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Self fertilization and seed set in *Trifolium repens* L. by in situ and in vitro pollination

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Summary. The frequency of seed formation has been determined from self-pollination in situ (by hand) and in vitro for *Trifolium repens*. Selfing in situ was measured over a period of 3 years in which plants were held either at 35 °C for 24 h post-pollination (1984 and 1985) or held at ambient temperatures (1986). Mean yield of self-seed per 100 florets was 2.8 in 1984, 5.2 in 1985 and 2.2 in 1986. This was based on over 15,000 selfings per year with seven varieties and a total of 166 genotypes. In general, seed set following self-pollination was low; 53% of all genotypes set less than one seed per 100 florets selfed. Selfing of 340 excised florets in vitro with six genotypes gave a mean of 30.6 seeds per 100 florets. Temperature treatments (post-pollination) had no significant effect on seed yield in vitro. Treatment of florets in vitro for 24 h post-pollination with 0.1% CO_2 increased the yield of self seed with three genotypes but had no effect on a fourth genotype.

Key words: Self compatibility – *Trifolium repens* – Carbon dioxide – Pollen pistil interactions – Temperature

Introduction

Development of varieties of *Trifolium repens* and *Trifolium pratense* has been based primarily on standard selection procedures which are appropriate for cross-pollinating species (Taylor and Smith 1979). Both species have strong systems of gametophytic self-incompatibility [SI] (De Nettancourt 1972). Hybridization between inbred lines could be controlled genetically by the SI system for production of double cross hybrids. Some combinations of partially inbred lines of *T. pratense* have indicated heterosis (Taylor et al. 1970) and significant non-additive genetic variance for vigour (Cornelius et al. 1977).

Inbred lines or partially inbred lines which are homozygous for alleles at the S locus are required for development of hybrid varieties based on the SI system. Production of homozygous genotypes by regeneration of plants from haploid cells followed by doubling of the chromosome number has proved unsuccessful thus far in the family *Leguminosae*. Alternatively, heat treatments of florets at anthesis have facilitated selfing in *T. pratense* (Kendall and Taylor 1969; Kendall 1973). Although high temperature treatments weakened SI with one genotype of *T. repens* (Chen and Gibson 1973; Gibson and Chen 1973), the system of SI is very strong in this species (Williams 1931). By pollination of florets in vitro, environmental and nutritional factors which may affect the SI reaction can be more easily manipulated and such techniques have been discussed previously for *T. pratense* (Kendall 1968, 1973; Kendall and Taylor 1969), *Brassica campestris, B. oleracea, Raphanus sativus* (Okazaki and Hinata 1987) and *Lycopersicon peruvianum* (Williams and Webb 1987). For *T. repens*, the technique of in vitro cross pollination of excised florets has been previously described in both intraspecific and interspecific hybridization (Richards and Rupert 1980).

The aims of the present study were to determine the frequency of selfing in several varieties and genotypes of *T. repens*, with hand pollination in situ and with excised florets self-pollinated in vitro. The effects of temperature on selfing in situ and in vitro are also presented and the effects of CO_2 supplementation with the in vitro system was determined.

Materials and methods

Plants of *T. repens* were raised from seed and were grown in 200 cm diameter pots containing potting compost. Plants were

Table 1. Effect of variety and post pollination temperature on seed formation following-self pollination in situ of *T. repens* in three seasons

Variety	No. geno-types	No. florets selfed	Seeds per 100 florets	
			Mean	Range
1984 (35 °C for 1 day post-pollination)				
Hs*	8	3,815	4.2	(0.9– 13.2)
Huia	5	1,461	1.2	(0.0– 2.8)
Kent	7	1,854	10.4	(0.0– 26.5)
Lirepa	4	1,878	1.1	(0.7– 3.3)
Milkanova	7	3,435	2.8	(0.4– 5.2)
Olwen	5	2,564	1.4	(0.4– 6.4)
Susi	2	948	2.2	(1.9– 2.3)
Total	38	15,955	–	–
Mean (1984)	–	–	2.8	–
1985 (35 °C for 1 day post-pollination)				
Hs*	11	3,477	9.7	(0.0–110.1)
Huia	7	851	4.1	(0.0– 33.1)
Kent	9	2,335	0.4	(0.0– 0.9)
Lirepa	6	1,988	16.3	(0.3– 94.6)
Milkanova	9	2,975	4.8	(0.7– 14.0)
Olwen	7	2 220	2.9	(0.3– 17.8)
Susi	11	4,447	1.0	(0.2– 2.7)
Total	60	18,293	–	–
Mean (1985)	–	–	5.2	–
Mean (1984+1985)	–	–	4.1	–
1986 Temperature 5 °–30 °C (ambient) post-pollination				
Hs*	8	2,259	0.5	(0.0– 2.2)
Huia	16	4,871	1.3	(0.0– 4.1)
Kent	10	3,113	1.7	(0.0– 6.3)
Lirepa	11	3,404	4.2	(0.0– 21.7)
Milkanova	10	3,205	4.2	(0.0– 7.4)
Olwen	5	1,446	0.7	(0.0– 1.2)
Susi	8	2,568	1.6	(0.3– 7.1)
Total	68	20,866	–	–
Mean (1986)	–	–	2.2	–
Overall total (1984, 1985, 1986)	166	55,114	–	–
Overall mean	–	–	3.4	–

* = Breeders line

maintained in insect-proof plastic houses for self-pollination in situ during the period May–August, temperature range (5°–30 °C). For temperature treatments in 1984 and 1985, pots were placed for 24 h post-pollination in a phytotron (Assab Company, Sweden) at 34°±1 °C with a light intensity 1,200 $\mu E \times m^{-2} \times sec^{-1}$. For in vitro self-pollination in the same period, florets were collected from glasshouse grown plants. Supplementary lighting was provided under glass from high pressure sodium lights in spring and autumn.

Self-pollinations were performed in situ on fresh florets in which the large standard petal was fully opened. Transfer of self-pollen to the stigma was made by depressing the keel petal of florets with a wooden cocktail stick. Approximately 30–50 florets per flower head were thus 'tripped' and remaining mature and immature florets were removed. Flower heads on each plant were numbered and yield of full seed after 4–5 weeks from each flower head was recorded separately with each genotype. All seeds were scarified using fine sandpaper prior to sowing.

For self-pollination in vitro, florets in which the large standard petal was just beginning to open were selected. In excising florets, as much floret-stalk as possible was taken. With these florets, rupture of anthers had just started or would occur within 24 h. Florets were placed in small plastic-weighted baskets and immersed for 20 min in a solution of calcium hypochlorite, which was prepared by mixing 1 part of (7% w/v) filtered calcium hypochlorite with 7 parts of sterile water. This was followed by three rinses in sterile distilled water. Pollination of each floret was performed separately. An incision was made in the calyx and along the length of the floret through the keel and wing petals. The stigma and style were then probed out through the incision. By similar incisions, anthers were removed from older florets of the same genotype in which rupture of anthers had begun. Pollen was transferred to the stigma by rubbing the stigma of surface-sterilised florets with these anthers. Each pollinated floret was transferred to a 5 × 6 cm petri plate (Sterilin) containing 6 ml of B5 medium (Gamborg et al. 1968) and dishes were wrapped with Parafilm M (American Can Co.). Medium contained sucrose (3% w/v) Difcobacto agar (0.8% w/v), and pH was adjusted to 5.8 prior to autoclaving. All cultures were maintained in a culture room at 22 °C±1 °C in a 16 h photoperiod (63 $\mu mol \times m^{-2} \times s^{-1}$) with Thorn warm white fluorescent tubes, except where otherwise stated. Alternative temperature regimes were provided for a duration of 24 h after pollination. After 2 weeks of culture, ovaries were dissected and the number of full seeds present was recorded. For germination, the testa of seeds were removed and embryos were recultured on B5 medium and scored for germination after a further 3–4 weeks. Plants were recultured on 0.5-strength B5 medium for 3–4 weeks before transfer to the greenhouse.

Elevated levels of CO_2 were supplied by injecting a measured volume of pure CO_2 into a dessicator in which florets were placed for 24 h after pollination. Control plates were handled similarly in an open-topped dessicator; plates were unwrapped with Parafilm during CO_2 treatment.

Self-pollinations were also peformed in situ using florets which had reached a developmental stage (described above) suitable for in vitro pollinations. In these cases all other florets on the flowerhead were removed and whole plants were maintained in the culture room for 24 h post-pollination. Thereafter they were returned to the glasshouse. At the same time, florets were taken from other heads on the same plants and self-pollinations were performed in vitro. A sample of 23–30 florets from each of four genotypes were selfed on the same day in vitro and in situ.

Results

Production of seed by self-pollination in situ was recorded for 166 genotypes among 7 varieties and results from 3 years are summarised in Table 1. Seed yield was quite variable among varieties and among different genotypes of the same variety. Less than one seed per 100 selfed florets was obtained with 53% of all genotypes. Overall, a mean of 4.1 seeds per 100 florets was obtained by holding plants at 35 °C for 24 h post-pollination whereas 2.2 seeds were obtained with plants maintained at ambient temperatures (Table 1). Seeds obtained from selfing in situ were sown and 52% germinated.

Table 2. Effect of variety and genotype on seed formation following self-pollination of *T. repens* in vitro

Variety (genotype)*	No. florets selfed	No. full seed	Seeds per 100 florets
Kersey (a)	128	26	20.3
Kersey (b)	20	5	25.0
Milkanova (a)	49	7	14.3
Milkanova (c)	12	2	16.7
Olwen (a)	102	54	52.9
Aran (a)	29	10	34.5
Total	340	104	–
Mean	–	–	30.6

* Different letters signify separate genotypes in each table

Table 3. Effect of temperature on seed-formation following self-pollination of *T. repens* in vitro

Temperature (°C)	Seeds per 100 florets selfed		
	Variety (genotype)		Mean (a+b)
	Olwen (a)	Kersey (b)	
22	53 (102)*	24 (20)	39.0
24	80 (10)	45 (20)	62.5
26	35 (20)	NT	35.0
28	25 (24)	47 (40)	36.0
30	42 (67)	19 (16)	30.5
32	44 (18)	19 (25)	26.0
34	16 (19)	22 (18)	19.0
Mean (all temperatures)	40.6	29.5	35.5

* Numbers in brackets are numbers of florets used per temperature treatment. Florets were self-pollinated in vitro and transferred to the indicated temperature for 24 h.
NT = not tested

For comparative purposes cross pollinations were performed on some genotypes. Seed set following cross pollination was 203, 186 and 229 per 100 florets in 1984, 1985 and 1986 respectively. An evaluation of seed set in non-tripped florets was also made. In 1986 three flower heads were marked and left untripped while other flower heads on the same plants were selfed normally. From 68 genotypes so tested, only one produced any seed (two seeds) from the three marked heads with untripped florets. This genotype also showed relatively high seed set on self-pollination (21.7 seeds per 100 florets selfed) (Table 1).

Production of seed by self-pollination in vitro with six genotypes is summarised in Table 2. Yield of seed varied with each genotype and a mean value for all genotypes of 30.6 seeds per 100 florets was obtained. With four genotypes, florets were chosen at the developmental stage used for in vitro pollination and a comparison of in vitro selfing was made with in situ selfing. From selfing in vitro the mean yield of seeds per 100 florets was 9.1 and from in situ selfing 1.7.

Table 4. Effect of CO_2 supplementation on seed formation following self-pollination in vitro of *T. repens*

Variety (genotype)	Temp (°C) during CO_2 treatment	Seeds per 100 florets selfed*	
		CO_2 concentration (v/v)**	
		0.03% (ambient)	0.1%
Milkanova (a)	22°	21.7 (23)	56.0 (25)
	30°	13.0 (23)	34.6 (26)
Milkanova (b)	22°	3.4 (29)	0 (30)
	30°	0 (27)	0 (24)
Kersey (a)	22°	34.6 (26)	50.0 (8)
	30°	17.2 (29)	44.0 (25)
Kersey (c)	30°	0 (22)	37.5 (16)
Mean no. seeds/ 100 florets	22°	19.9	35.3
	30°	10.1	29.0

* Numbers in brackets are numbers of florets used per treatment
** Florets were pollinated in vitro, transferred to CO_2 supplemented chamber (0.1%) for 24 h at temperature indicated and then returned to ambient CO_2 (22 °C)

A range of temperatures tested for 24 h post-selfing in vitro and the effects on seed yield with two genotypes are summarised in Table 2. A small increase was obtained at 24 °C for both genotypes; elevated temperatures (26°–34 °C) were without effect or were detrimental.

The effects of increasing the concentration of CO_2 for 24 h on selfing at two temperatures are given in Table 3. Raising the concentration of CO_2 from 0.03% (ambient) to 0.1% gave a significant ($P = 0.05$) increase in seed yield following selfing for three genotypes. However, with a fourth genotype (Milkanova b) seed set was very low and CO_2 supplementation had no effect (Table 4).

Compatible cross-pollinations made in vitro always gave at least one seed per floret. More than two seeds were never produced per floret in crossings. Germination of seeds obtained from all selfings made in vitro was 43%. Frequency of green seedlings ranged from 28%–100% depending on genotype. Over 90% of green seedlings survived transfer to the glasshouse.

Discussion

This study shows that self-incompatibility in *T. repens* is strong and seed set on selfing in situ is very low. Although there may be as many as six ovules per floret, a mean yield of only 3.4 seeds per 100 florets was obtained based on over 55,000 selfings in three years (Table 1). A similar yield was previously reported by Williams (1931) based on 12,000 selfings. Self-incompatibility is also

strong in *T. pratense*; no seed was obtained by in situ selfing from over 2,000 florets with ten genotypes, but every genotype gave some seed when flower heads were held at 40° for 2–3 days during anthesis (Kendall and Taylor 1969). A high degree of SI is a desirable character for production of intra-sterile inbred lines. Such lines may be used for hybrid seed production. With *T. pratense*, some evidence for the potential of hybrid variety production has been reported. Crosses involving first and second generation inbreds of *T. pratense* gave a significantly superior performance in some combinations (Taylor et al. 1970; Cornelius et al. 1977). In the present study, lines of *T. repens* homozygous for the SI alleles have been identified and plants with superior combining ability have been selected for further breeding work.

Variability in capacity for selfing was found among genotypes using in situ and in vitro pollination. In general, however, selfing in vitro gave a five-to tenfold increase in yield of seed relative to selfing in situ. With in vitro selfing, less mature florets were selected than for in situ selfings reported in Table 1. In such florets the SI mechanism may be less highly developed. However, higher yields of self seed were obtained from in vitro pollination when in vitro and in situ methods were directly compared using florets of similar maturity. This indicates that some features of the in vitro system may also have contributed to weakening of the SI response. Unlike sporophytic incompatibility where inhibition of pollen tubes is confined mainly to the stigma, inhibition in *Trifolium* spp. is progressive in both stigma and style (Knox et al. 1986).

Lessening of the SI response in vitro may result from several factors, including humidity, nutrition, the gaseous environment and injury responses resulting from floret excision. With *Phaseolus vulgaris,* germination of pollen within excised flowers was stimulated by exposure to high levels of humidity for 3–6 h (Weaver and Timm 1987). This response was observed in other species with enclosed flowers such as soybean and *Phaseolus lutanus,* but not for flowers with more exposed anthers such as tomato, cucumber, melon or pepper. Enclosure of flowers of *Lycopersicon peruvianum* also resulted in weakening of gametophytic SI and this occurred with flowers either cultured in vitro or still attached to the plant (Williams and Webb 1987).

Elevated temperatures (35 °C) after pollination gave higher seed set in situ than ambient conditions of variable temperature with *T. repens* (Table 1). Similarly, seed set was improved by selfing *T. pratense* during summer and in a growth chamber at 32 °C (Leffel 1963). Treatment of styles with high temperatures also enhanced seed formation on selfing of *Lilium* (Hopper et al. 1967; Campbell and Linskens 1984) and *Brassica* and *Raphanus* (Okazaki and Hinata 1987). With in vitro pollinations, however, 35 °C was detrimental and stimulation of selfing could not be demonstrated (Table 3). In excised pistils of *T. pratense*, elevated temperatures post-pollination inhibited growth of pollen tubes in both compatible and incompatible crosses, and differences between temperatures were less clear in self-pollinations (Kendall 1968). However, studies on treatments given prior to anthesis (40 °C for 3 days) with pistils and pollen gave an increase in the number of pollen tubes traversing treated styles, but had no effect on the growth of treated pollen growing in untreated styles (Kendall 1968). Breakdown of SI by high temperature treatments given prior to, or post-anthesis has been shown for some genotypes. Thus with one genotype of *T. repens*, 34 °C after pollination increased the percentage of embryo formation (Chen and Gibson 1973) and 30 °C increased seed yield from selfing (Gibson and Chen 1973). Similarly with *T. hybridum,* treatment of whole plants for 2–5 days (pre-anthesis) at 32.5 °C affected only the style and facilitated selfing (Townsend 1965, 1966). Subsequently, self-compatibility resulting from high temperature treatments was shown to be under the control of temperature-sensitive genes which interact with SI alleles of the S gene (Townsend 1971).

Supplementation with CO_2 enhanced seed production with three genotypes which could be selfed in vitro at ambient levels, but it did not weaken the SI response of one genotype which set seed at a very low rate at ambient levels. Further work with several genotypes and increasing concentrations of CO_2 should show if CO_2 may be used as a general method for weakening of SI. Increased frequency of selfing with the gametophytic SI system of *Lycopersicon peruvianum* by enclosure of florets may also be mediated by an accumulation of CO_2 (Williams and Webb 1987). In the sporophytic SI system, elevated levels of CO_2 weakened SI in cabbage (Nakanish and Hinata 1973), *B. napus* (Dhaliwal et al. 1979), *B. campestris* (O'Neill et al. 1984) and cauliflower (Palloix et al. 1985). Weakening of SI by elevated CO_2 was mediated through several responses in cauliflower including: increased adhesion of self pollen to stigmas, reduced formation of callose and increased percentage of pollen tube germination and penetration of the style (Palloix et al. 1985).

In conclusion, inbred lines of *T. repens* may be made by large-scale selfing in situ or more efficiently by in vitro pollination. Weakening of gametophytic SI by in vitro pollination offers a more useful method to manipulate developmental events and thereby facilitates studies on the biochemistry and molecular biology of SI (De Nettancourt 1972; Takayama et al. 1987). Selfing in vitro has not succeeded with all genotypes tested and we are currently attempting to achieve fertilisation by direct application of pollen to ovules in vitro.

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