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THEORETICAL AND APPLIED GENETICS

Pollination in vitro: effects on the growth of pollen tubes, seed set and gametophytic self-incompatibility in *Trifolium pratense* L. and *T. repens* L.

N. Leduc [1, 2], G.C. Douglas [1, *], M. Monnier [2] and V. Connolly [3]

[1] Agriculture and Food Development Authority (Teagasc), Kinsealy Research Centre, Malahide Road, Dublin 17, Ireland
[2] Laboratoire d'Histophysiologie Végétale, Université Pierre et Marie Curie, 12 rue Cuvier, 75005 Paris, France
[3] Agriculture and Food Development Authority, Oakpark Research Centre, Carlow, Ireland

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Summary. Growth of pollen tubes and seed set were compared after hand pollination in situ and in vitro in two self-incompatible species, *Trifolium pratense* and *Trifolium repens*. Adhesion of pollen grains to the stigma was greater in vitro for both species. After cross-pollination, in vitro culture gave a significant increase in the cumulative growth of pollen tubes in pistils of *T. pratense* compared to in situ conditions. After selfing in *T. repens*, pollen tube growth was significantly increased by in vitro culture of florets. Seed set after crossing in situ and in vitro was similar for both species. Seed set after selfing in vitro was not increased in *T. pratense*. Several genotypes of *T. repens* were classified as very good, good and poor selfers based on their capacity for seed set following selfing in situ. In vitro pollination increased self seed formation by 1.7-, 18.0- and 31.0-fold for each class, respectively. Ovules located nearest to the style were fertilized more often after selfing than after crossing.

Key words: Self-compatibility – *Trifolium repens* – *Trifolium pratense* – Pollen tube – In vitro culture

Introduction

In *Trifolium pratense* and *Trifolium repens* a gametophytic self-incompatibility system prevents inbreeding (De Nettancourt 1972). Inbred or partially inbred lines homozygous at the S-locus are necessary for the development of hybrid varieties in both these species. As the production of homozygotes by the diploidization of plants derived from haploid cells has not yet succeeded in the Leguminosae, methods have been developed to weaken the self-incompatibility (SI) system in these species and promote homozygosity by sexual reproduction. For this purpose the heat treatment of florets before pollination has proved partially efficient in *T. pratense* (Kendall and Taylor 1969; Kendall 1973) and in one particular genotype of *T. repens* (Chen and Gibson 1973; Gibson and Chen 1973).

In vitro culture has been used to provide controlled conditions in which to assess the effect of different factors and further weaken the SI system. In *T. pratense*, treating the stigmas with various chemicals prior to pollination, supplementing the medium with hormones, and varying the amounts of carbohydrates and salts did not affect the SI reaction; previous pollination of the excised florets in vitro also had no effect (Kendall 1968; Kendall and Taylor 1971). In *T. repens*, a higher seed yield was consistently obtained when excised florets were selfed in vitro. Temperature treatments post-pollination did not increase the seed yield, but CO_2 (0.1%) treatment for 24 h post-pollination was efficient for three out of the four genotypes tested (Douglas and Connolly 1989).

The aim of the present study was to measure the effect of in vitro culture on the SI reaction. The growth of the pollen tubes and the seed yield after compatible and incompatible hand-pollinations on the plant were compared with pollinations of excised florets in vitro, in both *T. pratense* and *T. repens*.

Materials and methods

Plants of several genotypes of *Trifolium repens* and *Trifolium pratense* (var 'granta') were grown in pots containing potting compost. Supplementary lighting was provided in the glasshouse in the autumn and spring to ensure a photoperiod of 16 h daylight per 24 h. For all in situ and in vitro pollinations, florets whose large standard petal had just started to open were select-

* Tho whom correspondence should be addressed

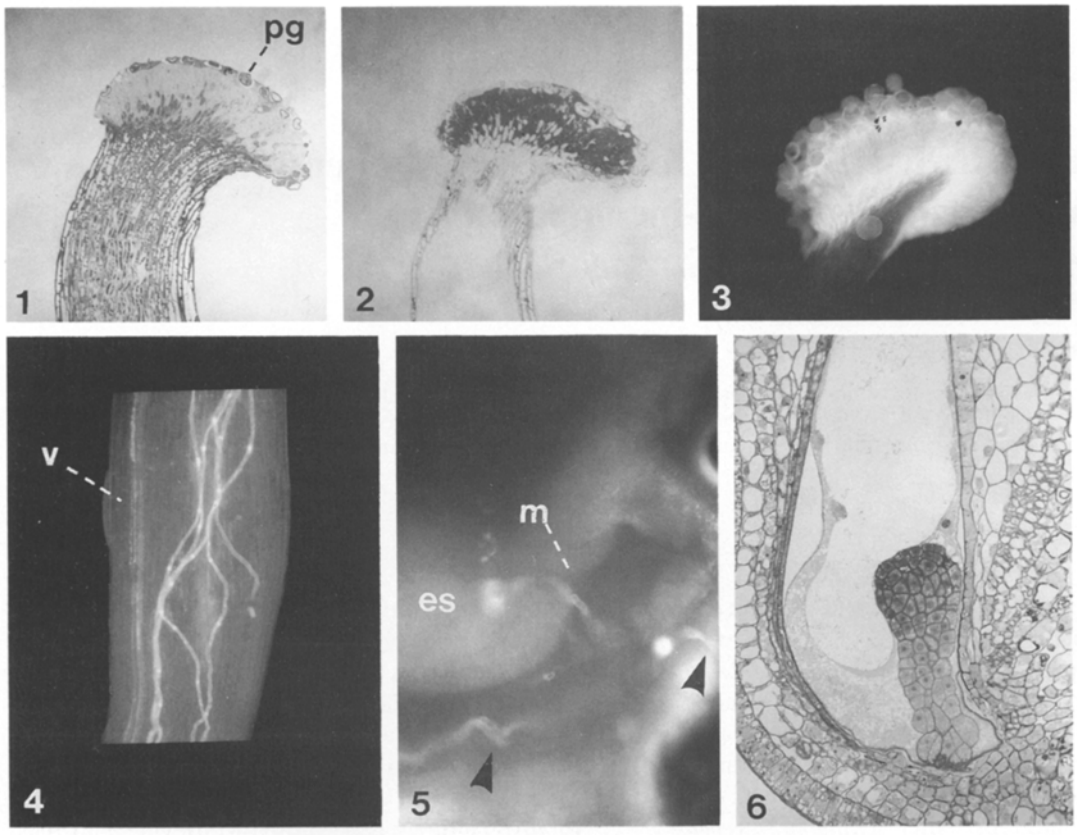

Fig. 1. Stigma of *T. repens* stained with Coomassie Blue for protein detection. Note staining of pollen grains (*pg*) and cuticle layer. Stigma fixed 5 h after self-pollination in vitro. × 80

Fig. 2. Stigma of *T. repens* treated with Sudan Black III for lipid detection. Note staining is confined to the stigmatic exudate in the subcuticular spaces. Stigma fixed 5 h after self-pollination in vitro. × 80

Fig. 3. Stigma of *T. pratense* showing autofluorescence (green) of stigmatic exudate and presence of pollen grains. × 80

Fig. 4. Fluorescence of pollen tubes and vascular bundle (*V*) in the style of *T. repens* stained with Aniline Blue. × 140

Fig. 5. Fertilization of an ovule of *T. repens*. Note pollen tube (*arrowheads*) travels along the placenta and funiculus. *m* micropyle, *es* embryo sac

Fig. 6. Longitudinal section of a globular embryo 4 days after self-pollination in vitro in *T. repens*. Note suspensor has three cells in width and free nuclear endosperm. × 250

ed. Anthesis would occur in these within 24 h. Pollination in situ involved opening the florets on the plant and transferring either self- or cross-pollen to the stigma.

For pollination in vitro, florets were collected with as much peduncle as possible. Sterilization was achieved by immersing the florets in a filtered calcium hypochlorite solution (1 part 7% w/v concentrated solution of calcium hypochlorite in 7 parts sterile water) for 20 min. Florets were rinsed 3 times in sterile water prior to transfer in vitro. The conditions of the in vitro culture have been described previously (Douglas and Connolly 1989). An incision was made along the calyx and the petals of the florets in order to probe out the style and stigma. Florets with stigmas already bearing pollen grains were discarded. Stigmas were pollinated by brushing mature anthers of the appropriate genotype on their surface.

The growth of pollen tubes in the pistil was observed at 5 h following pollination: florets pollinated in situ and in vitro were collected, and the pistils were excised and immediately immersed in NaOH (1 *M*) for one-half hour. They were then mounted in a drop of Aniline Blue solution (2 g KH_3PO_4 + 0.2 g Aniline Blue in 100 ml distilled water, pH = 8), gently pressed under a coverslip and observed using the fluorescence microscope.

For each floret observed, the number of pollen grains and number of tubes on the stigma was recorded as well as the number of pollen tubes through each optical section of the style. Pollen tubes just above the first ovule (upper ovary U.O.) and just under it (lower ovary L.O.) were also counted. From 5 to 15 pistils were observed for each genotype in each experiment, and the mean number of pollen tubes per stylar section and per pistil is presented in histograms (Figs. 7, 8, 9). The yield of full seed obtained in situ and in vitro is also given. Seeds were collected 4–5 weeks after pollination in situ and 10 days after pollination in vitro, as previously described (Douglas and Connolly 1989). Seed yield was expressed as number of seed per 100 florets pollinated.

Statistical analyses

In order to compare the growth of the pollen tubes under different conditions and simultaneously take into account the number

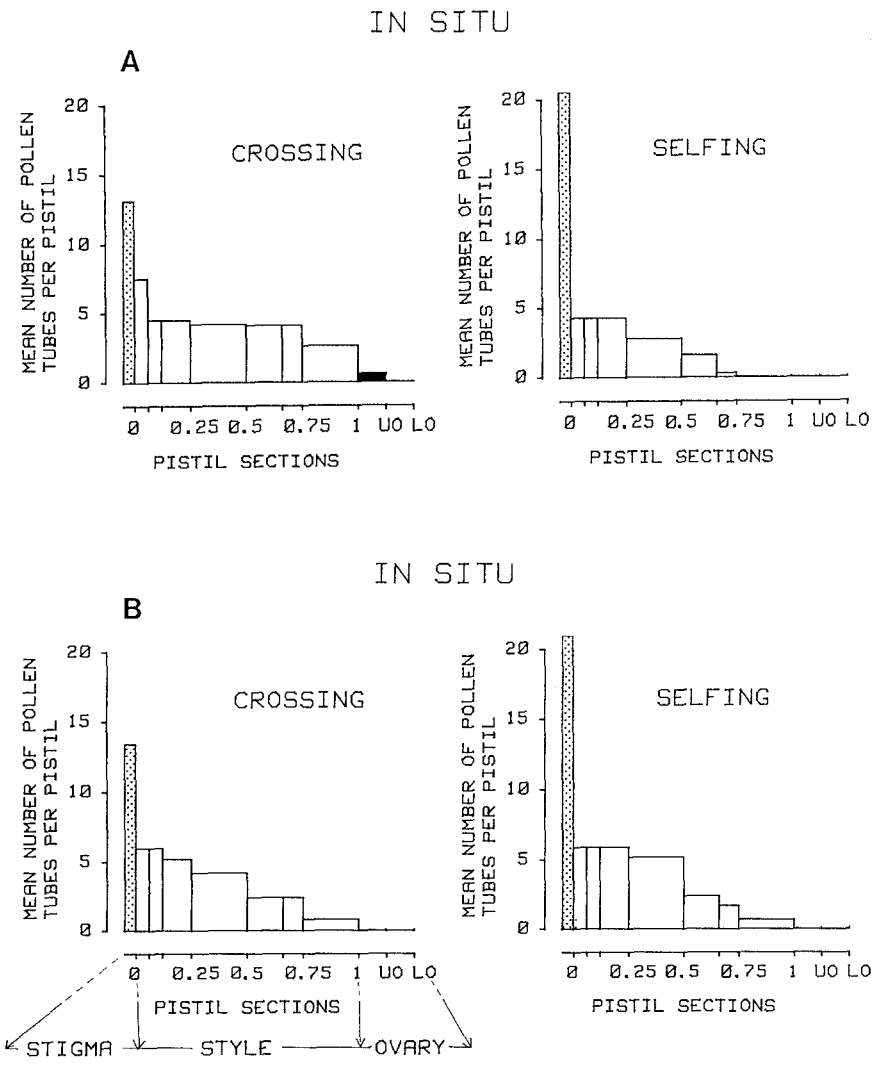

Fig. 7. Distribution pattern of pollen tubes in the stigma (*shaded column*), style (*white columns*) and ovary (*black column*) of *T. pratense*, 5 h after self- and cross-pollination in situ. Two genotypes were studied, A and B. The designation of stigma, style and ovary on the X axis is given for the lower left histogram and applies to all others in this fig. and in Figs. 8 and 9

and length of the tubes in the pistil, the length of the style was designated as one unit, i.e. 1.0, from the base of the stigma to the base of the style, and values were computed as described below. For each floret the number of pollen tubes found in each section of the pistil was multiplied by the length of this section (i.e. 0.06; 0.12; 0.25 ... 1.0) from the stigma to the base of the style. Lengths of 1.1 and 1.2 units were given to the upper and lower ovary sections. A total length of pollen tubes for each floret was obtained by adding the values calculated for each section of the pistil. For each experiment, a mean total length of pollen tubes per floret and a variance of the mean was calculated. Means were compared two by two using Student's test choosing a risk of 5%.

Histology

Sections 10 μm thick were prepared from stigmas and ovules of *T. repens* fixed in 3% glutaraldehyde and preserved in 0.05 *M* phosphate buffer prior to post-fixation with 2% osmium tetroxide. Tissues were dehydrated in an alcohol series and embedded in Emix Resin. Stigma sections were stained with Black Sudan III (a 70% ethanol solution saturated with black Sudan III powder) to reveal the presence of lipids and with Coomassie Blue (0.25 g Coomassie Blue powder in 10 ml methanol, 3 ml acetic acid, 87 ml distilled water solution) for the presence of proteins. Ovule sections were stained with Paragon (270 mg of Basic Fuchsin + 730 mg of Toluidine Blue powders in 100 mls of 30% ethanol solution). A gentle heating of the sections was necessary for staining with Coomassie Blue and Paragon.

Results

Anatomy

Histological staining of fresh and fixed stigmas of *T. pratense* and *T. repens* showed proteins on the surface of the pollen grains and in the cuticle layer of the stigma (Fig. 1). Longitudinal and transverse sections of stigmas of both species revealed long finger-like papillate cells surrounded by secreted material. This secreted material beneath the cuticle gave a negative reaction for the presence of proteins (Fig. 1), but a strong positive reaction for the presence of lipids (Fig. 2). The stigmatic secretion of both species produced a green autofluorescence in fresh material (Fig. 3). The styles of both species are solid

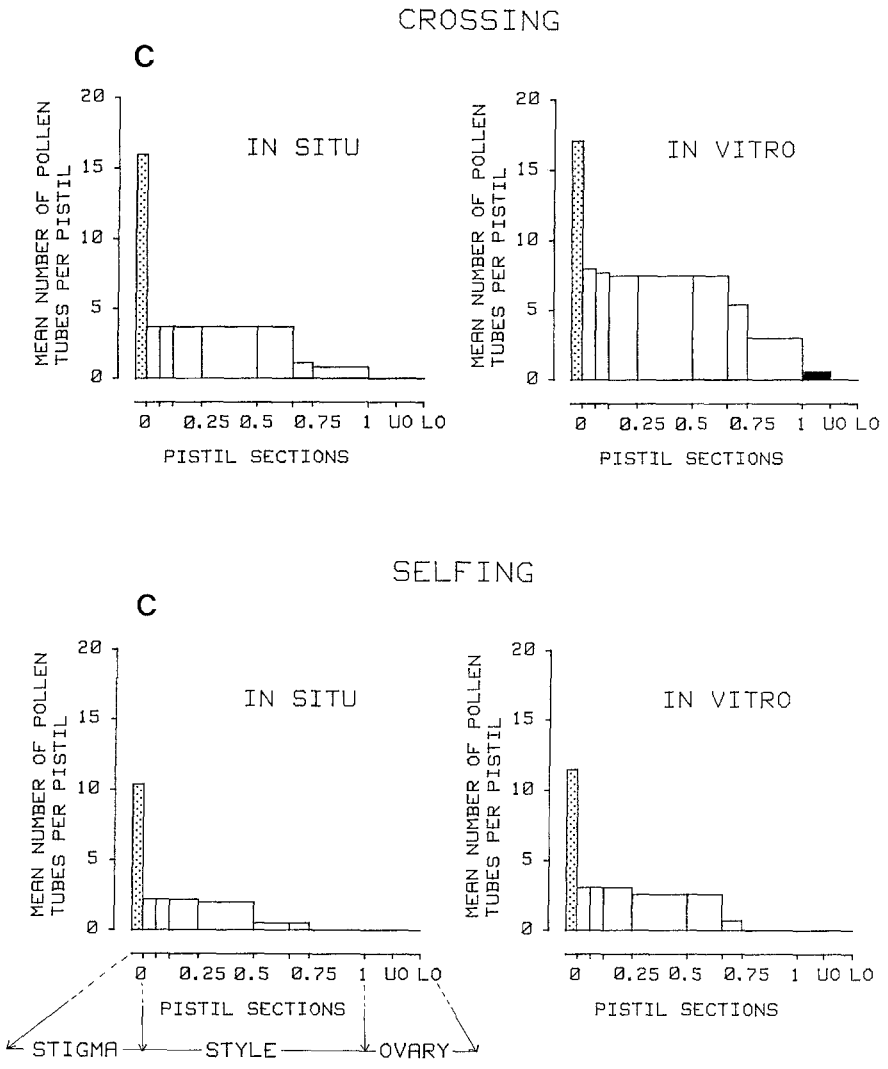

Fig. 8. Distribution pattern of pollen tubes in the stigma (*shaded column*), style (*white columns*) and ovary (*black column*) of *T. pratense*, genotype C, 5 h after pollination. Self- and cross-pollinations in situ and in vitro are marked

for a short distance beneath the stigma, and a hollow canal follows in which pollen tubes grow towards the ovary (Fig. 4). The fluorescence of the pollen tubes facilitated our quantification of their number and length, and our observation of fertilization (Fig. 5). Embryo development was compared after in vitro and in situ selfing: at 4 days after selfing, in vitro embryo development was normal (Fig. 6).

Pollen tube development

The total number of pollen grains adhering to the stigmas and observations on the development of pollen tubes in the pistils of *T. pratense* and *T. repens* were made 5 h after pollination.

In *T. pratense* between 23 and 29 pollen grains adhered to the stigmas after either self- or cross-pollination in situ. The germination and growth of the pollen tubes in the stigma, style and ovary is illustrated for two genotypes (A, B) in Fig. 7. In these genotypes the number of grains which germinated after selfing was almost double that obtained after crossing (Fig. 7). The growth of the pollen tubes of a third genotype (C) is illustrated in Fig. 8. Statistical analyses were made in order to compare pollen tube development after crossing with that after selfing. The cumulative lengths of the pollen tubes per pistil were computed from the number and the lengths of pollen tubes from all three genotypes of *T. pratense*. There was no statistical difference ($P<0.05$) in pollen tube length between self- and cross-pollinations for genotype B or C (Figs. 7, 8), whereas with genotype A, significantly fewer pollen tubes were present in the styles after self-pollination (Fig. 7).

The effects of in vitro conditions on the development of the pollen tubes in *T. pratense* was studied using genotype C (Fig. 8). Adherence of the pollen grains to the stigma in situ was similar after cross- and self-pollinations and was 23 ± 8 and 26 ± 15 grains per stigma, respectively. With in vitro culture this increased to 66 ± 21 and 73 ± 33 for cross- and self-pollinations, respectively.

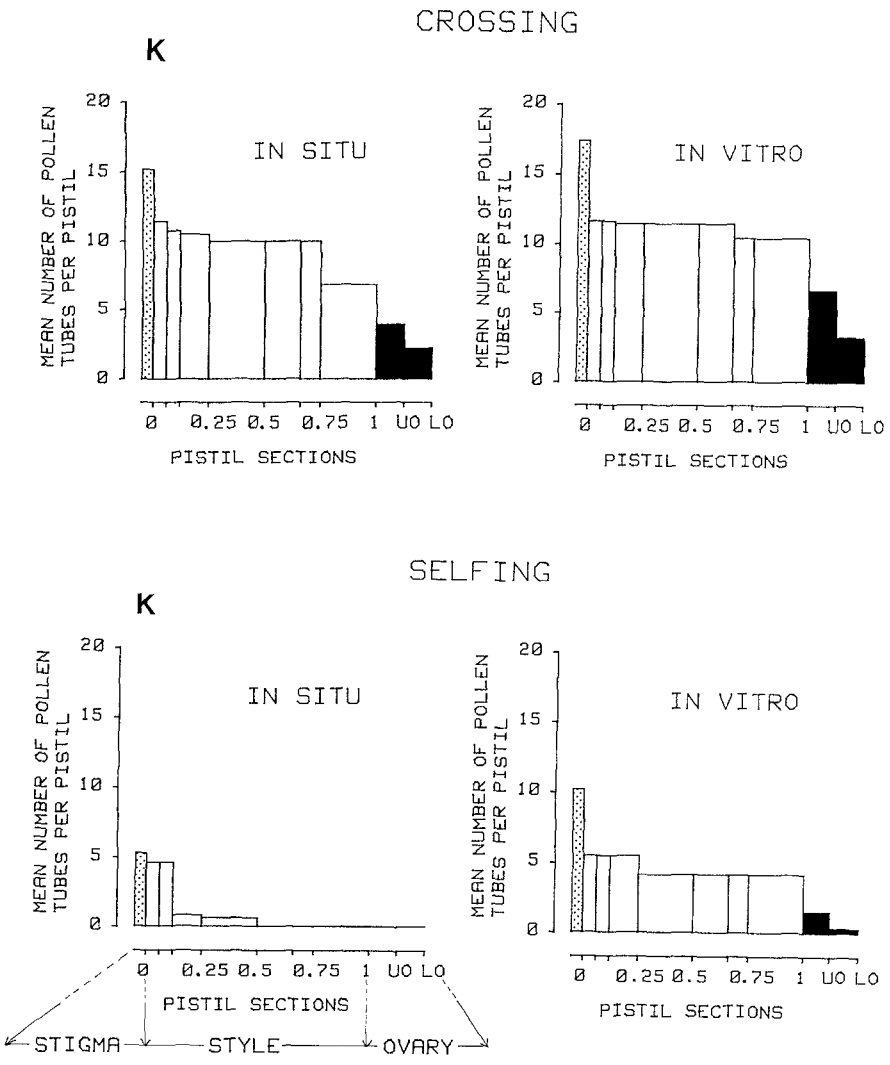

Fig. 9. Distribution pattern of pollen tubes in the stigma (*shaded column*), style (*white columns*) and ovary (*black columns*) of *T. repens*, genotype K, 5 h after pollination. Self- and cross-pollinations in situ and in vitro are marked

A significantly ($P \leq 0.01$) greater number and greater length of pollen tubes was observed in vitro after crossing (Fig. 8). However, with selfing, no significant difference was found between conditions in vitro and in situ.

Self- and cross-pollinations of *T. repens* were made in situ and in vitro, and the pattern of pollen tube development in genotype K is illustrated in Fig. 9. The number of pollen grains adhering to the stigmas in situ after cross- and self-pollination was 18 ± 8 and 10 ± 6, respectively. This increased slightly to 27 ± 15 and 18 ± 11 for in vitro culture. Self-pollination in situ resulted in a threefold reduction in pollen germination when compared to cross-pollination. Pollen germination after selfing was stimulated by in vitro conditions (Fig. 9). With both crossing and selfing almost all of the pollen grains which germinated on the stigma proceeded to penetrate the style (Figs. 4, 9). When the cumulative length of the pollen tubes in vitro and in situ were compared, no significant differences after cross-pollination were evident. With self-pollination a significantly ($P < 0.02$) greater cumulative length of pollen tubes was obtained by in vitro culture. Furthermore, no pollen tubes of *T. repens* passed the mid-point of the style 5 h after in situ selfing, whereas with in vitro culture a mean of 4 tubes was obtained at the mid-point and 1.3 penetrated into the ovary (Fig. 9).

Seed formation

Once the pollen tubes entered the ovary, they travelled along the placenta before reaching the micropyle (Fig. 5). The location of fertilized ovules (full seeds) in the ovary with reference to the base of the style was recorded after crossing and selfing *T. repens* in vitro. The percentage distribution of embryos found at each location in the ovary is illustrated in Fig. 10. After selfing, ovules closest to the base of the style (i.e. ovules 4 and 5) were most often fertilized, whereas after crossing, each ovule had an equal probability of being fertilized. This was confirmed statistically by comparing observed and expected patterns of distribution using the Chi-square test.

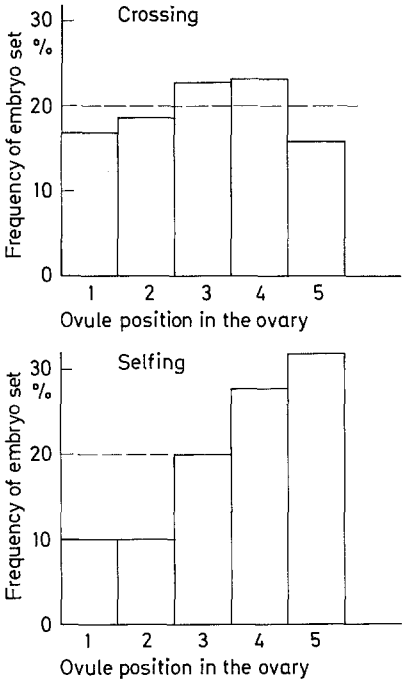

Fig. 10. Distribution of seed formation within the ovary after self- and cross-pollination of *T. repens* in vitro. Note ovule designated 5 is nearest to the base of the style and ovule 1 is nearest the peduncle. The *dotted line* shows the theoretical distribution where each ovule has equal probability to set seed

The yield of seeds obtained after cross-pollination in situ and in vitro are given for both species in Table 1. Cross-pollination with *T. pratense* in vitro resulted in 61 seeds per 100 florets; not more than 1 seed was found per ovary. The seed yield per 100 florets from cross-pollinations in situ was not tested but has been previously recorded to be 45–95 seeds per 100 florets (Williams 1931; Mackiewicz 1965). Cross-pollination of *T. repens* gave a similar seed yield in vitro and in situ. Although this species contains four–six ovules, not more than 3 seeds were obtained per cultured floret.

The capacity of *T. pratense* to produce seeds after self-pollination in vitro was tested using 183 florets from four genotypes. In these experiments only 1 seed was obtained; this seed failed to germinate. Seed set following selfing in situ was not tested.

The capacity for seed formation by in situ pollination has been reported for a large number of genotypes of *T. repens* (Douglas and Connolly 1989). In the present study selected genotypes (selfers) were classified according to their capacity for setting seed after selfing in situ as follows: very good, good, and very poor (Table 2). The effect of in vitro pollination on yield of seeds for each class is presented in Table 2. Variability existed among genotypes in each class, but in each individual genotype selfing in vitro gave consistently higher seed set than selfing in situ. The yield of seeds obtained with in vitro selfing was consistently higher by a factor varying from 1.7 to 31. One genotype which was a very good selfer in situ was also relatively very good in vitro. However, the in vitro method achieved the greatest improvement with very poor selfers. The ratio of seed set in vitro to in situ was highest in this class (Table 2). Usually only one seed per ovary was found after selfing in vitro (Fig. 6).

Table 1. Yield of full seeds by intraspecific cross-pollination of *T. pratense* and *T. repens* in situ and in vitro

Species	Full seed (yield 100 florets)	
	Method of pollination	
	In vitro	In situ
T. pratense	61 (64)	45–95 (200)[a]
T. repens	221 (109)	206 (930)

[a] Based on Williams' (1931) and Mackiewicz's (1965) observations; number in brackets is number of florets pollinated

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

Classification based on response in situ	Genotype	Seeds/100 Florets		Ratio In vitro
		In situ	In vitro	In situ
Very poor Selfers	A	0.31 (319)[a]	8.5 (35)	27.0
	B	0.30 (328)	3.1 (65)	10.0
	C	0.63 (315)	3.4 (29)	5.0
	D	0.34 (293)	10.8 (37)	31.0
	E	0.69 (722)	7.4 (54)	10.7
Good Selfers	F	1.58 (569)	28.6 (69)	18.0
	G	9.00 (332)	66.6 (18)	7.4
	H	2.00 (759)	0.0 (34)	–
	I	3.30 (695)	32.0 (25)	9.6
Very good Selfers	J	111.00 (191)	186.3 (22)	1.7

[a] Number of florets selfed are enclosed in brackets

Table 3. Effect of in vitro conditions on processes of pollination and seed set in *T. pratense* (genotype C) and *T. repens* (genotype K) relative to the response in situ

Processes	*T. pratense*		*T. repens*	
	Selfing	Crossing	Selfing	Crossing
Pollen adherance	I	I	I	I
Pollen germination	NI	NI	I	NI
Stylar penetration	NI	I	NI	NI
Tube growth in style and ovary	NI	I	I	NI
Seed set	NI	NI	I	NI

I, Improvement of the process; NI, no improvement of the process

A summary of all of the effects of in vitro conditions on the processes of pollination and seed set in *T. pratense* (genotype C) and *T. repens* (genotype K) is presented in Table 3.

Discussion

The growth of pollen tubes in the pistil is usually regulated so as to select out some tubes for fertilization and to reject the others. Two types of selection mechanisms may operate. The first is a general type which affects every pollen tube in the style and promotes (or permits) the growth of only the few that are selected for fertilization (Gawel and Robacker 1986; Malti and Shivanna 1985). This type can be found in both self-compatible and self-incompatible species. The second mechanism is more specific – to inhibit the growth of self-pollen SI or of pollen coming from another species (De Nettancourt 1972). We observed evidence of both a general selection mechanism and SI in *T. pratense* and *T. repens*, and we found differences between these two species. With *T. pratense* the zone of selection was most evident between the base of the stigma and the top of the style (Figs. 7, 8). Thereafter, there was a progressive selection along the length of the style towards the ovary. With *T. repens* a zone of selection was observed at the same location, but is was not as strong as in *T. pratense* since one-half to two-thirds of all pollen which germinated proceeded to grow into the style. A second zone of selection was evident between the base of the style and entry to the ovary in *T. repens* (Fig. 9).

Selection of pollen tubes by the SI mechanism was evident in each species. The SI system observed in *T. pratense* was characteristic of the gametophytic type where incompatible pollen grains germinate freely on the stigma and are progressively inhibited along the style (De Nettancourt 1972). On the contrary, with *T. repens* there was a strong inhibition of pollen germination on the stigma after selfing followed by further inhibition in the style. Atwood (1941) also observed stigmatic inhibition and *T. repens* may, therefore, be an exception in this respect among species with gametophytic SI (Knox et al. 1986).

The culture of florets in vitro increased the number of pollen grains adhering to the stigmas in cross- and self-pollinations of each species (Table 3). In addition, high humidity was shown to facilitate selfing and seed formation in *T. repens* (data not shown). Elevated humidity in vitro probably facilitated the binding of pollen to the stigma. The stigmatic cuticle in *T. pratense* and *T. repens* is broken upon pollination. The underlying material is very lipophilic, as has been shown previously for *T. pratense* (Heslop-Harrison and Heslop-Harrison 1982). The lipid nature of the subcuticle layer in both *T. pratense* and *T. repens* was shown by its strong positive reaction with Sudan Black III. Conditions of high humidity would enhance hydrophobic reactions and facilitate lipophilic interactions between stigmatic lipids and the lipophilic sporopollenin of the pollen exine. In *Brassica*, high humidity increases the adherance of pollen to stigmas. Palloix et al. (1985) and Ferrari et al. (1985) have proposed that the initial binding of pollen grains is consistent with van der Waals bonding forces between lipophilic molecules of the stigma and sporopollenin.

Seed set in vitro after crossing *T. pratense* gave only 61 seeds per 100 florets even though the cumulative growth of pollen tubes increased significantly under these conditions. However, *T. pratense* usually produces just one seed, and not all florets of this species set seed. Mackiecwicz (1965) obtained a yield of 45 seeds per 100 florets upon crossing 200 florets under experimental conditions. *T. pratense* is susceptible to embryo abortion (Povilaitis and Boyes 1960), and this may have been increased in vitro under conditions of high humidity (Ferdorschuck 1944). Seed set in vitro after crossing *T. repens* was identical to that observed in situ (about 2 seeds/floret), and in vitro conditions did not affect cumulative tube growth.

The SI reaction in *T. pratense* was not weakened in vitro and growth of incompatible pollen tubes was identical to that in situ. The level of seed set after selfing in vitro (0.5 seeds/100 florets) was similar to that reported by Williams (1931) for selfing this species in situ (0.1 seed/100 florets). Kendall and Taylor (1969) failed to obtain any self seed from among ten genotypes and 1,000 florets.

There is a considerable variation in self-compatibility among genotypes of *T. repens* (Douglas and Connolly 1989), but this is consistent with any population of outbreeders. It has been suggested that in some genotypes SI reactions have progressively disappeared during evolution, leading to self-compatibility. Such a model was proposed for *Gossypium hirsutum* (Gawel and Robacker 1986). It has been further suggested that the interaction between stylar tissues and pollen tubes in this species might be due to the residual action of genes once involved in SI. Qualitative or quantitative differences in gene expression among genotypes of *T. repens* may account for their different responses to selfing.

The SI response of *T. repens* was highly weakened in vitro. In vitro culture doubled the number of pollen grains germinating on the stigma, increased the cumulative growth of incompatible tubes and facilitated the entry of some tubes to the ovary. These events were coupled with a dramatic increase in seed set (Table 2) and were, therefore, interpreted to constitute part of the mechanism(s) of the SI system. Genotypes which were highly self-incompatible in situ were affected by in vitro conditions much more than genotypes that were relatively easy

to self-fertilize (Table 2). This suggests that those mechanisms of the SI response which were affected in vitro were probably important ones (Table 3). Other features of in vitro culture (medium, light, gaseous environment) might also have interfered with the SI response. Treatment with CO_2 in vitro weakens the gametophytic SI in some genotypes of *Lycopersicon peruvianum* (Williams and Webbs 1987) and *T. repens* (Douglas and Connolly 1987) and sporophytic SI in *Brassica oleracea* (Nakanishi and Hinata 1973; Palloix et al. 1985).

Ovules nearest the base of the style had a greater probability of producing seed after selfing than after crossing (Fig. 10). It is unlikely that differences in nutrient supply after selfing could account for this distribution since after crossing each ovule had an equal probability of producing seed. The propensity for seed formation by proximal ovules may indicate that SI reactions and/or selection pressures are experienced by tubes within the ovary and that this subsequently results in the fertilization of ovules nearest to the base of the style. In *Vicia faba*, where seed set after open-field pollination is low (2.86 seeds per pod), a greater frequency of fertilization and seed set has also been found for the ovule nearest to the style (Rowland and Bond 1983).

In conclusion, *T. pratense* and *T. repens* show differences in their regulation of the growth of compatible and incompatible (self) tubes. In addition, both the general selection response and the SI response are affected differently by in vitro culture. In vitro pollination is not suitable for *T. pratense*, whereas it provides a good system for studying the processes of cross- and self-fertilization in *T. repens* and is a good tool by which to produce inbred lines of *T. repens*.

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