6.5.2 Stigmatic Pollen Loads and Pollen Tube Growth

6.5.2.1 T. australe

Since *T. australe* flowers are very small, the total number of pollen grains on stigmas was counted to ensure that the hand-crossing technique was adequate for pollen deposition levels and to see whether pollen loads varied between treatments. In addition, the number of pollen grains that had germinated at the stigmatic surface and the number of pollen tubes that had grown to the base of the styles were assessed (Figure 6.24a). As might be expected, stigmas subjected to the outcross pollen treatment had significantly more pollen grains than the autogamous treatment ($F_{1,70} = 10.97$, P=0.0.002 sqrt transformed). The number of pollen grains that germinated on stigmas ($F_{1,70} = 3.78$, P=0.056) and the number of pollen tubes reaching the bases of styles ($F_{1,56} = 0.68$, P=0.412) was similar for self and outcross pollen. This indicates that the supplementing stigmas with outcross pollen had little influence on the level of pollen germination and pollen tube growth i.e. that outcross pollen tube growth is similar to self-pollen tube growth. Fluorescence microscope images of pollen on stigmas and pollen tubes in styles of *T. australe* are presented in Figures 6.26-6.28.

The above results suggest that pollen quality will not be influenced by density but stigmatic pollen loads (pollen quantity) may be. Thus, the same variables were assessed for stigmas that were left open to natural pollination and collected from sparse and dense plots (Figure 6.24b). Stigmatic pollen loads did not differ between sparse and dense plots ($F_{1,73} = 0.20$, P=0.656) and were similar to pollen loads on flowers that were bagged for autogamy ($F_{2,82} = 1.09$, P=0.342 log transformed), suggesting that there is little or no transfer of pollen among *T. australe* flowers left open to natural pollination. The number of pollen grains germinating on stigmas was also similar between sparse and dense plots ($F_{1,73} = 2.30$, P=0.134) but more pollen tubes were found in the bases of styles in flowers from sparse plots compared with dense ($F_{1,57} = 5.82$, P=0.019).

In addition to assessing actual counts of pollen grains, relevant proportions were calculated as follow;

GS:TP	(pollen Germinated on Stigma: Total Pollen on stigma) Proportion of total stigmatic pollen load (germinated + ungerminated grains) that germinated on the stigma *indicative of pollen viability/germinability
PTB:TP	(Pollen Tubes at Base of style: Total Pollen on stigma) Proportion of total stigmatic pollen load that produced pollen tubes which continued growing to the base of the style *indicative of overall pollen vigour
PTB:GS	(Pollen Tubes at Base of style: pollen Germinated on Stigma) Proportion of pollen germinated at the stigma which continued growing to the base of the style *indicative of vigour of germinated pollen

There was no difference for GS: TP between the outcross and autogamy treatments ($F_{1,62} = 0.11$, $P=0.742$) or between densities ($F_{1,68} = 3.78$, $P=0.056$) which further suggests that self pollen is as likely to germinate on the stigma as is outcross pollen. PTB: TP was significantly lower for outcross than self pollen ($F_{1,54} = 7.90$, $P=0.007$) and for flowers in dense plots compared with sparse ($F_{1,57} = 11.21$, $P=0.001$) indicating that pure self-pollen loads have an even greater potential to grow pollen tubes to the ovary than outcross pollen. PTB: GS was also lower for outcross pollen than for self pollen ($F_{1,56} = 5.83$, $P=0.002$) and although this was also lower in dense compared with sparse plots it was not significant ($F_{1,64} = 2.14$, $P=0.148$). Therefore, once self-pollen has germinated on the stigma it is more likely to continue growing to the ovary than is outcross pollen.

In summary, there is no evidence that density influences the amount of pollen deposited on stigmas and even if more outcross pollen was to be deposited on stigmas, there is no evidence that this would facilitate more or better quality seed; in fact outcross pollen (counter intuitively) appears to provide less opportunities for fertilisation as it produces a lower proportion of pollen tubes than does self-pollen.

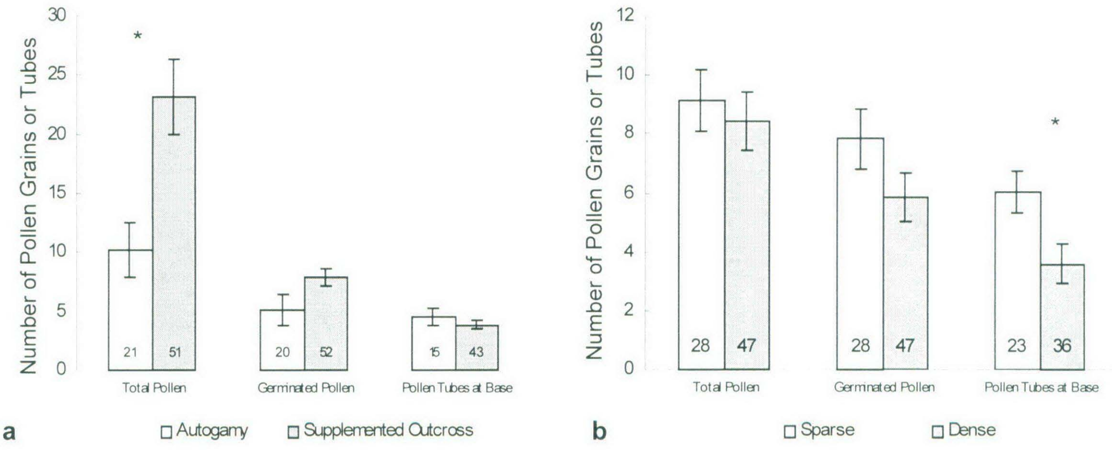

Figure 6.24 Mean number (±SE) of; total pollen grains on stigmas, pollen grains that had germinated on stigmas and pollen tubes observed at the base of pistils of *T. australe* from **a)** autogamous and supplemented outcross treatments and **b)** sparse and dense open flowers. N (in base of column)=number of stigmas. * Above columns indicate significant differences between treatments/densities (P<0.05).

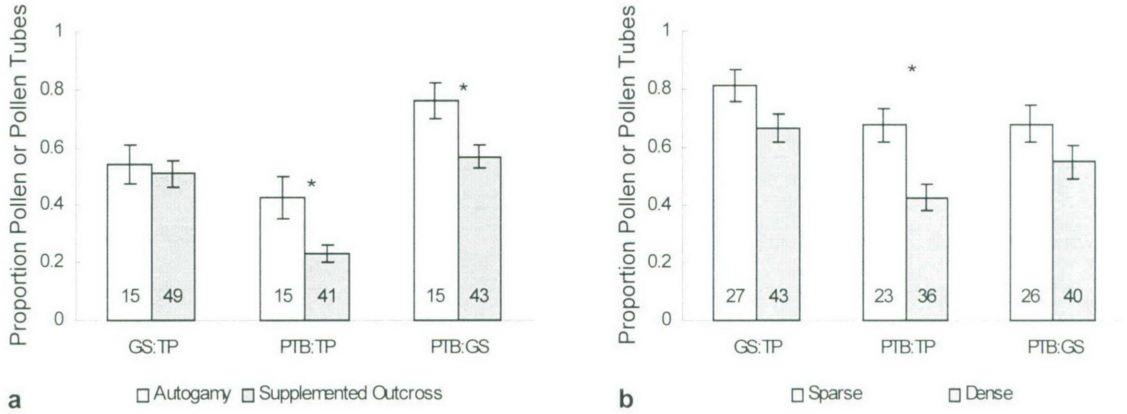

Figure 6.25 Mean proportions (±SE) calculated for pollen germination and pollen tube growth for *T. australe* **a)** autogamous and supplemented outcross treatments and **b)** sparse and dense open flowers. N (in base of columns)=number of pistils. * Above columns denote significant differences between treatments/densities (P<0.05).

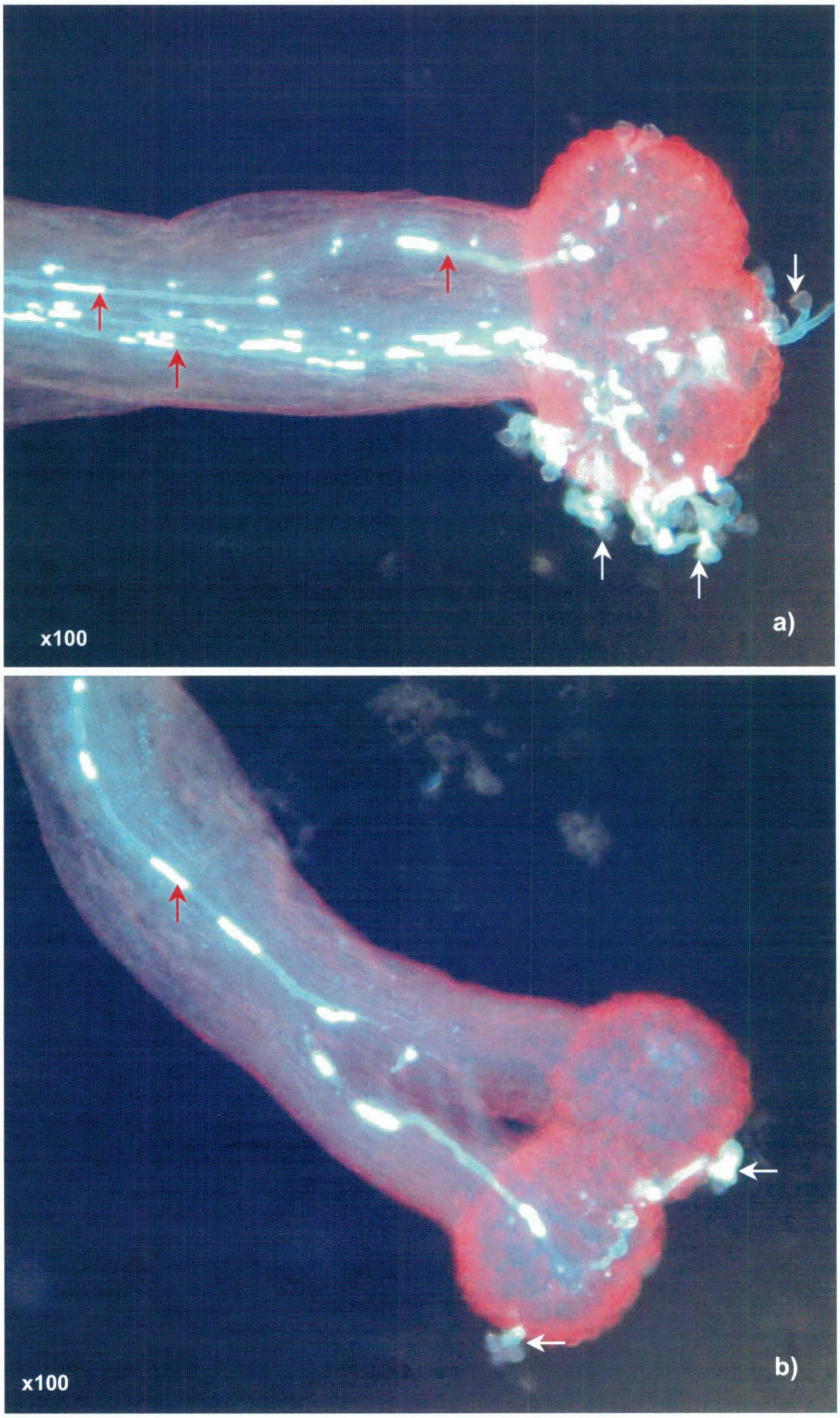

Figure 6.26 Pollen tube growth in *Thesium australe* arising from automatic self-pollination (bagged buds collected after floral opening). Passive pollen deposition can result in a) many pollen grains to b) few. (White arrows indicate germinating pollen; red arrows indicate pollen tube growth down the style).

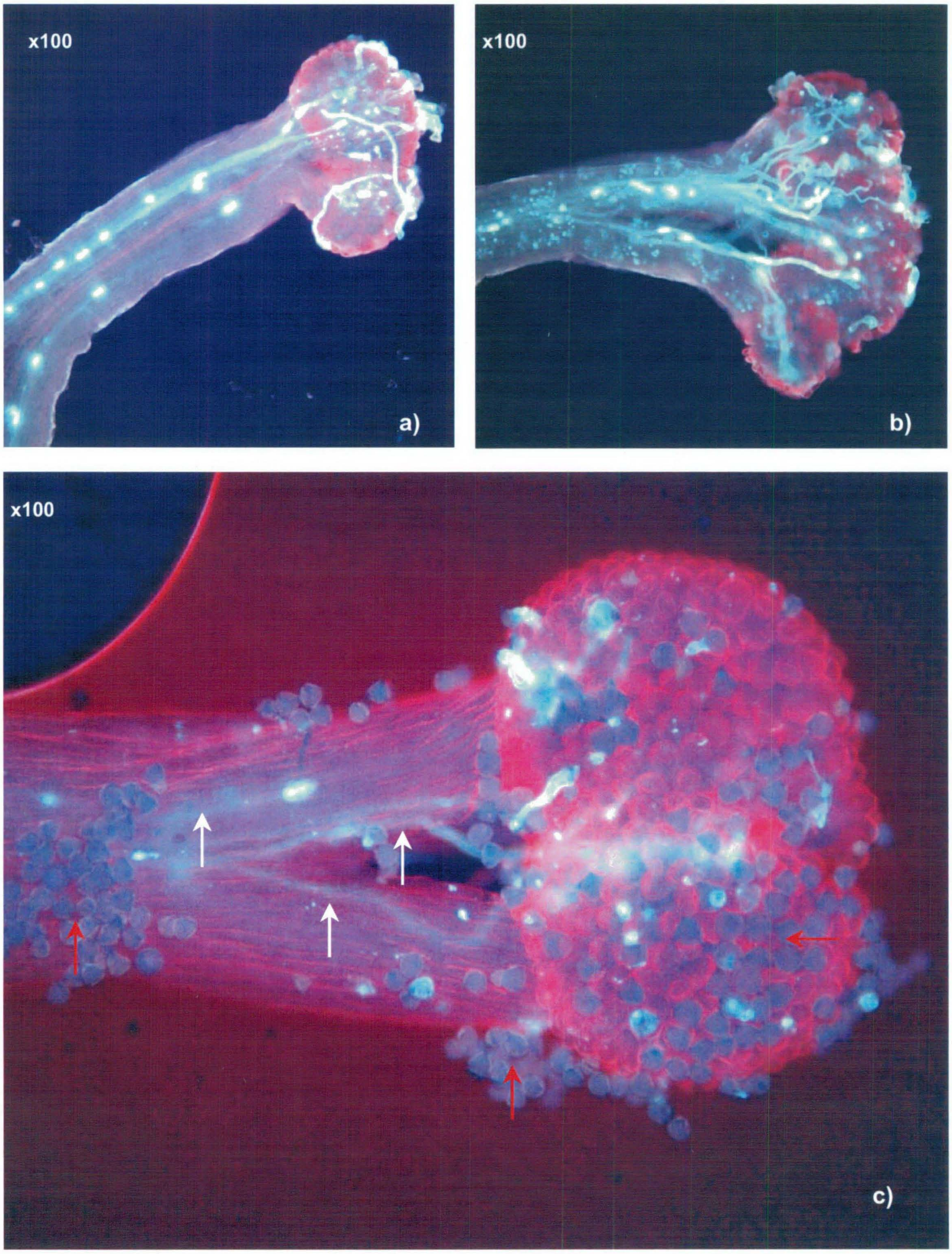

Figure 6.27 Outcross (hand supplemented) pollen germination and pollen tube growth in *Thesium australe* can range from a) & b) a relatively high proportion of germinations to c) relatively few germinations (clumps of ungerminated pollen indicated by red arrows, pollen tubes indicated by white arrows).

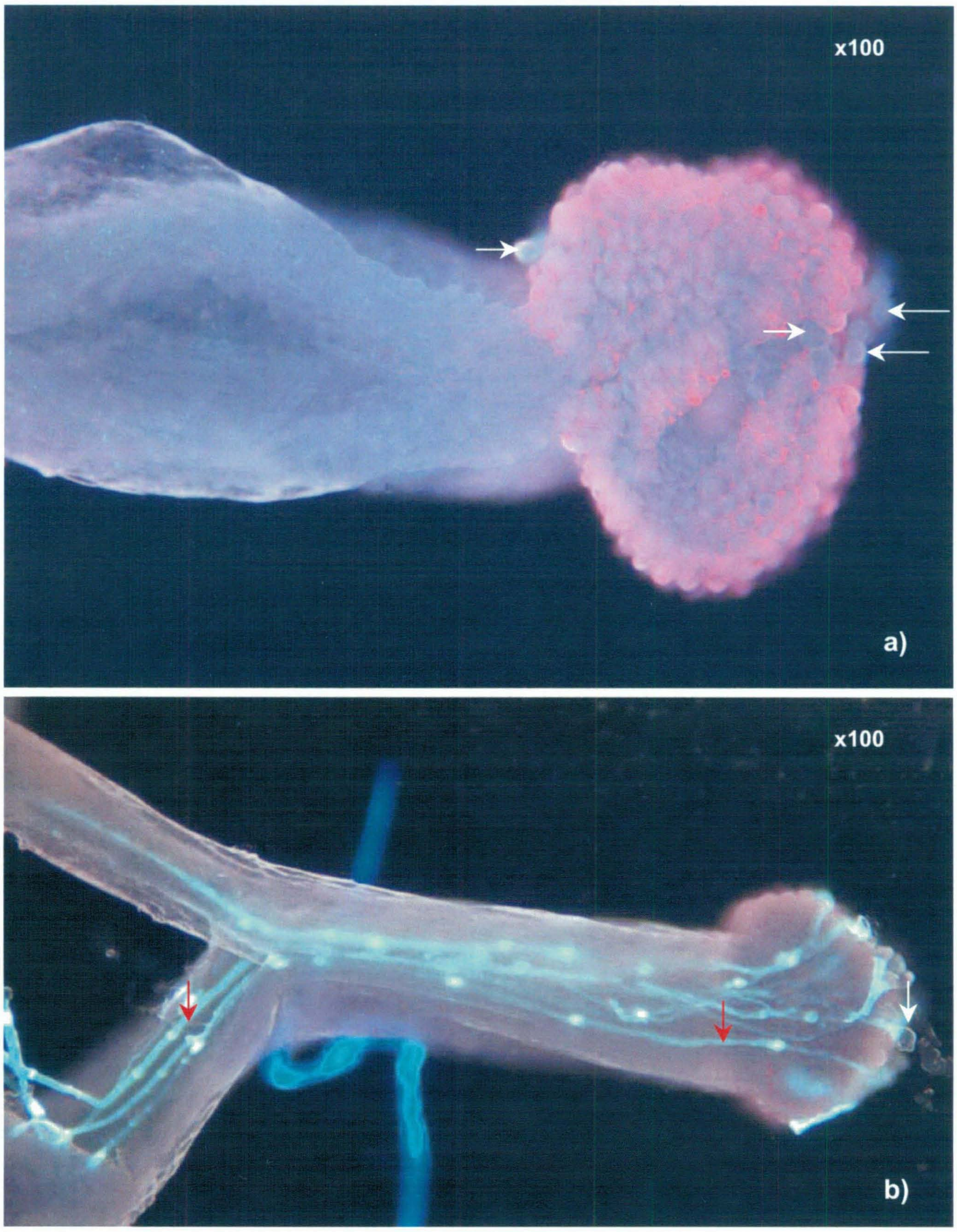

Figure 6.28 **a)** Ungerminated pollen grains (e.g. white arrows) on a stigma removed from a *Thesium australe* bud b) pollen germinating on stigma (e.g. white arrow) and **b)** pollen tubes (e.g. red arrows) growing down the style (flower left to open pollination).

6.5.2.2 W. luteola

Quantifying pollen germination on the stigma and pollen tube growth down the style proved extremely difficult to quantify for *W. luteola*. For both selfed and outcrossed treatments and for flowers left to open pollination (collected from sparse and dense plots), pollen loads, germination levels and pollen tube growth were profuse (Figure 6.29a) and scoring was altogether obscured. In addition, large indefinite numbers of pollen tubes tended to grow in thick cord-like arrangements down the centre of the style (Figure 6.29b & c) and individual pollen tubes were rarely distinguishable. Furthermore, *W. luteola* possesses fibres (possibly vascular bundles) that are present at the stigmatic surface and throughout the style and which fluoresce in a similar manner to pollen tubes, thus further confounding observations (Figure 6.29d). However, what can be established is that both outcross and self-pollen readily germinate in large proportions and can produce up to an estimated several hundred pollen tubes within styles. Thus, both self and outcross pollen are potentially capable of facilitating seed production, which is in line with the breeding system of *W. luteola* as addressed in Chapter 3. If there were differences in pollen loads and/or pollen tube growth between densities, they were indiscernible using the methods employed here. Investigation of this aspect for this species warrants further development of suitable protocols that will produce useful and quantifiable data.

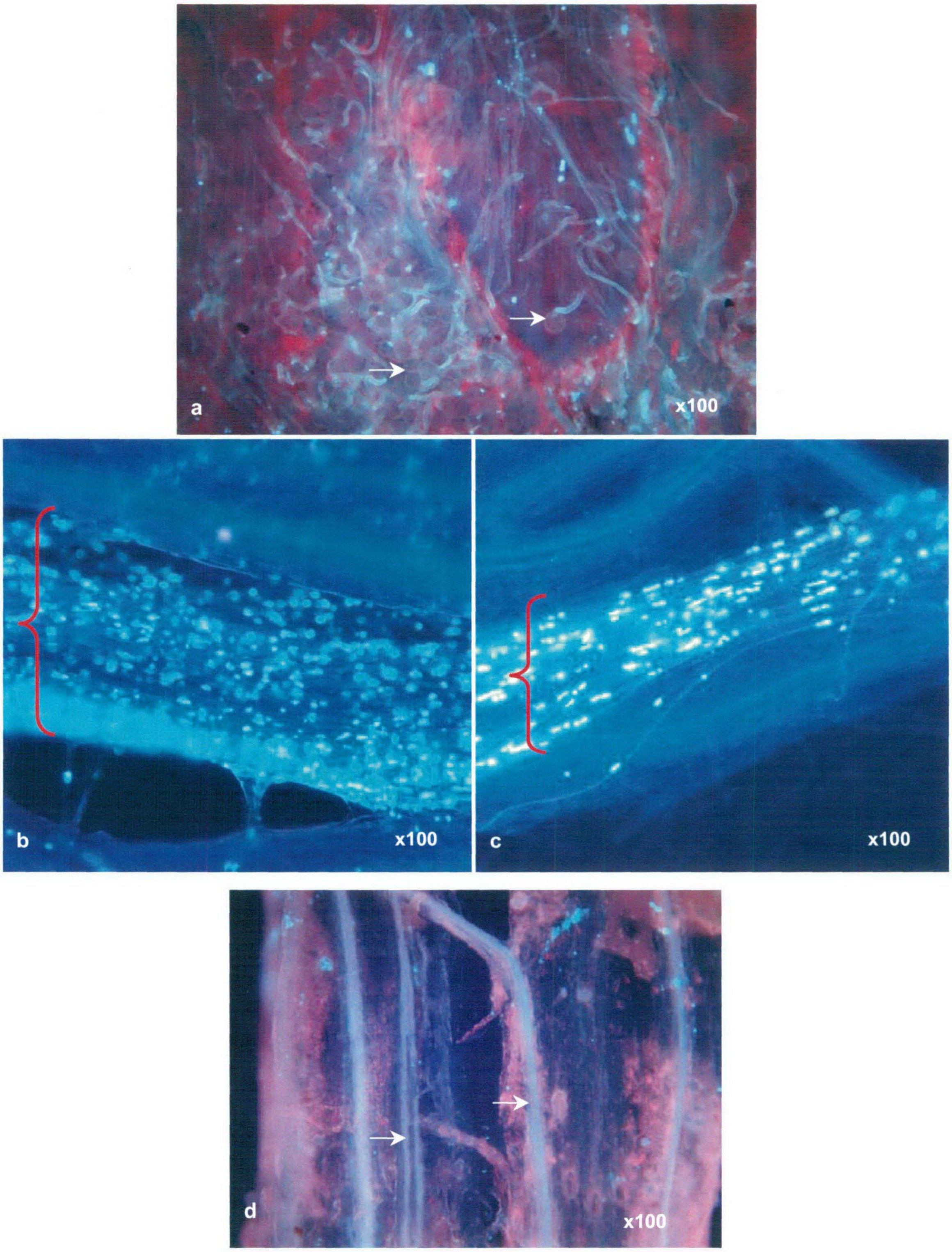

Figure 6.29 Pollen germination and pollen tube growth in *W. luteola* **a)** pollen germinating on a stigma (white arrows); the outline of a lobe can been seen fluorescing in red **b)** & **c)** pollen tubes grow in thick cords down the centre of the style (bracketed in red) **d)** fibres present in styles (white arrows).

6.5.2.3 D. sieberi

The number of pollen grains on stigmas whose flowers were bagged to exclude outcross pollen was counted to determine; 1) whether self-pollen is passively deposited on stigmas and if so, how many grains 2) if self-pollen germinates on the stigma and 3) if subsequent pollen tube growth could be detected. For comparison, stigmas that were supplemented with outcross pollen were assessed for; 1) the number of pollen grains on stigmas 2) germination rates and 3) the presence of pollen tubes. Refer to Figures 6.32 (self pollen) & 6.32 (cross pollen) for fluorescence microscope images of pollen and pollen tube behaviour in *D. sieberi*.

Self-pollen was found to adhere automatically to stigmas in small numbers. The amount of self-pollen on stigmas varied significantly among sites with the highest numbers observed at OAR ($F_{2,58}$ =3.81, P=0.028, log transformed). Supplementing stigmas with outcross pollen demonstrably increased stigmatic pollen loads compared with the self-treatment (~ 6-fold) and outcross pollen loads were similar among sites ($F_{2,55}$ =0.45, P=0.640) (Figure 6.30a).

Self-pollen was found to germinate on stigmas, but as expected, in very low proportions relative to outcross pollen. Proportions were similar among sites (self $F_{2,58}$ =0.23, P=0.794; outcross $F_{2,57}$ =2.87, P=0.065) therefore sites were pooled. A much higher proportion of outcross pollen grains germinated on stigmas than self-pollen ($F_{1,117}$ =235.30, P=0.000) (Figure 6.30b). In addition, pollen germination was encountered on 100% (N=58 stigmas) of the time on stigmas where outcross pollen had been applied, but germination only occurred on ~39% of stigmas with pure self-pollen loads (χ^2 = 49.23, P= 0.000 with Yates' correction).

Pollen tube growth was encountered significantly more often (χ^2 = 33.61, P= 0.000 with Yates' correction) in styles of flowers that had been supplemented with outcross pollen (30/58 styles ~52%), but was only encountered in 2 out of 60 (~3%) samples for the self-pollinated treatments. This strongly indicates that pollen-tube growth from self-pollen seldom occurs. The number of pollen tubes arising from outcross pollen ranged from 1 up to ~100, (mean = 6.9 ± 3.15) however counts were obscured by the style tissue and may be under-represented.

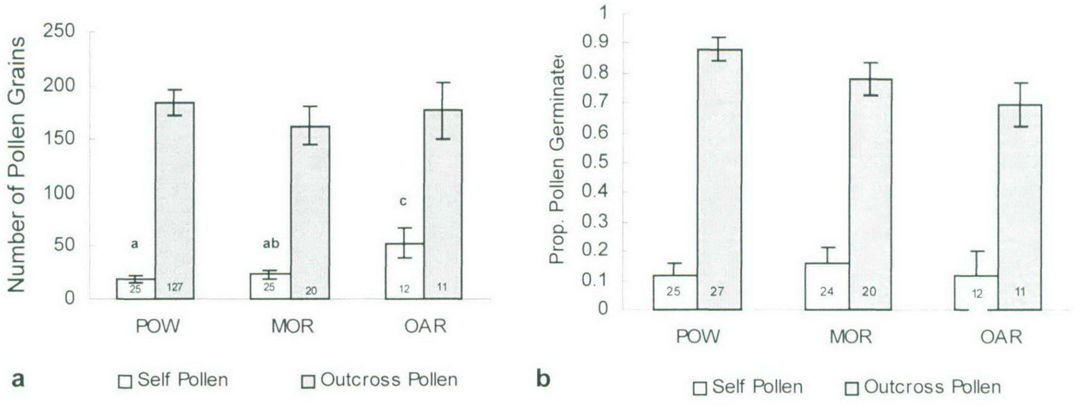

Figure 6.30 Self and outcross pollen on *D. sieberi* stigmas **a)** Mean number of pollen grains per stigma and **b)** proportion of self and outcross pollen germinating on stigmas N (inside base of columns)=number of stigmas. Letters above columns=significant difference among sites (P<0.05).

The above results allow the following assumptions to be made when addressing open pollen deposition rate on stigmas from sparse and dense plots. Firstly, if open pollen counts exceed those observed for the self-pollen treatment (i.e. on average are greater than ~50 grains/stigma), then the flower has likely received a visitor carrying pollen. Secondly, if the percentage of pollen germinating on the stigma is greater than ~15%, then a large proportion of that pollen is likely to be outcross pollen. In addition, if pollen tubes are often encountered in styles, this also indicates high outcross pollen loads as pollen tubes arising from self-pollen were very rarely encountered.

Total pollen counts from stigmas collected from sparse and dense plots (open) were intermediate between those recorded for autogamous and those for hand supplemented outcross pollen loads (summarised in Table 6.4). Flowers left to open pollination can receive up to 6 times more pollen (at POW) than they would in the absence of pollinators. Total pollen loads were slightly lower on stigmas from sparse plots than dense at two sites but there were no significant differences between densities (POW $F_{1,120}=0.07$, P=0.797; MOR $F_{1,105}=0.28$, P=0.600; OAR $F_{1,109}=0.51$, P=0.475) (Figure 6.31a). This suggests that quantity of pollen deposited on stigmas by pollinators is not influenced by density in this system.

In general, the proportion of pollen germinating on open stigmas (i.e. those collected from sparse and dense plots) (Figure 6.31b) was slightly lower than for stigmas that were treated with outcross pollen (Figure 6.30b). When the proportion of the total pollen load that was germinating on stigmas was assessed on a site by site basis for density effects, germination was always lower in sparse compared with dense plots, but this was not significant for any site (POW $F_{1,119}$ =1.87, P=0.174; MOR $F_{1,101}$ =3.16, P=0.079; OAR $F_{1,108}$ =0.13, P=0.717). However, when sites were pooled a significantly higher level of germination was revealed on stigmas from dense plots compared with sparse ($F_{1,332}$=3.93, P=0.048) (Figure 6.31b). Based on the fact that pure self-pollen loads germinate at a level of only ~15% (Figure 6.30b), this strongly suggests that 1) outcross pollen comprises the majority of the pollen load on open stigmas and 2) that stigmatic pollen loads in dense plots may contain relatively more outcross pollen than those in sparse plots.

To gauge whether the incidence of pollen germination on stigmas differed with density, the number of stigmas that presented germinating pollen was assessed for sparse and dense plots. Between 92% and 100% of stigmas from sparse and dense plots presented germinating pollen at POW & OAR and there was no significant difference between densities for these sites (POW χ^2 = 0.00, P= 0.977; OAR χ^2 = 1.92, P= 0.166 both with Yates' correction). However, only ~28% of stigmas from MOR sparse plots presented germinating pollen which was significantly less than the ~90% stigmas from dense plots (χ^2 = 41.00, P= 0.000 with Yates' correction) (Figure 6.31). This suggests that at this site, the vast majority of the pollen load on stigmas from sparse plots was self-pollen. The category "saturated" (as outlined in the methods section; stigmas with germinating pollen too numerous to quantify) was also assessed to determine the frequency of saturated stigmas between densities. Dense plots always had a higher percentage of saturated stigmas compared to sparse (Figure 6.31d) but this was never significant (POW χ^2 = 0.08, P= 0.781; MOR χ^2 = 0.07, P= 0.787; OAR χ^2 = 1.76, P= 0.185; all with Yates' correction). When sites were pooled, ~90% of stigmas from dense plots were saturated with germinating pollen compared with ~78% of those from sparse, again however, this was not significant (χ^2 = 2.21, P= 0.137 with Yates' correction).

Pollen tube growth to the base of the style was also analysed. The number of times that pollen tubes were identified growing down the style did not differ between densities for any site (POW χ^2 = 0.01, P= 0.906; MOR χ^2 = 0.490, P= 0.483; OAR χ^2 = 0.01, P= 0.906; all with Yates' correction). When data were pooled, around 50% of styles in both sparse and dense plots had pollen tubes. In addition, the number of pollen tubes counted in styles (which ranged from 1-14) was similar between densities for all sites (POW $F_{1,49}$ =0.85, P=0.361; MOR $F_{1,59}$=0.33, P=0.566; OAR $F_{1,60}$ =0.00, P=0.953). However, the difficulty encountered with scoring pollen tube growth (i.e. the obscuring nature of the style tissue) should be taken into account when interpreting these results as not all styles with pollen tubes would have been identified and not all pollen tubes could be counted.

Table 6.4 Summary of mean total pollen loads recorded at three sites on stigmas bagged for autogamy, stigmas that were supplemented by hand with outcross pollen and left open to natural pollination (means of those collected from sparse and dense plots). Autogamy and outcross supplemented material was collected from 10 individuals per site. Open material was from 20-40 individuals per site.

Site	Mean Number of Pollen Grains on Stigma (N)=number of stigmas		
	AUTOGAMOUS	OPEN	(Hand) OUTCROSS
POW	18.72 (25)	107.3 (102)	183.5 (27)
MOR	22.88 (25)	102.5 (106)	162.9 (20)
OAR	53.0 (12)	120.0 (111)	177.1 (11)

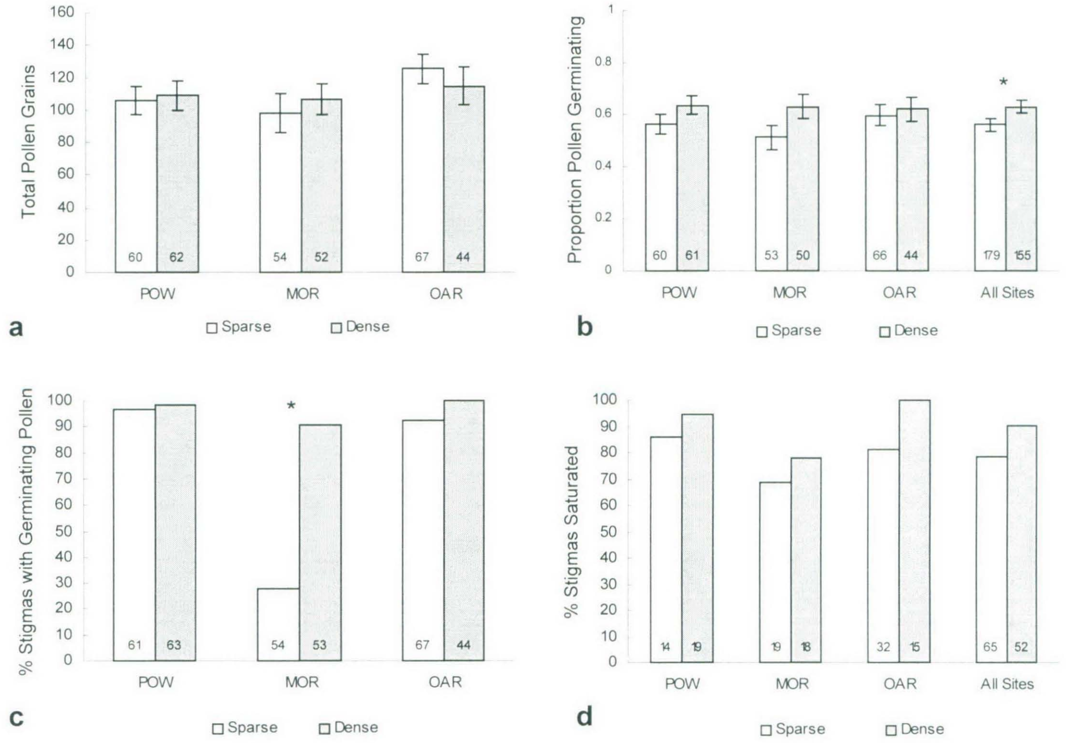

Figure 6.31 Pollen loads on *D. sieberi* **a)** mean total number (±SE) of pollen grains counted on stigmas (germinated + ungerminated) **b)** mean proportion (±SE) of the total pollen grains that had germinated **c)** % of stigmas assessed that bore germinating pollen and **d)** % of stigmas assessed that were saturated (at least 200 grains) with germinated pollen. N (in base of columns)=number of stigmas. *Above columns indicates significant difference between densities (P<0.05).

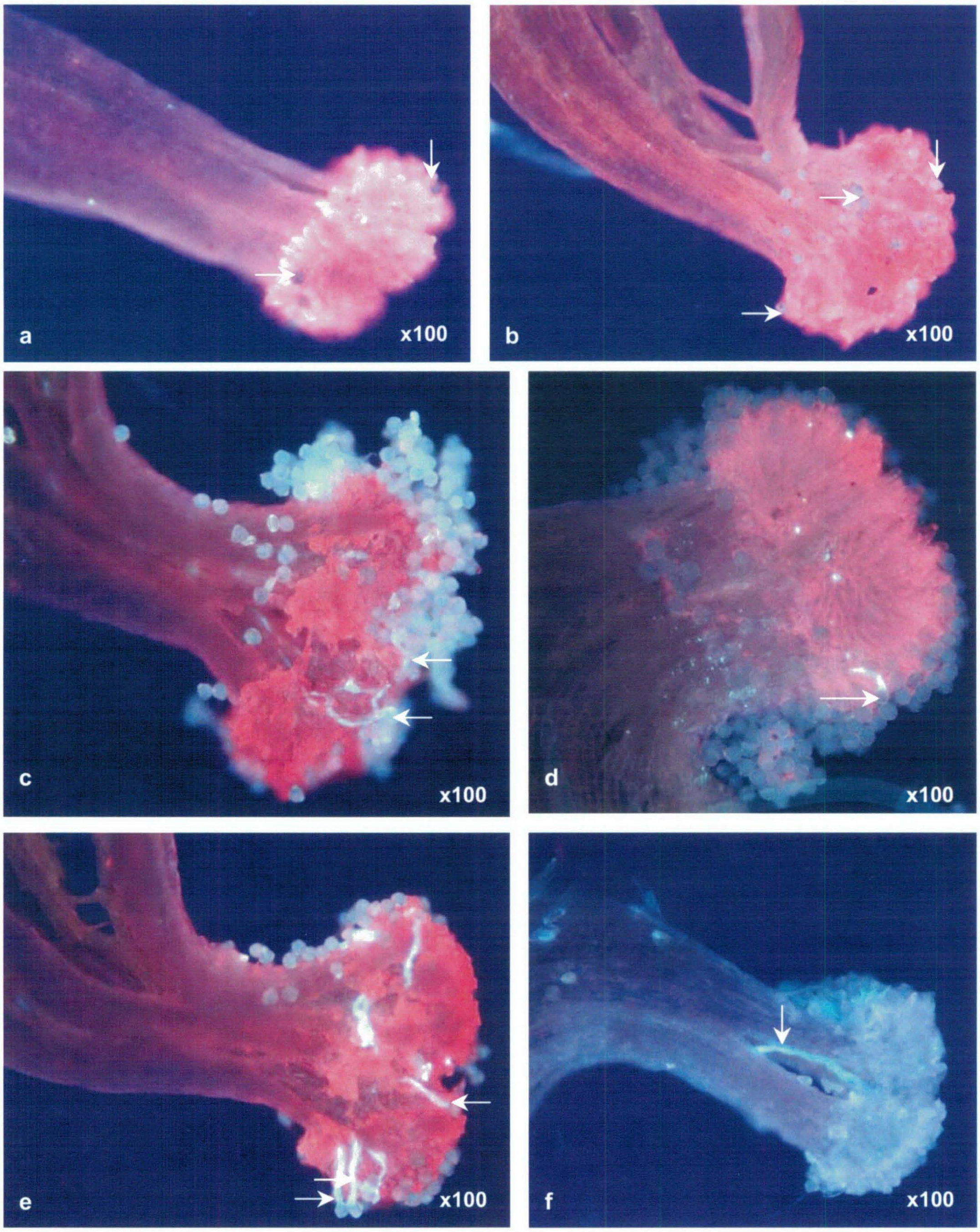

Figure 6.32 Self-pollen on *D. sieberi* stigmas **a)** & **b)** Pollen grains ranged from very few with no evidence of germination (arrows indicate ungerminated pollen grains) to **c), d)** & **e)** many pollen grains with low levels of germination (arrows indicate germinating pollen grains) and **e)** pollen tubes arising from self-pollen were rarely observed growing down the style (arrow indicates pollen tube).

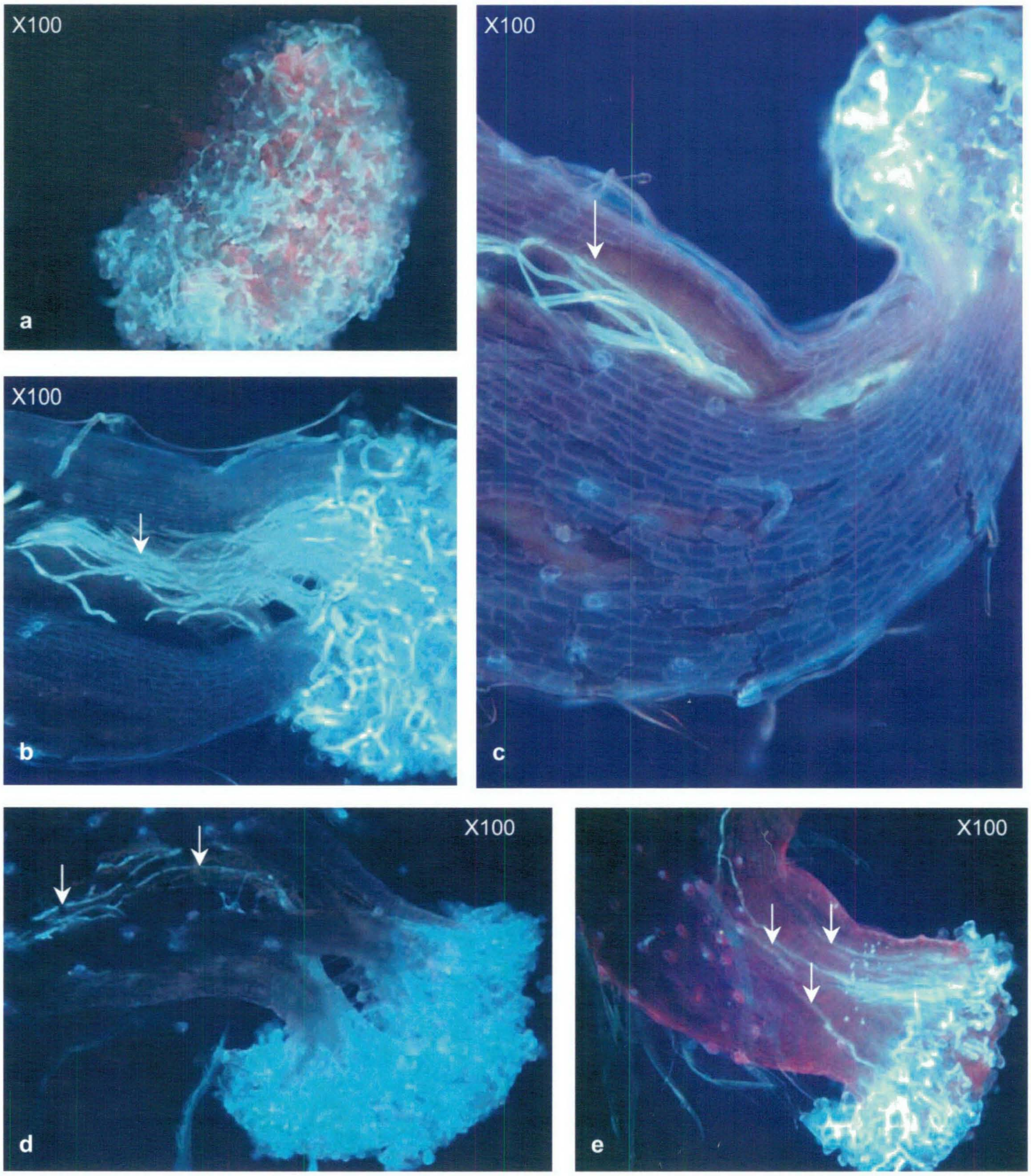

Figure 6.33 Outcross pollen on *D. sieberi* stigmas **a)** stigmas were often saturated with germinating pollen grains **b), c), d)** pollen tubes were often only visible if the style integument split during preparation **& e)** a rare example where pollen tubes were visible through a (translucent) style integument. (Arrows point to pollen tubes).

6.5.3 Results Summary

6.5.3.1 T. australe

Table 6.5 summarises pertinent fitness results for *T. australe*. Some maternal fitness differences were detected for seed viability. Seed arising from flowers left to open pollination displayed significantly higher levels of viability than autogamous seed. A density effect was also revealed where open seed from dense plots displayed greater levels of viability than those from sparse plots. Autogamous seed production however, was similar between densities. Levels of pollen germination were similar for self and outcross pollen but notably, self-pollen was more likely to grow pollen tubes to the base of the style. Furthermore, the proportion of total pollen on the stigma that produced pollen tubes which grew to the base of the style was significantly higher in material from sparse plots compared with dense plots.

Table 6.5 Summary of fitness responses for *T. australe*. S = Sparse, D= Dense. * = difference was significant (P<0.05).

Seed/Fruit Variables			**Pollen Variables**			
Fruit Weight	**Seed Viability**	**Seed Germ.**	**Pollen (Type)**		**Pollen (Density)**	
			Pollen Germ.	PT Growth	Pollen Germ.	PT Growth
S = D	Autog. < Open * Open Seed S < D * Autog. Seed S = D	None	Self = outcross	Self > outcross *	S = D	S > D *

6.4.3.2 W. luteola

Table 6.6 summarises pertinent fitness results for *W. luteola*. Very few density differences were manifest; variation occurred at the site level for offspring growth and survivorship with no clear trend for density. The UNE site was superior in terms of overall % germination and germination rate. Both self and cross pollen germinated at high levels, but unfortunately, pollen germination could not be quantified for density.

Table 6.6 Summary of fitness responses for *W. luteola*. S = Sparse, D = Dense; ↑ = Tended to be higher; P=POW, O=OAR; U=UNE, * = Significant; † = Significant for at least one measurement dav

	Seed Variables				**Offspring Variables**		**Pollen Variables**	
	Weight	**Viability**	**% Germ.**	**Germ. Rate**	**Growth**	**Surviv.**	**Type**	**Density**
Density Level	S = D	S = D	S = D	S = D	↑ D (P) ↑ S †(O&U)	↑ D (P) † ↑ S (O) † ↑ D (U) *	High levels of germ. & PT growth observed for both self & cross pollen	Not quantifiable
Site Level	Sites =	Sites =	UNE > POW & OAR *	UNE > POW > OAR *	(See above)	(See above)	NA	NA

6.5.3.3 D. sieberi

Tables 6.7 and 6.8 summarise pertinent maternal and paternal fitness results respectively for *D. sieberi*. Density effects for the seed and offspring data were variable but often significant; sites were also somewhat variable. Overall few effects were found. There were notable differences in the responses of GE1 compared with GE2. Also noteworthy were the differences between seed from 1- and 2- seeded fruits.

Pollen analyses were revealing with obvious tendencies for outcross pollen to be "fitter" both in terms of germinability and the production of pollen tubes. The trend was for dense plots to receive higher pollen loads and for there to be higher levels of pollen germination.

Table 6.7 Summary of maternal fitness responses for *D. sieberi*. ✓= Yes, X = No; ↑ = tended to be higher; S=Sparse, D=Dense; P=POW, M=MOR, O=OAR; 1-S=seed from 1-seeded fruits, 2-S=seed from 2-seeded fruits; * = significant; -no data

		Factor			
		Density	Site	GE1 vs GE2	1-seeded vs 2-seeded
Variable	Seed Weight	↑ S (P,M) * ↑ D (0) *	✓ S * ✓ D *	-	↑ 1-S *
	Seed Viability	X	X	↑ GE1	-
	% Germ.	X	X	↑ GE1	X
	Germ. Rate	X	-	X	↑ 2-S
	Offspring Growth	↑ S (P) * X (M,O)	✓	-	↑ 1-S (P) * X (M,O)
	Offspring Surviv.	X	X	↑ GE1	X
	Seed Weight vs Growth	Positive Relationship			
	Seed Weight vs Cotyledon Size	Positive Relationship			

Table 6.8 Summary of paternal fitness responses (pollen germination and growth) for *D. sieberi*. X = No; ↑ = tended to be higher; D= Dense; P=POW, M=MOR, O=OAR; * = significant, NA = not applicable; -no data

Variable	**Pollen Type**	**Density Effect**
Proportion of Pollen Load Germinating on Stigma	Cross > Self *	↑ D *
# Pollen Grains on Stigma	Open > Autogamy *	↑ D
% Stigmas with Germinating Pollen	Cross > Self *	↑ D (M) * ↑ D (P,O)
% Stigmas Saturated with Germinating Pollen	NA	↑ D
% Styles with PT Growth	Cross > Self *	X

6.6 DISCUSSION

The difficulties encountered with germinating the seed of *T. australe* meant that no seedlings were forthcoming and maternal fitness could only be measured for fruit and seed traits. Since reasonable germination rates have been achieved for other *T. australe* populations (~50% for Victorian populations (Scarlett & Parsons 1992)), the lack of germinability observed in this study for the New England Tablelands populations whilst using the same germination protocol, may be attributable to the effects of severe inbreeding depression. Inbreeding depression is further evidenced by the high incidence of fruit that was completely void of or only partially filled with seed (Chapter 5). However, assuming the tetrazolium viability testing technique was an accurate indication of seed viability, some germination would have been expected as overall, slightly more than half of the seeds tested positive for viability. It may be that for some reason, the germination protocol applied simply failed to break dormancy, and many of the seeds actually possessed the potential to germinate.

The results obtained for *T. australe* traits such as fruit weight and seed-fill levels have been ambiguous and have revealed little in the way of density effects; however seed viability (and therefore the potential for germination) differed significantly for both seed type (open versus autogamous) and density, indicating that this trait may reflect processes operating within the system which influence fitness. The fact that fruits arising from open pollination produced a higher proportion of viable seed (~70%) compared with autogamous fruits (~55%) indicates that autogamous seed is less fit than open seed. An explanation may be that open seed more often results from outcrossing which is often found to positively influence seed fitness (e.g. Dudash 1990; Sheridan & Karowe 2000; Vaughton & Ramsey 2006); however visitors (apart from a very few thrips and an ant) were not detected on *T. australe*. Abiotic vectors (e.g. wind) are unlikely given *T. australe*'s very low pollen production coupled with a floral morphology inappropriate to these pollen transfer mechanisms (Faegri & van der Pijl 1979). However, visitors may have been extremely cryptic (rare and/or mainly nocturnal) and their activity could have remained undetected during observation periods and the possibility that thrips or ants may play a role in promoting outcross pollination cannot be dismissed entirely. This reasoning may also help to explain the fact that higher viability was observed for open seed arising in dense (~85%) versus sparse (~50%) plots. It is plausible that if pollinators are present in the system, they are facilitating cross-pollination among individuals in dense plots but may not frequent more isolated plants; this might explain the reduction in viability of seed arising on sparse plants. Moreover, viability was statistically similar between densities for autogamous seed (but results may be confounded by small sample sizes). Alternatively, assuming a complete absence of pollinators and therefore that all open seed was produced via selfing, these results might be explicable in terms of resource availability. It may be that local plant density is moderated by the amount of resources available for 1) initial plant establishment and persistence (thus promoting patch density) and 2) maternal investment to reproduction (thus promoting the production of higher quality progeny within these plots) (see Cheplick & Sung 1998). However, if this was the case, differences in seed mass due to relatively higher maternal investment would have been expected yet, fruits arising in sparse plots were more often slightly heavier than those from dense plots and there were no indications that seed fill itself was density related (Chapter 5).

The propensity for the strongly self-compatible *T. australe* to produce numerous fruits that are only partially filled suggests high levels of inbreeding depression. This could be due to selfing and/or, assuming some cross pollen transfer is occurring within the system, it is possible that biparental inbreeding may be responsible as both selfing and biparental mating have been found to negatively influence offspring fitness (Levin 1984, 1989). The fruits of *T. australe* do not possess any obvious dispersal mechanisms, but as they are buoyant, are probably dispersed to some degree by water (Griffith 1992). Apart from the possibility that limited dispersal may occur via run-off during heavy rainfall, opportunity for extensive water-mediated transport within the study sites is restricted. Populations of species with limited seed dispersal are often found to comprise clusters of genetically similar/related individuals (Hardner *et al.* 1998) and dense *T. australe* patches are therefore likely to comprise close relatives. Little is known about the flight distance capabilities of thrips (Sakai 2001), however if pollen transfer is mediated by thrips (or ants), it is liable to be spatially limited. Thus, limited seed dispersal coupled with short pollen-transfer distances could be promoting biparental inbreeding depression (Gigord *et al.* 1998; Kelly & Willis 2002) in these *T. australe* populations; yet investigation of pollen loads and pollen tube growth offered little evidence that outcrossing was occurring to any degree or that, compared with self pollen, receipt of outcross pollen increased the potential for fertilisation.

Pollen tube growth of self and outcross pollen has been compared for several economically important *Santalum* species, however this appears to be the first assessment of this kind for *Thesium*. At the MOR population, both self and outcross pollen was found to germinate in similar proportions at the stigmatic surface (~55%), however there were some differences between pollen types when pollen tube growth down the style was assessed. Outcross pollen appeared to behave less vigorously than did self-pollen i.e. a lower proportion of both the total number of outcross grains and of those that had germinated at the stigmatic surface grew to reach the base of the style compared with self pollen. Sedgley (1982) reported no difference in pollen tube numbers reaching the ovary in *Santalum acuminatum* (Quandong) for either pollen type, thus self- and outcross pollen were equally as likely to fertilise an ovule, whereas Rugkhla *et al* (1997) observed a decrease in self-pollen (autogamous and geitonogamous) performance for *Santalum spicatum* and *S. album* compared with outcross. However, both of these studies incorporated more robust methods than those employed in this study. Due to time constraints, *T. australe* material was fixed within several hours post-hand pollination and it is clear from both Sedgley's (1982) and Rugkhla *et al*'s (1997) studies that pollen tubes can take 1-4 days to reach the ovary. In addition, the strength of stigma receptivity increased over time and flower age plays an important role in promoting pollen tube growth (at least in *Santalum*). More generally, among flower variability in pollen growth rates has been detected for some species (thus large sample sizes are preferred) and the presence of both pollen types on the stigma has been known to affect growth rates (Aizen *et al.* 1990). Therefore, the apparent reduced vigour displayed by *T. australe* outcross pollen, may simply be due to these factors not being accounted for. In the present study, self-pollen was likely to have been on the stigma for longer than outcross pollen and been given a "head start" in the pollen tube growth race. However, since pollen was found to germinate within the several hours prior to fixation, and if indeed outcross pollen is relatively fitter than self-pollen, comparatively higher levels of outcross pollen germination might have been expected.

Autogamous pollen loads on *T. australe* stigmas were very small (~10 grains) and hand-outcross pollination increased loads to only ~25 grains. When pollen loads from open flowers in sparse and dense plots were counted, they averaged <10 grains per stigma for both plot types. This suggests that little if any external pollen was being transferred to stigmas. Furthermore, stigmas from dense plots had fewer pollen grains, germinating pollen and pollen tubes than sparse plots and the proportion of pollen that germinated and produced tubes was also comparatively lower. Although this was not often significant, it is fair to assume that inter-floral pollen movement is limited (if not altogether absent) in these *T. australe* populations and that most seed results from autogamy and occasional biparental mating. Similar conclusions were drawn by Kephart (2004) who investigated progeny performance of the rare *Silene douglasii* var. *oraria* (Caryophyllaceae).

For the self-compatible and protandrous *W. luteola,* there was no evidence to suggest that density influences seed weight, nor did seed weight appear to confer any advantages to seedling emergence. Similar results were found for another protandrous species that produces copious small seeds (*Trachymene incisa* (Apiaceae)) when selfed versus outcrossed seed were compared (Davila & Wardle 2002). However, the results for *W. luteola* show that several seed traits differed significantly at the site, rather than at the local density level. Despite similar viability levels among sites, the seed from one site (UNE) significantly outperformed seed from the remaining sites in terms of percent germination and emergence rate. It appears therefore, that seed traits can display plasticity among these *W. luteola* populations. The UNE site is situated >30km south of the POW and OAR sites, and is therefore geographically (and probably genetically) distant from these sites, which are separated from each other by only a few kilometres. Furthermore, UNE's altitude is lower (UNE=1,070m, POW=1,270m, OAR=1,220m) and as a result, is subject to differing climatic conditions; perhaps there are latitudinal/altitudinal related affects operating that have promoted these among-site differences. Among population differences over a latitudinal gradients have been reported for a number of life history traits, both morphological (e.g. seed weight & final plant size) and phenological (e.g. days to emergence and onset of flowering), for another member of the Campanulaceae (*Campanula americana*) when grown under common environmental conditions (Kalisz & Wardle 1994). *Verbascum thapsus* (Scrophulariaceae) also displayed significant latitude related differences (Reinartz 1984). Since *W. luteola* seeds from the three sites were germinated under the same conditions, the results indicate that the differences found for *W. luteola* seed traits may have been selected for and are possibly due to among population genetic variation (see Primack & Kang 1989).

Results were inconsistent for density among and within sites for *W. luteola* seedling traits (i.e. survivorship and growth rate). There is a possibility that less than optimal functioning of the hotbed in which the seedlings were housed, may have contributed to these ambiguous results. In general, seedling survivorship at each of the census days was similar among sites and a similar decline in survivorship over time was also manifest across all sites. However, UNE seedlings from dense plots displayed a significantly higher proportion of survivorship for each census day and similar density differences also became significant in the later stages of growth for POW seedlings. Quite the reverse was found for OAR, where seedlings from sparse individuals had a higher rate of survival than those from dense individuals. Furthermore, seedling survival was not an indicator of faster growth rate; in fact seedlings from sparse plots were often larger at any given census date compared with their dense counterparts. Although there is some evidence that density may influence fitness (seedling survivorship and growth), the disparity among sites and densities for the variables measured indicates that more complex mechanisms may be operating at the site level, which extend beyond the simple assumptions regarding density-dependence. For example, genetic substructuring within populations may favour pollen transfer among close relatives in dense plots (biparental inbreeding), whereas sparse individuals could receive pollen from greater distances. Nevertheless, it appears that density-related fitness differences that were not detected at earlier life history stages (e.g. seed weight and germinability) may ultimately manifest later in the lifecycle (i.e. in seedling traits).

Unfortunately, no quantitative results could be gleaned from the *W. luteola* pollen investigations, except that pollen loads, pollen germination and pollen tube growth were high in both sparse and dense plots, and that both self- and outcross- pollen was capable of reaching the ovules. From the breeding system work, it is clear that self-pollen can fertilise ovules, but produces considerably less seed than outcross pollen. Self-pollen transfer is expected to be minimal in *W. luteola* due to its protandry and the fact that an individual usually produces only one to several flowers at any given time. The S: FR ratios (Chapter 5) indicate that a reduction in pollen quantity rather than quality is responsible for the minor decrease in seed production observed in sparse plots relative to dense. However, as outlined above there are indications that seed from sparse plots can be of higher quality than seed from dense plots, but this can vary among sites.

Seed weight was also not related to emergence rate for the self-incompatible *D. sieberi* but it was positively related to other traits later in the lifecycle. These relationships were particularly evident in the very early stages of seedling development; both cotyledon size and stem length in the first month or so of development, were significantly related to seed weight. These trends were still apparent but less inconsistently so, for later growth (up to 200 days). Thus, seed weight in *D. sieberi* may not confer an early germination advantage, but can promote larger seedlings that are likely to enjoy a competitive advantage over their conspecifics. Since increased seed weight can imply high genetic quality it was somewhat surprising to find that seed weights were often elevated in sparse plots compared with dense. Intuitively one would expect dense plots to produce higher quality, heavier seed as flowers are expected to receive higher quantities of outcross pollen (as they do, see below) from numerous conspecifics. Biparental inbreeding may be contributing to this scenario (as outlined for *T. australe*), but other factors that influence the incidence of one- and two-seeded fruits between densities, require further consideration.

Considering *D. sieberi's* strong self-incompatibility, it was not surprising that self-pollen germinated at a significantly lower level than outcross pollen and that pollen tubes were identified more often in styles of flowers that had been supplemented with outcross pollen; this underscores the importance of insect pollen vectors within the system. Although not always significant, general trends indicated that stigmatic pollen loads were of both higher quantity and quality in dense plots compared with sparse. Thus, it is obvious that the general trend for higher visitation rates to dense plots (Chapter 4) positively influenced pollen deposition to flowers in these plots. Therefore, for this species visitation rate and visitor behaviour have the potential to strongly influence the quantity and quality of seed produced. However, as detailed below, the subsequent effects may be counterintuitive.

According to Molano-Flores & Hendrix (1999), stigmatic pollen-load composition can be important for self-incompatible species and is likely to be influenced by pollinator behaviour. Since *D. sieberi* individuals present tens to many hundreds of flowers at any given time, the potential for geitonogamy is very high. High levels of self-pollen deposition can often have negative repercussions for seed set in self-incompatible species, (Ramsey & Vaughton 2000; but see Waites & Ågren 2004; e.g. Duncan *et al.* 2004a), which can result from factors such as; self pollen interference with outcross pollen function (e.g. *Polemonium viscosum* (Polemoniaceae) (Galen *et al.* 1989)) or a reduction in pollen quantity (e.g. *Eupatorium* spp. (Asteraceae) (Byers 1995). No doubt visitor behaviour, which increased geitonogamous visitation in sparse plots (Chapter 4), had an impact on pollen deposition and subsequently, the number of seeds maturing in fruits from sparse *D.*

sieberi individuals. Although the total number of pollen grains on stigmas did not differ significantly between sparse and dense plots, flowers in dense plots displayed higher levels of germinating pollen, which suggests greater amounts of outcross pollen is being transferred to flowers in dense plots relative to sparse. This implies that the pollen mix deposited on flowers in sparse plots comprises more self than outcross pollen, and that this is mediated by the pollinator behaviour observed on individuals within these plots. Indeed, more one-seeded fruits were produced in sparse plots relative to dense (see Chapter 5). However, seed arising from one-seeded fruits were consistently and significantly heavier than those from two-seeded fruits, thus explaining the unexpected higher mean seed weight observed in sparse plots. When reduced seed set but larger seed sizes were observed in small populations of both *Primula veris* and *Gentiana lutea,* Kéry *et al* (Kéry *et al.* 2000) suggested that this may simply represent a trade-off between seed number and seed size (but see Oostermeijer *et al.* 1994). However, since seed weight was found to confer a fitness advantage to *D. sieberi* seedlings, individuals in sparse plots appear to not only be producing larger seeds but also higher quality seeds. For *D. sieberi*, overall fruit production (FR: FL) is higher in dense plots but seed production (S: FR) is essentially equivalent between densities (Chapter 5). Thus, the higher incidence of one-seeded fruits in sparse plots may be especially important as whatever mechanism/s promote one-seeded fruit production could influence fitness dynamics at the population level via small-scale plant-pollinator interactions. Therefore, what would intuitively be construed as detrimental visitor behaviour for a self-incompatible plant, might actually to some degree be promoting fitness.

As pointed out by Richardson & Stephenson (1992) it is not unusual for positive influences of seed size to be revealed for early life history components such as germination, and detecting seed size effects on offspring performance components can be more difficult to track. Although researchers have found relationships between seed size and a wide spectrum of short and long-term fitness indicators (e.g. Halpern 2005), others have found that fitness differences may only become apparent with time (e.g. Fischer & Matthies 1998). No clear connection could be found between seed weight and germinability for any of the present study species. Furthermore, there was only limited evidence (albeit ambiguous) that differences in some components of offspring fitness may manifest later in the life cycle, at least for *W. luteola* and *D. sieberi.* Moreover, since some researchers have observed disparity between offspring performance under glasshouse versus natural environments had field experiments been included in the present investigations it is possible that the results obtained may have been less equivocal. Indeed, a combination of approaches proved informative when assessing fitness in *Costus* sp. (Zingiberaceae) (Schemske 1983) and *Sabatia angularis* L. (Gentianaceae) (Dudash 1990) and resource allocation in *Impatiens capensis* (Balsaminaceae) (Abrahamson & Hershey 1977). For both the protandrous *W. luteola* and the self-incompatible *D. sieberi*, easily demonstrable density effects on fitness may be confounded by mechanisms that were not investigated in this study. For instance, biparental inbreeding was suggested as a potential influence on offspring fitness for all of the study species; genetic substructuring of the study populations which can occur at the small scales utilised in this study (Griffin & Eckert 2003) may play an important role in determining density-dependent fitness responses. For the strongly self-compatible *T. australe* however, the maternal environment and resource availability could be more important in shaping offspring fitness in light of the apparent lack of outcross pollen transfer and high levels of autogamous seed production.