

# Ovule abortion in relation to breeding system in four *Trifolium* species

R. Dhar, N. Sharma\* and B. Sharma

Department of Botany, University of Jammu, Jammu 180 006, India

**The present investigation reports the findings on reproductive strategies in relation to ovule abortion of four *Trifolium* species, namely *T. dubium* Sibth, *T. fragiferum* L., *T. pratense* L. and *T. repens* L., forming natural populations in various parts of Jammu and Kashmir. The four species represent a spectrum of breeding systems. *T. dubium* is obligately selfed, *T. pratense* and *T. repens* are obligately outcrossed, while *T. fragiferum* practices mixed mating system. All these species suffer from ovule abortion, the rate and pattern however varies. In the two outcrosser species the rate of abortion is high, approaching 50%. In the obligately selfed species, it is invariably 50%. These three thus form one group. The species enjoying minimum ovule abortion and thus harnessing maximum reproductive potential is *T. fragiferum*, with mixed reproductive strategy.**

**Keywords:** Abortion, ovule, reproductive strategy, *Trifolium* sp.

TRIFOLIUMS are prolific flower producers but poor seed setters, because of the wide occurrence of ovule abortion. The loss of seed set accounts for more than 50% in many species<sup>1-4</sup>. Although a number of researchers have tried to work out the causes of ovule abortion in Trifoliums<sup>1,5-9</sup>, to our knowledge no studies have been carried out to correlate it to their breeding system. Sexual systems in Trifoliums are geared for a range of breeding systems, starting from total out-breeding to complete inbreeding<sup>10-12</sup>.

We studied the extent and pattern of ovule abortion in four *Trifolium* species forming natural populations in the study area and representing a spectrum of variation in the breeding system.

## Material

The present work is based on four species of genus *Trifolium* L., namely *T. dubium* Sibth., *T. fragiferum* L., *T. pratense* L. and *T. repens* L. abounding in different parts of Jammu and Kashmir (J&K). Details of their habit and floral features are given in Table 1. Frequent insect visitation was observed in three of four species studied, *T. dubium* being the only exception.

## Methods

For checking anther dehiscence and pollen shedding, flowers were picked at different timings before and after anthesis, their accessory parts carefully removed and essential whorls (stamens + pistils) stained in a drop of Lewis<sup>13</sup> stain (mixture consisting of 2 ml 1% aqueous acid fuchsin, 2 ml 1% aqueous light green, 40 ml lactic acid and 46 ml distilled water). Care was taken to retain the original form of anthers.

Stigma receptivity was checked by fixing stigmas of different ages in a mixture of three parts of absolute alcohol and one part of acetic acid (Cornoy's fixative) for 6–8 h. These stigmas were then washed in distilled water, stained in Lewis stain and mounted in lacto phenol (mixture consisting of lactic acid, distilled water, glycerine and phenol in the ratio 1:1:1:1). Only stigmas with germinating pollen grains attached to their surface were considered receptive. Shivanna and Rangaswamy's<sup>14</sup> schedule was followed for fluorescence microscopy. This was used to trace the path of pollen tubes in the pistil and to calculate the time taken by them to reach the ovary.

For evaluating the frequency and patterns of ovule abortion, pistils of each species collected from open pollinated flowers, 1–4 days after pollination were fixed in Cornoy's fixative for 6–8 h, washed in distilled water, stained with Lewis stain and mounted in lactophenol. These were then scanned for the position and number of ovules aborting within a carpel under normal HB Olympus microscope. Ovules turned flaccid or shrunk and reduced in size were treated as aborted. All these readings were then pooled to assess the number, position and percentage of the aborting ovules in a flower of each species.

The numbers of aborting ovules were tested for significance at various positions using one-way analysis of variance in *T. repens* and by applying Student's *t*-test in *T. fragiferum* and *T. pratense*.

In order to estimate the reproductive potential, inflorescences of the four species were tagged when they were in bloom. They were initially monitored for number of flowers and then for number of fruits. Fruits were later collected and studied for the number of seeds. From the figures thus collected, percentage fruit and seed set was calculated. Some inflorescences were bagged for autoseeding. Fruit and seed set on these bagged inflorescences has been treated as a measure of natural autogamy.

\*For correspondence. (e-mail: dpadha@rediffmail.com)

**Table 1.** Habit and some floral features of *Trifolium* species

Character	<i>T. dubium</i>	<i>T. fragiferum</i>	<i>T. pratense</i>	<i>T. repens</i>
Habit	Annual, herbaceous	Annual, herbaceous	Perennial, herbaceous	Perennial, herbaceous
Flower colour	Yellow	Pink	Pink to purplish	Creamy white
Stigma type	Wet	Wet	Wet	Wet
No. of stamens/flower	10 (9 + 1)	10 (9 + 1)	10 (9 + 1)	10 (9 + 1)
Average pollen count/flower	436 ± 25*	676 ± 105	2100 ± 26	3649 ± 95
No. of ovules/flower	2	2	2	4 ± 0.4 (3–6)
Average P/O ratio	218 ± 12	338 ± 85	1050 ± 130	902 ± 319

\*Mean ± SE.

**Table 2.** Some aspects of floral biology in *Trifolium* species

Character	<i>T. dubium</i>	<i>T. fragiferum</i>	<i>T. repens</i>	<i>T. pratense</i>
Time of anthesis <i>vis-à-vis</i> anther dehiscence	Coincides with anthesis	Coincides or precedes anthesis by 1–2 h	Precedes anthesis by about 2 h	Precedes anthesis by about 2 h
Anther dehiscence <i>vis-à-vis</i> stigma receptivity	Protogynous (0.5–2 h)	Protogynous (20–22h)	Stigma receptivity coincides with anther dehiscence	Protogynous (48 h)
Mode of pollen shedding	<i>In situ</i> pollen germination in about 97% flowers of an inflorescence	<i>In situ</i> pollen germination in 30–100% flowers of an inflorescence	Normal pollen dispersal through longitudinal slits	Normal pollen dispersal through longitudinal slits

## Results and discussion

As in other papilionoid legumes, in clovers also, expansion of the standard petal marks the anthesis, while the reproductive organs continue to remain concealed in the keel. Time of anthesis *vis-à-vis* anther dehiscence and stigma receptivity varies (Table 2). Anthers of almost all flowers of *T. dubium* ( $\bar{X}$  = 97%,  $n$  = 2262) and 68% flowers of *T. fragiferum* (30–100%,  $n$  = 3250) exhibit *in situ* pollen germination inside the anther locule and pollen tubes emerge in bundles to reach the receptive stigma, which is in proximity with the anthers. Pollen tubes travel down the style in bundles to reach the ovary.

In *T. dubium*, out of two ovules only the stylar ovule is fertilized. The peduncular ovule remains unfertilized and aborts (Figure 1 a). All the fruits turn out to be single-seeded. The pattern of ovule abortion in this species can authentically be termed as non-random. In *T. fragiferum*, both the ovules present in the ovary are fertilized (Figure 1 b). Both may mature into seeds or one may abort and other transform into seed. The rate of stylar ovule abortion is however much higher than the peduncular ovule (Table 3). Student's *t*-test classifies this abortion as non-random type.

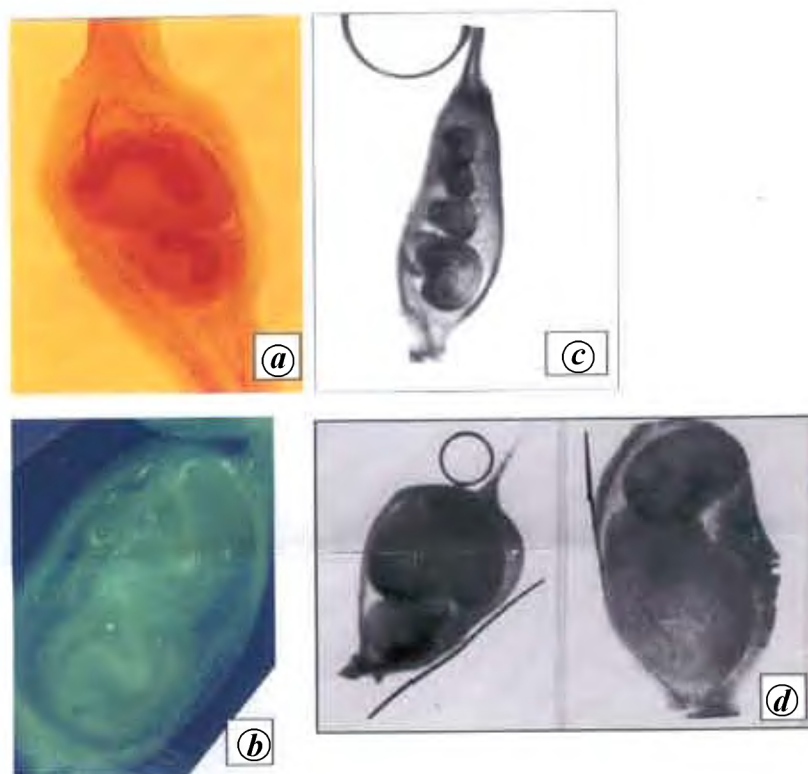
Both *T. pratense* and *T. repens* are self-incompatible. All the ovules of an ovary in both the species get fertilized, if adequately pollinated. The pollen tubes enter inside the ovules, following which the ovules increase in size. However, only some ovules develop, while others abort. In *T. pratense*, within 48 h of cross-pollination, one of the two ovules starts aborting (Figure 1 d). The ovule abortion is random and fruits of this species are invariably single-seeded.

In *T. repens* only one, two or at the best three ovules of the pistil mature into seed (Figure 1 c). Very rarely 4 or 5 ovules form seeds. Ovules present at the base of the style abort at a higher frequency followed by those present near the peduncular end. Ovules present at the middle positions abort least and hence contribute towards seed formation. Since ovary contains an average of four ovules and all these irrespective of their position experience some abortions, ovule abortion can be considered as random (Table 3). One-way ANOVA, however, suggests that difference in the rate of abortion at stylar, middle and peduncular ovule is statistically significant in the species ( $F$  = 13.61; d.f. = 3,76;  $P$  < 0.05). Stylar ovule consistently aborts at a higher frequency. This fact classifies ovule abortion in this species also, to the category of non-random.

The seed set percentage on open pollination varies in the four species. It is highest in *T. fragiferum* ( $\bar{X}$  = 63 ± 18.37) and least in *T. pratense* ( $\bar{X}$  = 15 ± 7.65; Table 2). Unassisted autogamy results in no significant change of seed output in *T. dubium*. In *T. fragiferum*, there is decrease in seed set on self-pollination (Table 3). Plants of *T. pratense* and *T. repens* do not set any seed on bagging.

The four taxa studied represent a spectrum of breeding systems. One end of this spectrum is occupied by *T. dubium* which sets seed by self-pollination, while the other end is occupied by *T. pratense* and *T. repens*, where the seed set is exclusively by cross-pollination. Plants of *T. fragiferum* combine inbreeding and out-breeding in varied proportions.

Flowers of *T. dubium* are bright yellow coloured, remain aggregated in dense spikes and seem structured for entomo-



**Figure 1.** Whole mounts of pistils of (a) *Trifolium dubium*, (b) *T. fragiferum*, (c) *T. repens* and (d) *T. pratense* showing patterns of ovule development.

**Table 3.** Rate and pattern of ovule abortion and percentage seed set in *Trifolium* species

Character	<i>T. dubium</i>	<i>T. fragiferum</i>	<i>T. pratense</i>	<i>T. repens</i>
Rate of ovule abortion (%)	50 <i>m</i> = 2262*	38 <i>m</i> = 3250	50 <i>m</i> = 3015	48 <i>m</i> = 4017
Patterns (%)				
Stylar	3	79.2	46	34**
Middle	—	—	—	15***
Peduncular	97	20.8	54	23
Average percentage seed set on				
Open-pollination	42 ± 1.72**** (27–50)***** <i>N</i> = 198 <i>n</i> = 9468	63 ± 18.37 (22–96) <i>N</i> = 144 <i>n</i> = 3964	42 ± 12.53 (16–68) <i>N</i> = 131 <i>n</i> = 5465	15 ± 7.65 (6–33) <i>N</i> = 120 <i>n</i> = 14,000
Bagging	41 ± 7.167 (9–49) <i>N</i> = 25 <i>n</i> = 750	55 ± 16.47 (23–83) <i>N</i> = 45 <i>n</i> = 898	0 <i>N</i> = 25 <i>n</i> = 945	0 <i>N</i> = 10 <i>n</i> = 450

*m*, Number of pistils scanned; \*Significant at  $t = 0.05$ ; \*\*One-way ANOVA ( $F = 13.61$ ; d.f. = 3.76;  $P < 0.05$ ); \*\*\*Mean % abortion of ovules at middle positions; \*\*\*\*Mean ± SE; \*\*\*\*\*Range; *n*, Number of flowers; *N*, Number of inflorescences.

phily; they attract meagre number of insects also. Even the few, however, act as casual visitors, as no pollen load was ever recorded from their bodies. Placement of anthers and stigma close to each other and closure of all these in the keel make conditions conducive for autogamy. The same gets assured when pollen grains germinate *in situ* in large numbers and about 400–500 pollen tubes clog the stigma instantly for siring just two ovules in the ovary<sup>2</sup>.

Flowers of *T. fragiferum* are predominately selfed, although they keep provision for outcrossing also. Reproductive apparatus in these flowers remains concealed in the keel; anthers and stigma are almost at the same level. Normal pollen dispersal takes place only in a proportion of flowers. In the rest, pollen grains germinate inside the pollen sac and pollen tubes thus produced come out through the dehiscence slit. Flowers are briskly visited by insects,

especially during morning hours and these do carry pollen from one flower to another. The flowers of the species being protogynous and insect-pollinated keep first provision for outcrossing, but ensure autogamy if the same fails. The species thus enjoys mixed mating system<sup>4</sup>. Insects in this species act both for inducing self-pollination and as agents of cross-pollination. *T. pratense* and *T. repens* show gametophytic self-incompatibility (GSI)<sup>15–17</sup> and hence are obligately outcrossed. Irrespective of variation in the breeding system, all the species suffer from ovule abortion. The rate and patterns vary. While in *T. dubium* and *T. pratense* one of the ovules present in an ovary aborts invariably resulting in 50% loss in reproductive potential, in *T. repens* and *T. fragiferum* the rate of abortion is less; although in the former, it is quite close to 50% (Table 3). Reproductive output of these *Trifolium* species varies and does not correlate with the breeding system. In nature, spikes of *T. fragiferum* set maximum number of seeds while those of *T. repens* the least (Table 3).

While percentage seed set on open pollination in general relates to a great extent to the rate of ovule abortion, in *T. repens* the difference is wide. This can be explained on two grounds:

- (1) Dependence on pollinators for successful pollination.
- (2) Insects pollinating stigma with self-pollen or with pollen carrying the same *S*-allele as in the pistil<sup>3</sup>.

Though ovule abortion leading to lowering of seed set/seedlessness has been reported in a number of plants species, the phenomenon has been studied in depth in only a few. Most of the studies have revolved around legumes<sup>18–21</sup>. A number of pre and post-fertilization mechanisms have been suggested to concert, creating a kind of 'genetic sieve' which leads to preferential development of certain embryo genotypes. While discussing P/O conflict as a cause of ovule abortion, Mohan Raju *et al.*<sup>20</sup> argue that the maternal plant tries to reduce abortion rate of developing seeds by increasing genetic relatedness among the siblings contained in a fruit. Going by this logic, ovule abortion should be less in self-pollinating species<sup>21</sup>. In fact, selfers are known to be high seed setters<sup>22–24</sup>, which is a direct evidence of their low abortion rates.

Data on four species of *Trifolium* having diverse breeding system reveals a typical pattern. While in the two out-crossers the rate of abortion is high and almost equal, the strict selfer of the group also gets clubbed with them. The only species left out is *T. fragiferum*, a species with mixed reproductive strategy. Although data on this small group of four species cannot be conclusive, this indicates the superiority of mixed mating strategy over obligate breeding systems.

1. Marshall, A. H., Michaelson-Yeates, T. P. T., Aluka, P. and Meredith, M., Reproductive characters of interspecific hybrids between *Trifolium repens* L. and *T. nigrescens* Viv. *Heredity*, 1996, **74**, 136–145.

2. Sharma, N. and Koul, A. K., *In situ* pollen germination and selective ovular abortion in *Trifolium dubium* Sibth. *Curr. Sci.*, 1996, **71**, 598.
3. Hagen, M. J. and Hamrick, J. L., Genetic variation and population genetic structure in *Trifolium pratense*. *J. Hered.*, 1998, **89**, 178–181.
4. Dhar, R., Sharma, N. and Koul, A. K., Intra population variation in site of pollen germination and pollination in *Trifolium fragiferum* L. *Phytomorphology*, 2002, **58**, 323–330.
5. Martin, J. N., Comparative morphology of some leguminosae. *Bot. Gaz.*, 2002, **58**, 154–167.
6. Erith, A. G., *White clover (Trifolium repens L.)*. A Monograph, Duckworth and Co, London, 1924.
7. Atwood, S. S., Controlled self and cross pollination of *Trifolium repens*. *Am. Soc. Agron. J.*, 1941, **33**, 538–545.
8. Rembert Jr., D. H., Ovule ontogeny, megasporogenesis, and early gametogenesis in *Trifolium repens* (Papilionaceae). *Am. J. Bot.*, 1977, **64**, 483–488.
9. Povilaitis, B. and Boyes, J. W., Ovule development in diploid red clover. *Can. J. Bot.*, 1960, **38**, 507–531.
10. Bissuel-Belaygue, C., Cowan, A. A., Marshall, A. H. and Wery, J., Reproductive development of white clover (*Trifolium repens* L.) is not impaired by a moderate water deficit that reduces vegetative growth: II. Fertilization efficiency and seed set. *Crop Sci.*, 2002, **42**, 414–422.
11. Martin, H. H., Leonard, W. H. and Stamp, D. L., *Principles of Field Crop Production*, Macmillan Publishing Co, Inc, New York, 1976, 3rd edn, pp. 659–675.
12. Bulinska-Radomska, Z., Morphological relationships among 15 species of *Trifolium* occurring in Poland. *Genet. Res. Crop Evol.*, 2000, **47**, 267–272.
13. Lewis, D., *Sexual Incompatibility in Plants*, Edward Arnold, London, 1979.
14. Shivanna, K. R. and Rangaswamy, N. S., *Pollen Biology – A Laboratory Manual*, Springer-Verlag, New York, 1992.
15. Williams, R. D. and Silow, R. D., Genetics of red clover (*Trifolium pratense* L.) compatibility I. *J. Genet.*, 1933, **27**, 341–362.
16. Williams, W., Genetics of red clover (*Trifolium pratense* L.) compatibility. II (a) Homozygous self-sterile SxSx genotypes obtained as a result of pseudo-fertility; (b) Self-fertility. *J. Genet.*, 1947, **48**, 51–68.
17. De Nettancourt, D., Self-incompatibility in basic and applied researches with higher plants. *Genet. Agrar.*, 1972, **26**, 163–226.
18. Ganeshaiah, K. N. and Uma Shaanker, R., Seed abortion in wind-dispersed pods of *Dalbergia sissoo*: maternal regulation or sibling rivalry. *Oecologia*, 1988, **77**, 135–139.
19. Rocha, O. J. and Stephenson, A. G., Effects of non-random seed abortion on progeny performance in *Phaseolus coccineus* L. *Evolution*, 1991, **45**, 1198–1208.
20. Mohan Raju, B., Uma Shaanker, R. and Ganeshaiah, K. N., Differential rates of seed abortion among trees of *Dalbergia sissoo*: role of post dispersal sibling competition. *Curr. Sci.*, 1995, **68**, 1114–1118.
21. Weins, D., Ovule survivorship, brood size, life history, breeding system and reproductive success in plants. *Oecologia*, 1984, **64**, 47–53.
22. Solbrig, O. T. and Rollins, R. C., The evolution of autogamy in species of the mustard grass genus *Leavenworthia*. *Evolution*, 1977, **31**, 265–281.
23. Schoen, D. J., The breeding system of *Gilia achilleifolia*. Variation in floral characteristics and outcrossing rate. *Evolution*, 1982, **36**, 352–360.
24. Sharma, N., Koul, A. K. and Kaul, V., Patterns of resource allocation of six *Plantago* species with different breeding systems. *J. Plant Res.*, 1999, **112**, 1–5.

Received 30 September 2005; revised accepted 5 April 2006