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## Late seed maturation improves the preservation of seedling emergence during storage in soybean<sup>1</sup>

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**ABSTRACT** - Long-term survival during dry storage or longevity is a pre-requisite to avoid deterioration, leading to loss of vigor. Longevity is routinely evaluated by the ability to germinate after storage. It increases progressively during seed maturation, after the acquisition of desiccation tolerance. However, the capacity to germinate represents only a part of the success of crop establishment. How seed maturation affects the resistance of several traits, as vigor, associated with seedling establishment, against deterioration was evaluated during seed filling and post-abscission phase of soybean BRS 284 seeds. Three new phenological stages between 7.1 and 7.2 (7.1.1, 7.1.2 and 7.1.3) were introduced to capture the rapid increase in seed longevity. Germination speed started to be affected at 7-14 days after storage depending on the stages. The delay on germination increased with maturation from 7.1.3 to dry mature seeds. The time to 50% loss of elongation capacity of both organs during storage was similar to that of loss of germination. Also, it increased steadily during seed maturation after mass maturity and harvest maturity stages, highlighting the importance of the late phase of seed maturation for building seed vigor.

Index terms: *Glycine max*, elongation, hypocotyl, longevity, maturation.

## Maturação tardia da semente preserva a emergência de plântulas de soja submetidas ao armazenamento

**RESUMO** - A sobrevivência durante o armazenamento ou longevidade é pré-requisito para evitar a deterioração, que leva à perda de vigor. A longevidade é rotineiramente avaliada pela habilidade da semente em germinar depois do armazenamento, que aumenta progressivamente durante a maturação depois da aquisição da tolerância à dessecação. No entanto, a germinação representa somente uma parte do processo de estabelecimento da planta. Foi avaliado como a maturação afeta a resistência de várias características, como vigor, relacionadas ao estabelecimento das plântulas em sementes de soja da variedade BRS 284. Três novos estádios entre 7.1 e 7.2 (7.1.1, 7.1.2 e 7.1.3) foram introduzidos para capturar o rápido aumento na longevidade das sementes. O armazenamento induziu uma queda na velocidade de germinação a partir de 7-14 dias de armazenamento, dependendo do estágio, e essa queda diminuiu com a maturação, a partir do estágio 7.1.3. O tempo para perda de 50% na capacidade de alongamento de ambos os órgãos foi similar àquele observado para capacidade de germinação. Além disso, esse tempo aumentou de forma constante durante o período de maturação, demonstrando a importância das fases finais de maturação da semente na construção do vigor.

Termos para indexação: *Glycine max*, alongamento, hipocótilo, longevidade, maturação.

### Introduction

*Ex situ* dry storage of seeds is unarguably the cheapest and safest way to preserve plant genetic resources and plant species against habitat losses and is paramount to the

production of high quality seeds. Long-term storage relies on the capacity of the seed to tolerate the loss of cellular water to a content in equilibrium with ambient air (desiccation tolerance) and the capacity to survive in the dry state for extended periods of time (longevity). Seed genebank data

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(USDA National Plant Germplasm System) show that soybean seeds are relatively short lived, even when they are stored in optimal conditions such -18 °C and 10% moisture (Walters et al., 2005). Deterioration during storage leads to loss of seed quality and vigor, thereby affecting seed supplies and marketing strategies. This problem is underscored during seed production when storage conditions are far from optimal and not controlled (França-Neto et al., 2010; Rao et al., 2017) or when the harvest is delayed (Diniz et al., 2013).

In soybean, longevity is progressively acquired during seed maturation from phenological stage 7.2 onwards, a few days after the acquisition of desiccation tolerance and shortly before end of seed filling and onset of maturation drying (Pereira Lima et al., 2017). Thereafter, longevity increases two-fold until stage R9 corresponding to dry mature seeds (Zanakis et al., 1994; Pereira Lima et al., 2017). There is increasing evidence that synthesis of protective mechanisms that enhance longevity are induced sequentially and increase progressively during late seed maturation (Probert et al., 2007; Righetti et al., 2015; Leprince et al., 2017). In the legume *Medicago truncatula*, LEA genes are induced during seed filling but a specific set of LEA polypeptides accumulate later in conjunction with longevity (Chatelain et al., 2012). In soybean, Pereira Lima et al. (2017) showed that the longevity increases concomitantly with increased level in transcripts encoding heat shock proteins and heat shock factors and increased content of raffinose family oligosaccharides (RFO), both contributing to resistance against accelerated ageing (Tejedor-Cano et al., 2010). Therefore, it is likely that developing seeds of soybean harvested at different maturity stages are endowed with different amounts of protective compounds.

In the literature, seed longevity is usually evaluated by the ability to germinate after storage (Walters et al., 2005; Hay and Probert, 2013; Leprince et al., 2017; Rao et al., 2017). In these assays, stored seeds are declared dead when the radicles fail to emerge out of the seed coats and grow in optimal conditions. However, germination represents only a part of the success of crop establishment. Other traits such as speed of germination, seedling emergence and absence of abnormal seedlings are also important to establish a crop in the field and can be used to evaluate seed vigor (Yagushi et al., 2014; Finch-Savage and Bassel, 2016; Marcos-Filho, 2016). Successful seedling emergence in epigeal species such as soybean depends on strong and fast elongation of the hypocotyl and the root system that is sustained by the mobilization of energy reserves and sugar mobilization until the embryonic stem reaches the light and cotyledons start photosynthesis (Lilley et al., 2012; Zhong et al., 2014; Pereira et al., 2015). After radicle emergence beneath the soil surface, initial growth

is essential to maintain contact with the soil moisture as the surface layers are drying out (Finch-Savage and Bassel, 2016). Seedling emergence depends also on the formation of an apical hook during elongation. This feature together with closed cotyledons protects the shoot apical meristem from damage when the extending hypocotyl pushes through the soil to reach the surface (Žádníková et al., 2015). Organ elongation during emergence is controlled by external factors such as light, temperature, oxygen and soil structure that are perceived by a complex signaling network involving auxin, ethylene, gibberellins and brassinosteroids (Sánchez-Bravo et al., 2008; Lilley et al., 2012; Zhong et al., 2014; Žádníková et al., 2015; Procko et al., 2016; De Wit et al., 2016). Seedling emergence is dependent on sowing practices such as depth (Finch-Savage and Bassel, 2016; Marcos-Filho, 2016) but can also be affected by heat stress during seed development (Dornbos and Mullen, 1991) and drying rate after harvest (Hartmann Filho et al., 2016). However, how maturation stage impacts organ elongation and seedling performance after seed storage remains poorly studied.

The objective was to evaluate whether the progress of seed maturation that increases longevity measured by germination also influences the life span of various traits that are important for seedling emergence, such as speed of germination, the appearance of normal seedlings and organ elongation after germination.

## Material and Methods

The cultivar BRS 284 was chosen as a standard genotype that it is recommended for altitude tropical climate. It belongs to an early maturity group with a plant cycle of 126 days and indeterminate growth. The work was performed at the experimental farm of UNESP, Botucatu, SP, Brazil (22° 49'S; 48° 25'W, Altitude 810 m). Seeds were sown on 14 December 2015 at a density of 18-23 seeds/m and 0.45 m between rows. On the day of sowing, seeds were treated with broad-spectrum fungicide Carbendazim + Thiram with a dose of 2 mL/kg of seeds of Vitavax Thiram 200 SC (Arysta Lifescience) and inoculated with *Bradyrhizobium* sp strains using Biomax Premium (Biosoja), at a dose of 1.6 mL/kg seeds. During the culture, phytosanitary controls were performed according to the recommendations (EMBRAPA, 2011). To monitor the seed development, 800 flowers were tagged. Pods were harvested manually and developing seeds were sorted into homogeneous lots according to different phenological stages based on the morphology characteristics of pods and seeds and seed age according to Fehr and Caviness (1977) adapted by Pereira Lima et al. (2017). The mean temperature and rainfall for the

experiment period were 23.3 °C and 1037 mm, respectively.

Upon harvest, immature seeds were immediately processed for physiological studies. They were submitted to fast drying to stop phenological processes from occurring during water loss. Seeds were placed on a screen in sealed container with silica gel at 15% relative humidity (RH) at 20 °C, which was monitored by a data logger. In these conditions, seeds reach 10% moisture within 1-3 days, depending on initial water content. Seed weight after drying was measured using eight replicates of 100 seeds. Water content was determined on four replicates of 20 seeds by oven drying at  $105 \pm 3$  °C for 24 hours and expressed as gram of water per gram of dry weight (DW). After drying, the seeds were placed over a saturated solution of NaCl (75% RH) at 35 °C in hermetically sealed boxes. From zero to 60 days, during storage, four replicates of 25 seeds were retrieved and imbibed in Petri dishes (150 x 15 mm) between two sheets of filter papers in the presence of 20 mL water with a 12 hours photoperiod at 25 °C. Germination was scored when seeds exhibited a protruding radicle of  $\geq 1$  mm in length. These germinated seeds were carefully placed with radicles pointing downwards on one line on three sheets of moist filter paper of 38 x 28 cm that were rolled and attached at the middle by a string. Rolls were put vertically inside a 2 L beaker containing 400 mL of water and covered with double layers of aluminum foil and incubated in the dark at 22 °C for seven days. Thereafter, hypocotyl and root length were measured, and abnormal seedlings were scored.

The experiment was performed in a factorial scheme of 6 x 2 consisting of six harvests for the different phenological stages (7.1, 7.1.1, 7.1.2, 7.1.3, 7.2, 7.3, 8.1, 9) and 2 traits (germination and root/hypocotyl length) in a randomized block design. Immature seeds from the six harvests from the same single stage were mixed together after drying. Germinated seeds were randomly selected to represent one roll. Each roll contained 4-15 germinated seeds and represented one replicate whereas the consecutive intervals during imbibition when germinated seeds were transferred into the roll represented one block. Each maturation stage involved three to 10 rolls and 2 to 5 blocks. Before variance analysis, normality and equality of variance between treatments were assessed by the Kolmogorov-Smirnov test and the Levene test, respectively, using XLSTAT (v 2018). When data did not pass these tests, data were transformed to a normal population with an unbiased standardization  $n-1$ . When significant effects were detected ( $P < 0.05$ ), pair-wise comparison was performed with the Tukey-Kramer test at 5% probability. To evaluate the effect of time, germination and elongation data were respectively subjected to sigmoidal regression and 2<sup>nd</sup> order polynomial regression analysis for which the significance of regression was assessed by the F test.

## Results and Discussion

In our previous work (Pereira Lima et al., 2017), seed longevity was assessed in detail from stage 7.2 onwards since only 20% of immature seeds collected at stage 7.1 were desiccation-tolerant. To assess the increase in seedling emergence during this time period, additional phenological stages between 7.1 and 7.2, representing 8 days of maturation, were included in this study (Table 1). During this period, developing seeds and pods started to lose their chlorophyll, which allowed discriminating three separate phenological stages (Table 1). Desiccation tolerance was acquired at stage 7.1.2 (Figure 1A) since immature dried seeds germinated to  $95 \pm 4\%$  within 3 days. Speed of germination, assessed by the time to reach 50% germination, was significantly higher at stage 7.1.2 (Figure 1B) and decreased slightly thereafter. It did not change significantly afterwards until the dry maturity stage.

To avoid the influence mechanical strength of the seed coat on elongation capacity of the radicle during germination, seeds with an emerged radicle ranging from 0.3 to 0.7 cm were preselected and transferred into darkness (Pierre et al., 2014). As 24 hours separated the first from the last germinating seeds (Figure 1A), three transfers of germinated seeds were performed successively. They were considered as randomized blocks because there was no significant difference between organ length according to the time of transfer as factor ( $P = 0.389$  for hypocotyls and  $P = 0.151$  for roots). A preliminary experiment using mature seeds was performed to estimate when to assess elongation capacity after transfer. The kinetics of elongation and changes in organ dry weight were assessed during 28 days in darkness (Figure 1C). Both hypocotyls and roots grew rapidly, their length being respectively 14.2 and 15.6 cm after 7 days of transfer. Afterwards, the elongation slowed down and a plateau was reached after 14 days. Such kinetics of elongation are in accordance with previous work on legume seeds (Pierre et al., 2014). For logistic reasons, 7 days of darkness was retained for further experiments considering that the elongation at that age represented 79% of the maximum value recorded at 28 days. Considering that organ growth in darkness is entirely heterotrophic and depends solely on the reserves present in the axis and cotyledons, dry weight of hypocotyl, root and cotyledons were also measured during the 28 days after transfer to assess whether the dry matter of the cotyledons would be limiting organ elongation in darkness (Figure 1C). After 7 days, the weight of both organs represented only 36% of the total dry weight of the seedlings. This value steadily increased to 60% due to the cotyledons depletion. Therefore, the reserves of the cotyledons are not a limiting factor for the growth of the

Table 1. Characterization of phenological stages of soybean seeds during maturation adapted from Pereira Lima et al. (2017).

Stage	DAF	Name	Seed (WC)	Morphology	
				Pod	Seed
7.1	58	Early maturation	1.57 ±0.01	Completely green.	Seed coat entirely green and yellow embryo axis. The seed consistency is hard.
7.1.1	62	Early maturation	1.49 ±0.01	Green with yellow spots.	Green seed coat with 25% of its surface yellow
7.1.2	63	Early Maturation	1.43 ±0.03	Green with yellow spots.	Green seed coat with 50% of its surface yellow
7.1.3	64	Early Maturation	1.39 ±0.02	Green with yellow spots.	Green seed coat with 75% of its surface yellow
7.2	66	Mass maturity	1.28±0.02	Yellow with green spots.	Seed coat mainly yellow with green spots in the middle.
7.3	68	Mid maturation/ maturation drying	1.26 ±0.05	Completely Yellow.	Seed coat completely yellow with a shiny surface. Seeds are detached from the fruit.
8.1	72	Harvest maturity	0.86 ± 0.16	Yellow with brown spots.	Yellow and completely opaque seed coat. Seed consistency is rubber.
9	76	Full maturation	0.18 ±0.02	Completely brown and dry.	Brown seed coat and dry seeds

DAF (days after flowering), WC; water content (gram of water per gram of dry weigh) at harvest. Data are the average of four replications of twenty seeds each (± SD).

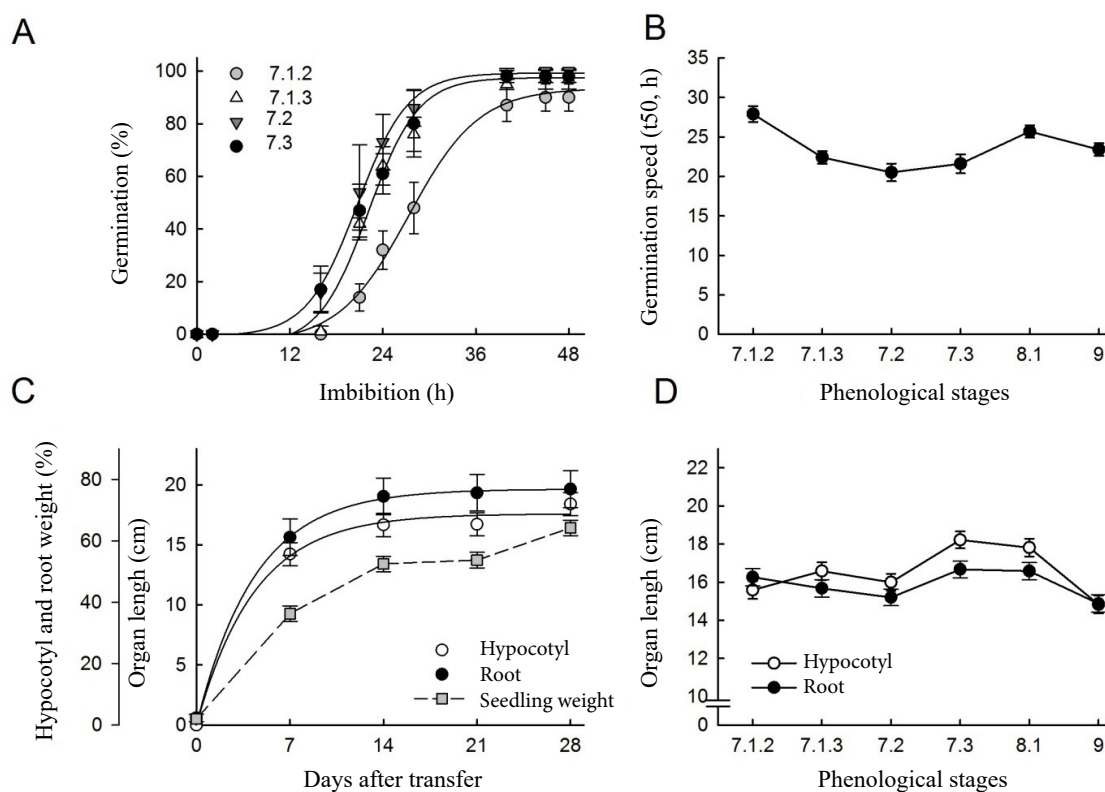


Figure 1. Evolution of vigor traits during late seed maturation. A) Germination percentages of indicated phenological stages. Data were fitted with sigmoid  $f=y_0+a(1+e^{-(x-x_0)^b})$  (adjusted  $r^2 > 0.986$ ). Only stages 7.1.2 to 7.3 are shown for clarity; B) Speed of germination calculated from the sigmoid function shown in panel A; C) Length of hypocotyl (○) and roots (●) after different time spans of incubation post-germination in the dark together with the weight proportion of the hypocotyl and root (data are expressed as percentage of total seedling weight including cotyledons, gray squares); D) Changes in hypocotyl (○) and root (●) length after 7 days of incubation post-germination in the dark during maturation. Data are the average (± SD) of 4 replicates of 25 seeds (A-B) and 10 replicates of 5 to 11 seeds (C-D).

organs in the dark. Between stages 7.1.1 and 7.2, the length of hypocotyls fluctuated around 16 cm then increased slightly to 18 cm at stage 7.3 (Figure 1D). Thereafter, it decreased significantly at stage 9. Root length was also around 16 cm and there were no significant changes during maturation. In legume seed such as soybean, elongation capacity of the cells is pre-established during embryogenesis (Sánchez-Bravo et al., 2008; Pierre et al., 2014). Thus in our material, elongation capacity following forced drying was already gained during seed filling.

The loss of germination capacity and changes of germination time of the survivors were assessed during storage in controlled conditions at 75% RH, 35 °C (Figure 2). Germination

percentages decreased in a sigmoidal way during storage (Figure 2A), as reported before (Chatelain et al., 2012; Pereira Lima et al., 2017). Immature seeds lost their capacity to germinate much faster than mature seeds. For example, developing seeds from stage 7.2 started to lose their viability 14 days after storage in contrast to 28 days for mature dry seeds from stage 9. Before germination capacity dropped below 50% during storage, the germination speed remained stable for 7-14 days of ageing according to the phenological stage then decreased during storage (Figure 2B). At stage 9, the time to 50% germination increased from 22 hours in unaged seeds to 75 hours after 42 days of storage. The increase

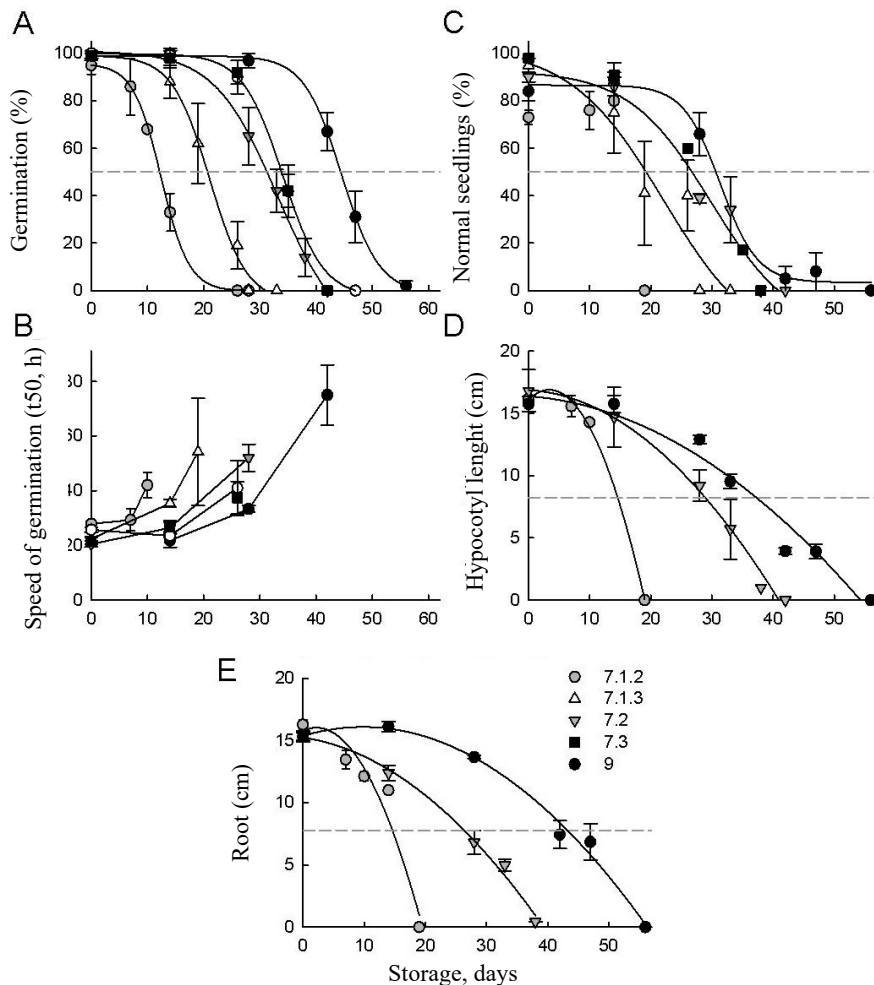


Figure 2. Profiles of the loss of seed vigor during storage. A) Percentage of germinated seeds. Data were fitted with sigmoid  $f=y_0+a(1+e^{-(x-x_0)^b})$  (adjusted  $r^2 > 0.986$ ). The broken line represents 50% germination for which the intersection with fitted sigmoid indicates the P50. B) Speed of germination (t50). Data are the average ( $\pm$  SD) of 4 replicates of 25 seeds. C) Percentages of normal seedlings. Data were fitted with sigmoid  $f=y_0+a(1+e^{-(x-x_0)^b})$  (adjusted  $r^2 > 0.988$ ) and represent the average ( $\pm$  SD) of 20-50 individuals before storage and 8-25 seedlings at the end of storage. D-E) Length of hypocotyl (D) and root (E) after 7 days of incubation post-germination in the dark. Data represent the average ( $\pm$  SD) of 3 replicates and were fitted a quadratic polynomial  $y=y_0+ax+bx^2$ , with adjusted  $R^2$  between 0.693 and 0.980 for hypocotyls and 0.780 and 0.996 for roots.

in seed germination time during storage might be interpreted as a mechanism to allow damage repair before engaging into organ growth. In support of this hypothesis, the DNA checkpoint kinase TAXIA TELANGIECTASIA MUTATED (ATM) and ATM-RELATED (ATR) involved in activating repair of DNA damage were found to delay the progression of germination and cell cycle during imbibition to allow repair of chromosome breakdown induced by artificial ageing (Waterworth et al., 2016). Whether storage in our conditions induced DNA damage in soybean remains to be investigated. However, a delayed germination also increases the risk for the seedling to be exposed to stressful conditions later during establishment that could perturb growth (Finch-Savage and Bassel, 2016; Zhu and Benková, 2016).

The percentage of normal seedlings decreased progressively during storage and that of abnormal seedlings increased (Figure 2C). Abnormalities included stubby and/or crooked organs often associated with a watery and translucent appearance. Twenty percent of immature seeds from stage 7.1.2 produced abnormal seedlings before storage and this value did not change during exposure to 75% RH, 35 °C. For the later stages, it was possible to fit the data with a sigmoidal function. The loss of normal seedlings occurred faster for seeds from 7.1.3 than later stages. Given the large variation among replicates within stages, the loss of normal seedlings during storage appeared to be similar from stage 7.2 and onwards (Figure 2C). Hartmann Filho et al. (2016) also noticed a decrease in the percentage of normal seedlings during storage and drying at temperature  $\geq 40$  °C when the seed moisture content decreased from 13 to 10%. The progressive increase in abnormal seedlings during storage suggests that ageing induces a decreased coordination in growth. In parallel to the occurrence of abnormal seedlings, the overall elongation capacity was also affected by storage (Figures 2D and 2E). There was a decline in both root and hypocotyl length as shown previously by Hartmann Filho et al. (2016) and Yagushi et al. (2014) on mature soybean seeds. However, the decline was much less important for mature seeds compared to immature seeds both for hypocotyls and roots.

From the regression analysis shown in Figure 2, the time to 50% loss of germination and capacity to elongate was calculated and named P50, as in Pereira Lima et al. (2017), and  $T_{H50}$  and  $T_{R50}$  for hypocotyl and root, respectively (Figure 3). During seed maturation, P50 increased almost linearly until dry mature seeds as previously shown by Pereira Lima et al. (2017). At stage 7.2, which corresponds to the end of seed filling, P50 value was 31 days. Thereafter it increased 1.5-fold until stage 9.  $T_{H50}$  also increased constantly during seed maturation, almost in parallel with P50. At stage 7.2, 7.3 and 9, it was slightly but significantly lower than P50.

The resistance of the root elongation capacity against ageing ( $T_{R50}$ ) also increased concomitantly with P50. Overall, loss of elongation capacity of the seedling was equally sensitive to ageing as germination. The fact that elongation starts before radicle emergence could indicate similar mechanisms of ageing on elongation capacity before and after germination. Nevertheless, the results in Figure 3 indicate that assessing loss of seed germination during ageing constitutes an easier way to test the effect of ageing compared to assessing the elongation of seedlings.

The continuous rise in the resistance of elongation against ageing until the dry mature state demonstrates that the late seed maturation program is also important for the developing seeds to acquire vigor traits beyond mass maturity and harvest maturity. This is in agreement with previous work on legumes (Righetti et al., 2015) and other species (Probert et al., 2007).

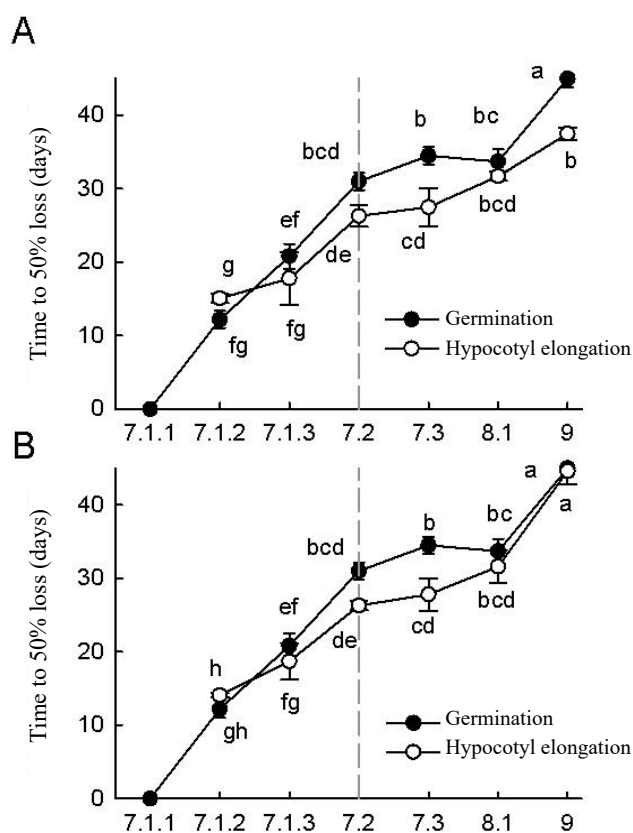


Figure 3. Comparison of the time to 50% loss of germination and seedling elongation [A – hypocotyl ( $T_{H50}$ ) and B – root ( $T_{R50}$ )] during maturation. Data were obtained from curve fitting analysis of Figure 2. Broken line indicates end of seed filling. Values significantly different (assessed by ANOVA,  $P < 0.05$ ) were ranked into groups as indicated by the respective letter using the Tukey-Kramer test ( $P < 0.05$ ).

The origin of this continuous increase is unknown but could be due to changes in cell wall content composition to maintain the properties necessary for elongation. In soybean, the cell wall arabinose:galactose ratio was associated with seed maturity (Stombaugh et al., 2003). Both compounds increased 1.4-fold in the axis during maturation drying together with a 100-fold increase in pectin esterase activity (Koch et al., 1999), indicating that cell properties are modified during the late stage of maturation. Galactose is also a key metabolite involved in RFO biosynthesis and longevity, although its role remains elusive (Leprince et al., 2017). Auxins play an important role in regulating hypocotyl and root growth (Žádníková et al., 2015; Procko et al., 2016) and this hormone could be important to regulate the deteriorative effects of seed ageing. In developing legume seeds, the transcriptome associated with the increase in seed longevity during maturation was linked with an enrichment of genes involved in auxin signaling (Righetti et al., 2015; Pereira Lima et al., 2017). Likewise, ethylene is needed for the seedling to respond to the soil and environmental conditions and reach the surface (Zhong et al., 2014; Zhu and Benková, 2016). In tomato and sweet corn, non-aged seeds produced 3 to 4-fold more ethylene than aged seeds and there was a strong positive correlation between the ability to produce ethylene and seed vigor (Siriwitayawan et al., 2003). In both soybean and *Medicago truncatula*, the transcriptomes associated with seed longevity during maturation contained several transcription factors involved in ethylene signaling (Righetti et al., 2015; Pereira Lima et al., 2017). Whether ethylene plays a role in the rate of the loss in elongation capacity warrants further investigation.

## Conclusions

The loss of elongation capacity of roots and hypocotyl during storage occurs at the same time as the loss of germination, indicating that germination speed is a good marker of maintenance of seedling vigor. Furthermore, the time to 50% loss of elongation capacity increases steadily during seed maturation after the mass maturity and harvest maturity stages, highlighting the importance of the late phase of seed maturation in building seed vigor. Considering the importance of organ elongation to penetrate the soil towards the surface in the subterranean environment, sowing too deep might be an issue for those seeds that are stored for periods of time longer than usual.

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