# CHAPTER 6.

# FITNESS COMPONENTS: OFFSPRING AND POLLEN

## 6.1 INTRODUCTION

The term "fitness", in a biological sense, is not readily definable and has been conceptualised in a variety of ways (see de Jong 1994). De Jong (1994) states "the main difference...is the contrast between a fitness concept that refers to the functioning of an organism (or genotype or trait), and a fitness concept that constitutes a technical term in population biology summarising numerical processes" e.g. a measures of fitness such as the intrinsic rate of increase (*r*); it is the former concept which is of significance here. According to Herrera (2000) variance in fitness can be divided into two main components; fecundity (number of progeny produced, as addressed in chapter 5 of this dissertation) and viability (probability of survival to maturity). Begon *et al* (1996) define fitness as "the contribution of descendents by an individual *relative* to the contribution made by others in its present population" which, coupled with Herrera's (2000) remarks, provides an appropriate context for the present chapter in which seed and seedling performance (maternal fitness) are investigated. In addition, since offspring fitness is often correlated with paternal fitness (Mena-Alí & Rocha 2005), it is important to consider male fitness (i.e. pollen removal, dispersal, deposition and pollen tube growth (Aguilar & Galetto 2004); thus several of these components are included in this investigation.

An informative seed character that can be used to assess fitness is seed size (or mass). Intraspecific variation in seed size is a common phenomenon (Halpern 2005) and has attracted much attention regarding its relevance to plant fitness and evolution (Gómez 2004). There are numerous explanations posited for intraspecific variability in seed size. For instance, experimental evidence suggests that resource availability can limit the ability of the parent plant to determine seed size, which can be dependent on spatial (within plant/within fruit) or temporal (across a season) factors. It is also possible that seed size variation is an adaptive trait, which may confer advantages to those species that persist in heterogenous environments (Vaughton & Ramsey 1997). Relevant to the present study is the premise that maternal provisioning of resources is preferentially afforded to offspring of higher genetic quality (Temme 1986). In general, outcrossing tends to favour the maintenance of heterozygosity compared with selfing, which increases homozygosity. Selfed individuals can be genetically inferior and consequently display reductions in

fitness (inbreeding depression), which may render them less capable of success in harsh or changeable environments (Dudash 1990). The pollen source (self versus outcross) can thus be an influential moderator of maternal investment during seed development, and can determine final seed mass, which can subsequently influence seed and seedling vigour.

Reduced fitness is a typical consequence of inbreeding depression (defined by Shi *et. al.* (2005) as "the decrease in performance resulting from the mating between close relatives"), for self-compatible species (Levin 1984) and is often detectable in the early stages of the plant life cycle. Inbreeding depression can manifest as diminished seed viability and seedling growth and survivorship (Schemske 1983; Menges 1991). Reduced progeny vigour due to inbreeding depression has been found for a number of species e.g. *Hibiscus moscheutos* (Malvaceae) (Snow & Spira 1993), *Banksia marginata* (Proteaceae) (Vaughton & Ramsey 2006) and *Sabatia angularis* (Gentianaceae) (Dudash 1990). Compared with selfing, outcrossing has been shown to increase fertilisation and seed production, as well as to produce heavier seeds of higher quality (e.g. Trager *et al.* 2005). Seed mass has been positively correlated with a number of fitness traits such as viability, germinability, time to germination and seedling growth and survivorship (e.g. *Prunus jenksinii* (Rosaceae) (Upadhaya *et al.* 2007); *Mesua ferrea* (Clusiaceae) (Arunachalam *et al.* 2003); *Quercus ilex* (Fagaceae) (Gómez 2004); *Lobelia inflata* (Campanulaceae) (Simons & Johnston 2000). Larger seeds are often more robust during seedling establishment and better equipped to combat environmental factors such as herbivory, competition and nutrient limitation (see Gómez 2004).

Given the relationship between pollen source and inbreeding depression, within and between plant visitor behaviour can be an important influence on both offspring quantity and quality, depending on a plant's breeding system. Moreover, since visitation can be influenced by local density, it is reasonable to expect that offspring fitness may consequentially be density-dependent. For self-compatible species, decreased pollen delivery or increased geitonogamous pollinations may not necessarily affect seed production, but may lower offspring fitness due to inbreeding depression (Colling *et al.* 2004) and for self-incompatible species seed production itself can be depressed (Field *et al.* 2005). Although the level of selfed seed production for completely self-incompatible species may often effectively approach zero, fitness reductions may still become apparent should mating occur with close relatives (biparental inbreeding). Levin (1984) found evidence for this when investigating the effects of interparental distance in the self-

incompatible *Phlox drummondii* (Polmoniaceae), where seed abortion rates were negatively correlated with interplant distance. Colling *et al.* (2004) speculated that hand-pollinations using pollen sourced from donors at a distance from the recipient plant may have improved both seed production and quality in the self-incompatible and rare *Scorzonera humilis* (Asteraceae).

Small population and density effects on various components of maternal fitness have been revealed for a number of plant species. Isolated individuals of the tree, *Enterolobium cyclocarpum* (Guanacaste) produced fewer seeds of lower quality than those persisting in continuous forest, potentially as a result of increased geitonogamy coupled with the possibility that a reduction in fruit yield resulted in low competition among developing fruits for resources, thus allowing for fruits to mature that would normally abort under more competitive circumstances (Rocha & Aguilar 2001). Menges (1991) found a positive correlation between germination success and population size in the self-compatible *Silene regia* (Caryophyllaceae), which was probably due to inbreeding depression and/or increased geitonogamy and decreased visitation rates within small populations by its hummingbird pollinator. However, effects on fitness are not always detected at these early stages of the plant life cycle. Fischer & Matthies (1998) found that offspring fitness (seedling survival and flower production) in *Gentianella germanica* (Gentianaceae) was related to population size, but only became significant some 17 months after seeds had germinated and had reached reproductive maturity. Similarly, Karron (1989) detected inbreeding depression only in the later stages of the lifecycle (seedling biomass) for two *Austragalus* species. Neutral effects on fitness have also been reported, for instance seed production, germination and seedling growth were independent of population size for the rare *Campanula cervicaria* (Campanulaceae) (Eisto *et al.* 2000).

Given the idiosyncrasies of different systems and the fact that fitness responses can manifest across different stages of the life cycle, practical time constraints imposed on studies can mean that offspring fitness components may remain undetected. In addition, discrepancies can be found between field and glasshouse results when measuring fitness components; often fitness reductions do not become apparent under the relatively benign conditions of the glasshouse (e.g. Kephart *et al.* 1999; Kephart 2004). Furthermore, among population variation in plant and seed morphological traits (see Kalisz & Wardle 1994) is also an important consideration.

Aspects of female function are the most commonly employed in studies that investigate reproduction and fitness, but male fitness responses are also important. Male fitness can be measured in terms of the number of pollen grains removed, dispersal distances, deposition rates and pollen tube growth and, although studies that include these variables are relatively few, there is evidence to suggest these factors respond to population degradation through habitat fragmentation (Aguilar & Galetto 2004). Pollen deposition is an important factor because not only the size of the pollen load, but also its composition (self and outcross pollen) can influence reproductive output. The size of the stigmatic pollen load was found to be particularly important to the probability of *Pachira quinata* (Bombaceae) flowers setting fruit (Quesada *et al.* 2001). Bat-mediated pollen deposition on stigmas of the weakly self-compatible tree, *Cebia grandiflora* (Bombacaceae), was higher in undisturbed habitats and promoted better fruit set than in disturbed areas (Quesada *et al.* 2003). In this instance, the authors contribute the reduction in fruit set to reduced visitation (smaller stigmatic pollen loads) and/or increased geitonogamy (greater levels of self-pollen deposition). Since many plants employ (and often rely on) animal vectors to transport pollen, density-dependent pollinator behaviour can be influential to male function. Thus, determining the amount and composition of pollen arriving at stigmas is an important initial step in understanding paternal contributions to fitness in density studies. Furthermore, investigating the overall viability of pollen in the system as well as the relative vigour of self and outcross pollen can be informative when estimating paternal contribution to reproduction.

There are many factors that affect the potential for a given microgametophyte to successfully fertilise an ovule (reviewed by Stephenson & Bertin 1983) and post-pollination male competition (which is also influenced via the mechanisms of "female choice") is one of the most experimentally tractable (readily observed via fluorescence microscopy (see for example Jefferies & Belcher 1974)). Variation among pollen germinability and pollen tube growth rates can indicate the relative fitness of self and outcross pollen; the fastest growing tube/s will ultimately be successful in fertilising the ovule/s (Erbar 2003). The differential in growth rates between self and outcross pollen is thought to be an influential factor in moulding self-incompatibility, since outcross pollen is often found to germinate more readily and/or grow at faster rates than self pollen e.g. *Dianthus chinensis* (Caryophyllaceae) (Aizen *et al.* 1990); yet other studies reveal little difference in vigour between the pollen types e.g. *Santalum acuminatum* (Santalaceae)

(Sedgley 1982). Furthermore, density-related effects on pollen tube growth have been observed, for example in *Shorea siamensis* (Dipterocarpaceae) (Ghazoul *et al.* 1998) where the results indicated a reduction in compatible pollen deposition to flowers of relatively isolated trees.

Maternal investment to fruit and seed production is a significant determinant of offspring fitness via its influence on seed size, which has been shown to be an important influence on the viability and vigour of progeny. The genetic quality of a given microgametophyte and the pollen tube it produces can determine its acceptance by the female sporophyte and the level of maternal investment given over to the development of the seed it sires. The pollination process is therefore a crucial step in regulating offspring (and therefore population fitness). Given the reliance of many plant species on animals for pollination and the propensity for plant-pollinator interactions to be density dependent, it is logical to expect that components of both female and male fitness will respond to local density.

## 6.2 AIMS

Utilising the same design outlined in the introductory chapters of this dissertation, this chapter investigates density-dependent affects on fitness for the three study species. Maternal fitness is addressed via investigating offspring fitness components including seed viability and germinability, and seedling survival and growth. Several aspects of male fitness are also addressed, including the comparison of stigmatic pollen loads (quantity and quality) between densities. The relative vigour of self and outcross pollen is also investigated and results are used to infer the incidence of these pollen types on stigmas in sparse and dense plots.

## 6.3 MATERIALS AND METHODS

### 6.3.1 Seed Germinability, Viability and Offspring Growth

#### *6.3.1.1* ***T. australe***

Scarification and the addition of $GA_3$ were found to increase germination in *T. alpinum* (Kuijt 1969). Therefore, in 2003, a preliminary germination trial, which consisted of nicking of *T. australe* fruits and watering with a 50mg/L gibberellic acid solution, was undertaken. In this trial, 30 fruits (collected from Black Mountain Cemetery in 2003 and by C. Gross from a site near Glen Innes NSW in 1996) were treated accordingly, housed in ice cube trays (bedding as for *D. sieberi* below), and placed in an incubator set at 25°C and 24 hr light. After 21 days, mould was becoming problematic and a 1g/L solution of Fongarid™ was applied to the fruits. After 23 days a single seed from the Glen Innes population germinated (Figure 6.1 b). $GA_3$ was reapplied to all fruits at 25 days and at 50 days the trial was abandoned as all fruits had succumbed to mould (i.e. were soft when squeezed with forceps).

An alternative germination protocol developed by Scarlett & Parsons (1992) for *Thesium australe* was subsequently trialed. Fruits were first weighed then subjected to immersion in 1M HCl for 24 hours, washed in detergent for 1.5 hrs, bathed in 5% sodium hypochlorite for half an hour and then cold treated for 3 months at 5°C. Three hundred fruits that were collected from plants bagged for autogamy and open pollination at Aberfoyle and Black Mountain Cemetery during May and July of 2004 were used for germination. Following pre-treatment, the fruits were placed in sterilised ice cube trays (bedding as for *D. sieberi* below) and placed into the incubator in September 2004. Fruits were checked for germination daily.

Fruit collected from sparse and dense plots at the Aberfoyle and Moray sites that were used to investigate *T. australe*'s S: FR ratio (Chapter 5) were assessed for fruit weight and seed viability. Fruits that arose on stems bagged for autogamy were used and were compared against fruits collected from plots that arose under natural open conditions. These fruits were collected in March 2005 and were stored in paper envelopes on silica gel until they could be processed in the laboratory. A subset of fruits was removed from each envelope. *Thesium australe* fruits retain a "crown" (persistent tepals) to varying degrees after maturation. So that fruits could be standardised for weighing, this crown (or its remains) was removed by scraping it away with a fingernail before weighing (Figure 6.1a).

After weighing, individual fruits were dissected to determine the presence of seed. Fruits were halved longitudinally and slightly off-centre to ensure an intact embryo was retained for tetrazolium staining. The seeds of *T. australe* can occupy the fruit to varying degrees; therefore seed fill was assessed to the nearest 0, 25, 50, 75 or 100%. Following this, one half of each of the seed-containing fruits was subjected to tetrazolium testing (0.1% concentration) for viability. Uptake of the stain was maximised as seed was in direct contact with the tetrazolium following dissection. The fact that *T. australe* fruits float meant that in order to keep track of individual seeds, staining had to be undertaken by placing the fruits between Kim-Wipe™ sheets that had been saturated with tetrazolium solution (Figure 6.2a). TCC (1970) was used to assist with determining viability. There was considerable variation in stain uptake (Figure 6.2b), and scoring was based on the protocols outlined in Table 6.1 below.

**Table 6.1** Scoring protocol for viability of *T. australe* seeds using tetrazolium staining. D=dark stain throughout, L=light stain throughout, 0=No stain, PS= patchy stain throughout with dark and light areas, PU= patchy stain throughout with unstained areas.

| Seed Structure | Stain Uptake | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Endosperm | L | D | L | D | 0 | L | L, D | 0 |
| Embryo | L | D | PS | D, L or PS | D, L or PS | 0 | PU | 0 |
| Viable Score | Yes | Yes | Yes | Yes | Yes | No | No | No |

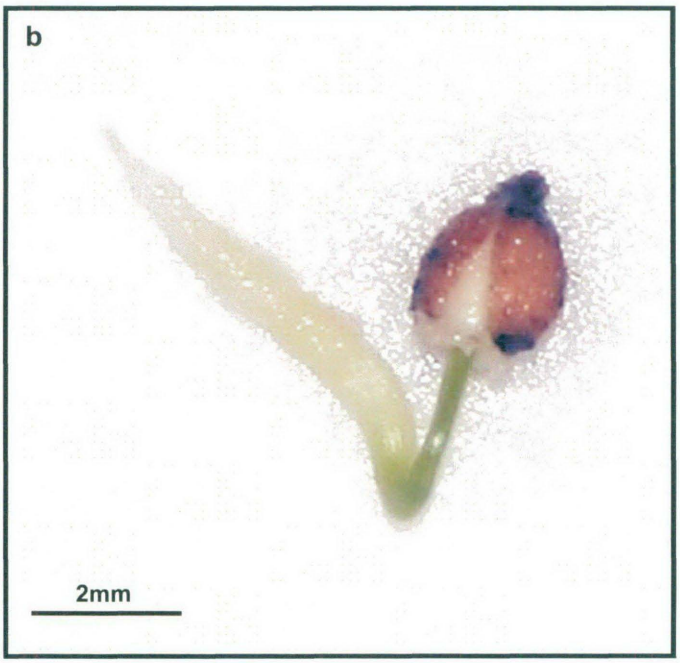




**Figure 6.1** **a)** Entire *Thesium australe* fruits and with the "crowns" (persistent tepals) removed prior to weighing **b)** a successful *T. australe* germination.

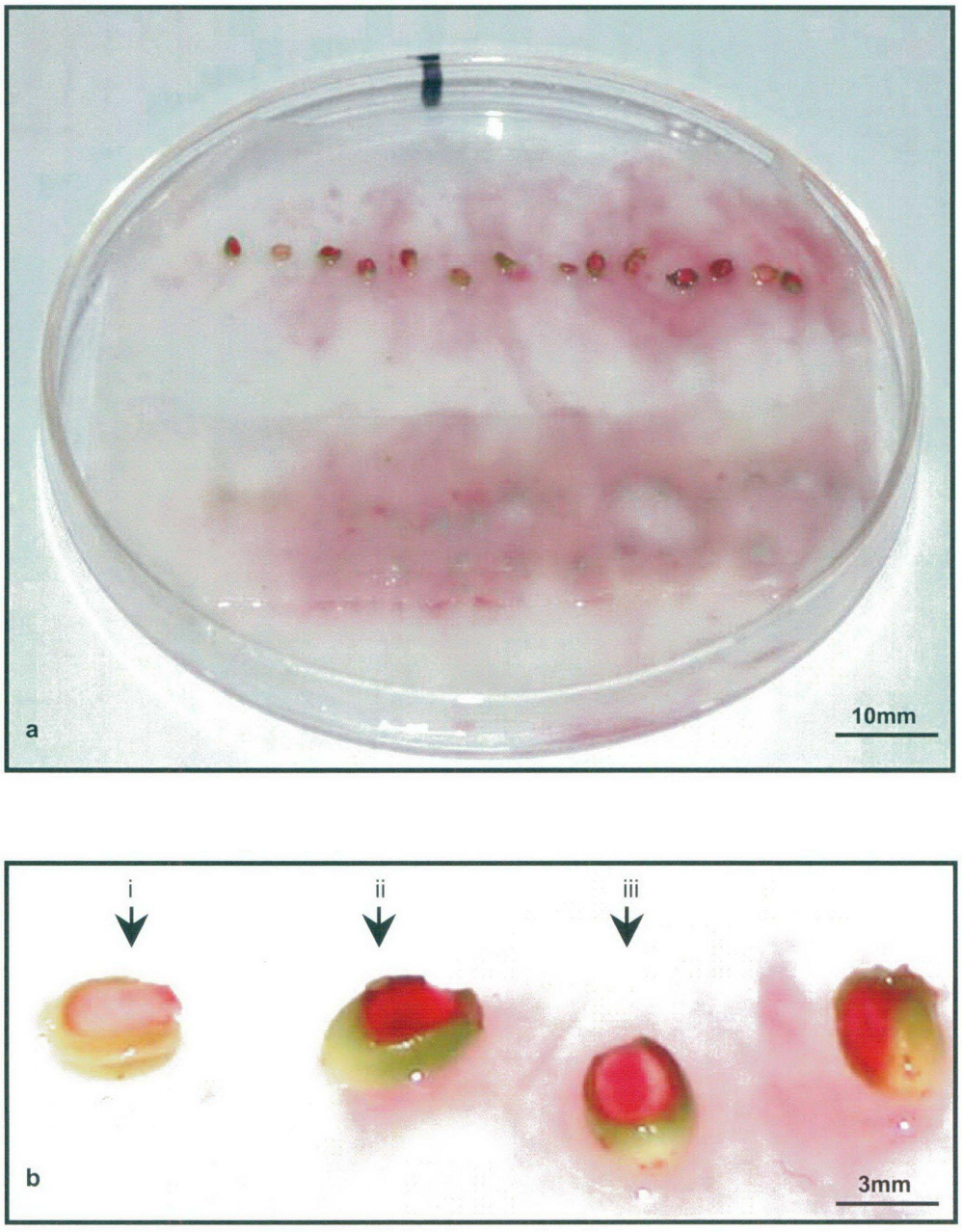



**Figure 6.2** *Thesium australe* tetrazolium staining **a)** staining method; dissected fruits placed between sheets of KimWipe™ and saturated with tetrazolium solution **b)** variation in staining i) light stain throughout ii) strong staining throughout iii) embryo strongly stained, but endosperm faint.

### *6.3.1.2 W. luteola*

To assess *W. luteola* seed fitness attributes and germinability, seed that arose from the fruits bagged on focal plants for the FR: FL and S:F R ratios (Chapter 5) were subjected to weighing and germination experiments. Fruits were opened and seeds counted under a dissection microscope with the help of a petri dish lined with graph paper and a clicker counter. The entire sample was weighed and an average seed weight was calculated per sample. A subsample of seed from each was subjected to a pilot germination experiment. Seeds were arranged in sterilised petri dishes on a circle of wettex sponged topped with filter paper, watered and place in the incubator set at 30°C/15°C, 12/12 hr day/night. Seeds were checked daily for radicle emergence using a handheld lens (x10 magnification). Radicle emergence was deemed to have occurred when the testa split at one end and the radicle was exposed. The number of days it took for cotyledons to emerge was also noted. Germination for this experiment was skewed towards several plots at the UNE site and many seeds across all sites failed to germinate after 36 days. Therefore, tetrazolium testing was then undertaken and a high level of viability was revealed. The germination experiment proper was subsequently undertaken.

The germination experiment for *W. luteola* (Figure 6.4 a & b) was initiated using the same methods as for the pilot experiment but day temperature was reduced to 20°C from 30°C following Morgan (1998) and a larger quantity of seed was utilised. During this experiment, radicle and cotyledon emergence was checked at 3, 6, 12, 18, 24, 36 with cotyledon emergence monitoring occurring further at 43, 50 and 57 days. After 57 days, the germination experiment was concluded and up to 15 germinates from each fruit sample were planted out into seed raising trays with Searle's™ native potting mix. Trays were placed in a hotbed set at ~25°C under automatic mist sprays (Figure 6.4c). Seedlings were monitored for fitness attributes every ten days, including stem growth (measured as the distance between cotyledon node and the node of youngest leaves) and number of leaves produced. This was undertaken for 40 days; further monitoring was not possible when the majority of seedlings were lost due to malfunction of the hot bed. All ungerminated seeds were subjected to tetrazolium testing (see below).

Tetrazolium testing of fresh *W. luteola* and of ungerminated seed remaining after the germination experiment was undertaken. Samples of fresh seed were shaken into discrete piles on a Styrofoam tray lined with several layers of tetrazolium soaked KimWipe ™. The samples were then covered in several more layers of soaked KimWipe material.

To score the seeds, each sample was cut out from the tray, the top layer of material removed and placed in a petri dish on a dissection microscope (Figure 6.3). Individual seeds were scored using a transmitted light source to observe stained embryos. In addition, embryos were squeezed out from within the seedcoat by applying light pressure to end of the seed, so that the level of embryo staining could be better visualised. The scoring process for the ungerminated seed was the same, but seeds were covered in tetrazolium solution whilst still in their germination bedding. Seed were scored as viable if their embryos were obviously pink.

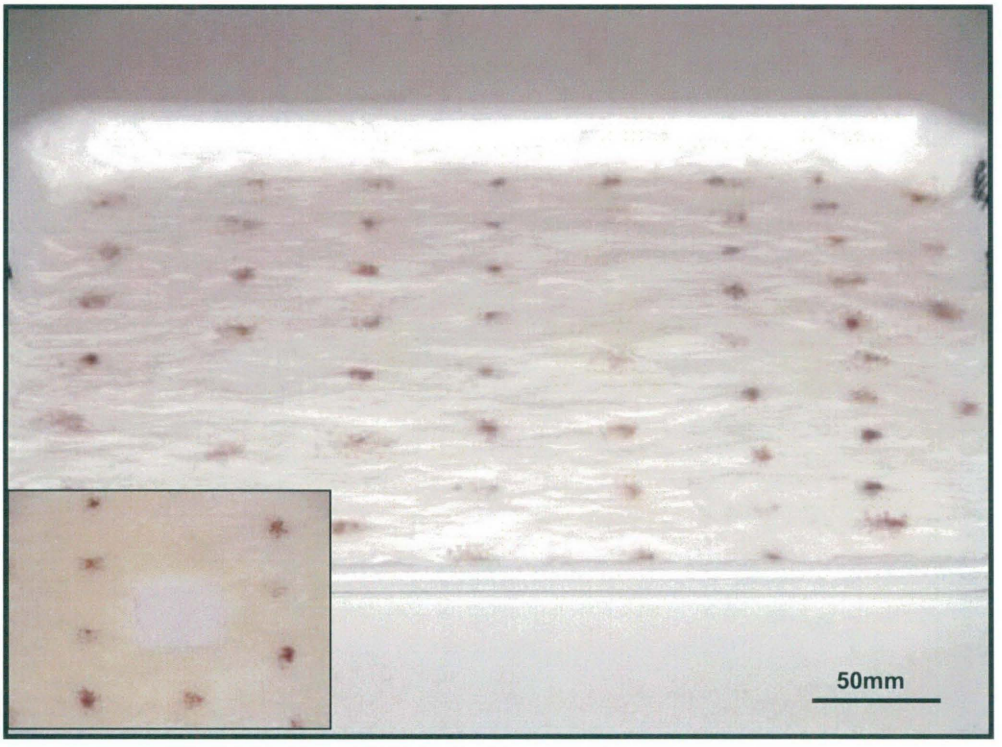



**Figure 6.3** *Wahlenbergia luteola* tetrazolium staining. Seed samples were placed between Kim Wipe™ sheets on a foam tray and saturated with tetrazolium solution. A square of material containing the seed sample was then cut out (see insert) and transferred onto a petri dish for scoring.

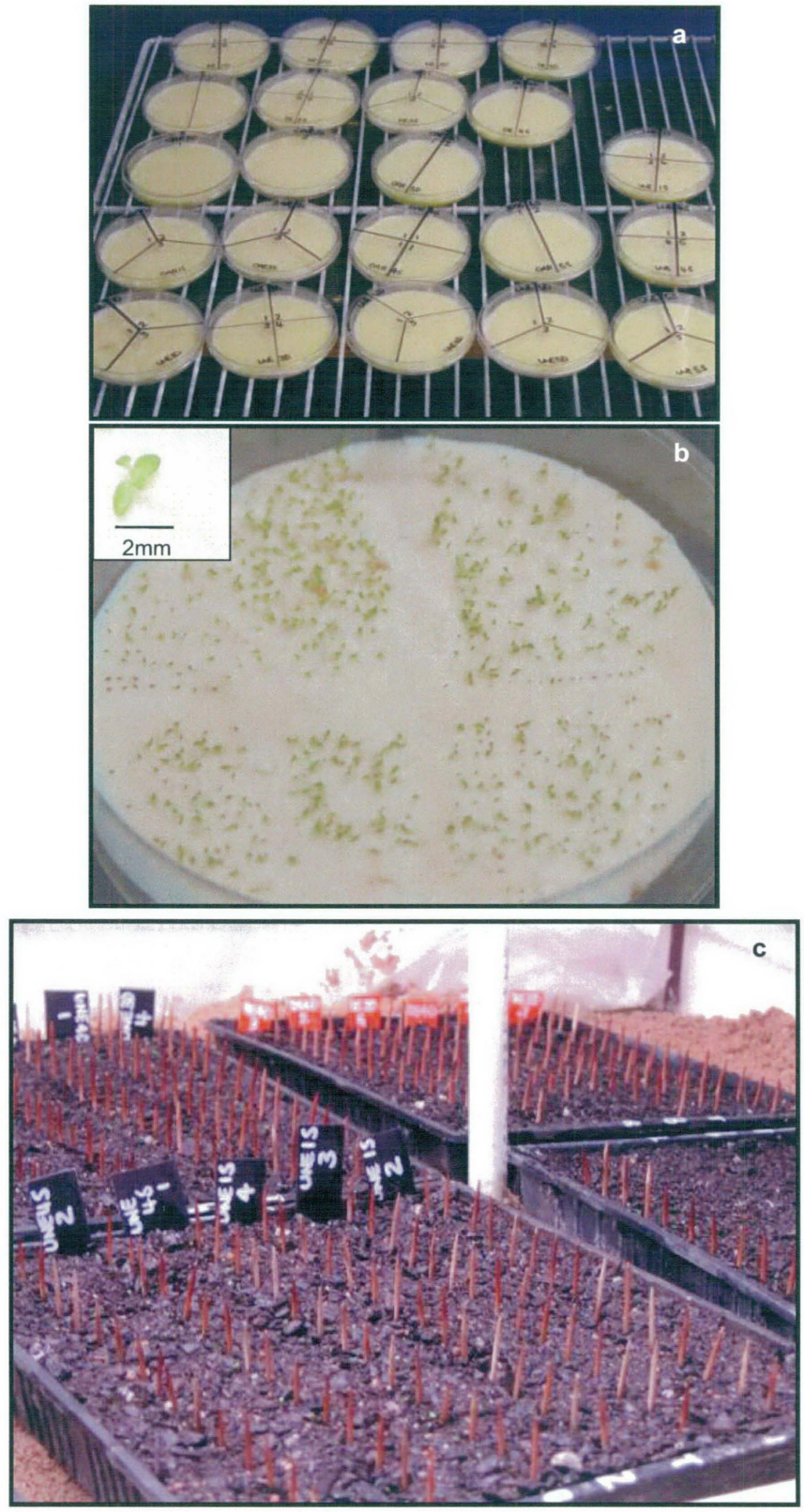



**Figure 6.4** *Wahlenbergia luteola* **a)** germination experiment **b)** germinates **c)** Some of the germinates that were transferred to a hot bed for growth development monitoring; toothpicks marked their positions in the trays.

### *6.3.1.3 D. sieberi*

Fruits of *D. sieberi* produce either one or two seeds. Two germination experiments (GEs) were undertaken. The first (GE1) utilised seed that resulted from the FP fruit to flower ratio stems (2004) (Chapter 5). These fruits had dehisced in the bag prior to collection, therefore it could not be determined whether seed had resulted from a one or two-seeded fruit. Stems on focal plants had also been bagged to check for fruit predation (Chapter 5). These stems were collected when the fruit had mostly matured, but prior to seed dehiscence. Because fruits could be manually opened, the number of seeds produced within could be scored. Therefore, seeds resulting from one and two-seeded fruits in predation bags were germinated (GE2) to check for possible fitness differences related to the number of seeds produced by a fruit. All seeds were individually weighed pre-germination.

Germination Experiment 1

Seeds resulting from the stems collected from focal plants in Dec 2004 for the fruit to flower ratio experiment were placed into an envelope and stored in a container with silica gel. The envelope for each focal plant therefore contained fruits from 5 stems except for M1S where only four stems produced fruit (fruit predation in the 5$^{th}$). No seed was available for DE 2S, as all fruits produced were predated. Trials indicated that *D. sieberi* germinates relatively easily on the windowsill after covering with boiling water, soaking overnight and nicking with a scalpel the following day.

Plastic ice cube trays were used to house the seeds during the germination process. Each seed was allotted its own numbered "cube" so it could be tracked for traits to be measured (Figure 6.6 a). The trays were sterilised by rinsing with a hot water and bleach solution. Each cube was then lined with absorbent cotton wool and topped with a square of blotting paper. Fourteen seeds for each focal plant (except DE2S) for each site were randomly chosen by using the first fourteen "healthy" (not wrinkled, chewed etc) seeds to be shaken from the packet. (Total N=406, DE= 70 dense, 56 sparse, MOR & OAR = 70 dense and 70 sparse). Seeds were individually weighed and placed on top of the blotting paper square in their allotted cubes. Each seed and its bedding was then covered with boiling distilled water to sterilise the seed and its bedding. The seeds were then left to soak over night. The following day, each seed was nicked on the end distal to the eliasome using a scalpel. Excess water was then drained off using a pipette and trays were covered

with cellophane bags to prevent desiccation and placed in an incubator which was set to 30°C/15°C, 12/12 hr day/night. Seeds were checked daily for imbibition and cotyledon and radicle emergence. After emergence, the length of the longest cotyledon (Figure 6.6b) was measured every two days. After 10 days following radicle emergence, germinates were transplanted to tubes containing Searle's™ native potting mix with an identification tag, then transferred to the glasshouse for offspring fitness measurements.

Seeds that did not germinate after 25 days were subjected to tetrazolium testing to assess their viability (Figure 6.5). Tetrazolium solution was added directly to the containers in which the seeds had been placed for germination (after removal of excess moisture from the bedding), left overnight and scored the following day.

Germination Experiment 2

Three *D. sieberi* stems per focal plant at all sites were bagged to score the level of predation (Chapter 5) and were collected in January 2005. Because fruits had not yet dehisced, they could be used to assess the number of one and two-seeded fruits; the resulting seed was used for the germination experiment. Seeds from the three bags were pooled as a single sample for each focal plant. Seeds were pre-treated in the same manner and germinated under the same conditions as for GE1. However, as opposed to using ice cube trays, seeds were germinated in petri dishes. Each sterilised petri dish was lined with a circle of sterilised dishwashing sponge and topped with a circle of Whatman™ filter paper. Seeds from one and two seeded fruits from a given focal plant were placed in the same petri dish (labelled accordingly) upon the filter paper, covered with boiling distilled water and nicked the following day. They were then placed in the incubator. The same variables were measured and transfer to the glasshouse was undertaken as for GE1. Seeds that did not germinate after 25 days were subjected to tetrazolium testing to assess their viability as outlined above.

Germinates resulting from germination experiments 1 and 2 were placed in the glasshouse as outlined above (Figure 6.6d). Temperatures in the glasshouse ranged from 10°C to 30°C and seedlings were watered twice daily by an array of mist sprays. Each seedling was measured for survival, growth and number of leaves at 30, 45, 60, 75 and 200 days after its transfer to the glasshouse. Seedlings were scored as having died if a) no evidence of the seedling remained in the tube or b) the seedling was brown and withered. If green was evident, the seedling was scored as alive. To quantify growth, a ruler was

used to measure the distance between the base of the cotyledons and the uppermost tip of the seedling (Figure 6.6c). A scar is evident at the location of the cotyledons on older seedlings, therefore providing a consistent point of measurement throughout the experiment. Only true leaves were counted and cotyledons were not included. Therefore, if a seedling had not developed beyond cotyledon stage when data was collected, it received a score of 0mm growth (and 0 true leaves).

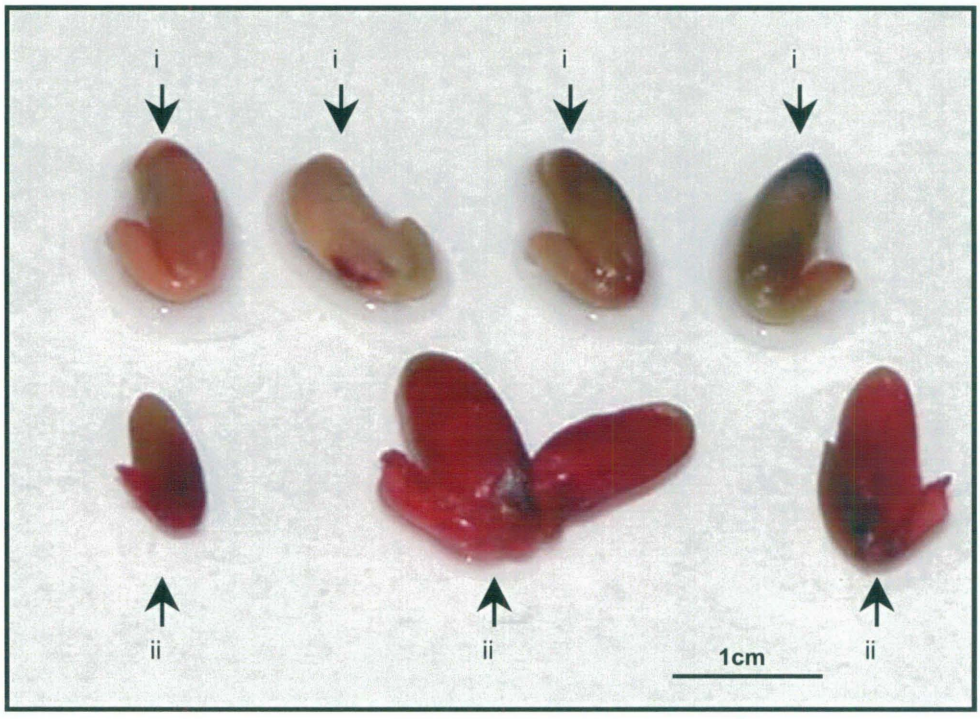



**Figure 6.5** *Dillwynia sieberi* ungerminated seeds, which were tested for viability using tetrazolium, i) scored as non-viable ii) scored as viable.

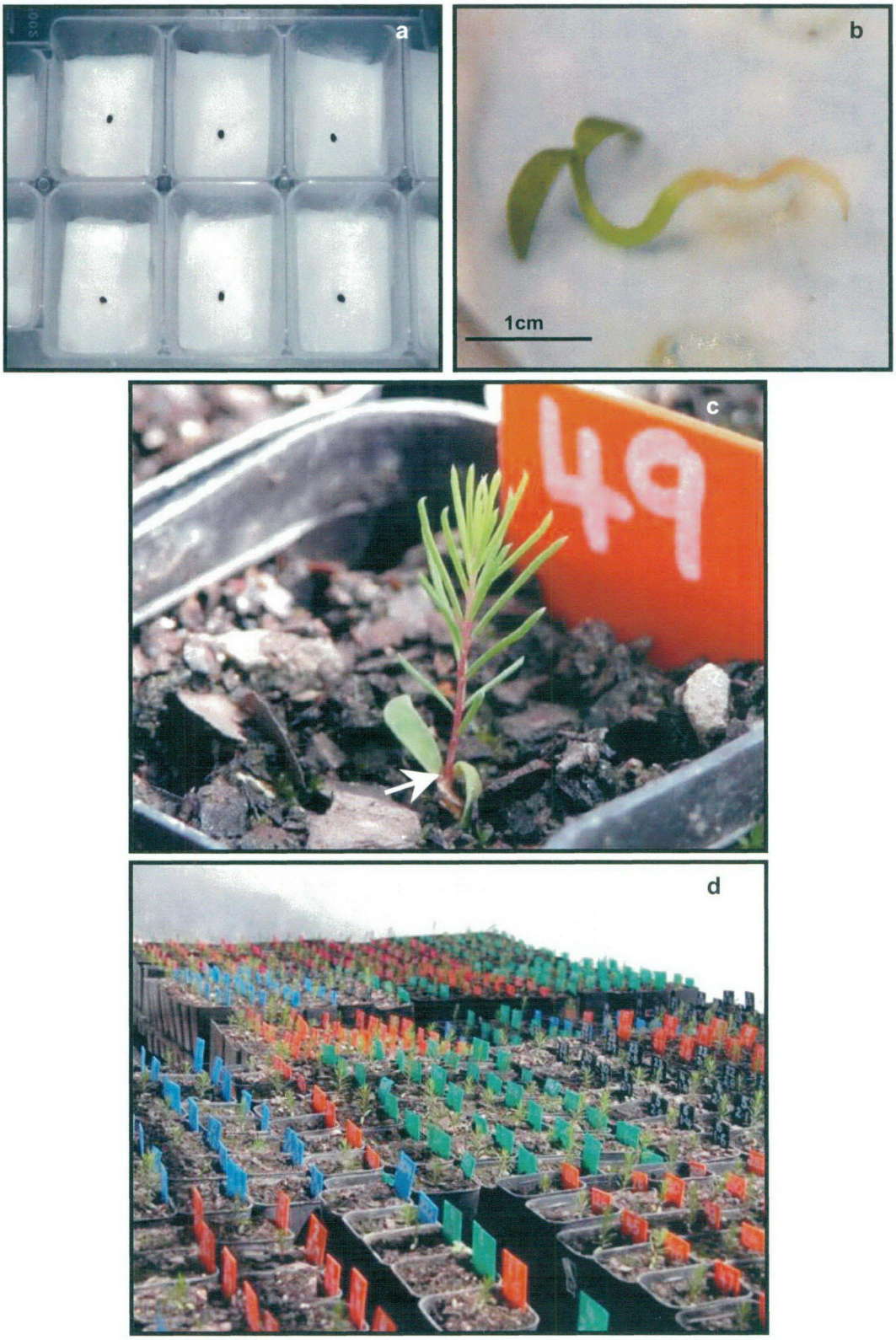



**Figure 6.6** **a)** *Dillwynia sieberi* seeds housed in ice-cube trays for Germination Experiment 1 **b)** a germinate from Germination Experiment 2 **c)** seedlings in glasshouse; arrow indicates point of cotyledon attachment; cotyledons leave scars which were used for consistency when measuring stem length **d)** seedlings from both GE1 and GE2 in the glasshouse.

### 6.3.2 Stigmatic Pollen Loads and Pollen Tube Growth

A simple assessment of stigmatic pollen loads, pollen germination and pollen tube growth using fluorescence microscopy techniques was undertaken for the three study species. Material was fixed and stored in FAA (5% formalin: acetic acid: alcohol) (Kearns & Inouye 1993) and later transferred to aniline blue stain. Slides were then prepared for viewing under the fluorescence microscope (see below for individual methods). The aniline blue solution was prepared by dissolving 0.01875 g of aniline blue into 150ml of 1M solution of $KH_2PO_4$ (made by dissolving 9.11g of the salt into 1L $H_2O$). (Decolourisation should occur within 2 hours; using a magnetic stirrer speeds up this process). The pH of the solution was then adjusted to 8.5. Material was viewed using ultraviolet light under an Olympus BH-2 microscope with a Mercury lamp (HBO 100W/2). The microscope set up consisted of a 20UV-W excitation filter, L435 barrier filter and a BH-DMU dichroic mirror.

For each species, standards using selfed and outcross pollen on field plants were produced to use as benchmarks for comparison of pollen tube growth against material collected from within sparse and dense plots. All material was placed in Epindorph tubes with FAA fixative immediately on collection in the field and stored until processing could be undertaken in the laboratory. It should be noted that for all species, assessment of stigmatic pollen loads/germination assumes that at least some pollen is likely to have been lost from the stigmatic surface during processing for fluorescence microscopy and results should be treated with this in mind. For all species, the general approach was to detect and quantify germinated and non-germinated pollen grains on the stigmatic surface and pollen tube growth down the style.

#### *6.3.2.1 T. australe*

All pollen tube material was collected from MOR. Autogamous standards were prepared by bagging stems with buds (3 stems on each of 10 plants) and returning several days later to collect open flowers. Only autogamy was assessed; hand-self pollen tube growth was not investigated. To check for outcross pollen tube growth, 10 plants with open flowers were randomly chosen; 62 flowers in total were treated. Each open flower on these plants was supplemented by hand with outcross pollen. Donor pollen was collected from a minimum of three different plants situated at least 5m away from the recipient plant. Since *T. australe* flowers are very small and contain relatively little pollen, entire

flowers were used as the pollination tool. Outcross-pollinated flowers were collected 2-3 hours post hand-pollination. Collection of flowers from sparse and dense plots (subject to open natural pollination) was undertaken as they became available; appropriate material was characterised simply as having open petals and no withering apparent.

Entire flowers were placed and stored in FAA fixative. In the laboratory, flowers were removed from the FAA and rinsed in distilled $H_2O$. All superfluous structures were removed with the assistance of a dissection microscope. Entire pistils were then placed in Epindorphs with aniline blue to stain up overnight. The following day, pistils were placed on slides, squashed under a coverslip and sealed with clear nail varnish. Pistils were then viewed under the fluorescence microscope. Pollen on the stigmas of *T. australe* (germinated and ungerminated) were readily observed and counted. The number of pollen tubes that reached the base of the style was also scored for material in which this was visible.

#### *6.3.2.2 W. luteola*

Pollen tube material was collected from all three *W. luteola* sites (POW, OAR and UNE). Outcross standards at each site were produced by randomly selecting flowers on up to ten individuals whose stigmas were open and supplementing them by hand with outcross pollen. Donor pollen came from a minimum of 3 individuals at least 5m away from the recipient plant. Pollen was transferred to the recipient stigmas using fine forceps. Flowers were tagged and collected 3-4 hours later. To produce the selfed standards, flowers on up to ten individuals at each site were tagged and bagged and checked regularly for stigmatic opening. Similar problems arose regarding stigmatic opening as for the selfing treatment during the breeding system experiment (Chapter 3) and pollen was similarly applied when the stigmatic surface had "cracked". Again, material was collected 3-4 hours later. Flowers were collected from sparse and dense plots (open to natural pollination). After removing excess petals and sepals, pistils were placed in FAA and stored until processing.

It was established that removal of the ovary from the stigma and style facilitated slide preparation and observation under the microscope. Therefore after removal from the FAA fixative, pistils were rinsed in distilled $H_2O$ and superfluous material was removed with the assistance of a dissection microscope, leaving only the stigma and style for examination. This component was placed in aniline blue overnight (as for *T. australe*

above) so that pollen on stigmas and pollen tube growth down styles could be observed and scored.

#### *6.3.2.3 D. sieberi*

Pollen tube material was collected from all three *D. sieberi* sites (POW, MOR and OAR). Self and outcross standard material was gathered at each site from ten plants (chosen outside of the designated sparse and dense plots). On each plant three buds of roughly the same age, on two separate stems were allocated as a self and an outcross treatment; all other flowers and buds were removed from the stem and stems were bagged. Buds were checked daily for opening. Outcross pollen was transferred to the recipient stigmas using fine forceps when flowers were one day old. Donor pollen was sourced from a minimum of 3 individuals greater than 5m away from the recipient plant. After cross-pollination, standard petals were marked with a paint dot so they could be recognised for collection the following day. Buds bagged for the self-treatment were left bagged until floral opening. Once flowers had opened, standards were similarly marked with a paint dot to identify them for collection the following day. After removal of excess floral material, pistils were placed in FAA as for the other species.

Flowers open to natural pollination were collected from sparse and dense plots at each of the three sites. Focal plants and their near neighbours were used as the source of material. Five stems per plot that were in bud were tagged. Stems were checked regularly for floral opening and, when a bud opened it was marked with a paint dot on the calyx for collection the following day. Therefore all open flowers were accessible to pollinators for a least one day before collection. After removal of excess floral material pistils were placed in FAA as for the other species.

Pistils of *D. sieberi* are hard making them difficult to squash for slide preparation. Therefore, soaking them overnight in a 1M NaOH solution was trialed in an attempt to soften them, but had little effect. Subsequently, pistils were longitudinally dissected in a "butterfly" manner but this caused too much damage to the material and the technique was abandoned. Finally, pistils were prepared by placing the entire pistil in aniline blue and stained over night (as for *T. australe* and *W. luteola*). When preparing slides the following day, the top few millimetres of the stigmatic end of the pistil was excised (at the point where the pistil began to bulge). This reduced the amount of tissue and removed hairs,

which obscured observation of pollen-tube growth. Nevertheless, pollen tubes were not readily observed in *D. sieberi* unless the pistil happened to split during squashing.
Scoring was based on the following questions;

1. Was pollen present on the stigma; if so was it germinating?
2. Could pollen tubes be identified in the style; if so how many?
3. How many pollen grains were germinating on the stigma?
4. How many pollen grains on the stigma failed to germinate?
5. What proportion of pollen grains germinated on the stigma?

If pollen grains (ungerminated or germinated) were too numerous to individually score, the stigma was deemed "saturated" with germinated pollen and the pollen count in this situation was arbitrarily assigned a score of 200. This was based on the fact that the total number of countable pollen grains never exceeded ~130. Pollen tubes in the style were scored as present or absent and those that were visible were counted. Data was assessed on both a categorical and quantitative basis.

## 6.4 Statistical Analyses

Analysis was undertaken using Statgraphics® *Plus* Version 5.1, 2001. One-way ANOVAs (type III sum of squares) were employed after data were checked for normality using Cochran's test. In addition, standard kurtosis and skewness values were checked to confirm normality. Non-normal data was transformed as necessary before analysis. Tukey's (HSD) was used to subsequently discriminate among the means. If data could not be adequately transformed and normalised, a Kruskal-Wallis non-parametric comparison of the medians was undertaken; differences among medians were determined using box and whisker plots (median notch) as generated by the software package. Simple linear regressions and Chi-square analysis were used where appropriate.

## 6.5 RESULTS

### 6.5.1 Seed Germinability, Viability and Offspring Growth

#### *6.5.1.1 T. australe*

Seed Germinability

A single germinate arose (Figure 6.1b) from the 30 fruits for which the initial trial protocol was applied. Although Scarlett & Parson's (1992) protocol elicited around 50% germination success from Victorian material (Scarlett, N.H. unpublished data, 1985), no germinates resulted from the second germination experiment using this protocol in this study. Attempts to germinate *T. australe* were then abandoned and offspring fitness attributes could not be measured for this species. However, it was found that the majority of the fruits that were subjected to the second germination experiment were empty of seed; this is an obvious problem, as without sampling destructively, one cannot determine seed content before subjecting fruits to germination experiments. This prompted further investigation to see if *T. australe* fruits could be assessed for seed content by weighing and to measure the incidence of empty fruit and seed viability. Fruit weight was also analysed as a fitness component and related to site and density (below).

Fruit Weight

Fruit weight was assessed using the material collected from MOR and ABR in 2005 (i.e. as for S: FR ratios Chapter 5) and data was pooled for site. The weight of *T. australe* fruits ranged from 0.5-8.8 mg (Figure 6.7). As might be expected, there was a steady increase in mean weight with percent seed fill. Although the mean weight of each % seed fill category differed significantly from one another ($F_{4,614}$=367.66, P=0.000 SQRT transformed), mean fruit weight cannot be used as an indicator of seed content with any surety, due to the large fruit weight range around each % seed fill category (Figure 6.7).

Fruit arising from sparse plots were heavier than those from dense at ABR, but this was significant only for open fruits (ABR open $F_{1,290}$ =5.93, P= 0.016; ABR autogamy $F_{1,154}$ =3.09, P= 0.081). Weights for open and autogamous fruits at MOR were similar between densities (MOR open $F_{1,144}$ =0.00, P= 0.959; MOR autogamy $F_{1,53}$ =0.06, P= 0.813). There was no difference in weight between fruit types at ABR, and when data were pooled for this site, fruits were significantly heavier in sparse plots than in dense plots ($F_{1,446}$ =9.01, P= 0.003). Alternatively at MOR, autogamous fruits weighed significantly

more than open fruits overall ($F_{1,199}$ =15.60, P= 0.000) but when assessed separately for density, this was only significant for dense plots ($F_{1,159}$ =12.70, P= 0.001) (Figure 6.8). In summary, a density effect was found for open but not autogamous fruits at ABR, but the effect at MOR was that of fruit type where, unexpectedly, autogamous fruit were heavier than open fruit.

Overall, fruits from MOR weighed less ($\bar{x}$ =2.12±0.07 mg, N=448) than those from ABR ($\bar{x}$ =3.01±0.06 mg, N=201). This is likely due to the fact that fruits at MOR had a lower rate of 100% seed fill and a higher rate of fruits that were void of seed compared with ABR (see Chapter 5), although variation in fruit size between sites would have to be considered to confirm this.

Seed Viability

Seed viability, tested using tetrazolium staining, revealed an increase in the incidence of viability with % seed fill (Figure 6.9), indicating an increase in fitness with seed size. When seed arising from autogamous and open fruit were assessed independently, the proportion of viable autogamous seed was found to be lower at both sites, but this was significant only for ABR ($F_{1,220}$=6.75, P=0.010; MOR $F_{1,69}$=1.91, P=0.172) (Figure 6.10a).

The viability of open and autogamous seed from sparse and dense plots were analysed separately to check for density effects (Figure 6.10b). At both sites, open seed from dense plots displayed higher viability than those from sparse plots (ABR $\chi^2$ = 16.33, P=0.000; MOR $\chi^2$ = 4.14, P=0.042 both with Yates' Correction), however autogamous seed was similar between densities for both sites (ABR $\chi^2$ = 1.72, P=0.189; MOR $\chi^2$ = 0.07, P=0.791 both with Yates' Correction).

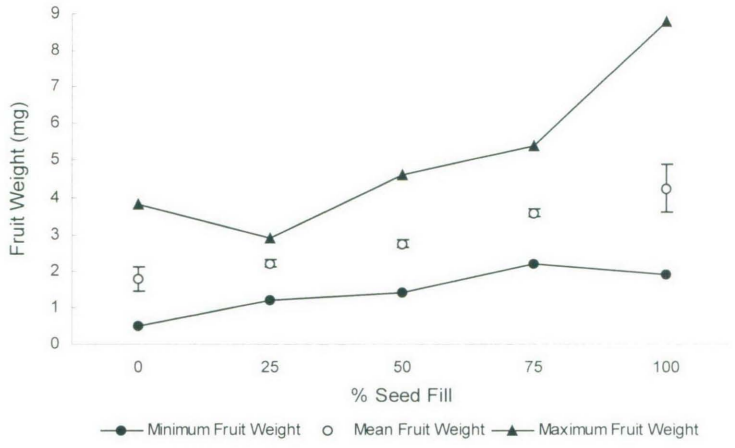



**Figure 6.7** Mean (±SE) and minimum and maximum weight (mg) of *T. australe* fruits from ABR and MOR for 0, 25, 50, 75 and 100% seed fill.

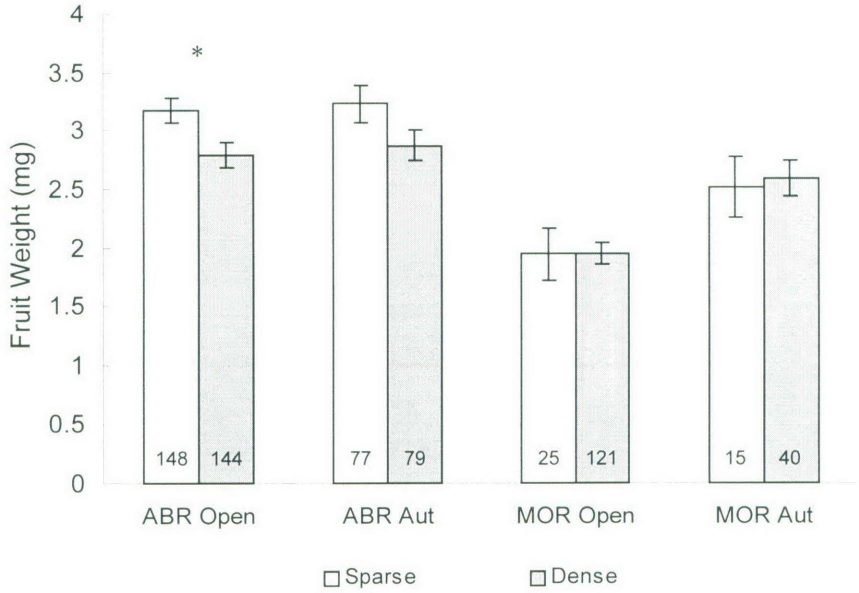



**Figure 6.8** Mean (±SE) fruit weight (mg) of *T. australe* open and autogamous fruits across two sites and densities. N (in base of columns)=number of fruit. * Denotes significant difference between densities.

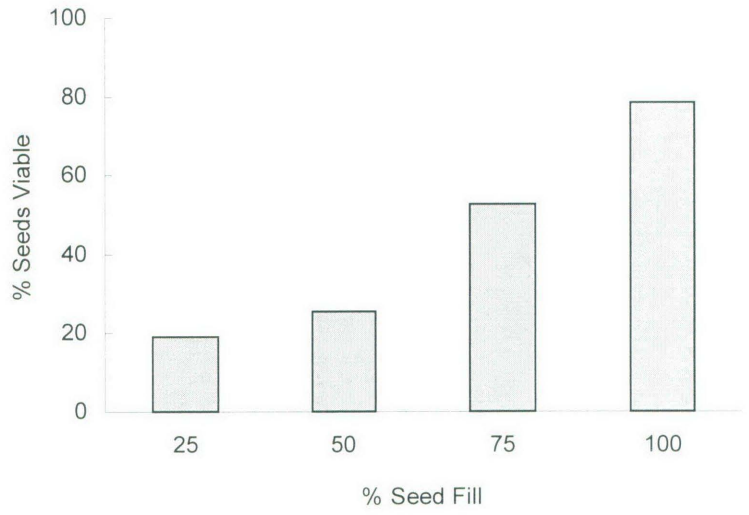
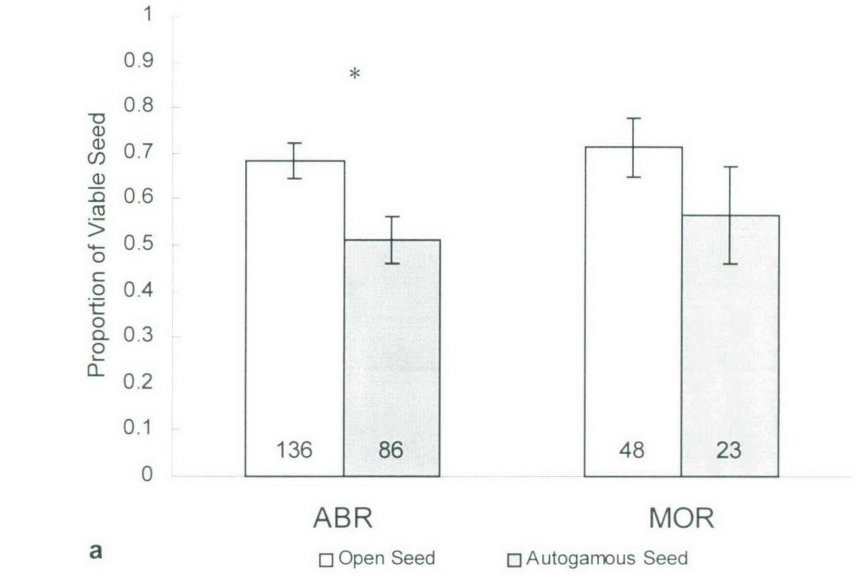



**Figure 6.9** Percentage of seeds viable in each seed fill category (i.e. fruit filled to the nearest 25%, 50%, 75% and 100% with seed) for *T. australe*.



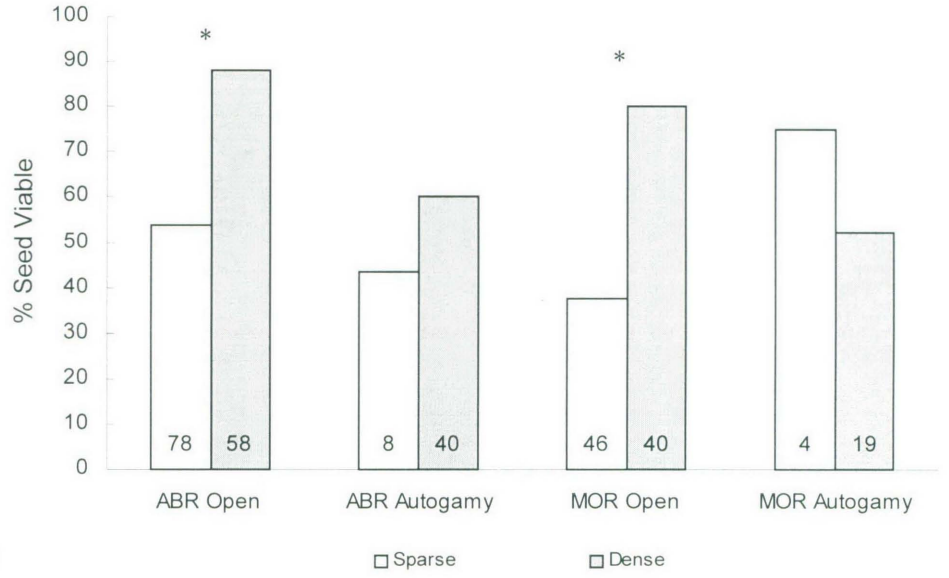




**Figure 6.10** **a)** Mean proportion (±SE) of open and autogamous *T. australe* seeds that were viable * Above columns = significant difference between seed types **b)** Percent viability of open and autogamous seed by density at two sites. * Above columns = significant difference between densities. N (in base of columns)=number of seeds.

### *6.5.1.2 W. luteola*

Individual Seed Weights

Seed weights were estimated using fruits collected from FPs. The entire seed sample for each fruit was weighed and the number of healthy seeds was counted. To estimate the weight of an individual seed in a given fruit, the weight of each seed sample was divided by the number of seeds contained in that sample. Mean individual *W. luteola* seed weights ranged from 0.037mg at OAR to 0.153 also at OAR. There was no influence of density on individual seed weight (POW $F_{1, 37}$=0.04 P=0.834; OAR $F_{1,11}$=1.75 P=0.212 LOG transformed; UNE $F_{1,21}$=0.44 P=0.515) and densities were pooled, however no significant difference was found among sites (H=5.709, P=0.0576 comparison of medians) (Figure 6.11).

When mean individual seed weight was regressed against the number of healthy seeds contained within a fruit, a weak negative relationship was found, where individual seed weight decreased with an increase in the number of seeds contained in a fruit ($y=0.000022-8.377x10^{-9}x$, P=0.0196, $R^2$=8.46%, r=-0.29).

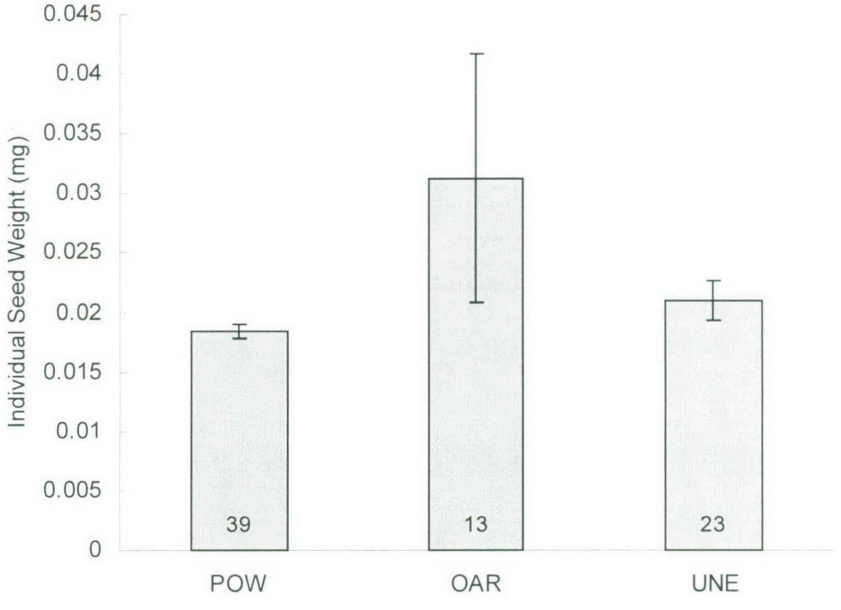



**Figure 6.11** Mean weight (mg) (±SE) of individual *W. luteola* seeds arising on FPs across three sites. N (in base of columns)=number of fruits.

Seed Germinability

*Wahlenbergia luteola* seed was not considered to have successfully germinated until cotyledon emergence, as cotyledon emergence did not always follow radicle emergence (Figures 6.13). The proportion of seed that successfully germinated by day 57 did not differ between densities (POW $F_{1,25}$=0.00 P=0.961; OAR $F_{1,9}$=0.05 P=0.85; UNE $F_{1,18}$=0.00 P=0.986). However, UNE displayed significantly higher levels of germination in both densities compared with POW and OAR (sparse $F_{2,25}$=11.75 P=0.000; dense $F_{2,27}$=20.75 P=0.000) (Figure 6.12).

There were some losses due to mould attack during the experiment. This did not appear to be related to the germination substrate, since 2 to 4 replicates were housed in the same petri dish and mould attack did not affect all replicates in the same dish. However, the OAR material appeared to be more readily affected than the other sites; therefore this variable was analysed. No significant difference was found between density for any site (POW $F_{1,13}$=0.19 P=0.670 LOG transformed; OAR $F_{1,9}$=0.18 P=0.681; UNE $F_{1,6}$=3.45 P=0.113 LOG transformed), but when densities were pooled, mould attack was found to be significant among sites ($F_{2,28}$=7.01 P=0.003 LOG transformed) with OAR (~16%) suffering a significantly greater level of attack than UNE (~0.7%). Loss at POW was ~3%, but this was not significantly different to OAR. Therefore, a large proportion of the lack of germination at OAR could be contributed to mould attack.

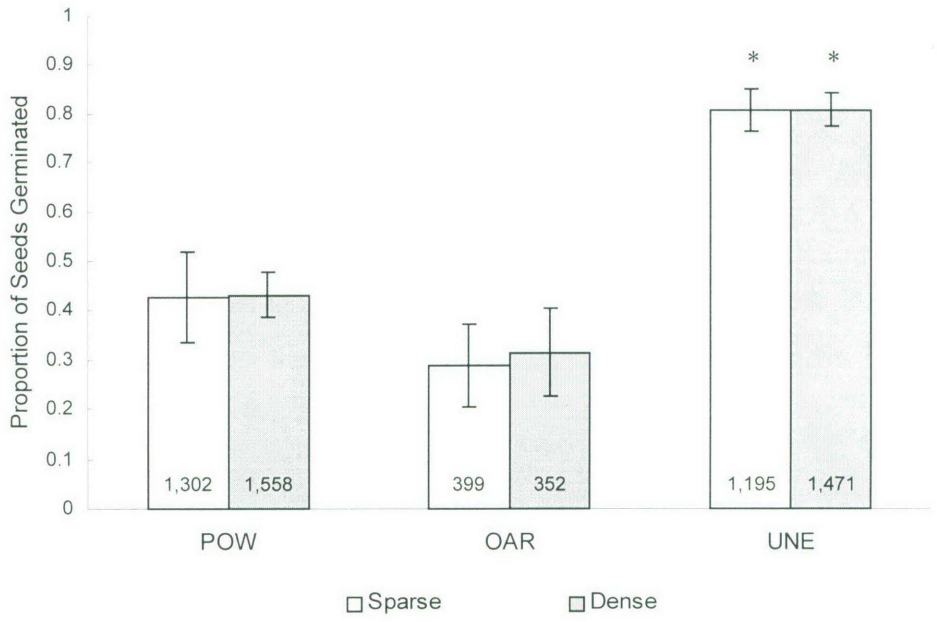



**Figure 6.12** Mean proportion of *W. luteola* seeds (±SE) that successfully germinated (i.e. progressed to cotyledon emergence) for two densities and three sites N (in base of columns)=number of seeds. * Above columns=significant difference among sites for density (P<0.05).

Radicle and Cotyledon Emergence Rates

The time between radicle emergence and cotyledon emergence varied greatly and post-radicle cotyledon emergence frequently failed to occur altogether. For instance, at day 36 only 38% of OAR seeds that had produced a radicle had also produced cotyledons, compared with 57% at POW and 84% at UNE. Figure 6.13a shows the proportion of seeds that did not produce a radicle over 36 days for the three *W. luteola* sites. There was no difference between densities within sites for this variable. However, a higher proportion of seeds from the UNE site produced radicles and produced them more rapidly than POW and OAR seeds on all monitoring days. For instance, at day three ~70% of seeds from UNE had produced a radicle and only ~20% at POW and 0% at OAR had reached this stage.

A similar pattern was observed for cotyledon emergence, which also occurred more rapidly and in greater numbers at UNE compared with the remaining sites (Figure 6.13b). Around 60% of seed had produced cotyledons by day 12 at UNE, yet only ~20% at POW and ~10% at OAR were at this stage on this day. Again, there were no significant differences within sites for density and densities were pooled. UNE cotyledon emergence was in significantly higher proportions than OAR and POW on all monitoring days, except for days 43 and 50, where POW was also significantly higher than OAR; by day 57 POW and OAR were again statistically similar.

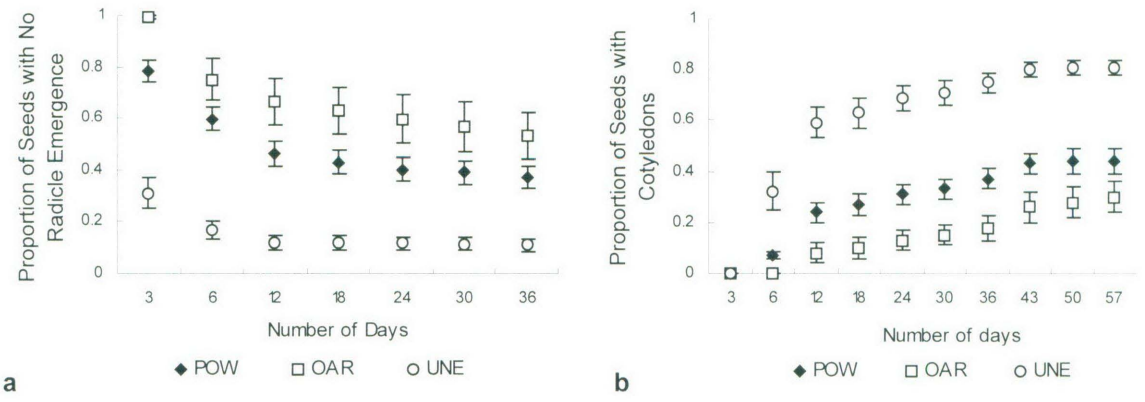



**Figure 6.13** **a)** Mean proportion (±SE) of *W. luteola* seeds from three sites that had not produced a radicle over 36 days **b)** Mean proportion of *W. luteola* seeds ±SE from three sites that had produced cotyledons over 57 days.

Seed Viability

The germination experiment was discontinued after 57 days, and many seeds remained that had either failed to show any signs of germination or had produced radicles but had failed to produce cotyledons. These seeds were subsequently subjected to tetrazolium viability testing. In addition, fresh seed (from the same plants) was also subjected to tetrazolium testing to estimate viability. Results are presented in Figure 6.14. Data was pooled for density, as there was no effect of density on viability at any site either in fresh seed (POW H=2.09, P=0.149; OAR $F_{1,8}$ =0.00, P=0.971; UNE $F_{1,20}$ =0.30, P=0.592 LOG transformed) or ungerminated seed remaining from the germination experiment (POW, $F_{1,26}$ =0.06 P=0.811; OAR $F_{1,8}$ =0.52, P=0.491; UNE $F_{1,6}$ =0.42, P=0.541). POW had the highest level of viability for fresh seed followed by OAR and then UNE, but there was no difference among sites (H=2.841, P=0.242).

When the viability of ungerminated seed was analysed UNE had a significantly lower level of viable seeds than the remaining sites ($F_{2,55}$ =20.64, P=0.000 SQRT transformed). This is most likely due to the fact that UNE displayed the highest level of success during the germination experiment and the majority of viable seed from this site had already germinated and were therefore underrepresented in the test sample. For POW and OAR seeds however, which were slower to produce cotyledons, many viable seeds may still have been in the sample tested at 57 days when the experiment was concluded. Had the germination experiment been extended beyond this time, it is likely that a greater number of successful germinations might have been scored for these two sites.

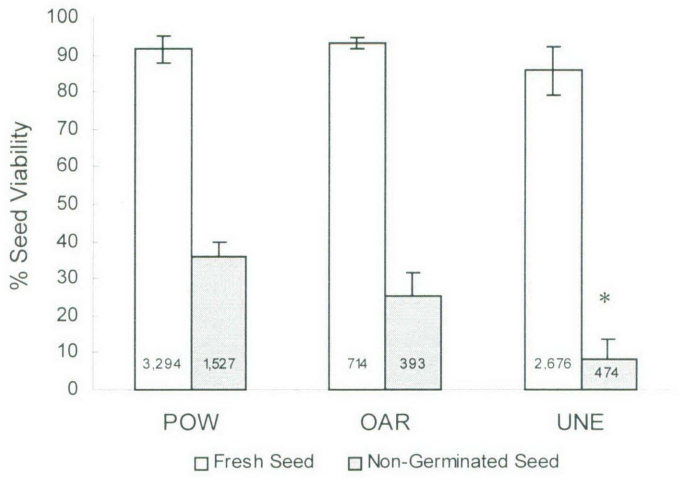



**Figure 6.14** Mean % viability (±SE) of fresh *W. luteola* seed arising from FPs and of ungerminated seeds remaining after the germination experiment. N (in base of columns)=number of seeds. * Above columns=significant difference among sites for seed type tested (P<0.05).

To assess whether seed weight imparted a fitness advantage in terms of days until radicle and cotyledon emergence, mean individual seed weights were regressed against mean proportion of seeds without radicle emergence at 3 days and proportion of seeds whose cotyledons had emerged at 6 and at 57 days. No significant relationship was revealed for these variables (radicle emergence 3 days $r^2$=0.87%, P=0.687; cotyledons 6 days $r^2$=0.002%, P=0.985; cotyledons 57 days $r^2$=1.88%, P=0.553). This suggests seed weight was not related to either radicle or cotyledon emergence.

Offspring Survivorship & Growth

To see whether progeny survivorship was related to density, seedlings were monitored for 40 days at 10-day intervals. Seedling survivorship was consistently and significantly higher for dense progeny than for sparse at UNE. This was also similar for POW, but the relationship did not become significant until 30 days for this site. OAR dense progeny on the other hand, were less likely to survive than sparse, but this was only significant at 40 days (Figure 6.15) (See Appendix G for analysis summary).

Very little growth was observed for *W. luteola* progeny over the first 20 days. In general, seedlings from POW grew more slowly than those from OAR and UNE. The mean height of POW seedlings from dense plots was greater at each monitoring day than those from sparse plots, but this difference was never significant (10d H=0.371, P=0.542; 20d $F_{1,71}$ =0.44, P=0.511 LOG transformed; 30d $F_{1,105}$ =1.93, P=0.167 LOG transformed; 40d $F_{1,106}$ =1.91, P=0.170 LOG transformed). Alternatively, for OAR and UNE, mean seedling heights were always greater for sparse progeny. This was significant only at 30 days for OAR (10d $F_{1,13}$ =1.58, P=0.232 LOG transformed; 20d $F_{1,33}$ =0.01, P=0.923 LOG transformed; 30d $F_{1,42}$ =5.97, P=0.019; 40d $F_{1,21}$ =1.72, P=0.204) and at 30 and 40 days for UNE (10d H=3.83, P=0.05; 20d $F_{1,81}$ =0.01, P=0.921 LOG transformed; 30d $F_{1,99}$ =4.62, P=0.034 LOG transformed; 40d $F_{1,106}$ =8.37, P=0.005 LOG transformed) (Figure 6.16).

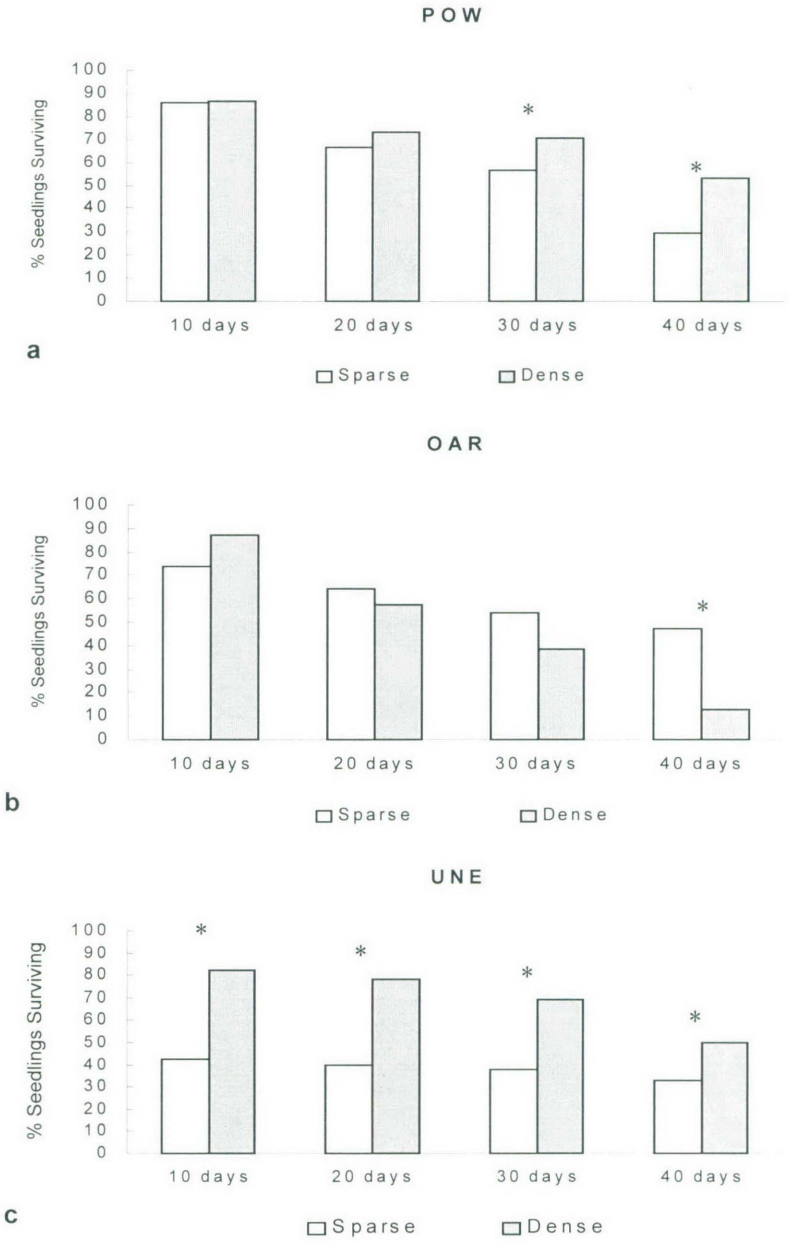



**Figure 6.15** **a)** Percentage of *W. luteola* seedlings from sparse and dense plots that survived up to 10, 20, 30 and 40 days for **a)** POW **b)** OAR & **c)** UNE. * = Significant difference between densities ($P<0.05$).

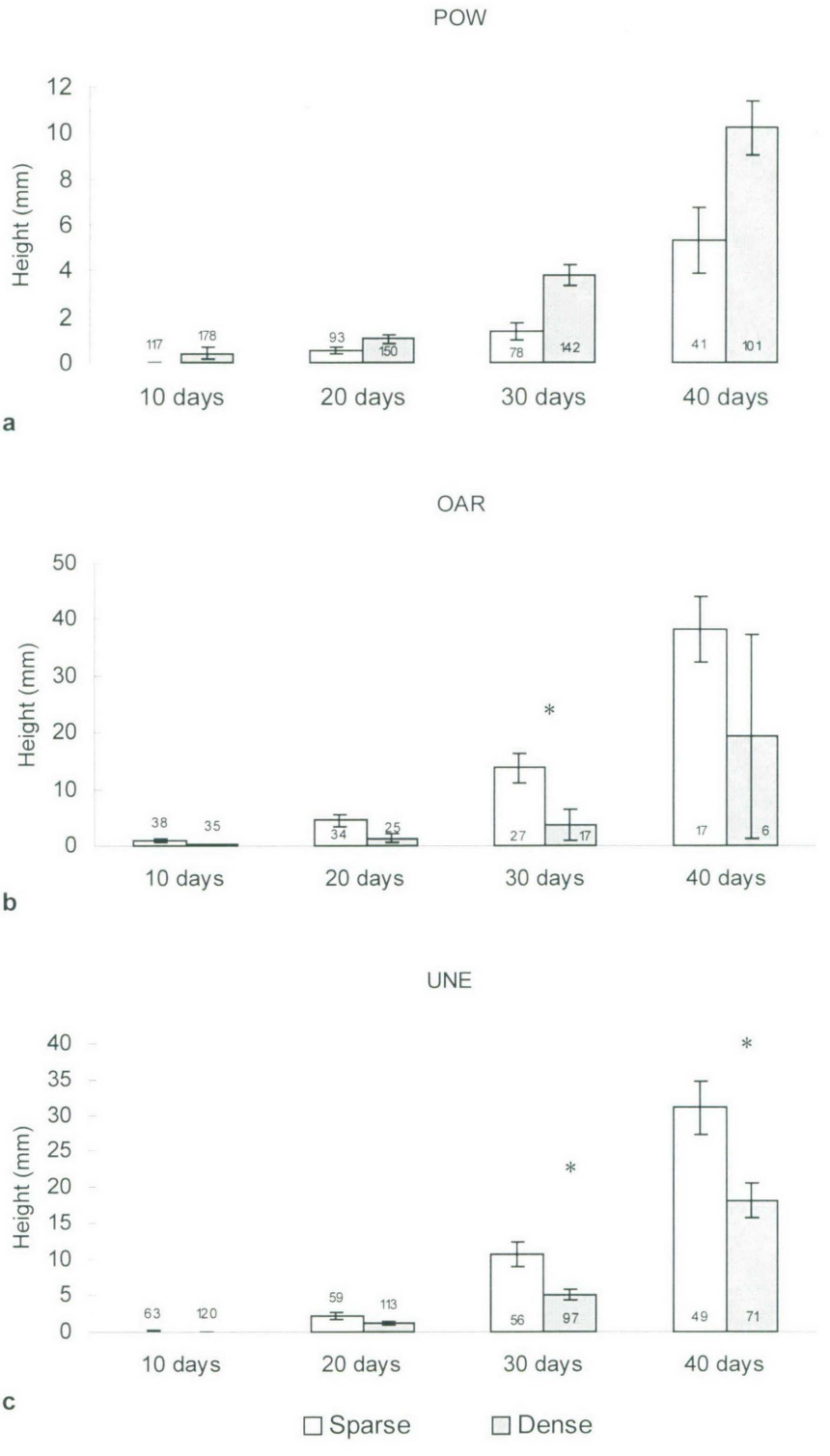



**Figure 6.16** Mean growth rates (±SE) of *W. luteola* seedlings over 40 days from **a)** POW **b)** OAR & **c)** UNE. N=number of seedlings measured * = Significant difference between densities ($P<0.05$).

### *6.5.1.3 D. sieberi*

Seed Weight

To see whether seed weight as a measure of maternal fitness was influenced by density, weights of seed used for GE1 and GE2 were assessed. Seeds from FPs (GE1) in sparse plots were significantly heavier at POW ($F_{1,301} = 13.09$, P=0.000) and MOR ($F_{1,186} = 32.95$, P=0.000). However, at OAR dense plots produced significantly heavier seed than sparse (H = 4.80, P=0.028 comparison of medians). In addition, seed weight varied among sites for sparse (MOR>OAR>POW $F_{2,407} = 36.8$, P=0.000) and dense plots (OAR>MOR>POW $F_{2,407} = 34.29$, P=0.000) (Figure 6.17).

To further dissect this finding and, since the level of maternal resources given over to seed may vary with the number of seeds contained within a fruit, the weights of the two seed types (i.e. from 1- or 2-seeded fruits) was also investigated. Seeds from 2-seeded fruits were consistently and significantly lighter than those from 1-seeded fruits across all sites (POW t=2.163, P=0.032; MOR t=6.31, P=0.000 LOG transformed; OAR H=870.5, P=0.000 comparison of medians) (Figure 6.18). The higher mean weight of seeds arising in sparse plots (Figure 6.16), is possibly influenced by the fact that more 1-seeded fruits (the seed of which is heavier on average than 2-seeded fruits) are produced in sparse plots than in dense (Figure 5.15-Chapter 5).

To investigate whether seed weight confers a fitness advantage to offspring, seed weight was regressed against several variables. Early germination should impart an advantage to seedlings, but weight had no bearing on days until radicle emergence or days until cotyledon emergence. However, cotyledon size at 10 days after emergence was positively correlated with seed weight for GE1 and GE2 (Figure 6.19). Thus, heavier seeds produce larger cotyledons. Furthermore, seed weight was positively related to growth (main stem length) at least at day 30 and day 45 for GE1 and GE2 (Figure 6.20). This relationship was less consistent in later stages of growth.

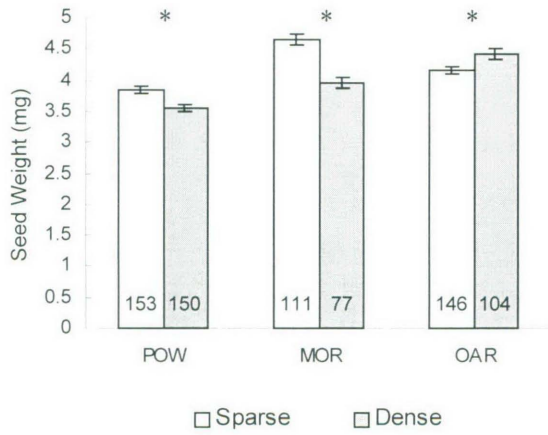



**Figure 6.17** Mean weight (mg) (±SE) of seed arising on focal plants (GE1) in sparse and dense plots from three sites. N (in base of columns)=number of seed. * Above columns=significant difference within site for density (P<0.05).

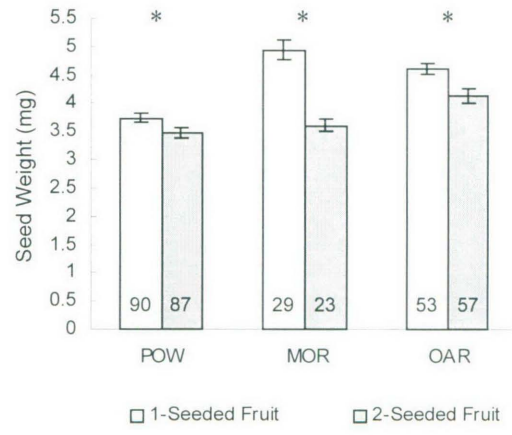



**Figure 6.18** Mean weight (mg) (±SE) of seed arising from 1- and 2-seeded fruits (GE2) from three sites. N (in base of columns) = number of seeds. *Above columns=significant difference within site for seed type (P<0.05).

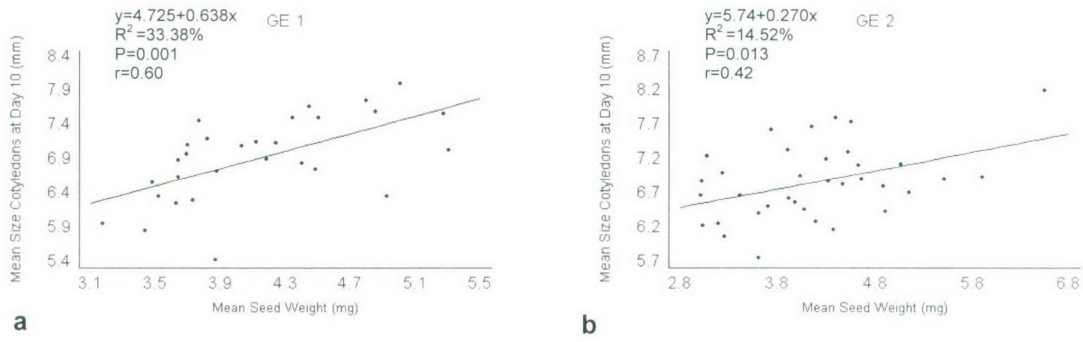



**Figure 6.19** Relationship between mean seed weight and mean cotyledon length (mm) as measured 10 days after cotyledon emergence for seeds subjected to **a)** GE 1 and **b)** GE 2.

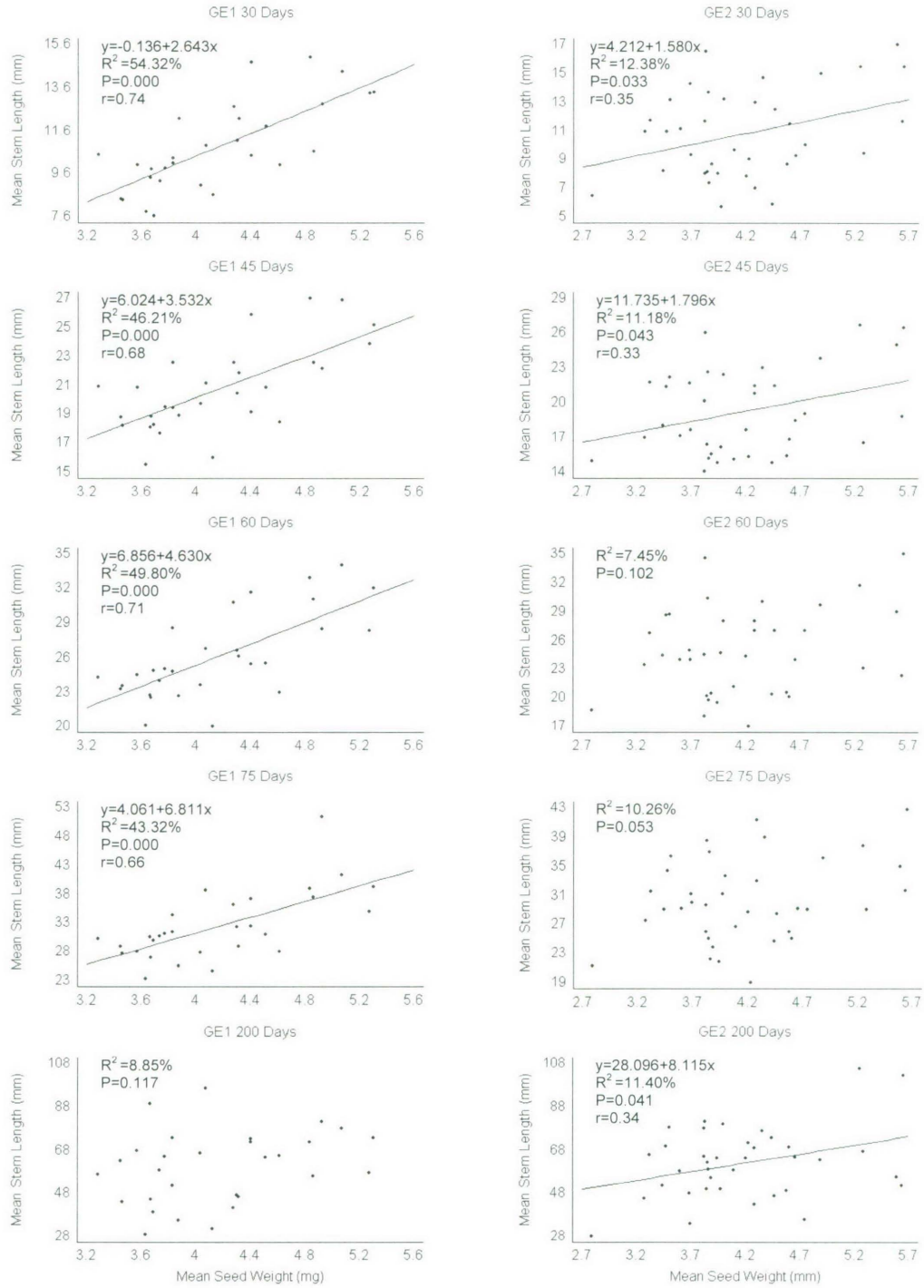



**Figure 6.20** The relationship between mean seed weight and mean growth (stem length) of *D. sieberi* seedlings at 30, 45, 60, 75 and 200 days for two germination experiments (GE1 & GE2).

Seed Germinability

Overall germination was very high across all sites and densities for *D. sieberi* for Germination Experiment 1 (GE1). On some occasions, a radicle was produced, but spontaneously broke off at the site of emergence and development did not progress (10.2% of seed for GE1 and 5.03% for GE2). These seeds were considered to have not successfully germinated, thus successful germination was defined as a seed that not only produced a radicle, but also went on to produce cotyledons. Only 0.74% of seeds from GE1 failed to produce a radicle at all, which was considerably less than the 18.88% recorded for GE2. For GE1, percent successful germination did not differ significantly among sites for sparse ($\chi^2$ = 1.81, P=0.404) or dense plots ($\chi^2$ = 3.45, P=0.178) and there was no difference between densities when sites were pooled (sparse=90.16%; dense=92.34%) ($\chi^2$ = 0.36, P=0.0.548 with Yates' correction for continuity), giving an overall germination rate of 91.25%. In Germination Experiment 2 (GE2), overall successful germination was somewhat lower (than for GE1) (MOR 86.4%; OAR 84.4%; POW 54.8%). Therefore, there appeared to be no effect of density or site on the germinability of *D. sieberi* seeds for either germination experiment. Lower levels of germination in GE2 compared with GE1 are possibly due to the fact that fruits were collected somewhat earlier for GE2, as predehiscent fruits for the purposes of assessing the occurrence of 1- and 2-seeded fruits were required. Thus, the seeds from GE2 were possibly slightly immature compared with those collected for GE1, which had been left until fruit dehiscence was complete. Alternatively, it could reflect a seasonal difference between 2004 and 2005 seed production.

Percent successful germination of seed arising from 1- and 2-seeded fruits did not differ between seed types or within sites, but both seed-types had significantly lower levels of germination at POW (Table 6.2).

Radicle and Cotyledon Emergence Rate

There was no difference within site between densities for mean number of days until radicle or cotyledon emergence, with the exception of Moray (GE2) where cotyledons from seeds in dense plots took significantly longer to emerge than those from sparse (however, small sample size may be influencing this result) (H=4.93, P=0.026 Kruskal Wallis comparison of medians). This indicates that the rate of seed germination is not related to density. Seeds resulting from 1- and 2-seeded fruits were also assessed for days

until radicle and cotyledon emergence (Figure 6.21). Radicle emergence was somewhat faster for 2-seeded, but this was not statistically significant within any site (POW $F_{1,116}$ = 0.70, P=0.404; MOR $F_{1,47}$ = 2.27, P=0.138; OAR $F_{1,105}$ = 0.46, P=0.498). Cotyledon emergence was faster for 2-seeded at two sites, but only significant for one (POW $F_{1,93}$ = 8.67, P=0.004; OAR $F_{1,85}$ = 1.52, P=0.220 sqrt transformed). At the third site seeds from 1-seeded fruits produced cotyledons faster than 2-seeded, but this difference was not significant (MOR $F_{1,34}$ = 0.45, P=0.508 log transformed). In general, it appears that germination (radicle and cotyledon emergence) occurs more rapidly in seeds that arise from 2-seeded fruits, but there is variability among sites for both seed types (see Appendix H for analysis summary).

**Table 6.2** Percent successful germination of *D. sieberi* seed arising from 1- and 2-seeded fruits three sites.

| | % Germination (N)=Number of Seeds | | | |
|---|---|---|---|---|
| **Seed Type** | **POW** | **MOR** | **OAR** | **Among Site Differences** |
| **1-Seeded** | 50.6 (89) | 80.8 (26) | 83.3 (54) | $\chi^2$=19.12 P=0.000 |
| **2-Seeded** | 59.8 (87) | 91.3 (23) | 82.5 (57) | $\chi^2$=13.75 P=0.001 |
| **Within Site Differences** | $\chi^2$=1.16 P=0.282 | $\chi^2$=0.41 P=0.520 | $\chi^2$=0.00 P=1.00 | |

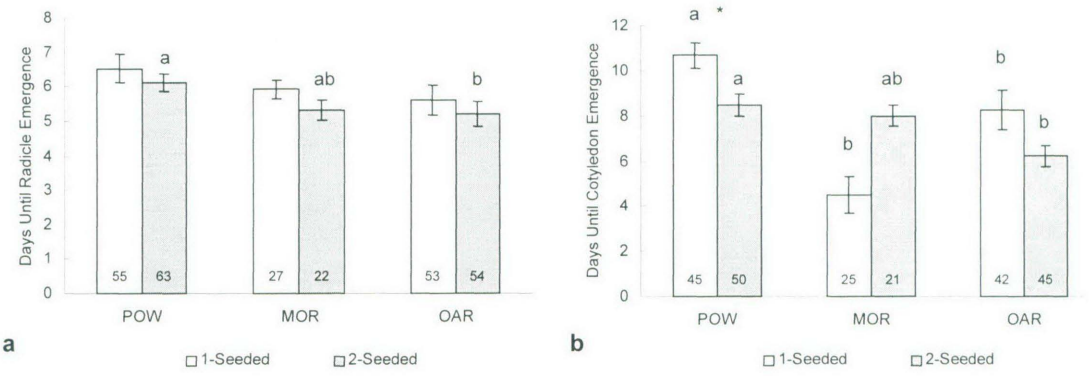



**Figure 6.21** Mean days until emergence (±SE) for *D. sieberi* seed arising from 1- and 2-seeded fruits at 3 sites **a)** days until radicle emergence **b)** days until cotyledon emergence N (in base of columns)=number of seeds. Letters above columns denote differences among sites for seed type. * Above column pairs=significant within site for seed type (P<0.05).

Viability of Ungerminated Seed

When the ungerminated *D. sieberi* seeds remaining from GE2 were tested for viability, only 4 out of 60 seeds were scored as viable; 3 of these were from 1-seeded fruits. Therefore, virtually all viable seeds germinated under the conditions imposed during the experiment. This suggests that GE2 seed had a much lower level of viability overall than GE1 seed, possibly for similar reasons as outlined above. The three ungerminated seeds remaining after GE1 were not subjected to tetrazolium staining.

Survival and Growth Rate of Progeny

Survival of seedlings from GE1 was very high (~90%) across all sites and densities (Table 6.3). Chi-square analysis revealed no significant differences for survival among sites for density (sparse $\chi^2$ = 15.36, P=0.119; dense $\chi^2$ = 8.79, P=0.552) or among sites when densities were pooled ($\chi^2$ = 11.65, P=0.310). Seedlings from GE2 displayed a lower survivorship (~82%) (Table 6.2) across all sites and densities. This was significantly lower than survivorship of seedlings from GE1 ($\chi^2$ = 15.16, P=0.01). There were no differences among sites for density (sparse $\chi^2$= 15.57, P=0.05; dense $\chi^2$ = 5.78, P=0.449) or among sites when densities were pooled ($\chi^2$= 13.61, P=0.093). Additionally, seedlings arising from 1-seeded (84.21%) and 2-seeded fruits (81.10%) were each as likely to survive as the other ($\chi^2$ = 7.28, P=0.12). Thus, density and site did not appear to influence seedling survival.

**Table 6.3** Cumulative percentage mortality and overall survival of seedlings transferred to the glasshouse for GE1 and GE2 for the 200 day period over which growth rate and survival were monitored.

| | **Cumulative Mortality Over 200 Days** (%) | | | | | |
|---|---|---|---|---|---|---|
| | **30** | **45** | **60** | **75** | **200** | **Total Survival** (%) |
| **Germination Exp. 1** | 2.71 | 4.07 | 4.61 | 4.88 | 8.94 | **91.06** |
| **Germination Exp. 2** | 8.71 | 10.78 | 10.78 | 11.61 | 17.43 | **82.57** |

Growth Rates of *D. sieberi* Progeny (GE1)

At POW and MORE there was a general trend for progeny from sparse plots to grow more rapidly than those from dense, but this was not always significant. For instance at POW, the trend only became significant at day 75, remaining significant until day 200 but at MOR was significant at day 10 only (Figure 6.22). At OAR growth rate was similar between densities until day 200, where dense progeny possessed significantly longer main stems than those from sparse plots (Figure 6.22). (See Appendix F for analysis summary).

Growth Rates of *D. sieberi* Progeny (GE2)

The mean growth rate of seedlings arising from 1- and 2-seeded fruits was assessed to check whether seed size conferred advantages to offspring. A significant difference in growth rates was found at POW, with 1-seeded progeny growing consistently faster than 2-seeded up until day 75; by day 200, the difference was no longer significant. This pattern was not observed at MOR or OAR (Figure 6.23) (see Appendix F for analysis summary).

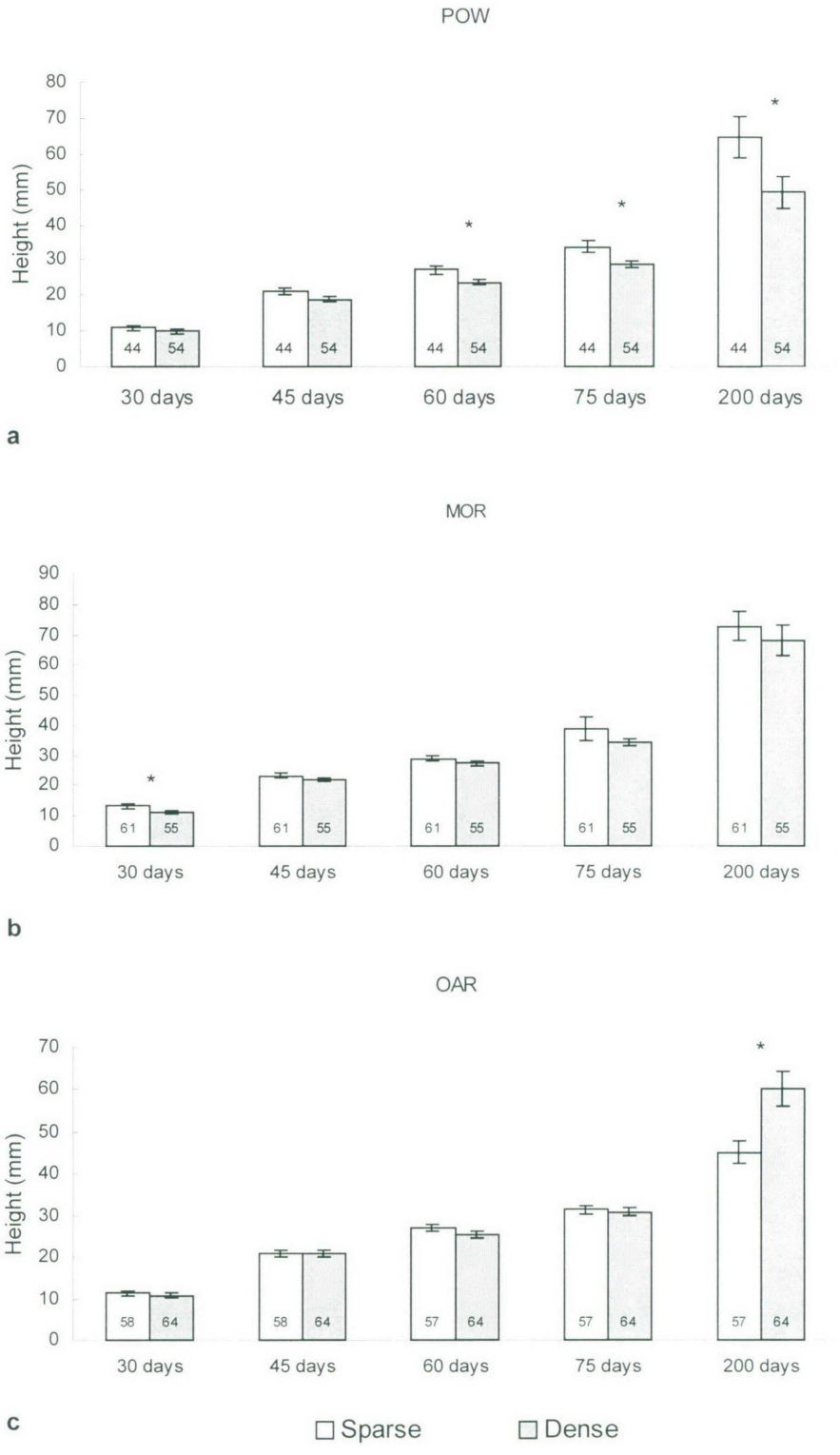



**Figure 6.22** Mean growth rate (mm) (±SE) of *D sieberi* progeny over 200 days for GE1 (seed arising on focal plants) at **a)** POW **b)** MOR **c)** OAR. N (in base of columns)=number of seedlings. *=Significant difference between densities ($P<0.05$).

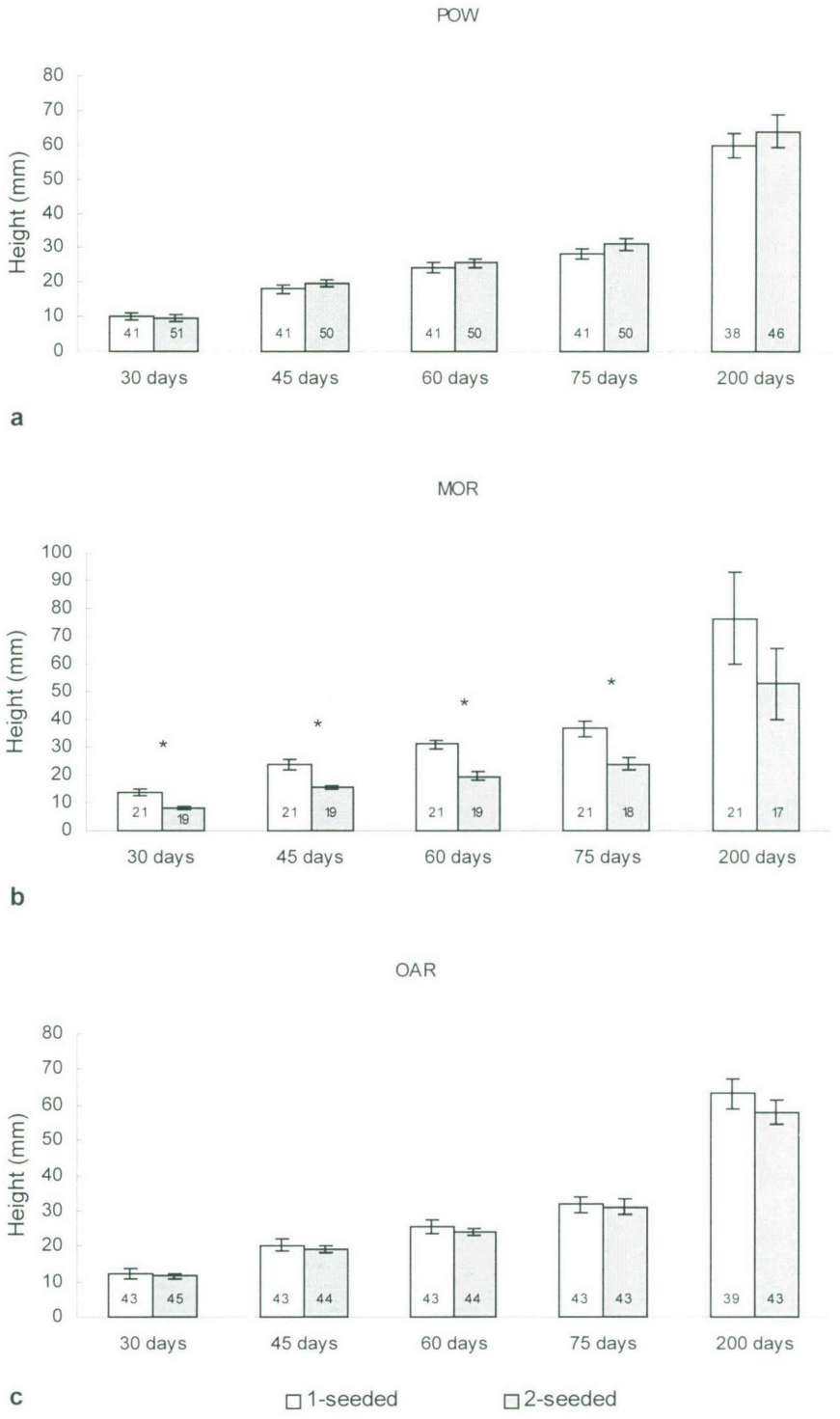



**Figure 6.23** Mean growth rate (mm) (±SE) of progeny arising from 1- and 2-seeded fruits over 200 days at **a)** POW **b)** MOR **c)** OAR. N (in base of columns)=number of seedlings. *=Significant difference between 1- and 2-seeded progeny ($P<0.05$).