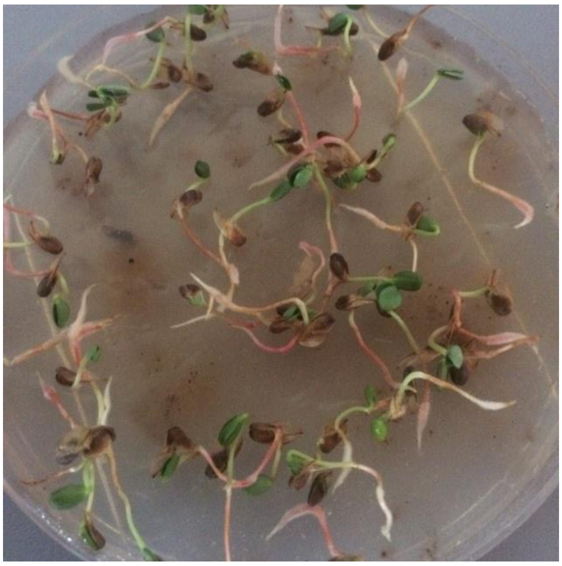
Storage Longevity of Belah (*Casuarina pauper*) seeds:

2020 Progress Report B



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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 at the Ginkgo mine (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, some species can have a short shelf life (only remain viable for 1-2 years), even when stored in controlled conditions.

Belah (*Casuarina pauper*) is a major component of the environment surrounding the Ginkgo and Snapper mines and as such the re-establishment of this species is considered a high priority. However, seeds of this species have been difficult to obtain on a regular basis, with mass seed set only observed every five or so years. Prior to storage within the Ginkgo Seed Store, Belah seeds have been sealed within plastic bags that have been treated with CO^2. The CO^2 treatment is commonly used to limit insect predation by displacing Oxygen and suffocating any insects and larvae within seeds. Storing the seeds in sealed bags is also used to prevent gas and moisture exchange, limiting oxidation and maximising shelf life.

If Belah seeds lack the ability to maintain viability during storage (age rapidly) within the Ginkgo Seed Store (GSS), the additional lack of regular seeding events may create a 'bottleneck' in returning this important species to the rehabilitated landscape. As such, a seed longevity trial on this species began in 2016 to determine the storage capabilities of this species and has included three batches to date (ONS 321, ONS 322 and ONS 366).

Previous analyses indicated that significant aging had occurred during storage within the GSS for ONS 321 and ONS 366. However, the significant aging that occurred for ONS 321 was found to have been caused by the 3-year re-test sample having been taken from a bag that had been opened and partially used during seed mixing, then re-sealed only with tape. The opened bag was used in order to limit the number of bags that were opened prior to sowing. However, when a fresh sample was taken from a previously unopened, sealed bag it revealed that the viability for the majority of the ONS 321 batch had actually remained stable after 4 years in storage.

As the bag state had influenced the results had ONS 321, further tests have now been conducted on ONS 322 and ONS 366 using fresh samples from previously unopened, sealed bags.

Below is a summary of these results as well as the current recommendations moving forward:

- The additional re-tests on ONS 322 and 366, using seeds from sealed bags, were not found to be significantly different from the existing 2020 results. As these results differ to ONS 321, it is expected that ONS 321 was the only batch where samples had been taken from previously opened bags.

- It had previously been recommended that Belah seeds should only be stored for only 1-2 years, therefore creating a potential bottleneck in being able to return this species to the rehabilitated landscape. Based on current data from ONS 321 and ONS 322, however, it appears that Belah seeds can be capable of storage on a short to medium term basis (minimum of 4 years), providing that they are stored within heat-sealed plastic bags that have been treated with CO^2.

- In contrast, the ONS 366 batch indicated a significant decline in viability within 2 years of storage. The ONS 366 results must be treated with caution, however, as initial viability was based on basic 'cut-tests', rather than germination tests. As such, the initial viability of this batch was likely to have been lower than used in the current analyses, with some seeds categorised as viable unlikely to have been actually capable of germination (lacked vigour). Unfortunately, it therefore cannot be accurately determined how much (or indeed if any) significant aging has actually occurred for ONS 366.

- The results for ONS 321 and ONS 322 within the first two years of the study should also be treated with caution. This is due to the low number of replicates and total seeds used during these tests skewing results (i.e. showing an apparent increase in viability for ONS 322). The low initial viability (~40%) for these two batches has also reduced the strength of analyses. It is therefore recommended that further batches are added to the study, as seed becomes available, in order to clarify if significant aging can indeed occur during the first two years of storage.

- As ONS 321 results showed that viability loss has occurred when bags of seeds have been unsealed; oxidation and high humidity levels within the Ginkgo Seed Store are likely to have contributed to aging of Belah seeds. As such, all future Belah re-test samples should be kept within heat-sealed plastic bags, treated with CO^2. After annual sampling, re-test sample bags should then be re-treated with CO^2 and resealed using a heat sealer.

- In order to maximise shelf life during storage it is also imperative that when Belah bags are opened and partially used during seed mixing they are immediately re-treated with CO^2 and re-sealed with a heat sealer.

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1. Background

Tronox Mining Australia Ltd. (Tronox, previously Cristal Mining Australia Ltd.) currently conducts broad-scale rehabilitation at the Ginkgo and Snapper mines, located in western New South Wales. Rehabilitation at these mines is being 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 at the Ginkgo mine (two air-conditioned shipping containers held at ~20°C and ambient humidity). While these conditions are generally considered adequate for short term seed banks, with seed used within 3-5 years, some species can have a short shelf life (only remain viable for 1-2 years), even when stored in controlled conditions (Quarmby 2017b, Quarmby 2019a, Quarmby 2019b, Quarmby 2019c).

Belah (*Casuarina pauper*) is a major component of the environment surrounding the mines and as such the re-establishment of this species is considered a high priority. However, seeds of this species have been difficult to obtain on a regular basis, with mass seed set only observed every five or so years (B. Isaacs and T. Zwiersen, pers. comm.). Prior to storage at the Ginkgo Seed Store (GSS) Belah seeds are placed in thick polypropylene (plastic) bags, treated with CO^2, then sealed with a heat sealer. The CO^2 treatment is commonly used to limit insect predation by displacing Oxygen and therefore suffocating any insects and larvae within seeds (Zwiersen, pers. comm). Storing the seeds in sealed bags also prevents gas and moisture exchange, limiting oxidation and maximising shelf life.

In 2016 a seed longevity trial began on this species, in order to determine how long Belah seeds should be stored within the GSS without significant loss in viability (i.e. seed longevity). The trial initially included two batches collected in November 2015 (ONS 321 and ONS 322). However, after 1 year of storage ONS 321 was considered stable, yet ONS 322 supposedly increased in viability (Quarmby, 2017a). As viability is greatest at the time of dispersal and can only decrease, this result showed that the number of seeds used in the initial tests (150 seeds, 3 replicates of 50) were not adequate for accurately assessing variation within batches. Analyses have also been weakened by low initial viability (40% or less) for both batches. As such, all subsequent tests have been conducted using 400 seeds (8 replicates of 50 seeds).

A third batch of Belah (ONS 366) was added to the study in 2019. While this batch was collected in December 2016, it was not initially included in the study. Therefore, an initial germination test on fresh seed was not conducted. As the cut-test results on ONS 366 indicated an initial viability that was approximately 20% higher than ONS 321 and ONS 322, it was subsequently added during the 2019 re-tests (Quarmby 2017a, Quarmby 2017b, Quarmby 2019a).

After 2 years of storage ONS 321 and ONS 322 were found to be capable of storage for at least 2 years in storage without a significant decline in viability (Quarmby 2017b). After a third year in storage the viability of the two batches were found to differ, with ONS 322 remaining stable, however ONS 321 reportedly declining in viability (Quarmby 2019a). In contrast to the ONS 321 and ONS 322, ONS 366 was found to have significantly declined in viability within 2 years of storage (Quarmby 2019a), yet remain stable after a further year in storage.

The aging that occurred for ONS 321, however, was found to have been caused by the 3-year re-test sample having been taken from a bag that had been opened and partially used during seed mixing, then re-sealed only with tape (Quarmby 2020). The opened bag was used in order to limit the number of bags that were opened prior to sowing. However, when a fresh sample was taken from a previously unopened, sealed bag it revealed that the viability for the majority of the ONS 321 batch, stored within sealed bags, had actually remained stable after 4 years in storage.

As the bag state had influenced the results had ONS 321, further tests have been conducted on ONS 322 and ONS 366 using fresh samples from previously unopened, sealed bags. This report details the results of these additional tests and current storage recommendations.

2. Methods

2.1 Seed lots & tests

The additional test on ONS 366 was conducted using seed from a previously unopened, sealed bag. However, only a previously opened, bag was located for ONS 322. This bag had been re-sealed with a heat sealer, but did not appear to have been re-treated with CO^2. Despite this, the bag was still considered appropriate for testing as the results for this batch to date had not shown a significant decline in viability.

2.2 Test parameters

Viability was assessed using germination tests, whereby 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). Although tests prior to 2019 were conducted for 6 weeks (42 days), these results have indicated that minimal seeds (<2 seeds) germinated after 4 weeks (28 days) (Quarmby 2017a, Quarmby 2017b). As such the 2019 and 2020 tests were conducted for only 4 weeks (28 days). At the completion of the germination test any non-germinated seed were dissected to determine if they were still viable (healthy embryo) or considered non-viable due to aging (unhealthy embryo) or lacking any embryonic material.

2.3 Analysis

The effect of storage on viability (seed health at completion of germination test) and viability adjusted germination (germination results of only viable seeds) was analysed on results after 4 weeks using a generalized linear model, with mixed effects (glmer). Comparisons between storage lengths within 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).

3. Results

The re-test results to date for the three Belah batches are shown in Figure 1 and Table 1 (Appendix 1). As per the previous 2020 progress report (Quarmby 2020) both ONS 321 and ONS 322 are capable of storage for at least 4 years. In contrast, the viability of ONS 366 reportedly reduced by 83% within 2 years of storage, then remained stable after a further year in storage.

As previously reported (Quarmby 2020), the results for ONS 321 indicating significant decline of 97.5% in viability during the third year (and subsequently the fourth year) of storage within the GSS. However, these results were found to be misleading as both samples had been taken from an opened bag. These results were nullified when an additional test after 4 years, taken from a previously unopened, sealed bag showed that the viability had in-fact remained stable during storage.

The additional re-test samples from sealed bags for ONS 322 and ONS 366 were not found to significantly differ from the existing 2020 re-test sample results. This was not unexpected for ONS 322 as viability had remained stable to date, and that the additional test sample had been taken from a previously opened (then re-sealed) bag. However, the results for ONS 366 confirm that the current viability of ONS 366 is 10.25%, and that the 2 year re-test samples were likely to have been taken from unopened, sealed bags.

Overall, viable seeds were able to germinated readily, regardless of storage duration or batch (Figure 1 and Table 1 (Appendix 1)). Any apparent differences between storage years within individual batches were not considered significant.

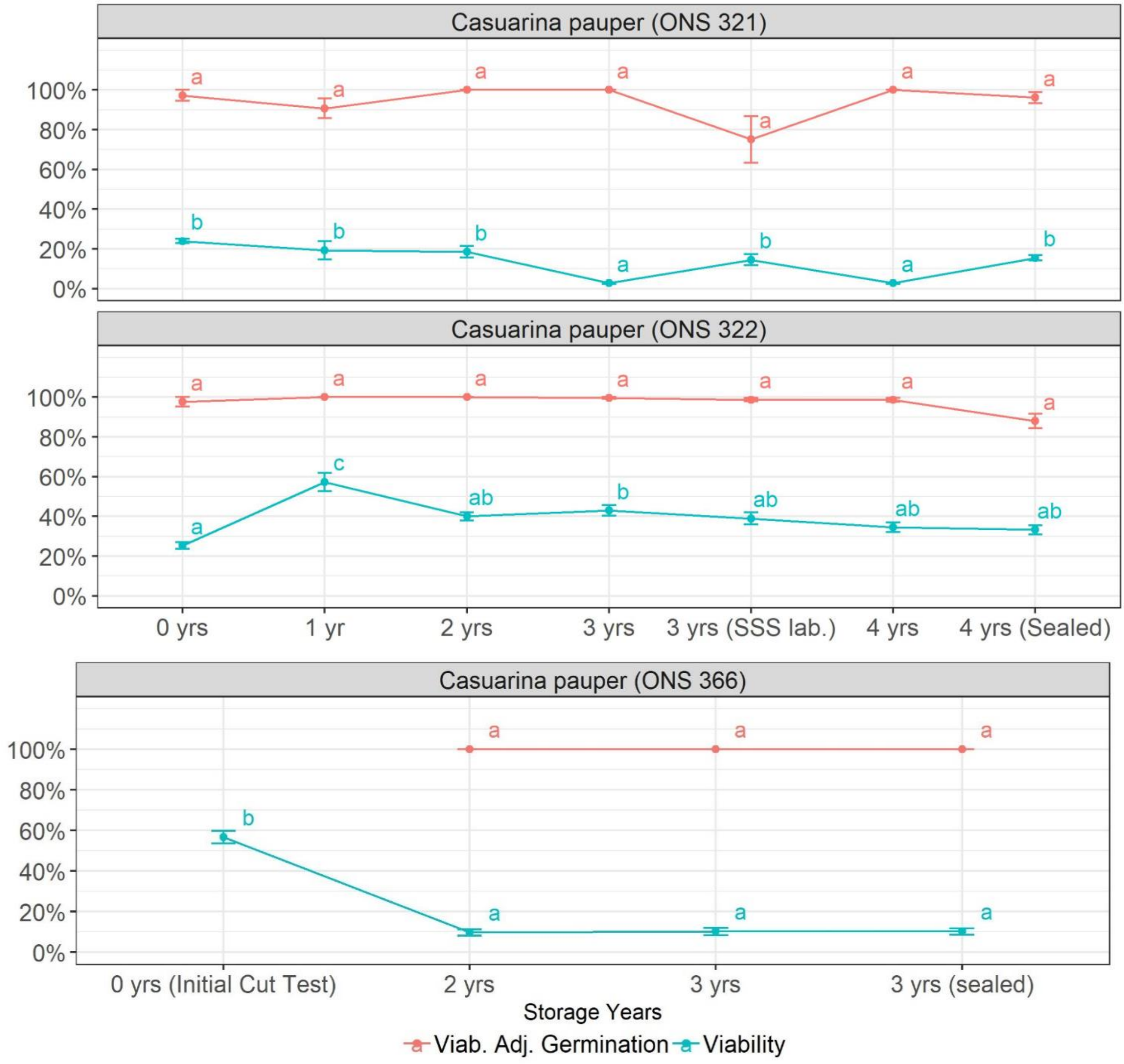

Figure 1. Viability and Viability Adjusted Germination test results for each of the Belah batches ± St. Error. Letters indicate significant differences (p<0.05)

4. Discussion

It had previously recommended that Belah seeds should only be stored for only 1-2 years (Quarmby 2019a, Duncan, 2019), therefore creating a potential bottleneck in being able to return this species to the rehabilitated landscape. However, based on the current data from ONS 321 and ONS 322, Belah seeds appear to be capable of storage on a short to medium term basis (minimum of 4 years), providing that they are sealed within plastic bags that have been treated with CO^2.

Although the rate of aging differed for ONS 366, indicating a significant decline in viability within 2 years of storage, these results must be treated with caution. This is due to the initial viability reflecting initial 'cut-test' results, with seeds that contained a firm and cream endosperm and embryo categorised as viable. However, not all of the seeds categorised as viable during cut-tests on ONS 321 and ONS 322 were found to be capable of germination during the germination tests on fresh seeds (data not presented here). As such, it is plausible that this would have also been the case for ONS 366, had a germination test been conducted on fresh seed. Unfortunately, it therefore cannot be accurately determined how much (or indeed if any) significant aging has actually occurred for ONS 366 during the first two years of storage; or if it was possible that only 10% of the seeds were ever capable of germination, under the test conditions used for this study.

Although the viability of ONS 321 and ONS 322 appears to have remained stable across all four years tested to date, the performance for these batches over the first 2 years should also be created with caution. These tests were only conducted using 3 replicates of 50 seed and resulted in underrepresenting and overrepresenting the viability of ONS 322. The lowest viability was recorded for fresh seed, with the highest viability after 1 year of storage, noting that viability will be the greatest at the time of dispersal and can only decrease thereafter. Although the replicates have been subsequently increased to 8 replicates of 50 seed, the analyses for both batches have been further weakened by low initial viability (~40%), with less than 20 seeds from each replicate of 50 actually containing an embryo, due to the presence of seed mimics (seeds containing non-embryonic woody tissue). As such, further work should be carried out on fresh batches on Belah seed, when available, using 8 replicates of 50 seed, in order to clarify if significant aging can indeed occur during the first two years of storage within sealed, plastic bags.

The longevity of Belah seeds has also been investigated by Duncan *et al*. (2019), also using seeds from the ONS 321 batch. The study reported a decline in viability of less than 20% after 1 year but a loss of 75% after 2 years. However, differences in results may have been caused by differing storage conditions and viability tests, with Duncan *et al*. (2019) storing seeds within paper bags, at a temperature between 10-20˚C and a humidity of 40-50%. Duncan *et al*. (2019) also assessed viability using tetrazolium staining, rather than germination tests, and used 100 seeds for each test period, compared to 150 or 400 seed tested here.

It is expected that the main factors that have influenced the storage capabilities of Belah seeds to date are the type of storage bag and storage conditions. When seeds are stored within sealed, non-porous plastic bags potential gas and moisture exchange is limited, therefore maximising shelf life. However, when seeds are stored in porous bags (i.e. as per the Duncan *et al.* (2019) study), or unsealed plastic bags (as per the ONS 321 re-test sample), oxidation and moisture exchange can occur, increasing the rate of aging. If seeds are exposed to relative humidity (RH) levels above 40% it can cause rapid seed ageing, limiting seed longevity (Delouche *et al*, 1973, Ralph, 2006, Martyn *et al.,* 2009, Pakeman *et al.,* 2012; Suma *et al*., 2013). As humidity levels within the GSS are greater than 40% year-round, with some spikes to near 70% RH (Appendix 2, Figure 1), it is likely to have caused the rapid seed aging of the ONS 321 sample taken from an opened bag. However, while bags remain sealed, Belah seeds will not be affected by the high humidity within the GSS. In order to maximise viability during storage within the GSS it is therefore imperative that Belah seeds are continued to be stored in sealed plastic (non-porous) bags. If these bags are opened during annual seed mixing, they must be resealed immediately in order to limit subsequent aging.

Although further batches of Belah seed should the added to the study to clarify storage behaviour within the first two years of storage, potential variation in storage capabilities between individual batches is not unexpected. This can be attributed to varying environmental factors experienced by the parent plants during seed development (e.g. water supply, temperature, mineral nutrition and light). These factors have been reported to affect seed longevity of other species (Stephenson, 1981; Waller *et al.,* 1983, Mayer and Poljakoff-Mayber

1989). ONS 366 experienced increased rainfall experienced by during August and September prior to maturation in December, compared to ONS 321 and ONS 322 (see Figure 1, Appendix 3). The varying developmental conditions have therefore likely attributed to the increased initial viability of ONS 366, and may also be partly responsible for the potential difference in aging of ONS 366 during storage (if it has indeed occurred), compared to ONS 321 and ONS 322.

5. Summary and Recommendations

- The additional re-tests on ONS 322 and 366, using seeds from sealed bags, were not found to be significantly different from the existing 2020 results. As these results differ to ONS 321, it is expected that ONS 321 was the only batch where samples had been taken from previously opened bags.

- It had previously been recommended that Belah seeds should only be stored for only 1-2 years, therefore creating a potential bottleneck in being able to return this species to the rehabilitated landscape. Based on current data from ONS 321 and ONS 322, however, it appears that Belah seeds can be capable of storage on a short to medium term basis (minimum of 4 years), providing that they are stored within heat-sealed plastic bags that have been treated with CO^2.

- In contrast, the ONS 366 batch indicated a significant decline in viability within 2 years of storage. These results must be treated with caution, however, as initial viability of ONS 366 was based on basic 'cut-tests', rather than germination tests. As such, the initial viability of this batch was likely to have been lower than used in the current analyses, given that some seeds categorised as viable may not have been actually capable of germination (lacked vigour). Unfortunately, it therefore cannot be accurately determined how much (or indeed if any) significant aging has actually occurred for ONS 366.

- The results for ONS 321 and ONS 322 within the first two years of the study should also be treated with caution. This is due to the low number of replicates and total seeds used during these tests skewing results. The low initial viability (~40%) for these two batches has also reduced the strength of analyses. It is therefore recommended that further batches are added to the study, as seed becomes available, in order to clarify if significant aging can indeed occur during the first two years of storage.

- As ONS 321 results showed that viability loss has occurred when bags of seeds have been unsealed; oxidation and high humidity levels within the Ginkgo Seed Store are likely to have contributed to aging of Belah seeds. As such, all future Belah re-test samples should be kept within heat-sealed plastic bags, treated with CO^2. After annual sampling, re-test sample bags should then be re-treated with CO^2 and resealed using a heat sealer.

- In order to maximise shelf life during storage it is also imperative that when Belah bags are opened and partially used during seed mixing they are immediately re-treated with CO^2 and re-sealed with a heat sealer.

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Appendix 1 Seed Test Data

Table 1 Summary test results for each Belah (*Casuarina pauper*) batch. Numbers in brackets indicate standard error, and dissimilar letters indicate significant differences (P< 0.05) between pre-treatments

Collection	Storage Length	Viability % (± St. Error)	Viab. Adj. Germination % (± St. Error)
ONS 321	0 yrs	24.00 (± 1.15) b	97.22 (± 2.78) a
	1 yr (U)	19.33 (± 4.67) b	90.74 (± 4.90) a
	2 yrs (U)	18.50 (± 2.87) b	100.00 (± 0.00) a
	3 yrs (O)	2.75 (± 0.37) a	87.50 (± 8.18) a
	3 yrs (SSS) (O)	14.50 (± 2.80) b	75.15 (± 11.71) a
	4 years (R)	2.75 (± 0.37) a	81.25 (± 13.15) a
	4 years (S)	15.50 (± 1.30) b	96.11 (± 2.68) a
ONS 322	0 yrs (initial cut test)	25.33 (± 1.76) a	97.62 (± 2.38) a
	1 yr (U)	57.33 (± 4.67) c	100.00 (± 0.00) a
	2 yrs (U)	40.00 (± 2.16) ab	100.00 (± 0.00) a
	3 yrs (S)	43.00 (± 2.67) b	99.52 (± 0.48) a
	3 yrs (SSS) (O)	39.00 (± 3.07) ab	98.74 (± 0.83) a
	4 years (R)	34.50 (± 2.47) ab	98.57 (± 0.95) a
	4 years (S)	33.25 (± 2.36) ab	88.02 (± 3.62) a
ONS 366	0 yrs (initial cut test)	56.25 (± 2.74) b	NA
	2 yrs (U)	9.75 (± 1.62) a	100.00 (± 0.00) a
	3 years (R)	10.25 (± 1.79) a	100.00 (± 0.00) a
	3 years (S)	10.25 (± 1.53) a	100.00 (± 0.00) a

*Bag state: S: Sealed O: Open, R: “re-test sample bag” (calico), U = unknown

Appendix 2. Seed Storage Conditions

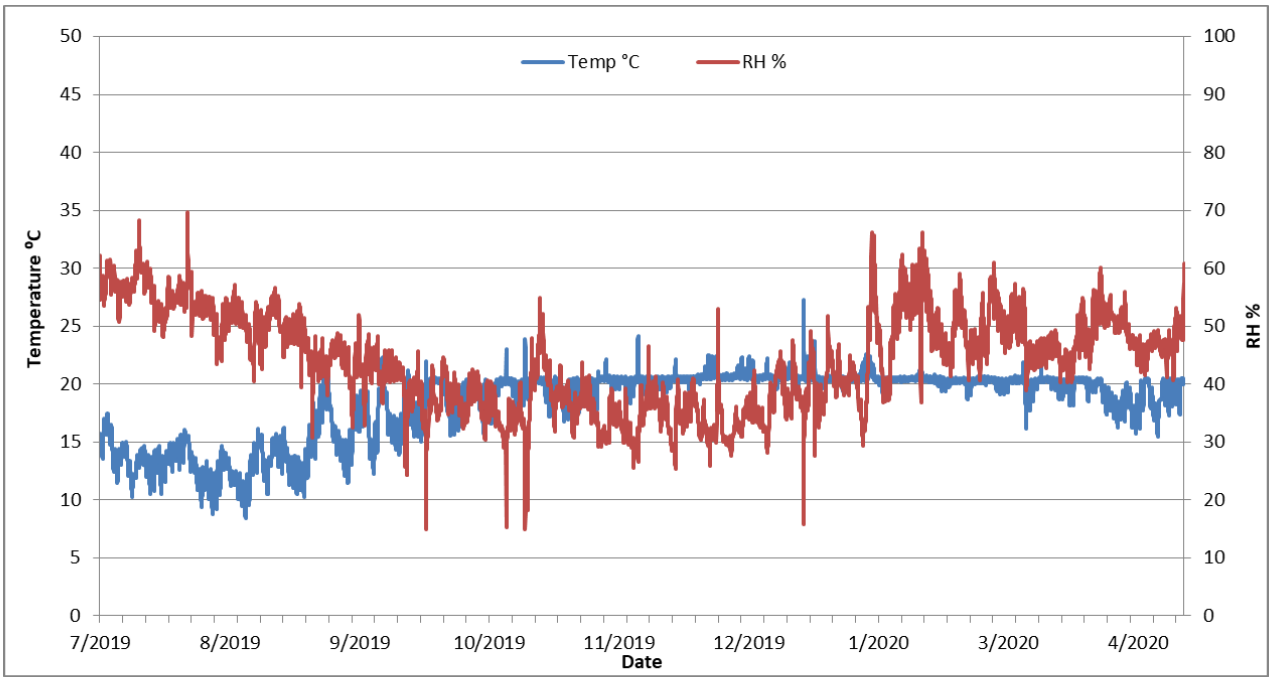

Figure 1 Ginkgo Seed Storage conditions (Temperature & Relative Humidity), Container 1

Appendix 3. Rainfall data

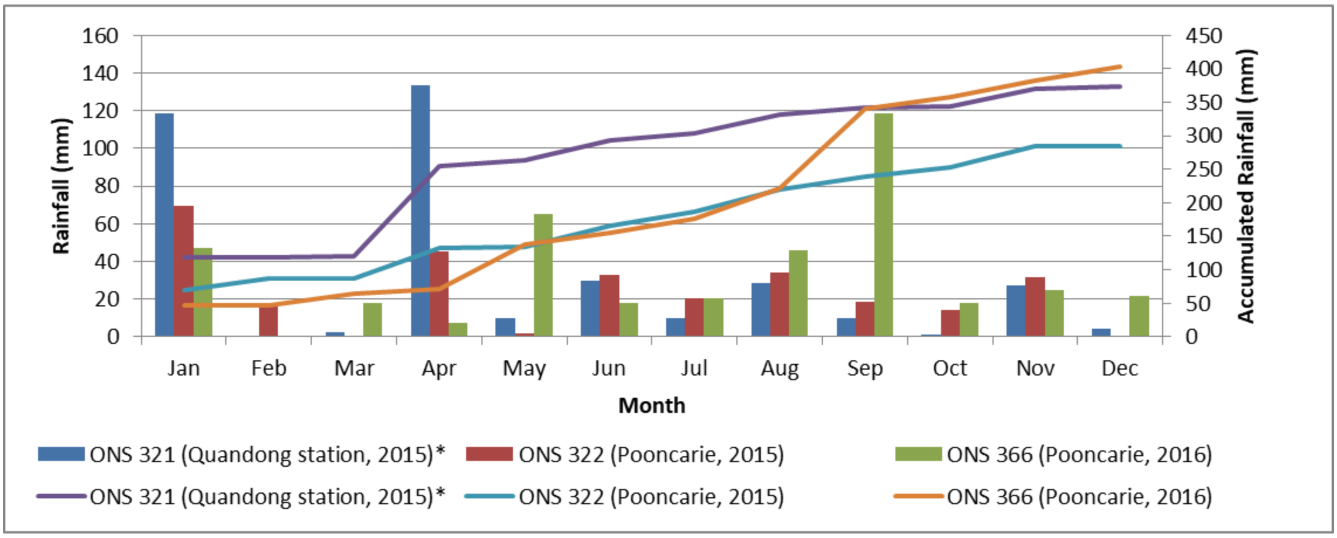

Figure 1 Monthly rainfall data during seed development for each batch. * Data averaged from nearby weather stations *(data provided from Bureau of Meteorology (2019))*