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# Pre-emergence processes limit seedling recruitment in two direct-seeded *Acacia* spp.

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**Keywords**

Direct Seeding, Life-history Stage Transition, Recruitment Bottleneck, Seedling Survival, Seed Mortality

**Abstract**

The use of direct seeding for revegetation often results in poor recruitment outcomes. For many species, it is unclear where recruitment bottlenecks occur in the transitions between early life-history stages and how soil moisture conditions affect these bottlenecks. Thus, we asked: (1) which life-history stage transitions are most limiting to seedling recruitment? and (2) how do soil moisture levels affect recruitment? Using a field-based trial, we quantified the recruitment process from a seed to seedling for two woody *Acacia* species. Using a novel technique, in which seeds were confined to germination caches (small baskets), along with the use of germination bags, we assessed pre-emergence processes at regular intervals. Seeds sown directly into the soil in adjacent rows were intensively monitored to assess post-emergence processes. To investigate the effects of soil moisture on seedling recruitment, a glasshouse experiment assessed transitions between life-history stages under three different soil moisture treatments. In the field, the transition between a seed and germinated seed limited recruitment more than all other life-history stage transitions combined, with 32%–50% of seeds not germinating for the two species. Approximately one third of seeds of both species died prior to

germinating, with few seeds remaining dormant. In the glasshouse trial, seed germination increased with increasing soil moisture, however, so did the extent of seed death. Our results suggest that the transition from a seed to germinated seed was the most limiting bottleneck to recruitment, mostly due to seed mortality rather than dormancy processes, with pathogen attack the most likely cause. As increased soil moisture both promoted germination but also seed mortality, understanding the optimal soil moisture thresholds that maximise germination and the transition to an established plant is essential in maximising direct seeding outcomes. Identifying the life-history stage transitions most limiting to plant recruitment may allow management to target specific bottlenecks in order to improve direct seeding outcomes.

# 1. Introduction

Anthropogenic pressures are leading to major losses of biodiversity and ecosystem functioning and services globally (Fischer & Lindenmayer, 2007; Foley et al., 2005). Active restoration through revegetation can help reverse some of these losses (Munro et al., 2012). Direct seeding is an important revegetation technique, with the potential to be much cheaper than planting (e.g. Ede et al., 2018; Palmerlee & Young, 2010), however, seedling recruitment rates from direct seeding are often low (Grabau et al., 2011; Hallett et al., 2014), even when seed lots have high viability (Clarke & Davison, 2001). For example, a recent meta-analysis of planting and direct seeding outcomes found that on average only 18% of seed sown in direct seeding resulted in a seedling (Palma & Laurance, 2015). Rates of cabinet germination have also been found to be unreliable in predicting emergence with Gibson-Roy et al. (2007) reporting some grass species had >75% germination *in vitro* but <3% emergence in the field. The ability of direct seeding to be a reliable revegetation tool, both in terms of recruitment success and financial benefits, therefore requires identification of the key early life-history stage transitions limiting

recruitment in order to predict direct seeding outcomes and target management efficiently (James et al., 2013).

During the early recruitment stages, seeds and seedlings are highly vulnerable, particularly after direct seeding in which seeds may be sown at, or near, the surface. Initial predation of seeds by ants and granivores may reduce seed numbers (Davidson & Morton, 1984), and pathogen attack may also contribute to seed mortality (Leishman et al., 2000). Desiccation during imbibition and radicle emergence may occur if soil water potential falls below a threshold due to high temperatures and/or low rainfall and similarly, young seedlings may die when soil moisture reaches permanent wilting point (McKenzie et al., 2004). After germination, the successful emergence and survival of seedlings may also depend on overcoming edaphic factors such as surface crusting and waterlogging (Mayence et al., 2017; Ruiz Talonia et al., 2017) along with stressors relating to drought (Hallett et al., 2014). Furthermore, biotic factors such as competition with other seedlings and extant vegetation (Davis et al., 1999), herbivory (Hanley, 1998) and diseases such as those caused by *Phytophthora* spp. and *Pythium* spp. (Cahill et al., 2008; Reid et al., 2013; Roux & Wingfield, 1997) all potentially limit plant recruitment.

Soil moisture is a key driver of early plant life-history processes. The accumulation of time under a specific soil water potential is crucial for seed germination and subsequent processes (Bradford, 2002; Huarte & Benech-Arnold, 2007). Sowing seeds into soil moisture conditions that are optimal for germination, emergence and seedling survival will maximise seedling recruitment rates (Abbott & Roundy, 2003). Understanding the soil moisture conditions required by a seedling to transition through the early life-history stages can help to inform management to increase seedling recruitment (Atondo-Bueno et al., 2016). For example, watering seedlings through summer, in combination with seedling guards, increased survival and height for some myrtaceous species in a Mediterranean-type woodland (Ruthrof et al., 2013). Data is lacking, however, on how soil moisture influences seed to seedling transitions.

Due to the difficulty of studying below-ground life-history stages in soil or potting media, studies investigating recruitment bottlenecks in the transition from a seed to emergent seedling are limited. Consequently, identifying key drivers contributing to recruitment failure may be missed, particularly if processes below the surface (pre-emergence) are limiting (Larson et al., 2015). For example, the transition from germination to emergence was found to be the most critical bottleneck to establishment of grassland species in Oregon, USA, with an average of 72% of seeds germinating but as few as 7% of germinants emerging, likely due to seasonal effects post sowing (James et al., 2011). Similarly, a study of seed of three species of *Acacia* sown under a range of sowing depths and soil types found that seed either did not germinate, or germinated and subsequently died before emergence, likely due to low soil moisture and surface crusting (Mayence et al., 2017). The findings from these studies suggest key drivers of plant recruitment failure may often be incorrectly attributed to a lack of germination and/or post-emergence (aboveground) processes.

In order to ascertain which life-history stage transitions best explain low rates of recruitment in the field, we used novel methods to study two woody *Acacia* species with hard seed coats, commonly sown in revegetation activities in south-eastern Australia. We asked: (1) which life-history stage transitions are most limiting to seedling recruitment? and (2) how do soil moisture levels affect recruitment? We hypothesised that: (1) below-ground processes would represent the greatest bottleneck to seedling recruitment; and (2) that higher soil moisture content would increase seed germination and seedling recruitment.

# 2. Methods

## 2.1. Study species and seed information

The two study species were *Acacia paradoxa* DC. and *A. verticillata* (L'Her.), both woody shrubs commonly used in revegetation projects across south-eastern Australia. *A. paradoxa* is distributed over a wider geographic range than *A. verticillata*, which is confined to cooler regions of SE Australia. Both species have hard seed coats, with the oblong seeds of *A. paradoxa* varying between 3.5–5 mm long (with a seed weight of ca. 55 seeds/g), and the elliptic seeds of *A. verticillata* varying between 3–4 mm long (ca. 139 seeds/g). All seed was sourced from the same seed lot during October 2016.

Pre-treatment to overcome physical (hard-seeded) dormancy typical of *Acacia* spp. was applied. Pre-sowing treatments involved submerging the seeds in just boiled water for 1 minute, then removing the seeds and leaving them to soak in cool smoke water (Regan Smokewater 1:10 dilution) overnight to imbibe (Greening Australia 2003; see also Ede & Greet 2021). Seeds were imbibed in smokewater instead of spraying them with smokewater, as is common practice in the direct-seeding process in revegetation projects across Victoria, Australia. All seed was treated prior to sowing, except where specified in an experiment comparing germination of treated and untreated seed.

A germination viability trial was undertaken using cabinet incubation of seeds, however, the trial was compromised by a pathogenic attack despite standard protocols being used and other concurrent trials being unaffected. Seed appeared in excellent condition, and any obviously damaged seeds were not included in the experiments. Given the high germination rates described in the results section, and the fact we were able to inspect each seed, we believe the viability was as high as can be expected for the two species. We discuss viability and study limitations in the discussion section.

## 2.2. Field trial

### *2.2.1. Site and environmental conditions*

Our field site was located at The University of Melbourne's Burnley campus in Melbourne, Australia (-37.830070°, 145.024300°, 13 m elevation) and was representative of potential sites that may undergo revegetation works. The soil on site is a sandy loam with increased organic matter from a pine bark-based compost introduced in past experiments and the site is fenced off from larger herbivores. Irrigation was set up with four overhead sprinklers which were used only twice to apply ca. 3 mm of water during particularly dry periods.

Mean annual rainfall for the area is 663 mm and mean monthly rainfall from April-October (study period) is 54 mm (Bureau of Meteorology 2020). Monthly mean maximum and minimum temperatures for April-October are 15.7 and 8.5 °C, respectively (Bureau of Meteorology 2020). Soil moisture was measured weekly with a Hydrosense II soil moisture probe (Campbell Scientific®) beside each of the 12 sown rows. Air and soil temperatures were recorded at 30 minute intervals with ibuttons® (Maxim Integrated™), with 4 placed 30 mm above, and 6 placed 30 mm below, the soil surface.

### *2.2.2. Field trial environmental conditions*

Total rainfall for the field trial over 6 months was 290 mm with 123 mm falling in the first month after sowing, 2.7 times the historical average. The high rainfall in month 1 was followed by low rainfall in month 2 (25 mm) and rainfall 3 times below the historical average in month 3 (16 mm) (Bureau of Meteorology 2020). The weekly estimated volumetric water content (VWC), averaged across the six months, was 19.9% with the lowest, 12.7%, and highest, 30.5% occurring in the first month (Figure 1). Ambient air temperatures ranged from a mean minimum of 8.6 $^{o}$C to a mean maximum of 16.7 $^{o}$C with the highest daily maximum temperature recorded 30.9 $^{o}$C towards the end of the experiment, and the lowest daily minimum of 0.8 $^{o}$C occurring during month 4 (Bureau of Meteorology 2020). Temperature

ranges 30 mm above and below ground showed greater variation than ambient air temperatures and above-ground temperatures had a wider range than below-ground temperatures, which were more consistent over the study (Table 1).

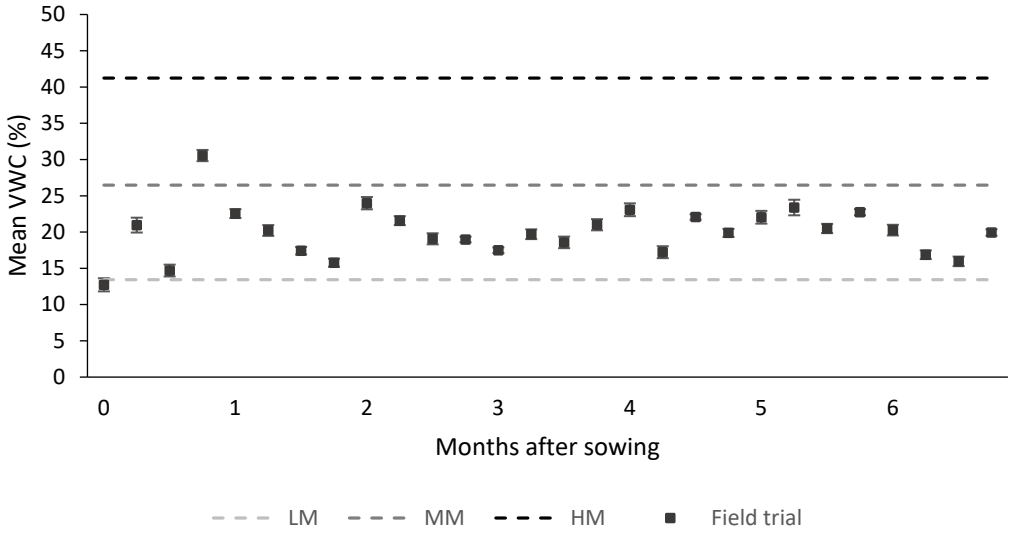



Figure 1. Mean VWC during the 6-month field trial and treatment means taken from the glasshouse soil moisture experiment for; low moisture (LM, 13.4 ± 0.1%); medium moisture (MM, 26.5 ± 2.4%); and high moisture (HM, 41.2 ± 0.4%). Mean ± 1SE are shown for the field.

Table 1. Daily mean temperature ranges 30 mm below and 30 mm above the soil surface during the field trial, measured using temperature loggers (ibuttons®).

| | Daily mean range (°C) | Daily minimum range (°C) | Daily maximum range (°C) |
|---|---|---|---|
| **Above ground** | 4.1–26.4 | -3.5–23.4 | 9.9–40.5 |
| **Below ground** | 4.1–24.4 | 0.7–21.6 | 10.1–33.5 |

### *2.2.3. Site preparation and experimental design*

In April 2017, a 14 × 8 m site was sprayed with the broad-spectrum herbicide glyphosate three weeks prior to being cultivated with a rotary cultivator. The site was raked free of dead weeds and levelled with a hand operated lawn roller. Throughout the experiment, weeding was maintained across the site using a Dutch hoe between rows while weeding within rows was conducted carefully using pruning snips to limit soil disturbance. The experiment was run for 12 weeks during the austral autumn and winter (5 April to 29 June 2017) to measure early life-history stage transitions - both pre- and post-emergence - that may be limiting plant recruitment in the field (Figure 2b–f). In addition, ongoing assessment of post-emergence life-history stages was conducted to 26 weeks (4 October 2017).

### *2.2.4. Emergence rows*

Within the field site, 12 rows were prepared by creating V shaped furrows using a rake. Each row was 3 m long and the furrow 20–30 mm deep. The allocation of species to rows was randomised, with six replicate rows sown with each species. Within each row, 100 seeds were sown evenly by hand and buried just beneath the soil surface. Rows were hand watered at a rate of 9 L per row directly after sowing.

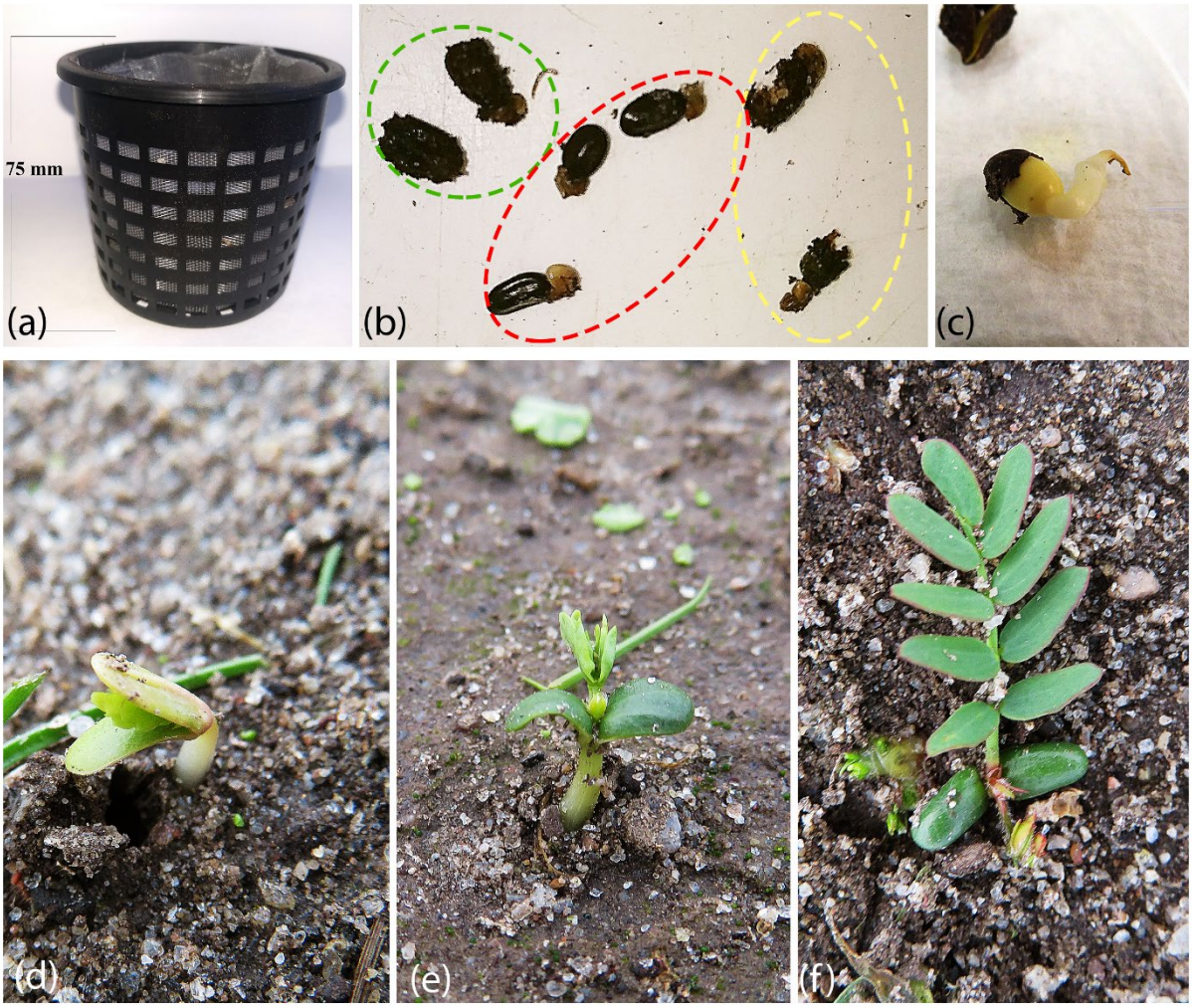



Figure 2. (a) Germination cache, and different seed conditions and seed and seedling early life-history stages: (b) ungerminated seed circled in red, and dead seed circled in green (soft seeds) and yellow (broken seed); (c) pre-emergent germinated seed with radicle; (d) emerged seedling, before cotyledons have unfurled; (e) emerged seedling with unfurled cotyledons; and (f) established seedling, with fully unfurled true leaf and leaflets.

157 Post-emergence life-history stages were assessed using a seedling census, which was conducted every
158 two days for the initial 12 weeks, followed by a weekly census for another 14 weeks. Seedlings were
159 scored as either emerged, cotyledons or established (Figure 2d–f). The location of each seedling was

identified with a unique position along a tape measure and individual seedlings were monitored throughout the experiment.

### *2.2.5. Germination caches*

To assess pre-emergence life-history stages that occur beneath the soil surface, a novel approach was used with a series of germination caches (Figure 2a) being deployed adjacent to the sown rows. These were established to assess seed germination and emergence over time. Each cache consisted of a round 80 × 75 mm hydroponic basket lined with a fine nylon mesh. An 80 mm hand operated augur was used to create a row of 4 holes adjacent to each emergence row at a 65 mm depth to place each cache, leaving a 10 mm lip above the soil to ensure seeds were secure. Each cache was filled with soil from the site and 10 seeds of the same species sown in the adjacent emergence row were buried just beneath the soil surface. The positions of the four caches along each row were randomised by harvest date.

This technique was used in preference to germination bags as it was important that germinated seeds could establish as viable seedlings, which could emerge and grow *in situ* for a period of time after germination without impediment. The caches both provided this environment to grow while allowing easy extraction of all seed/seedling material for quantification.

Over the first 12 weeks, caches were monitored every two days for signs of emergence, with emerged seedlings and any seedling mortality recorded. During the experiment, four harvests were conducted at the three weekly intervals, which involved removing one cache per row for assessment. This was done by carefully removing emerged seedlings, then gently washing the soil through a sieve and searching for the remaining seeds to quantify germinated, ungerminated and dead seed (Figure 2b–c). Note that, herein, we use ‘dead seed’ to refer to seed that appeared dead, however, without viability data, we cannot be sure if all seed was viable to begin with. We discuss this further in the discussion section.

Further assessment of ungerminated seed was conducted by placing these seeds in sterilised petri dishes on filter paper moistened with Mancozeb fungicide (5 ml dilution with 1 L of water) and then placing the petri dishes in growth cabinets on a day/night cycle of 25/10 °C for six weeks following harvests. Seeds were then scored as able to germinate (residual germination) or remaining ungerminated.

### *2.2.6. Germination bags*

During the initial harvest of the germination caches it became obvious that some seeds had been lost from the caches. In order to determine whether these seeds had been lost due to their removal from the caches or due to their breakdown within the caches, and if seed pre-treatment had an effect, germination bags of seeds were buried adjacent to each of the 12 rows. The bags were made of a very fine nylon mesh that was stapled closed after the seeds were inserted. For each species, six bags of treated (see section 2.1), and six bags of untreated seeds were deployed. Ten seeds were placed in each bag, except for untreated *A. paradoxa* seed, which had six seeds per bag due to limited availability of seed.

Two bags each of treated and untreated seeds per species were harvested after 6 weeks and the remainder were harvested at 12 weeks. Seeds were scored as germinated, ungerminated and dead seed. As the bags prevented the removal of intact seeds, and no bags had been compromised during the experiment based on inspection, this information was valuable in understanding the number of seeds lost to processes in the soil, such as microbial induced decay.

## 2.3. Glasshouse pot-based soil moisture experiment

### *2.3.1. Glasshouse experimental design*

A temperature-controlled glasshouse at The University of Melbourne's Burnley campus in Melbourne, Australia, (-37.828591°, 145.020334°) was used for an experiment to investigate the effects of soil moisture on seed germination and seedling emergence and survival to 3 months. Within the glasshouse, temperatures were maintained between 10°C (night) and 25°C (day).

Seeds were sown into three soil moisture treatments (low, medium, and high; see below) and monitored for 12 weeks (26 May to 18 August 2017). There were 3 harvest dates, and the trial used a randomised complete block design with 6 blocks that had 1 replicate per species per treatment per harvest (108 pots in total). Rectangular plastic pots measuring 90 × 85 × 85 mm were used with drainage holes covered by a fine nylon mesh. Each pot was filled with 320 g of a potting mix containing 71.5% medium pine bark, 14.3% washed coarse sand and 14.3% sieved coir. The air-filled porosity, water-holding capacity and bulk density of the substrate were determined for five additional pots using methods outlined in the Australian Standard for potting mixes (AS 3743-2003; Standards Australia, 2003). Pot water-holding capacity was converted to volumetric water content (VWC) by multiplying gravimetric water content by the bulk density of the substrate. Each pot was evenly sown with 10 treated (see section 2.1) seeds of one of the two study species, just below the mix surface.

### *2.3.2. Soil moisture treatment and conditions*

Three soil moisture treatments were used: low moisture (LM), 25% of pot capacity (ca. 12% VWC); medium moisture (MM), 55% of pot capacity (ca. 27% VWC); and high moisture (HM), 85% of pot capacity (ca. 42% VWC). To maintain treatments, every 2–3 days a random sample of pots (1 pot per treatment per block, total of 18 pots) was weighed and the weights averaged before calculating the required water to add. All pots were then watered to bring them back to their respective treatment

VWC. Once per fortnight, all pots were weighed and watered individually to recalibrate the soil moisture content of each pot. The location of blocks within the glasshouse was randomly cycled fortnightly.

Soil moisture, maintained from day five after initial calibration, had a mean VWC range of: 12.3 to 14.6% for LM; 23.4 to 28.7% for MM; and 38.2 to 44.0% for HM. Soil moisture levels in the field were generally in the range between the LM and MM glasshouse treatments when compared over the 6 months (Figure 1).

#### *2.3.3. Seedling census*

Over the 12 week experiment, a census was conducted of each pot every 2–3 days. Emerged seedlings were recorded and position noted to allow them to be monitored over time. Seedlings were scored following the same life-history stages as seedlings in the field trial rows. To capture pre-emergence life-history stages, 36 pots were harvested at four weekly intervals, representing 1 pot per treatment per species from every block, using the same technique as harvests of the field trial germination caches.

### 2.4. Statistical analysis

Investigation of summary statistics across harvests in the field trial (caches and bags) and glasshouse experiment revealed that the data from the final harvests in the field and in the glasshouse (12 weeks after sowing in both cases) were the most informative, and these data are presented here.

To address our hypothesis that below-ground processes would represent the greatest recruitment bottleneck, life-history stage transitions, and differences between treated and untreated seed in bags, in the field trial were assessed using generalised linear mixed models (GLMM) with a beta distribution (R package *glmmTMB*; Brooks et al. 2017). To model data with different subsample sizes from both germination caches (10 seeds per cache) and rows (100 seeds per row), or between germination bags with different subsample sizes, counts at each life-history stage were standardised by dividing counts by

246 total seeds sown per cache, row or germination bag to calculate a proportion. As beta regression
247 requires values >0 and <1, values of 1 were changed to 0.9999 and values of 0 changed to 0.0001. A
248 GLMM was constructed separately for each species, as this was of most interest in this study (rather
249 than inter-specific differences). For models of the field trial data, the proportion of individuals was
250 treated as the response variable and life-history stage as a fixed factor and row as a random factor. For
251 the germination bag data, the proportion of individuals at each life-history stage was treated as the
252 response variable and treatment (treated or untreated seed) a fixed factor and row a random factor.
253 Post hoc pairwise comparisons were made for the field trial model using Tukey's honestly significant
254 difference (Tukey's HSD) with the *emmeans* package (Lenth 2021). Raw data (as percentages) and
255 standard errors are presented in graphs and tables, with unstandardised effect sizes (raw group
256 differences) and *P*-values from beta regression (germination bags) or Tukey's HSD (field trial) provided in
257 the text.

258 To investigate temporal emergence patterns in the field trial, survival analysis was used to estimate
259 survival curves, in this case the proportion of seedling emergence at any given day, with 1 minus
260 Kaplan−Meier curves using R packages *survival* (Therneau 2020) and *survminer* (Kassambra et al., 2021).
261 A log-rank test was used to test for a significant difference between the survival curves of the two
262 species.

263 In the glasshouse trial, the number of geminated seeds that failed to emerge was minimal for both
264 species with no pre-emergent seeds found with a dead radicle or hypocotyl, therefore counts of
265 germinated and emerged seeds were combined as 'germinated' seed for statistical analysis of pre-
266 emergence processes. To test our hypothesis that soil moisture would increase germination and
267 recruitment, we modelled the effect of different soil moisture levels at each pre-emergent life-history
268 stage (germinated, residual, ungerminated and dead) using generalised linear models (GLM) with a
269 poisson distribution. For these models, soil moisture treatment (Low, Medium, or High) was treated as

the explanatory variable and counts at each life-history stage the response variable with Tukey's HSD tests used to identify significant differences among soil moisture treatments. Seedlings in the glasshouse trial were allocated to two post-emergent life-history stages (emerged and survived) given the shorter duration of this study (3 months) compared to 6 months for the field trial where four stages were identified. To identify bottlenecks in the transition from germinated to survived, a GLM for each species was constructed with soil moisture treatment and life-history stage (germinated, emerged and survived) the predictor variables and individual counts the response variable, with an interaction term between treatment and life-history stage included. Models were checked for overdispersion and no obvious overdispersion was detected. As with the field trial, species were modelled separately.

All analysis was performed in R Studio (R Core Team, 2017) with a significance level of $\alpha = 0.05$.

# 3. Results

## 3.1. Field trial

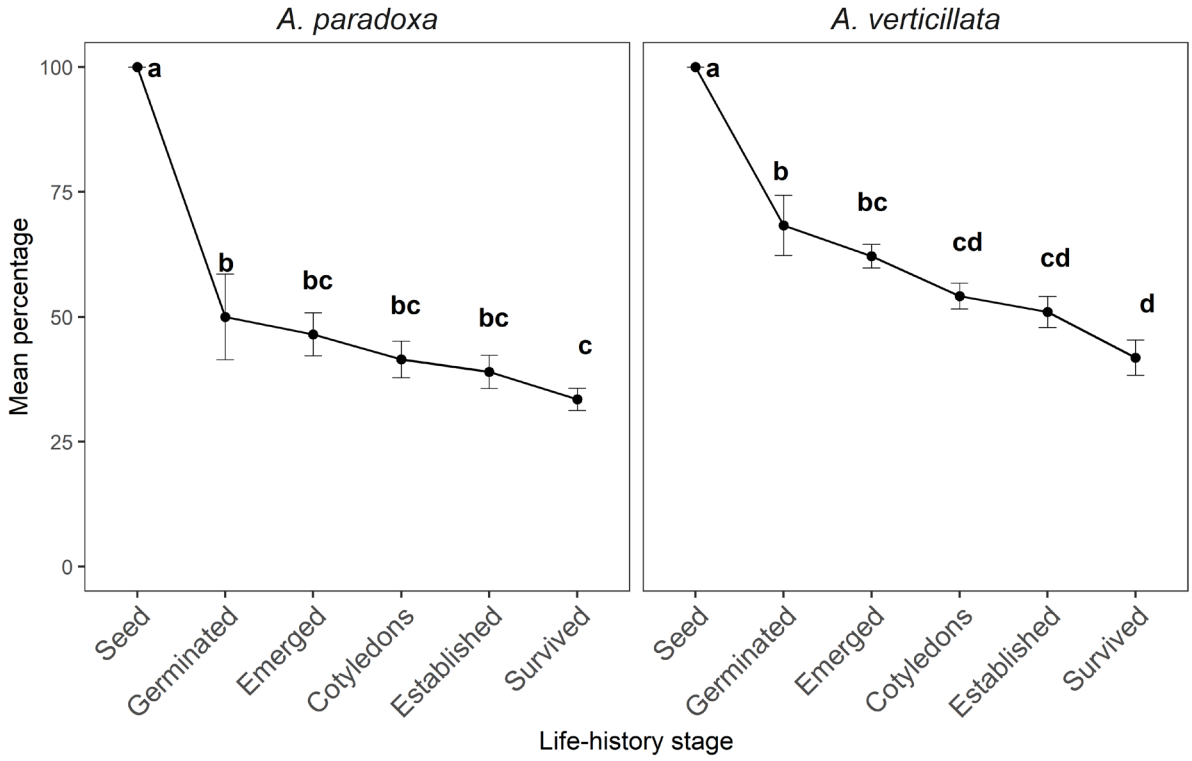
### *3.1.1. Transitions between life-history stages*

At the end of the six month study period, on average 33.5 ± 2.2% and 41.8 ± 3.5% of the 100 seeds sown in each row had transitioned through to a survived seedling for *A. paradoxa* and *A. verticillata* respectively (Figure 3). The transition from a (treated) seed to a germinated seed represented the single most limiting life-history stage transition, resulting in significantly fewer individuals and contributing more to recruitment failure than all other life-history stages combined for both species (Figure 3). Specifically, half of *A. paradoxa* seeds failed to germinate (100−50.0% = 50%, Tukey's HSD $P < 0.001$), while a third of *A. verticillata* seed did not germinate (100−68.3% = 31.7%, Tukey's HSD $P < 0.001$).

Once germinated, survival for both species gradually declined across subsequent life-history stages, with no single transition causing a significant recruitment bottleneck. For *A. paradoxa*, 33.0% of germinated

292 seed had died at 6 months while *A. verticillata* had a slightly steeper decline resulting in the death of

293 38.8% of germinants (Figure 3).




294

295 Figure 3. The mean percentage of individuals surviving to each life-history stage from germination caches

296 (Germinated) and rows (Emerged−Survived) for *Acacia paradoxa* and *A. verticillata*, sown in the field trial. Values

297 for germinated seed (Germinated) are from germination caches harvested at 12 weeks after sowing (*n*=6). Values

298 for emerged seedlings (Emerged) to seedling survival at 6 months after sowing (Survived) are from rows (*n*=6).

299 Means ± 1SE are shown. Different letters indicate significant differences ($P < 0.05$) between life-history stages from

300 Tukey's HSD *post hoc* tests.

301 *3.1.2. Pre-emergence processes in the field*

302 Seed germination rates from caches were high with 50.0 ± 8.6% and 68.3 ± 6.0% of sown seeds

303 germinating for *A. paradoxa* and *A. verticillata*, respectively (Table 2; Figure 3). Seed death accounted

304 for approximately a third of the non-germinated seeds for both species. While all *A. verticillata* seed was

either germinated or dead, 6.7 ±% of the remaining *A. paradoxa* seed germinated under growth cabinet conditions (residual germination) and 10.0 ± 6.3% did not (ungerminated seed) (Table 2).

The proportion of dead seed in the germination bags (27.5–35%) was comparable to that found in the caches (Table 2). This result indicates that the seed which could not be found during the cache harvests had decayed within the caches prior to harvest, rather than having been removed from the caches by animals or insects.

The effect of the pre-treatment of seeds of these two *Acacia* species differed markedly between species. There were no significant differences between treated and untreated seed for *A. paradoxa*. In contrast, *A. verticillata* had 70 ± 4.1% germination for treated seeds compared with no germination for untreated seed (70−0% = 70%, Beta regression $P < 0.001$) and 65 ± 6.5% of untreated seed remained ungerminated with no seed remaining ungerminated for the treated seed (65−0% = 65%, Beta regression $P < 0.001$) (Table 2). For both species, rates of seed death were similar for both treated and untreated seed.

Table 2. The percentage of seeds germinating (Germinated), residual germination (Residual), seeds remaining ungerminated (Ungerminated) and seed that was dead or unable to be found at all (Dead seed) in germination caches and in mesh bags buried in soil in the field trial. Seeds in caches were treated while seeds in bags were either treated or untreated. Treated seed was heat and smoke water treated. Residual germination was not tested in mesh bags and ND (Not Determined) is shown. Means ±1SE are shown.

| Source | Species | Germinated (%) | Residual (%) | Ungerminated (%) | Dead seed (%) | Sample size |
|---|---|---|---|---|---|---|
| Cache | *A. paradoxa* | 50.0 ± 8.6 | 6.7 ± 3.3 | 10.0 ± 6.3 | 33.3 ± 10.2 | 6 |
| Bag, treated | *A. paradoxa* | 30.0 ± 12.25 | ND | 42.5 ± 6.3 | 27.5 ± 13.8 | 4 |
| Bag, untreated | *A. paradoxa* | 20.8 ± 10.5 | ND | 45.8 ± 15.8 | 33.3 ± 6.8 | 4 |
| Cache | *A. verticillata* | 68.3 ± 6.0 | 0.0 | 0.0 | 31.7 ± 6.0 | 6 |
| Bag, treated | *A. verticillata* | 70.0 ± 4.1 | ND | 0.0 | 30.0 ± 4.1 | 4 |
| Bag, untreated | *A. verticillata* | 0.0 | ND | 65.0 ± 6.5 | 35.0 ± 6.4 | 4 |

### *3.1.3. Post-emergence processes in the field*

At the end of the study period (6 months), 46.5 ± 4.3% and 62.2 ± 2.4% of the sown seed had emerged for *A. paradoxa* and *A. verticillata*, respectively (Figure 4). Temporal emergence patterns varied significantly between species (log-rank test $P < 0.001$) with most (89%) *A. verticillata* seedlings emerging in the first two months. In contrast, emergence of *A. paradoxa* represented a bet hedging strategy with a slower, more linear rate of emergence across the study period, with new emergence concluding only two weeks prior to the study's conclusion (Figure 4).

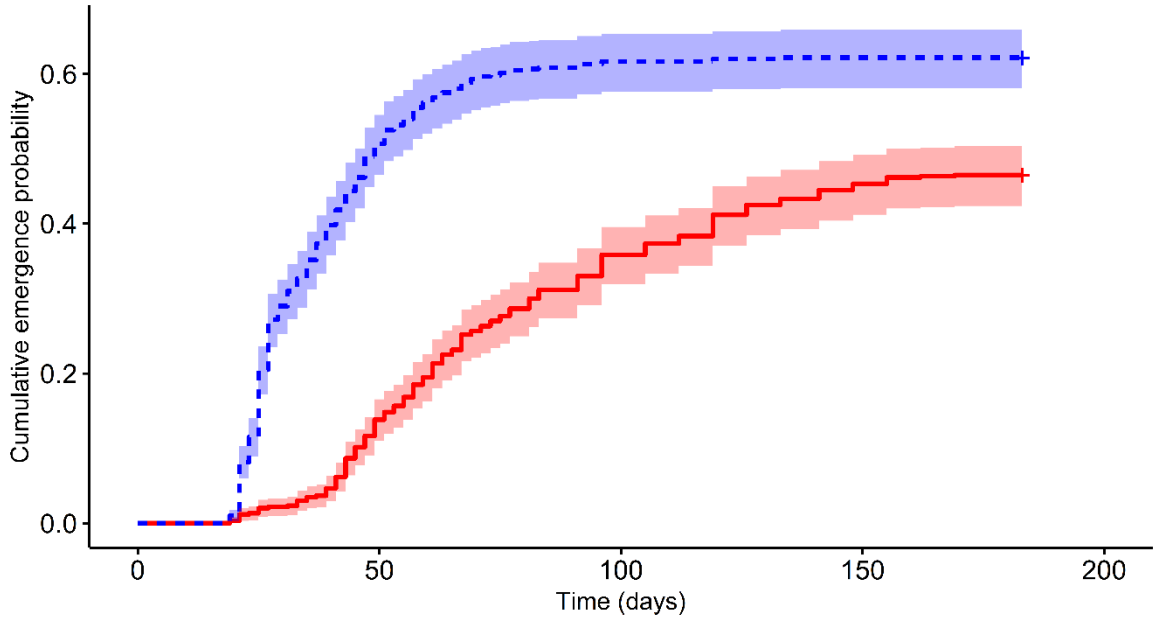
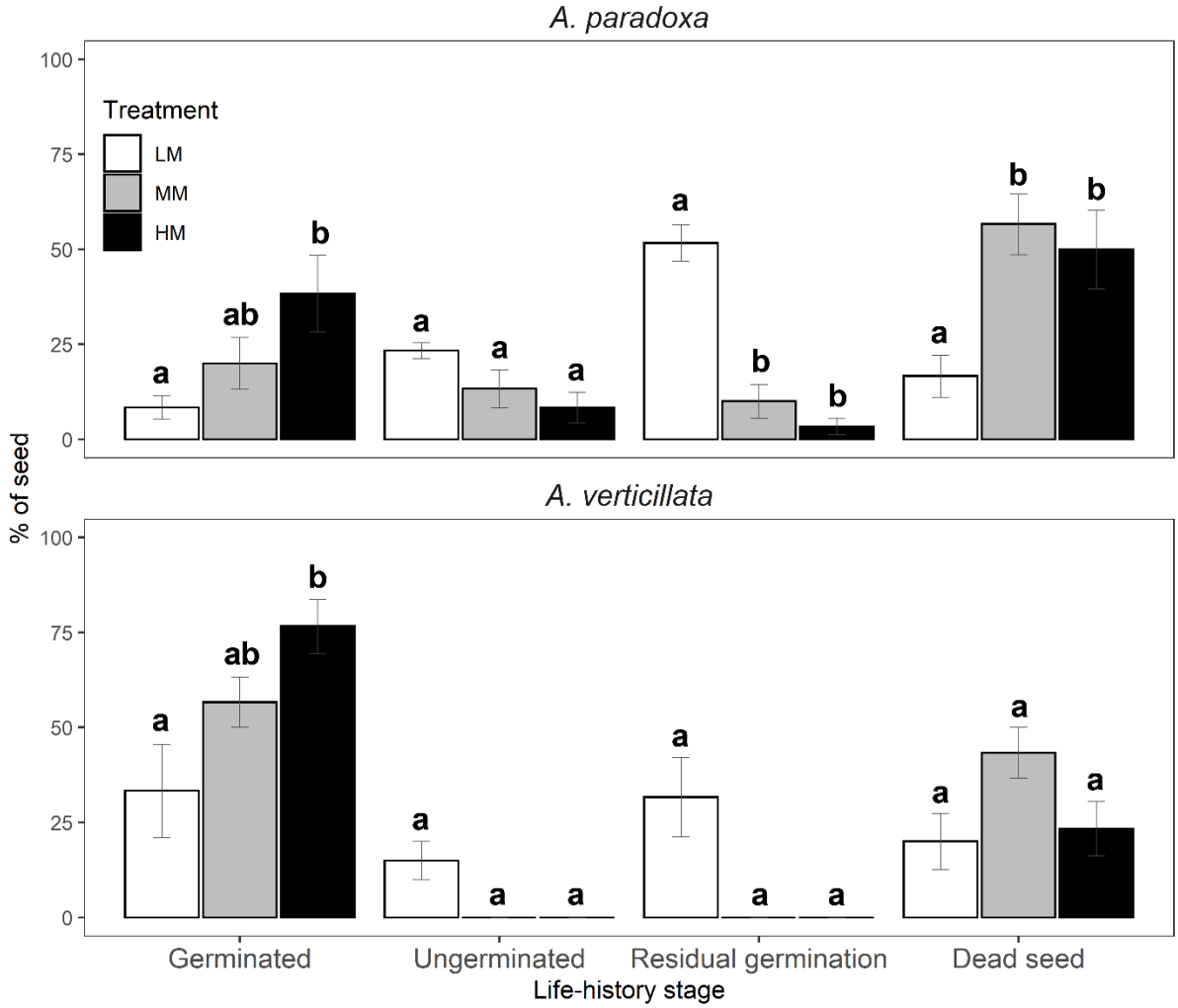



Figure 4. Cumulative emergence probability (1−Kaplan–Meier) and 95% confidence intervals for *Acacia paradoxa* (red solid line) and *A. verticillata* (blue dashed line). Survival curves of the two species are significantly different ($P < 0.001$) based on a log-rank test.

## 3.2. Glasshouse experiment

### *3.2.1. Pre-emergence processes in the glasshouse*

Both species showed similar responses to soil moisture treatments across life-history stages, although absolute values differed between species (Figure 5) with, for example, higher germination rates in *A. verticillata* than *A. paradoxa*, consistent with the field experiment. The proportion of germinated seed increased with increasing soil moisture, with germination greater under HM than LM for both species (38.3−8.3% = 30%, Tukey's HSD $P = 0.006$ and 76.7−33.3 = 43.4%, Tukey's HSD $P = 0.005$ for *A. paradoxa* and *A. verticillata*, respectively). Correspondingly, the proportion of seed that remained ungerminated or exhibited residual germination was highest under LM conditions (Figure 5). However, LM conditions resulted in less seed death (Figure 5), particularly for *A. paradoxa* seed with 56.7 ± 8.0% seed death at MM and 50.0 ± 10.3% at HM, around three times higher than the proportion of dead seeds at LM (16.7 ± 5.6%) (56.7−16.7% = 40.0%, Tukey's HSD $P = 0.007$ and 50.0−16.7% = 33.3%, Tukey's HSD $P = 0.002$ for MM and HM, respectively). The relationship between soil moisture and seed death was less clear for *A. verticillata*, with somewhat more seeds dead in MM, however, differences between the treatments were not significant (MM and HM) or weakly non-significant (MM and LM, 43.3−20.0% = 23.3%, Tukey's HSD $P = 0.069$).




350

351 Figure 5. Mean percent of seeds that germinated, remained ungerminated, were ungerminated but germinated
352 following cabinet incubation (Residual) or were dead for (a) *Acacia paradoxa* and (b) *A. verticillata* treated under
353 either low (LM), medium (MM) or high (HM) soil moisture treatments for 12 weeks in the glasshouse. Mean ± 1SE
354 of n=6 pots are shown. Different letters indicate significant differences ($P < 0.05$) between soil moisture
355 treatments within life-history stages from Tukey's HSD *post hoc* tests.

356 *3.2.2. Post-emergence processes in the glasshouse*

357 Overall, survival of seedlings once emerged was high (Table 3) with between 73–100% of seedlings
358 surviving for both species under all treatments except for *A. verticillata* seedlings under LM where only

359 46.1% survived to 3 months; however, no significant differences were detected between seedling
360 germination, emergence and survival at 3 months and there was no interaction between treatment and
361 life-history stage.

362 Table 3. The total number of sown seeds that emerged, the mean percentage of emergence and the mean
363 percentage of emerged seedlings that survived to 3 months, grown in the glasshouse under either low (LM),
364 medium (MM) or high (HM) soil moisture treatments. Data are means of 6 replicates of 10 seeds per species ± 1SE.

| Species/Treatment | Total emergence | Mean emergence (%) | Mean percent survived (%) |
|---|---|---|---|
| *A. paradoxa* | | | |
| LM | 5 | 8.3 ± 3.1 | 75.0 ± 25.0 |
| MM | 8 | 13.3 ± 4.9 | 91.7 ± 8.3 |
| HM | 22 | 36.7 ± 9.2 | 88.9 ± 8.2 |
| *A. verticillata* | | | |
| LM | 19 | 31.7 ± 13.0 | 46.1 ± 15.6 |
| MM | 33 | 55.5 ± 8.2 | 72.7 ± 8.4 |
| HM | 46 | 76.7 ± 7.2 | 100.0 ± 0.0 |

365 # 4. Discussion

366 The ability to predict the outcomes of direct seeding, and identify any limiting stages to plant
367 recruitment, is essential to the successful application of direct seeding as a tool for revegetation and
368 restoration. Our hypothesis that there would be bottlenecks to plant recruitment in the early life-history
369 stages was supported for both species in the field trial with seed mortality predominantly occurring in
370 the pre-emergence stage prior to germination, while the post-emergence stages contributed less to
371 recruitment failure. As was also hypothesised, germination and emergence increased with increasing
372 soil moisture in the glasshouse trial, however, seed mortality also increased.

## 4.1. Pre-emergent processes driving recruitment failure

The application of a novel technique through the use of germination caches allowed us to easily study pre-emergence processes associated with normal seedling development. We found that seed mortality prior to seed germination was the single most limiting transition for the two study species, although we could not rule out seed being unviable also, as discussed below. Few seeds were found at the germination stage and no seeds were observed with an unhealthy radicle or hypocotyl indicating seed mortality happened prior to radicle emergence, in contrast to other studies (James et al., 2011; Mayence et al., 2017). Initial concerns that seeds were being lost from the caches through physical removal by animals or insects were allayed by observation of seed loss in the germination bags, which were sealed to prevent physical loss. Seed loss rates were comparable between the caches (mean 32.5%) and bags (31.5%), indicating that seed loss was a result of seed breakdown and decay within the caches. This decay was unrelated to the pre-treatment of the seeds, as untreated and treated seeds in germination bags had similar rates of decay.

Higher soil moisture both promoted germination, but also increased seed mortality, under glasshouse conditions, particularly for *A. paradoxa*. Seed that did not germinate in potting mix under the low moisture treatments had high levels of residual germination, indicating tolerance to desiccation (Long et al., 2015). In contrast, residual germination of seed from medium and high moisture treatments was low (*A. paradoxa*) or absent (*A. verticillata*). This may indicate that the higher moisture treatments were at a soil water potential threshold at which more seeds imbibed but could not complete radicle emergence (Bewley & Black, 1994). Soil water potential has an important influence on the ability of seeds to imbibe water and the rate at which they germinate, with interspecific differences in thresholds that cause steep declines in germination and time to maximum germination (Bradford, 2002; Fenner & Thompson, 2004). For example, soil water potential below -0.75 MPa caused a significant decrease in germinability, and an increase in time to maximum germination for *Acacia harpophylla* under lab conditions (Arnold,

Kailichova & Baumgartl 2014). In our study, the month post sowing was the wettest across the study period and most likely contributed to high germination rates compared to *Acacia* species in other studies (e.g. Hallett et al., 2014).

Pathogen attack is a likely cause for much of the seed mortality across both our study components. High soil moisture due to wetter conditions has been found to increase the risk of pathogen attack on some grass seeds (Schafer & Kotanen, 2003), while seeds in wetlands have been found to be more susceptible to pathogen attack than seed of the same species in uplands (Blaney & Kotanen, 2001). Applying fungicide monthly to buried seeds of a Canadian species of birch was found to double rates of germination compared to no fungicide application (O'Hanlon-Manners & Kotanen, 2004; see also Leishman et al., 2000). These studies suggest the relationship with soil moisture and pathogens is a plausible explanation for seed mortality in our field and glasshouse trials. Further research on optimal fungicide treatments to prevent seed mortality is needed, however, it is important to consider potential deleterious effects to other soil biota from such fungicide treatments.

While we have assumed seed mortality of viable seed was the greatest bottleneck to recruitment, there may have been a certain percent of unviable seed that we were unable to quantify without viability data. While the major bottleneck to recruitment in this study was still at the seed life-history stage, there may be both unviable seed and seed mortality at play. This lack of viability data may be more representative of revegetation projects where seed viability testing of every seed lot and every species is not feasible. If the seed to germinated seed transition is the most limiting to recruitment, however, then understanding both the seed viability, and seed mortality rates would allow seeding rates to be corrected for unviable seed, and viable seed treated to maximise germination. This will allow greater control over early plant communities resulting from direct-seeding revegetation.

## 4.2. Post-emergence processes

Compared with many other studies, we observed much higher rates of field emergence (46 and 62% for *A. paradoxa and A. verticillata*, respectively). One of the novel aspects of our study is the frequency with which plant emergence and survival was assessed, both in the field (every two days for the first three months after sowing and then at weekly intervals), and in the glasshouse (every 2–3 days). Several studies have reported low emergence rates for *Acacia* spp. during initial post-sowing assessments, for example, 21% at three months (Geeves et al., 2008), 10% at 11 weeks (Clarke & Davison, 2001), 5% at two months (Jusaitis & Polomka, 2008) and 13% at around four months (Hallett et al., 2014). Given the time between sowing and initial assessment in these studies, it can be hypothesised that true seedling emergence may in fact have been higher, and that a lower frequency of assessments may not be adequate to determine true emergence rates and identify recruitment bottlenecks in the early post-sowing days. This is important as management options and research hypotheses may not be targeting the key stages limiting plant recruitment.

In the field trial, 28 and 33% of emerged seedlings died across the first six months for *A. paradoxa* and *A. verticillata*, respectively. The frequency of assessments during the field trial allowed for some causes of seedling mortality to be observed more clearly. Seedling damage consisted of seedling biomass missing above the stem, missing cotyledons in the early days post-emergence and missing leaflets (data not shown), probably caused by herbivory from invertebrates such as snails and slugs, and possibly red legged earth mites. Damping off also affected seedlings in the field which may have been caused by pathogens such as *Pythium* spp.. In the glasshouse, low moisture reduced survival of *A. verticillata* seedlings due to desiccation with this effect most obvious at the last harvest when seedlings that managed to emerge may be relying less on seed-stored resources (Baskin & Baskin, 1998; Bewley & Black, 1994). In the field trial, seedling desiccation was observed in the final months as temperatures began to increase, likely due to the surface of the sandy soil being prone to drying and the colder season

443 slowing seedling growth which reduces the depth at which soil moisture can be extracted by roots
444 (Raven et al., 2013). Soil properties can affect where recruitment bottlenecks may occur, for example,
445 sand may promote higher emergence while clay may increase survival in summer conditions (Hallett et
446 al., 2014). Investigation of these potential causes of mortality would help maximise seedling survival in
447 the early months post-sowing.

448 ## 4.3. Seed treatment

449 In direct seeding projects, seeds with hard seed coats are routinely treated before sowing either by hot
450 water treatment, scarification, smoke or a combination of treatments, to break down the hard seed coat
451 and enhance rates of germination (Clemens et al., 1977). Our study found similar rates of seed death for
452 treated and untreated seed in the field indicating treatment with hot water and smoke did not increase
453 the vulnerability of seeds to seed death. Our results confirmed that such treatment is crucial for
454 germination of *A. verticillata*. In contrast, treatment had virtually no effect for *A. paradoxa*, which
455 continued to emerge over nearly six months, whether treated or not. Fewer *A. paradoxa* seeds may
456 have imbibed during seed pre-treatment or they may be tolerant of cycling between desiccation and
457 imbibition before conditions are appropriate for germination. That 52% of ungerminated seeds were
458 able to germinate in cabinets after 12 weeks in the low moisture treatment indicates that this is the
459 case. Given the high rates of dead seeds under high and medium moisture treatments, it may be more
460 beneficial to sow untreated *A. paradoxa* seeds that germinate based on environmental cues. The
461 outcomes from this study highlight that a good understanding of the optimal pre-treatment, or lack of
462 treatment, combined with soil moisture conditions, of any species being sown in direct-seeded
463 revegetation projects is important.

## 4.4. Conclusions, future research and management implications

In this study, we found that seed mortality was a bottleneck to plant recruitment with around a third of seeds dying, or unviable, at the pre-emergence stage under field conditions for both species. In the glasshouse, higher soil moisture increased germination but also seed mortality for *A. paradoxa*, while lower soil moisture left more than 50% of seed still able to germinate. *A. verticillata*, however, benefitted from higher soil moisture overall. This highlights the importance of understanding species-specific requirements that inform sowing conditions to maximise germination and emergence. Further research should look at species-specific interactions between soil moisture content and seed mortality under field conditions across different soil types and seasons representative of future direct seeding projects. Given that climate change is predicted to change future weather patterns, research is also needed into how different future soil moisture and temperature scenarios are likely to affect seed and seedling life-history stages (e.g. seed germination; see Cochrane, 2020). Furthermore, quantifying the life-history stages of species sown together and representative of direct-seeding seed mixes is important in understanding how bottlenecks to recruitment may change and shape direct-seeded plant communities. Constraining seeds to caches, allowing seedlings to develop freely and making seed/seedling harvesting easier, is also recommended as a suitable technique to study pre-emergence processes in the field.

The greater frequency of assessments in our study, relative to other direct seeding studies, allowed us to assess the full extent of seedling emergence and quantify post-emergence life-history stages and recruitment bottlenecks. Understanding the most vulnerable transitions during seedling establishment under different conditions allows management actions to target those stages to increase recruitment. The results of our study build on those of previous studies investigating both pre-emergence and post-emergence recruitment bottlenecks for species used in direct seeding projects, and highlights that a

487 robust understanding of the transitions between all life-history stages is key to improving revegetation
488 outcomes for all species used in direct seeding.

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