

*RESEARCH ARTICLE*

Short Title: Jooste et al.—Germination spectrum in *Oxalis* seeds from Cape Flora

***Oxalis* seeds from the Cape Flora have a spectrum of germination strategies**

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## **Abstract**

**PREMISE OF THE STUDY:** Seed germination strategy has profound ecological and evolutionary consequences, with transitions between germination strategies receiving renewed recent attention. *Oxalis* from the Cape Flora, South Africa, has seeds with two contrasting germination strategies: orthodox and recalcitrant. The morphological gulf between these strategies (and potential intermediate morphologies) has been poorly quantified, with questions regarding their ecological function and evolution. We reconsidered this binary classification, emphasizing potential intermediate states.

**METHODS:** Seed physiological traits were used to assign strategies to 64 *Oxalis* species. We tested for morphological/phenological signal corresponding to defined strategies with cluster, principal component, *K*-means clustering and discriminant analyses.

**KEY RESULTS:** We show that an intermediate germination strategy does exist among Cape *Oxalis*, with two possible morphological groups within each strategy. These could reflect a continuum of germination states, where an ancestral orthodox strategy evolved towards a maximally recalcitrant peak, with a mosaic of intermediate states reflected in extant taxa.

**CONCLUSIONS:** Environmental factors may affect germination strategy and distribution throughout the Cape because recalcitrant and intermediate species are confined to the winter rainfall region. They occupy specialized niches and may face adverse impacts under predicted climate change (hotter and drier winters), meriting focused future conservation.

**KEY WORDS:** endosperm; germination strategy; intermediate germination; orthodox germination, Oxalidaceae; recalcitrant; seedlings.

Across all life, many species produce dormant eggs or seeds that do not hatch or germinate immediately after release, despite being fully mature and exposed to favorable environmental conditions (Evans and Dennehy, 2005). Instead, they hatch or germinate at intervals over a long time, allowing bet-hedging against future catastrophes. Despite the numerous benefits associated with a dormant state, many species reduce or eliminate the dormant stage due to trade-offs associated with population growth (Ellner, 1985; MacArthur and Wilson, 1967). Venable (2007, p. 1088) elegantly stated that “the best a non-germinating seed can do is survive, while a germinating seed may either die without leaving any descendants or make 100’s or even 1000’s of new seeds.”

All angiosperms depend on seeds to ensure the dispersal of their progeny in space and time and consequently germination strategies and post-germination traits are subjected to strong selection pressures (Donohue et al., 2010; Huang et al., 2010; Dayrell et al., 2016). Many plants have developed mechanisms to control seed germination, so that seed dormancy is broken only when conditions are favorable (Nikolaeva, 1969, 1977; Baskin and Baskin, 1989). Seeds with this germination strategy are defined as orthodox seeds (Ellis and Roberts, 1981). A selection of unrelated angiosperms has done away with a dormant period, in a strategy known as recalcitrance (Roberts, 1973; Chin and Roberts 1980; Pammenter and Berjak, 1999). Recalcitrant seeds are desiccation sensitive and have high water content and well-developed embryos when shed from fruits (Crocker, 1916; Martin, 1946; Grushvitzky, 1967; Roberts, 1973; Pammenter and Berjak, 1999; Floyd and Friedman, 2000; Forbis et al., 2002).

Further study proved this binary classification to be too narrow because some seeds vary substantially in their morphology and physiological responses to desiccation (Normah et al., 1986; Farrant et al., 1989; Connor et al., 1996; Hong and Ellis, 1996; Daws et al., 2004, 2006). Consequently, a third category of intermediate seeds was introduced (Ellis et al. 1990). These seeds have high water content when shed and are capable of withstanding considerable desiccation, although not to the same extent as orthodox seeds (Ellis et al., 1990; Hong and Ellis, 1996). It is currently estimated that 89% of angiosperms have orthodox seeds, 5% have recalcitrant seeds, and only 1%

have intermediate seeds, while the strategies of the remainder are unknown (Tweddle et al., 2003; Gold and Hay, 2008a; Wyse and Dickie, 2017).

Many authors have suggested that a continuum between two states, namely extreme orthodoxy and maximal recalcitrance, may be favored above two or three discretized strategies (Berjak and Pammenter, 1997; Sun 1998; Pammenter and Berjak, 1999; Kermode and Finch-Savage, 2002; Berjak et al., 2004; Berjak and Pammenter, 2008). The continuum concept accommodates the documented within- and between-species physiological and morphological variation and effects of seed provenance on seed desiccation tolerance (Daws et al., 2004, 2006). Plasticity of seed morphological, phenological, and physiological traits is regarded as an important source of variation influencing the shift between different germination strategies (Clauss and Venable, 2000; Venable, 2007).

A strong association between seed germination strategies and habitat preference has been documented (Roberts and King, 1980; Von Teichman and Van Wyk, 1994; Berjak et al., 2004), consistent with theoretical expectations favoring recalcitrance in less changeable environments (Dayrell et al., 2016). On seasonal time scales, plants with orthodox and intermediate seeds are characteristic of temperate or arid habitats with strong seasonality (Jurado and Flores, 2005; Baskin and Baskin, 2014), but also occur (if at very low frequency) in all other habitat types (Hong and Ellis, 1996). Germination of recalcitrant seeds is usually initiated immediately or soon after shedding (Farrant et al., 1985, 1986); therefore, these seeds are commonly associated with aseasonal and moist environments, such as tropical, subtropical and wetland habitats (Tweddle et al., 2003). As a result of the primary bet-hedging benefits of orthodoxy in stochastic environments, over longer time scales, geologically and climatically stable environments would be expected to have relatively greater proportions of recalcitrant species (Dayrell et al., 2016).

One such example of a climatically stable system is the botanically rich Greater Cape region of southern Africa (Cowling et al., 2014). Climatic conditions for the Greater Cape range from seasonal winter rainfall in the southwest, aseasonal rainfall in the east, and semi-arid conditions with scant

winter rainfall to the northwest (Manning and Goldblatt, 2012; Snijman, 2013). Within the predominantly winter rainfall region, plants rely on the seasonal availability of water for their orthodox seeds to germinate (Keeley and Bond, 1997). In contrast to what is known for other climatically stable systems (Dayrell et al., 2016), only two known Cape lineages have species with recalcitrant seeds, namely, members from the monocotyledonous tribe Amaryllideae (Amaryllidaceae) (Snijman and Linder, 1996; Berjak et al., 2004) and many species from the eudicot genus *Oxalis* (Oxalidaceae) (Hildebrand, 1884; Salter, 1944; Brink, 2017).

*Oxalis* is the largest geophytic genus in the Cape (ca. 210 species) (Proches et al., 2006; Manning and Goldblatt, 2012) and therefore contributes a large diversity component to the Cape Flora. *Oxalis* seeds have classically been defined as endospermous (orthodox) and exendospermous (recalcitrant) (Hildebrand, 1884; Salter, 1944). Recalcitrant-seeded taxa represent the majority of *Oxalis* in southern Africa (approximately 60%; Salter, 1944). Although Salter (1944) mostly treated the presence or absence of endosperm as a binary variable, he noted a “tendency to a transition’ toward the exendospermous seed type among a few endospermous-seeded *Oxalis* species. Reportedly, eight endospermous species displayed various intermediate structural and behavioral traits, which suggest that a strictly orthodox or recalcitrant classification may not adequately reflect germination strategies among Cape *Oxalis* (Salter, 1944; Brink, 2017).

The high species diversity and intriguing seed biology of the Cape *Oxalis* provide an ideal model system to study and explore the diversity of germination strategies. We aimed to re-assess the binary classification of germination strategies for Cape *Oxalis* and to investigate putative intermediate states using seed physiological traits. We also aimed to determine which seed and seedling morphological and developmental traits are associated with each of the germination strategies. Information on traits that consistently distinguish between germination strategies might shed light on different selective regimes driving the evolution of seed germination strategies within Cape *Oxalis*.

## **MATERIALS AND METHODS**

### **Sample collection**

Sixty-four Cape *Oxalis* species with a wide taxonomic distribution (Salter, 1944; Oberlander et al., 2011) were selected based on phylogenetic placement and availability (Appendix S1; see Supplemental Data with this article). Seeds were collected from various field localities throughout the Cape region of South Africa and from plants in the Stellenbosch University Botanical Garden *Oxalis* research collection. Due to the need for large numbers of seeds that could not be generated in a research collection context, we could not explicitly account for maternal effects and so variation in wild- vs. garden-collected seed at least partly reflects this effect. However, there is no obvious pattern of bias or change in variance between the wild- vs. garden-collected seeds in our analyses (data not shown), so we consider variation due to maternally inherited effects minor compared to natural between-species variation. Seeds were considered mature once they spontaneously explosively dehisced from their outer testa (Salter, 1944), and only mature seeds were used in all germination experiments. Such mature seeds were harvested from capsules, and seeds were exposed to experimental treatments starting on the day of harvest. MO-accessions used in this study correspond to herbarium and living collection samples maintained at Stellenbosch University, South Africa.

### **Seed physiology**

We used three physiological measures to place seeds on an orthodox/recalcitrance spectrum. The Kew 100-seed test for desiccation tolerance (adapted from Pritchard et al. [2004] and summarized as a schematic diagram in Gold and Hay [2008b]) and the “seed storage category screening” (adapted from Hong and Ellis [1996]) were used to determine seed desiccation tolerance, to assess seed moisture content at shedding and to assess critical moisture contents of all desiccation-tolerant species. The 100-seed test for desiccation tolerance (Pritchard et al. [2004]) was specifically designed for studies assessing small sample sizes of as few as 100 seeds per species. As proposed for the 100-seed test for desiccation tolerance, samples of 100 seeds per species were used and subdivided into smaller batches depending on

experimental treatment. An initial (control) germination test was conducted on 26 seeds per species, where the germination success (% germination) of seeds were assessed when germinated at ambient air temperature on moist filter paper in petri dishes. A desiccation treatment was conducted by drying 32 seeds with 5g silica crystals in sealed plastic bags. A moisture-stored treatment was conducted by placing another 32 seeds on wet filter paper in sealed plastic bags to maintain high humidity (bags were opened every second day for aeration). The seeds of both experimental treatments were then incubated with an ecologically representative day/night light cycle (13 h light, 11 h dark) at 25°C for 2 weeks.

Germination success from each experimental treatment was then assessed by placing 13 seeds per species on moist filter paper in petri dishes at ambient air temperatures. One petri-dish (90 × 15 mm) was used per species, but seeds were equally dispersed with at least 1 cm space between seeds. Germination was defined as the splitting of the tegmen (lignified tegmens split into multiple (usually five) segments, while nonlignified tegmens split into two segments). The germination success of all treatments (initial control, desiccated and moisture-stored) was recorded daily for 5 weeks. All data were plotted as germination progress curves. Because not all exendospermous species could withstand desiccation, germination progress curves alone could not be used to define strategies among *Oxalis* and were not further explored. Consequently, we included an assessment of initial moisture content and critical moisture content of seeds.

Ten untreated seeds were used to determine initial moisture content at shedding by calculating the mass difference between fresh (day 1) and oven-dried (2 weeks at 40°C) seeds (eq. 1 from Reeb et al. [1999]). A sample of six seeds per experimental treatment was used to determine seed moisture contents after each treatment.

To assess the critical moisture content of all desiccation-tolerant species, 13 seeds per desiccated experimental treatment were dried for two additional weeks (under the same desiccation treatment conditions described above). After the incubation, a germination test was conducted using seven seeds,

while the remaining sample of six seeds was used to determine seed moisture contents. As we worked with a very limited supply of seeds, we did not assess ability of seeds to survive freezing, and we reduced the number of desiccation treatments to two treatments, as suggested by Hong and Ellis (1996). These authors suggested that seeds should be dried to about 12% and 5% moisture content, which we were able to achieve after 2 and 4 weeks of desiccation. According to the literature, orthodox seeds typically have critical moisture contents below 7% and intermediate seeds above 8%, while recalcitrant seeds span a wide range (20 to 96%) depending on the oil content (Ellis et al., 1989; Probert and Longley, 1989; Pritchard, 1991; Hong and Ellis, 1996; Pammenter and Berjak, 1999).

### **Seed and seedling morphology**

We aimed to determine whether morphological signal among seed and seedling data correlated with assigned germination strategies (using physiological traits) using an independent morphological and developmental *Oxalis* seed and seedling data set that was compiled for all studied species. A daily digital image record was taken to document the sequence of development until seedlings reached maturity, using five seeds per species from the initial control germination treatment. Morphological data were collected for all of these individual seeds from the day that seeds were shed and harvested, until seedlings reached maturity. Seedling maturity was defined as 1 day after the leaflets of the first foliar leaf of the seedling had fully emerged and unfolded. A total of 71 morphological seed and seedling traits were studied (Appendix S1), which included 32 qualitative (discrete, unordered), three qualitative (discrete, ordered) and 36 quantitative (continuous) traits. Continuous traits were measured to scale from images imported to ImageJ (Abràmoff et al., 2004).

The seeds of 20 endospermous *Oxalis* species did not germinate throughout the duration of this study (the first growing season after shedding); however, these seeds were viable as they successfully germinated within the following growing season (Brink, 2017). Germination data and seedling morphological traits from these species were not included in our study, as these seeds were used in another study. Based on the available morphological and



developmental data for the studied species, three separate data sets were constructed, namely a seed, seedling, and combined seed and seedling data set. Due to the lack of germination, the seed morphological traits of the 20 aforementioned species were included in the seed data set (which included data for all 64 *Oxalis* species), but excluded from the seedling and combined seed and seedling data sets (which included data for only 44 *Oxalis* species). These three separate data sets were used to assess our assignment of pre-defined germination strategies based on physiological traits and to compare our results to previous studies on the seed (Obone, 2005) and seedling (Brink, 2017) morphology of Cape *Oxalis*. Additionally, embryo development (relative size and pigmentation) and presence of endosperm were assessed by sectioning fresh seeds lengthwise. A Leica M125 stereomicroscope, Leica MC 170 HD camera, and LAS CORE software (Leica, Heerbrugg, Switzerland) were used to document these seed sections. Because we often had a limited sample of seeds, it was not possible to assess these traits for all species.

### **Cluster, principal component, and K-means clustering analyses**

All data were analysed using the R statistical environment version 3.4.1 (R Core Team, 2014). Cluster analyses (CA) and principal component analyses (PCA) were implemented to assess major sources of variation in discrete and continuous seed (13 traits), seedling (58 traits), and combined (71 traits) datasets. These analyses were conducted to determine whether species or strategies cluster together based on morphological and developmental traits. Data for five replicates per species were included in all analyses (Appendices S2 and S3). The Gower's method was used to calculate distances to center and scale data for the CAs and data for the PCAs were centered and scaled with the built-in scale function of the FactoMineR package (PCA function [Lê et al., 2008]). The mean clustering method was applied for the CAs that were conducted with the Dendextend (Galili, 2015) package. The FactoMineR (PCA function) and Factoextra (fviz\_pca\_ind function [Kassambara and Mundt, 2016]) packages were used for PCAs.

The NbClust (Charrad et al., 2014) package was used for *K*-means clustering (Ward, Silhouette, and Gap statistical methods) to determine the optimal

number of clusters in the data (the majority rule was used to determine the best number of clusters based on the results from the three methods). Predefined physiological germination strategies (as determined in the previous section) were mapped onto these clusters for each data set. Additionally, suboptimal clusters in the data were explored, but this did not aid in elucidating patterns among groups.

### **Discriminant analyses**

Discriminant analyses (DA) were implemented to test whether morphological and developmental traits were predictive of membership to each of our assigned physiological germination strategies. Continuous and ordered discrete data of the seed (8 traits), seedling (31 traits) and combined (39 traits) datasets were centered and scaled with the inbuilt scale function (`dudi.pca` from the `ade4` package (Dray and Dufour, 2007)) for DAs. Categories proposed in the seed physiology section of this work were used as a priori grouping variables in DAs. Statistical analyses to test support for groups were done with the use of one-way multivariate analysis of variance (MANOVA) and Pillai's tests and Monte-Carlo Permutation tests with 9999 replicates (Dray and Dufour, 2007).

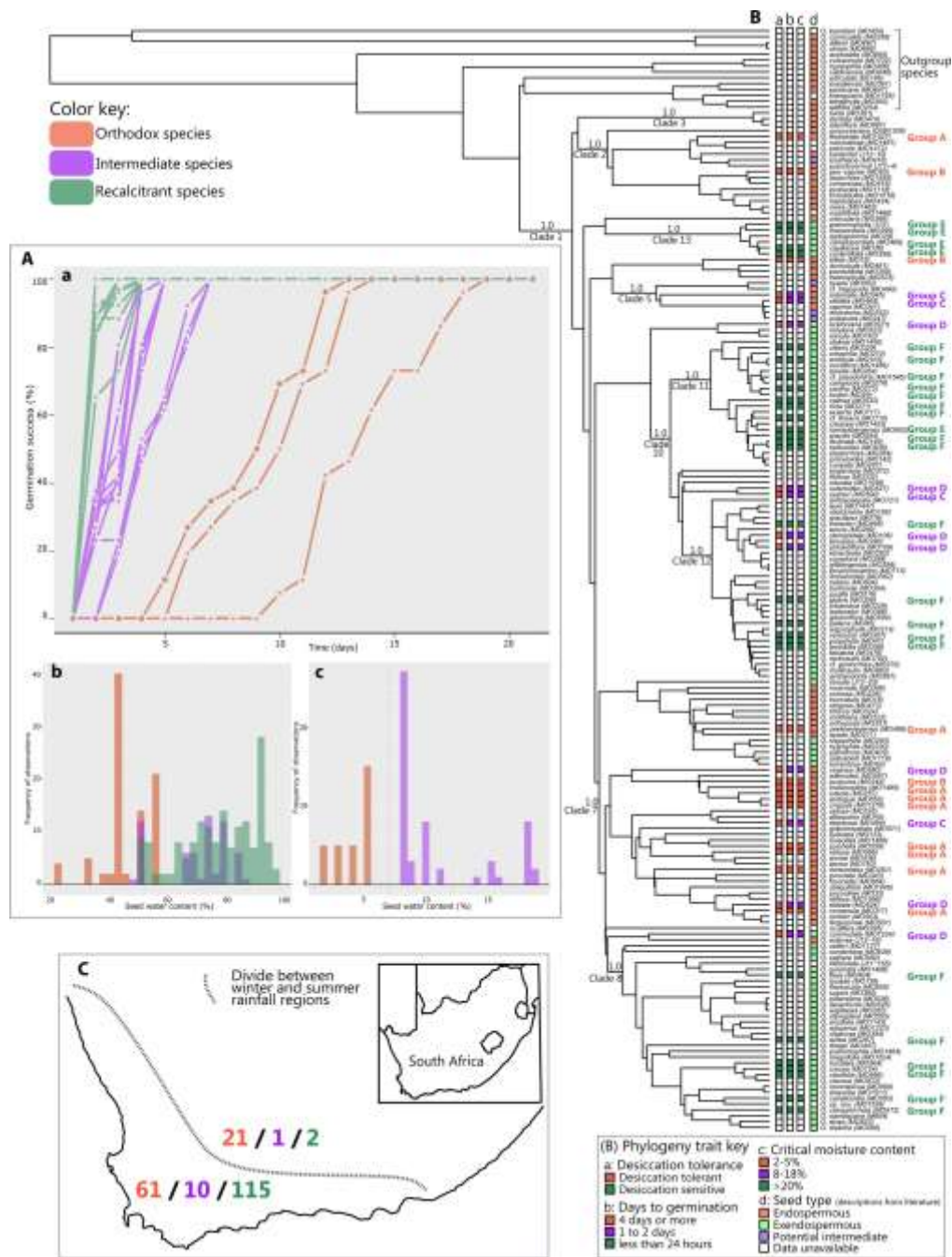
## **RESULTS**

### **Seed physiology**

Many of the endospermous seeds of *Oxalis* species (24.2% of our initial sample) did not germinate during the time period of this study. These species included *O. ambigua* Jacq., *O. convexula* Jacq., *O. crispula* Sond., *O. fenestrata* Dreyer, Roets and Oberl., *O. lichenoides* T.M.Salter, *O. luteola* Jacq., *O. melanosticta* Sond., *O. obtusa* Jacq., *O. obtusa* var. *atrata* T.M.Salter, *O. cf. pes-caprae* (project number MO1632), *O. pulchella* Jacq., *O. purpurea* L., *O. zeekoevleyensis* R.Knuth). Failure to germinate within the same year as collection has been recorded for some of these species by Brink (2017). Morphological or phenological data of seedlings could therefore not be documented for these species. However, the seeds of all species were viable, as they successfully germinated in the subsequent growing season

(Brink [2017] and personal observation [M. Jooste, L.L. Dreyer]). We suggest that these seeds have a longer and possibly mandatory delay in germination. Among the remaining species, 22.6% had desiccation-tolerant seeds, and 53.2% had desiccation-sensitive seeds. For most of the species with desiccation-tolerant seeds, seeds germinated within 1 to 2 days after shedding, while seeds for three species had a quiescent period of at least 4 to 7 days before germination (Fig. 1A-a). All desiccation-sensitive seeds germinated within 1 day after shedding. Unexpectedly, seeds of some typical exendospermous, recalcitrant species (Salter, 1944) proved to be desiccation-tolerant (including *O. commutata*, *O. eckloniana* C.Presl, *O. phloxidiflora* Schltr., *O. stenopetala* T.M.Salter, *O. suteroides* T.M.Salter, and *O. zeyheri* Sond).

Seed moisture content (mc) at shedding had substantial overlap between desiccation-tolerant (22.2 to 86.3% mc) and desiccation-sensitive (50 to 96.2% mc) species (Fig. 1A-b). The critical seed moisture content (cmc) of desiccation-sensitive seeds could not be determined, because all seeds lost viability after a 2-week desiccation period. This loss indicated that recalcitrant seeds cannot tolerate substantial water-loss, and we would expect that these seeds have very high cmc values. Cmc of desiccation-tolerant seeds showed a distinctive divide between species that were able to survive only one desiccation treatment (8 to 19% cmc) and species that were able to survive both desiccation treatments (2 to 5% cmc) (Fig. 1A-c).



**Figure 1.** The phylogenetic, physiological, and geographic distribution of orthodox, recalcitrant, and intermediate seed germination strategies in southern African *Oxalis*. (A) Physiological traits of seed germination strategies. (a) Germination progress curves of orthodox, intermediate, and recalcitrant seeds (seeds exposed to the initial germination test from the Kew 100-seed test). (b) Comparative percentages of seed moisture content at shedding between orthodox, intermediate, and recalcitrant seeds. (c) Critical seed moisture contents for desiccation-tolerant orthodox and intermediate seeds. The vertical dashed line represents the critical moisture content classically used to distinguish between orthodox and intermediate seeds (Ellis et al., 1989; Probert and Longley, 1989; Pritchard, 1991; Hong and Ellis, 1996; Pammenter and Berjak, 1999). (B) Phylogenetic distribution of characters/germination strategies. (a) Desiccation tolerance; (b) number of days to germination and (c) critical seed moisture content; (d) presence/absence of endosperm in *Oxalis* seeds, as described in the literature. Posterior probability values and major clade names

corresponding to Jooste et al. (2016) are indicated at relevant nodes. Groups labelled at right correspond to groups identified in the text and following figures. Details of phylogeny reconstruction are available in Jooste et al. (2016). (C) Geographic distribution of germination strategies. The number of species from each strategy is indicated with representative colors. The divide between the winter (below the dashed line) and summer (above the dashed line) rainfall regions is indicated with a dashed line (Snijman, 2013).

## **Defining seed germination strategies based on seed physiology**

Here we used a composite of three physiological traits to classify seeds into three categories: orthodox, recalcitrant, and intermediate (Appendix S4).

These traits included seed desiccation tolerance, time from shedding to germination and lowest critical seed moisture contents for desiccation-tolerant species (Fig. 1A and 1B). Consequently, orthodox *Oxalis* seeds were defined as seeds that could survive a desiccation period of 4 weeks without loss of viability, had critical moisture contents between 2 and 5% and a minimum quiescent period of at least 4 days (up to a year) before germination.

Recalcitrant seeds were defined as seeds that could not survive a desiccation period of 2 weeks and were therefore unable to tolerate water loss and germinated within 1–2 days after shedding. Seeds lying between these two categories were defined as intermediate.

Among the studied species, 28.6% had orthodox seeds, 53.9% had recalcitrant seeds, and 17.5% had intermediate seeds. All orthodox species were endospermous, and all recalcitrant species lacked endosperm (Salter, 1944). The new intermediate group included five species previously described as endospermous (*O. depressa* Eckl. and Zeyh., *O. dilatata* L.Bolus, *O. imbricata*, *O. stellata* Eckl. and Zeyh., *O. virginea* Jacq.) and six species described as exendospermous (*O. commutata*, *O. eckloniana*, *O. phloxidiflora*, *O. stenopetala*, *O. suteroides*, *O. zeyheri*) (Salter, 1944) (Fig. 1B).

## **Exploring morphological groupings among germination strategies**

### ***Cluster analyses***

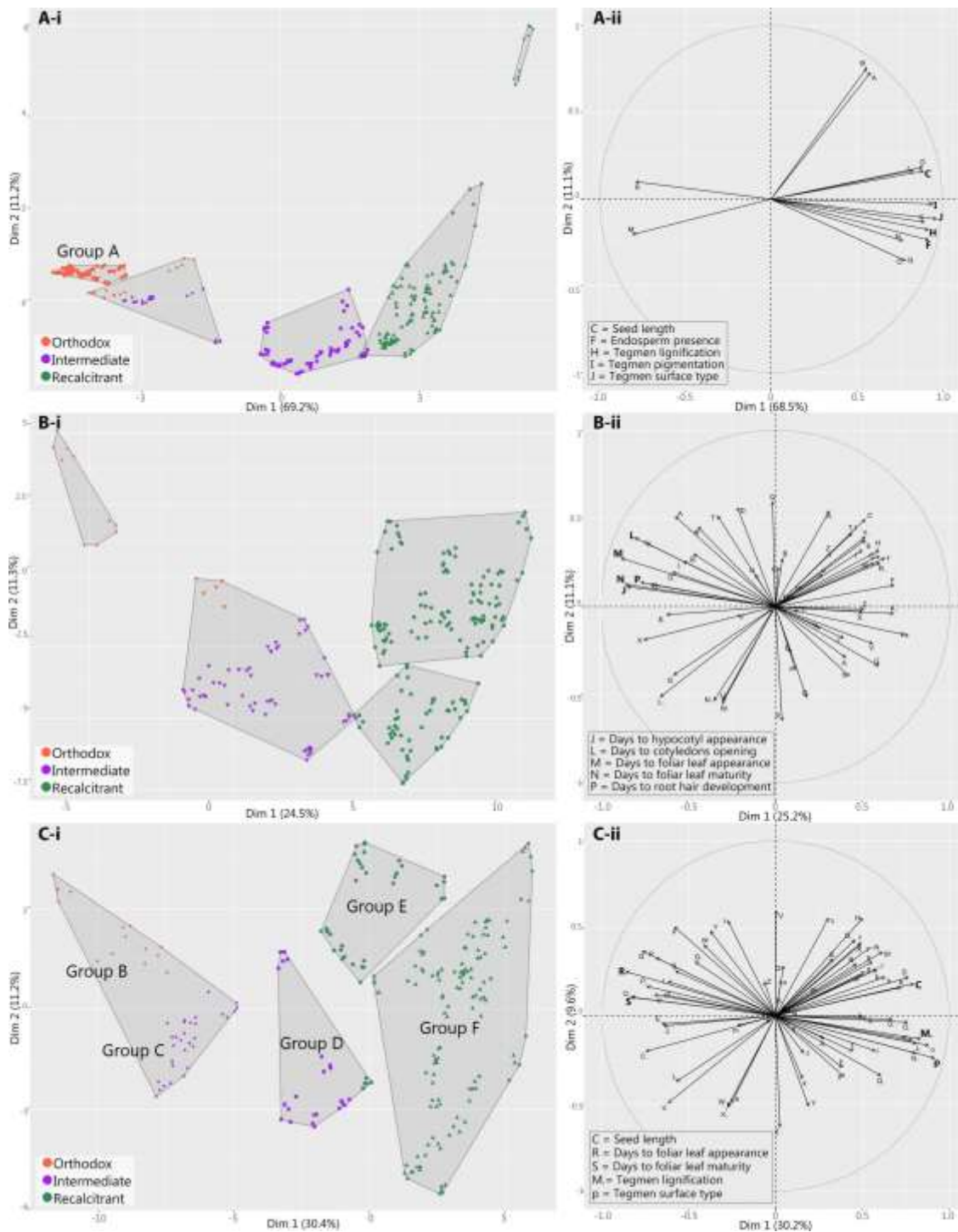
The majority of within-species replicates formed distinct clusters. Analysis of seed morphology indicated at least four clusters that loosely corresponded to the three seed germination strategies as defined above (Appendix S2 A).

However, there were a few odd placements (e.g., *O. bifida*, *O. virginica*, and *O. zeyheri*) and overlap among strategies. This unclear pattern may be due to the relatively few traits included in the seed data set. Seedling morphology data revealed three clusters that corresponded to our predefined strategies, with one odd placement (*O. cf. pallens*) (Appendix S2 B). Analysis of the combined seed and seedling data set clearly separated our predefined categories, with one cluster corresponding to orthodox species, one to recalcitrant species and two clusters with intermediate species (Appendix S2 C).

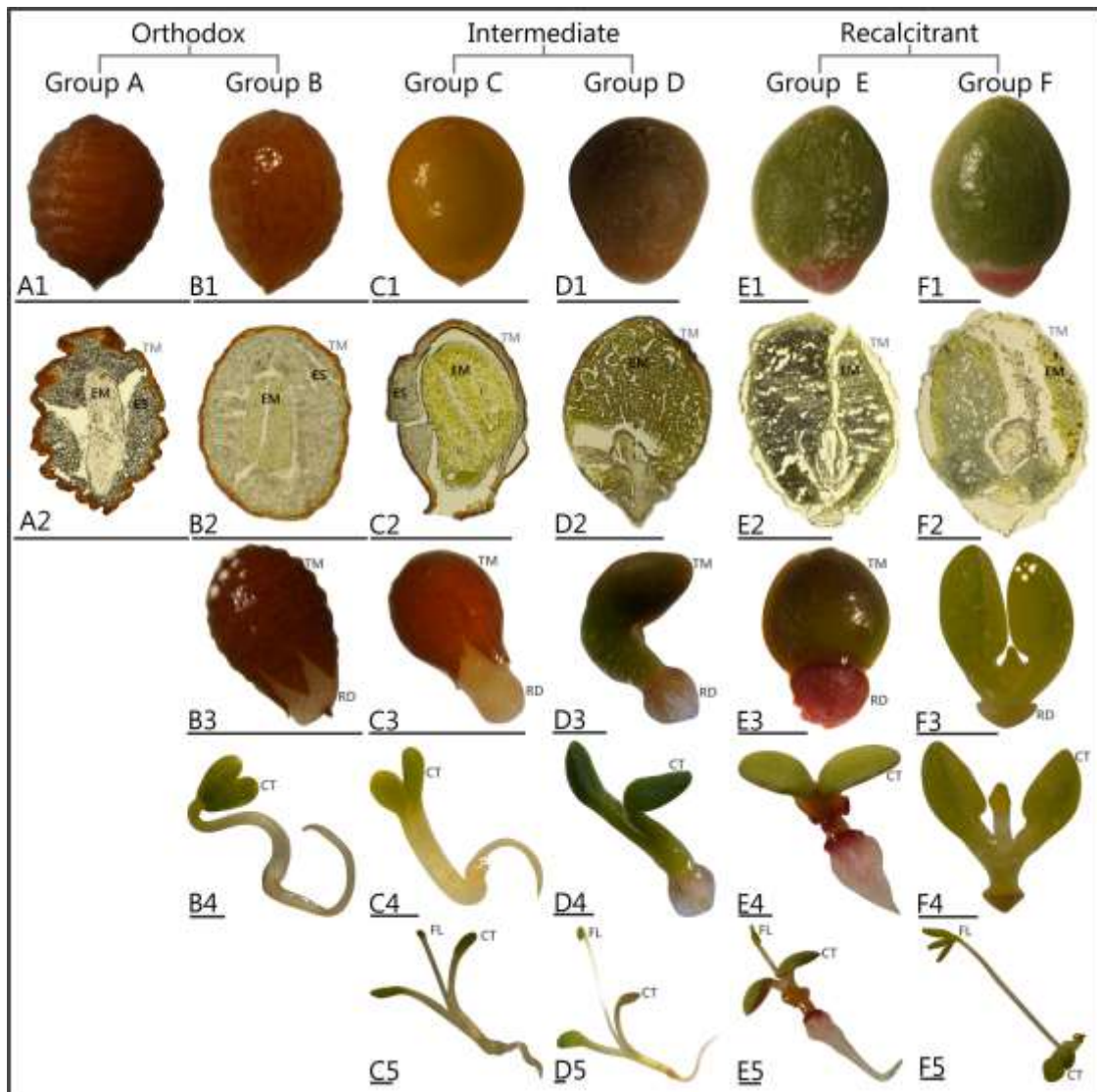
### ***Principal component analyses (PCA) with K-means optimal clustering***

The five within-species replicates formed distinct clusters in the PCAs (Appendix S3), with a few notable exceptions. PCA and *K*-means clustering showed at least three clusters corresponding to germination strategies within each of the seed, seedling, and combined seed and seedling morphological data sets.

In the seed data set, the first two principal components explained 69.2% and 11.2% of the variation in the data. The *K*-means cluster analyses identified five optimal clusters (Fig. 2A). The orthodox species with seeds that did not germinate formed the first coherent cluster (subsequently referred to as Group A; Fig. 3A, Table 1). The second cluster included orthodox species that germinated within the first growing season and a few intermediate species that appear to be morphologically most similar to other orthodox taxa. The third cluster included intermediate and two recalcitrant species (*O. glabra* and *O. cf. pallens*), indicating overlap among our predefined strategies. The fourth cluster included recalcitrant species and one intermediate species (*O. stenopetala*) in the region of overlap, while the last cluster included recalcitrant species only.



**Figure 2.** PCA with *K*-means clustering with the optimal number of clusters among discrete and continuous *Oxalis* seed and seedling morphological and developmental data. Support for three main germination strategies is evident among the (A) seed, (B) seedling, and (C) combined seed and seedling data sets. Individual factor maps with optimal clusters (i) and variable factor maps (ii). Five traits with the strongest grouping effects of each of the respective data sets are indicated in bold text with a key to these selected variable names provided. A key to all variable factor names is provided in Appendix S6a–c.



**Figure 3.** Seed morphology corresponding to six morphological groups (Groups A to F) from three germination strategies among Cape *Oxalis*. Each column contains photos of representative seeds and seedlings from each group. (1) Seed with tegmen, (2) seed cross section (TM = tegmen, ES = endosperm, EM = embryo), (3) germinating seed (RD = radicle), (4) seedling with opening cotyledons (CT = cotyledons), (5) seedling with emerging first foliar leaf (FL = foliar leaf). Scale bars = 1 mm. All seeds/seedlings oriented with radicle pointing to bottom (rows 1, 2) or bottom right (rows 3–5) of figure. A key to all species names is provided in Appendix S7.

In the seedling data set, clusters corresponded to germination strategies and the first two principal components explained 24.5% and 11.3% of the variation in the data. The *K*-means cluster analyses revealed four optimal clusters (Fig. 2B). The first cluster included only two of the three orthodox species that germinated (*O. bifida*, *O. purpurea*). The second cluster included



**TABLE 1.** (A)Physiological, (B) seed morphological, and (C) seedling morphological traits associated with germination strategies and subgroups identified among Cape *Oxalis*. NA = not applicable.

	Orthodox		Intermediate		Recalcitrant	
	Group A	Group B	Group C	Group D	Group E	Group F
<b>(A) Physiological traits</b>						
Desiccation tolerance or sensitivity	NA	Tolerant	Tolerant	Tolerant	Sensitive	Sensitive
No. of days to germination	NA	4–9	1–2	1	1	1
Critical seed moisture content (%)	NA	2–5	8–18	8–18	NA	NA
<b>(B) Seed morphological traits</b>						
No. of seeds per capsule	25-100	50-90	5–50	20–45	5–30	5–25
Endosperm	Present	Present	Present	Absent	Absent	Absent
Embryo pigmentation	None	Green	Green	Green	Green	Green
Dry single seed mass (mg)	0.03–0.07	0.03–0.91	0.03–0.10	0.10–0.12	0.78–3.54	0.10–0.82
Seed width (mm)	0.59–1.08	0.61–1.46	0.65–1.06	0.78–1.73	1.27–1.80	1.11–2.44
Seed length (mm)	0.65–1.76	0.85–1.50	0.90–1.37	1.30–2.24	1.67–2.27	1.14–3.95
Tegmen lignification	Yes	Yes	Yes	No	No	No
Tegmen pigmentation	Yes	Yes	Yes	Yes	Semi-transparent	No
Tegmen surface type	Irregular	Irregular	Irregular	Smooth, velvety	Smooth, papery	Smooth
Hypocotyl protrusion visible	No	No	No	Yes	Yes	Yes

**(C) Seedling morphological traits**

Days to hypocotyl emerge	NA	4–22	3–8	1–5	1–2	1–2
Days to root hairs emerge	NA	5–23	3–13	2–6	2–8	2–8
Days to cotyledons open	NA	8–30	6–10	2–5	1–4	1–4
Days to first foliar leaf emerge	NA	10–29	8–19	3–18	1–3	1–3
Germination sequence	NA	Radicle-first germination	Radicle-first germination	Simultaneous radicle, root development	Simultaneous radicle, root development	Foliar-leaf-first germination
Hypocotyl width (after cotyledons opened, mm)	NA	0.34–0.55	0.26–0.64	0.43–1.04	0.43–1.65	0.52–1.44
Root lengths, (after cotyledons opened, mm)	NA	3.33–14.5	0.54–5.15	0.42–12.04	0.50–4.07	1.08–13.95
Hypocotyl length (after foliar leaf matured, mm)	NA	0.58–6.12	0.12–0.59	0.17–0.96	1.30–2.24	1.30–0.94
Cotyledon petiole length (mm)	NA	0–1.47	0.41–3.67	0.87–5.23	0–0.65	0–1.92
Cotyledon shape	NA	Elliptical	Elliptical	Elliptical	Ovate	Ovate
Stomatal position on cotyledons	NA	Amphistomatic or hypostomatic	Amphistomatic	Amphistomatic or epistomatic	Amphistomatic or epistomatic	Amphistomatic or epistomatic

the other orthodox species (*O. pes-caprae*), all intermediate species, and an overlap with one recalcitrant species (*O. cf. pallens*), again indicating some overlap among our predefined strategies. The two remaining clusters included recalcitrant species only.

In the combined data set clusters, the first two principal components explained 30.4% and 11.2% of the variation in the data. The *K*-means cluster analyses showed four distinct clusters (Fig. 2C). The combined seed and seedling data set was deemed the most robust and representative because it included the most traits and species (even though the orthodox species that did not germinate had to be excluded). Descriptions and interpretations of all traits important in explaining distribution of data from the seed, seedling, and combined data sets are included as Appendix S5.

The first cluster included distinct groups of orthodox (hereafter Group B; Fig. 3B, Table 1) and intermediate (Group C, endospermous intermediate; Fig. 3C, Table 1) species, and corresponded to a similar cluster found in the seed data set. Although *K*-means clustering placed these taxa in one cluster, the clear gap between orthodox and intermediate morphologies in this cluster supports its division into two groups. All Group B and C seeds have lignified tegmens, are endospermous at seed release, and have green-pigmented embryos, but physiologically respond differently to desiccation. The second cluster (Group D, exendospermous intermediate, Fig. 3D, Table 1) held the remaining intermediate and one recalcitrant species (*O. cf. pallens*). The two remaining clusters included recalcitrant species only, namely, one small cluster that consisted of species from one subsection (section *Angustatae* subsection *Pardales* sensu Salter (1944), except for *O. kamiesbergensis* T.M.Salter, subsequently referred to as Group E; Fig. 3E, Table 1), while the other included the remaining recalcitrant species (subsequently referred to as Group F; Fig. 3F, Table 1). All three data sets agreed on separating recalcitrant species from the other clusters, although precise clustering among recalcitrant taxa differed across data sets.

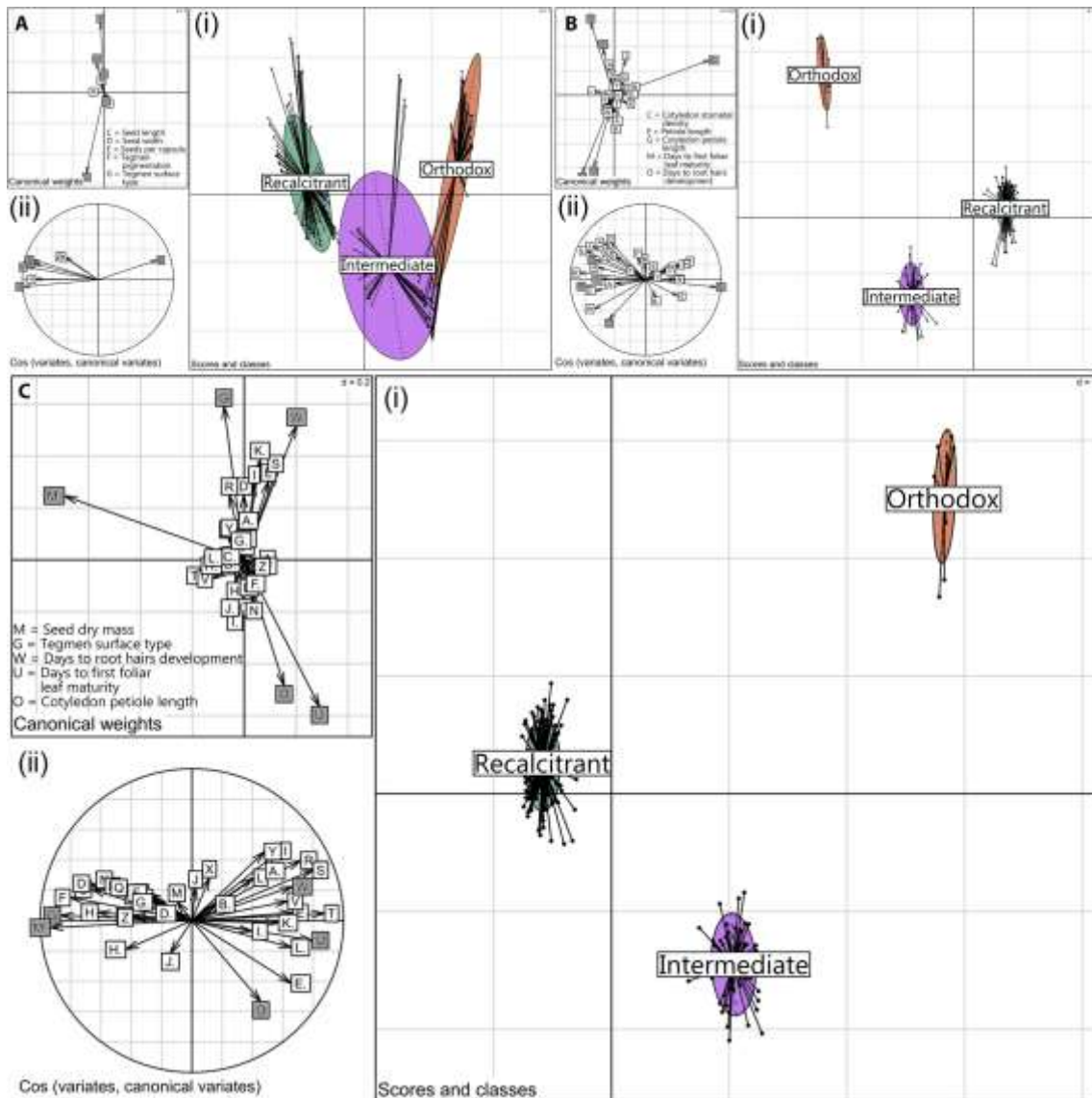
The number of seeds per capsule and cotyledon shape determined the spread of data across the first principal component, and cotyledon petiole length and

hypocotyl width were the most important traits across the second principal component. The most important traits separating clusters included morphological traits such as tegmen surface texture, tegmen lignification, presence or absence of endosperm, and embryo pigmentation, and phenological traits such as number of days until cotyledons opened, until root hair development, and until the first foliar leaf became visible (Fig. 2C). The traits that determined the spread of clusters for this data set were largely the same as those identified among the separate seed and seedling data sets.

### ***Confirming germination strategies with discriminant analyses (DA)***

Discriminant analyses of the seed data set to determine whether morphological and developmental seed and seedling traits were predictive of our three proposed seed germination strategies showed that species primarily clustered according to seed germination strategies (MANOVA and Pillai's test ( $F_{2, 1.156} = 52.352$ ,  $p < 0.0001$ ), Monte-Carlo permutation test (mean observations = 0.1444564,  $p < 0.0001$ ), Fig. 4A). However, there was a detectable overlap between the orthodox and intermediate groups, while the recalcitrant seeds formed a separate cluster. The DA of the seedling data set also indicated that species clearly clustered according to seed germination strategies (MANOVA and Pillai's test ( $F_{2, 1.883} = 107.58$ ,  $p < 0.0001$ ), Monte-Carlo permutation test (mean obs = 0.06072856,  $p < 0.0001$ ), Fig. 4B), this time without any overlap between any of the three groups.

Clustering according to germination strategies was also evident from the DA using the combined seed and seedling data set (MANOVA and Pillai's test ( $F_{2, 1.912} = 111.55$ ,  $p < 0.0001$ ), Monte-Carlo permutation test (mean obs = 0.04902814,  $p < 0.0001$ ), Fig. 4C), again without overlap between groups. Among the combined data set traits important in separating strategies along the first axis were tegmen surface type, tegmen permeability, and the number of days until the first foliar leaf appears. The separation of recalcitrant and intermediate strategies was strongly influenced by seed dry mass (measured after cotyledons opened and after the first foliar leaf matured). Orthodox and intermediate strategies were strongly influenced by tegmen surface type and the number of days until the root hairs developed.



**Figure 4.** Discriminant analyses indicating that morphological and developmental *Oxalis* seed and seedling traits were predictive of membership to each of our assigned physiological germination strategies. Support for three germination strategies is evident among the (A) seed, (B) seedling, and (C) combined seed and seedling data sets. Individual factor maps (i) are used to visualize the spread of data, and variable factor maps (ii) are used to assess the specific continuous and ordered discrete traits that explain these groupings. Five traits with the strongest grouping effects of each of the respective data sets are shaded in grey with a key to these selected variable names.

Our results for seed data are mostly consistent with Obone (2005) and Brink (2017). For seeds, the results of Obone (2005) and our study support a primary split between orthodox and mostly recalcitrant taxa. Although our results include some typically orthodox taxa in the recalcitrant cluster, these could be due to broader taxonomic sampling, or partially non-overlapping sets

of seed characters. For seedling data, in contrast to a clear pattern separating orthodox from recalcitrant/intermediate taxa in our study, Brink (2017) found a somewhat more complex pattern. Cluster analyses showed two distinct recalcitrant clusters and two orthodox clusters, one consisting of *O. bifida* and the other containing all other orthodox taxa. However, PCAs showed a close similarity between all orthodox taxa, and there was clear morphological support separating the orthodox and the recalcitrant strategy, consistent with our results.

In summary, all data sets showed that germination strategies were associated with suites of seed and seedling morphological and developmental traits. The majority of traits identified in the separate seed and seedling data sets were also important in separating strategies in the combined data set. These data and analyses provide strong, independent evidence in support of the recognition of three germination strategies among Cape *Oxalis*, as well as subgroups among each strategy.

## **DISCUSSION**

Here we have shown that the division of *Oxalis* germination strategies into two discrete types, namely orthodoxy and recalcitrance, is an oversimplification. Many species occupy positions intermediate between these two states. We have identified three germination strategies, defined according to three seed physiological traits, namely, desiccation tolerance, time from shedding to germination, and lowest seed critical moisture content. We have also shown that there is morphological and phenological support underpinning these physiologically defined strategies. Analyses of morphological data revealed two potential subgroups among each of the three seed germination strategies (Groups A–F). Even though we have identified six groupings among the three germination strategies, it is important to note that we have sampled approximately 27% of the 230 *Oxalis* taxa in southern Africa and that increased sampling might blur the boundaries between these defined strategies and groups. Thus, the data could just as likely represent a continuum of states.

It is of interest that there is very little pattern corresponding to the current taxonomy (Salter, 1944) or to phylogeny (Fig. 1B) among the three strategies

or six groups. A recent study that assessed seedling morphological traits of Cape *Oxalis* showed that orthodox species of widespread taxonomic affinity clustered together regardless of relatedness (Brink, 2017). This result was interpreted as a possible signal of stabilising selection (Brink, 2017). The same study found possible morphological signals for convergent evolution of recalcitrance in unrelated lineages. However, our findings indicated that all three strategies included species from multiple, unrelated lineages. The balance of evidence thus suggests a complex pattern of convergence on germination strategy within Cape *Oxalis*. No paper has examined the evolution of germination strategy in Cape *Oxalis* in an explicit phylogenetic context—such a study will have to await germination data in the ca. 73% of unsampled *Oxalis* taxa.

Potential adaptive significance of morphological traits associated with strategies

Several seed traits varied distinctly among the three germination strategies indicative of classic quantity–quality trade-offs. Orthodox species (Groups A and B) produce capsules with many seeds, that are very small (low seed length and mass) and have an endosperm. This strategy may be low risk for orthodox species because seeds have the ability to maintain quiescence, survive desiccation, and can consequently be effectively dispersed through space and time until they encounter favorable germination conditions (Evans and Dennehy, 2005). Recalcitrant species (Groups E and F) seem to follow the exact opposite strategy, where species produce few seeds per capsule, but with high seed mass and contain no endosperm upon release from the capsule. The embryos of recalcitrant seeds are large and ready to germinate upon release, which coincides with the fact that these seeds are dispersed and germinate in the wettest season of the Cape. Species with intermediate seeds (Groups C and D) are intermediate in number, size, and amount of endosperm relative to the orthodox and recalcitrant strategies. These intermediate species therefore benefit by having relatively large seeds with relatively well-developed embryos and the presence of endosperm, which affords them the opportunity to either germinate immediately upon release when conditions are favorable or

maintain quiescence for a few days or weeks until conditions are more favorable.

Orthodox species (Group B) have seedlings that germinate with their hypocotyl emerging from the seed, followed by substantial root growth, then root hair development. These species form long roots with a long, thin hypocotyl. Cotyledons only unfold once the root and root hairs are well established. The first foliar leaf develops and matures at a much later stage. This sequence of development is similar to the classical sequence of development documented among the majority of angiosperm seedlings (Esau, 1960). Intermediate species (Groups C and D) display various sequences of development where either the hypocotyl or root hairs emerge from the seed, but all seedlings reach maturity much more rapidly than orthodox species. Recalcitrant species (Group E) display root-first germination. The hypocotyl and root hairs emerge and develop, followed by cotyledons unfolding, and the development and maturation of the first foliar leaf. The majority of recalcitrant species (Group F) displays a strategy of inverse germination, where cotyledons and the first foliar leaf develop rapidly and appear to sustain rapid growth of the seedling, until the hypocotyl, root hairs, and roots subsequently emerge. This is a remarkable phenomenon where seedlings are capable of rapid growth and development temporarily without well-established roots to supply the seedling with nutrients. Many of these recalcitrant species produce large amounts of mucilage upon germination. Preliminary investigation of microbes within the mucilage (under sterile control and various experimental conditions) revealed the presence of both bacteria and fungi. Subsequent research is aimed at investigating potential associations between recalcitrant *Oxalis* species and mucilage-dwelling microbes.

### **Potential evolutionary trends among germination strategies**

The orthodox strategy is regarded as the ancestral state among Oxalidaceae and *Oxalis* species, while recalcitrance appears to have had multiple independent origins within the Cape (Salter, 1944; Oberlander et al., 2011, Fig. 1B). Our results suggest that orthodox and recalcitrant strategies may be viewed as two extreme states, connected physiologically through the



intermediate strategy and morphologically through at least two intermediate groups. We suggest that seed germination has evolved from the ancestral orthodox state toward a derived, maximally recalcitrant peak. Even though we have opted for discrete groups in explaining the variety of intermediate morphologies evident among our sampled taxa, the orthodox (Group B), intermediate (Groups C and D), and recalcitrant (Groups E and F) groups could represent an over-simplification of the reality. It is possible that all of the aforementioned groups could represent a continuum of states, bounded by typical orthodoxy and maximal recalcitrance, and connected evolutionarily by taxa containing a mosaic of morphologically and physiologically intermediate seed and seedling characters, some of which are still represented among extant Cape *Oxalis* species.

Many authors have proposed a general evolutionary trend of increasing embryo size (development) and decreasing endosperm to acquire recalcitrant seeds among angiosperms (Martin, 1946; Berjak and Pammenter, 1997; Sun 1998; Forbis et al., 2002; Kermode and Finch-Savage, 2002; Berjak et al., 2004). Based on this hypothesis, one would expect that a transition from orthodoxy to maximal recalcitrance among Cape *Oxalis* species would be a step-by-step assembly of traits associated with recalcitrance, where certain physiological and morphological traits (such as desiccation tolerance and consequently increased embryo size and decreased endosperm) are lost or acquired. As possible descendants of the various steps in this process, taxa in the intermediate strategy (Groups C and D) might provide information on the assembly of the recalcitrant strategy and the selective pressures leading to its establishment.

Absence of a strong phylogenetic signal of recalcitrance (and associated morphological and phenological traits) among angiosperm clades has been attributed to convergent evolution of traits in response to environmental conditions (Lord et al., 1995; Rees, 1996; Forbis et al., 2002). Berjak and Pammenter (2008) stated that if desiccation sensitivity is a derived trait, there must be selective advantages to losing desiccation tolerance, even though it is such an important functional trait. These selective advantages would include either direct fitness advantages, such as competition avoidance or higher

population growth rates (discussed in more detail below), or ecological trade-offs within a niche under specific environmental conditions (sensu Grubb (1977)). Fitness advantages could benefit seeds at the stage of shedding, dispersal, germination, and/or seedling establishment and growth.

### **Environmental factors affecting germination strategies**

Tweddle et al. (2003) proposed that seeds shed in seasonal environments will be highly influenced by two important environmental factors, temperature and water availability. Using the presence or absence of endosperm as a rough proxy for orthodoxy or recalcitrance, the vast majority of exendospermous *Oxalis* species (121 of 123) are restricted to the winter rainfall region of the Cape (Salter, 1944, Fig. 1C). Exendospermous *Oxalis* species flower and set seed during the early winter months (May to June) (Dreyer et al., 2006), therefore ensuring that seeds can take advantage of high seasonal water availability and presumably maximising the amount of time for growth and establishment. Endospermous, i.e., mostly orthodox *Oxalis* species are distributed more evenly in both the winter (63 species) and summer (28 species) rainfall regions of southern Africa (Salter, 1944, Fig. 1C). Similar to the recalcitrant *Oxalis* species, the majority of intermediate species (10 of 11) are also restricted to the winter rainfall region (Fig. 1C) with only one endospermous intermediate species from the summer rainfall region. African *Oxalis* most likely originated in the Cape region (Oberlander et al., 2011), and recalcitrance is also likely to have evolved there, given the almost complete confinement of exendospermous taxa to the Cape region. Thus, it is likely that the decrease in reliable winter rainfall moving east might create a significant barrier to the establishment of recalcitrant seedlings, and thus of recalcitrant taxa, outside of the Cape. At the least, this difference in geographic distribution of germination strategies strongly implies a close linkage of recalcitrance to the winter rainfall Cape region.

The summer rainfall region often experiences freezing (especially during the winter months), with relatively low humidity compared to the winter rainfall region (Manning and Goldblatt, 2012; Snijman, 2013). Desiccation-tolerant seeds that are capable of surviving below-zero temperatures would be favored

in these habitats (Tweddle et al., 2003) or would be forced to adopt a desiccation-avoidance strategy (Pammenter and Berjak, 2000). Recalcitrant and intermediate *Oxalis* seeds have a high moisture content, indicating that these seeds would not be able to survive freezing, due to ice formation in their embryos (Ellis et al., 1990). Even though we did not assess freezing ability directly, we would predict that orthodox *Oxalis* seeds would remain viable under sub-zero temperatures, as is typical for all orthodox seeds (Ellis et al., 1990). These environmental factors would prevent the successful establishment of recalcitrant and intermediate species in summer rainfall regions of the Cape, consequently limiting species with these strategies to the winter rainfall region. We therefore predict that both reliable seasonal rainfall (available moisture) and minimum air temperature would influence and/or determine the distribution of *Oxalis* species throughout the two rainfall regions of the Cape. Dussert et al. (2000) proposed that dispersal methods, fruiting phenology and habitat-related descriptions are required to fully understand factors affecting or determining germination strategies.

### **Fitness advantages associated with different germination strategies**

Seed quiescence is regarded as a bet-hedging strategy to spread the risk of unsuccessful reproduction in unpredictable or stochastic environments (Cohen, 1966; Venable 2007; Poisot et al., 2011; Moreira and Pausas, 2012). Due to the ability of orthodox seeds to survive desiccation, these seeds would be capable of avoiding unfavorable conditions and would be able to establish large seeds banks. Seeds may remain viable for long periods (species-specific responses), allowing species to “select” the optimal time to initiate germination (Linkies et al., 2010; Baskin and Baskin, 2014). Desiccation-tolerant seeds are more likely to be dispersed in space and in time and are therefore able to minimize competition between siblings (Cheplick, 1992). Dreyer *et al.* (2006) reported that orthodox *Oxalis* species flower for a relatively long period. Orthodox *Oxalis* seeds consequently experience less climatic constraint because seeds that are shed late in the season are able to remain quiescent (and viable) until the following growing season (Dreyer et al., 2006).

Recalcitrant seeds have the ability to germinate immediately or soon after shedding, which may be advantageous in particular scenarios. If the seeds are shed under predictably favorable environmental conditions, such as the wet winter months of the Cape, germination success immediately after seed dispersal will be highly likely and loss of quiescence may become favorable (Kermode and Finch-Savage, 2002). These seeds are metabolically active when shed, which enables them to germinate, establish, and reach maturity much more rapidly than orthodox seeds (Kermode and Finch-Savage, 2002). Rapid germination of recalcitrant seeds decreases the time that seeds are exposed to post-shedding predation or microbial decay and increases the amount of growth (seedling biomass) before seedlings are exposed to unfavorable conditions (Tweddle et al., 2003). Recalcitrant seeds do not produce large amounts of endosperm or lignified tegmens, possibly indicating a more efficient utilization of resources in comparison with orthodox seeds (Berjak and Pammenter, 2008). Recalcitrance could, however, come with the cost of decreased growth rates or high mortality rates if seeds are shed when the environmental is unfavorable (low humidity and low available moisture) (Farnsworth, 2000; Tweddle et al., 2003). Given the short flowering period and early flowering peak of exendospermous species (Dreyer et al., 2006), suboptimal environmental conditions during the flowering period might have major repercussions for recalcitrant *Oxalis* recruitment.

Intermediate seeds are capable of both desiccation tolerance and rapid germination due to their well-developed embryos upon release. The investment in lignified tegmens and endosperm may be costly, but these structures ensure that the well-developed and metabolically active embryos are able to survive periods of desiccation (Ellis et al., 1990). These seeds have the benefit of immediate germination if environmental conditions are favorable, or delay germination until conditions become favorable. This view is, however, challenged by the comparative rarity of species with an intermediate germination strategy.

## CONCLUSIONS

Here we have shown that the division of *Oxalis* germination strategies into two discrete types, dormancy or recalcitrance, is an oversimplification. Many

species occupy positions intermediate between these two states and an intermediate germination strategy does exist among Cape *Oxalis*, with two possible morphological groups within each strategy. These could reflect a continuum of germination states, where an ancestral dormant strategy evolved towards a maximally recalcitrant peak, with a mosaic of intermediate states reflected in extant taxa. Environmental factors may affect germination strategy and distribution throughout the Cape because recalcitrant and intermediate species are confined to the winter rainfall region. They occupy specialized niches and may face adverse impacts when winters become hotter and drier, meriting focused future conservation.

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## **AUTHOR CONTRIBUTIONS**

L.L.D., K.C.O., and M.J. designed the research. M.J. conducted most of the research, with some field assistance from L.L.D. Data were analyzed by M.J. All authors contributed equally to data interpretation. M.J. wrote the paper, and all co-authors commented on two drafts of the final paper.

## **DATA ACCESSIBILITY**

The data sets generated during the current study are included as supplementary files of this article (Appendices S1–S7).

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