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A comparative study on seed physiology and germination requirements for 15 species of *Eucalyptus*

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Abstract Seed physiology of 15 *Eucalyptus* species of interest for cut foliage plantations was unknown and therefore evaluated. The viability and vigour of seeds and germination potential of 15 *Eucalyptus* species was determined by using a tetrazolium (TZ) staining test, and the results were compared to a germination test. In a separate experiment, seeds of each lot were subjected to either 0 or 4-week cold stratification at 4 ± 1 °C to investigate their potential stratification requirement. After stratification, seeds were then allowed to germinate at 22 ± 1 °C with 16 h lighting per day for 36 days. Seed viability and vigour were checked by evaluating % root, cotyledon and first true leaves emergence, and the speed of emergence, in the germination test. The germination percentages varied with the species. Seed stratification with the interaction of seed species lots significantly affected both viability and vigour. The seed viability of the different species ranged from 9 to 100% and 2 to 100%, for the TZ test and germination test, respectively, with a high correlation (R^2 = 0.89) between the two. Physiology tests revealed that cold stratification of seed was not required for the 15 species to maximise their germination potential and growth in Irish and British climate.

Keywords Seed physiology · *Eucalyptus* · Viability · Vigour · Tetrazolium · Stratification

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1 Introduction

Eucalypt species are important for an expanding cut foliage sector in Ireland as they provide contrast in colour and shape with the flowers in a bouquet (Forrest and Moore 2008; Whelton 2012). The cut flower market in Europe is worth €14.5 billion of which foliage accounts for €1.3 billion (Brooker 2000; Toumi et al. 2016; Eurostat 2020). Currently, *Eucalyptus subcrenulata, E. parvula, E. glaucescens*, and *E. cinerea* are grown commercially in Ireland for foliage (http://www.forestproduce.ie/) but novel and interesting foliage species are always being sought. This requires continually evaluating potentially interesting species. Successful candidates will need to germinate and grow well in North West European conditions if they are to be considered for cut foliage plantations.

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Viable mature seeds of most eucalypts germinate under favourable conditions without stratification. However, some species germinate better when cold stratified, particularly those which are from alpine areas, such as *Eucalyptus delegatensis* and *E. pauciflora*, while for others, it is essential (Bell et al. 1995). Periods of 1 to 3 weeks cold stratification increased germination percentage of seed lots of *E. delegatensis*, particularly those which were collected from higher altitudes (Close and Wilson 2002). The need for several weeks of chilling to release dormancy may reflect the nature of the cold winter environment where they grow (Afroze and O'Reilly 2013).

The physiological quality of the seed determines its viability and vigour (Bell et al. 1995). However, there is not always information available on the seed viability and vigour of many eucalypt species offered by respected suppliers such as the Commonwealth Scientific and Industrial Research Organisation (CSIRO) (https://www.csiro.au/en/research) or Milligans Seeds (https://www.milliganseeds.co.nz/). Seeds lots, which have a larger number of sound full seeds, and uniform seedling emergence, are considered more vigorous (Bell et al. 1995). This can be ascertained by measuring morphological developments such as the speed of emergence of the root, cotyledon and first true leaves, which will give an indication of their physiological quality (Harrison et al. 2014).

Early screening of viability of different eucalypt species through germination tests is important as it may indicate how they are likely to perform once planted out (Williams et al. 2007; Brasil 2013). The tetrazolium (TZ) test is also routinely used to check seed viability of tree and other species before the actual germination tests. In addition to viability, it provides valuable information about seed vigour (Wood et al. 2005; Guedes et al. 2010; Flores et al. 2011; Marcos Filho 2015). Seed vigour is defined as "the sum total of those properties of the seed, which determine the level of activity and performance of the seed or seed lot during germination and seedling emergence" (Perry 1978; Perry and DA 1980). A quantitative assessment of vigour using a TZ test, based on formazan extraction and quantification using spectrophotometry, has been described (Harty et al. 1972; Norton 1985). TZ testing to determine the vigour index can be useful, especially for seeds of important crops such as common beans, corn, sunflower, and wheat (Das and Sen-Mandi 1988; Wood et al. 2005).

The aim of this study was to screen 15 eucalypt species selected for their cold hardiness and potential suitability to North West European climatic conditions as potential candidates for cut foliage. There has been no work done on them in terms of germination and vigour, therefore experiments were carried out to determine: (1): seed viability and vigour using a TZ test and germination test and (2): the effect of cold stratification (4 ± 1 °C) on seed germination and germination speed. The results will provide information on their potential suitability for cut foliage and other plantations in temperate North West European locations.

2 Material and methods

2.1 Seed material

15 *Eucalyptus* spp. were selected based on both their foliage characteristics from a cut-foliage perspective and their cold tolerance for growth in the Irish climate. The species are; *E. rubida* H. Deane & Maiden., *E. urnigera* Hook., *E. nitida* Hook., *E. moorei* Maiden & Cambage., *E. morrisbyi* Brett., *E. crenulata* Blakely & Beuzev., *E. cordata s*sp. *cordata* Labill., *E. cordata s*sp. *quadrangulosa* Labill., *E. coccifera* Hook., *E. mitchelliana* Cambage., *E. pauciflora s*sp. *niphophila* (Maiden & Blakely) L.A.S.Johnson & Blaxell., *E. subcrenulata*., *E. parvula* L.A.S.Johnson & K.D.Hill., *E. glaucescens* Maiden & Blakely, and *E. cinerea* F.Muell. ex Benth (Table 1). Four species in this study are native to alpine forests in Australia (*E. coccifera, E. mitchelliana, E. subcrenulata* and *E. pauciflora* ssp. *niphophila*). Seeds of each species were obtained from Milligan Seeds (https://www.milliganseeds.co.nz/) in Southland, New Zealand. The origin and provenance of these seed lots, where known, are given in Table 1. Seeds were harvested in 2016, delivered in March 2017 and subsequently stored dry at 4 ± 1 °C until used for experiments, which were carried out from July to November 2017. During storage, the seed moisture content was maintained at 8–10 °C Seed purity of each species was maintained close to 100% by separating the seeds from any inert materials through sieving before the viability test. The seeds were considered 'small' to 'very small', with a range

Table 1 Summary description of the 15 *Eucalyptus* species with their classification, seed lot origin and seed mass (g)

Eucalyptus species and sub species	Subgenus	Section	Seed mass (g) (1000 seeds)	Seed lot origin	Natural distribution
E. rubida	*Symphyomyrtus*	*Maidenaria*	2.08	Local New Zealand land race[a]	NSW
E. parvula	*Symphyomyrtus*	*Maidenaria*	0.3	Kybean, NSW, Australia	NSW
E. moorei	*Eucalyptus*	*Eucalyptus*	0.88	Origin is unknown	NSW
E. glaucescens	*Symphyomyrtus*	*Maidenaria*	1.95	The southern tablelands of NSW, Australia	NSW, Vic
E. cinerea	*Symphyomyrtus*	*Maidenaria*	0.79	Information has not been provided	NSW, Vic
E. morrisbyi	*Symphyomyrtus*	*Maidenaria*	0.73	Cremore, NSW	Tas
E. urnigera	*Symphyomyrtus*	*Maidenaria*	4.08	Local New Zealand land race	Tas
E. cordata ssp. *quadrangulosa*	*Symphyomyrtus*	*Maidenaria*	6.35	Bruny Island, Tas, Australia	Tas
E. cordata ssp. *cordata*	*Symphyomyrtus*	*Maidenaria*	5.28	Local New Zealand land race	Tas
E. subcrenulata (Mt Field)	*Symphyomyrtus*	*Maidenaria*	6.3	Mount Field, Tas, Australia	Tas
E. nitida	*Eucalyptus*	*Eucalyptus*	2.08	Local New Zealand land race	Tas
E. coccifera (Mt Field)	*Eucalyptus*	*Eucalyptus*	3.73	Mount Field, Tas, Australia	Tas
E. mitchelliana (Mt Buffalo)	*Eucalyptus*	*Eucalyptus*	5.88	Mount Buffalo, Vic, Australia	Vic
E. pauciflora ssp. *niphophila* (Mt Bogong)	*Eucalyptus*	*Eucalyptus*	2.1	Mount Bogong, Vic, Australia	Vic, NSW
E. crenulata	*Symphyomyrtus*	*Maidenaria*	1.6	Local New Zealand land race	Vic

NSW New South Wales, *Tas* Tasmania, *Vic* Victoria

[a]Local New Zealand land race means the seed was collected from trees planted in New Zealand from an unknown source

of mass from 0.3 to 6.4 g per 1000 (equivalent to 0.3 to 6.4 mg per seed). Seed mass of each species lot was determined by weighing four replications of 100 seeds per replicate. In the experiments, seeds were counted for each replication.

2.2 Cold stratification and seed germination

Seeds of each species were surface sterilised with 70% ethanol (v/v) for 5 min and subsequently in a 7% (w/v) calcium hypochlorite (70% active chlorine) solutions for 20 min to produce aseptic seedlings according to the sterilisation protocols of Azmi et al. (1997). The seeds were then rinsed three times in sterile distilled water. Batches of seeds for each species were given a cold stratification treatment for 4 weeks or left untreated. Each treatment had six replications. Thirty seeds per replication were placed into 90 mm Petri dishes on two layers of sterilized Whatman #No. 1 filter paper moistened with 5 mL sterile distilled water. The Petri dishes were sealed by Parafilm™. Petri dishes containing seeds for cold stratification were placed in the fridge at 4 ± 1 °C for 4 weeks before the germination test. Water was added to the dishes as necessary to keep the filter paper moist during the stratification period. The relative humidity inside the Petri dishes was close to 100%.

After the cold stratification period of 4 weeks, the treated seeds together with the control seeds were placed in a growth cabinet for germination at 22 ± 1 °C. The experiment consisted of 15 species, two treatments (cold stratified or not), each with six replicates of 30 seeds per replicate. The growth cabinet was equipped with 16 h photoperiod using Philips® cool white fluorescent tubes giving a photon flux density of 58.6 µmol m^{-2} s^{-1}. The germination test was run for 36 days. Newly germinated seeds were counted every 3 or 4 days for 36 days. A seed was considered to have germinated when the radicle protruded about 1.5–2.5 mm. The number of seeds that germinated precociously during cold treatment

(4 ± 1 °C) was determined before the Petri dishes were placed in the growth cabinet. Percentage germination (root emergence) was calculated from these data. Data were also recorded for 'days after sowing for the first cotyledons to open' and 'days after sowing for the first true leaves to emerge' during the 36 days of incubation. Days after sowing when 50% root, cotyledon and first true leaves emerged were also recorded i.e., progression of emergence. The evaluations were performed individually for each stage of the progression of emergence.

2.3 Tetrazolium test for seed viability

Tetrazolium (TZ) seed viability percentages of each eucalypt species were determined by staining with tetrazolium chloride (1%, w/v) (TZ) solution using three replications of 25 seeds. To obtain a 1.0% solution, one gram of tetrazolium salt (2,3,5-triphenyl tetrazolium chloride—TTC) was dissolved in phosphate buffer solution and the volume made up to 100 mL with distilled water. The pH of the buffer solution was 7.0. The seeds of each species (approx. 0.05 to 0.1 g) were then soaked in 50 mL of 1% TZ solution for 24 h at 25 ± 1 °C, thereafter excess solution was drained off and the seeds were washed in distilled water, split lengthways between the cotyledons, and observed under the microscope and classified as viable or non-viable according to Nery et al. (2007). Seeds were considered viable when the embryo showed a uniform shiny pink colour, and the cotyledons had more than 50% of the surface pink coloured, and tissue with normal aspect (Fig. 1a). Non-viable seeds were those with a white colour in all parts and with soft tissues or the cotyledons having less than 50% of the surface pink coloured, characterizing dead tissue.

2.4 Tetrazolium test for vigour

Tetrazolium seed vigour for each species was estimated according to a method (colorimetric determination of formazan) described by Kittock and Law (1968) on the basis of colour intensity of the stained embryo or seed. In general, the higher the intensity of the extracted formazan, the greater the vigour (Kittock and Law 1968). For the TZ vigour test, the viable stained seeds in each of the replicates for each species were soaked in 10 mL of 2-methoxyethanol (anhydrous, 99.8%) solvent for 4 to 6 h with occasional stirring until the extraction of red coloured, stable, non-diffusible formazan (the reduced form of TTC-salt) was complete i.e. the axis become colourless. The extract was decanted and the intensity of colour was read at 480 nm in a Spectronic 20 colorimeter (Thermo Scientific™) using 2-methoxyethanol as the blank. A standardised TZ vigour characteristic for each species was calculated by dividing the TZ vigour value by the number of viable seed in each replicate and calculating the average TZ vigour per seed for each species.

2.5 Data analysis

Data analysis for tetrazolium and stratification experiments was done using SAS® 9.4 analysis of variance and the PROC GLM (general linear model) procedure (SAS 1999). Germination data scored in percentages were subjected to arcsin transformation before analysis and then converted back to percentages for

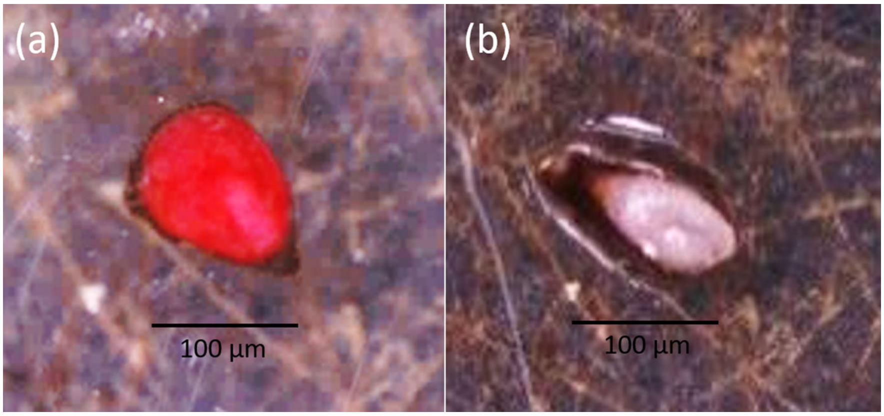

Fig. 1 Tetrazolium (TZ) viability test **a** live (viable seed) and **b** dead (non-viable seed) of *Eucalyptus cinerea*

presentation in the tables (Snedecor and Cochran 1968). Least square means were obtained by using PROC Glimmix (Generalised Linear Mixed Models) procedure. In the stratification and germination tests, the effects of seed species lots, stratification, emergence types and their interactions on percentage emergence were analysed. The effect of stratification on the emergence of root, cotyledon, and first true leaves of 15 eucalypt species were also estimated using the T test. The SigmaPlot 14.0 software was used to plot each lot's emergence response curves.

3 Results

3.1 Effect of species seed lots and cold stratification on seed germination and vigour

Species seed lot and stratification had a significant effect on the germination results. We also found significant differences for the three types of emergence (% radicle, % cotyledon and % true leaves) as well as significant interactions between all three main factors (Table 2).

No precocious germination was observed during 4 weeks of cold stratification. Four weeks of cold stratification significantly reduced germination (root emergence) overall but the impact was more severe on some species compared to others (Fig. 2). In one case cold stratification increased germination (*E. mitchelliana*) while in others the effect was minor (*Eucalyptus parvula, E. rubida, E. morrisbyi and E. coccifera).*

Cold stratification for 4 weeks significantly affected the seedling development and seed vigour, from the emergence of root to the first true leaves across the species compared to seeds germinated without stratification (Fig. 3). The effect was more pronounced on the % of true leaves emerging than on % radicle emergence or cotyledon emergence, particularly for *E. parvula, E. cordata* ssp. *cordata*, *E. cordata* ssp. *quadrangulosa*, *E. glaucescens*, *E. morrisbyi, E. moorei and E. subcrenulata.* These species germinated well when stratified but failed to progress to a greater extent into first true leaf stage during cold stratification. However, all the cold stratified treatments had reduced emergence across the board compared with those, which were not stratified (Fig. 3b).

Table 2 Significance levels of the seed lot(s) (L), stratification (S), types of emergence (E) and the interaction between seed species lots, stratification and emergence

Sources of variation	DF	F-value	Pr > F
Species seed lot(s) (L)	14	340.38	< 0.0001
Stratification at 4 ± 1 °C (S)	1	581.26	< 0.0001
Emergence type (E)	2	91.21	< 0.0001
L × S	14	12.78	< 0.0001
L × E	28	2.06	0.0013
S × E	2	20.32	< 0.0001
L × S × E	28	2.07	0.0013

3.2 Speed of seedling emergence

The germination of species seed lots followed by their development into the seedling stage was evaluated for unstratified seed for up to 36 days to compare their ability to elicit a good germination response. The 15 Eucalypt species tested were categorised into three groups based on the similarity of their responses in terms of radicle, cotyledon and true leaves emergence (Table 3; Fig. 4).

The germination experiments revealed that a time of four to ten days was adequate to evaluate germination characteristics for most of the species at 22 ± 1 °C. However, germination speed was two to ten days slower in different species when a 4-week cold stratification was applied compared to controls (data not shown). The fastest root emergence time was 2 to 3 days, observed for non-stratified seeds of *Eucalyptus rubida, E. parvula, E. crenulata, E cinerea, E. cordata* ssp. *quadrangulosa* and *E. morrisbyi* (Fig. 4). Some species such as *Eucalyptus rubida, E. cinerea, E. crenulata*, and *E. urnigera*; however, showed development of first true leaves stage proportionally better than other species in response to stratification.

3.3 Tetrazolium measurement of seed viability and vigour

Significant differences ($P < 0.0001$) in both seed TZ viability and TZ seed vigour were detected between

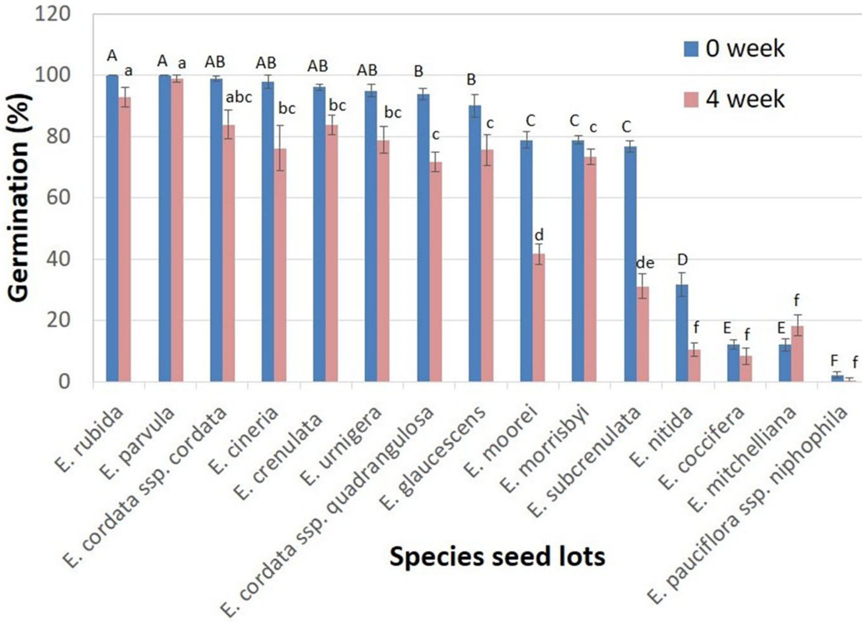

Fig. 2 Effect of cold stratification at 4 ± 1 °C for 4 weeks or no stratification (controls) on root emergence of 15 *Eucalyptus* species seed lots after 36 days. Least square (LS) means for 4 weeks and 0 week stratifications are 3.74 and 2.0, respectively. The same capital letters on the error bars indicate that the species seed lots are not significantly different from each other at $P < 0.05$ at 0 week cold stratification. The same small letters on the error bars indicate that the species seed lots are not significantly different from each other at $P < 0.05$ at 4 weeks cold stratification. Data are means of six replicates for each type of stratification

the 15 Eucalypt species tested following treatment with 1% TZ (Table 4). TZ vigour per 1000 seeds varied between 12.98 and 1.74.

The lowest seed mass (0.3 g) was observed in *E. parvula* followed by *E. morrisbyi* and *E. cinerea*, however, their vigour and viability were much higher compared to their seed mass, particularly seed vigour for *E. morrisbyi* (9.3 g) and *E. cinerea* (7.44). The TZ viability of *E. parvula*, *E. cinerea* and *E. morrisbyi* was 100%, 96%) and 80%, respectively. In contrast, *E. subcrenulata* (6.3), *E. mitchelliana* (5.88), *E. coccifera* (3.73) and *E. nitida* (2.08) had bigger seeds compare to their vigour, vigour of *E. subcrenulata* and *E. mitchelliana* was 2.78 and 3.98, respectively. The highest seed mass was observed in *E. cordata* ssp. *quadrangulosa* (6.35) and *E. subcrenulata* (6.30), followed by *E. mitchelliana*, (5.87), *E. cordata* ssp. *cordata* (5.28), *E. urnigera* (4.08) and *E. coccifera* (3.73). However, seed vigour was also observed highest in *E. cordata* ssp. *quadrangulosa* (12.98). Seed viability of this species was 98 (24.5 viable seeds). The lowest TZ vigour score per seed was for *E. nitida* and *E. moorei* at 0.002 (data not shown). The seed vigour per seed of *E. parvula* was only 0.004. Some species, which had medium bigger seeds, had high viability (*E. rubida*, *E. cordata* ssp. *cordata and E. urnigera*) and some had low viability, for example, *E. mitchelliana*.

3.4 Tetrazolium vs. germination

Depending on the species, the % germination ranged between 2 and 100% (Fig. 5a). Seven species lots (*E. parvula*, *E. rubida*, *E. cinerea*, *E. crenulata*, *E. urnigera*, *E. cordata* ssp. *cordata* and *E. cordata* ssp. *quadrangulosa*) exhibited germination rates of 95% or higher while two (*E. coccifera* and *E. pauciflora* ssp. *niphophila*) exhibited the present study, a TZ viabilityd rates of 12% or lower; with eight species with germination rates between these two extremes. The TZ test resulted in viability values more or less similar to the germination test except for *E. mitchelliana* (Fig. 5a). For this species, viability (% root emergence) was much lower in the germination test than in the TZ test (% viability). Lowest TZ and germination values

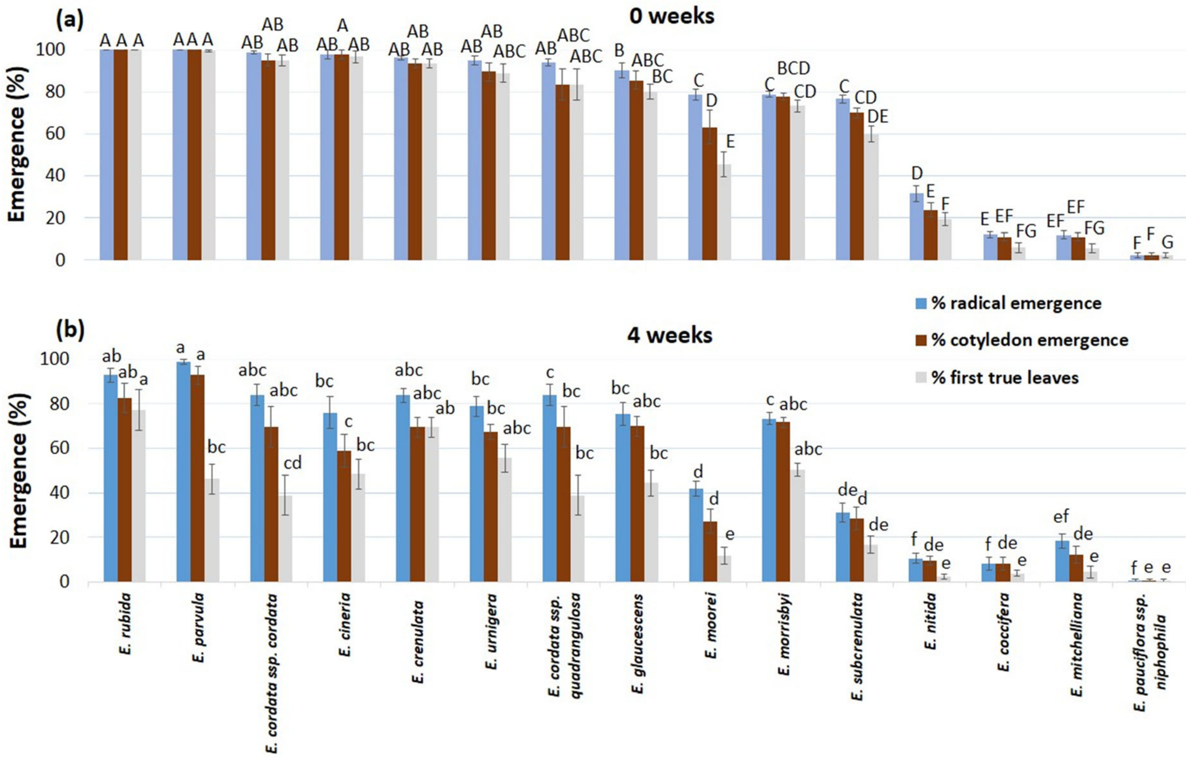

Fig. 3 Effects of (**a**) no stratification, and (**b**) 4 weeks stratification on % radicle emergence, % cotyledon emergence and % first true leaves for 15 Eucalyptus species after 36 days. The same capital letters on the error bars **a** indicate that the species seed lots are not significantly different from each other at $P < 0.05$ at 0 week cold stratification. The same small letters on the error bars **b** indicate that the species seed lots are not significantly different from each other at $P < 0.05$ at 4 weeks cold stratification. Data are means of six replicates for each type of stratification

Table 3 Categorisation of vigour of 15 *Eucalyptus* species based on % radicle, cotyledon and first true leaves emergence

Vigour	High	Medium	Low
Root emergence	> 85%	50–85%	< 50%
Cotyledon emergence	> 80%	50–70%	< 25%
First true leaves emergence	> 75%	40–70%	< 20%
Species	*E. cinerea*	*E. subcrenulata*	*E. nitida*
	E. crenulata	*E. morrisbyi*	*E. coccifera*
	E. rubida	*E. moorei*	*E. mitchelliana*
	E. parvula		*E. pauciflora* ssp. *niphophila*
	E. urnigera		
	E. glaucescens		
	E. cordata ssp. *cordata*		
	E. cordata ssp. *quadrangulosa*		

Six replications were evaluated for each type of emergences for each species

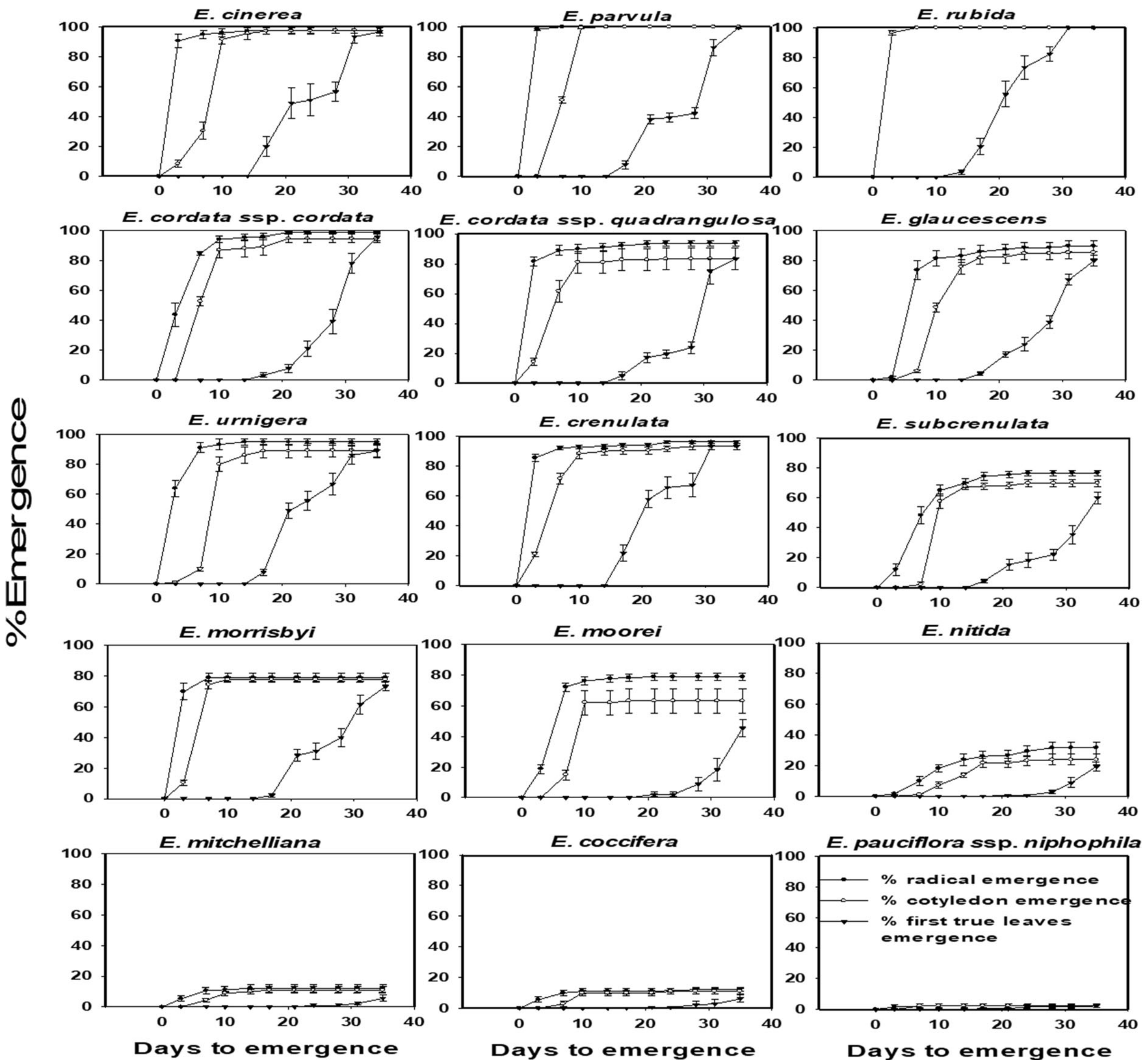

Fig. 4 Percentage emergence of root, cotyledon and first true leaves for 15 *Eucalyptus* species (without cold stratification) over a 36-day period. Data are means of six replicates

were obtained for *E. pauciflora* ssp. *niphophila*, followed by *E. coccifera.* We found a strong correlation (r = 0.945) between TZ viability score and % germination (Fig. 5b) with 89% of the germination test seed viability accounted for by the TZ viability measurement. This positive correlation is also suggesting that the species which are highly viable in the TZ test can also exhibit high viability in the germination test and, vice versa.

3.5 Correlations between TZ seed viability, vigour and seed mass

No correlations were observed between the TZ seed viability and the seed mass (Fig. 6a) or between TZ vigour and seed mass (Fig. 6b) across the 15 species, with some of the heavier, larger seeds having lower vigour than smaller lighter seeds.

Table 4 TZ viability and vigour of 15 *Eucalyptus* species seed lots using tetrazolium (TZ) tests

Species seed lots	TZ viability (average no. of TZ positive seeds out of 25)	Recorded vigour of TZ positive seeds	Average TZ vigour per viable seed (× 10^3)	Average seed mass (g) (× 10^3)
E. rubida	25 a	0.14 bc	5.7 bcdefg	2.08 f
E. parvula	25 a	0.10 bcd	4.09 cdefg	0.3 i
E. cordata ssp. *cordata*	23.5 ab	0.14 bc	5.99 bcdef	5.28 c
E. cineria	24 a	0.18 b	7.44 bc	0.79 h
E. crenulata	23.25 bc	0.16 b	7.04 bcd	1.6 g
E. urnigera	24.5 a	0.16 b	6.38 bcde	4.08 d
E. cordata ssp. *quadrangulosa*	24.5 a	0.32 a	12.98 a	6.35 a
E. glaucescens	23.5 ab	0.19 b	8.04 bc	1.95 f
E. moorei	21 cd	0.04 de	1.89 fg	0.88 h
E. morrisbyi	20 d	0.19 b	9.3 ab	0.73 h
E. subcrenulata	21.5 cd	0.06 cde	2.78 efg	6.3 a
E. nitida	8.5 f	0.01 de	1.74 g	2.08 f
E. coccifera	2.5 g	0.01 e	3.08 efg	3.73 e
E. mitchelliana	14.5 e	0.06 de	3.98 defg	5.88 b
E. pauciflora ssp. *niphophila*	2.25 g	0.01 e	6.56 bcde	2.1 f

Equal letters in the column do not differ by Tukey test ($P < 0.05$). Data are means of four replicates

4 Discussion

Although a germination test is the best indication of the potential of a seed lot to emerge under field conditions, tetrazolium-based tests are fast, reliable and very useful in the processing, handling, storing and marketing of large quantities of seed lots in a short time, especially for testing dormancy and vigour (Elias and Garay 2004; Patil and Dadlani 2009; Oliveira et al. 2014). No information was available so far on the viability and vigour of the 15 Eucalypt spp. investigated in this study, so comparative seed germination and vigour data was generated for the first time using standard germination and tetrazolium (TZ) based tests. Eight species (*E. cinerea, E. crenulata, E. rubida, E. parvula, E. urnigera, E. glaucescens, E. cordata* ssp. *cordata, E. cordata* ssp. *quadrangulosa*) germinated well, three germinated medium (*E. subcrenulata, E. morrisbyi, E. moorei*) while four (*E. nitida, E. coccifera, E. mitchelliana, E. pauciflora* ssp. *niphophila*) germinated very poorly. Although the exact reason for poor germination is not known, it could be associated with the age or physiological state of the seed lots or handling of the seeds (Bell et al. 1995). Furthermore, germination of the seeds of different eucalypts is generally variable depending on environmental factors (Grose and Zimmer 1957; Souza and Cardoso 2000; Domingues-Junior et al. 2019). However, it was clear that the seed species lot was the most important factor affecting seed viability and seed vigour in both TZ and germination tests. In this study, the TZ viability test correlated highly with germination, indicating that it is an acceptable alternative method for predicting germination performance. TZ test results usually show similar or higher viability compared with actual germination tests in a number of studies (Bell et al. 1995; França-Neto and Krzyzanowski 2019; Teixeira et al. 2020).

Bell et al. (1995) and Harrison et al. (2014) stated that seed lots which have larger seeds usually contain more stored food reserves so tend to exhibit higher viability or vigour than those that have smaller seeds. The current study disagree with their statement because this was not the case in this study. *E. parvula* and *E. cinerea* have very small seeds compared to other eucalypt species however, their viability and

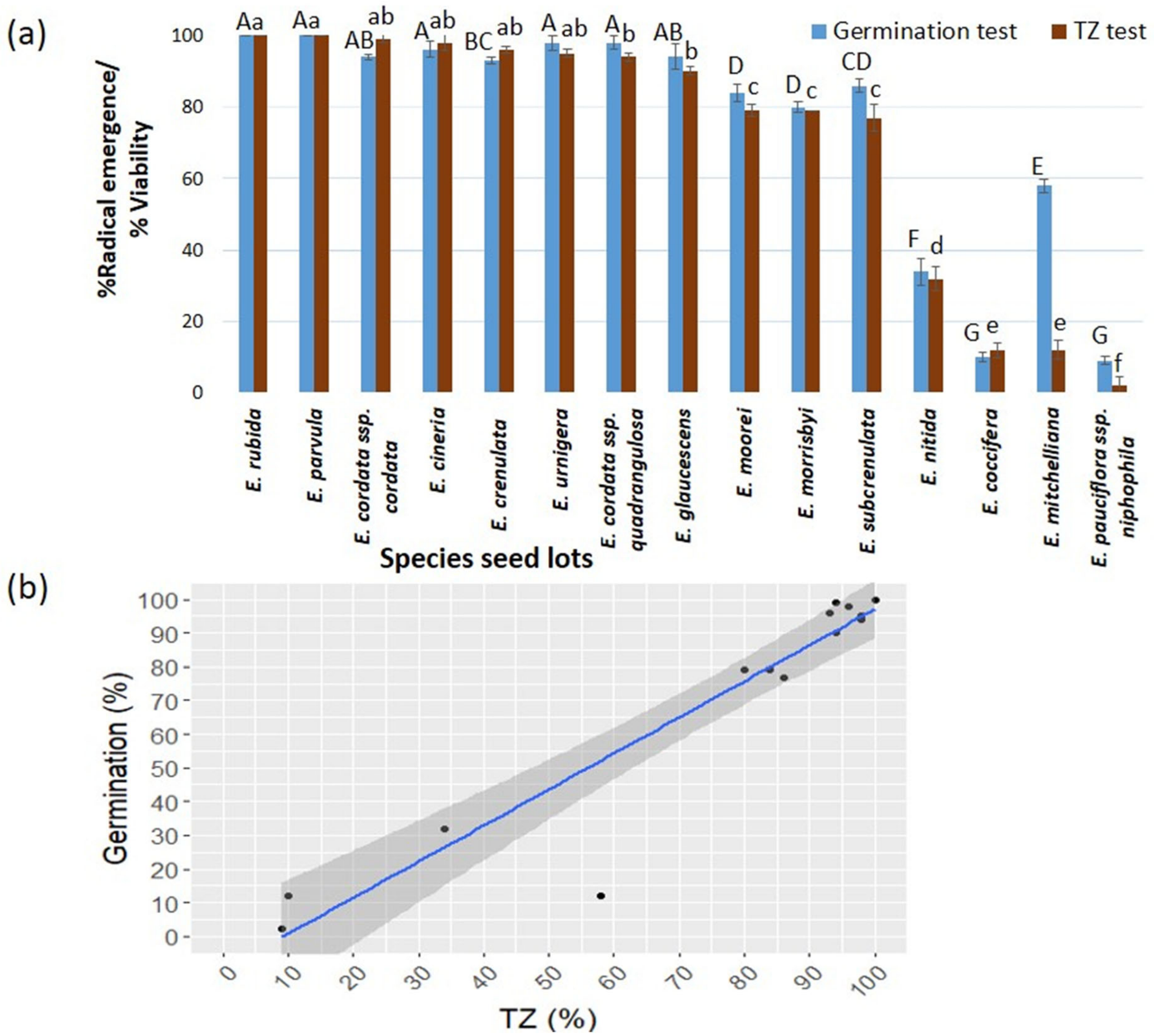

Fig. 5 **(a)** Comparison between TZ (Tetrazolium) test (% viable) and Germination test (% root emergence) for 15 *Eucalyptus* species seed lots. The same capital letters on the error bars indicate that the species seed lots are not significantly different from each other at $P < 0.05$ at germination test. The same small letters on the error bars indicate that the species seed lots are not significantly different from each other at $P < 0.05$ at TZ test. Data are means of four replicates ± standard error, and **(b)** correlation between % viable from the TZ test and % radicle emergence from the germination test for 15 *Eucalyptus* species seed lots. Correlation coefficient, $R^2 = 0.89$

germination percentages were among the highest compared with larger-seeded species, for example, *E. mitchelliana, E. nitida*, *E. subcrenulata*, and *E. urnigera*. A significant negative correlation between seed size and percentage germination was observed in black cherry (*Prunus serotina* Ehrh.) but that was confined to seeds of a single species (Pitcher 1984). França-Neto and Krzyzanowski (2019) stated that the differences between TZ viability and germination test results are usually smaller in high quality seed than in low quality seed. This may suggest that some of the seed lots used in this study may have been of poor quality, especially *E. subcrenulata, E. mitchelliana* and *E. pauciflora* ssp. *niphophila, which* all had higher TZ viabilty scores compared to germination scores. França-Neto and Krzyzanowski (2019) also stated that the differences between TZ viability and germination test results were smaller with large-seeded crops than

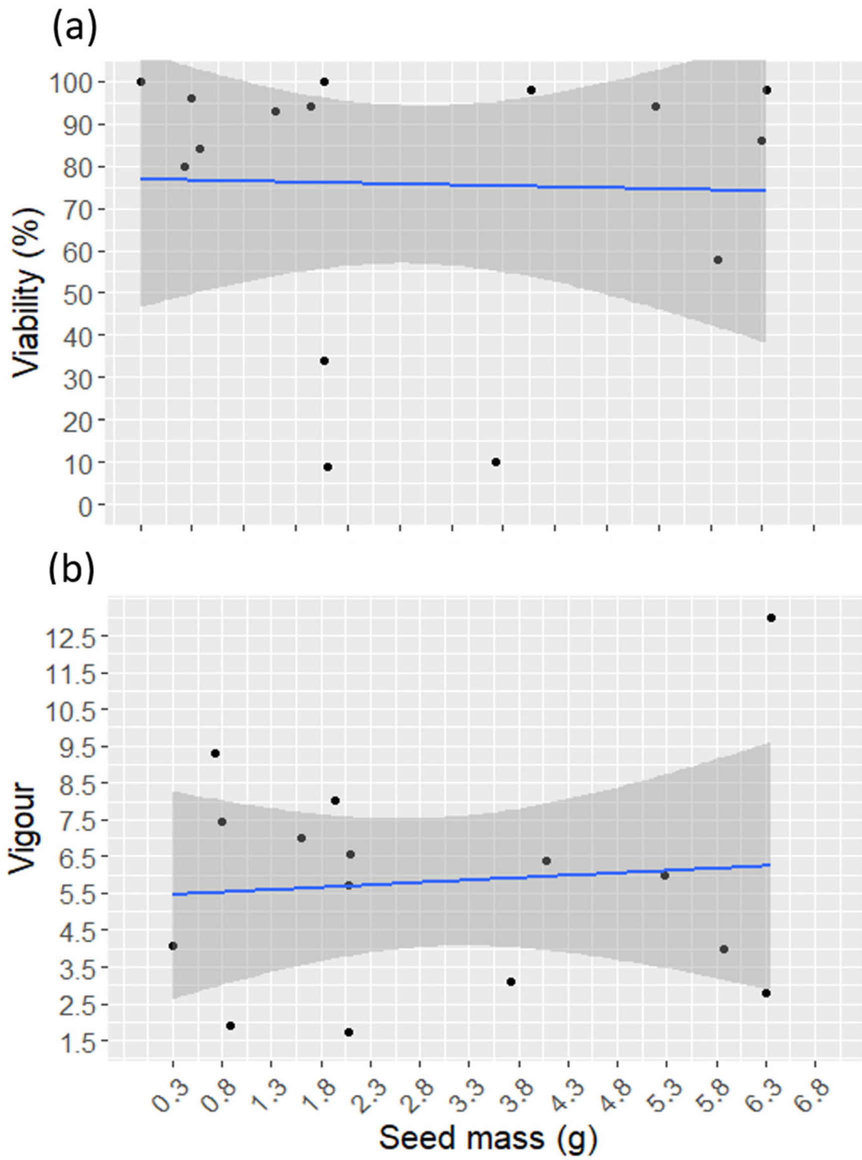

Fig. 6 **a** Correlation between seed mass and TZ seed viability, and **b** correlation between seed mass and seed vigour using TZ test

with small-seeded crops, but this was not the case in this study.

Cold stratification of seeds prior to germination tests affected the 15 species examined differently, with little or no impact on root emergence for some species, e.g. *Eucalyptus parvula, E. rubida, E. morrisbyi*, *E. coccifera* and *E. pauciflora* ssp. *niphophila*, while for most of the others, e.g. *E. cordata* ssp. *cordata, E. cinerea, E. crenulata, E. urnigera, E. cordata* ssp. *quadrangulosa, E. glaucescens, E. moorei, E. subcrenulata and E. nitida*, cold stratification reduced germination rates compared to unstratified seeds. Cold stress not only reduces the yield but also restricts the geographical distribution of many plants, environmental conditions greatly influence the accumulation of many proteins and thus dissecting the dynamics of proteome in response to any external stimuli (Jan et al. 2019). In contrast, germination of seeds of one species, *E. mitchelliana*, increased following a four-week cold-stratification period.

Eucalyptus tereticornis presented the best performance under cold stratification (Schimpl et al. 2018). In other studies, Zohar et al. (1975) observed that seeds of *Eucalyptus occidentalis* germinated well without cold stratification and the seed germination rate of *E. grandis* was inhibited at temperatures lower than 17 °C (Souza and Cardoso 2000). High germination performance was observed in *E. ovata* in Tasmania without any stratification (Wood 2012; Harrison et al. 2014). Growth of eucalypts in plantations in the UK is likely to benefit from warm temperature, provided that it is not accompanied by extreme periods of cold (Leslie et al. 2013; Affonso et al. 2018). For *E. delegatensis*, increased germination percentages in warm conditions compared to cold stratified condition appears related to the ability of the seeds to sense the native environment (Battaglia 1993). On the other hand, chilling or cold stratification greatly increased the rate of seed germination for other trees such as *Sorbus aucuparia* (Afroze and O'Reilly 2013).

Cold moist stratification generally overcomes dormancy in seeds that are native in the alpine forests of Australia. However, three of the four alpine eucalypt species that were studied in this present study did not respond well to the cold stratification treatment with only *E. mitchelliana* benefitting from it. The cold stratification period of 4 weeks prior to the germination test, used in this study, may have induced some physical dormancy in seeds (Afroze and O'Reilly 2017) and delayed the softening of the seed coat that inhibited and/or slowed down root emergence. All seeds of a cohort may not have germinated readily under the combination of moisture and cool temperature, however, this would leave some viable seeds dormant to germinate at a later warmer temperature. The permeability of the various seed coats may be affected and the water absorption by the seed may be decreased if the temperature is low (Souza and Cardoso 2000; Afroze and O'Reilly 2016). In addition, moisture in the filter paper inside the Petri dishes penetrated the seed coat more readily in non-stratified seed coats (Schopfer and Plachy 1985) than those that were cold stratified which may result in lower levels of germination.

Growth and physiological traits in this study showed significant differences among 15 taxa of eucalypts, similar to the findings for several eucalypt species (Ngugi et al. 2004; da Silva et al. 2016). However, no correlation between TZ viability and TZ vigour between the species. The TZ vigour value of some species; (e.g. *Eucalyptus parvula* and *E. rubida*) was two to three times lower than for *E. cordata* ssp. *quadrangulosa* although all three species had similar TZ viability scores. In contrast, Ma et al. (2019) observed a positive correlation between TZ viabilities and vigour however they were studying different batches of a single species. The TZ seed vigour test is not an effective measure of vigour when comparing different species, especially where there are significant differences in seed size, as with the 15 species of eucalypts studied here. Comparisons of the results of the tests of vigour, viability and the seed mass (g) of each species lot in this study indicated that seed vigour and viability are not always correlated with seed size. Thus, other unknown factors might have had an effect on seed viability and seed vigour.

In relation to the germination speed, most of the highly viable species in this study reached their maximum germination percentage by 3 to 7 days, similar to the germination speed of other eucalypts, for example, 3 days for *Eucalyptus camaldulensis* and *E. tereticornis* and 5 days for *E. grandis* and *E. radiata* (Brasil 2013). In general, days to cotyledon emergence mirrored the root emergence response, regardless of the species. Some species developed into first true leaves stages proportionately better than other species in response to stratification. This is probably because some species may have been sensitive to cold and carried over this effect and died surviving even if they grew in a suitable condition. The growth potential of *Eucalyptus nitens* was impressive (in the field) but trees failed to survive (Leslie 2003), highlighting the sensitivity to cold of this species.

The germination response of the 15 different eucalypt species examined varied considerably. The seed lot of *Eucalyptus pauciflora* ssp. *niphophila* used in this study was collected from alpine areas of Australia and was found to be the least viable species in both non-stratification and 4 week cold stratification tests. Close and Wilson (2002) stated that seed germination of *E. pauciflora* and *E. delegatensis*, collected from higher altitude provenances, were enhanced after a wet, cold stratification. It is likely that genetic and provenance differences played an important role in this study. The germination response may vary between provenances of the same species due to genetic or environmental factors (Bischoff et al.

2006; Rix et al. 2012). Considerable variation was observed in *Eucalyptus camaldulensis* morphology between the provenances in Australia (Bell et al. 1993). A high degree of genetic variation is present both between and within species because of the wide range of the habitats in Australia (Brooker 2000). Intra specific variation in widespread species is more often related to the provenance's environmental conditions (Ngugi et al. 2004). Cold stratification did not improve the seed germination of *E. ovata* from high altitude provenances but there was significant provenance differences in seed mass and the germination traits (Harrison et al. 2014). This suggested that seeds from different provenances differed in germination vigour. Thus it is important to carefully select seed provenance, in conjunction with the evaluation of germination and vigour. Other factors such as handling, processing and storage may also affect the germination response as well as the state of seed maturity or weather conditions during seed maturations (Tanaka 1984; Gosling et al. 2009).

In the present study, a TZ viability method was established showing a high correlation with the actual germination response of 15 eucalypt species. No positive effect of cold stratification in terms of germination speed or percentage germination was observed for these 15 eucalypt species except for *E. mitchelliana*. Different species exhibited different germination capacities. Of the 15 Eucalypt spp. tested, based on their seed viability and vigour, this study showed that a number of *Eucalyptus* species; *Eucalyptus rubida, E. parvula, E. cinerea, E. cordata* ssp. *quadrangulosa, E. cordata* ssp. *cordata, E. urnigera*, and *E. crenulata*, originating from different provenances of Australia, could be considered for further cut-foliage trials in terms of their growth and establishment in Ireland. For some species with low germination results in this study (*E. nitida, E. coccifera, E. mitchelliana* and *E. pauciflora* ssp. *niphophila*), further work is needed to identify provenances or seed lots that with optimised germination performance

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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