Clovers

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The clover species described in this chapter are the true clovers belonging to the genus *Trifolium*. The species of major agricultural importance in the United States are red clover (*T. pratense* L.), white clover (*T. repens* L.), crimson clover (*T. incarnatum* L.), and alsike clover (*T. hybridum* L.). Crimson clover is an annual species, and the others are perennial. These clovers are thought to have originated in Asia minor or southeastern Europe. They are grown extensively for hay, pasture, and soil improvement throughout the eastern half of the United States and under irrigation in the Pacific and adjacent states.

I. PARENTAL MATERIAL

The genus *Trifolium* consists of about 240 species divided by taxonomists into about 16 sections (Hossain, 1961). Little or no gene transfer among sections has been possible. In the largest section of *Trifolium*, hybridization has chiefly centered around improvement of white clover. The species has been crossed successfully with *T. nigrescens* Viv., *T. xeroccephalum* Fenzl., *T. isthmocarpum* Brot., *T. occidentale* D. E. Coombe, and *T. uniflorum* L. (Trimble and Hovin, 1960; Kazimierski and Kazimierska, 1968, 1970; Gibson and Beinhart, 1969; Chen and Gibson, 1970; Pandey, 1957). The only verified hybrids with red clover were obtained with the diploid annuals *T. diffusum* Ehrh. and *T. pallidum* Waldst. & Kit. (Taylor et al., 1963; Schwer and Cleveland, 1972; Armstrong and Cleveland, 1970). Alsike clover has been hybridized with Kura clover (*T. ambiguum* L.), but the hybrids did not flower (Keim, 1953). No verified interspecific crosses involving crimson clover have been obtained.

Seeds of *Trifolium* are available from the Regional Plant Introduction Stations at Geneva, New York (perennials), and Experiment, Georgia (an-
nuals). A recent survey of germplasm in *Trifolium* indicated that seeds of about 85 species are available in the two locations and exploration for new species is underway (Taylor et al., 1977).

II. PLANT CULTURE

A. Field

The clovers generally flower and produce seeds best under soil conditions of medium fertility, medium pH levels, good drainage, and of moderate to heavy texture. White clover will tolerate relatively infertile conditions and sandy soils, whereas red clover succeeds best on non-sandy soils. Alsike clover is adapted to wet soils and will tolerate flooded conditions for short periods.

Soil treatments with phosphorus, potassium, and calcium to obtain the best vegetative growth of clover generally is thought to be adequate for seed production. On certain soils, the addition of minor elements, such as boron and sulfur, may increase seed yields. Nitrogen applications should be avoided because they intensify vegetative growth and lodging under field conditions. The amount of nitrogen fixed by *Rhizobium* species growing symbiotically with clovers is sufficient for adequate flowering and seed production.

Although adequate moisture is necessary for optimum growth of clovers, too much moisture or high relative humidity during flowering and seed set generally is detrimental. Cloudy, rainy weather reduces the number of heads of red clover per unit area, interferes with ovary development, and retards the pollination activity of bees.

Density of 70 to 80 plants/m² generally is adequate for seed production. Seeding rates vary with size of seed and range from 1 kg/ha for white clover to 20 kg/ha for crimson clover.

Nursery populations usually are transplanted or sown from 0.3 to 1 m within and between rows depending upon cultivation equipment available. Plantings may be clean cultivated or overseeded with grasses such as Kentucky bluegrass (*Poa pratensis* L.) or tall fescue (*Festuca arundinacea* Schreb.) to control weeds. Herbicides commonly used to control weeds under clean cultivation include Treflan, CIPC, Balan, and 2,4-D-B applied at rates indicated on labels of the products.

Common nursery designs used for open-pollinated seed production include completely randomized designs, with or without replication. However, for polycross nurseries, randomized complete blocks, Latin squares, or lattice designs are used (Chapter 5). Replicated plantings usually are not necessary for maintenance of seed of ecotypes and mass-selected cultivars.

B. Growth Chamber and Greenhouse

Seeds may be germinated in flats of soil, vermiculite, or in petri dishes 2 to 3 months prior to the time plants are needed for selfing or crossing. Seed scarification is often necessary because many of the clover seeds pos-
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sess hard coats, particularly during the first year after harvest. Seeds may be scarified in any convenient manner provided the embryo and cotyledons are not damaged. One method is to rub the seeds lightly between two sheets of fine-grit emery paper until scarification is accomplished. If the seeds are then placed on a watered paper disc in a covered petri dish, they usually begin to imbibe water in a few hours. Seeds that remain unswollen may be removed for further scarification. Viable seeds that swell but fail to germinate often may be induced to do so by sprinkling activated charcoal on each seed. Seedlings should be transplanted into disinfested soil or other media to eliminate damping-off and other disease organisms and weed seeds. Methyl bromide has been used as a disinfestant, but often results in incomplete killing of white clover seeds. Steam sterilization of some soils under pressure will cause puddling and release of toxic elements, particularly manganese.

Vegetative increase of genetic materials is often necessary for hybridization. The perennial species and some annual species may be increased vegetatively by rooting stolons, rhizomes, stems, roots, or crowns, or by division of crowns. Rooting media include vermiculite, perlite, peat, and sand. Crown cuttings of red clover have been rooted successfully during the winter under short photoperiods in a mixture of equal parts of sedge peat, soil, and sand, and transplanted directly with the rooting medium. Annual species are rooted most successfully in a vegetative stage inasmuch as senescence occurs after flowering.

Most clover species appear to be long-day plants. However, clovers from different latitudes require different photoperiods, and interactions of temperature and day-length requirements for flowering are common. Many annual and some perennial species will flower if sown in a greenhouse at ambient temperatures and exposed to moderately long day lengths. Crimson clover and other annuals will flower earlier if exposed for 2 to 3 weeks to 10 to 15 °C and transferred to a higher temperature (Knight and Hollowell, 1958). Most white clovers will flower under 14 to 16 hour photoperiods without vernalization (Beatty and Gardner, 1961).

A general procedure to obtain flowering in 4 to 6 weeks in a greenhouse is as follows: Plants are brought into a greenhouse in the late fall or early winter depending on the vernalization requirement for the species. They are transplanted into a moderately organic soil of adequate fertility to insure good flowering. Plants are subjected to a continuous low temperature (13 to 16 °C) for approximately 4 to 5 weeks at a 10 to 12-hour photoperiod. Temperature is increased to 18 to 24 °C and day lengths to 14 to 16 hours.

III. FLORAL CHARACTERISTICS

The clovers have a perfect, leguminous flower consisting of a calyx, a corolla, 10 stamens, and a pistil (Fig. 1). The calyx tube terminates in five lobes or teeth. A standard petal, two wing petals, and two keel petals unite at the base to form the corolla tube which is white or cream colored in white clover, reddish pink in red clover, crimson in crimson clover, purple in T. purpureum L., and yellow in hop clover (T. campestre L.).
Inside the corolla tube are nine stamens and one stigma united into a sexual column. The tenth stamen is free (Fig. 1). The number of ovules per ovary commonly varies from one to four, but may be as many as 10. Flowers are grouped in heads or short spikes and may be either sessile or stalked. At maturity, petals usually are indehiscent and either reflexed (white clover) or erect (red clover). In some species, the calyx enlarges to a bladder-like structure as seeds develop (strawberry clover, *Trifolium fragiferum* L.). Flowers per head vary from one for *T. uniflorum* L. and only a few for subterranean clover, *T. subterraneum* L.) to over 100 per head in many species. The ovules mature within or slightly extruded from the calyx and are indehiscent or dehiscence by ventral seams or hardened lids.

About 30% of the clover species are self-incompatible and cross-pollinated by bees, and 70% are self-pollinated (Taylor et al., 1977). Some species (e.g., crimson clover) normally cross-pollinate, but set considerable

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**Fig. 1—Idealized diagram of typical clover flower.**
seed upon selfing (Knight and Hollowell, 1973). Small flowers are often characteristic of self-pollinated species, and large flowers of cross-pollinated species. The flowers of red and zigzag clover (T. medium L.) are so large that honeybees (Apis mellifera) may avoid these species if other nectar and pollen sources are available. Bumblebees (Bombus spp) are efficient pollinators of the large-flowered clover species.

Among the self-pollinated species, pollination and fertilization may occur before the flowers open. For cross-pollinated species such as red clover, the stage of bloom for optimum seed set appears to be when each flower is about half open. Stigma receptivity and pollen viability continue, but decline throughout a period of 10 days. Red clover flowers first open in the middle of and on the topmost heads of the main stem. The length of time between pollination and fertilization of the egg cell in diploid red clover is between 28 and 32 hours. The first division of the zygote occurs 20 to 33 hours after fertilization (Mackiewicz, 1965).

IV. ARTIFICIAL HYBRIDIZATION AND SELF-POLLINATION

A. Equipment

Equipment used for crossing and selfing includes curved or straight pointed forceps, cuticle scissors, magnifying glasses (usually 10×), small jewelry tags, bamboo stakes, rafia or plastic ties for staking and tying plants, and instruments for transferring pollen (Fig. 2 and 3). Toothpicks to which a small piece of black emery paper has been glued, a sharp pointed lead pencil, or a small folded card are all satisfactory for pollen transfer. This equipment may be obtained from florist supply companies.

B. Preparation of the Female

Prior to flowering, especially in the field, the heads of each plant are protected against pollinating insects with bags of fine muslin, about 9 × 14 cm, which can be closed with a draw string. Bagged heads are supported with wires or stakes appropriate for the height of the plant. For easy manipulation, heads are trimmed to 15 or 20 newly opened flowers in the centers of the heads.

Emasculature is usually not necessary in red and white clover because of self-incompatibility controlled by the gametophytic S-allele system. However, in crimson clover and some self-fertile stocks of red and white clover, emasculation is desirable. Emasculation of self-pollinated species is quite difficult because the flowers may be small and tightly packed in the head. Furthermore, the anthers may dehisce at a very early stage, sometimes even before the petals are extruded beyond the calyx.

White and alsike clover can be emasculated by removal of the corolla. The underside of the corolla is gripped with forceps at a point midway between the tip of the calyx and the tip of the standard. The corolla tube and the attached anthers are removed leaving the pistil intact. This operation has been found effective for emasculation for all florets, except those which
are fully opened or immature. For self-compatible genotypes, water is sprayed on stigmas to kill pollen that may have dehisced prior to removal of the corolla.

For species such as red clover in which removal of the corolla also removes the stigma, other emasculation procedures have been devised. One technique is to slit the corolla and calyx longitudinally on the underside, and remove the corolla and staminal column intact without disturbing the stigma. Another method is to remove the wing and keel petals of newly opened flowers in the center of the flowering head by grasping with forceps the uppermost part of the wings and pulling upward. Then, the heads are immersed in 66.5% ethanol for 10 to 20 sec and rinsed with water (Bassiri and Smith, 1972). Each investigator should determine methods of emasculation for a given clover species or particular genetic stocks before using them routinely in genetic and breeding investigations.

C. Pollination

The optimum stage of bloom to pollinate most clover species is shortly after the flowers open. The specific time of day for pollination appears to be unimportant. Cross-pollination of red clover flowers that are half open usually results in the highest seed set. Even 10 days after flower opening, approximately 40% of the flowers produce seeds. Wilted flowers should not be pollinated. The maximum length of time pollen will remain effective is not known for most clover species.

In manual pollinations, the pollen is removed from the plant used as the male by inserting the pollinating instrument (toothpick, etc.) between the standard and the keel and applying downward pressure. This causes the

Fig. 2—Removal of flowers from a head of red clover, with the use of magnifying glass.
staminal column to strike the toothpick (Fig. 3). The pollen is checked at this time to see that it is moist and yellow rather than dry and white. Pollen is transferred on the toothpick to stigmas of plants used as females. Pollen from unemasculated female plants will tend to dilute that of the plant used as male and usually only 5 to 10 flowers can be effectively cross pollinated. One collection of pollen will usually pollinate 10 to 15 emasculated flowers. For reciprocal crosses, pollen is collected and applied alternately between paired heads of different plants using the same toothpick or folded card. After all flowers of a particular cross have been pollinated, a small jewelry tag is looped and secured over the stem immediately under the head. Tags are labeled as to parentage using an indelible pencil to prevent loss of the record when the plants are watered. Heads are kept free of water for at least the first 24 hours to prevent abortion of pollen. Before proceeding to the next cross, hands, forceps and other pollinating equipment are washed with alcohol and rinsed with water. If pollinating instruments are to be reused, they are set aside for several days after washing to prevent contamination.

Seeds of red clover can be produced on excised stems as a convenience for crossing at different locations. Stems bearing freshly-opened flowers that have not been crossed by bees are brought to the greenhouse or laboratory from the field. Stems are severed just above the crown and cut ends are immersed in water. In the greenhouse, stems are shortened and inserted in vials containing a 2% sucrose solution prior to making cross pollinations (Kendall and Taylor, 1969).

Self-incompatible clovers may be self-pollinated by three methods: spontaneous (no manipulation); tripping of individual florets by use of a toothpick, card, or lead pencil; or by rubbing the heads between the thumb and fingers. Tripping or rubbing usually produces more seeds than spontaneous selfing.

Red clover plants grown at high temperatures often produce more self seed than those grown at low temperatures. Likewise, exposure to 32 C for 1 to 2 days temporarily changes alike clover from self-incompatible to self-
compatible (Townsend, 1968). A technique to increase self seed set of red clover was developed by Kendall and Taylor (1969). Heads, either excised or on intact plants, may be treated when in bud with some petal color showing. Heads are inserted in a chamber maintained at 40 C and with the lower part of the stems maintained at 25 C. After the flowers open at 40 C, the heads are removed from the chamber and the florets are selfed by tripping with a toothpick. Excised heads or intact plants are maintained at 25 C until seeds mature. Average seed set per head on highly self-incompatible clones may range up to 9.0 with the procedure.

D. Factors Affecting Efficiency

Most of the clovers flower over a rather long period of time and matching flowering dates usually is not a problem. Extremely early flowering genotypes may be maintained under short photoperiods or flowering stems may be removed to delay flowering. To prevent senescence, self-pollinated species should not be allowed to flower until ready for use.

Genetic markers for identifying hybrid seeds or plants have been summarized for white clover by Gibson and Hollowell (1966). Dominant characters include: red leaf, red flecking, red midrib, and white ‘v’-marking, cyanidin red corolla, and black seed. Recessive characters are blush-colored corolla, and nonclasping bracts.

Red clover leaf characters include leaf marking that is dominant over nonmarking and yellow leaf-mark that is dominant over white. Red pigment on stems and stipules is conditioned by two dominant factors, but classification is difficult because the intensity of color is influenced by external conditions, especially light (Wexelsen, 1932). White flower-petal is conditioned by three recessive nonallelic genetic factors. Each of the genes in the homozygous recessive condition is epistatic to all other flower colors, except a dominant factor that causes a variegated flower color (Williams, 1935).

Simply inherited recessive characters in crimson clover include white flower color (Sandal, 1955), glabrous leaf, petiolulate leaf attachment, and multifoliolate leaves (Knight, 1969). The inheritance of characters in all clovers may be complicated by different genetic backgrounds and should be investigated in the stocks on hand before attempting their use as genetic markers in controlled crosses.

V. NATURAL HYBRIDIZATION

The following procedure is used for crossing clovers with honeybees. Bees are maintained in 4 to 10-frame hives in an apiary until the clover begins to bloom in the cages. Blooming heads are removed to prevent contamination from pollen carried by bees, and hives are moved into the cages, preferably at night when most bees are in their hives. Bees are fed a 1:1 by
volume solution of granulated white sugar and water or commercially prepared bee food until the clover blooms profusely in the cage. Red clover in small cages in Kentucky have yielded up to 560 kg of seed per ha.

Clover may be cross-pollinated with bumblebees by the following technique: Bumblebees are captured by swiftly enclosing a mason jar or other container over the bee. The bees are washed by adding lukewarm water to the jar and shaking to remove and abort pollen. Bees are placed directly into the cage, where after drying, they will begin to cross-pollinate the flowers. Four to six bees generally will pollinate two plants depending on the number of flowers present. In sunny weather, bumblebees usually will live 10 days to 2 weeks, but in wet, cool weather they may die in a few days, and fresh bees should be introduced to complete the crossing. Bumblebees in a glass bee house will be considerably more active and live longer than those outside. Outdoor lights over cages may induce uniform and abundant flowering for pollination of white clover by honey and other bees.

Clover may be cross-pollinated outdoors without cages, but great distances are required particularly to isolate small areas. Volunteer plants of clover are also a hazard, and the isolation area must be observed daily before bees are active. Red clover plants with relatively few blooms usually are heavily contaminated with pollen from the outside when isolated at distances up to 457 m. On the other hand, plot sizes of 274 to 366 m² in heavy bloom may be effectively isolated (98 to 99% purity) from other clover at 457 m (Williams and Evans, 1935).

Crossing in the self-incompatible clovers may be controlled by planting propagules of single clones in fields of the species that serve as pollen parents. It is also possible to convert red clover lines to genetic male sterility for crossing. However, bee visitation to male sterile plants is at a minimum, and little seed is produced (Smith, 1971).

Large-scale crossing in the clovers has been most successfully controlled by the gametophytic S-allele system. Many variations have been suggested, but only one scheme has been used to produce a double cross hybrid (Anderson et al., 1972). By this method non-inbred clones of red clover (Iₐ) are selfed and first generation selfs (I₁) are tested to isolate homozygous S-allele genotypes. Crosses then proceed according to the following diagram:

<table>
<thead>
<tr>
<th>Material</th>
<th>Clonal identification and S-alleles expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inbred line with homzygous S-alleles</td>
<td>S₁S₁, S₂S₁, S₁S₂, S₁S₃</td>
</tr>
<tr>
<td>Single cross: one genotype each</td>
<td>S₁S₁, S₂S₂</td>
</tr>
<tr>
<td>Double cross: four genotypes</td>
<td>S₁S₁, S₁S₂, S₁S₃, S₁S₄</td>
</tr>
</tbody>
</table>
The inbred lines are vegetatively propagated to obtain an isolated field for each single cross. Seeds from the single crosses are blended and sown in an isolated field to obtain double-cross seed. The double-cross is controlled by the S-allele system and the product is true double-cross seed, except for occasional selfs or intra-line crosses. Theoretically, generations beyond the double cross should have reduced forage yields and are not recommended. 

The principal disadvantage of the method is the high cost of the single-cross seed because of the necessity of establishing large fields of the four inbred clones with low seed yields.

One hybrid produced by this method was tested throughout the United States, but forage yields were not greater than those of the synthetic cultivar 'Kenstar'. Similar single-cross or double-cross hybrids, while theoretically possible in other cross-pollinated diploid clovers, have not been produced to date.

VI. SEED DEVELOPMENT, HARVEST, AND STORAGE

Pollinated clover flowers usually begin to wilt in about 2 days, but non-pollinated heads will remain unwilted and receptive up to 10 days after blooming. Clover seeds may mature as early as 21 days after pollination, but under humid conditions, ripening may take somewhat longer.

Harvested seeds are usually dried for a few days either at room temperature or in a dryer at temperatures not exceeding 45°C. For crosses in which only a few heads are involved, a simple method of threshing is to manually rub seeds from florets between two corrugated rubber mats. For somewhat larger quantities of heads, one useful machine for threshing is the Forsberg seed scarifier in which the emery paper inside the drum is replaced with corrugated rubber. Still larger quantities may be threshed with a 90-cm belt thresher. If very large quantities of heads with stems are to be threshed, a small combine may be used. The Clipper Cleaner (model 2-B) with two screens, and the larger four to six-screen models are available to clean threshed seed. A set of hand-testing screens is often useful for preliminary screening, and a South Dakota seed blower may be used for final cleaning. A Tornado blower-vacuum will clean the machine between lots. Most of this equipment is available from Ferrell-Ross, Oklahoma City, Ok.; Burrows Equipment Co., Evanston, Ill.; or Seedburo Equipment Co., Chicago, Ill. Seed threshing and cleaning equipment are matters of individual choice, and many different methods are available.

Often it is desired to identify clover seeds as to female parent. The seeds of red clover vary from white, yellow, brown, and deep purple. Seeds must be mature for full expression of color, and weathered seeds are likely to be brown. Many other species do not have variable seed colors. Variation within species should be determined prior to threshing and cleaning, if maintenance of genetic identity is likely to be a problem.

Clover seeds may be stored for long periods at 0°C or lower without loss of viability. Red clover seeds lost little viability over a 10-year period when stored in a freezer (Rincker, 1974). Other investigators have stored clover seeds at 40% relative humidity and 4 to 5°C for up to 5 years without great loss of viability.
VII. TECHNIQUES FOR SPECIAL SITUATIONS

Many of the special techniques which have been developed for *Trifolium* are concerned with interspecific hybridization. Chromosome doubling has been shown to increase the success of interspecific hybridization and to increase fertility by formation of amphiploids. Stem cuttings or seedlings are inverted in 0.2% aqueous colchicine for 2 to 6 hours, rinsed in tap water, and transplanted to pots filled with disinfested soil. Doubled branches are identified by the presence of stainable pollen at anthesis for sterile diploid hybrids and by cuboidal pollen (4x) verses rod-shaped pollen (2x) for fertile diploid hybrids. Tip cuttings of doubled branches may be rooted and expected chromosome numbers should be verified.

Nitrous oxide treatment, applicable only to fertile materials, can be applied to red clover and related species for 24 hours at 6 bars atmospheric pressure beginning 24 hours after cross pollination. Up to 49% tetraploids were produced by the treatment in *T. alpestre* L., 71% in *T. pratense*, and 79% in *T. rubens* L. Doubled plants and sectors may be identified by the same methods as used in colchicine-doubled materials.

To overcome post-fertilization barriers, embryos have been cultured on artificial media (Hovin, 1962). Excised embryos are placed in an aqueous Nitsch solution containing 2% sucrose and maintained until trifoliolate leaves form. Seedlings are transferred to a 0.7% agar-solidified Nitsch medium containing 1% sucrose. After 2 to 3 weeks, surviving seedlings are transferred again to an aerated nutrient solution with sucrose. The last transfer is to disinfested soil which is watered with modified Nitsch solution until the plants are well established.

REFERENCES


