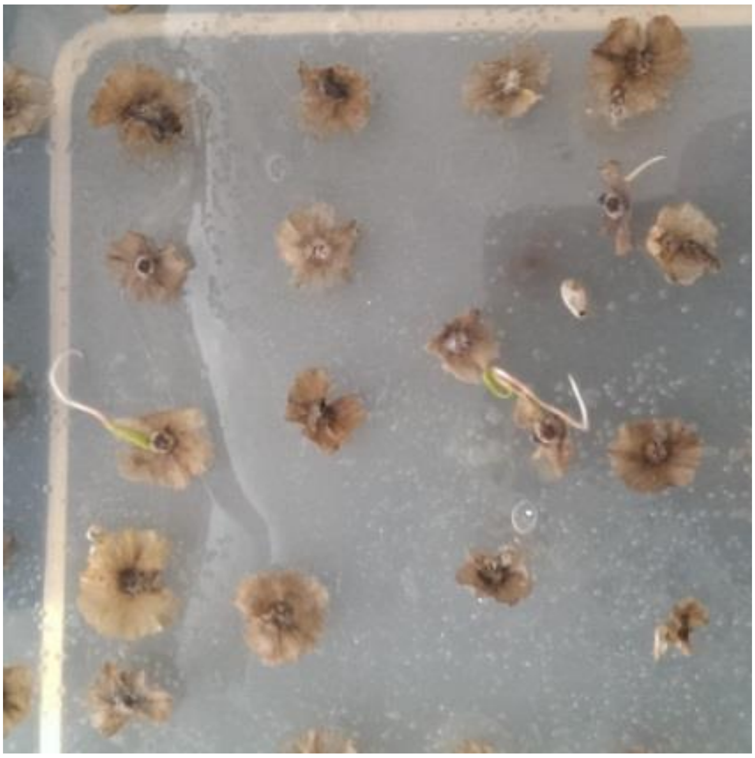
Swainsona Seed Services

Storage Longevity of *Maireana sedifolia* (Pearl Bluebush) seeds: 2020 Progress Report



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Report Title: Storage Longevity of Pearl Bluebush (*Maireana sedifolia*) seeds: 2020 Progress Report

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Executive Summary

Tronox Mining Australia Ltd. (Tronox, previously Cristal Mining Australia Ltd.) currently conducts broad-scale rehabilitation at Ginkgo and Snapper mines, located in western New South Wales. A large component of rehabilitation is the broadcasting of native seed.

Prior to broadcasting, seeds are stored within controlled conditions (two air-conditioned shipping containers held at ~20°C and ambient humidity). These conditions are generally considered adequate for short term seed banks, with seed used within 3-5 years. However, trials have shown that some species can have a short shelf life (only remain viable for 1-2 years), even when stored in these controlled conditions (Quarmby 2017b Quarmby 2019a, Quarmby 2019b, Quarmby 2019c).

Initial investigations on single collections of *Maireana pyramidata* (Black Bluebush) (ONS 279) and *M. sedifolia* (Pearl Bluebush) (ONS 309) indicated that both Bluebush species showed significant losses in viability, with minimal seed remaining viable after 2 years in storage. It was therefore recommended that *M. pyramidata* and *M. sedifolia* should not be stored beyond 1 year, collecting fresh seed every year to cover only the required quantities for that rehabilitation season. While *M. pyramidata* is available for collection most years, *M. sedifolia* seeds infrequently and the lack of storage capability may create a 'bottleneck' in being able to return this species to the rehabilitated landscape.

A further 6 batches of *M. pyramidata* have since been tested and were all considered capable of storage in the Ginkgo Seed Store for at least 1-2 years, contrasting the results from the original batch (ONS 279). Given the variation in results in the *M. Pyramidata* batches, a further five *M. sedifolia* batches have been added to the study.

A summary of the current findings and current recommendations for *M. sedifolia* are provided below:

- The initial seed fill, viability and germination (viability adjusted) was found to vary across the 5 batches included in this study to date, with ONS 309 having the highest seed fill and ONS 443 having the lowest seed fill. Differences in seed fill and viability were likely due to the environmental conditions experienced by the parent plants prior to and during seed development, with sustained moisture across multiple months maximising initial seed fill and viability. Note that environmental conditions cannot be controlled and will continue to cause variation between individual batches.

- The longevity of all *M. sedifolia* batches tested to date (including Duncan *et al*., 2019) have significantly declined in viability within 2 years of storage. ONS 309 was considered to have aged at a greater rate than ONS 399, however, ONS 309 results may have been affected by increased seed age, with seeds potentially 12 months old during the initial tests. ONS 309 results are also less robust than the ONS 399 batch due to the low number of replicates that were tested. These factors may have also attributed to the results by Duncan *et al.* (2019), with low numbers of seeds used during tests (100 seeds).

- Based on the data to date, it is still recommended that *M. sedifolia* seeds should not be stored beyond 12 months. As seed availability is sporadic, with seed not available every year, the current lack of storage capability beyond 1-2 years continues to be considered as a potential 'bottleneck' in returning this species to the rehabilitated landscape.

- The humidity with the Ginkgo seed store has been shown to be above 40% for several months of the year. It is therefore likely to have reduced the shelf-life of *M. sedifolia* batches to date. As such it is recommended that a dehumidifier is installed to reduce humidity to ~15% year-round. Once this occurs, the storage performance of future *M. sedifolia* batches should be monitored to ascertain the potential increases in shelf-life.

- The storage capability may also be improved if *M. sedifolia* seeds are stored in plastic lined woven Polypropylene (WPP) bags, rather than porous, WPP bags that allow moisture and gas exchange. To this end it is recommended that the current trial should be expanded to determine the effect of storage bag on the ONS 433, ONS 444 and ONS 449 batches.

- Unless the shelf life can be improved (as per above recommendations) other options, such as tubestock planting, may be required as substitutes to broadcasting seed in order to return this keystone species to the rehabilitated landscape.

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1. Background

Tronox Mining Australia Ltd. (Tronox, previously Cristal Mining Australia Ltd.) currently conducts broad-scale rehabilitation at Ginkgo and Snapper mines, located in western New South Wales. To date rehabilitation has been achieved by (a) storing and returning stockpiled subsoil and topsoil, (b) mechanically broadcasting seed (direct seeding) and (c) planting tubestock for selected species.

Prior to broadcasting, all seeds are stored within controlled conditions (two air-conditioned shipping containers held at ~20°C and ambient humidity). However, it has been reported that the seeds of chenopod species (e.g. Bluebush) have a short longevity (only remain viable for 1-2 years), even when stored in controlled conditions. As such, a trial began in 2015 to determine how long the seed of important chenopod species could be stored within the Ginkgo seed stores before significant loss of viability.

This initially included single collections of *Maireana pyramidata* (Black Bluebush) (ONS 279) and *M. sedifolia* (Pearl Bluebush) (ONS 309). After just one year in storage both Bluebush species showed significant losses in viability, with minimal seed remaining viable after 2 years in storage. It was also shown that both collections used in the trial had high proportions of immature seeds, which may have contributed to the rapid decline in viability.

As a result of the above findings it was initially recommended that *M. pyramidata* and *M. sedifolia* should not be stored beyond 1 year, each year collecting only the required quantities for that rehabilitation season. While *M. pyramidata* is available for collection most years, *M. sedifolia* seeds infrequently and the lack of storage capability may create a 'bottleneck' in being able to return this species to the rehabilitated landscape.

In 2016 four additional Bluebush species (*M. georgei, M. pentatropis, M. trichoptera* and *M. turbinata)* were added to the trial. While all species had small declines in viability during storage, each of these species have been deemed capable of storage for at least 3 years (Quarmby, 2019).

Since 2017 a further 6 batches of *M. pyramidata* have been added to the trial in order to clarify if the trends seen in the ONS 279 batch holds true with varying seasons and/or populations. However, results to date have shown that only the ONS 279 batch rapid declined in viability during storage, with all other collections tested capable of storage in the Ginkgo Seed Store for at least 1-2 years.

Another batch of *M. sedifolia* (ONS 399) has now been in storage for 2 years. As such, the viability of this batch has been re-tested in order to check if the viability has decreased at a similar rate to the ONS 309 batch. A further 3 batches, all collected in 2020, have also been added to this study, however, only the baseline tests (initial tests on fresh seed prior to storage) have been conducted to date.

This report details the baseline (initial) results for all batches tested as part of this study to date, as well as a comparison of storage traits for ONS 309 and ONS 399 after 2 years of storage.

2. Methods

2.1 Seed lots & test samples

Table 1 below presents the *M. sedifolia* batches in this study, as well as the size and number of replicates used. *M. sedifolia* seeds are dispersed within an indehiscent fruit (bract), with one seed per bract. Viable non-dormant seeds are capable of germinating from within the bract (Quarmby 2015), therefore all tests have been conducted using the intact fruit.

The tests on ONS 309 were carried out using 3 replicates of 50 seed. However, all subsequent batches have been tested using 8 replicates of 50 seed. This is due to analyses using 3 replicates of 50 seed considered weak, with results considered with caution.

Table 1. Initial viability and number of seeds tested for each batch and re-test year

Batch Number	Collection location	Collection Date	Age at time of first germination test	Number of replicates and seeds per test		
				0 years	1 year	2 years
ONS 309	Ginkgo	Apr 2014*	12 months*	3 x 50*	3 x 50	3 x 50
ONS 399	Riverland S.A.	Jan 2018	6 months	8 x 50	8 x 50	
ONS 343	Mid North S.A.	Mar 2020	2 months	8 x 50		
ONS 344	Riverland S.A.	Mar 2020	2 months	8 x 50		
ONS 349	Riverland S.A.	Mar 2020	4 months	8 x 50		

*date recorded, but may have been Apr 2015

2.2 Germination Tests

All germination tests seeds were plated on a medium of 1% water agar and placed in a germination cabinet under Winter conditions for Pooncarie (17°C day/ 7°C night and 14 hours of light). All tests prior to 2019 were conducted for 6 weeks (42 days), however, as initial results indicated that minimal seeds germinated after 2 weeks (14 days) (Quarmby 2015, Quarmby 2017) subsequent tests have been conducted for a maximum of 4 weeks (28 days). At the end of the germination test any non-germinated seed were dissected to determine seed fill and health of embryos present (viable seeds).

2.3 Analysis

Differences in the seed fill, viability (post-germination) and viability adjusted germination (germination of only viable seeds only) of fresh seed between individual collections were analysed using a generalized linear model (glm). The effect of storage on viability (post-germination) and viability adjusted germination (germination of only viable seeds only) within collections and between collections was analysed using a generalized linear model with mixed effects (glmer).

Comparisons between storage lengths and/or collections were analysed using a Tukey's HSD post-hoc test using the "glht" function in the "mult-comp" package of R software. (R. 2020, version: 3.4.30) software.

3. Results

2.4 Initial seed fill, viability and germination results

The initial seed fill, viability and germination (viability adjusted) of the individual *M. sedifolia* batches are presented in Figure 1 and Table 1 (Appendix 1). Seed fill varied significantly (P>0.05) from 59% (ONS 443) through to 89% (ONS 309) between batches, as indicated by the dis-similar letters in Figure 1.

While seed fill was highest in ONS 309, many of these seeds perished during germination tests, and therefore were considered non-viable (52% viability). Despite ONS 399, ONS 444 and ONS 449 having a lower seed fill than ONS 309, the viability of ONS 444 and ONS 449 was considered the similar to ONS 309, with the viability of ONS 399 considered greater than ONS 309. ONS 443 was found to have a significantly lower seed fill and initial viability than all other batches presented in this report. Both ONS 309 and ONS 443 were found to have higher proportions of immature seeds that were considered dead (embryo dark green/black in colour) during initial cut tests (33% and 25% respectively, compared to less than 20% for all other batches (Figure 1 (Appendix 1)).

The germination of viable seeds was also found to vary significantly between batches. ONS 399 had the lowest germination (74%), followed by 86% for ONS 309, and greater than 97% of viable seed germinating in all other batches. Note that the apparent significant differences or similarities in germination between batches have been exacerbated by the varying levels of viability between batches (as described above). Overall, the seeds of *M sedifolia* are considered non-dormant due to their high germination results, mainly occurring within 14 days of plating (data not shown).

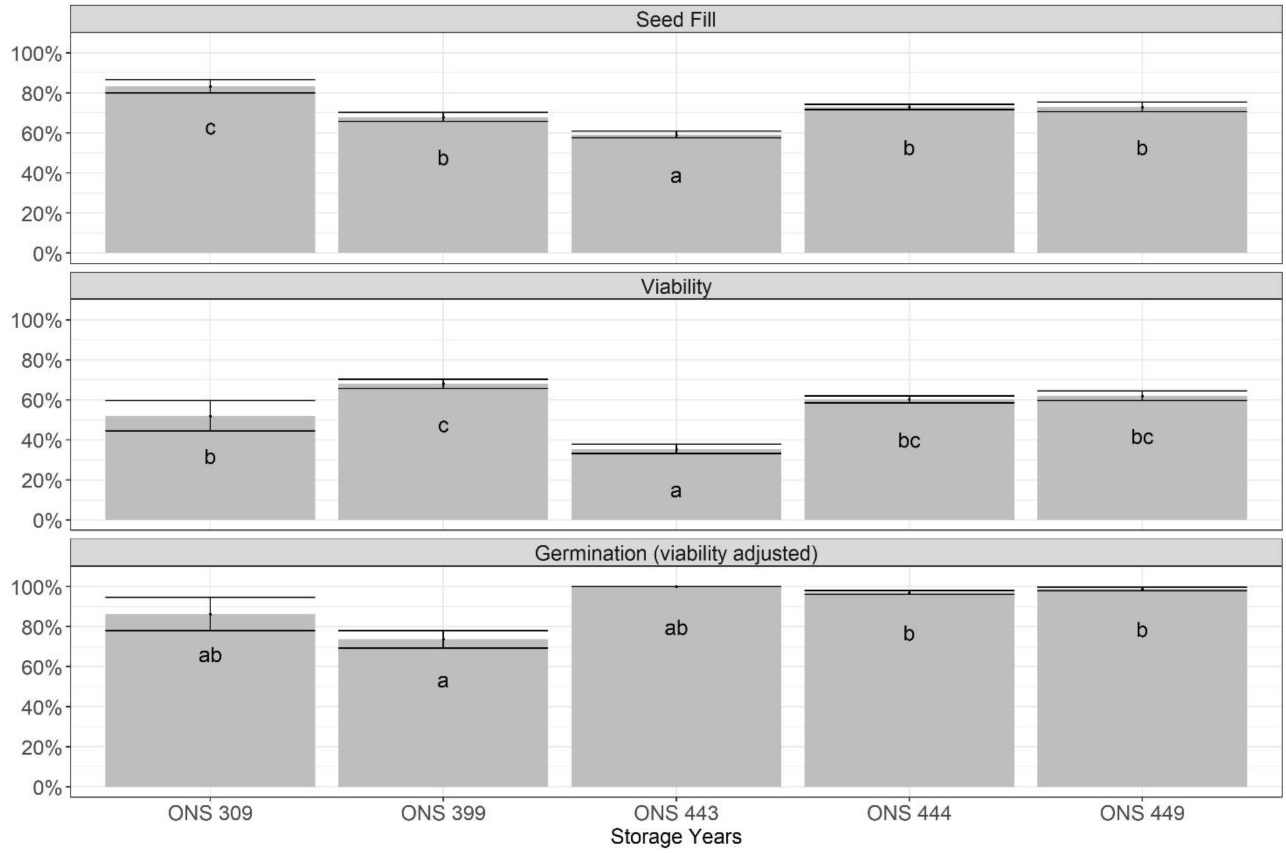

Figure 1. Initial Seed Fill, Viability (post germination) and Germination (viability adjusted) test results ± St. Error. Dissimilar letters indicate significant differences (P< 0.05) between collections.

2.5 Effect of Storage on viability and germination

The effect of storage on viability for ONS 309 and ONS 399 is shown in Figure 2 and Table 2 (Appendix 1). The viability of both batches was found to decline significantly within 2 years of storage, however, the rate of decline was considered different between the two batches. After two years in storage minimal seeds from the ONS 309 batch were considered viable, yet 17.75% of seeds were considered to still be viable for ONS 399.

Storage was not found have any effect on the germination of viable seeds for ONS 309, however, ONS 399 was considered to have increased in germination during storage (Figure 2, Table 2 (Appendix 1)). The apparent change in germination for ONS 399 is likely due to the reduction in number of viable seeds accentuating differences, rather than a real change in germination rates. Viable seeds germinated readily, regardless of storage length or batch, with germination mainly occurring within 14 days of plating (data not shown).

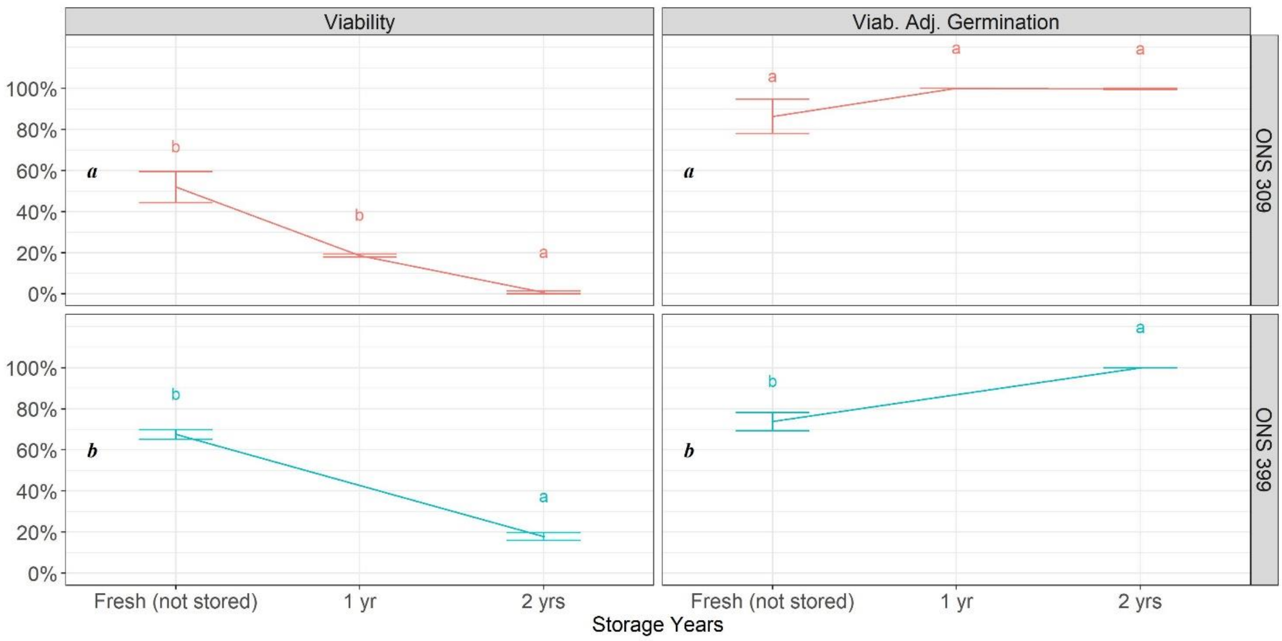

Figure 2. Effect of Storage on Viability (post germination) and Germination (viability adjusted). ± St. Error. Dissimilar letters indicate significant differences (P< 0.05) between collections and storage length.

4. Discussion

The initial seed fill, viability and germination was found to vary significantly between individual batches. This is most likely due differing to environmental conditions experienced by the parent plants during seed development, such as water supply, temperature, mineral nutrition and light (Waller *et al.,* 1983; Mayer and Poljakoff-Mayber 1989; Wotton, 1993). While *Maireana sedifolia* generally produces seeds in response to summer rainfall events (T. Zwiersen, pers. comm, Quarmby, pers. obs.), the timing and amount of rainfall differed for the various batches tested in this study as shown in Appendix 2. As ONS 443 also had a higher proportion of non-viable seeds compared other batches it suggests that several rainfall events across a few months, rather than within a single month, are required for sustaining seed development.

Based on temperature and humidity data within the Ginkgo Seed stores (see Appendix 3) it is likely that the storage conditions, especially high humidity, has impacted the storage capabilities of *M. sedifolia* seeds. Cool, dry conditions are essential for maximising seed longevity during storage, with seed aging rapidly increased when RH is above 40% (Delouche *et al*, 1973, Ralph, 2006, Martyn *et al.,* 2009, Pakeman *et al.,* 2012; Suma *et al*., 2013). However, data loggers within the GSS have shown during cool months the RH within the GSS ranges mostly between 40-60% RH, with some spikes to near 70% RH (Appendix 3, Figure 1). It is therefore recommended that dehumidifiers are fitted to the Ginkgo Seed Stores, allowing a RH of ~15% year-round, increasing shelf life of all species within the GSS.

Until now *M. sedifolia* seeds have been stored in large woven polypropylene (WPP) bags. The WPP bags allow moisture and gas exchange with the surrounding environment, therefore the seed moisture has reflected the humidity within the seed stores. The WPP bags have also enabled oxidation, further increasing the rate of seed aging. As plastic lined polypropylene bags are available a trial is recommended comparing the difference in storage length with type of storage bag (lined WPP versus non-lined WPP bag), using the three 2020 batches (ONS 443, ONS 443 and ONS 449).

Duncan *et al.* (2019) have also investigated the storage longevity of M sedifolia seeds. In their study seeds were stored in paper bags at a temperature between 10-20˚C and a humidity of 40-50%. They also reported a rapid decline in viability during storage, with approximately 20% loss after 12 months and minimal viability after 2 years of storage. As seeds were also stored in porous bags (paper), these results also support the idea that moisture levels and gas exchange (i.e. humidity and oxidation) may be responsible for declines in viability during storage. While this data supports the trends shown in this study, the Duncan *et al.* (2019) results should be taken with caution due to the low number of seeds tested (100 seeds for each test period, compared to germination tests and 150-400 seeds per test for this study) and differing test method (tetrazolium staining, rather than germination tests).

Although all *M. sedifolia* batches tested to date (including Duncan *et al*., 2019) have shown significant aging within 2 year of storage, it should be noted that the increased rate of aging for ONS 309 may have been affected by increased seed age, with seeds potentially 12 months old (according to records) when initially tested. The increased age may also account for the high proportion of dead (non-viable) seeds during the initial tests, compared to all other batches. However, as discussed previously, the results for this batch are taken with caution, due to the low number of replicates that were tested.

Based on the data to date, it is recommended that *M. sedifolia* seeds should be used with 12 months of collection. As seed availability is sporadic, with seed not available every year, the current lack of storage capability beyond 1-2 years is still considered a 'bottleneck' in returning this species to the rehabilitated landscape. However, it is expected that storage life can be improved if the humidity is reduced within the seed stores, and if seeds are stored in lined WPP bags moving forward.

5. Recommendations

A summary of the current findings and current recommendations are provided below:

- The initial seed fill, viability and germination (viability adjusted) was found to vary across the 5 batches included in this study to date, with ONS 309 having the highest seed fill and ONS 443 having the lowest seed fill. Differences in seed fill and viability were likely due to the environmental conditions experienced by the parent plants prior to and during seed development, with sustained moisture across multiple months maximising initial seed fill and viability. Note that environmental conditions cannot be controlled and will continue to cause variation between individual batches.

- All *M. sedifolia* batches tested to date (including Duncan *et al*., 2019) have shown a significant decline in viability within 2 years of storage. ONS 309 was considered to have aged at a greater rate than ONS 399, however, ONS 309 results may have been affected by increased seed age, with seeds potentially 12 months old during the initial tests. ONS 309 results are also less robust than the ONS 399 batch due to the low number of replicates that were tested. These factors may have also attributed to the results by Duncan *et al.* (2019), with low numbers of seeds used during tests (100 seeds).

- Based on the data to date, it is still recommended that *M. sedifolia* seeds should not be stored beyond 12 months. As seed availability is sporadic, with seed not available every year, the current lack of storage capability beyond 1-2 years is still considered a 'bottleneck' in returning this species to the rehabilitated landscape.

- As the humidity with the Ginkgo seed store has been shown to be above 40% for several months of the year, it is therefore likely to have reduced the shelf-life of *M. sedifolia* batches. As such it is recommended that a dehumidifier is installed to reduce humidity to ~15% year-round. Once this occurs, the storage performance of future *M. sedifolia* batches should be monitored to ascertain the potential increases in shelf-life.

- The storage capability may also be improved if *M. sedifolia* seeds are stored in plastic lined WPP bags, rather than porous, WPP bags that allow moisture and gas exchange. To this end it is recommended that the current trial should be expanded to determine the effect of storage bag on the ONS 433, ONS 444 and ONS 449 batches.

- Unless the shelf life can be improved (as per above recommendations) other options, such as tubestock planting, may be required as substitutes to broadcasting seed in order to return this keystone species to the rehabilitated landscape.

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Appendix 1 Seed Test Data

Table 1. Initial seed fill, viability (post germination) and germination (viability adjusted) test results ± St. Error. Dissimilar letters indicate significant differences (P< 0.05) between collections.

Collection Number	Seed Fill % (± St. Error)	Viability % (± St. Error)	Germination % (± St. Error)
ONS 309	83.33 (±3.33) c	52.00 (±7.57) b	86.32 (±8.29) ab
ONS 399	68.00 (±2.30) b	68.00 (±2.30) c	73.75 (±4.37) a
ONS 443	59.25 (±1.64) a	35.50 (±2.26) a	100.00 (±0.00) ab
ONS 444	73.00 (±1.36) b	60.25 (±1.67) bc	97.08 (±0.98) b
ONS 449	73.00 (±2.36) b	62.00 (±2.39) bc	98.79 (±0.84) b

Table 2. Effect of storage on viability (post germination) and germination (viability adjusted) for individual batches ± St. Error. Dissimilar letters indicate significant differences (P< 0.05) between storage lengths.

Batch Number	Treatment	Viability % (± St. Error)	Germination % (± St. Error)
ONS 309	Fresh Seed	52.00 (±7.57) b	86.32 (±8.29) a
ONS 309	Stored 1 year	18.67 (±0.67) b	100.00 (±0.00) a
ONS 309	Stored 2 years	0.67 (±0.67) a	99.67 (±0.33) a
ONS 399	Fresh Seed	67.50 (±2.35) b	73.75 (±4.37) b
ONS 399	Stored 2 years	17.75 (±1.94) a	100.00 (±0.00) a

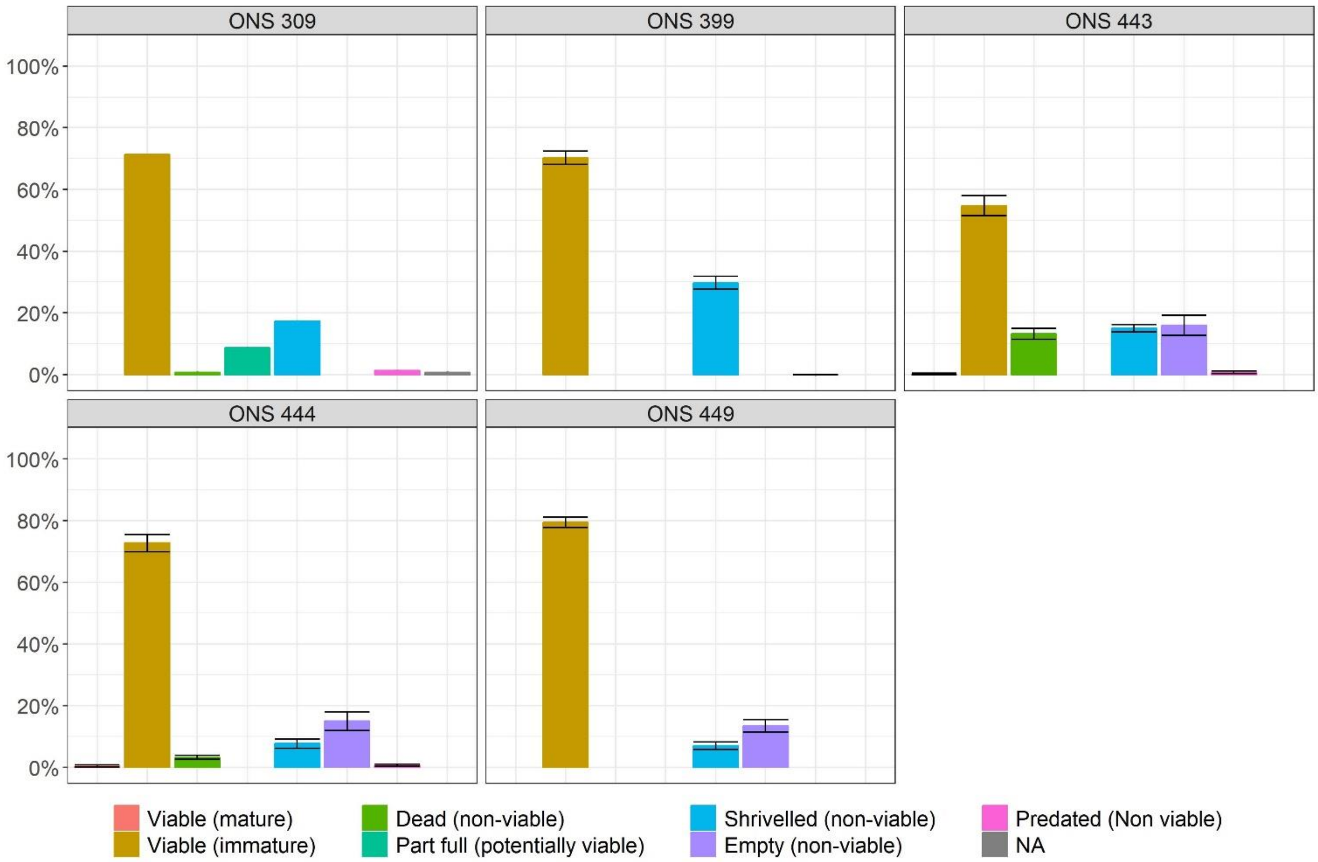

Figure 1. Pre-Germination Viability Test Categories (Cut Test results) ± St. Error

Figure 2. Post Germination Viability Test Categories

Appendix 2. Rainfall data

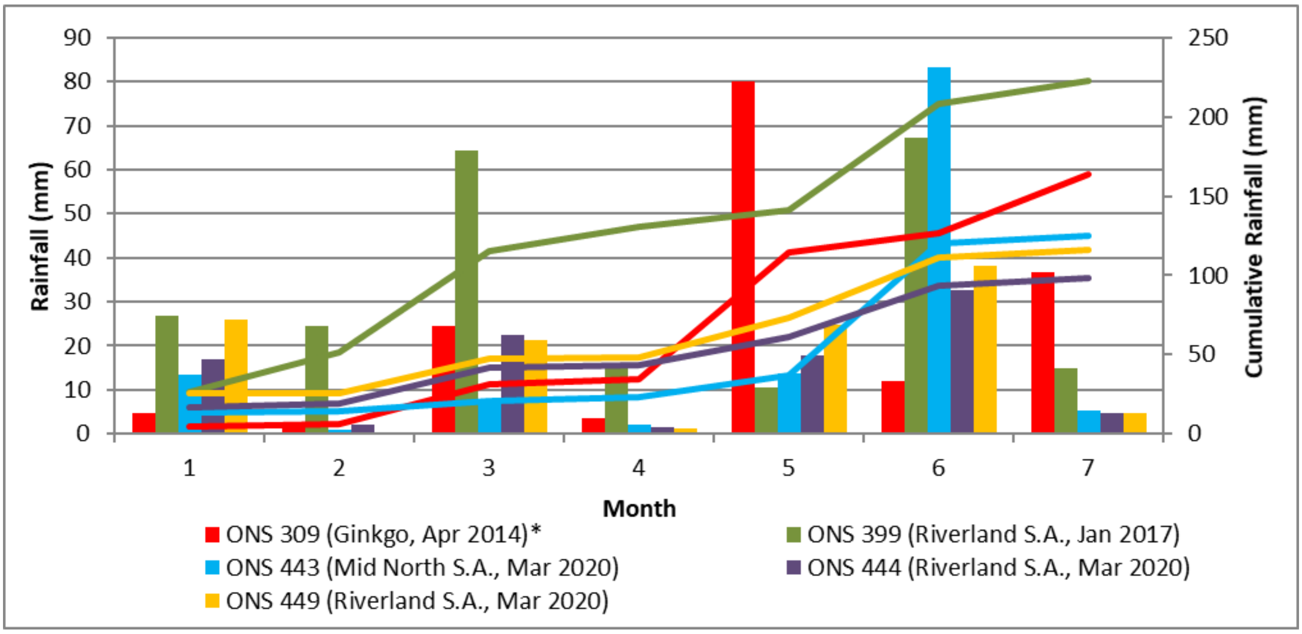

Figure 1 Monthly rainfall data during seed development for each batch (Bureau of Meteorology, 2020).
*** Data taken from Ginkgo Mine Weather station (Weathermation, 2020).**

Appendix 3. Ginkgo Seed Storage Conditions

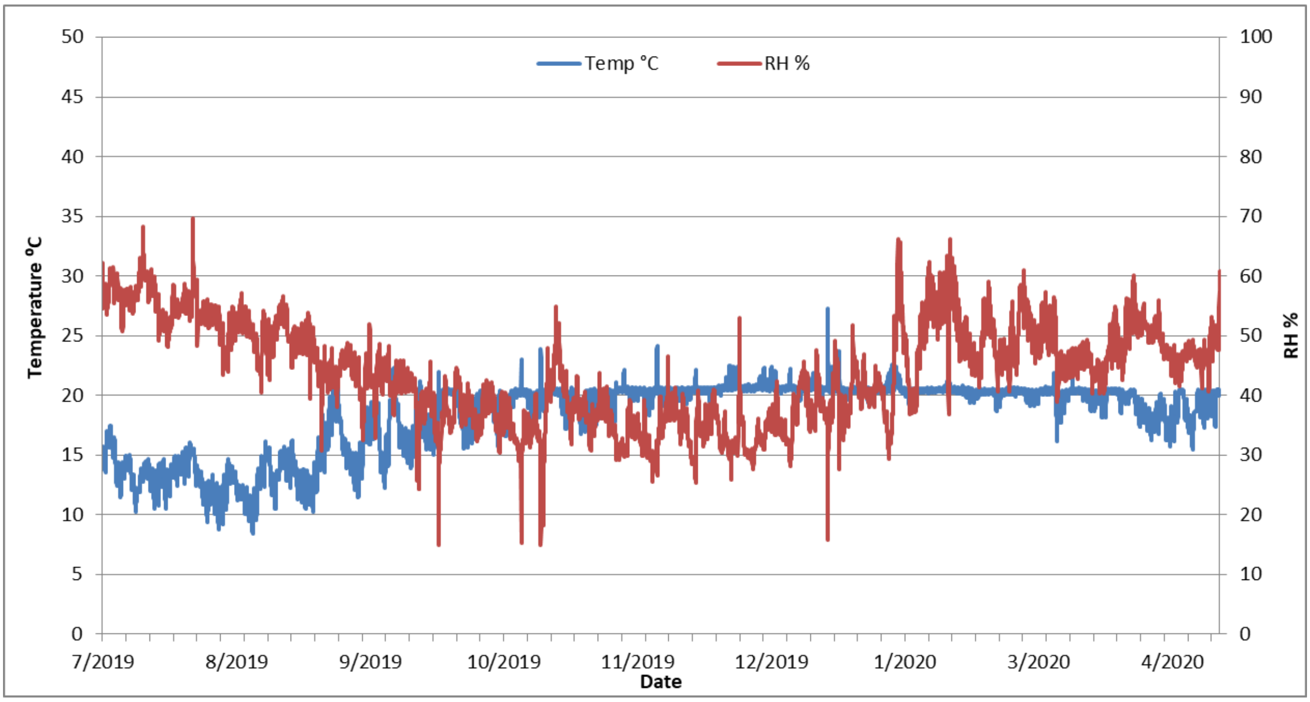

Figure 1 Ginkgo Seed Store conditions (Temperature & Relative Humidity), Container 1 (southern seed store)

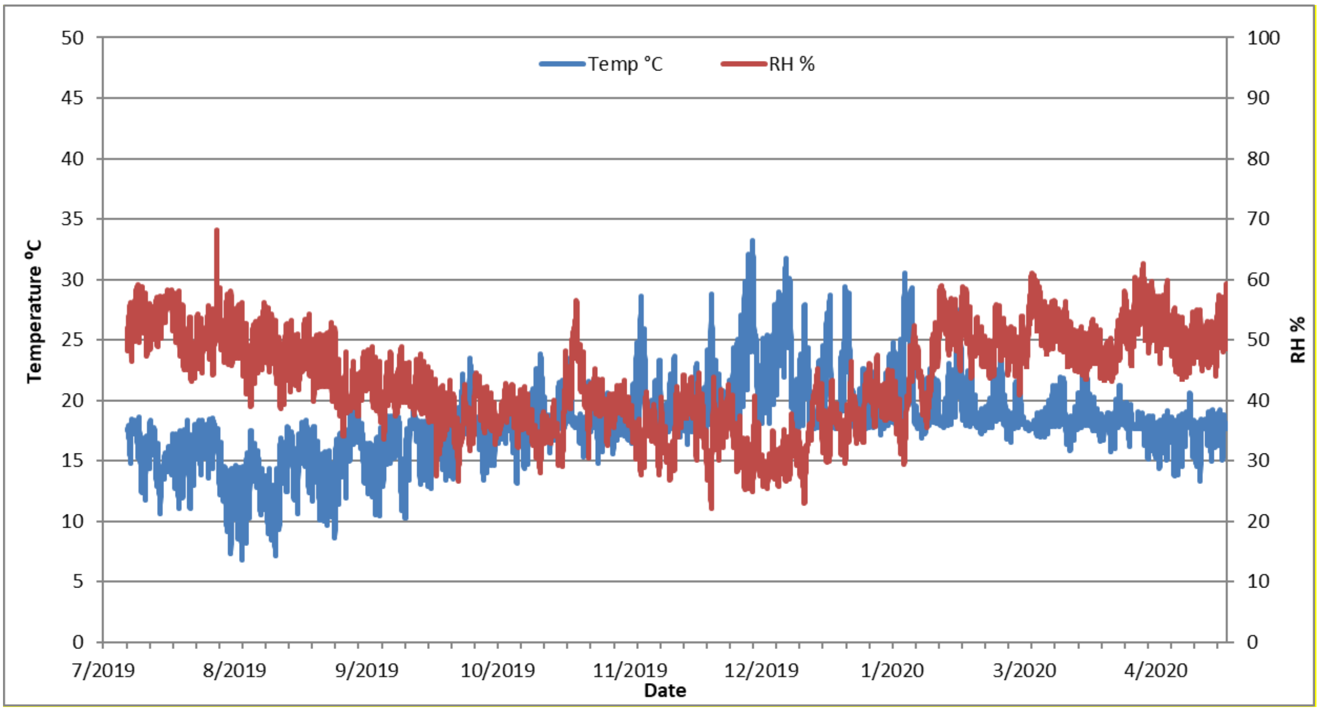

Figure 2 Ginkgo Seed Store conditions (Temperature & Relative Humidity), Container 2 (northern seed store)