pollinations may have been rescued. A total of 17 embryos were rescued not including plant regeneration from embryo-derived callus. The final in vitro experiment evaluating cultural efficiency produced 15 of the rescued embryos whereas the other two embryos were rescued during earlier experiments involving twice as many experimental units. In other words, the optimal culture sequence was about 15 times more efficient than the initial culture treatments examined.

The in vitro embryo rescue method developed during this study should be appropriate for the rescue of other interspecific hybrids of red clover with only minor modification, since all conditions and factors have been optimized experimentally with the use of red clover alone or as one parent. A different interspecific hybrid may possess an increased level of fertility or other desirable characteristics. The embryo rescue procedure should also be useful for advancing back-

cross generations of the hybrid where sterility is a problem, if sufficient numbers of embryos are produced. In the present case this may entail thousands of backcross pollinations.

The leaf-mark trait is expressed more weakly in heterozygotes than in homozygotes (Wexelsen 1932) and this may account for its weak expression in the hybrid. The calyx veination of the staminate parent was expressed in the hybrid and also verified the transfer of paternal genes to the hybrid. This trait appears to be inherited by dominance.

The almost complete lack of meiosis in the *T. sarosiense* × *T. pratense* hybrid suggests that there may be a genic rather than a chromosomal basis for hybrid sterility (Dobzhansky 1951, Stebbins 1958). That is, the gene content of the two genomes may be sufficiently different that they fail to function in a coordinate manner during gametogenesis or meiosis. This is contrasted with chromosome structural differences which lead to irregular meiotic pairing as a cause of sterility. Chromosome number doubling to the amphidiploid level, providing a basis for the pairing of homologues, generally overcomes chromosomal sterility (Dobzhansky 1951). Chromosomal sterility was encountered and overcome by ploidy manipulation in red clover hybrids with annuals obtained during previous hybridization efforts as reported in the literature. The occurrence of other floral abnormalities in the *T. sarosiense* × *T. pratense* hybrid supports the argument in favor of genic sterility. However, since the amphidiploid version of this hybrid has not been obtained, the possibility of chromosomal sterility being present can not be ruled out.

Chromosomal sterility is most common among closely related plant species (Stebbins 1958). The presence of genic sterility should indicate that the genomes are related to a lesser degree. Following this argument it is concluded that *T. sarosiense* and *T. pratense* are probably distantly related. But there must be some link in evolutionary history since a fairly high level of hybrid embryo formation was available for embryo rescue. Post-zygotic barriers between red clover and two annual species were not as severe as in the present case, and chromosome structural differences were observed in the former cases. It seems that *T. pratense* is more closely related to the annual species of *Trifolium* Subsect. *Trifolium*. These arguments also support the claim that annuals and perennials related to red clover may have evolved by different mechanisms (Quesenberry and Taylor 1976).

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