UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

FCA
FACULDADE DE CIÊNCIAS AGRONÔMICAS
UNESP | BOTUCATU

DENISE PUNTEL BASSO

CONTRIBUTION TO THE CHARACTERIZATION OF THE ACQUISITION OF THE EMERGENCY VIGOR DURING SEED MATURATION

Botucatu

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ABSTRACT

The stable production of high vigorous seeds regardless of the environment is a key lever to increase crop production. Seed vigor is defined as the sum of the physiological properties that lead to homogenous and vigorous seedling establishment. It includes longevity, defined as the capacity to remain viable for long periods during dry storage and the capacity to elongate after germination. However, how these traits are acquired during seed development and how the environment impacts their acquisition remain poorly understood. Yet this information is important to determine the harvest stage corresponding to maximum vigor. Using agronomy and physiological approaches on soybean and *Medicago truncatula*, we confirm that longevity is progressively acquired during seed maturation. In soybean, our data suggest that the climate influenced longevity whereas in Medicago, heat applied during seed maturation had no significant impact. This work also showed that HEAT SHOCK FACTOR A2.2, a homologue of HSFA9 and hub gene involved in seed maturation does not play a role in seed longevity but acts as negative regulator of embryonic dormancy. Longevity is evaluated by the ability to germinate after storage, which represents only a part of the success of crop establishment. How seed maturation affects the loss of seedling establishment capacity during storage was evaluated in soybean using an experimental system set up to assess elongation capacity. The pattern of acquisition of elongation capacity during maturation varied between crop years and growth conditions. The time to 50% loss of elongation capacity 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 in building seed vigor.

Keywords: Maturation. Elongation. Heat shock factor. Vigor. Seed quality.

RESUMO

A produção de sementes altamente vigorosas, independentemente do ambiente, é uma alavanca fundamental para aumentar a produção agrícola. O vigor das sementes é definido como a soma das propriedades fisiológicas que levam ao estabelecimento homogêneo e vigoroso de plântulas. Inclui a longevidade, definida como a capacidade de permanecer viável por longos períodos durante o armazenamento a seco e a capacidade de alongamento após a germinação. No entanto, como essas características são adquiridas durante o desenvolvimento de sementes e como o ambiente impacta sua aquisição permanecem pouco compreendidas. No entanto, essa informação é importante para determinar o estágio de colheita correspondente ao vigor máximo. Utilizando abordagens agronômicas e fisiológicas em soja e *Medicago truncatula*, confirmamos que a longevidade é progressivamente adquirida durante a maturação das sementes. Em soja, nossos dados sugerem que o clima influenciou a longevidade, enquanto em Medicago o calor aplicado durante a maturação de sementes não teve impacto significativo. Este trabalho também mostrou que o FATOR DE CHOQUE DE TÉRMICO A2.2, um homólogo de HSFA9 que está envolvido na maturação de sementes, não desempenha um papel na longevidade das sementes, mas age como regulador negativo da dormência embrionária. A longevidade é avaliada pela capacidade de germinar após o armazenamento, o que representa apenas uma parte do sucesso do estabelecimento da cultura. Como a maturação de sementes afeta a perda de capacidade de estabelecimento de plântulas durante o armazenamento foi avaliada em soja usando um sistema experimental montado para avaliar a capacidade de alongamento. O padrão de aquisição da capacidade de alongamento durante a maturação variou entre os anos agrícolas e as condições de crescimento. O tempo para perda de 50% da capacidade de alongamento durante o armazenamento foi semelhante ao da perda de germinação. Além disso, aumentou de forma constante durante a maturação das sementes após a maturação em massa e nos estágios de maturação da colheita, destacando a importância da fase tardia da maturação das sementes na construção do vigor das sementes.

Palavras-chave: Maturação. Alongamento. Fator de choque térmico. Vigor. Qualidade de sementes.

RÉSUMÉ

La production de semences vigoureuses est un levier pour augmenter les rendements. La vigueur est définie comme la somme des propriétés physiologiques conduisant à l'établissement homogène et vigoureux du peuplement végétal. Elle comprend la longévité, définie comme la capacité à rester viable pendant le stockage et la capacité de la plantule à s'allonger après germination. Cependant, comment ces caractéristiques sont acquises au cours du développement de la graine et comment l'environnement influence leur acquisition restent mal compris. Ces informations sont importantes pour déterminer le stade de récolte correspondant à une vigueur maximale. En utilisant des approches agronomique et physiologique sur le soja et *Medicago truncatula*, nous montrons que la longévité est progressivement acquise au cours de la maturation. Chez le soja, le climat influence la longévité de manière complexe alors que chez Medicago, la chaleur pendant la maturation ne l'impacte pas significativement. Nous montrons également que HEAT SHOCK FACTOR A2.2, un homologue de HSFA9 impliqué dans la survie à l'état sec ne joue pas de rôle dans la longévité chez Medicago mais agit comme régulateur négatif de la dormance. La longévité se mesure par la perte de la germination pendant le stockage et ne représente qu'une partie du succès de l'établissement de la culture. Donc, l'influence de la maturation sur la perte de capacité d'établir une plantule lors du stockage a été évaluée chez le soja. L'acquisition de la capacité d'élongation pendant la maturation varie entre les années de culture et selon les conditions de croissance des plantules. Le temps nécessaire pour diminuer la capacité d'élongation de 50% pendant le stockage augmente constamment pendant la maturation après la maturité de masse. Ceci démontre l'importance des phases tardives de la maturation dans l'élaboration de la vigueur germinative.

Mots-clés: Maturation. Élongation. Heat shock factor. Vigueur. Qualité des semences.

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LIST OF ABBREVIATION

ABA	Abscisic Acid
ABI3	ABA-insensitive 3
ABI5	ABA-insensitive 5
AWT	Associated wild type.
BR	Brassinosteroids
cDNA	Complementary acid desoxyribonucleic
CK	Cytokinin
CDT	Controlled deterioration treatment
DAF	Days after flowering,
DNA	Acid desoxyribonucleic
DW	Dry weight
DNase	Desoxyribonuclease
ETH	Ethylene
GA	Gibberellin
RH	Relative humidity
HSF	Heat Shock Factor
HSFA9	Heat Shock Factor A9
HSP	Heat Shock Protein
IAA	Indole acid
INRA	Institut de Recherche en Horticulture et Semences
IRHS	Institut de Recherche en Horticulture et Semences
K_2CO_3	Potassium carbonate
LiCl	Lithium chloride
LEA	Late embryogenesis abundant
M	Molar
MeOH	Methanol hydroxide
MM	Maturity mass
NaCl	Sodium chloride
PCA	Principal component analysis
PCR	Polymerase chain reaction
PM	Physiological maturity
RFO	Raffinose family oligosaccharides

RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-PCR
SAM	Shoot apical meristem.
SD	Stardard deviation
SE	Standard error
SUC	Sucrose
TD	Desiccation tolerance
UNESP	University of São Paulo “Júlio de Mesquita Filho’’
UV	Ultra violet
WC	Water content
WT	Wild type

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GENERAL INTRODUCTION

Legume crops are important to the global agriculture since it provides a sustainable solution to food and feed protein security. Significant efforts are being made to increase yield and quality of legumes and to identify climate-resilient genotypes with improved grain characteristics (CONSIDINE et al., 2017). Soybean is one of the most important commodities worldwide, being a rich source of protein and oil for a multitude of end-products for feed and food (GUNSTONE, 2011) With the continued increase in world demand for sources of plant oil and proteins, soybean production has spread rapidly to tropical regions. In Brazil the annual growth of soybean production in the last 20 years was 3.5 million tons, representing an increase of 13.4% each year due to an increase in productivity and in the cultivated areas (JUNIOR et al., 2017).

The production of highly vigorous seeds is crucial to achieve crop production efficiency through seed physiological quality. The term “physiological quality” describes the overall value of a seed lot. It is defined as the sum of physiological properties that leads to homogeneous and fast germination, uniform seedling establishment and greater tolerance of stressful conditions during germination (vigor) and remain viable for long periods during storage in dry state (longevity) (BEWLEY et al., 2013). Also, the fast establishment of normal seedlings in the field also allows the efficient control of weeds and avoids the introduction of pathogens. However, a tropical environment such in Brazil is problematic for soybean as seeds have a short lifespan during storage. Knowledge on the acquisition of these different seed quality traits during development is important to determine the ideal harvest point to obtain seeds with maximum physiological potential.

Seed physiological quality is acquired sequentially during seed development and maturation, but in many plant species the different physiological quality traits are not acquired at same time. The capacity to germinate is acquired prior to maximum dry weight. This is followed by the development of desiccation tolerance. Seed longevity is progressively acquired, after seed filling, until the seed reaches a dry state (CHATELAIN et al., 2012, RIGHETTI et al., 2015, LIMA et al., 2017). During this period of development, late embryogenesis abundant (LEA) protein, raffinose family oligosaccharides (RFOs), heat shock proteins (HSP) accumulate, which confer protection against denaturation and deleterious changes in the

conformation of macromolecules during drying and in the dry state (ROSNOBLET, 2007; BUITINK and LEPRINCE, 2008; CHATELAIN et al., 2012, VERDIER et al., 2013, RIGHETTI et al., 2015). Some works suggest that HSP can improve seed vigor (KAUR et al., 2015).

Also the content of LEA, RFO serves as an indicative of seed maturity and vigor (JALINK et al., 1998; SINNIAH et al., 1998; VANDECASTEELE et al., 2011; DE SOUZA VIDIGAL et al., 2016). Longevity is also conferred by a range of antioxidants that protect the seeds against oxidative damage during storage, such as glutathione (KRANNER et al., 2006), tocopherols (MÈNE-SAFFRANÉ et al., 2010, VOM DORP et al., 2015), and flavonoids that are present in the seed coat (DEBEAUJON et al., 2000, DE GIORGI et al., 2015). However, the regulatory mechanisms controlling the acquisition and regulation of seed longevity and vigor remains largely unknown.

In parallel with the longevity, seed vigor continues to increase after severing the connection with the mother plant, which occurs at or shortly after mass maturity (maximum seed dry weight) and does not necessarily coincides with harvest maturity (when seeds can be safely harvested without mechanical damage (DEMIR and ELLIS, 1992; ELLIS at al.,1987, reviewed in FINCH-SAVAGE and BASSEL, 2016, BEWLEY et al., 2013). In the same way, how the vigor and seedling establishment is acquired during maturation remains poorly understood.

The position of the seed on the mother appear to be an effect on seed quality both the seed attributes (weight, shape) and physiological quality (vigor, viability, longevity). However, there is no consensus in the literature about this. The differences in quality attributes may be associated with time to pod set, differences in seed maturity or in time left for seed ageing on the plant. In addition, the environment and external conditions around the pod and internal characteristics (e.g. local sink-source relations) may contribute to position effects on physiological seed attributes (ILLIPRONTI et al., 2000).

With climate change, the impact of the maternal environment on the acquisition of seed vigor becomes highly relevant. Climate change is predicted to affect global agricultural production with adverse effects of high temperature and irregular rains, challenging researchers to find out adaptation strategies for crops (VALLIYODAN et al., 2017). Environmental conditions during reproductive growth of soybean can cause differences in seed yield and quality (EGLI et al., 2005; PUTEH et al., 2013)

but how they influence acquisition of seed vigor remains poorly understood. To achieve a stable production of high vigor seeds, it is necessary to gain further knowledge into the mechanisms governing seed vigor during seed development by taking into account the maternal effects.

So, in order to overcome challenges in agriculture, studies about molecular and physiological mechanisms of seed maturation, dormancy and longevity, and seed vigor can serve as a model to explain how these mechanisms work in many species, therefore, helping breeding programs to increase their chances to improve quality to crops worldwide.

The objective of my PhD is to study the main events related to the acquisition of seed quality (longevity and vigor) that occur during late seed maturation in legumes when the developing seeds prepare for the dry stage. For this purpose, we used soybean seeds grown in two consecutive years under field conditions in Brazil and *Medicago truncatula* grown under controlled optimal conditions and at high temperature.

The work presented here is divided into five parts. A first chapter of bibliographical synthesis exposes current knowledge of seed maturation events and its regulation. In the chapter 2, using soybean we evaluated the impact of the maternal environment on seed quality by comparing the effect of slow and fast drying on the survival in the dry state (desiccation tolerance and seed longevity) at different stages of maturation. Taking advantage of the contrasting climate data of 2015 and 2016, we also evaluated the impact of the environmental conditions on the acquisition of seed longevity and the impact of maturation on the thermal dependence of germination.

In chapter 3 using soybean, the work documents how and when organ elongation during seedling establishment is acquired during seed maturation. We studied whether the progress of maturation, the seed position on the mother plant has an influence on hypocotyl elongation and whether elongation capacity is affected during storage.

The aim of chapter 4 was to assess the role of HSFA2.2 from *Medicago truncatula* and HSFA9 from Arabidopsis in the regulation of seed vigor. HSFA.2.2 is the homologue of the sunflower HSFA9 (PRIETO-DAPENA et al., 2006) whose role in seed vigor has been suggested. For this purpose, we obtained and characterized mutant seeds from both species. Since the role of HSF and their

targets, HSP are central to heat response of cells. In Medicago, heat stress reduces seed longevity (RIGHETTI et al., 2015). Therefore, the effect of high temperatures on seed maturation was also studied in *Mthsfa2.2.* seeds. The corresponding mutants were characterized for seed quality characteristics including dormancy and longevity as well as response to phytohormones.

CHAPTER 1
LITERATURE REVIEW

In this review we will show general aspects of soybean seed production and stages of seed development. We will discuss events related to the acquisition of seed physiological quality and its regulation. In addition, we will present information related to role of the maternal environment on seed quality (seed position on the mother plant and environmental factors affecting seed quality). Finally, we also present information related to the role of Heat Shock Proteins (HSP) and Heat shock transcription factor (HSF) on seed quality.

1.1 Soybean seed production and stages of development

Soybean (*Glycine max* (L.) Merrill.) (2n = 40 chromosomes) is an herbaceous and autogamous plant belonging to the Fabaceae family. Soybean is one of the main commodities produced in the world. Currently, Brazil is the second largest soybean producer in the world, preceded by the United States. The world production of soybean in the years 2016-2017 was 351.311 million tons, with a planted area of 120,958 million of hectares (USDA, 2017). In Brazil, cultivated soybean represents an estimated area of 33,909 million of hectares with a production of 114,075 million tons and an average yield of 3,4 kg ha^{-1}, 17.2% higher than the previous harvest (CONAB, 2017).

Soybean grains are rich in proteins and lipids. They are widely used by the agroindustry, including animal feed, production of vegetable oil, chemical industry and foods. Soybean is also used as alternative source of biofuel (SEDIYAMA et al., 2009).

The success of the soybean cultivation depends on several factors but the seed plays a crucial role. For a high agronomic performance, the seeds possess attributes of genetic, physical, physiological and sanitary qualities. The seed physiological quality is a collection of properties inherent to a seed lot that allow a high and uniform level of germination, which will produce uniform, vigorous seedlings, ability of the seeds to undergo complete desiccation without loss of viability and contributes to seed survival during storage, considered an indispensable element to generate vigorous plants, with superior performance in

the field, tolerance to potential stresses in different environmental conditions. Low vigor or deteriorated seeds result in less vigorous seedlings and with little or no chance of success in seedling establishment (FINCH-SAVAGE and BASSEL, 2016). Therefore, seed production aims at maximum economic productivity, based on the perfect integration between the plant, the production environment and the management. It is important that the seed producer knows the physical characteristics of the plant, such as its phenological stages, its nutritional value, water, thermal and photoperiodic requirements, for a correct field management (SEDIYAMA et al., 2009).

Figure 1. Characterization by phenological stages on the plant and pods according to the description of Fehr and Caviness (1977) with adaptations by Lima et al. (2017).

Source: Oliveira Junior et al. (2016).

The characterization by phenological stages on the plant and seeds allow detailing the description of the plant cycle with respect to the utilities in each stage. Soybean development is characterized by two distinct growth phases. The first is

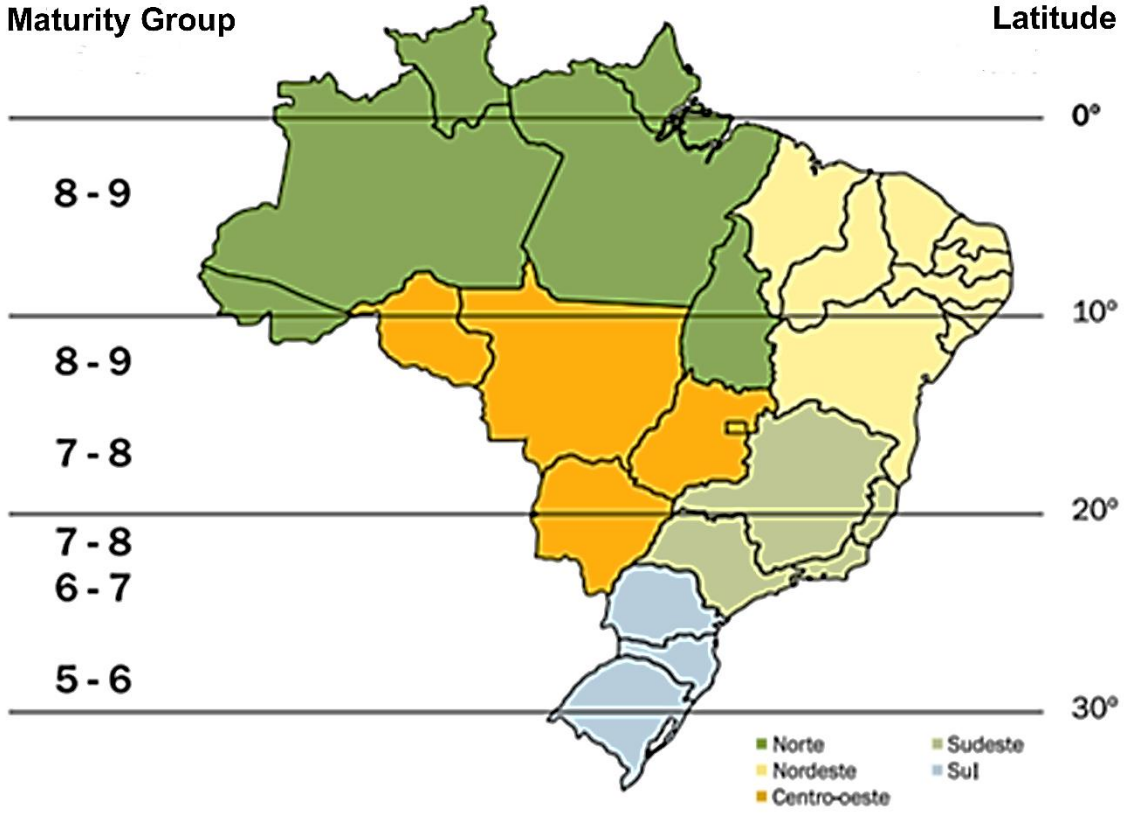
the vegetative stages (V) that cover development from emergence through flowering. The second is the reproductive (R) stages from flowering through maturation (Figure1). After germination of the seed in the soil, the first two stages are designated as VE (emergency) and VC (cotyledon stage), the vegetative stages are determined by counting the number of nodes on the main stem, beginning with the unifoliolate node, that have or have had a fully developed leaf. The V stages are designated numerically as V1, V2, V3... (n), which (n) can be represents the number for the last node. (FEHR and CAVINESS, 1977). The reproductive stage is characterized by the onset of flowering (R1), where the plants have at least one open flower at any node; R2 indicates full bloom (the plants have an open flower in the node immediately below the highest node with a completely unrolled leaf). Stages R3 and R4 comprise the development of the pod where in R3 development begins and R4 when the pod is fully developed, 3/4 of an inch long at one of the four upper nodes on the main stem with a fully developed leaf. From the stage R5 begins the development of the seed inside the pod.

Lima et al. (2017) subdivided the R5 stage between R5.1 and R5.5, at this stage the grain filling occurs progressively. The stage R6 the grains are completely developed and green leaves. The stage R7.1 and R7.2 is the beginning of maturation called early maturation with Start to 50% ate 75 % of yellowing leaf and pods, R7.3 with half of maturation and more than 76% of leaves and pods yellow. The stage R8.1and R8.2 is full maturation with more than 50% defoliation before harvest. R9 is harvest maturation, considered point of harvest (LIMA et al., 2017).

Both phases (vegetative and reproductive growth) are essential for the production of seed yield, but they are not equally important and the distribution of time between vegetative and reproductive growth can affect the yield potential of the crop and how efficiently the crop exploits the available resources (reviewed by EGLI, 2011). According to Wright and Lenssen (2013), in soybean seeds the transition between the stages within the reproductive phase (R1 to R8) takes much longer compared to the vegetative phase, ranging from 3 days to develop from R1 to R2, 15 days to develop from R5 to R6 and 18 days to develop from R6 to R7. In soybean the transition from vegetative to the reproductive growth stages depends largely on plant responses to relative changes in light and dark periods (photoperiod) (KANTOLIC, 2008) and temperature (HESKETH et al., 1973). The sensitivity to photoperiod varies with the genotype (RODRIGUES et al., 2001) and

with the developing stage (SETIYONO et al., 2007). Since the length of the day varies with latitude, the soybean has been adapted to grow in specific latitudes. Based on the response to the photoperiod and the area of adaptation, soybean cultivars, are classified based on the maturity group (Figure 2). Relative maturity is a rating designed to account for all of the factors that affect maturity date and number of days from planting to maturity. These factors include variety, planting date, rainfall and latitude (ALLIPRANDINI et al., 2009). This classification allows the ideal growth and production potential that are only achieved when soybeans are grown in their region of adaptation (CAVASSIM et al., 2013).

Figure 2. The distribution of relative maturity groups for soybean cultivars in Brazil (ALLIPRANDINI et al., 2009).



Source: EMBRAPA (Portfólio Embrapa de Cultivares de Soja), 2016.

The maturity can be responsible for the variation in vegetative duration within a species, while variation among species reflects species characteristics and environmental effects. The differences among species at the same duration are not surprising since maximum vegetative mass is also affected by growth rate

(reviewed by EGLI, 2011). Growth, development and yield of soybeans depend on genetic potential of a cultivar and its interaction with the environment.

The environmental conditions (water and temperature) can delay or advance the phenological stages of the crop, altering the result of the final product, production and quality of the seed produced. Duration of grain filling is shortened at high (over 30 °C) temperatures in wheat, is probably a consequence of accelerated development, and since the rate of grain filling does not increase proportionately to the larger number of cells produced there is diminished grain weight (yield) at maturity (BEWLEY and BLACK, 1994).

The total water requirement in the soybean crop, for maximum yield, varies between 450 and 800 mm/cycle, depending on the climatic conditions, crop management and cycle duration of the cultivar. Water is indispensable, from germination to seedling emergence and from flowering to seed filling (FARIAS et al., 2007; EMBRAPA, 2014). The loss of productivity under water deficit conditions depends on the soybean phenological stage, duration and intensity of water shortages (DOSS and THURLOW, 1974, DESCLAUX et al., 2000). Desclaux et al. (1996) evaluated the effects of water stress at various stages of development in soybean plants. A drought stresses led shorter reproductive phases because of a speeding up of organ emergence and a decrease in the number of nodes. This decreased the phenological lags between axis nodes a this suggest a quicker sequence between phases. Besides, a smaller accumulation of dry matter in seeds growing under stress during pod filling could be explained by the reduction of the filling period. Water stress during seed filling reduce soybean yield and shortening the seed filling period (DE SOUZA et al., 1997).

Soybean is best suited to regions where temperatures range from 20 °C to 30 °C and the ideal temperature for its development is around 30 °C. Seed germination and seedling emergence are favored by temperatures between 25 °C and 30 °C, in most genotypes. Soil temperature below 10 °C results in delayed of germination and subjects the seeds to the action of soil pathogens. Seeds sown in soil with a temperature around 40 °C do not germinate and this information is important, because in certain regions, soils at 5 cm depth have a temperature above 45 °C, which will prevent their germination. Other criteria to consider for high yields are the maturity group, genotype, the crop rotation and the properties of the

soil, such as fertility, drainage and topography, control of weed, pests and diseases (EMBRAPA, 2014).

The rate of plant development for any variety is directly related to temperature, so the length of time between the different stages will vary as the temperature varies both between and within the growing season. These changes and variability may have significant impacts on phenology, growth and yield of soybeans. Effective strategies to adapt agricultural production require deep understanding of soybean maturity groups.

1.2 Seed development

Seed development is a complex process involving the integration of genetic, metabolic and physiological pathways with environmental cues (DANTE et al., 2014). According to Egli (2006) the growth and development of the seeds comprise three phases, where the first phase is initiated in fertilization and characterized by cell proliferation; Phase II is defined as seed filling, presenting a large increase in dry weight associated with cell enlargement and accumulation of storage compounds; in phase III is observed a reduction of the dry weight associated with desiccation and dormancy.

The seed development is divided into three phases (I corresponding to embryogenesis, II, corresponding to seed filling, and III late maturation).

a) Embryogenesis:

Fertilization determines the beginning of the process of seed formation (Phase I).

Seed formation begins with the fusion of male and female gamete, a process known as fertilization. Fertilization can occur when both male and female gametophytes are full mature. Fertilization can occur in a dual fusion process called double fertilization. When the pollen grain reaches the stigma, it germinates by sending out a pollinic tube, which grows in the style, into the embryonic sac. The tube nucleus degenerates thereafter, and the two pollen sperm cells enter the embryo sac to make a diploid fusion (2N) and the polar nucleus forms a triploid (3N) endosperm, after this fusion, a period of reorganization happens, the vacuole adjacent to the zygote gradually disappears. The still undivided zygote usually elongates along the horizontal axis (COPELAND and MCDONALD, 2004).

After the suspensor has been formed, there is an intense division that characterizes the development of the embryo. From this stage, in dicotyledons, there is a flattening of the upper extremity and an intense cell division in the right and left region of the pro-embryo forming the beginning of the cotyledons. In them, the plumule differs, and in the lower region lies the hypocotyl-radicle axis (MARCOS-FILHO, 2015). The developmental pattern of the embryo and legume endosperm is characterized by exalbuminous (cotyledonary). The true endosperm, is just found in the angiosperms; it is come from the endosperm cell which has triple fusion nucleus. Exist two kinds of endosperm development that occur in monocots and dicots: a) nuclear, where several divisions of the nuclei happen before cell wall formation; b) cellular, where cell walls being formed soon after the mitotic division (BEWLEY et al., 2013).

Reserves are stored in adjacent tissues in the endosperm during its development, as well new products reserves. So, the embryo becomes enveloped, according to its associated reserves source, which can be useful during maturation stages (BEWLEY et al., 2013).

In most dicotyledons seeds, the endosperm is almost completely consumed during development of the embryo. In these seeds, the reserves are stored in the cotyledons. In soybean seeds, the embryo is formed by two cotyledons and embryo axis, which are responsible for 90% of the soybean seed weight (MARCOS-FILHO, 2015).

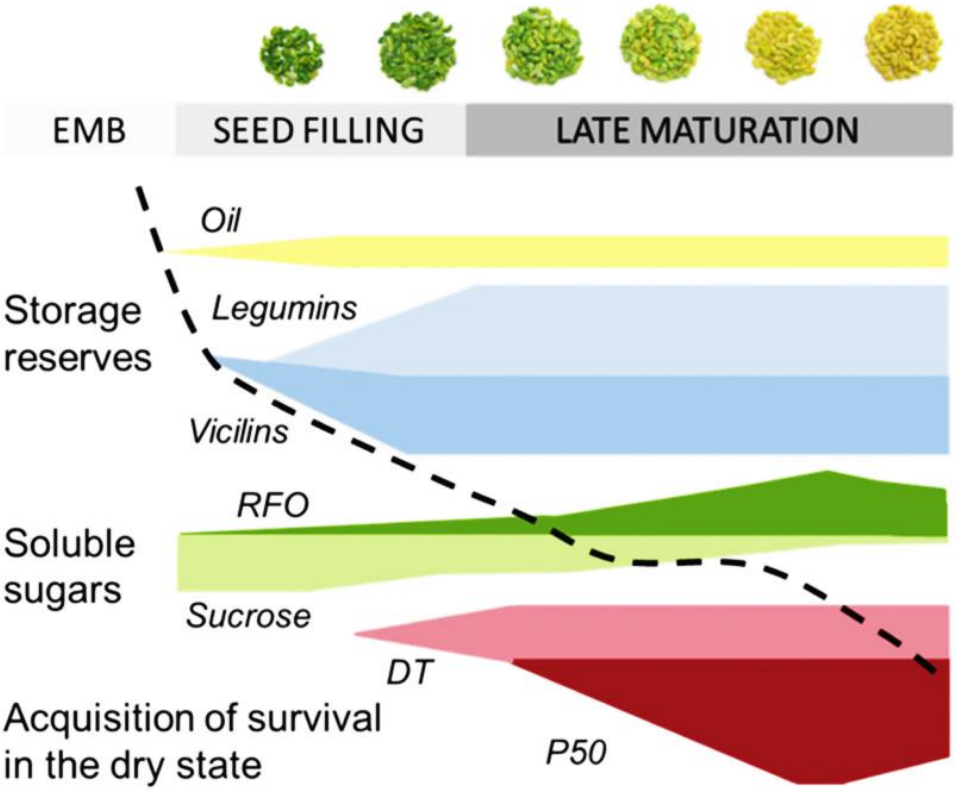
b) Seed filling

Phase II represents the seed filling during which cells are expanding as they synthesize storage protein and oil. The reserve accumulation results in a significant increase in dry weight during this phase a slight decrease of water content (BARLOW et al., 1980). During phase II, most endosperm cells or cotyledons generated in phase I accumulate storage compounds. Thus, phase II is characterized by cell growth due to cell expansion and a peak in the water content of the seed (EGLI, 2006). Dornbos and McDonald (1986). The mass and composition of the cultivar Williams 79 during seed development that the stages R5 and R7 corresponded to the initiation of seed filling and to physiological maturity, respectively. Among these phases, the water content (% of fresh weight) decreases steadily, although the total amount of water per embryo is still

increasing, with ABA being the most abundant hormone at this stage, reaching maximum levels during the period of maximum increase weight of the seed.

The storage compounds found in most mature seeds accumulate during seed filling (Figure 3), they are principally storage proteins, oil and carbohydrates. These reserves are of major importance for they support early seedling growth when degraded upon germination and, therefore, participate in crop establishment (GALLARDO et al., 2003). Seeds of legume species, such as soybean (*Glycine max*), are an important protein and oil source, around 38% and 20%, respectively (LEE et al., 2015) depending on species, genotype, and environment. The storage compounds accumulate is very important, but the processes that coordinate their accumulation at specific stages during seed development are not well understood.

Figure 3. Main physiological and biochemical events related to seed filling and late seed maturation are overlapping in *Medicago truncatula* (LEPRINCE et al., 2017). The broken line indicates the loss of water during development. DT, desiccation tolerance; EMB, embryogenesis; P50, half-life of viability loss during storage; RFO, raffinose family oligosaccharide content.



Source: Leprince et al. (2017), adapted from Righetti et al. (2015).

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c) Late seed maturation and dry state

During phase III, seed development ends with a drying phase of maturation represented by the loss of water leading to the quiescent state after seed filling where phase the accumulation of reserves ceases. During this phase the seeds present a low level of metabolic activity, which preserves the viability of the seed. In addition, during this period there is a decrease of the inhibitors of germination, alteration of the membranes and degradation of the protein that improves the vigor (FINCH-SAVAGE and LEUBNER-METZGER, 2006).

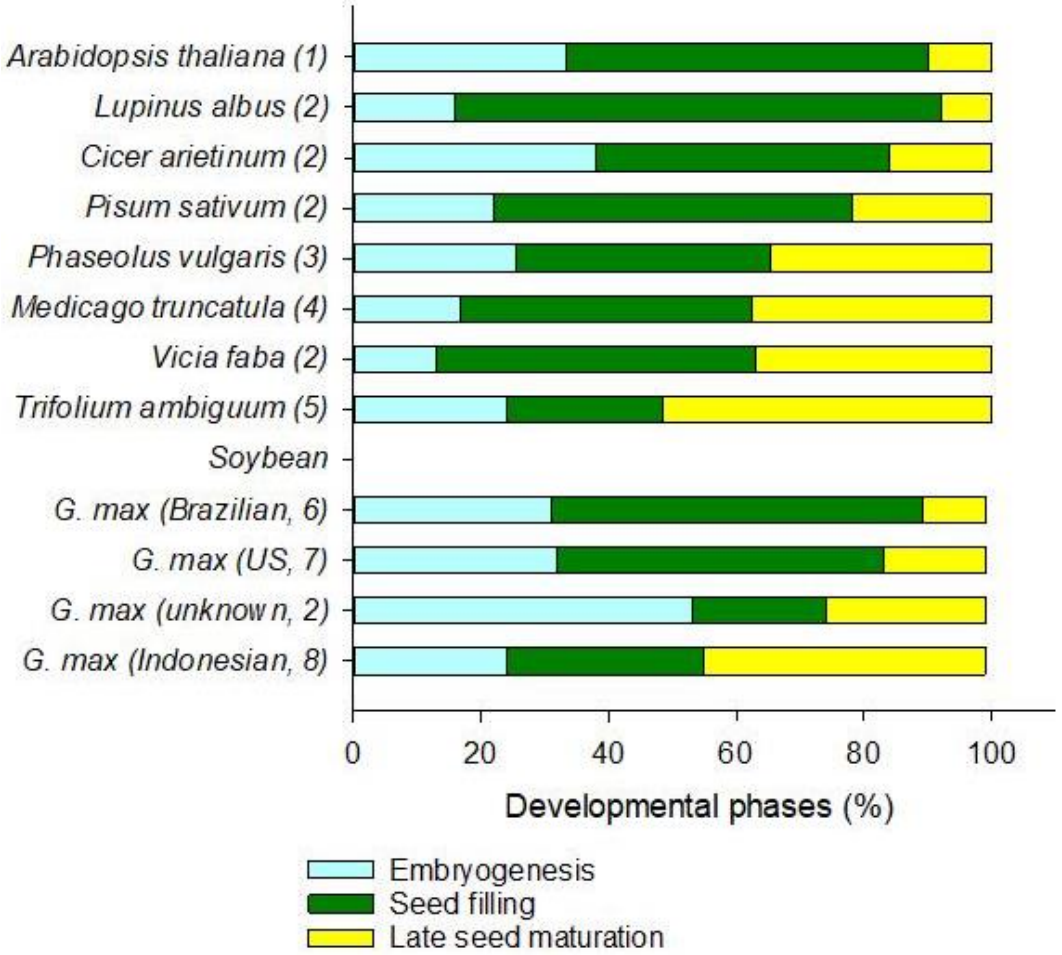
When seeds enter in the late maturation phase germination and desiccation tolerance are already acquired. In soybean seeds longevity is progressively acquired coinciding with the end of the seed filling (LIMA et al., 2017). To illustrate the importance of the late maturation phase, Leprince et al. (2017) compared its relative length in time during seed development across legume species. There is no consensus as to when late seed maturation starts, and the easiest morphological criterion is the end of seed filling and when seed water content drops below the embryo and/or endosperm ceases to expand and accumulates storage reserves. Leprince et al. (2017) shows that using this criterion, the relative time span of the late seed maturation within the legume family varies tremendously from one species to another, ranging from 8% to 50% of the total developmental time in *Lupinus* and *Trifolium*, respectively. Arabidopsis exhibits a late maturation phase that is among the shortest, providing an explanation as to why this phase has not yet received a great deal of attention at the molecular level. Late seed maturation also varies within a species, as shown for soybean. Depending on the cultivar and growth condition, it varies between 10 (Brazilian cultivar grown in Botucatu, Brazil)

and 46%, corresponding an Indonesian genotype grown in greenhouse in the UK) (Figure 4).

RFO sugars accumulate at the final stages of maturation, concomitant with a decrease in Suc (DJEMEL et al., 2005; ROSNOBLET, 2007). In legumes, RFO sugars have been as an easily available energy reserve for seedling establishment (VANDECASTEELE et al., 2011). Vandecasteele et al. (2011) suggests that it is not the absolute amount of RFOs, but rather the conversion of Sucrose (Suc) into RFO in maturing seeds (reflected in the ratio Suc/RFO in mature seeds), that is linked to seed vigor during germination and seedling establishment in legume seeds. Therefore, the variation in sugars and vigor traits could because by slight differences in the extent to which the genetic program governing seed maturation.

In *Medicago truncatula*, as a model plant for legume, the loss of chlorophyll is associated with late maturation. In most plant species, the amount of chlorophyll is directly related to the degreening process during the late stages of seed maturation, before the seed reaches the dry state (JALINK et al., 1998; NAKAJIMA et al., 2012; TEIXEIRA et al., 2016).

Figure 4. Relative importance of the three phases of seed development. Data are taken from the literature showing the evolution of fresh and dry weight and water content during seed development. The switch from embryogenesis to seed filling was defined as the onset of the dry weight increase. The late seed maturation starts when seed filling ceased and seed water was approximately 55% (fresh weigh basis). Seed development was considered terminated when seed moisture content was less than 20%.



Source: Redrawn from Leprince et al. (2017) with data from 1) Baud et al. (2002); 2) Ellis et al. (1987); 3) Sanhewe and Ellis (1996); 4) Chatelain et al. (2012); 5) Hay et al. (2010); 6) Lima et al. (2017); 7) Obendorf et al. (2009); 8) Zanakis et al. (1994).

On the molecular level, a number of mechanisms have been discovered that influence survival in the dry state. They include the synthesis of protective molecules, such as non- reducing sugars (HOEKSTRA et al., 2001), late embryogenesis abundant (LEA) proteins (HUNDERTMARK et al., 2011b, CHATELAIN et al., 2012), heat shock proteins (HSP) (PRIETO-DAPENA et al., 2006, ROSNOBLET et al., 2007), various other stress proteins (SUGLIANI et al., 2009) and a set of antioxidant defenses against oxidative stress such as tocopherols (SATTLER et al., 2004) and flavonoids present in the testa (DEBEAUJON et al., 2000). By acting synergistically, four types of mechanisms

can be observed, attenuating the deleterious effects of desiccation: a) prevention of oxidative damage (antioxidant compounds, - tocopherols and glutathione); b) avoiding structural stress (cell wall modification, intracellular membrane and cytoskeletal reorganization, and chromatin condensation); c) stabilization of membranes and proteins (non-reducing sugars, abundant proteins of late embryogenesis and heat shock proteins) by the formation of hydrogen bonds with polar residues of proteins and membrane phospholipids that keep molecules dry in a physical state similar to that observed in the presence of water and d) efficient systems for detoxification and repair of damaged DNA and proteins after rehydration (CROWE et al., 1992; HOEKSTRA et al., 2001; LEPRINCE and BUITINK, 2010; GAFF and OLIVER, 2013; TAPIA and KOSHLAND., 2014).

One of the first researchers to describe the process of seed maturation was Willard (1925). This author working with soybean related the point of maturity with leaf abscission, and leaf and pod colors.

Shaw and Loomis (1950), considered the seed physiological maturity using as criterion the maximum dry weight. This stage was also called “relative maturity” by Aldrich (1943) and “morphological maturity” by Anderson (1955), all of them had as criterion the maximum dry weight (MARCOS-FILHO, 2015). Harrington (1972) suggested that developing seeds achieve maximum viability and vigor at physiological maturity. When the seed potential is the highest, that which corresponds to the so-called physiological maturity (maximum germinability, longevity and vigor) eg. maximum seed quality.

However, physiological maturity does not always correspond to maximum dry weight. Ellis and Pieta Filho (1992) showed that the maximum physiological potential occurs after physiological maturity and proposed the term “mass maturity” to denominate the point of maximum dry mass. According to the review Carvalho and Nakagawa (2012), the physiological maturity, within each species, can vary in relation to the moment of its occurrence, depending on the cultivar and the environmental conditions.

The definition of physiological maturity (PM) and mass maturity (MM) is not clear in the literature. Some researchers consider the physiological maturity is the time as the highest seed quality (viability, vigor, longevity), others consider that the highest quality coincides with the maximum dry weight, where the seed is disconnected from the mother plant. Currently, research shows that seed quality

may continue to increase even after the seeds have reached maximum seed dry weigh.

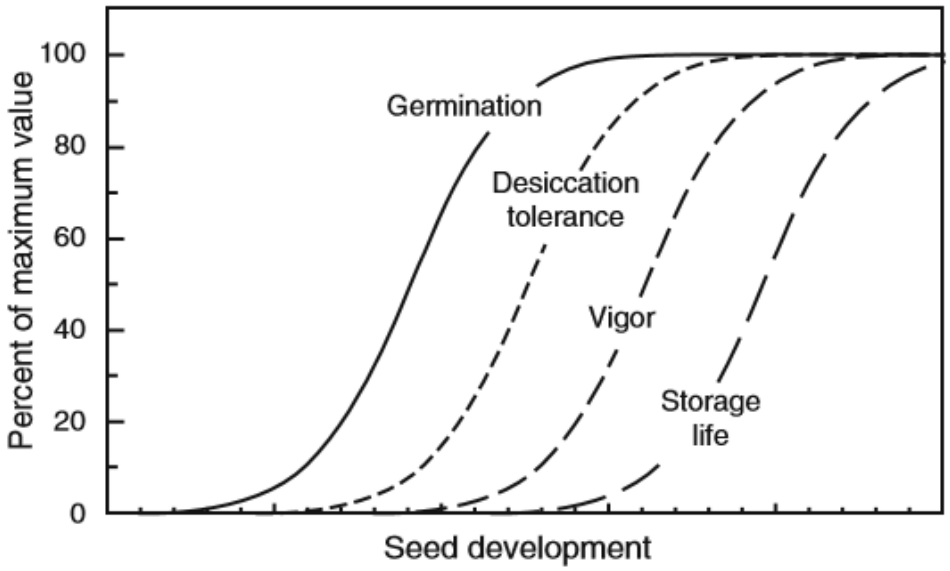
Ideally, seed crops, like soybean, should be harvested when maximum physiological quality is acquired, but his is very difficult to achieve, because is no consensus in the literature as to when the maximum quality occurs during seed development. Usually, the seeds are harvested when it has water content compatible with the mechanized harvest in order to avoid mechanical damage.

Physiological maturity (PM), mass maturity (MM) are three important terms which are closely related to seed quality, but there is still no exact definition in the literature about these traits.

1.3 Acquisition of physiological quality during development

The capacity to germinate, to withstand the desiccation, to perform fast and uniform growth of the seedlings and to remain viable for long periods are attributes of seed quality are not acquired in the same time. In general, the ability of the seeds to germinate (at least in some conditions) develops before the maximum dry weight. During development, the seeds acquire desiccation tolerance and maximum viability after dehydration. The vigor of the seeds is obtained after the components of viability and tolerance to desiccation; and longevity is not acquired until the end of the complete seed development (Figure 5) (BEWLEY et al., 2013).

Figure 5. Pattern of development of seed physiological quality traits.



Source: Bewley et al.(2013).

1.3.1 Germinability

Germinability can be defined as the ability of an immature seed to germinate. Germinability is acquired early during the seed filling in the middle of the development phase. Lima et al. (2017) show that soybean seeds at 36 DAF (stage R5) already have ability to germinate (Figure 6). Similar observations were reported by Blackman et al. (1992) at 34 DAF for soybean seeds. Zanakis et al. (1994) found that germinability in soybean seeds increases between 25 and 40 days DAF. The seeds acquire the ability to germinate when fresh before they acquire desiccation tolerance. However, in cereals such as barley and wheat immature seeds germinated to higher levels when they were dried than fresh (ALDRIDGE and PROBERT, 1993), this probably being related to the presence of seed dormancy.

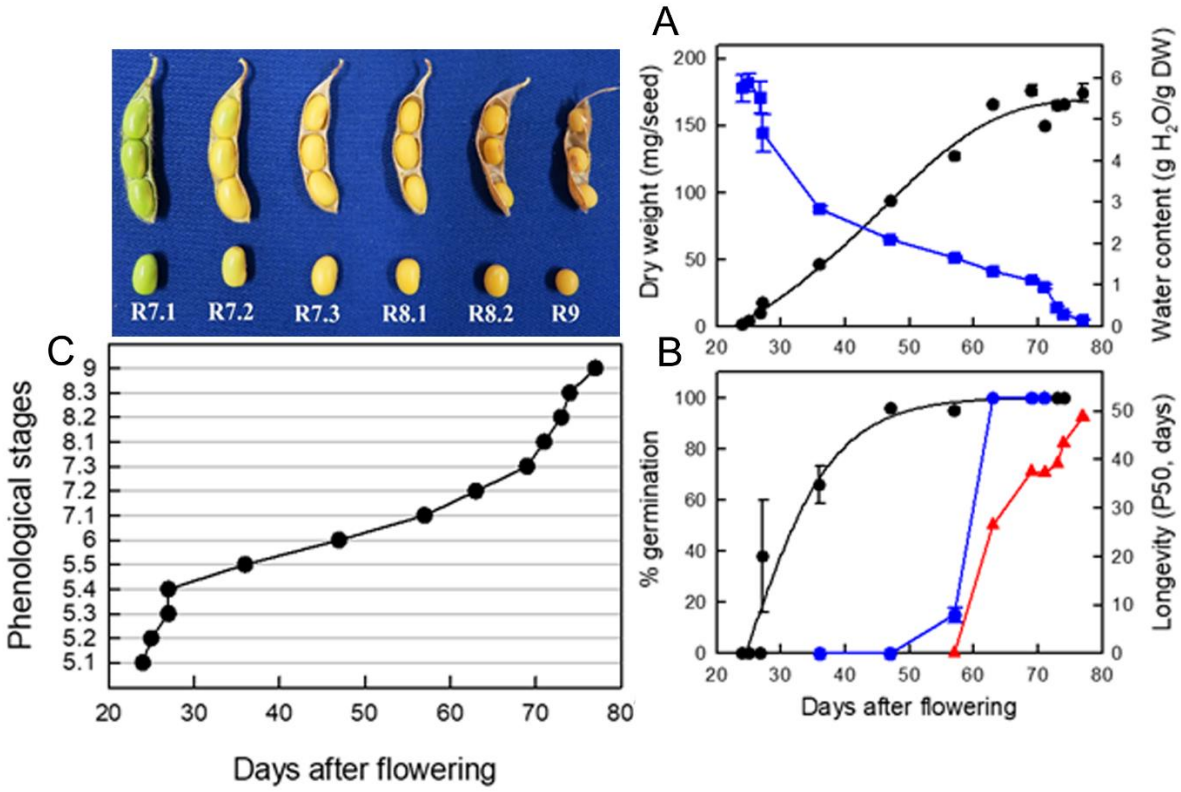
1.3.2 Seed desiccation tolerance

Desiccation tolerance (DT) is defined as the ability to survive to a rapid drying that brings the seed moisture in equilibrium with ambient air without irreversible damage. This corresponds to the removal of almost all cellular water, which corresponds to approximately 10% moisture, depending on the species and the amount of oil reserve. In crops that shed dry seeds, desiccation tolerance is acquired (reviewed in LEPRINCE and BUITINK, 2010) halfway during seed filling before the onset of maturation drying. It should be emphasized that the speed of enforced drying is a key element to determine when DT is acquired in immature seeds. Indeed, during the progressive loss of water, metabolic processes can still occur. Therefore, a slow drying allows the developmental program that increases the level of tolerance to drying compared to the stage when immature seeds were harvested (BLACKMAN et al., 1991). This is particularly true for large seeds that take long to dry out. In soybean using a moderate drying rate where loss of water occurred within 3 days, DT was acquired towards the end of seed filling which coincides with the stage R7.2, i.e. 63 DAF (LIMA et al., 2017).

In orthodox seeds, the acquisition of DT during development is resilient to both environmental and genetic perturbations. This means that developing seeds always acquire their DT regardless of the growth conditions of the mother plants.

For example, in *Medicago truncatula*, DT was acquired during seed filling both in cold, heat or water stress conditions (RIGHETTI et al., 2015).

Figure 6. Physiological characterization of soybean seed maturation. (A) Evolution of seed dry weight (back circle) and water content (blue square). Data are the means (± SE) of 3 to 5 replicates of 20 seeds. (B) Acquisition of germinability (●, black circle) and desiccation tolerance (blue circle), evaluated after fast drying to 10% moisture and longevity (red triangle) as assessed by P50, (C) Phenological stages during seed development in soybean seeds (LIMA et al., 2017).



Source: Lima et al. (2017).

Desiccation tolerance is an integral part of the development of on orthodox seeds and consequently a set of mechanisms is involved in the protection and repair must be synthesized under the control of specific regulators while repression of oxidative metabolism is necessary to avoid stress and oxidation during drying (LEPRINCE and BUITINK, 2010). Desiccation tolerance cannot be attributed to a simple protection mechanism; rather, it appears to be a multifactorial phenomenon in which each component is equally critical, acting synergistically (LEPRINCE et al., 1993).

Abscisic acid (ABA) regulates many agronomical important aspects of plant development, including the accumulation of nutritive reserves, the induction of

seed desiccation tolerance and dormancy (reviewed by LEUNG and GIRAUDAT, 1998). This can be demonstrated using mutants that are deficient and/or insensitive to ABA (OOMS et al, 1993, DELAHAIE et al., 2013). Seed maturation is controlled by regulators that interact in a complex way and includes the binding of Leafy cotyledon factor (LEC1) and the three B3 domain contents (ABSCISIC ACID INSENSITIVE (ABI3), FUSCA (FUS3) and LEC2) (GIRAUDAT, 1992; NAMBARA et al., 1992; LUERSSEN et al., 1998; STONE et al., 2001). Strong allelic mutants of these genes also produce desiccation-sensitive seeds probably because these regulators are important for the transition from embryogenesis to seed maturation. For the sake of clarity, the role of ABA signaling pathway during seed development will be discussed in the next sections in relation to seed longevity and dormancy, respectively.

1.3.3 Seed longevity and hormonal control

The review below will only focus *on ex situ* dry storage. Seed longevity is defined as the time-span during which seeds remain viable during dry storage (SANO et al., 2016).

During late maturation, the longevity is progressively acquired, increasing 30 to 50 times until to reach the maximum before or close to the dry state, depending on the species, genotype and the environment (HAY et al., 2015; PROBERT et al., 2007; RIGHETTI et al., 2015). In species like Medicago (CHATELAIN et al., 2012, VERDIER et al., 2013) and rice, the long maturation phase allows a clear separation in time of the induction of longevity from the acquisition of DT and end of seed filling. In contrast, in species where the late maturation is short such as soybean (LIMA et al., 2017) and *Arabidopsis thaliana* (RIGHETTI et al., 2015), longevity is acquired rapidly after DT but still during the seed filling phase. When immature seeds are dried slowly or in an uncontrolled fashion, it is difficult, if not impossible to interpret whether longevity is acquired at the end of seed filling or later. When submitted to drying immature seeds can suffer from the immediate effects of water loss and/or the longer-term effects during storage. For example, in an experiment where pea seeds were dried very slowly, Walters et al. (2001) demonstrated that the predominant stress from slow drying was ageing due to metabolic imbalance as the drying seeds stayed too long between 1 and 0.3 g

water/g DW. By analogy, testing longevity during seed maturation using a slow drying rate might induce artifacts that confound desiccation tolerance and longevity.

The seed longevity decreases rapidly during storage under high relative humidity and high temperature it is well known. This is possibly due to increased fluidity of the cytoplasm that in turn promotes irreversible aggregation of denatured proteins. Oxidation of cellular molecules such as lipids, cell membranes, DNA, RNA and proteins (OSBORNE and BOUBRIAK, 1994; BAILLY, 2004; RAJJOU and DEBEAUJON., 2008; RAJJOU et al., 2008), mediated by reactive oxygen species (ROS), also affects seed longevity (HARRISON and MCLEISH, 1954; JUSTICE and BASS., 1978; GROOT et al., 2012).

Low temperature and low seed moisture content prolong seed life span during storage as these conditions allows the cytoplasm to form a glassy state, i.e. an amorphous solid-like matrix where the deteriorative reactions are severely slowed down (WALTERS, 1998; WALTERS et al., 2005; BUITINK and LEPRINCE, 2004). This trait is an important factor in the preservation of seed viability and quality during dry storage and an essential parameter to ensure fast and homogenous seedling establishment to ensure high yield. The vitreous state prevents harmful events, such as the Maillard reaction, where the raffinose is associated with protection from oxidative damage, possibly by elimination of hydroxyl radicals (NISHIZAWA et al., 2008).

The deteriorative reactions occurring during ageing are complex and poorly understood and it is so far impossible to relate accumulation of damage with the seed death. Complexity is revealed by the range of volatiles that emanate from ageing seeds during storage and include molecules raising from deranged metabolism such as ethanol, acetaldehyde and others of unknown origin such as pentane, 2-heptanone, *n*-hexylformate, 2-propanol, 2-propenenitrile (MIRA et al., 2010, COLVILLE et al., 2012). All cellular compartments are targeted by ageing since lipids, proteins, nucleic acids and probably cell walls are affected during storage (reviewed in SANO et al., 2016). Oxidation and peroxidation is the most commonly reported mechanism of ageing (reviewed in SANO et al., 2016). However, additional mechanisms of ageing must exist because seeds stored in liquid nitrogen (WALTERS et al., 2004) or under vacuum (HAY et al., 2013) also loose progressively their viability during storage, albeit at a very low rate.

Mechanisms involved in protecting seeds during desiccation are also involved in maintaining the viability during storage (SANO et al., 2016), which makes several authors think that longevity and desiccation tolerance are the same phenomenon. In legumes, LEA proteins, heat shock proteins (HSP) and non-reducing sugars accumulate concomitantly with the acquisition of longevity (CHATELAIN et al., 2012; VERDIER et al., 2013, RIGHETI et al., 2015, LIMA et al., 2017). LEA proteins are small hydrophilic, largely unstructured and thermostable proteins that are synthesized during mid- to late maturation. Some LEA proteins like EM1 and EM6 are seed-specific but others such as dehydrins can be induced in vegetative tissues upon osmotic stress. They are thought to have a range of protective functions against desiccation with different efficiencies, including ion binding, antioxidant activity, hydration buffering, and membrane and protein stabilization (TUNNACLIFFE and WISE, 2007; BATTAGLIA et al., 2008; AMARA et al., 2012). A direct role of LEA in longevity was demonstrated in Arabidopsis seeds where the repression of three seed-specific dehydrins resulted in a decreased longevity (HUNDERTMARK et al., 2011b). In developing Medicago seeds, certain LEA proteins were correlated with desiccation tolerance but most of them were correlated with seed longevity (CHATELAIN et al., 2012). In dry mature seeds, the most abundant soluble non-reducing sugars are either sucrose, representing 60-80% of total soluble sugars or RFO that accumulate particularly in legume seeds. Seeds can become desiccation-tolerant without any accumulation of RFO, including soybean (OBENDORF et al., 2009, BILYEU and WIEBOLD et al., 2016). Rather, the increase of RFO during maturation goes in parallel with the acquisition of seed longevity (VERDIER et al., 2013; LIMA et al., 2017). The role of non-reducing sugar in seed longevity is not clear. In vitro, they can protect membrane and proteins by providing OH groups in the dry state, thereby replacing water molecules (reviewed by HOEKSTRA et al., 2001). They can also form a glassy state upon drying. However, the properties of such glass differ from those measured in dry seeds (reviewed by BUITINK and LEPRINCE, 2004). The role of HSP, which also appear late during seed maturation will be discussed later in a separate section.

In addition, antioxidants such as glutathione (KRANNER et al., 2006), tocopherols (SATTLER et al., 2004) and flavonoids present in the integument (DEBEAUJON et al., 2000) also play an important role in longevity by alleviating

oxidation. However, how the accumulation of these antioxidants is regulated during seed maturation in connection with induction of longevity remains to be assessed.

Early genetic studies indicated that ABA was involved in the control of both DT and longevity (OOMS et al., 1993; DELAHAIE et al., 2013). ABA insensitivity in Arabidopsis aba insensitive3 (abi3) mutants was correlated with reduced dormancy, intolerance to desiccation and rapid viability loss during dry storage. ABI3 is one of the main transducers of the abscisic acid (ABA) hormone signal, which is necessary for the expression of maturation genes, such as seed storage protein genes (PARCY et al., 1997; KROJ et al., 2003), late embryo abundant (LEA) genes (GIRAUDAT et al., 1992), genes with antioxidant functions (HASLEKKÅS et al., 2003) and heat shock protein (HSP) (KOTAK et al., 2007). Also seeds of mutants abscisic acid insensitive3-5 (abi3-5) and leafy cotyledon1-3 (lec1-3) alleles had impaired seed maturation and quickly lost seed viability during storage (SUGLIANI et al., 2009).

Transcription factors that regulate LEA gene expression are ABI3, ABI4 and ABI5 (BIES-ETHEVE et al., 2008; REEVES et al., 2011) (Figure 7). The proteome of abi3-5 and lec1 seeds that had a reduced longevity were affected in the accumulation of 12S seed storage proteins, LEA proteins and HSP, including HSP70, HSP90 and small HSP while chloroplastic proteins were upregulated (SUGLIANI et al., 2009). ABI3 interacts with ABI5 to regulate expression of downstream genes, whereas ABI4 and ABI3 control the induction of ABI5 (BOSSI et al., 2009; CUTLER et al., 2010). However, in Arabidopsis, mature seeds of abi4 and abi5 mutants are desiccation tolerant and their longevity are not affected (ZINSMEISTER et al., 2016). Previously, it was demonstrated that ABI5 binds directly to the promoters of the EM1 and EM6 genes, and Arabidopsis abi5 mutants exhibited reduced accumulation of the corresponding proteins during maturation (FINKELSTEIN and LYNCH, 2000; CARLES et al., 2002). According with Zinsmeister (2016) ABI5 appears to be a prominent regulator of late seed maturation in *Medicago truncatula* and *pea* by coordinating the acquisition of seed longevity and dormancy by controlling the production of LEA proteins (EM/EM6, D-34, SBP65), the repression of photosynthesis-associated nuclear genes, and induction of raffinose synthase genes thus producing RFO. While a homologue of ABI5 exists in soybean, it is not known whether it regulates seed longevity.

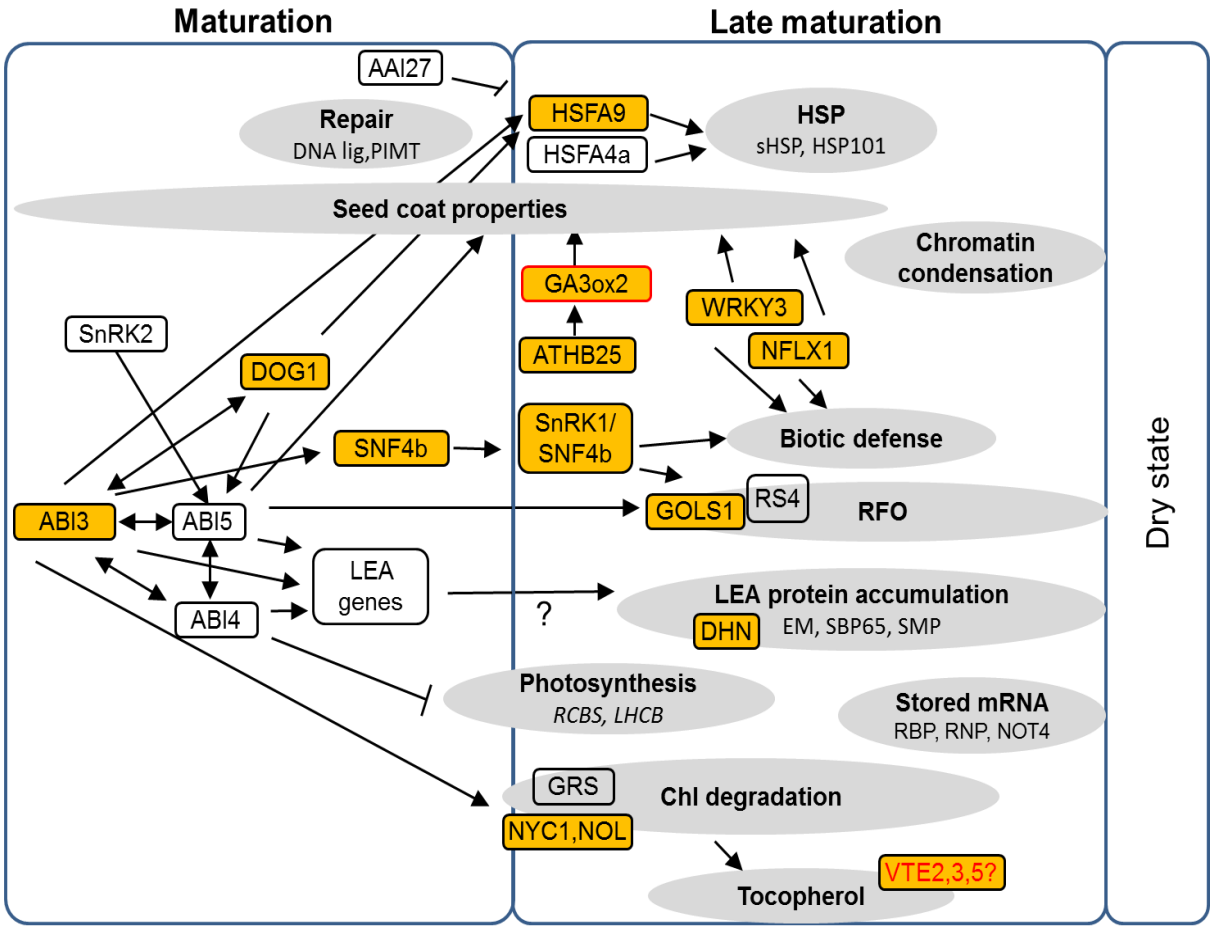
Transcripts of this gene were not differentially regulated during acquisition of seed longevity (LIMA et al., 2017).

It has been hypothesized that gibberellins also control seed longevity. Resistance against controlled deterioration was increased in seeds from GA3-treated plants or the quintuple DELLA mutant that shows constitutive gibberellin responses (BUESO et al., 2014; SANO al., 2016).

Gibberellins are required for normal formation of seed coat epidermal cells, Mutants in gibberellin biosynthesis and receptors exhibit developmental defects, with reduced halos of mucilage being released on imbibition (KIM et al., 2005) but whether longevity was impacted was not investigated. Interestingly, the transcriptome of developing seeds of soybean (LIMA et al., 2017) and *Medicago truncatula* (RIGHETTI et al., 2015, ZINSMEISTER et al., 2016) showed that the transcription factors whose expression was correlated with longevity were related to GA and auxins. The putative role of the transcription factors in inducing longevity remains to be investigated. Finally, a heat shock factor, HSFA9 acting downstream of ABI3 was also involved in seed longevity and is described in section below.

In Arabidopsis, seed longevity and dormancy appeared to be negatively correlated. High storability was correlated with shallow seed dormancy, and low storability correlated with high levels of seed dormancy (NGUYEN et al., 2012). However, such correlation does not exist in *Medicago truncatula* (ZINSMEISTER et al., 2016).

Figure 7. Factors involved in the induction of seed longevity during maturation according to Diagram of key events and players in the establishment of longevity during maturation and late maturation of a dicot seed. Most of the data are from Arabidopsis and *M. truncatula* as model plants. *Grey ellipses indicate confirmed compounds, enzymes, or biochemical pathways that are activated or synthesized during late maturation. Orange boxes correspond to transcription factors, enzymes, or proteins whose mutants have been shown to exhibit reduced longevity. White boxes indicate players that participate indirectly in seed longevity or whose role remains to be evaluated. AAI27, AUXIN-RESPONSIVE PROTEIN27; ABI, ABA INSENSITIVE; CCD, CAROTENOID CLEAVAGE DIOXYGENASE; DHN, DEHYDRIN; DOG1, DELAY OF GERMINATION1; EM, EARLY METHIONINE; GA3ox2, GA3 OXIDASE2; GOLS1, GALACTINOL SYNTASE1; HSFA9/4a, HEAT SHOCK FACTOR A9/4a; HSP, HEAT SHOCK PROTEINS; LEA, LATE EMBRYOGENESIS ABUNDANT; LHCB, LIGHT HARVESTING COMPLEX PROTEIN B; NFXL1, NUCLEAR FACTOR-X1-LIKE1; NOL, NYC1-LIKE; NYC1, NON YELLOW COLORING1; PIMT, PROTEIN REPAIR L-ISOASPARTYL METHYLTRANSFERASE1; RBP, RNA BINDING PROTEIN; RNP, RIBONUCLEOPROTEIN; RS4, RAFFINOSE SYNTHASE4; SBP65, SEED BIOTINYLATED PROTEIN 65; SGR, STAY- GREEN; SMP, SEED MATURATION PROTEIN; SNF4b, SUCROSE NONFERMENTING SUBUNIT4b; SNRK2, SUCROSE NONFERMENTING-RELATED KINASE 2; VTE6, VITAMIN E DEFICIENT6 (LEPRINCE et al., 2017).



Source: Leprince et al. (2017).

1.3.4 Seed dormancy and hormonal control

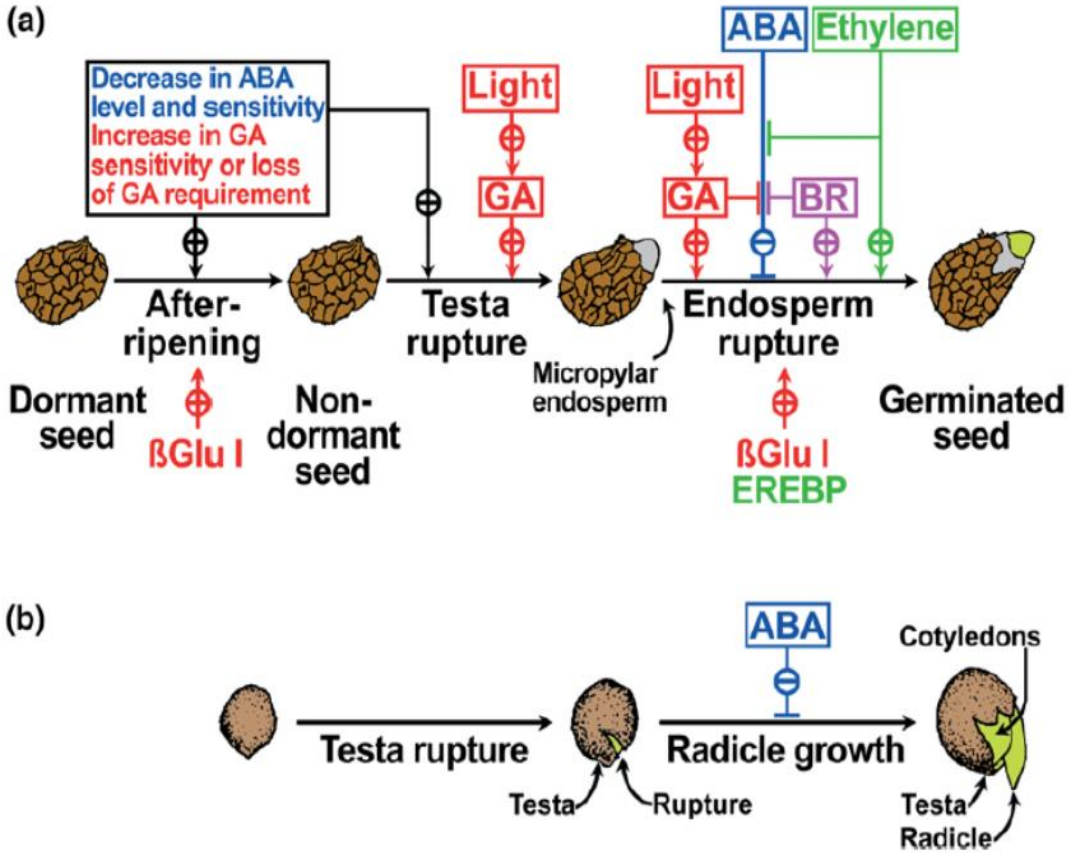
Primary seed dormancy is acquired during seed maturation and reaches a high level in freshly harvested seeds and maintained for a certain period that allows seeds to survive under unfavorable conditions and prevents pre-harvest sprouting, and thus an important aspect of plant fitness (DONOHUE et al., 2005; GRAEBER et al., 2012). Finch-Savage and Leubner-Metzeger (2005) define seed dormancy as an innate seed property that defines the environmental conditions in which the seed is able to germinate. According to Baskin and Baskin (2004) there are five classes of dormancy: physiological dormancy (PD), morphological dormancy (MD), morphophysiological dormancy (MPD), physical dormancy (PY) and combinational dormancy (PY + PD). In this review will only focus on physiological dormancy.

The seed dormancy is determined by genetics with a substantial environmental influence which is mediated, at least in part, by the plant hormones ABA and GA. Seed dormancy is crucial to plant survival and ensures that seeds germinate only when environmental conditions are optimal. It is an adaptive trait in numerous seed-plant species, enabling wild plants to survive under stressful conditions in nature (reviewed in BENTSINK and KOORNNEEF, 2008). The strategy extends the possibilities of survival of the species and avoids earlier seed germination under inappropriate conditions. There are not many studies that describe when dormancy is acquired during maturation. In *Medicago truncatula*, it is acquired during the late maturation
phase (ZINSMEISTER et al., 2016).

ABA is important in inducing and maintaining seed dormancy and inhibiting germination. GA neutralizes the inhibitory effects of ABA by releasing dormancy and promoting germination (Figure 8). The dormant state is characterized by a balance in the biosynthesis and catabolism of ABA and GA, instead of the absolute amount of these two hormones. Thus, the dormant state is characterized by increased ABA biosynthesis and GA degradation. The ABA content is regulated mainly by two ABA biosynthetic enzymes, zeaxanthin epoxidase (ZEP) and 9-cisepoxycaretonoid dioxygenase (NCED), and an enzyme that catalyzes the predominant ABA catabolic pathway, ABA 8'- hydroxylase (ABA 8'OH). The inhibitory effects of ABA on seed germination is by delaying the radicle expansion and weakening of micropylar endosperm, as well as the enhanced expression of

transcription factors, which may adversely affect the process of seed germination (reviewed in GRAEBER et al., 2010, 2012).

Figure 8. Hormonal interactions during the regulation of seed dormancy release and germination of Nicotiana (a) and Brassica (b) model species. (a) Nicotiana sp. seed germination is two-step: testa rupture followed by endosperm rupture. *Dormancy release and germination promotion occur during seed after-ripening (dry storage at room temperature for several months) or via the light-gibberellin (GA) pathway during imbibition. Abscisic acid (ABA) inhibits endosperm rupture but not testa rupture. GA, ethylene and brassinosteroids (BRs) promote endosperm rupture and counteract the inhibitory effects of ABA. (b) *Brassica napus* seed germination is one-step. The mature seeds of these species are without an endosperm and so testa rupture plus initial radicle elongation result in the completion of germination. ABA does not inhibit testa rupture, but inhibits subsequent radicle growth (SCHOPFER and PLACHY, 1984; for a review, see KUCERA et al., 2005). (FINCH-SAVAGE and LEUBNER-METZEGER, 2006).



Source: Finch-Savage and Leubner-Metzeger, 2006

Gibberellins stimulate the synthesis and production of the cell wall hydrolases, resulting in the germination of seeds (APPLEFORD and LENTON, 1997; YAMAGUCHI, 2008) *ga1* mutant in Arabidopsis that is defective in GA synthesis needs supplement of exogenous GA to germinate (DEBEAUJON et al., 2000).

The dormant state is characterized by the transcription of genes with an overrepresentation of ABA-responsive elements (ABRE) in their promoters and of genes for transcription factors that bind to the ABRE. GA-deficient mutants, such as ga1 and ga2, show strong seed dormancy and fail to germinate without exogenous GA treatment (LEE et al., 2002; SHU et al., 2013). Besides ABA and GA, auxin, nearly all other phytohormones are also likely involved in modulation of seed dormancy and germination, including ethylene (ET), brassinosteroids (BRs), jasmonic acid (JA), salicylic acid (SA), cytokinins (CKs), and strigolactones (SLs) (CORBINEAU et al., 1988; BABIKER et al., 1993; SUGIMOTO et al., 2003; RAJJOU, 2006; TOH et al., 2012; ZHU et al., 2013).

Various environmental cues determine the appropriate timing for seed germination, also by mediating the ABA/GA balance. Light is a major environmental factor during seed germination, increasing the expression of GA anabolic genes, GA3ox1 and GA3ox2, and repressing the expression of GA2ox2, a GA catabolism gene (CHO et al., 2012). The GA biosynthesis gene, GA20ox, was identified as a quantitative trait locus associated with seed dormancy and PHS tolerance in rice, barley and wheat, suggesting that reduced GA signaling can increase seed dormancy in these cereals (LI et al., 2004; SPIELMEYER et al., 2004; APPLEFORD et al., 2006).

1.3.5 Seed vigor and seedling establishment

In the above sections, we discussed how DT, dormancy and longevity are progressively acquired during seed maturation**.** These traits characterize seed vigor, being the sum of properties that determine the activity and performance of seed lots of acceptable germination in a wide range of environments. A vigorous seed lot will be able to perform well under environmental conditions that are not optimal for the species (ISTA, 2014). Seed vigor is a concept used in the seed industry to define seed performance in the field, namely the rate and uniformity of seed germination and seedling growth, emergence ability under unfavorable environmental conditions, and the retention of these characteristics after storage (FINCH-SAVAGE and BASSEL, 2016). Therefore, vigor is important not only for field performance but also for storage potential. Nowadays there is no universally accepted single test for assessing seed vigor of a given species. Seed vigor can

continue to increase after the seed lose the connection with the mother plant and so physiological maturity can occur after mass maturity (maximum seed dry weight) and the precise time of maximum vigor will differ between species (TEKRONY and EGLI, 1997).

A more accurate determination of this performance potential is possible if seeds are first subjected to rapid ageing under controlled conditions. There are two accepted methods for this: accelerated ageing (TEKRONY, 1993) and controlled deterioration (POWELL, 1995).

Thus, ageing is a key characteristic that is both a cause of differences in vigor and a basis for vigor testing. Other validated ISTA vigor tests, the electrical conductivity test and the radicle emergence test, are also related to physiological changes that occur during ageing (ISTA, 2014). These tests are based on a large body of physiological evidence linking seed vigor difference to ageing both before and after seed harvest. MCDONALD (1975) grouped vigor tests into three groups: a) Physical tests – determine seed characteristics such as size and mass; b) Physiological tests - using germination and growth parameters (there are two types of these tests. First type, when germination is done under favorable conditions (standard laboratory germination, and test of growth intensity). Second type, when seed is exposed to unfavorable environmental conditions (cold test, accelerated aging test) c) Biochemical tests – are considered as indirect methods for estimation of seed value. These are Tetrazolium test, conductometric measurements, enzyme activity and respiration.

Harvesting at the proper time is a key factor that contributes to obtaining high vigor seeds. Harvesting too early may result in immature seeds that have poor vigor (BEDANE et al., 2006). The major impacts of variation in seed vigor manifest through a negative direct effect on seedling emergence and therefore an indirect effect on yield (FINCH-SAVAGE, 1995). The high-vigor seeds germinate uniformly and rapidly and grow more resistant seedlings, then better field performance and higher yield are expecting. Considering the importance of seedling establishment in crop yield, we describe below some features regarding the elongation of hypocotyls.

The process of seedling establishment starts with the heterotrophic growth of the newly emerged radicle below and above the soil surface. It is characterized by the downward growth of the root, and the upward growth of the epi/hypocotyl.

Seedling establishment is completed when the young plantlet becomes autotrophic by relying on its own photosynthesis. Post germinative growth relies on the seed storage reserves that were synthesized during seed filling. In soybean the efficiency of reserve mobilization is associated with seedling vigor (PEREIRA et al., 2015) whereas in Arabidopsis, degradation of oil reserves is not an absolute requirement for seedling establishment (KELLY et al., 2011). The growth strategy in the dark is a sustained and rapid elongation to reach light above ground, a process called skotomorphogenesis. During elongation, the apical hook and the closed cotyledons protect the apical meristem from the contact with the soil particle. When the organ reaches the surface and perceive light, the photomorphogenic program is induced, leading to inhibition of the hypocotyl growth, cotyledon expansion and greening (reviewed in DE WIT et al., 2016).

The hypocotyl of *Arabidopsis thaliana*, has been studied extensively. It is a very plastic organ, strongly influenced by both external and internal cues known to regulate cell elongation, such as light, hypoxia, gravity, temperature, soil structure and hormones. Hypocotyl growth depends on the internal turgor pressure in its individual cells, which causes the extension of the epidermis cell walls, up to 100-fold compared to dry seeds. However, more complex cellular factors are involved. In sunflower, hypocotyl growth is due to cell division followed by cell enlargement of the peripheral wall(s) and turgor maintenance via a tightly regulated catabolism of imported sucrose (KUTSCHERA and NIKLAS, 2013). These authors also suggested that this sugar serves both as a metabolite and a signaling molecule originating from the cotyledons. In soybean did not find any information. In the legume *Medicago truncatula*, elongation is controlled by the epidermal cell layer and depends both on cell elongation and cell division (PIERRE et al., 2014). However, this depends both on the genotype and environmental conditions such as cold. At the cellular level, growth is controlled by the characteristics of cell wall loosening due to modification of pectins and cellulose, turgor pressure, the properties of the cytoskeleton, vesicular trafficking of auxin transporter. Auxins, cytokinins, ethylene, gibberellins and brassinosteroids are involved in hypocotyl elongation and the response of this organ to the soil environment (reviewed in VANSTRAELEN and BENKOVA, 2012; KUTSCHERA and NIKLAS, 2013). Auxin from the apex appears to be responsible for hypocotyl growth, since decapitation of seedlings strongly reduces growth, which was restored after the application of

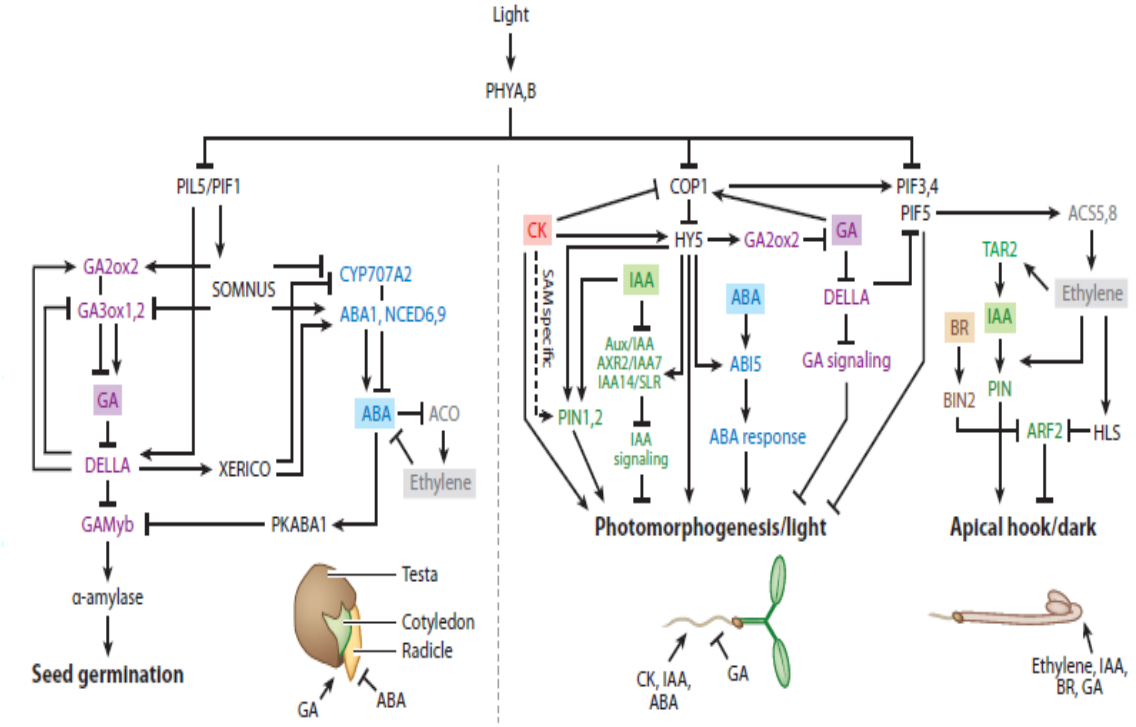
exogenous IAA to the cut surface (ORTUÑO et al., 1988; ORTUÑO et al., 1990, BRAVO el al., 2008). There are indications that the auxin signal responsible for regulation of cellular growth in the outermost layers, the epidermis and cortex, originates from the endodermis. Gendreau et al. (1997) showed that the mechanism for elongation of hypocotyls in continuous darkness essentially differs from that in uniform light in that hows an acropetal movement of cell growth. This implies that in darkness first the basal cells elongate and that the elongation zone moves towards the apex with time. However, in sunflower, seedlings either in darkness or in light auxin-mediated elongation ceases after 1 day, but the hypocotyl continues to grow afterwards, indicating that other hormones are important (reviewed in KUTSCHERA and NIKLAS, 2013)

In the dark, hypocotyls become very long as compared to those of light-grown seedlings. This is the most appropriate response to quickly reach light above the soil surface and de-etiolate. Cytokinins have a similar effect, through induction of ethylene biosynthesis by stabilizing ACC synthase and consequently reducing hypocotyl elongation in the dark (VOGEL et al., 1998; CHAE et al., 2003). Although gibberellin (GA) is known to be a very prominent growth stimulator, exogenous GA does promote hypocotyl elongation in etiolated seedlings but not in the light (COWLING and HARBERD, 1999). GA also promotes the formation of the apical hook, a structure that protects the shoot meristems and young leaves when the seedling pushes through the soil (Figure 9). Decreasing the amounts of GA prevents the etiolated phenotype of dark-grown seedlings. The GA-promoting effect on apical hook formation is linked to ethylene biosynthesis (reviewed in VANSTRAELEN and BENKOVA, 2012).

Ethylene is now emerging as an important regulator involved in adapting the hypocotyl growth to mechanical light and oxygen stress during seedling establishment in Arabidopsis (reviewed in ZHU and BENKOVA, 2016). In legumes such as peanut and bean seeds, exogenously applied ethylene also inhibited hypocotyl and root elongation in the dark in a dose-dependent manner (GOESCHL, 1975). In Arabidopsis, compacted soil induces ethylene accumulation and inhibition of hypocotyl elongation, which apparently facilitates penetration through the soil structure. Ethylene also plays a role in synchronizing growth with the initial exposure to light when the seedling reaches the soil surface (reviewed in ZHU and BENKOVA, 2016). Finally, upon hypoxia, ethylene promotes the maintenance of

the apical hook during seedling growth. In soybean, ethylene production decreased by 45% with etiolated seedlings exposed to red light, a treatment that promoted hypocotyl growth (SAMINY, 1978). 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; LIMA et al., 2017). Whether ethylene plays a role in the rate of the loss in elongation capacity warrants further investigation.

Figure 9. Hormonal networks underlying light-regulated plant development encompassing seed germination (left) and light (photomorphogenesis)- and dark-grown seedlings (right). *Arrows and inhibition lines represent positive and negative interactions, respectively. Components of the auxin (green), cytokinin (CK, red), abscisic acid (ABA, blue), gibberellin (GA, purple), brassinosteroids (BR, brown), and ethylene (gray) are highlighted. Diagrams at the bottom represent schematic drawings of a germinating seedling (left) and light- and dark-regulated morphogenesis (right) with the impact of the different hormones summarized (VANSTRAELEN and BENKOVA, 2012).



Source: Vanstraelen and Benkova, 2012.

1.4 Role of the maternal environment on the seed vigor

1.4.1 The position of seeds on mother plant

Whether seed attributes and physiological quality are related to the pod position on the mother plant and its position within the pod is debated in the literature. Soybean seeds borne on different nodes of the stem that vary from down part to upper part of the plant are subjected to positional effects (BENNETT et al., 2003). Soybean seeds harvested from bottom and middle position showed higher germination, while seeds from the top position resulted in poor germination (KHALIL et al., 2010). These results contradicts the findings of McDonald et al. (1983) who reported that seeds from top position exhibited higher germination compared with seeds from bottom position. The contradiction may be due to environmental differences in which soybeans were grown. McDonald et al. (1983) grew soybeans in the field in which the bottom pods were likely to have more potential disease problems than the seed from the bottom in the present growth chamber study, because the bottom pods developed at high temperature and humidity. Larger seeds were obtained from bottom position, followed by middle position, while top position produced the smaller seeds. Smiciklas et al. (1992) showed that seeds from the top part of plant had better seed quality when drought was applied at R5-R7 stages and that seed from the bottom part were more sensitive to the reduction of seed quality when drought occurred at the reproductive stages R5 or R6.

Several factors could affect the development of seeds at the top of the plant differently than those at the bottom of the canopy and therefore could be responsible for differences in seed composition at maturity. Differences in seed maturity or in time left for seed ageing on the plant may explain differences in physiological quality. Keigley and Mullen (1986) already suggested that seed maturity is an important factor explaining seed position effects but little is known about the relations between the duration of pod growth, development, maturation and ageing and physiological seed quality attributes for individual seeds.

The first factor that causes difference in seeds is the flowering in the indeterminate soybean plants as used in the present study occurs first at lower nodes, thus, there is the potential for seeds lower in the canopy to develop over a

longer period. However, while there is a lot of information about node position and flowering, there are few reports that have documented differences in duration of the seed fill period as a function of node (RABOY and DICKINSON, 1987). As a second factor we have the external conditions around the pod (e.g. temperature, relative humidity, light) and internal characteristics (e.g. local sink-source relations) may contribute to position effects on physiological seed attributes. The seeds lower in the canopy develop under altered environmental conditions in terms of temperature, irradiance, light quality and humidity, which are recognized to impact soybean seed composition (CARRERA et al., 2009; CARRERA et al., 2011).

A third factor is the contribution of remobilization of reserves, including minerals, from leaves that may vary among minerals and with node position oil content and fatty acid composition vary between positions of seeds on the stem axis (GULERIA et al., 2007, 2008). This difference in the oil and protein content at various nodal positions of the stem axis has been described to be due to variation occurring in nutrients and assimilates supply and other factors at each position. Seed filling influenced by different node position of seed influences the germination potential of seed; where soybean seeds at the basal nodes showed higher germination percentage whereas vigor index was higher at apical positions (SHARMA et al. 2009). According to the results of Illipronti et al. (2000), under normal temperature conditions, the viability was higher for seeds produced on the branches from seeds produced on the main stem.

1.4.2 Environmental factors affecting seed quality

Stressful environmental conditions of the mother plant affect each stage of seed development. Because seeds are end product for animal feed and food, major efforts have been made at understanding the impact of environment on seed yield and nutritional quality (DELOUCHE, 1980; reviewed in WANG and FREI, 2011). Here we will focus on the impact of seeds on different traits characterizing seed vigor.

Temperatures during early growth stages are a major limitation for crop area distribution and yield. The prevailing environmental conditions from the time of seed sowing until harvest have influence on seed quality. In *Medicago truncatula* the high temperature influences the duration of the maturation period of the seeds,

consequently the acquisition of longevity (RIGHETTI et al., 2015). High temperatures during soybean seed development frequently results in seed with poor germination, increased incidence of pathogen infection, and decreased economic value. Soybean seed weight, germination, vigor and yield are decreased when the air temperature exceeds 33 °C during reproductive growth (DORNBOS and MULLEN, 1991). Protein and lipid levels decreased markedly when air temperature exceeds 40° C during reproductive stages (THOMAS et al., 2003). Gibson and Mullen (1996) showed that high day and night temperature reduced soybean seed germination and seedling vigor. High temperature or drought stress imposed during soybean seed development can alter the seed coat morphology leading to negative effects on seed germination rate and vigor (DORNBOS and MULLEN, 1991; EGLI et al., 2005; SMITH et al., 2008).

Water stress imposed during seed development reduced seed size (DE SOUZA et al., 1997). Water stress during later phases of reproductive stages decreased the duration of the seed filling stage (MECKEL et al., 1984), which was strongly correlated with seed try matter accumulation (FREDERICK et al., 1991; DESCLAUX and ROUMET, 1996). Desclaux and Roumet (1996) also indicated that drought stress imposed during node emergence decreased node number and accelerated the development of reproductive stages. The most critical time of water stress are thought to be from late flowering to early seed development (CALVINO et al., 2003). Frederick et al. (2001) noted that drought stress between initial flowering (R1) and seed filling (R5–R7) decreased the vegetative growth, seed number, and yield on branches, because most branch growth occurred between initial flowering and beginning of seed filling.

Field weathering may cause negative effects on the viability and vigor or soybean seeds (TEKRONY et al., 1980). Weathering damage occurs when dry seed became exposed to heavy rain, temperature fluctuations and high relative humidity at pre-harvesting (FRANÇA-NETO et al., 2000). The weathering damage is a result of soybean seed exposure to alternate cycles of high and low moisture before harvest and is considered one of the main causes associated to reduced quality of the soybean seeds (HUTH et al., 2016).

Environmental stress effects on seed development include: shortened embryogenesis and seed filling duration, accelerated leaf senescence and reduced photosynthetic rate, resulting in shortened seed maturation and may lead to a

limited source for the supply of assimilates to the developing seed (NGUYEN et al., 2016) and poor seed quality.

1.5 Heat Shock Proteins (HSP) and HSF

Throughout evolutionary time, plants have been confronted with adverse environmental conditions, often extreme and stressful such as low or high temperatures, high salt content or water deficiency which require special adaptations to tolerate these stresses (BANIWAL et al., 2004). Almost all types of stress induce the production of a group of proteins called heat-shock proteins (HSPs) or stress-induced proteins. HSPs are highly conserved proteins that play a range of functions, including cytoprotection, intracellular assembly, protein folding, and translocation of oligomeric proteins (reviewed in HIGHTOWER, 1991). Which play a central role as molecular chaperones not only for protection against stress damage but also stabilization of protein structures, cellular homeostasis and assistance in the protein refolding of proteins under stress (KOTAK et al., 2007, KAMPINGA and CRAIG, 2010). Heat shock proteins (HSPs) are divided into several classes according to their molecular weight, include HSP70 (DnaK), the chaperonins (GroEL and HSP60), HSP90, HSP100, and the small HSP (sHSP) families (WANG et al., 2004).

HSPs are expressed not only in response to environmental stresses (with heat stress) but also in several development programs, including pollen maturation, and seed maturation. In this latter program, they may play a protective role in seed longevity (ZUR NIEDEN et al., 1995; WATERS et al., 1996; WEHMEYER and VIERLING, 2000, LEPRINCE et al., 2017). In soybean seeds, the increase in longevity during the phase of maturation correlated with an increase in transcript levels of sHSP genes (sHSP 17.6 a, sHSP 17.6 b, and sHSP21) (LIMA et al., 2017). In rice, the OsHSP18.2 transcript was found to markedly increase during the late maturation stage, being highly abundant in dry seeds and it is an exceptional candidate to improve seed vigor and longevity in rice seeds, protecting vulnerable cellular proteins during maturation drying, desiccation and aging in seeds (KAUR et al., 2015). In Arabidopsis, Hsp101 is also regulated during seed development in the absence of stress, in a pattern similar to that seen for LEA proteins accumulates during mid-maturation and is stored in the dry seed (HONG

and VIERLING, 2001). In Arabidopsis mutants where HSP101 levels were 10-fold lower than wild-type, seedling elongation was affected at 38°C and above. HSP70 has a wide spectrum of functions common to all chaperone proteins and participates in shaping the structure of newly synthesized native proteins, restoring of partially denatured proteins, and degradation of irreversibly damaged protein molecules. HSP70 transcripts also increased during seed maturation in Arabidopsis with any stress (SUNG et al., 2001). HSP90 appears to play a role in seedling emergence. When treated with geldamycin, an inhibitor of HSP90 activity, hypocotyl length was reduced compared to untreated ones. Furthermore, these authors also showed that the reduction was strongly dependent on the genotype (SANGSTER et al., 2008).

In plants, the heat-shock response, synthesized of HSP and some developmental processes are controlled by a gene family of transcription factors known as the heat-shock transcription factors (HSFs). Plants contain the highest number of HSFs among eukaryotes. HSFs are essential for the transcription of HSP coding genes that are active in response to sub-lethal heat stress leading to increased tolerance against a subsequent, otherwise lethal, heat shock (HSU et al., 2010). Ectopic over-expression of BhHsf1, a HSF from the resurrection plant *Boea hygrometrica* also increased the tolerance of seedlings against heat stress but also reduced cotyledon growth under normal conditions. There are three classes of HSF in plants, named A, B and C Class A members contain the conserved oligomerization DNA-binding and an AHA activation domain, a nuclear localization domain. Class B and C lacks the action domain and may serve as co-regulators. HsfA9 is the unique HSF among the 21 members of the HSF family as it is exclusively expressed in the seed maturation phase and not during other stages of development or during heat or other stresses, emphasizing that HSFs may present specific individual functions under different conditions (KOTAK et al., 2007; ALMOGUERA et al., 2012). Level of HsfA9 transcripts increased during seed maturation (KOTAK et al., 2007). It activates transcription of specific small heat shock protein (sHSP) genes and is involved in the control of a genetic program that regulates seed longevity (PRIETO-DAPENA et al., 2006; ALMOGUERA et al., 2009) and seed vigor. Overexpression of the sunflower *HaHSFA9* in tobacco seeds leads to an increase in sHSP and HSP101, increased resistance against ageing induced by controlled deterioration (PRIETO-DAPENA et al., 2006). In transgenic seedlings, overexpression of HaHsfA9 induces

thermotolerance and tolerance against oxidative damage of photosynthetic membranes (ALMOGUERA et al., 2012) In *Medicago truncatula* (VERDIER et al., 2013, RIGHETTI et al., 2015) and in soybean (LIMA et al., 2017), transcripts of several HSF homologues of HSFA9 were found to increase during late maturation during the acquisition of seed longevity.

HSFA9 acts also in concert with other transcription factors, such as HSFA4 and DREB2A. Seeds overexpressing *HaHSFA4a* and *HaHSFA9* resisted accelerated aging better than seeds that overexpress *HaHSFA9* only. However, overexpression of *HaHSFA4a* alone did not induce tolerance against different types of abiotic stress tolerance in seedlings (PERSONAT et al., 2014). In addition, HSFA9 also exerts cross talks with ABA signaling pathway both in developing seeds and in vegetative tissues submitted to abiotic stress (KOTAK et al., 2007). In mesophyll protoplasts, ABI3 was able to activate he HsfA9 promoter, leading to HSFA9 synthesis and expression of HSP. Therefore, a complex network of HSFs under the control of ABA probably is part of the regulatory networks controlling seed vigor.

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CHAPTER 2
THE ACQUISITION OF SEED VIGOR IS AFFECTED BY ENVIRONMENTAL AND MATERNAL EFFECTS

ABSTRACT

The maturation of seeds is a very important process in seed development because it is during this phase that most vigor traits that are acquired, such as survival in the dry state (desiccation tolerance and longevity and dormancy). These traits are acquired sequentially following a genetic program that received a lot of attention. It is increasingly recognized that maternal environment is also influencing the acquisition of dormancy and longevity, mostly in wild species. The objective of this work was to document the impact of drying rate, environment and seed position on the acquisition of seed longevity and thermal dependence of germination in field grown soybean. Our data support the hypothesis that the acquisition of longevity is an autonomous embryonic program that does not depend entirely on the connections with the vascular tissues of the mother plants. The impact of high temperatures can be beneficial for the longevity program whereas they are detrimental to seed filling. We found also that stage R7.2 represent a key stage during maturation where the germination and desiccation tolerance is acquired, yet remaining susceptible to the environmental conditions in terms of longevity

Keywords: Drying. Germination. Heat. Longevity. Temperature.

2.1 INTRODUCTION

Producing highly vigorous seeds is a key lever to increase crop production. “Seed physiological quality” or “seed vigor” is defined as the sum of the seed physiological properties that leads to homogenous and vigorous germination and uniform seedling establishment during optimal and stressful conditions reviewed in Bewley et al. (2013) e Marcos-Filho (2015). It also includes longevity, defined as the capacity to remain viable for long periods of time during storage in the quiescent dry state (longevity) (LEPRINCE et al., 2017).

In orthodox seeds, physiological quality is acquired sequentially during their development. The capacity to germinate is achieved prior to maximum dry weight but is impeded during development by endogenous ABA and the maternal tissues such as the seed coat and/or pericarp according to the species (BEWLEY et al., 2013; RIGHETTI et al., 2015). Desiccation tolerance, defined as the ability to survive rapid drying to a moisture content in equilibrium with ambient air (i.e. ± 10% moisture, (LEPRINCE and BUITINK, 2010) is established midway through development after germinability and during seed filling (VERDIER et al., 2013). According to Lima et al. (2017), soybean seeds developing in the field acquire their germinability between 25 and 57 DAF (stage R7.1). Desiccation tolerance is gained between 57 and 63 DAF at stage R7.2, coincidentally with the end of seed filling and detachment of the mother plant. In parallel, capacity to germinate at low temperature and in salt (100 mM NaCl) is also achieved at that stage (ROSSI, 2016). Physiological maturity corresponds to the point when all these characteristics are fully acquired (BEWLEY et al., 2013). However, it the acquisition of longevity does not necessarily coincide with physiological maturity. Longevity is still increasing afterwards during the later stages of seed maturation when the developing seeds undergo drying (reviewed in ZANAKIS et al., 1994; PROBERT et al., 2007; BEWLEY et al., 2013, VERDIER et al., 2013; LEPRINCE el al., 2017). In soybean, longevity increases two-fold between stages R7.2 and R9 (LIMA et al., 2017). However, in practice, soybean seeds are harvested at a specific moisture content corresponding to a compromise between avoiding seed losses due to fruit shattering or losses of quality due to mechanical operations during harvest and accelerated deterioration on the mother plant or during post-harvest handling. In soybean, harvest maturity occurs before maximum longevity (LIMA et al., 2017). The period between these different maturity criteria is critical to obtain seed lots of maximal performance because the developing seeds remain attached to the

mother plant or enclosed of the pod tissues and thus exposed to biotic and abiotic factors that may influence various aspects of its vigor.

Environmental conditions during the reproductive growth and biotic factors such as the position of the seed on the mother plant and infection by pathogens can alter directly or indirectly the acquisition of physiological quality and seed longevity according to the species and genotype (DELOUCHE, 1980; LI et al., 2017). Collectively, these conditions are termed maternal environment when they act via the mother plant. Stress conditions such as high temperature, water deficits, inadequate photoperiod or the combination of these conditions are detrimental for seed vigor. For example, Righetti et al. (2015) observed in *Medicago truncatula* seeds that a temperature 6°C above or below the growth optimum applied after flowering reduced the longevity by 30% at maturity. In Arabidopsis, high light intensity and low temperature (15°C) during seed maturation were highly correlated with longevity and also induced increased contents in galactinol and raffinose respectively (HE et al., 2014). In soybean, temperatures above 30°C during seed filling and water stress reduced soybean seed germination and seedling vigor (EGLI et al., 2005) and shifted seed weight distributions towards a larger proportion of small seeds (DORNBOS and MULLEN, 1991; GIBSON et al., 1996). In these experiments, the longevity was not studied. Furthermore, in several wild species, environmental conditions to which mother plants were exposed before fecundation impacted the longevity of their progeny (KOCHANEK, 2010, 2011, reviewed in LEPRINCE et al., 2017). For example, in *Plantago cunninghamii*, plants that were grown under dry cold conditions then moved to optimal conditions at flowering produced seeds with a longevity twice as high as those produced from plants exposed to warm and wet conditions before transfer to optimal conditions at flowering.

The position of the seed on the mother plant has complex effects on seed composition and physiological quality (BEWLEY et al., 2013). In soybean, data are not consistent between studies as to whether the position has a significant effect on seed vigor (ILLIPRONTI et al., 2000; BENNET et al., 2003; SHARMA et al., 2013). Factors that might contribute to these positional effects remain unclear but could include the microclimate around the seed/pod and reaching the seeds. The microclimate could impact the seed drying rate in the pod during late maturation, thereby affecting the duration before reaching the dry quiescent stage, whereas

competition between pods could restrict the flow of assimilates to the embryo. Both factors could lead to differences in the final amount different set of protective compounds that accumulated during maturation, thereby altering longevity. However, before testing this hypothesis, it is necessary to evaluate the importance of drying rate and seed position on seed longevity during maturation.

Seeds were sown respectively on 28 November in 2014 and 14 December 2015 at a density between 18-23 seeds/m and 0.45 m between rows. Plants were grown according to standard cultural techniques at the experimental farm. Briefly, soil fertilization was adapted according to the physico-chemical analysis of table 1. 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 and inoculated with *Bradyrhizobium* sp strains using the c.p Biomax- Biosoja, at a dose of 1.6 mL/ kg seeds. During the culture, phytosanitary controls were performed according to the recommendations by EMBRAPA (2011).

The loss of water at the end of maturation requires the protective mechanisms against possible structural changes. Among them, HSPs play a protective role that limits the aggregation of proteins and prevents their folding. They accumulate gradually during seed maturation (WEHMEYER et al., 1996) under the effect of a cascade of complex regulations involving HSF- type transcription factors and the ABA signaling pathway (KOTAK et al., 2007). Several lines of evidence suggest that HSP may play a role in seed longevity (PRIETO-DAPENA et al., 2006; LIMA et al., 2017). Therefore, based on these data, we reasoned that if developing seeds gradually accumulate HSP at the end of seed maturation (LIMA et al., 2017), the ability to germinate at high temperature would increase concomitantly with the advancement of maturity and acquisition of longevity. If proven correct, this hypothesis could lead to a high temperature germination test as proxy for seed longevity testing.

Thus, the objectives of this work were to compare the effect of slow and fast drying on the survival in the dry state (desiccation tolerance and longevity) at different stages of maturation, to evaluate the impact of the environmental conditions on the acquisition of longevity using field grown seeds obtained from three consecutive years and to study the impact of maturation on the thermal dependence of germination.

2.2 MATERIAL AND METHODS

2.2.1 Plant material, growth conditions and seed sampling

The cultivar BRS 284 was chosen as a standard genotype that it is recommended for altitude tropical climate, namely dry winter and hot and rainy summer. BRS 284 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, Brazil (22º 49''S; 48º 25''W, Altitude 810m). The mean annual temperature and rainfall is 20.3 °C and 1600 mm, respectively (CUNHA and MARTINS, 2009).

For our experiments, temperature was measured with an electric thermograph during the crop cycle and rainfall data were obtained from the Department of Natural Resources – Sector of Climatology, UNESP-Botucatu-SP (Figure 1). The soil in the experimental area is classified as a "distroferric red nitosol" (EMBRAPA, 2006). The physico-chemical characteristics of the soil before sowing are presented in Table 1.

Figure 1. Variations in maximum (open circles), medium (black triangle) and minimum temperature (black circles) and daily precipitation (red square) at the experimental field from sowing to harvest during the crop years 2014/2015 (A) and 2015/2016 (B).

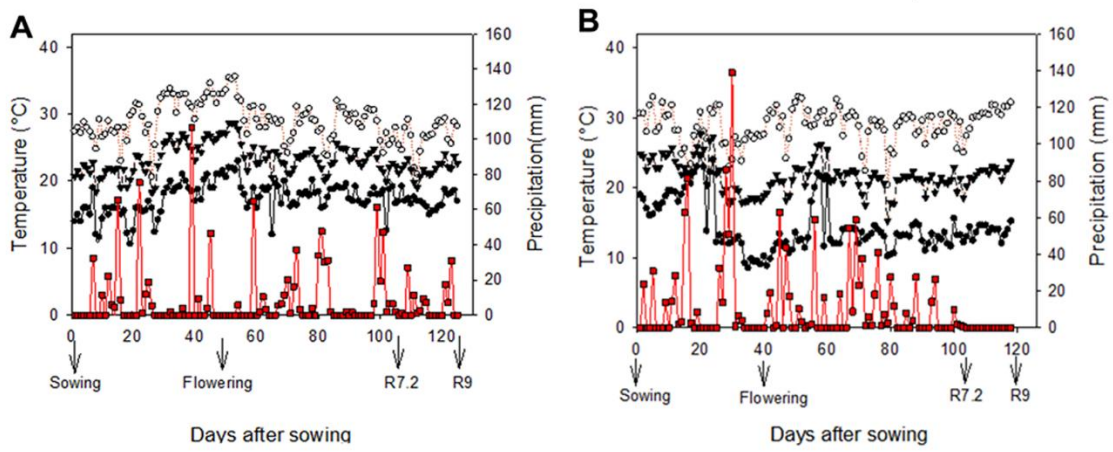


Table 1. Physico-chemical characteristics of the soil at 0-0.20 m depth before sowing. SOM: soil organic matter, P resin: Potential acidity; CEC: cation exchange capacity; BS: base saturation.

Year	pH	SOM	P resin	H^+Al	K^+	Ca^{2+}	Mg^{2+}	CEC	BS
		g/dm^3	mg/dm^3	$mmol_c/dm^3$					

2014	4.8	23	25	48	2.9	22	10	84	36
2015	4.9	23	14	58	7.2	35	15	116	58

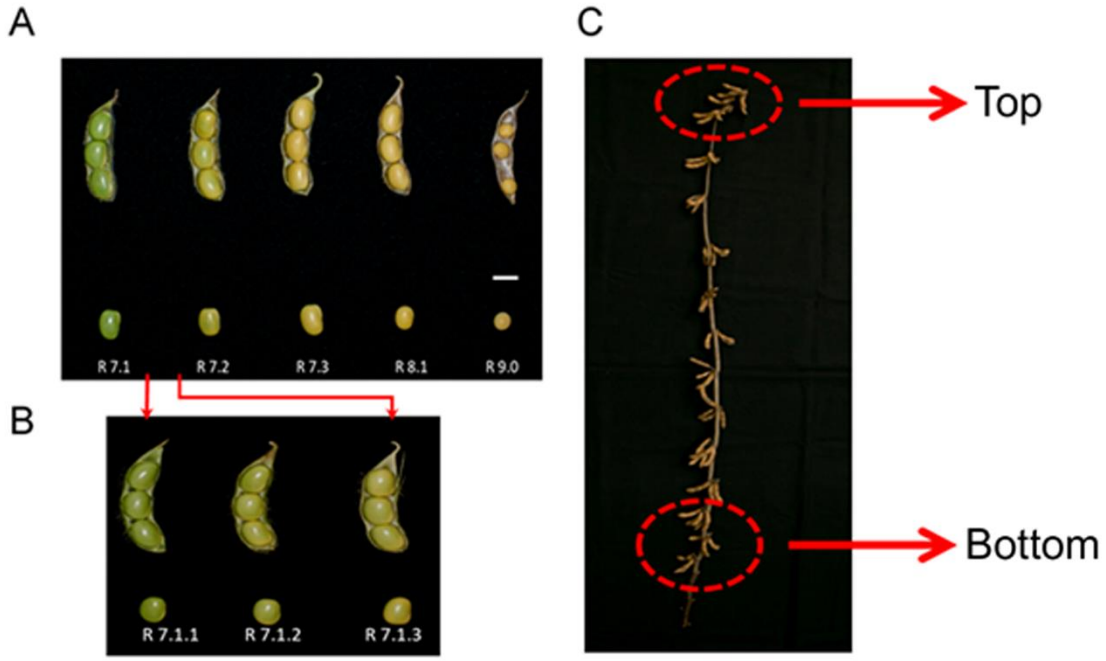
To monitor the seed development, 800 flowers were tagged. Pods were harvested manually and developing seeds were sorted into homogeneous lots according to different phenological stages based on the morphology characteristics and seed age (Table 2). Immature seeds were immediately processed for physiological studies. At maturity, seeds were also harvested and separated according to their position on the mother stem. Seeds at the first basal (named bottom thereafter) and last 3 apical nodes (top, Figure 2) were kept for analysis.

Table 2. Characterization of the phenological stages of soybean seeds during maturation according to LIMA et al. (2017) with adaptations.

Stage	**DAF**	**Name**	**Seed water content (DW)**	**Pod**	**Morphology Seed**
R 7.1	58	Early maturation	1.57 ±0.01	Completely green.	Seed coat entirely green and yellow embryo axis. The seed consistency is hard.
R7.1.1	62	Early maturation	1.49 ±0.01	Green with yellow spots.	Green seed coat with 25% of its surface yellow
R7.1.2	63	Maturation	1.43 ±0.03	Green with yellow spots.	Green seed coat with 50% of its surface yellow
R7.1.3	64	Maturation	1.39 ±0.02	Green with yellow spots.	Green seed coat with 75% of its surface yellow
R 7.2	66	Mid maturation/ end of seed filling	1.28±0.02	Yellow with green spots.	Seed coat mainly yellow with green spots in the middle.
R 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.
R 8.1	72	Full maturation	0.86 ± 0.16	Yellow with brown spots.	Yellow and completely opaque seed coat. Seed consistency is rubber.
R 9	76	Harvest maturity	0.18 ±0.02	Completely brown and dry.	Brown seed coat and dry seeds

DAF (days after flowering), DW (Dry weight) at harvest. Data are the average of four repetitions of twenty seeds each (± SE).

Figure 2. A) Pod and seed development of soybean as described in Lima et al. (2017). B) Phenological stages added for the purpose of this work. C) Stem of a mature plants showing seeds at stage R9.



Photograph:Denise Puntel Basso, 2018.

2.2.3 Physiological characterization

a) Seed drying

Fast drying was performed by placing the seeds on a screen in a sealed container with silica gel at approximately 15% RH at 20°C. For the slow drying, seeds were placed in closed containers successively at the following decreasing RH using salt solutions at 20°C: 1 day at 95% RH (LiCl) according to protocol described in GOLD and HAY (2014), 2 days at saturated solutions in 75% RH (NaCl), 2 d at 45% RH (K_2CO_3) and 2 d at 15% RH using silica gel. A data logger was used to monitor temperature and humidity relative during drying.

b) Seed weight and water content determination

Seed weight after drying was measured using 8 replicates of 100 seeds. Water content was determined on four replicates of 20 seeds by oven drying at 105 ± 3° C for 24 hours and expressed as gram of water per gram of DW.

c) Desiccation tolerance and longevity determination

After drying seeds were placed over a saturated solution of NaCl (75% RH) at 35°C in hermetically sealed boxes for different time spans. At different intervals during storage, four replicates of 25 seeds (crop 2016) and three replicates of 25 seeds (crop 2015) were imbibed in Petri dishes (150x15 mm) between two sheets of filter papers in the presence of 20 ml water with a 12 h photoperiod at 25°C. Viability was assessed by recording germination which was scored when seeds exhibited a protruding radicle of ≥ 1 mm in length. To estimate the life span, survival curves were obtained by fitting a sigmoidal equation to the data sets. From the fit, P50 was calculated, defined as the time (days) at which the seed lost 50% of viability during storage.

d) Temperature effect on germination.

Two to four replicates of 25 seeds were imbibed in pleated paper with 80 ml of water in closed plastic boxes (170x65 mm) and placed in the dark at different temperatures. Boxes were placed in a random design in the germination cabinet and a data logger was used to monitor temperature. The germination percentage was scored daily by counting seeds with protruding radicle of ≥ 1 mm in length. Scoring was done under green safe light at room temperature, one box at a time to avoid excessive cooling and heating compared to the germination temperature.

e) Tetrazolium test

Two replicates of 50 seeds were imbibed using paper roll moistened with water, the seed were placed between two sheets of filter papers at 25°C during 16 hours, after that, the seed were placed in Beckers being totally submerged by the tetrazolium solution (2,3,5 triphenyl tetrazolium chloride) with concentration of 0.0075%. The seeds were kept under these conditions during 3 hours at 35 °C in the dark. The methodology and the evaluations of the test were according to França-Neto (1998).

2.2.3 Statistical analyses

Analysis of variance was conducted to assess significant differences between treatments ($p< 0.05$) using SISVAR version 7.0 (FERREIRA, 2008). Post hoc multiple comparison were performed using the Tukey test ($p<0.05$). Principal component analysis (PCA) was performed with matrix correlation using Minitab 17

to simultaneously explore correlations between longevity, climatic variables and positional location. Climatic variables were the sum of days when the temperature was above 30 °C and the precipitation in mm (total amount of accumulated rainfall). To assess the impact of different maternal environments, they were calculated from different intervals during the culture and seed development, namely from sowing to flowering, flowering to harvest and maturation to harvest.

PCA was used to identify linear combinations of variables in a high-dimensional space best representing the variance that is present in the data. This is achieved by considering each variable to be an axis in a high-dimensional space. Individuals, or groups of individuals, can be represented as points in this space. PCA identifies a linear combination of the original variables, called principal component that accounts for the largest amount of the experimental variability. Once this first principal component is set, PCA finds successive orthogonal principal components that explain the maximum amount of the remaining variance given that the orthogonality constraint is met. Finally, the original data and the original variables can be projected in this new space defined by the principal components.

2.3 RESULTS

2.3.1 Influence of the drying regime on longevity

Successive exposure of immature seeds to decreasing RH over 5 days led to a slow decrease in the seed water content that was similar between stages (Figure 3, closed symbols). During the first 24 h of drying, the water loss represented only 3 to 6% of the original weight.

Thereafter water content decreased linearly at a rate of 3.6mg water/h. For seeds harvested at R7.2, it took 144 h to reach a water content of 10% (dry weight basis) (Figure 3). In comparison, when seeds were exposed to silica gel, their water loss was exponential. During the first 24 h, seeds lost water at a rate of 79 mg/h and it took about 66 h for R7.2 seeds to reach 10%. It is noteworthy that i*n planta*, it takes approximatively 8 days (192 h) for seeds of stage R7.2 to reach stage R9 (Table 2). After drying, no significant difference in the seed dry weight were found between stages (Figure 3).

Figure 3. Changes in water content of immature soybean seeds harvested in 2015 at indicated phenological stages during maturation. Seeds were submitted to slow (closed symbols) and fast drying (open symbols).

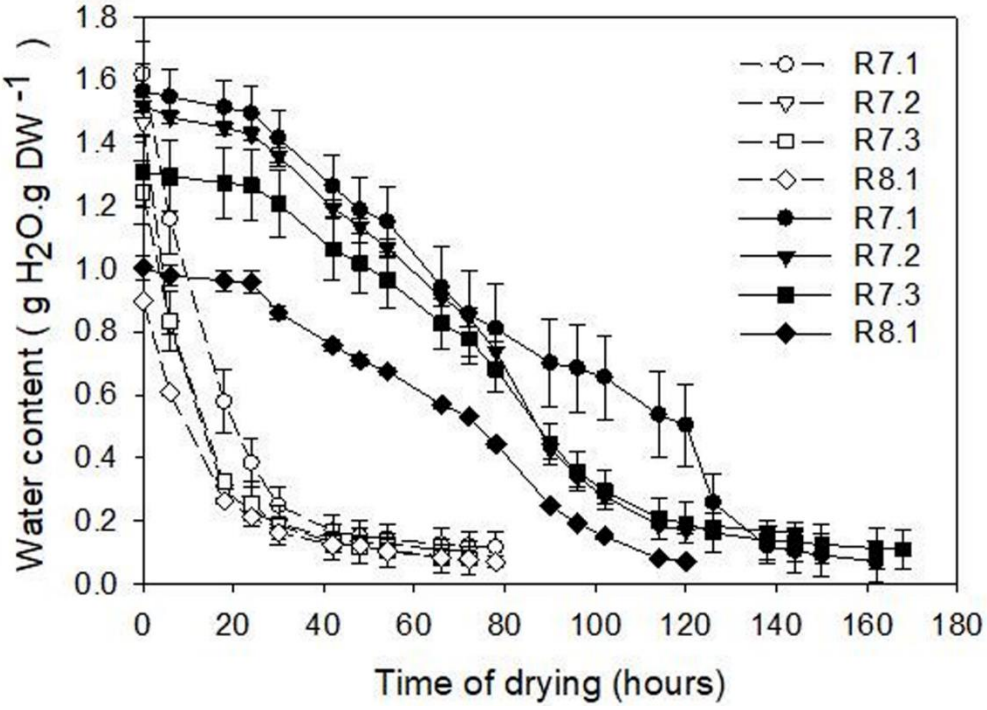


Figure 4. Evolution of desiccation tolerance following fast (open symbols) and slow (closed symbols) during seed maturation. Data (n = 3 replicates of 25 seeds) are expressed based on phenological stages (A) and seed age (B) which takes into account both the date of harvest and the drying time. Data from 2015 seeds.

Next, we compared the effect the drying regimes on the acquisition of seed longevity. During storage at 75%RH, 35°C, seed started to die much earlier when they were immature compared to mature seeds (Figure 5). To quantify longevity, data were fitted with sigmoidal curves and P50 values (defined as the time which stored seed lose 50% of viability) were calculated (Figure 6). P50 increased progressively throughout seed maturation once the seeds were desiccation tolerant (Figure 6A). When the data are expressed on the basis of phenological stages, P50 values were always lower after fast drying compared to slow drying, suggesting that the rapid loss of water was detrimental to the acquisition of seed longevity. However, when data are expressed based on the age of the developing seed, the acquisition of longevity was identical regardless of the drying rate, except for 64d- old seeds (Figure 6B). Furthermore, if seeds older than 73 d are given time to advance in their maturation process due to the slow drying rate, longevity keeps increasing. Lima et al. (2017) observed that the detachment of seeds from the mother plant occurs between stage R7.1 and R7.2. Altogether, this suggests that the advancement of longevity during maturation drying can occur independently from the mother plant.

Figure 5. Changes in germination percentage of immature seeds harvested at indicated phenological stages and dried slowly (A) and fast (B). The seeds were stored at 35 °C and 75% relative humidity (RH) from crop season 2014/2015.

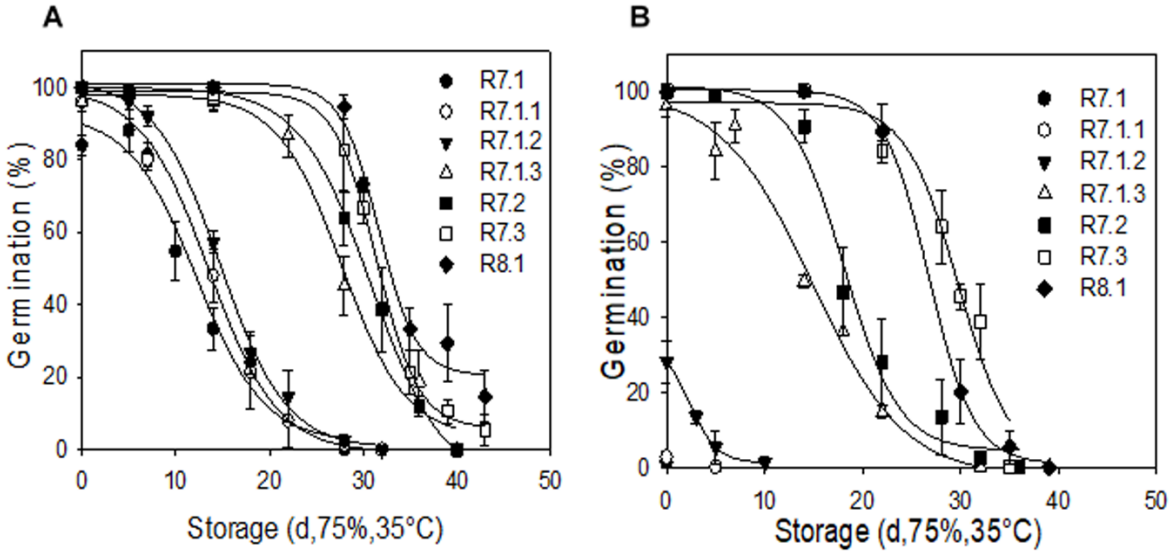
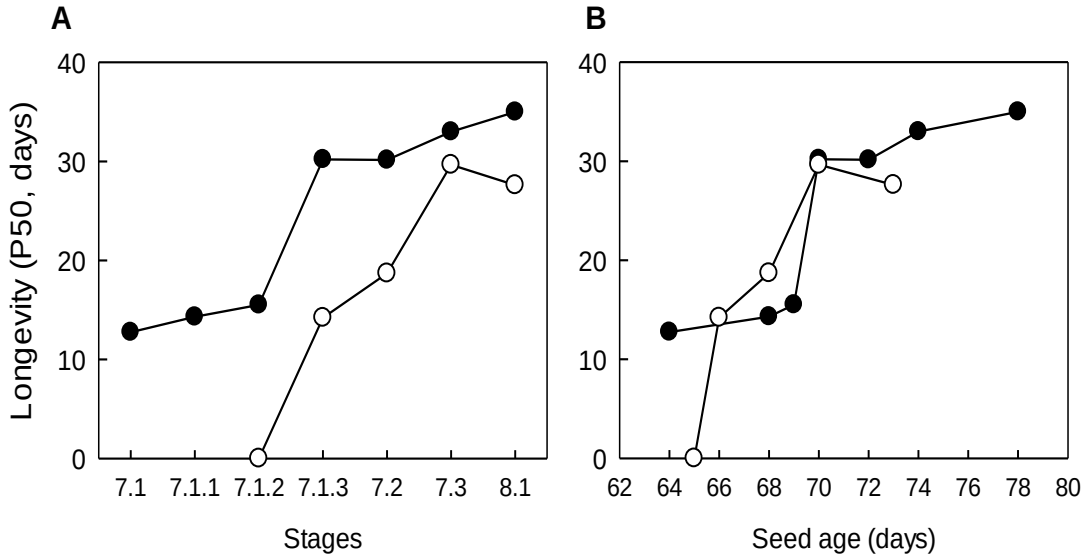


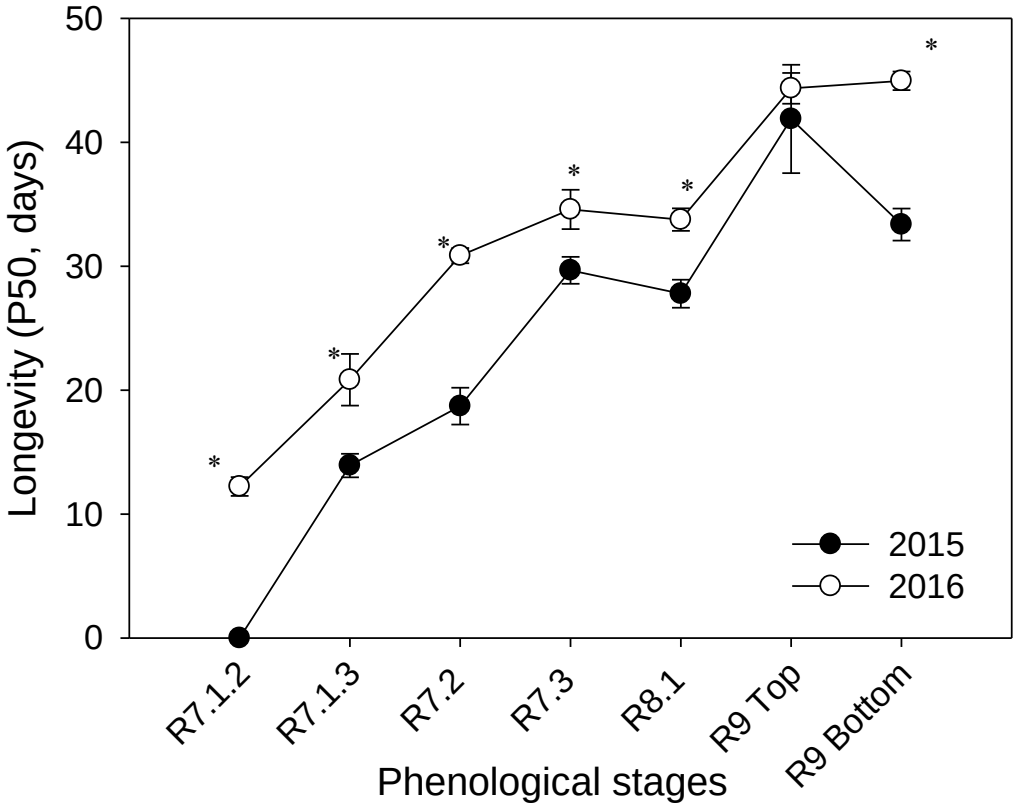
Figure 6. Evolution of longevity following fast (open symbols) and slow (closed symbols) during seed maturation. P50 were calculated from Fig. and are expressed based on phenological stages (A) and seed age (B) taking into account both the date of harvest and the drying time.



2.3.2 Impact of the environmental conditions on the acquisition of seed longevity

Lima et al. (2017) observed a slight variation in the acquisition of seed longevity between crop years 2013 and 2014. Here we expanded these observations by assessing whether variations between years could be attributed to environmental conditions throughout the culture by taking advantage of climate data available for 2015 and 2016. Here, changes in P50 during maturation were assessed after fast drying using phenological stages in two consecutive crop seasons 2014/15 and 2015/16. Figure 7 shows that the pattern of acquisition of longevity was identical between years, characterized by a progressive increase in P50 until full maturity at R9. However, for the same stages, differences were found between years. Developing seeds harvested in 2016 consistently exhibited a higher longevity than those of 2015 (Figure 7). Interestingly, the amplitude of this differences between years decreased progressively during maturation and disappeared at stage 9 for seeds harvested from the top nodes. Significant differences were found for bottom nodes between years. In 2015, P50 was 33 d compared to 44 d in 2016.

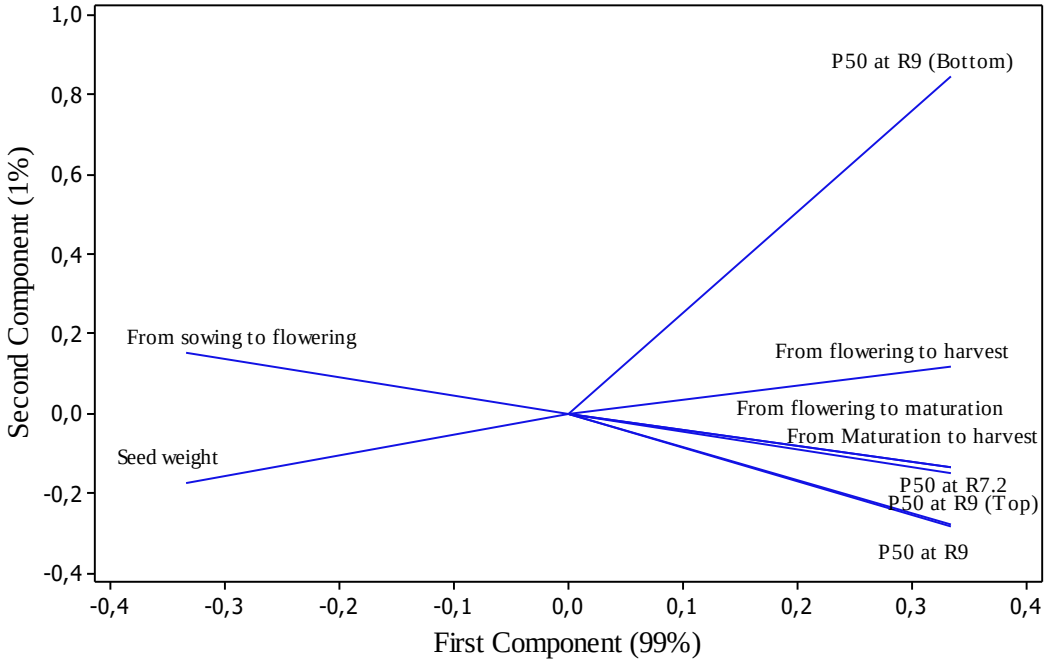
Figure 7. Changes in seed longevity during seed maturation in crop years 2014/2015 (black symbols) and 2015/2016 (open symbols). R9 Top and R9 Bottom correspond to dry mature seeds that were harvested respectively from the 3 apical and basal nodes. Bars represent the standard error established from survival curves using 3 replicates of 25 seeds for 2015 and 4 replicates of 25 seeds for 2016. The means were compared with ANOVA at 5 % of probability. Significant values between years are indicated by an asterisk.



To investigate how pre-zygotic and post-zygotic environments influenced longevity, we performed a PCA analysis**.** For longevity, we choose stages R9 and R7.2, corresponding to a maturation stage where P50 values are roughly 50% of that found in mature R9 seeds (Figure 8). As seed variable, we used seed weight at harvest to reflect the seed filling, P50 values calculated from seeds harvested in 2015 and 2016 at these two stages and P50 values obtained from seeds harvest at the top and bottom nodes of plants grown in 2015 and 2016 (Figure 8). As climate variable, we used the number of days when the temperature was above 30°C, a temperature considered to be stressful for the germinative vigor in soybean (Figure 8) (EGLI et al., 2005; CHEBROLU et al., 2016) and the accumulation of rain fall (total amount of precipitation) (Figure 9). To discriminate he environment

during the vegetative growth from that during seed development, these climate values were calculated respectively from the sowing and flowering dates those corresponding to flowering and harvest. This latter variable was also split in two to investigate more precisely the effect on the environment during the latter stages of seed maturation: from flowering to maturation (i.e. stage 7.2) and from maturation to harvest.

Figure 8. Principal Components Analysis (PCA) showing the relationships between seed longevity at stages R7.2, R9, R9 bottom, R9 top, seed weight and the sum of days when the temperature was above 30°C for the crop seasons 2014/2015 and 2015/2016.



For heat as climate variable, the first and second component explained respectively 99% and 1% of variability among treatments. Examining these relationships simultaneously showed that there is a clear separation between seed weight and heat during the vegetative growth (from sowing to flowering) on one side of the first component and the longevity and heat during the reproductive phase (from flowering to harvest) on the other side of the first component. This indicates that both seed traits were affected by the number of days above 30°C. Seed weight was close to “heat during vegetative growth” but opposite to “heat during reproductive growth”, indicating respectively a positive and negative correlation (Figure 8). This suggests that high temperatures experienced during vegetative

growth could indirectly enhance seed filling via a positive impact that remain to be identified. However, high temperatures during seed filling is probably felt by the developing seeds as a stressful condition that could limit the rate of the seed filling as previously observed in plants grown in phytotrons (SPEARS et al., 1997). The impact of high temperatures on the acquisition of longevity was different from seed weight (Figure 8). Heat during vegetative growth was negatively correlated to longevity values at stage R7.2 and R9 but close to high temperature during seed maturation. In contrast to seed weight, temperatures above 30°C favor high longevity (Figure 8).

Along the second component, longevity from seeds harvested at the bottom of the plant was clearly separated from those harvested at the top of the plant (Figure 8). The observation that the P50 vector from the R9 bottom seeds is orthogonal to those of both seeds at R9, R9 Top and temperature is indicative of an absence of correlation. This suggests that another environmental factor is responsible for this variation in longevity between seeds at the lower part of the plant.

When rainfall before or after flowering was used as climate variable, the first component and second component explained respectively 99% and 1% of the variability within treatments (Figure 9). Seed weight appeared to be separated from longevity along the first component like for the number days with a temperature above 30°C. However, in contrast to temperature, the seed weight vector was not greatly influenced by the rainfall during most of the plant cycle. Since plants were irrigated during the culture, it is unlikely that plants suffered from water deficits, which might explain the little or no effect on seed filling and longevity. Interestingly, the vector corresponding to rainfall occurring during the late stage of seed maturation was in the opposite direction of the longevity vectors at stage R7.2, R 9 and of seeds harvested on top of the plants, suggesting that high moisture at the latter stages of maturation is detrimental to seed longevity. Longevity of seeds harvested at the bottom of the plants separated from the other longevity vectors, like for temperature and was orthogonal to all the rainfall vectors, suggesting lack of correlation. The end of the 2015 season was particularly humid (Figure 1) and we verified whether bottom seeds suffered a so-called weathering effect that is well described in the literature Moore (1973), Pereira e Andrews (1976), Tekrony et al. (1980), França-Neto and Henning (1984) and Forti et al. (2013). Seeds from the bottom nodes exhibited characteristic wrinkles on the cotyledons (Figure 10A), in the region

opposite the thread. Furthermore, 100 seeds were selected and submitted to the tetrazolium test. After coloring 29% of seeds revealed the presence of lesions of intense red coloration on region opposite the thread to such wrinkles (Figure 10B).

Figure 9. Principal Components Analysis (PCA) and respective vectors considering P50 from the stages R7.2, R9 and R9 top and R9 bottom and the accumulation of rainfall (at different times for the crop seasons 2014/2015 and 2015/2016. The seeds from the R9 top and R9 bottom where from the crop season 2015/2016 with the respective vectors.

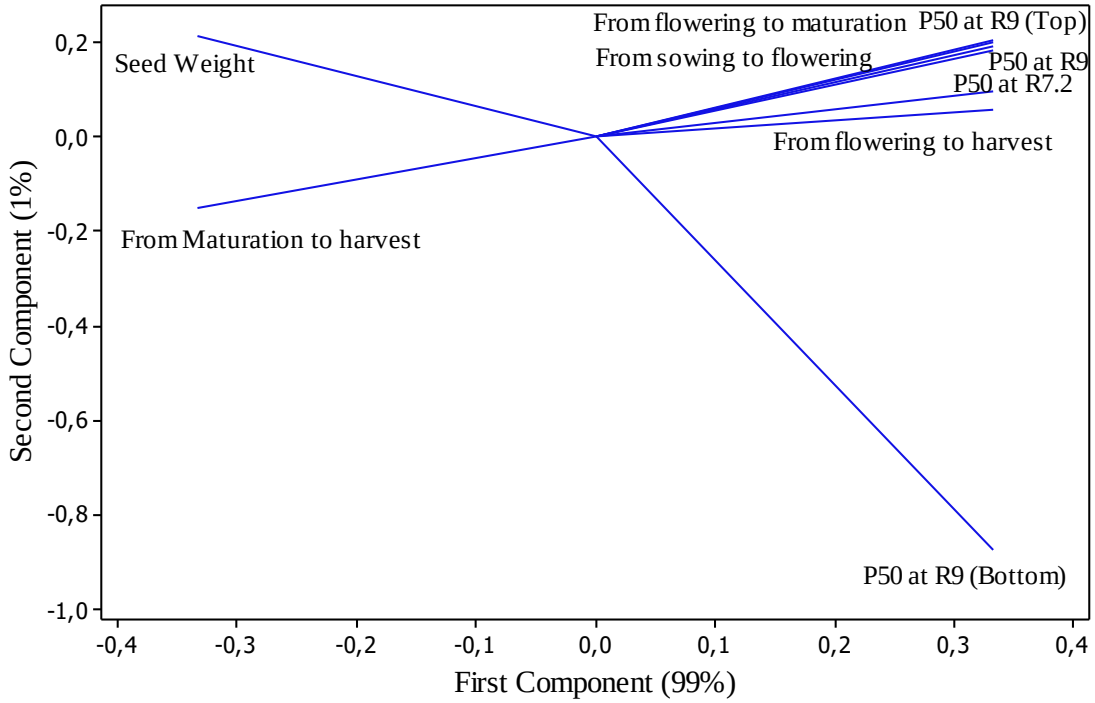
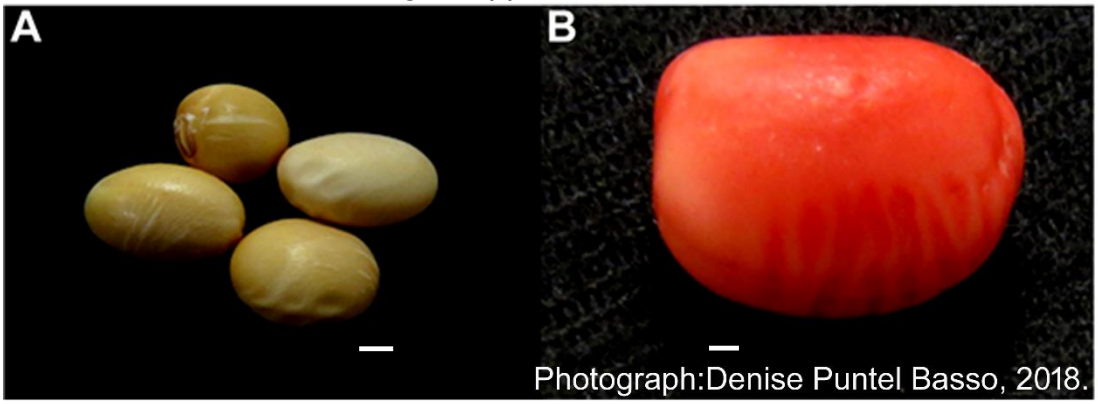


Figure 10. A) Wrinkled seeds harvested from bottom nodes at stage R9 in 2015. B) The Photo of a tetrazolium stained seeds showing lesions such as striations of intense red coloration in the region opposite the thread. White bar = 1cm.



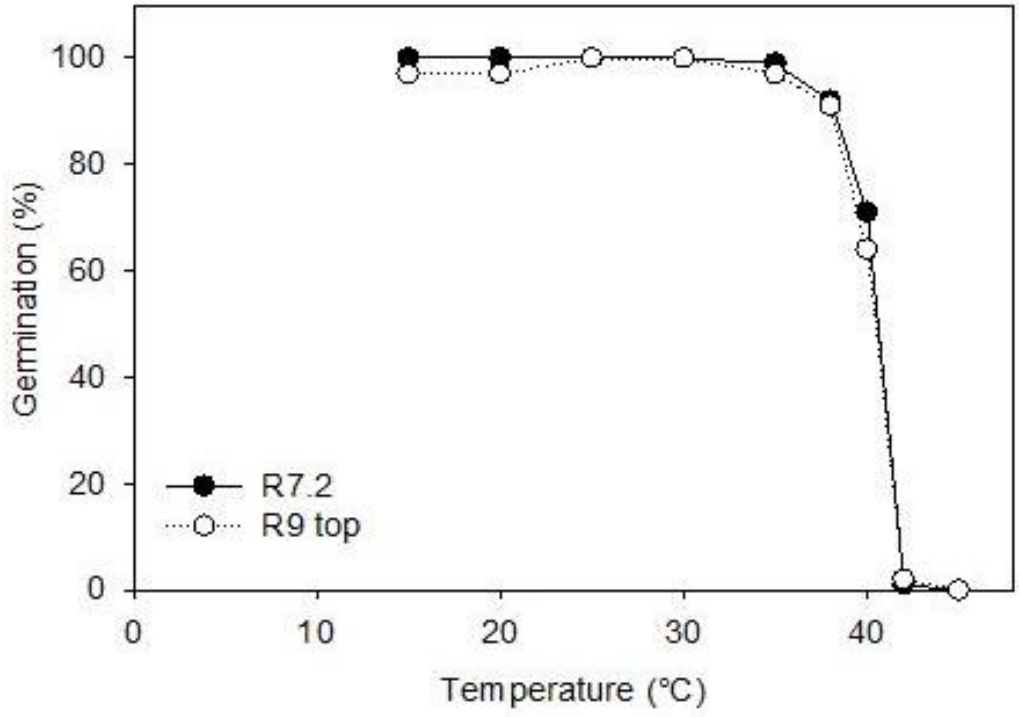
2.3.3 Acquisition of the thermal dependence of germination during seed maturation

We observed that temperatures above 30°C during seed development do not appear detrimental to the acquisition of longevity. Here, longevity was assessed at 35°C. American varieties can germinate at temperatures as high as 38°C but are very sensitive to low temperature below 10-15°C. However, this strongly depends on the genotype (CHEBROLU et al., 2016). These observations prompted us to examine when germination at high temperature is acquired during seed development in our material and whether this characteristic could be used as vigor test reflecting the level of seed maturity. Since, the impact of temperature on germination was not known for our cultivar, we first tested a range of temperatures from 15 to 45°C on germination of seeds harvested at stage 7.2 and 9 (Figure 11). All seeds germinated when imbibed between 15 and 35 °C in the dark. From 38°C and higher, there was a sharp reduction in germination percentage, being lethal at 42°C for both crop seasons (Figure 12). When investigating the thermal dependence of germination, it is customary to calculate the optimal temperature (To) for germination at maximum speed, the base temperature (Tb), corresponding to the temperature at which no seed germinate and the thermal time (θT) corresponding to the degree-days to accomplish germination, based on the hypothesis that the germination speed increases linearly with temperature and is directly proportional to the difference between experimental T and Tb, up to To (FINCH-SAVAGE et al., 2015). For this purpose, we first calculated the germination speed by fitting 3rd or 4th order sigmoidal curves to the relation between time of incubation and percentages of germination (data not shown).

From these curves, the time to 50% germination (t50 in h) were obtained and germination speed was calculated as 1/t50. Tb, To and θT values were obtained through regression analysis of the reciprocal of the times for 50% germination, with Tb corresponding to the intercept on abscissa, 1/θT the slope of the regression line until To, and To as the cross-point between the regression equation fitting the low and high temperature range, respectively. For both R7.2 and 9 seeds, germination speed increased with temperature until To. The regression analysis gave similar values for all thermal parameters with Tb = 4-5°C, To = 33-34°C and θT = 382 °h. Considering the limited amount of the seeds and no difference

between years following this preliminary analysis, we did not pursue further experiments to obtain more precise values of To for each stages. Based on these results, we investigated the impact of maturity on germination at two sub-optimal temperatures (15 and 25°C) and at one supra-optimal temperature (38 °C) for seeds harvested in the crop seasons 2014/2015 and 2015/2016.

Figure 11. Effect of temperature on germination percentage of seed lots harvested at R7.2 and R9 (top nodes of the plant) from the crop 2015/2016. Data are the average of two replicates of 50 seeds.



Germination at 15°C, there was no significant effect of the treatment between stages (Table 3). Seeds germinated 100% (Figure 13A). At 25°C, germination percentages were significantly lower for immature seeds from stage R7.1.2 compared to later stages in 2015, but there was no difference between stages in 2016 (Table 4). Germination at 38°C enhanced the heterogeneity of the seed lots as shown by the size of the standard error (Figure 13C). Nevertheless, table 4 shows that immature seeds gained progressively the capacity to germinate at this high temperature (38°C) between R7.1.2. and R7.2.

Figure 12. Effect of temperature on germination speed of seeds harvested at stage R7.2 (open symbols) and R9 (closed symbols). *Two replicates of 50 seeds are shown for each stage. Data were fitted with linear regressions as follows: stage R7.2, low temperature range: y= 0.00262x-0.0114 (r2=0.905), high temperature range: y = -0.008 +0.362 (r^2=0.782); stage R9, low temperature range: y= 0.00221x-0.0134 (r^2=0.945), high temperature range y=-0.00783+0.0337 (r^2=0.765).

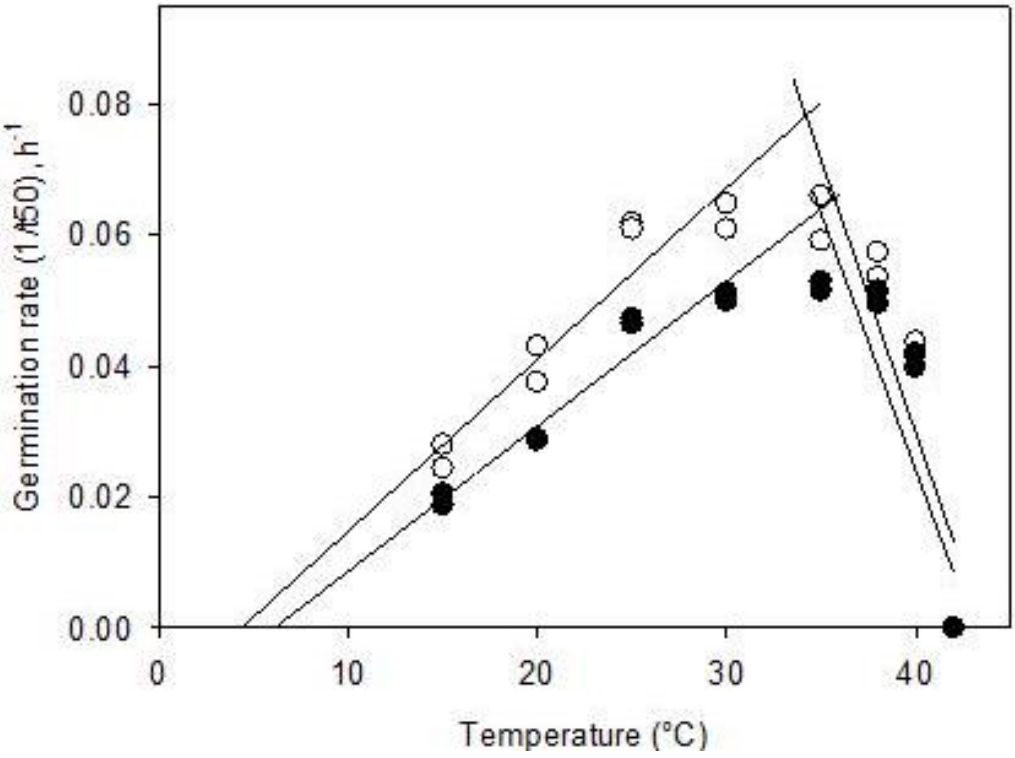


Table 3. Analysis of Variance showing P values for principal effects and Interaction between phenological stages and years for the germination and germination speed variables.

	Variables	Treatment		
Temperature		Year	Stage	Year x stage
15 °C	Germination	0.669^{ns}	0.757^{ns}	0.218^{ns}
	Germination speed	<0.0001	<0.0001	<0.0001
25 °C	Germination	<0.0001	< 0.0001	< 0.0001
	Germination speed	0.9158^{ns}	<0.0001	<0.0001
38 °C	Germination	<0.0001	< 0.0001	< 0.0001
	Germination speed	0.4864^{ns}	0.0433	0.0233

*ns = non significant < 0.05.

Figure 13. Relation between phenological stages during maturation and germination percentages (A-C) and germination rate (D-F) at 15 °C (A, D) 25 °C (B, E) and 38 °C (C, F) for the crop years 2014/2015 (black symbols) and 2015/2016 (open symbols). Data are the average of 4 replicates of 25 seeds (±SE).

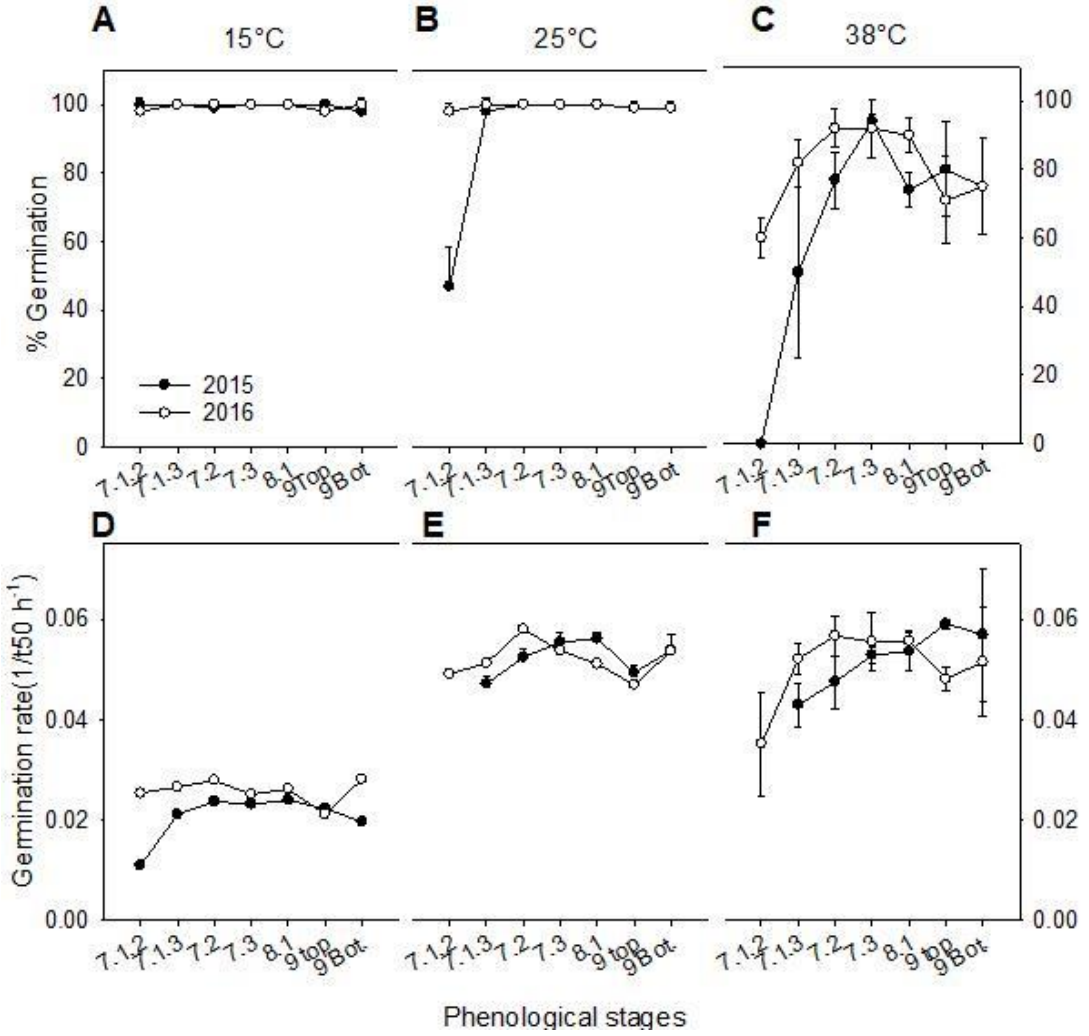


Table 4. Significant effects of maturation of soybean on germination for seasons 2015 and 2016. Data are the average of 4 replicates of 25 seeds.

	Temperature			
	25°C		38°C	
	Years			
Stages	2015	2016	2015	2016
R7.1.2	47,0 Bb	98,0 Aa	0Cb	60,0 Ba
R7.1.3	98,0 Aa	100 Aa	50,0Bb	82,0Aba
R7.2	100 Aa	100 Aa	77,0Aa	92,0 Aa
R7.3	100 Aa	100 Aa	94,0Aa	92,0Aa
R8.1	100 Aa	100 Aa	74,0Ab	90,0Aa
R9 Top	99 Aa	99,0 Aa	80,0Aa	71,0Aba
R9 Bottom	99 Aa	99,0 Aa	74,0Aa	75,0 Aba

Data not followed by the same letter differ between stages and between years, the capital letters are compared in the same column and the lowercase letters are compared in the same line. Multiple pairwise comparisons were performed using test Tukey at p <0.05.

Germination speed at 15°C in 2015, seeds of R7.1.2 germinated lower when compared to later stages (Table 5). However, in 2016, seeds of R9 top exhibited lower germination speed. At 25°C, recommended temperature for soybean germination according to Brasil (2009), the germination speed was greater between stages R7.2 e R9, however in 2016, the most vigorous maturation stage was R7.2.

When seeds were subjected to high temperatures in 2015, maturity stages earlier as R7.1.2 the germination speed was affected by heat, being lethal at 38 °C, and the most vigorous maturation stages were between R7.3 and R9. In 2016, we had the same trend as 2015 (Table 5).

Table 5. Significant effects of maturation of soybean on germination speed for seasons 2015 and 2016. Data are the average of 4 replicates of 25 seeds.

	15°C		25 °C		38 °C	
	Years					
Stages	2015	2016	2015	2016	2015	2016
R7.1.2	0,011 Cb	0,025Ba	-	0,0490 DC	-	0,042 D
R7.1.3	0,021Bb	0,026Aba	0,0471Da	0,051 BCDa	0,0426Db	0,052 ABCa
R7.2	0,023ABb	0,028Aa	0,0523BCb	0,058 Aa	0,0484BCDa	0,056ABCa
R7.3	0,023ABb	0,025 Ba	0,055ABa	0.0537 ABCa	0,053ABCa	0,055ABCa
R8.1	0,024Ab	0,026Aba	0,056ABa	0,051BCDa	0,053ABCa	0,055ABCa
R9 Top	0,022Aba	0,021Ca	0,049DCa	0,046Da	0,059Aa	0,047DCb
R9 Bottom	0,024Ab	0,028Aa	0,053ABCa	0,053ABCa	0,057Aba	0,052ABCa

Data not followed by the same letter differ between stages and between years, the capital letters are compared in the same column and the lowercase letters are compared in the same line. Multiple pairwise comparisons were performed using test Tukey at p <0.05. Absence of means indicates no T50 between stages.

2.4 DISCUSSION

The influence of the maternal environment is increasingly recognized as an important factor that regulates seed traits such as seed weight and composition in crop species. Maternal environment comprises biotic factors that control the supply of assimilates and hormones to the developing seeds and the fruit tissues that control the rate of water loss at the end of maturation. It also includes abiotic

factors, such as temperature, light and water availability that can act on the mother plant and on the developing seeds. Climate conditions during seed development affects seed dormancy and seed longevity in wild species (GUTTERMAN et al., 2000; KOCHANEK et al., 2010, 2011) such as Arabidopsis (HE et al., 2014) and *M. truncatula* (RIGHETTI et al., 2015) when grown in controlled environments. Using field grown seeds, our study provides indirect evidence that the maternal environment also influences in a complex way the acquisition of seed longevity and vigor, measured as germination capacity at 38°C. As key components of this environment, we identified the drying rate and the microclimate around the developing pod (Figure 4), heat that occurred both during the vegetative and reproductive phase (Figure 8).

Our study extends the findings of Lima et al. (2017) on the acquisition of desiccation tolerance and longevity in field grown soybean. These authors showed that desiccation tolerance was acquired during seed development between stages R7.1 and R.7.2 for both 2013 and 2014 harvest. The time lapse between R7.1 and R7.2 is 8 days (Table 2). Here, we incorporated new phenological stages between R7.1 and R7.2 stages to better describe the acquisition of desiccation tolerance and longevity during seed maturation (Figure 4 and 5). In very immature seeds that they were isolated from the maternal tissues, desiccation tolerance was acquired earlier during maturation when they were dried slowly compared to fast drying (i.e. within 2 days, Figure 4B). This suggests that slow drying induces the acquisition of desiccation tolerance, irrespective of the presence of the mother plant. This is similar to the observations by Blackman et al. (1991, 1992) who showed that drying increased the desiccation tolerance of the immature soybean seeds concomitantly with the accumulation of soluble sugars and heat stable proteins. Therefore, several metabolic changes may occur during slow drying within the immature seeds, favoring acquisition of desiccation tolerance at earlier stages of development.

We confirmed that longevity is progressively acquired during seed maturation at the end of seed filling (Figure 7). The addition of phenological stages allowed us to better determine the onset of this acquisition which was shortly after stage R7.1. The onset does not strongly depend on the drying rate when p50 values were corrected for the drying time. In other words, slow drying mimicked the environment *in planta*. Slow drying of immature seeds has also been shown to increase

storability in several wild plant species (PROBERT and HAY, 2000; HAY and SMITH, 2003) and there is convincing evidence that such treatments allow the continuation of natural ripening events after post-abscission (PROBERT et al., 2007). Consequently, our data reinforces the contention that late seed maturation is a distinct and necessary phase in the production of high quality soybean seeds as previously suggested by ZANAKIS et al. (1994). Altogether, we postulate that the acquisition of longevity is an autonomous embryonic program that does not depend entirely on the connections with the vascular tissues of the mother plants, being lost at or shortly before R7.2. Further work is necessary to understand the mechanisms triggering the embryo into a seed longevity program and which protective factors are implicated in this program. Thus, two corollaries to the hypothesis of an autonomous longevity program need also to be examined: 1) how the developing seeds resort on their own metabolism to synthesize the necessary protective compounds such as oligosaccharides that were found to increase concomitantly with longevity (LIMA et al., 2017) and 2) whether the abiotic environment impact such metabolism. Testing this hypothesis is warranted by the observation that temperature during seed filling is one of the main factor influencing tocopherol content (CARRERA and SEGUIN, 2016), an antioxidant compound involved in seed longevity in oily seeds fluctuates according to the temperature during seed filling.

2.4.1 The impact of environmental conditions and the effect of the mother plant on the acquisition of seed longevity

In many species, factors such as age of the mother plant, position of the seed in the fruit, and canopy can affect seed traits (GUTTERMAN, 2000). Some of them may be of genetic (PLATENKAMP and SHAW, 1993) or phenotypic origin or may due to an interaction of both, caused by the local conditions under which the seeds matured (GUTTERMAN, 1999, 2000). The conditions consist of a combination of the microenvironment experienced by seed due to its position on the parental plant and the abiotic environment of the plant, both factors influencing the speed of seed maturation (ILLIPRONTI et al., 2000). Our principal component analysis confirmed previous works on soybean showing that heat during seed development measured

here as number of days above 30°C negatively impacts seed filling (EGLI et al., 2005; PUTEH et al., 2013).

Our analysis also revealed a complex relationship between heat and longevity. During seed development, the biplot (Figure 8) shows that heat was associated with longevity on the first component, opposite of seed weight, suggesting that these high temperatures are beneficial for the longevity program whereas they are detrimental to seed filling. This is consistent with the findings of Zanakis et al. (1994) using Indonesian varieties of soybean and of Whitehouse et al. (2015) in a range of rice accessions of the japonica varieties. Interestingly in this latter species, seed longevity could be doubled when immature seeds close to maturity were initially dried at 45°C instead of 15°C. According to Whitehouse et al. (2015) when developing rice seeds experience some desiccation, “substantial accumulation of seed longevity” is observed, depending on the genotype and environment. Our results are also consistent with the study by Probert et al. (2009) who screened the longevities of 195 wild species from environments ranging from tropical forests to cold desert. This survey showed that seeds from hot, dry environments tend to exhibit a higher longevity than those from cool, wet conditions.

It is noteworthy that our work is different from studies where soybean plants were grown in controlled conditions at much more stressful conditions than ours such as a temperature regime of *ca.* 32°C/28° night during seed development (KEIGLEY and MULLEN, 1986; ILLIPRONTI et al., 2000; CHEBROLU et al., 2016;). Here on average, the day/night temperatures from flowering onwards were 26°/17 for 2015 and 29°C/13°C for 2016. Therefore, we cannot exclude that more stressful conditions might also have a negative impact on seed longevity as observed in other cold legume species such as *Medicago truncatula* (RIGHETTI et al., 2015) and in *Vicia faba* (LI et al., 2017). Therefore, it would be interesting to expand our study with different genetic material and establish a cause-effect relationship between heat (day or night) during seed development and acquisition of longevity and determine the breakpoint temperature when heat negatively impacts longevity.

Another intriguing observation from our biplot (Figure 8) is that the differences in P50 across the years were attributable to the temperature during the vegetative growth with heat being negatively correlated with longevity. In recent years, experimental evidence on wild species showed that seed longevity is affected by the environment that the plant experiences before fecundation (KOCHANEK et al. 2010,

2011, reviewed in LEPRINCE et al. 2017). These authors suggested that a plastic parental response is passed on to offspring seeds and changes their longevity, even though the seeds developed on plants that were removed from their environment before fecundation. In wild species, this parental response may reflect a bet-hedging strategy that maximizes the species dispersal since the probability of having different fractions of seeds with different lifespans is increased (KOCHANEK et al., 2011). In crops, it is expected that such bet-hedging strategy would have been eliminated by breeding during domestication but we speculate that our study might reveal some traces of it. To the best of our knowledge, a putative impact of the maternal environment before seed set has only been observed in *Vicia faba* on seed vigor (assessed by accelerated aging in 100% at 42°C) but not on seed longevity. Further work is necessary to confirm our findings and explore a putative negative the interaction between vegetative growth before seed set and longevity.

The PCA also suggests that the position of the seed on the mother plant lacks any correlation with seed longevity. This is consistent with the work of Illipronti et al. (2000) showing that seed position was less important than time to pod set to explain differences in seed vigor at maturity. A difference in seed longevity from the top and the bottom part of plant was apparent when high temperatures and rain occurred from seed maturation to harvest (Figure 7 and 8). These weather conditions occurred only in 2015 characterized by a cumulative rainfall of 113.6 mm between R7.1.2 at R9 in contrast to 0.5 mm in 2016 (Figure 1 A and B). In soybean, such conditions are known to cause field weathering which causes physical alterations by successive expansions and contractions of the seed volume due to the oscillations of the relative humidity and temperature (FRANÇA-NETO and HENNING, 1984). This wrinkles the seed coat tegument opposite the hilum. Indeed, we observed a considerable number of seeds from the bottom part of the plant with wrinkled teguments (Figure 10). Therefore, we suspect that a wet and humid microenvironment was created at the bottom of the plants where seeds had already reached R9 while those on the top nodes were still under development and not exposed to such microclimate.

2.4.2 Acquisition of germination capacity at high temperature during seed maturation

During seed maturation various physiological traits are acquired that are important for seedling establishment in the field. These traits that constitute seed vigor are very important to farmers and the seed industry which expresses the need to harvest seeds at the right maturity stage. Rossi (2016) showed that the capacity to germinate in salt was acquired during maturation of soybean seed. A similar conclusion can be reached from this work. Here we found that capacity of soybean seeds to tolerate heat during imbibition increases with seed maturation, being maximum at stages 7.3 and 8.1 and declining slightly thereafter independently of the crop season. Thus, field weathering impacts only the factors conducive to seed longevity but not stress tolerance during germination. We also found there was no difference in the capacity to germination under high temperature when the position of the mother plants was considered. This is similar to previous soybean data from Keigley and Mullen (1985) and Ilipronti et al. (2000). In their studies, exposure to increasing lengths at a 32°C/28°C temperature regime during seed development resulted in a linear decline in seed germination but no significant interaction was found between this effect and the plant position. In conjunction with the desiccation tolerance data, we conclude that stage R7.2 represent a key stage during maturation where the germination and desiccation tolerance is acquired yet remaining susceptible to the environmental conditions in terms of longevity.

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CHAPTER 3
IMPACT OF MATURATION ON THE ACQUISITION OF ORGAN ELONGATION DURING SEEDLING ESTABLISHMENT AND IMPLICATIONS ON VIGOR

ABSTRACT

To emerge out of the soil, seed organs need to elongate. Therefore, the growth capacity of the radicle and hypocotyl are important for seed vigor. While there is extensive work on the mechanisms of hypocotyl growth, there is little information as to how growth capacity is acquired during seed maturation and influenced by pre- and post-harvest factors. The aims of work were to test how and when elongation is acquired during seed maturation of soybean and whether seed position on mother plant and seed storage influence the elongation capacity. We found that the elongation capacity was acquired early during maturation and the increase of hypocotyl growth during seed maturation after mass maturity is important to late phase of seed maturation in building seed vigor, however it is dependent on year of culture and ways to measure. We found that hypocotyl growth follows the same pattern as viability, but reduction is more sensitive to storage than survival. No effect of the seed position on the seed vigor. However, a significant difference was found between years, suggesting that the culture may influence the seedling behavior.

Keywords: Elongation. Hypocotyl. Longevity. Radicle. Seed vigor. Storage.

3.1 INTRODUCTION

The majority of crops produced in the world agriculture begin with the sowing of the seeds to establish a new plant in the field. Successful seedling establishment is the first critical step for crop production and determines the success or failure of the future harvest. Seedling establishment is a complex transition during which dry seeds, carrying the full genetic complement from the crop, imbibe in the soil to produce a young plant (FINCH-SAVAGE and BASSEL, 2016). It can be divided broadly into three successive phases: germination, leading to radicle emergence, heterotrophic growth leading to the emergence of the hypocotyl or epicotyl out of

the soil according to the species and autotrophic growth and morphogenesis leading to a young plant. Since soybean has an epigeal germination, the epicotyl might also participate to the seedling establishment.

Light does not penetrate the soil to a depth of more than 1–2 mm, meaning that after sowing, seedlings grow in dark conditions in the soil for a crucial period until seedlings emerge from the soil surface and start photosynthesis. From the onset of germination, seed reserves are converted into soluble metabolites that are transported to the embryo axis for further metabolism and seedling growth (PIERRE et al., 2013). Being undergrown for the first part of their existence, those organs will rely on their own storage reserves to ensure heterotrophic growth. The heterotrophic growth of the hypocotyl and/or epicotyl is a standard model system in physiological studies to understand the mechanisms of elongation and how they respond to the environment. Therefore, there is a wealth of works showing that the hypocotyl is a very plastic organ, strongly influenced by both external and internal cues known to regulate cell elongation, such as light, oxygen and soil structure that are perceived by a complex signaling network involving auxin, ethylene, gibberellins and brassinosteroids (VANDENBUSSCHE et al., 2005; SANCHEZ-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). However, how this plasticity is important for the success of the establishment of the culture is far from understood.

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).

The size of an organ relies on cell division and/or cell elongation. In some species, it has been shown that hypocotyl growth in the dark is due to elongation of epidermal and cortical cells (GENDREAU et al., 1997; GALLI, 1988; KUTSCHERA, 2000; RAZ et al., 2001; VERSCHT et al., 2006). Seedling growth occurs through cell division and cell elongation. Growth of the whole plant is compartmentalized into zones, namely meristems where cell division occurs and elongation zones where cell expansion occurs via turgescence and cell wall deformation. Kutschera and Niklas (2007) elaborated the epidermal-growth-control theory of stem elongation.

Hypocotyl growth depends on internal turgor pressure in its individual cells, which causes the extension of the epidermis cell walls. In *A. thaliana*, hypocotyl basal epidermal cells reached up to 1 mm in length, representing a 100-fold increase in length compared to embryo cells in dry seeds before imbibition (GENDREAU et al., 1997).

Seed vigor is a set of desirable characteristics that will guarantee the crop establishment. Therefore, many tests were designed to capture and predict vigor in the laboratory. These tests evaluate specific characteristics as biochemical aspects, while others try to detect physiological differences or detect tolerance to abiotic stress. These tests include electrical conductivity, seedling growth measurements, cold test, accelerated aging and controlled deterioration (MARCOS-FILHO, 2015). The relationship between laboratory test results and seedling field emergence is directly influenced by environmental conditions and sowing procedures. The ability of laboratory tests to estimate the potential of seedling emergence decreases as environmental conditions become less adequate (TORRES et al., 2004). Nowadays there is no universally accepted single test for assessing seed vigor of a given species. For this reason, research on seed testing and the identification of factors that affect seed performance have been considered priorities among seed scientist in comparison to other quality attributes (MARCOS-FILHO, 2015b). In contrast, the emergence out of the soil surface and the ensuing autotrophic growth pertaining to seed vigor has been overlooked so far.

During development, seeds progressively gain the ability to germinate and the capacity to produce a seedling (BEWLEY et al., 2013). Seed vigor then progressively increases progressively and reaches a maximal level called physiological maturity (PM). There is no relationship between mass maturity and PM since vigor continue to increase after severing the connection with the mother plant. PM will differ between species but within a species, there is no consensus as to when it occurs during maturation because of a lack standardized method with a high predictive value of seed vigor (DORNBOS, 1995A; TEKRONY and EGLI, 1997; STILL and BRADFORD, 1998; BEWLEY et al., 2013).

The analysis of the transcriptome of developing soybean seeds during later maturation (LIMA et al., 2017) suggested that the growth capacity of the radicle and hypocotyl may be important for seed vigor. Indeed, transcripts encoding transcription factors associated with auxin and developmental cell fate during

organ growth and differentiation were abundant during the late maturation phase when longevity was acquired. However, for soybean seed there is a little information as to how growth capacity is acquired during seed maturation and the influence by pre- and post-harvest factors. For instance, is not completely known when the elongation capacity is acquired during soybean seed maturation and the influence of storage condition and the seed position on the mother plant.

Therefore, the objectives of this work are to document how and when elongation is acquired during seed maturation of soybean, to assess whether seed position on the mother plant and storage after harvest influences hypocotyl elongation. For this purpose, we designed an experimental system to measure organ length independently of the radicle emergence. We tested the influence of light and darkness of elongation. The preliminary design elaborated with the crop of 2015 was further improved with that of 2016.

3.2 MATERIAL AND METHODS

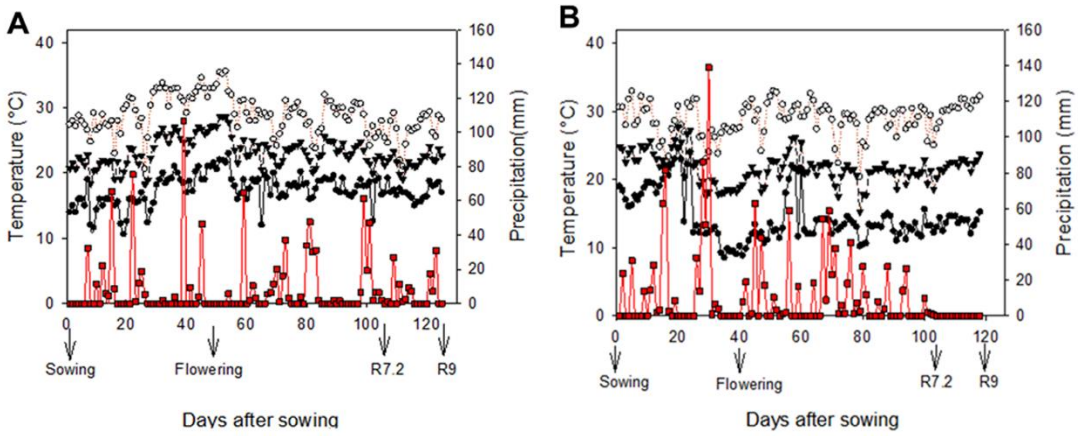
3.2.1 Plant material, growth conditions and seed sampling

The cultivar BRS 284 it is recommended for altitude tropical climate, namely dry winter and hot and rainy summer. 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, Brazil (22º 49''S; 48º 25''W, Altitude 810m). The mean annual temperature and rainfall is 20.3 °C and 1600 mm, respectively (CUNHA and MARTINS, 2009). During the crop cycle, temperature was measured with an electric thermograph and rainfall data were obtained from the Department of Natural Resources – Sector of Climatology, UNESP-Botucatu-SP (Figure 1). The soil in the experimental farm is classified as a “distroferric red nitosol” (EMBRAPA, 2006).

Seeds were sown respectively on 28 November in 2014 and 14 December 2015 at a density between 18-23 seeds/m and 0.45 m between rows. Plants were grown according to standard cultural techniques at the experimental farm. Briefly, soil fertilization was adapted according to the physico-chemical analysis. 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 and inoculated

Bradyrhizobium sp strains using the c.p Biomax- Biosoja, at a dose of 1.6 mL/ kg seeds. During the culture, phytosanitary controls were performed according to the recommendations by EMBRAPA (2011).

Figure 1. Variations in maximum (open circles), medium (black triangle) and minimum temperature (black circles) and daily precipitation (red square) at the experimental field from sowing to harvest during the crop years 2014/2015 (A) and 2015/2016 (B).



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 and seed age (Table 1). Immature seeds were immediately processed for physiological studies. At stage R9, dry seeds were harvested and separated according to their position on the mother stem. Seeds at the first 3 nodes basal (named bottom thereafter) and last 3 apical nodes (top) were kept for analysis. The subdivision of the R9 stage allowed higher precision in obtaining information related to the characterization of soybean seeds at the last stage of seed maturation facilitating the adoption of measures management practices related to the crop at harvesting time.

a) Seed drying

Fast drying was performed by placing the seeds on a screen in a sealed container with silica gel at approximately 15% RH at 20°C. For the slow drying, seeds were placed in closed containers successively at the following decreasing RH using salt solutions at 20°C: 1 d at 95% RH (LiCl) (GOLD and HAY., 2014), 2 d at 75% RH (NaCl), 2 d at 45% RH (K_2CO_3) then 2d at 15% RH using silica gel. A data logger was used to monitor temperature and humidity relative during drying.

Table 1. Characterization of the phenological stages of soybean seeds during maturation according to (LIMA et al., 2017) with adaptations.

Stage	DAF	Name	Seed water content (DW)	Pod	Seed
R7.1.2	63	Maturation	1.43 ±0.03	Green with yellow spots.	Green seed coat with 50% of its surface yellow
R7.1.3	64	Maturation	1.39 ±0.02	Green with yellow spots.	Green seed coat with 75% of its surface yellow
R 7.2	66	Mid maturation/ end of seed filling	1.28±0.02	Yellow with green spots.	Seed coat mainly yellow with green spots in the middle.
R 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.
R 8.1	72	Full maturation	0.86 ±0.16	Yellow with brown spots.	Yellow and completely opaque seed coat. Seed consistency is rubber.
R 9	76	Maturity	0.18 ±0.02	Completely brown and dry.	Brown seed coat and dry seeds

DAF (days after flowering), DW (Dry weight) at harvest. Data are the average of four repetitions of twenty seeds each (± SE).

b) Measurements of hypocotyl, root and epicotyl length

After postharvest treatments (drying, storage), seeds were first imbibed in two sheets of filter paper in Petri dishes (150x15 mm) with 20 ml of water. They were incubated at 22°C with photoperiod of 12 h light. At three successive time intervals, germinated seeds having an emerged radicle of > 1 mm were placed into 3 sheets of moist filter of 38x28 cm. Sheets were rolled and attached by a string (Figure 2). The seed distribution on the paper was positioned radicles facing down in a line. Each roll contained 4-15 germinated seeds, with a total of 3-10 rolls per stage. Rolls were put vertically inside a 2L Becker containing 450 ml of water so that the bottom of the rolls was dipping into it but not its top half. To avoid excessive evaporation, Beckers were covered with saran film when seeds were exposed to light and totally wrapped in double layers of aluminum foils and covered with black

plastic bag for those incubated in the dark, in this system the maximum capacity was 10 rolls (Figure 3A). They were incubated for up to 28 d at 22°C in the dark or in 12 h in light using Tubular Fluorescent Lamp 20W T1 with afluence rate of 30 µmol m^{-2} s^{-1} at bench level. For experiments using seeds harvested in the season 2016, the objective was to homogenize the environment in which the seedlings grew and allow more air and space for the organs to grow Figure 3B. Rolls were put vertically inside a 0,5 L Becker containing 250 ml of water, Beckers were placed inside a plastic tube (55x10 cm) covered with aluminum foil and incubated in the dark. In this system the maximum capacity was 4 rolls (Figure 3B). The length of the hypocotyl, root and epicotyl were measured after three, seven and twenty-eight days after transfer.

c) Storage experiment

Immature dried or mature seeds were placed over a saturated solution of NaCl (75% RH) at 35°C in hermetically sealed boxes. At different times during storage, seeds were imbibed, and organ length was measured as described above.

Figure 2. Experimental design used to assess elongation capacity.

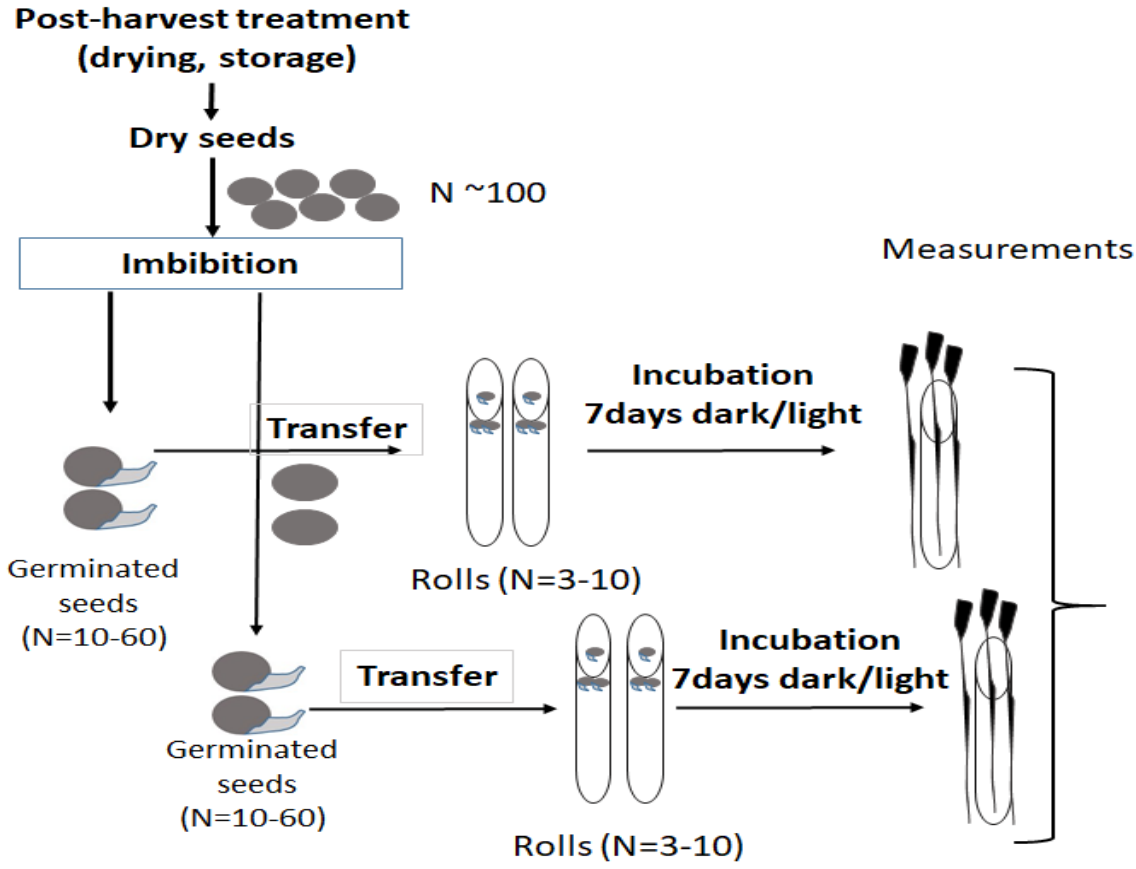
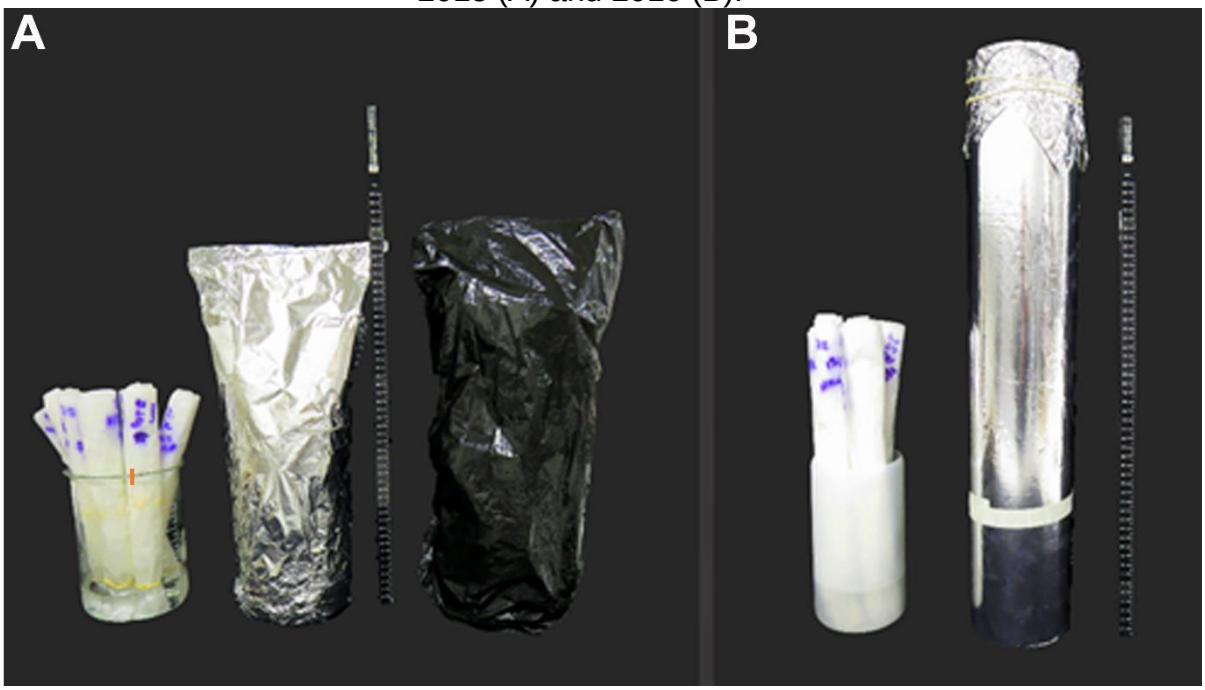


Figure 3. Experimental design used to measure seedling growth in the dark in 2015 (A) and 2016 (B).



3.2.2 Experimental design and statistical analysis

The experimental design consisted of completely randomized harvest of 7 phenological stages. Seeds were mixed before imbibition and germinated seeds were selected 2 to 3 times for transfer. Three to 10 rolls were used per stage and were separated in different beakers in a randomized fashion. For all measured variables, normality and equal variance between treatments were assessed by the Kolmogorov-Smirnov test and the Levene tests, respectively using XLSTAT (v2018). When samples did not pass these tests, data were transformed to a normal population with an unbiased standardization n-1 using XLSTAT (default setting). An ANOVA was applied using the model:

$$Length = A\ x\ stage + B\ x\ treatment + C\ x\ stage\ (i) x\ treatment\ (i) + E$$

Where treatments were either drying rate, storage time, light/dark during incubation, year of harvest, E residuals. Between 79 and 90 % of the variation in length was explained when ($p<0.05$). Pair-wise multiple comparison was then performed using the Tukey- Kramer test at 5% probability.

3.3 RESULTS

3.3.1 Kinetics of organ elongation of germinated seeds

First, we tested different times of incubation after transfer to best capture the elongation capacity of the different organs. For this purpose, the kinetics of elongation of the seedling organs were measured during 28 days using seeds harvested at different stages during maturation and submitted to fast drying. Speed and homogeneity of seedling emergence depends both on the capacity of the embryonic axis to elongate during germination and the capacity of hypocotyl and root to elongate afterwards. To distinguish these processes, seeds were first allowed to germinate and seeds with an emerged radicle were transferred into paper rolls standing upright and incubated in the dark. At 3, 7 and 28 d, a batch of rolls were retrieved for measurements and discarded afterwards. Therefore, each time point is independent of each other. The length of the hypocotyl increased linearly over the first 7 days after transfer (Figure 4A). Thereafter, the increase rate was slower for the next 14 days. This pattern of elongation appeared to be similar for all development stages studied. Roots elongated rapidly and reached their final length 7 d after transfer (Figure 4B). The epicotyl only appeared after 14 d after transfer (Figure 4C). Therefore, the kinetics of elongation could not be monitored for this organ. Considering the heavy logistics of these experiments and that maximal root length was reached after 7 d after transfer, we choose this time point for the rest of the experiments.

3.3.2 Effect of drying during seed maturation on hypocotyl elongation

The influence of fast and slow drying regimes on hypocotyl growth in the presence of light and in the dark was studied for seeds from the crop year 2015 across maturation stages (Table 2). Compared to slow drying, hypocotyls and roots were significantly shorter by 15-25% in seeds that were dried fast, regardless of the light conditions during elongation. The treatment light *vs.* dark across different stages also showed significant differences, the organ being shorter by 30%. Furthermore, the interaction between drying, light and phenological stages was also significant, the three treatments being necessary to explain the variability of

the data. There were no statistical differences between drying rates and phenological stages when the length ratio between organs was compared, suggesting that both organs were equally affected.

Figure 4. Time course of elongation of hypocotyl (A), root (B) and epicotyl (C) after transferring germinated seeds of indicated phenological stages into darkness at 22°C. Data represent the average (± SE) of 7-10 replicates of 4-11 germinated seed.

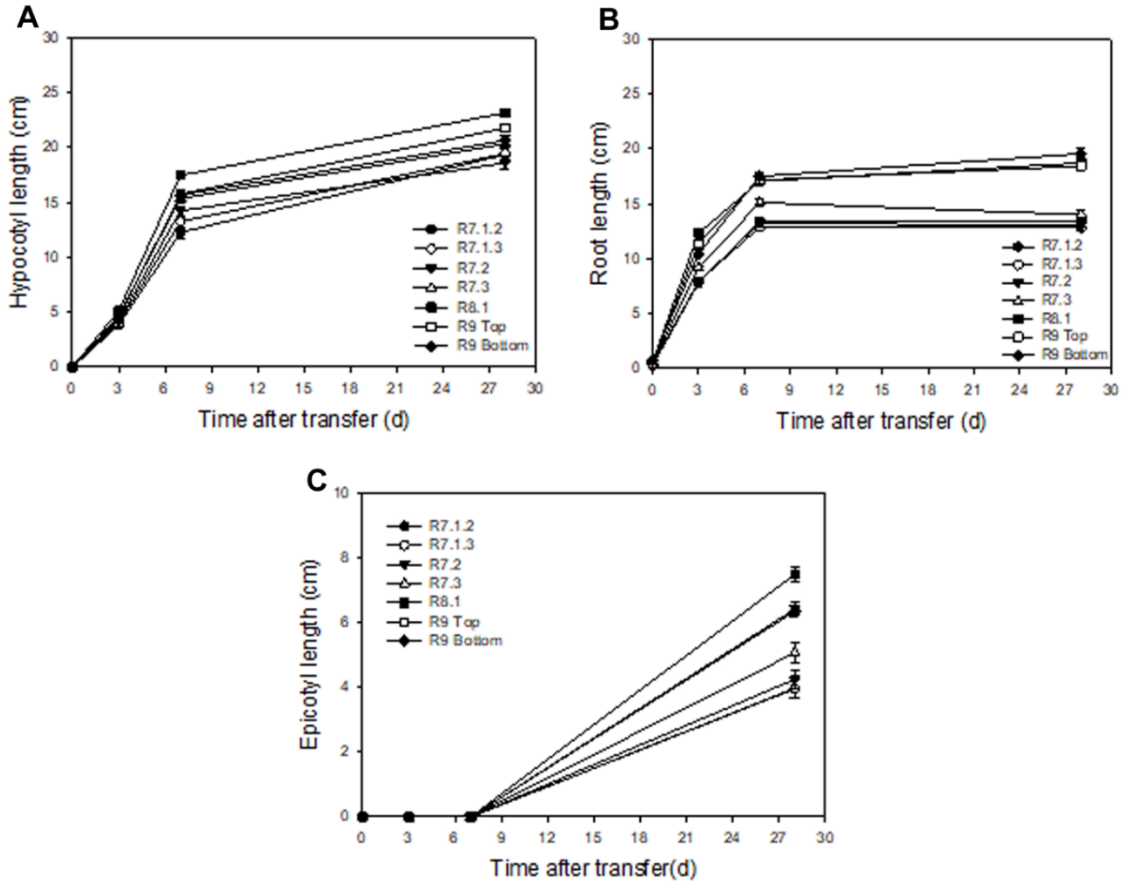
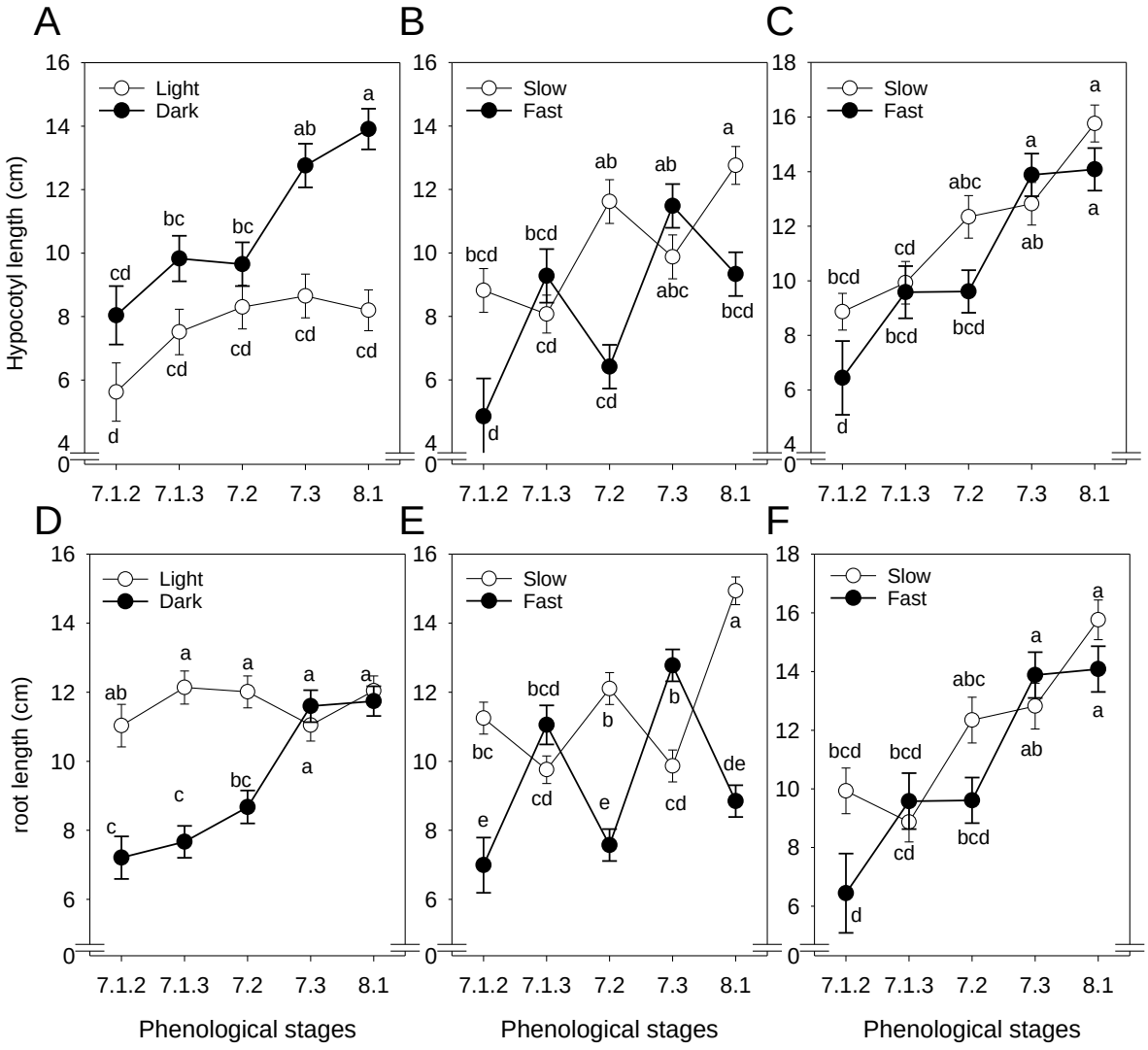


Table 2. Drying rate influences elongation capacity drying maturation. Results of the ANOVA analysis of the effect of drying rates as treatment on hypocotyl and root elongation across all phenological stages of maturation. Comparisons between treatments were performed using the Tukey test. (*) Data for roots measured in darkness were transformed to normalize the data. Diff, difference.

Elongation condition	Organ	Drying rate		Difference	p> Diff
		Slow	Fast		
Dark	Hypocotyl	11.7 ± 0.5	9.9 ± 0.6	1.82	0.023
	Root	10.3 ± 0.3	8.6 ± 0.4	0.55*	0.003
Light	Hypocotyl	8.6 ± 0.3	6.6 ± 0.4	1.98	0.001
	Root	12.3 ± 0.4	10.3 ± 0.3	2.04	0.002

When each treatment was examined separately across developmental stages, there was an effect of maturation on elongation capacity during maturation (Figure 5). In the dark, the length of hypocotyl (Figure 5A) and root (Figure 5D) significantly increased respectively 1.8- and 0.6 fold from stages R7.1.2 to R8.1. However, such difference was no longer apparent when seedlings were incubated in the light. When the drying rate was compared, results were inconsistent (Figure 5 B, C). After slow drying, there was a slight but significant increase in both hypocotyl and root length when stage R7.1.2 was compared with stage 8.1. However, after fast drying, this trend was not found as large variations were observed between stages (Figure 5B, E). Since rolls from stage R7.2 and R8.1 contained 30-55% of abnormal seedlings, we re-examined the data by removing those abnormal seedlings for all the stages before statistical analyses (Figure 5 C, F). In this case, the increase in elongation capacity was clearly detectable, with seedlings from stages R7.3 and R8.1 performing much better after drying compared to younger stages. For example, hypocotyl length after fast drying increased 2.2-fold when seeds developed from stage R7.1.2 to R8.1. For the next experiments, only elongation in the dark was used since it the differences between stages were best highlighted. However, we kept in the analysis the abnormal seedlings since the origin of these seedlings was unknown.

Figure 5. Elongation capacity increases with maturation. A, D) Effect of light during elongation on hypocotyl (A) and root (D); B-E) effect of slow and fast drying regimes prior to germination on hypocotyl (B) and root length (E); C-F) Same as B-E except that only the population that develop into a normal seedling was taken into account. Immature seeds were harvested in 2015 at indicated phenological stages. Organs were measured 7 days after transfer of germinated seeds. Data are the mean (±SE) of 3-to 4 replicates of 10-15 seedlings. When shown, letters indicate significant differences between stages and indicated treatments (P<0.05). Test were performed by ANOVA and posthoc pairwise multiple comparison using the Tukey method.

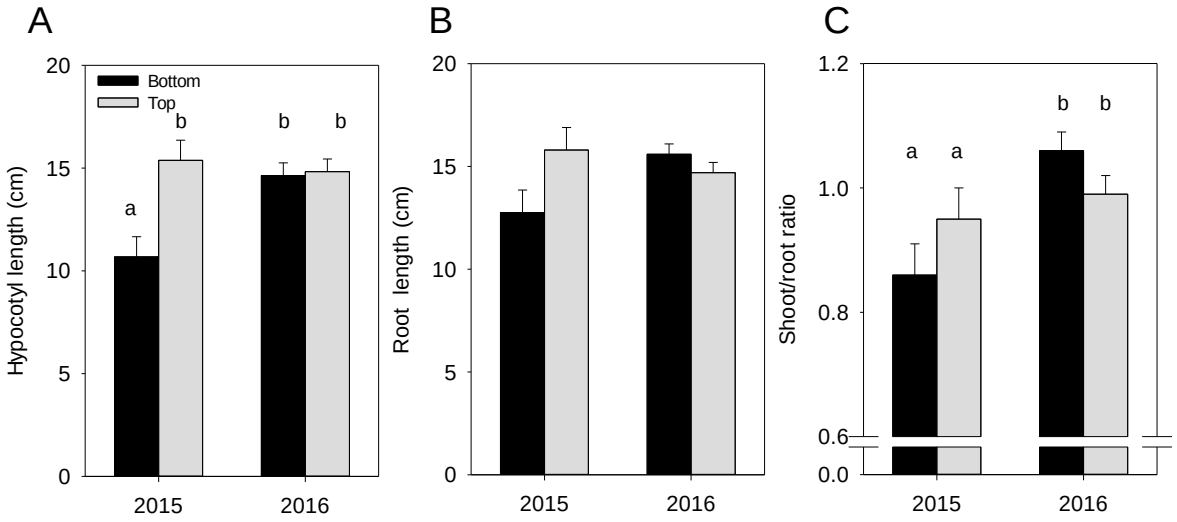


3.3.3 The effect of maturation on seedling elongation from two crop seasons

Whether the crop year and the seed position on the mother plant had an influence on root and hypocotyl elongation was tested in mature seeds at the stage R9 (Figure 6). Seeds were selected from the bottom and top 3 nodes of the mother

plants. No significant difference (P<0.05) in hypocotyl length between seed positions was found for 2016. Likewise, no significant difference between crop years was found for seeds harvested at the top of the plant (Figure 6A). Similar results were found for the root length (Figure 6B). In contrast, hypocotyls harvested in 2015 from the bottom nodes were significantly smaller than those from the top nodes (Figure 6B). A similar trend was observed for roots although differences were not found significant at 5%. The decrease in elongation capacity in bottom seeds of 2015 was attributed to a humid and warm conditions that induced the so-called field “weathering”, characterized by cycles of rehydration/dehydration in dry seeds before harvest (see chapter 1). The shoot/root ratio was not affected by the field weathering, suggesting that each organ is equally affected (Figure 6 C). However, a significant difference was found between years, suggesting that the climate conditions during seed development or the difference in experimental design between 2015 and 2016 may influence the seedling behavior. For the rest of the study, we choose seeds harvested at the top position to characterize stage R9.

Figure 6. Effect of seed position (bottom 3 nodes, black bars; top 3 nodes grey bards) and year of harvest on the elongation of hypocotyl (A) and length (B) and on shoot/root length ratio (C) of dry mature seeds (R9). Organs were measured after 7 days in the dark after transfer of germinated seeds. Data are the mean (±SE) of 4-to 13 replicates of 10-15 seedlings. When shown, letters indicate significant differences between treatments (p<0.05).



To assess whether the culture had an influence on the acquisition of elongation, the experiment presented in Figure 7 was repeated using seeds submitted to fast drying seeds and transfer in the dark after germination. Surprisingly, the data obtained in 2016 did not confirm those of 2015 both for hypocotyls and roots (Figure 7 A and B). The comparison between immature stages shows that the elongation capacity of hypocotyl and root was 2- to 3-fold higher in 2016 compared to 2015. However, in 2016, the capacity of the hypocotyl to elongate was already gained at stage 7.1.2 as it did not increase afterwards (Figure 7). Similar results were found for roots (Figure 7B). Since over 100 seedlings were measured for each stage in 2016, we took the opportunity to check whether there was an interaction with the speed of germination. Indeed, in our experimental design, we kept records of whether the rolls corresponded to the first, second or third selection of germinated seeds, each selection being separated by 17-24 h. As shown in table 3, no significant differences were found between early and late germinators, suggesting that organ elongation is not dependent on incipient germination rate.

Figure 7. Comparison of the crop year on elongation capacity at indicated phenological stages. For hypocotyls (A) and roots (B). Immature seeds were harvested in 2015 (open symbols) and 2016 (closed symbols) and fast dried. Organs were measured after incubation for 7 days in the dark after transfer of germinated seeds. Data are the mean (±SE) of 3-to 4 replicates of 10-15 seedlings. When shown, letters indicate significant differences between stages and indicated treatments (P<0.05). Test were performed by ANOVA and posthoc pairwise multiple comparison using the Tukey method.

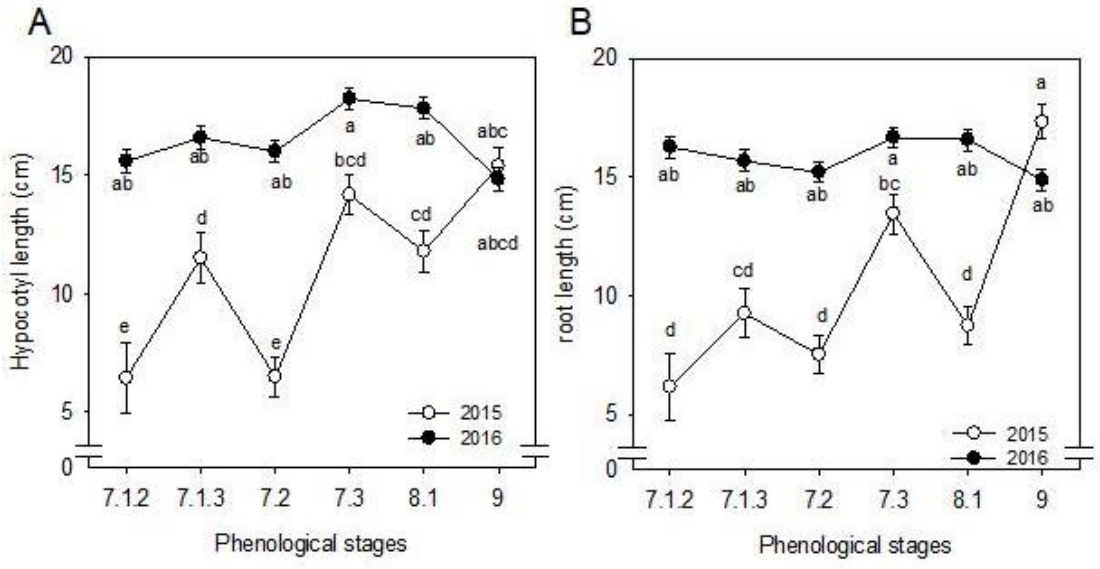


Table 3. Results of the ANOVA analysis of the effect of germination rate as treatment on hypocotyl and root elongation across phenological stages of maturation. Comparisons between treatments were performed using the Tukey test. Diff, difference.

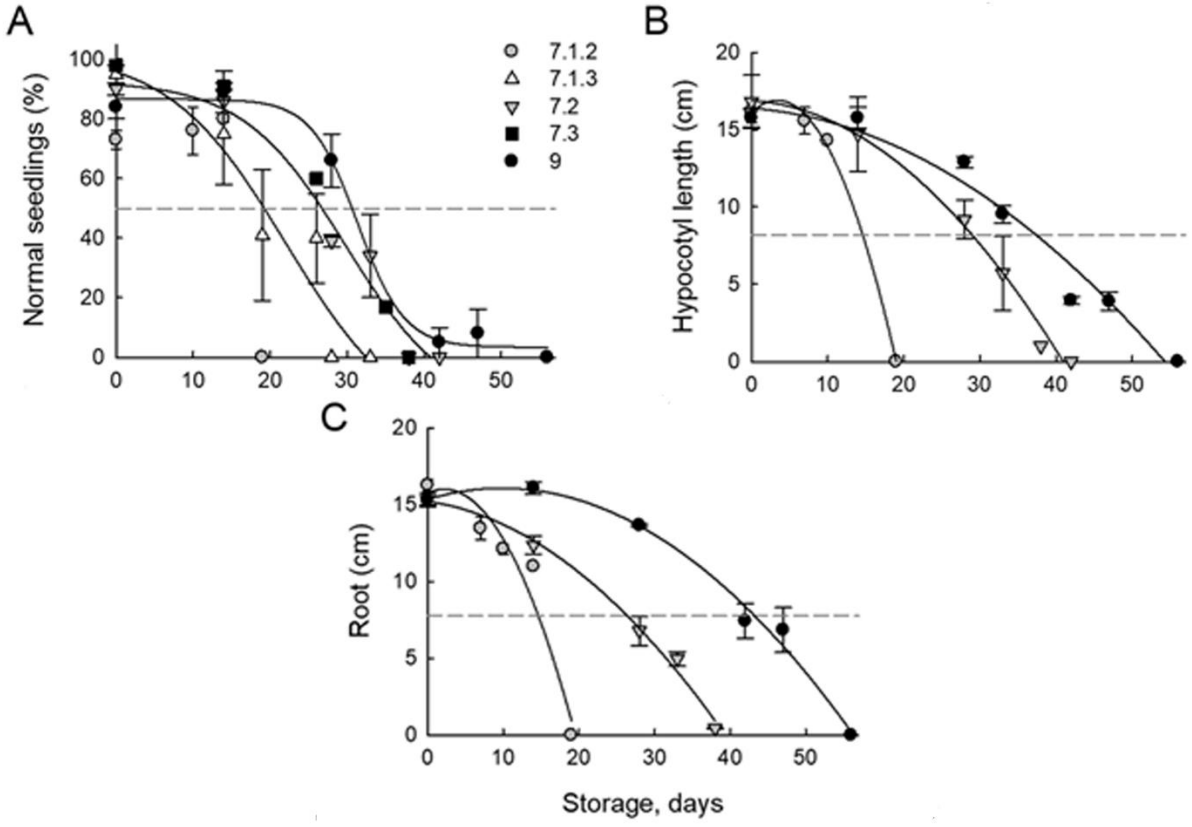
Organ	Early germinators	Late germinators	P>diff
Hypocotyl	16.8 ± 0.4	16.4 ± 0.3	0.389
Root	16.2 ± 0.5	15.6 ± 0.3	0.151

3.3.4 The influence of storage on the elongation capacity during seed maturation

To test whether elongation capacity was affected during storage seeds were stored 75% RH, 35°C. After storage, germinated seeds were transfer for 7 days in the dark before measuring hypocotyl length (Figure 8).

The percentage of normal seedlings decreased progressively during storage and that of abnormal seedlings increased (Figure 8A). 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 8A). Next to abnormal seedlings, the overall elongation capacity was also affected by storage (Figure 8B-C). There was a decline in both root and hypocotyl length during storage, as shown previously by Hartmann Filho et al. (2016) and Yagushi et al. (2014) on mature soybean seeds. The decline was much less steeper for mature seeds compared to immature seeds both for hypocotyls and roots.

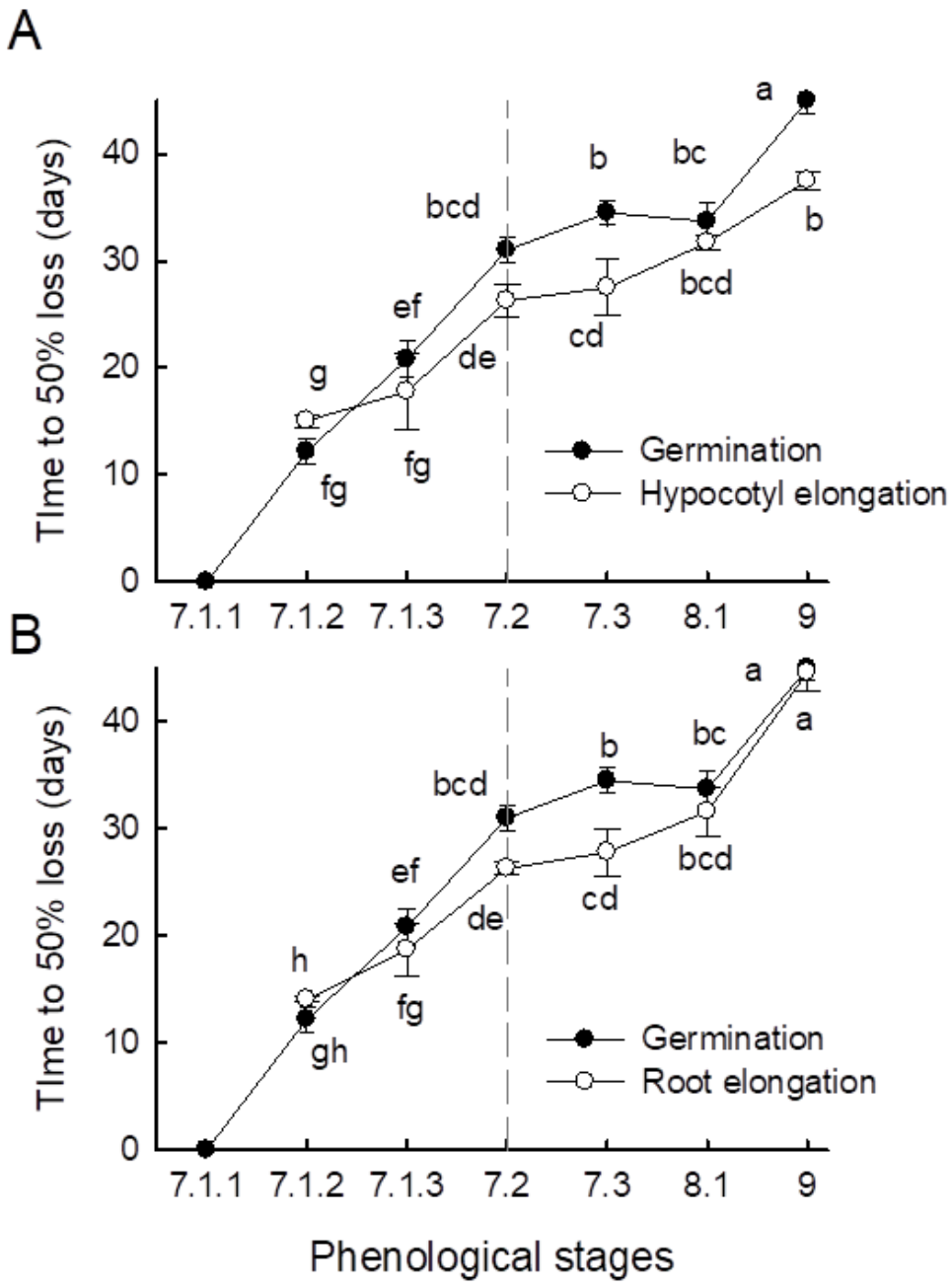
Figure 8. Profiles of the loss of elongation capacity during storage. A) Percentages of normal seedlings. Data were fitted with sigmoid f=y0+a(1+e(-(x-x0)/b)) (adjusted r2 > 0.988) and represent the average of 3 replicates (± SD) of 20-50 individuals before storage and 8-25 seedlings at the end of storage. D-E) Length of hypocotyl (B) and root (C) after 7 days of incubation post-germination in the dark. Data represent the average (± SD) of 3 replicates and were fitted a quadratic polynomial y=y0+ax+bx2, with adjusted R2 between 0.693 and 0.980 for hypocotyls and 0.780 and 0.996 for roots. The broken line represents 50% loss for which the intersection with fitted sigmoid indicates the TH50 and TR50.



From the regression analysis shown in Figure 8, the time to 50% loss of capacity to elongate was calculated and named and T_H50 and T_R50 for hypocotyl and root, respectively (Figure 9). These values were compared to the P50 characterizing longevity (see chapter 1) During seed maturation, P50 increased almost linearly until dry mature seeds. At stage R7.2, 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 R7.2, R7.3 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

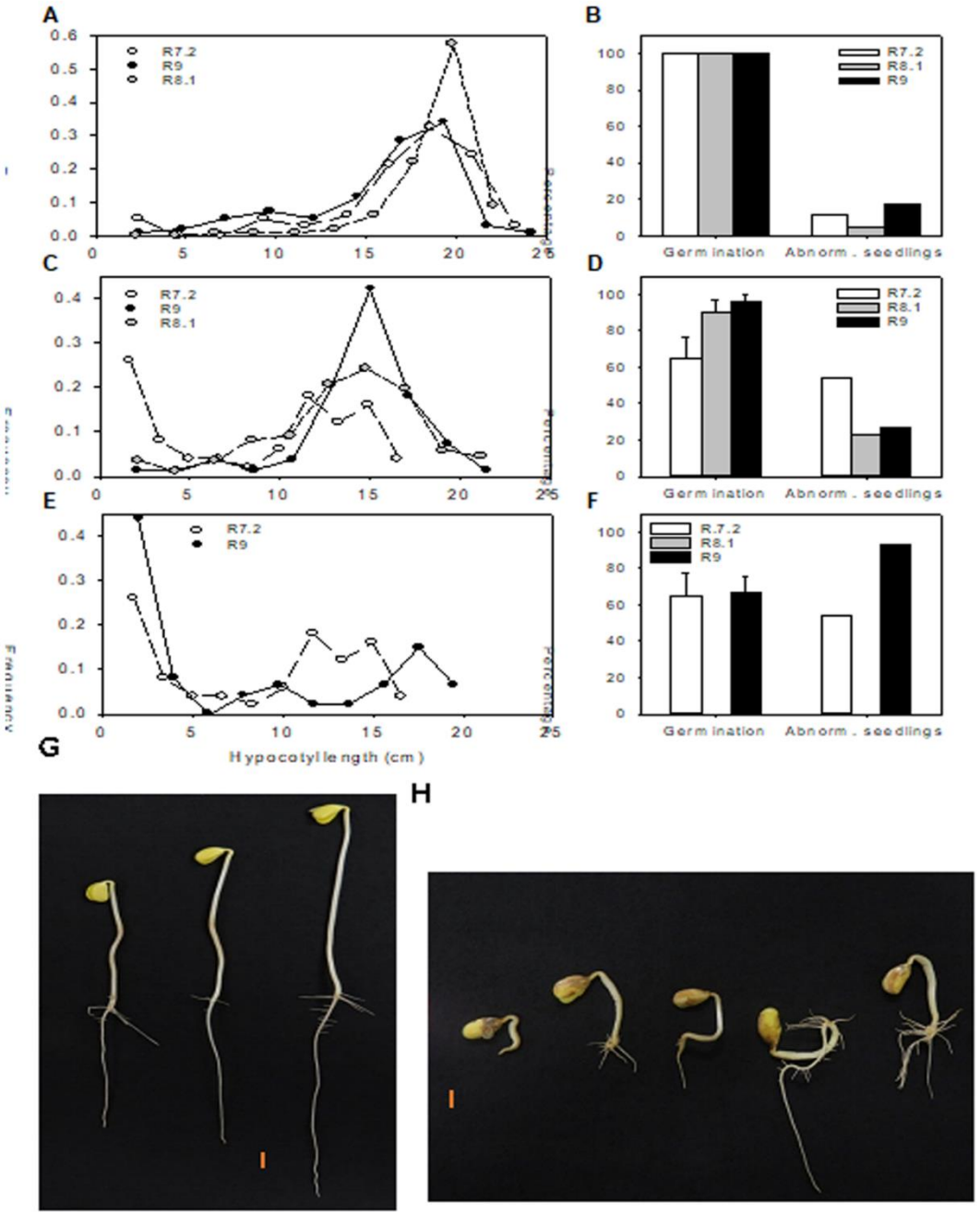
capacity before and after germination. Nevertheless, the results in figure 9 indicate that assessing loss of seed germination during ageing constitutes an easier way to test the effect of ageing compared to the elongation of seedlings.

Figure 9. Comparison of the time to 50% loss of germination and seedling elongation [A – hypocotyl (TH50) and B – root (TR50)] 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 ($p < 0.05$).



We monitored the changes in the frequency distribution of hypocotyl length of several stages together with the % of abnormal seedling during storage of seeds harvested at stage R7.2, R8 and R9. Before storage, the frequency distribution was homogenous, with a peak around 18-19 cm (Figure 10 A). The percentages of abnormal seedlings were below 20% for all stages, albeit significant (χ^2 $p<0.05$), with stage 8.1 exhibiting the least amount of abnormal seedlings. After 28 d of storage, two observations were made on the frequency distribution. Firstly, the peak of distribution decreased to lower values at around 15 cm for stages R8.1 and 9 and to around 12 cm for R7.2. Secondly, very small seedlings with a hypocotyl length below 5 cm became the most preponderant population at stage R7.2. In parallel, the percentages of abnormal seedlings at that stage increased to 50% whereas those of stage R8 and R9 increased slightly but not significantly. To assess whether the increase in the proportion of seedlings with low hypocotyl length could be due to abnormal growth, we compared the frequency distribution of seedlings from stage R7.2 and stage R9 that exhibited a similar survival rate, namely 65% germination (Figure 10 F). At stage R9, nearly all seedlings of stage 9 were abnormal and exhibited a high proportion (0.5) of very small hypocotyls (Figure 10E). Furthermore, this proportion was much higher than that of R7.2 (0.27). The percentage of abnormal seedlings at stage 9 was also significantly higher than at stage R7.2 (Figure 10F). Interestingly, we did not find a significant proportion of seedlings with a hypocotyl with a length between 5 and 10 cm. Therefore, we conclude that storage has a complex effect on elongation, altering first the capacity to grow then inducing abnormality with little growth. The morphological features associated with abnormal seedlings can be seen in the photographs showing deformed growth suggesting an abnormal response to gravitropism and/or absence of growth of the primary root (Figure 10).

Figure 10. Characterization of the population of seedlings of mature seeds (R9) before storage (A-B, G) and during storage at 35°C, 75% RH (C-F, H). A, C, E). Distribution of hypocotyl lengths of indicated stages before storage (A), after 28 days of storage (C) and after obtaining 65% of germination (E), regardless of the time of storage (namely 28 days for R7.2 and 42 for R9). For each stage, the frequency distribution was obtained from a population of 90-100 seedlings. B, D, F) Percentages of germination and abnormal seedlings of indicated phenological stages before storage (B), after 28 days of storage (D) and after obtaining 65% of germination (F), regardless of the time of storage. G-F Photographs showing normal seedlings before storage and abnormal seedlings after 14 days of storage.



3.4 DISCUSSION

Seed vigor is one of the factors that determine the speed and uniformity of seedling and to have high potential for upward shoot growth in soil of increasing impedance. For this, hypocotyl elongation is a crucial process for elevating the cotyledons and epicotyl to the soil surface ensure seedling emergence (FINCH-SAVAGE et al., 2010). Due to the importance of the hypocotyl on seedling establishment, we evaluated the elongation of soybean seedling through the hypocotyl and root length during maturation in two seasons 2015 and 2016.The capacity of elongation of the hypocotyl and root was higher in 2016 compared to 2015, where was already gained at stage R7.1.2 and it did not increase afterwards (Figure 7).

The contrasting result in elongation capacity between 2015 and 2016 is intriguing and was not solved in this work. one likely explanation is the difference in experimental design between both years that may have affected seedling elongation according to the stage of maturation. In 2015, seedlings were put in conditions that might have been stressful. A considerable number of rolls were tightly packed into a small closed environment resulting in a hypoxic condition and perceived as obstacle for growth. In 2016, measures were taken to allow seedlings to grow in sufficient space (see Figure 3). Elongation of the hypocotyl can be reduced by a range of stresses such as cold (PIERRE et al., 2014, YOUSSEF et al., 2016). Ethylene and auxin synergistically regulate plant growth and developmental processes, including root growth, root hair formation, and lateral root development (WANG et al., 2013). Konings and Jackson (1979) reported that a certain amount of endogenous ethylene is necessary for root growth, but that excess ethylene decreased cell elongation in the roots. According to Taiz (2004), injury can induce ethylene biosynthesis, as well as physiological stress caused by anoxia, cold temperature, drought and mechanical wounding. In dark-grown seedlings, ethylene causes inhibition of root growth, short and swelling of the hypocotyls, and aggregated apical hook called, ‘triple response’ (GOESCH, 1975; WANG et al., 2013;). In lupin (*Lupinus albus L.)*, ethylene reduced the length and increased the diameter of hypocotyls by modifying the cell growth pattern, stimulating lateral cell expansion and cell wall thickness, while reducing cell elongation (SANCHEZ-BRAVO et al., 1992). Therefore, we speculate that

conditions used in 2015 and 2016 would have allowed different growth conditions and the release of different amounts of ethylene. Interestingly, in soybean seeds rates in ethylene release decreased as developing seeds progressed in maturity (SAMARAH et al., 2016). In the transcriptome associated with late maturation in soybean seeds, transcripts encoding five different ethylene receptors and two enzymes involved in the biosynthesis of ethylene were strongly up-regulated (LIMA et al., 2017). Therefore, this might explain the discrepancy why maturation had an impact on elongation rate in 2015 but not 2016. Further work is necessary to evaluate whether the acquisition of elongation capacity during maturation strongly depends on the seedling environment.

3.4.1 Seed position on the mother plant does not influence hypocotyl elongation

The results showed that there was no difference between seed position in the mother plant in the hypocotyl and root growth, the shoot/root ratio showed not affected by the field weathering, suggesting that each organ is equally affected. Ahmed et al. (2010) also observed that pod position in three portions top, middle and bottom did not show influence on seed germination, moisture content, seedling dry weight and vigor index in soybean seeds. However, a significant difference was found between years and this was attributed to a field weathering effect. Therefore, environmental conditions may influence the seedling behavior. External conditions around the pod (e.g. temperature, relative humidity, light) and internal characteristics may contribute to position effects on physiological seed attributes. Differences in seed maturity or in time left for seed ageing on the plant may explain differences in physiological quality. Keigley and Mullen (1986) already suggested that seed maturity is an important factor explaining seed position.

3.4.2 The influence of storage on the elongation capacity during seed maturation

Among the several processes by which the soybean seeds have to pass until the next crop, the storage plays a very important role, particularly in Brazilian tropical and subtropical climatic conditions. It is exactly the storage phase that

worries the seed producers who aim at minimizing the speed of the deterioration process (FRANÇA-NETO et al., 2010; DANTAS et al., 2012; RAO et al., 2017). 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 the harvest is delayed (DINIZ et al., 2013). Here, we found that storage induced a decline in the capacity for the hypocotyl to elongate and this decline was less pronounced as maturation progressed (Figure 8). This 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). 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; 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., 2003b). 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; LIMA

et al., 2017). Whether ethylene plays a role in the rate of the loss in elongation capacity warrants further investigation.

3.4.3 Storage has a complex effect on organ elongation

Stored seeds are declared dead when the radicle fails to emerge out of the seed coats and grow in optimal conditions. However, this represents only a part of the success of crop establishment. Other traits such as 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, 2015b).

The percentage of normal seedlings decreased progressively during storage and that of abnormal seedlings increased. Abnormalities included stubby and/or crooked organs often associated with a watery and translucent appearance. As percentage of abnormal seedlings were inferior to 20% for all the stages before storage. After 28 days of storage the R7.2 stage showed 2 fold increase in abnormal seedling than the following stages. However, after obtaining 65% of germination at the R9 stage almost all of the seedlings were abnormal and showed high proportion of small seedlings. Hartmann Filho et al. (2016) also noticed a decrease in the percentage of normal seedlings during storage and drying at temperature ≥ 40 °C when the seed moisture content decreased from 13 to 10%. The progressive increase in abnormal seedling during storage suggests that ageing induces a decreased coordination in growth. Next to abnormal seedlings, the overall elongation capacity was also affected by storage. 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.

Seed vigor depends on the efficiency of the metabolic processes of synthesis, supported by efficient respiratory activities. Therefore, the availability of accumulated reserves during maturation are important since during the deterioration there is the loss of the capacity to use these reserves (MOHAMMADI et al., 2011; LEE et al., 2012; PARRISH and LEOPOLD, 1978). Decreasing the synthesis of polysaccharides, lipids, proteins, nucleic acids, can decrease the production of ATP (FERGURSON et al., 1990; MARCOS-FILHO, 2015). Heterotrophic seedling growth occurs as a result of the utilization and catabolism

of stored reserves such as fats, carbohydrates and proteins. The cotyledons are the source of sucrose, which is exported and translocated via the phloem into the growing regions of the developing seedling (KUTSCHERA, 2000).

Thus, aged embryos may be related to a decline in the activity of various cytoplasmic components, including factors related to the initiation of cell-stretching metabolism. These biochemical changes lead to an increase in the proportion of seedlings with morphological abnormality, the release of solutes and the sensitivity of seeds to pathogenic microorganisms (CRUZ-GARCIA et al., 1995).

Seed aging is a complex process that impacts elongation and can occur via many possible avenues hence it is likely that multiple genes, biochemical pathways and products accumulated during seed development contribute to their vigor. Understanding the mechanisms involved in the loss of quality during seed ageing and the processes involving the reduction of hypocotyl, root and their morphological abnormalities may allow the identification of groups of markers for the assessment of seed vigor.

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CHAPTER 4
HEAT SHOCK FACTOR A2.2 REGULATES GERMINATION QUALITY DURING SEED MATURATION OF *Medicago truncatula*

ABSTRACT

Dormancy and longevity, two complementary seed quality traits are important for agriculture and conservation of biodiversity and also key adaptive traits that contribute to seed lifespan and dispersal. So far efforts are targeted to identify and understand the molecular mechanisms regulating dormancy rather than longevity. Heat Shock Factors are versatile transcription factors that regulate gene expression in response to thermal stress but also to developmental cues. Several members are particular among the HSF class A family in that they are exclusively expressed in the late stage of maturation when seed dormancy and longevity are acquired and regulate desiccation tolerance and accelerating ageing. This work tested whether MtHSFA2.2, a homologue of HSFA9 and hub gene involved in seed maturation plays a role in seed longevity in *M. truncatula*. Storage experiments with seeds of *Mthsfa2.2* insertion mutants did not validate the hypothesis, although several targets of HSFA were down-regulated in mutants. However, it turned out that MtHSFA2.2 is a negative regulator of seed dormancy, acting independently of the maternal environment and likely controlling GA catabolism and ABA sensitivity.

Keywords: Dormancy. Heat Shock Factors. Longevity. *Medicago truncatula.*

4.1 INTRODUCTION

During development, seeds acquire a set of physiological characteristics that are both essential to ensure the dispersion of the species and crucial for the establishment of seedlings in the field. These characteristics include dormancy and seed longevity, which play complementary roles in maintaining the embryo in a protected state to ensure germination under optimal conditions (Reviewed in BEWLEY et al., 2013; LEPRINCE et al., 2017). Both traits vary markedly among plant species with important consequences in plant phenology, establishment in the field, yield and *ex situ* preservation of the genetic diversity. Dormancy is an adaptive

trait that inhibits freshly matured seeds to germinate under otherwise favorable conditions or out of the appropriate season. During storage and/or dispersal, mature seeds progressively lose dormancy over time, a process known as after-ripening. Longevity is defined as the ability to survive for extended periods of time during dry storage for seed crops and in soil seed banks for wild species (Reviewed in SANO et al., 2016; LEPRINCE et al., 2017).

Longevity depends on the ability to undergo complete desiccation without loss of viability. Dormancy and longevity are acquired progressively during seed maturation under the control of the maternal environment and abscisic acid (GRAEBER et al., 2012; MAC GREGOR et al., 2014; ZINSMEISTER et al., 2016). However, the extent to which both traits share the same regulatory pathways is unknown. Obviously, they can be separated since dormancy has been eradicated during domestication in crop species without necessarily impacting longevity. While major efforts are made in understanding dormancy (Reviewed in FINCH-SAVAGE and LEUBER-METZER et al., 2006; GRAEBER et al., 2012), the regulatory mechanisms controlling the acquisition of seed longevity during germination is largely unknown.

Abiotic stress can cause aggregation or denaturation of proteins, which can impede their normal cellular function (JACOB et al., 2017). Plants like other organisms cope with abiotic stresses by producing heat shock proteins (HSP), which play a central role as molecular chaperones for stabilization of protein structures, cellular homeostasis and assistance in the protein refolding of proteins under stress. During maturation, orthodox seeds undergo a substantial loss of water and require protective mechanisms to cope with possible structural damages. There is increasing evidence that the synthesis of HSP might be an important in this respect. In *M. truncatula*, the expression of many genes encoding HSPs were found to correlate with the acquisition of desiccation tolerance and dormancy like *MtHSP70*, *MtHSP90, MtHSP101 and* small HSPs *MtHSP18.2*, *MtHSP17.4, MtHSP17.6* (VERDIER et al., 2013). In developing soybean seeds, the acquisition of seed longevity was correlated with an increase of three sHSP transcripts, *sHSP17.6a*, *sHSP17.6b*, and *sHSP21* (LIMA et al., 2017). In rice, *OsHSP18.2* transcripts increased during the late maturation and were highly abundant in dry seeds (KAUR et al., 2015). Furthermore, Arabidopsis and rice seeds overexpressing *OsHSP18.2* displayed an improved tolerance against deterioration when seeds were stored at

100% RH and 45°C or against osmotic stress during germination (KAUR et al., 2015). In cabbage, the content of protein HSP17.6 that accumulated in dry seeds as found to be positively correlated with germination under water stress and after accelerated ageing (BETTEY and FINCH-SAVAGE, 1998). In tobacco, a heat shock treatment or the over-expression of *NtHSP18.2*, *NtHSP17.6*, and *NtHSP18.3* in seeds overcame the inhibitory effect of light on germination (KOO et al., 2015). Altogether, this suggests that HSP might play an important role in seed vigor.

The main regulators of HSP expression are heat stress transcription factors (HSFs) (BANIWAl et al., 2004; WANG et al., 2004; SCHRAMM et al., 2006). In Arabidopsis and soybean, HSFs represent a family of 21 and 38 members respectively. HSPs are expressed not only in response to environmental stresses (like heat stress) under the control of HSF but are also developmental regulated during seed maturation without stress. In addition, some HSF factors are exclusively expressed during seed maturation and not in response to heat or other stresses in vegetative tissues (KOTAK et al., 2007). Transcripts of several HSF from the class A increase during maturation, concomitant with the acquisition of desiccation tolerance or longevity, as was found for soybean (LIMA et al., 2017), *Medicago truncatula* (VERDIER et al., 2013) and Arabidopsis (KOTAK et al., 2007). A cause-effect relationship between longevity and HSFA has been suggested by overexpressing *HaHSFA9* in tobacco seeds. Transgenic seeds exhibited increased resistance to controlled deterioration and activation of various HSPs (HSP101, sHSP-CI, sHSP-CII, and plastid sHSP) (PRIETO-DAPENA et al., 2006). *HaHSFA9* repression in tobacco seeds using an active repressor version HSFA9-SRDX resulted in the reduction of seed-specific sHSP proteins and a decreased tolerance against controlled deterioration but did not affect desiccation tolerance (TEJEDOR-CANO et al., 2010). Interestingly, in Arabidopsis, HSA9 is regulated by the transcription factor ABI3, involved in ABA signaling pathway and controlling desiccation tolerance (KOTAK et al., 2007).

In *M. truncatula* the construction of a gene regulatory network of transcription factors preferentially expressed in seeds and concomitantly with the acquisition of desiccation tolerance or longevity during seed maturation resulted in 3 modules characterizing the desiccation tolerance, longevity and abscission (VERDIER et al., 2013). Interestingly in this network *MtHSFA2.2,* the putative ortholog of HSFA9

was found as the connecting node between the longevity and desiccation tolerance module. Furthermore, in our laboratory a transcriptome analysis of *M. truncatula* roots overexpressing 35S::*MtHSFA2.2* revealed an enrichment in transcripts coding HSP20-like, HSP70 and HSP17.II (a target gene of HSFA9 identified by Kotak et al., 2007) as well transcripts encoding galactinol synthase, responsible for the synthesis of RFO and a couple of transcripts involved in ABA and gibberellin metabolism (ZINSMEISTER et al., unpublished data).

The objective of this work was to investigate the role of HSFA2.2 from *Medicago truncatula* and HSFA9 from Arabidopsis in the regulation of seed vigor. For this purpose, we obtained and characterized mutant seeds from both species. Since the role of HSF and their targets, HSP are central to heat response of cells. In Medicago, heat stress reduces seed longevity (RIGHETTI et al., 2015). Therefore, the effect of high temperatures on seed maturation was also studied in *Mthsfa2.2.* seeds. The corresponding mutants were characterized for seed quality characteristics including dormancy and longevity as well as response to phytohormones. Our results suggest the implication of MtHSFA2.2 as a negative regulator of but not in longevity in *M. truncatula* whereas HSFA9 is not critical to expression of seed vigor in Arabidopsis.

4.2 MATERIAL AND METHODS

4.2.1 Plants material and growth conditions

4.2.1.1 Identification of mutants Mthsfa2.2 *in* M. truncatula

Two independent Tnt1 insertion lines in *M. truncatula,* NF10440 (*Mthsfa2.2-1*) and NF13157 (*Mthsfa2.2-2*) in the R108 background were ordered via the Noble Foundation. Homozygous lines were screened by PCR with the primers listed in Table 1. For each allele wild type control plants were simultaneously selected based on the absence of the Tnt1 insertion in the HSFA2.2 gene and were called associated wild type. The *Mthsfa2.2-1* line was backcrossed once with the pollen of the wild type R108, and the segregating lines were screened to select *Mthsfa2.2-1_BC1* homozygous mutants (named thereafter *Mthsfa2.2-1*) as well as a control line wild type for *HSFA2.2,* named associated WT (aWT).

4.2.1.2 Identification of hsfa9 *mutants in Arabidopsis*

Three alleles of Arabidopsis *HSFA9* (At5g54070) were ordered from the Salk institute: Salk_054859, Salk_062453 N562453 and Salk_108809 (N608809). Homozygous lines were screened by PCR. The T-DNA left border sequence was used for PCR amplification with LBb1.3As primer available from the T-DNA express website for Arabidopsis mutants together with genes specific primers (Table 1). Mutants were identified as homozygous for only one allele SALK_062453.

Table 1. List of primers used for experiments performed by PCR (Tnt1 insertion selection). The primers were designed with the help of the Blast Primer Software.

(to be continued)

Species	Mutant allele/ Gene	Gene number	Forward (5'-3')	Reverse (5'-3')
Medicago truncatula	Mthsfa2.2-1	Medtr4g126070	GTTACGACGGTGAAATCGGTG	GAGCTGGCGAATGAAGCTAGA
	Mthsfa2.2-2	Medtr4g126070	CGGTGGTTACGACGGTGAAA	ATAGGTGTTGAGCTGGCGAA
	MtGOLS2	Medtr1g084670.1	AGTGGTTGCAGTGTTACCTGA	ACTGAGTTTGGTTTTCTGGAGGA
	MtHSP70	Medtr4g130540.1	TGCTGGTGCTATTTCGGGTT	GCACGTTCTGTTCGCCTTTT
	MtHSP18.2	Medtr4g091590.1	CCAAGTTTCTTTGGTGGCCG	GCAGAAAGTGCAGAATTGGGA
	MtHSP17.5	Medtr5g064060.1	CTTCCCTACCACCAACGAAA	GCGATGCCAGGTATCATTCT
	MtLEA EM1	Medtr7g069250	TGGGAACAAAAGGAGGGCAG	AGCACGTTGTTCACCAGACT
	MtHONSU	Medtr3g068200	ACTTCGGTGTTTTCGACGGT	CGAGCAAAGCCTTGTTGCAT
	MtABA8ox	Medtr8g072260	TTGGAAATGGGGTCCATGCATGTC	TCCGTTCTTTTCACCGACCACA
	MtGA2ox1	TC120578/Medtr2g070870	TGGTCCTGCAAATCCATTTGGG	CCAACATCACCATTGGGTCCAATC

Table 1. List of primers used for experiments performed by PCR (Tnt1 insertion selection). The primers were designed with the help of the Blast Primer Software. (Continuation)

Species	Mutant allele/ Gene	Gene number	Forward (5'-3')	Reverse (5'-3')
Arabidopsis thaliana	MtGA2ox2	TC11927311/ Medtr4g096840	AGGGTAAA TCACTACG CGGCATGT	GGTCAGTA TGCTCCCC AAATCCAA
	MtGA3ox1	TC131874	AGATCACC CTCTGCAC CTTCATCA	TGCATGG GAGTCAG GCAAAGTT
	SALK_05485 9	At5g54070	TTATCGCCT ATGTAACC GTCG	GGAGGGA AGAAACAT TTGCTT
	SALK_062453	At5g54070	TCCTCTTCG CTTTGTCAA GAG	GCAAAACC GGTAACAT AGCAA
	SALK_108809	At5g54070	TTATCGCCT ATGTAACC GTCG	GGGTTTTA AAAAGGTC GATTCA
	ACTIN 11	Medtr2g008050	GCTGACCG TATGAGCAA GGA	TGCCAAGA TAGACCCA CCAA
	MSC27 (Mt TCTP)	Medtr2g436620	GTTGAAGTA GACATTGG TGCTAAC	AGCTGAGT CATCAACA CCCTCAT

4.2.1.3 Growth conditions

Plants of *Medicago truncatula* were grown in pots containing a sterile mix of vermiculite (10 plants/pot) at 20°C/19°C, with a 16 h light photoperiod at 200 µmol photons m $^{-2}$s^{-2}. To impose a heat stress during seed development, some plants were transferred to a growth room at 26°C with similar light conditions when 10 flowers had appeared. At the abscission stage the pods were dried for 3 days at a relative humidity of 44% generated by saturated solution of K_2CO_3 at 20°C. The seeds were manually removed from the pods and stored at 4°C or -20°C if not otherwise indicated. *Arabidopsis* plants were grown at 20°C/18°C with a photoperiod of 16 hours. The seeds were harvested at maturity and then dried for three days at 44% RH, generated by saturated solution of K_2CO_3 at 20 °C.

4.2.2 Physiological tests

4.2.2.1 Germination test

Mature seeds of *M. truncatula* produced in the two growth environments (20°C and 26°C) were scarified with sandpaper in order to remove hard seediness. Subsequently, 3 replicates of 30 seeds each were imbibed onto a filter paper moistened with 4 mL of water and placed in Petri dishes at 20°C in the dark (CHATELAIN et al., 2012). For *A. thaliana* 3 replicates of around 200 seeds were imbibed onto a filter paper with 1 mL of deionized water in 45 mm Petri dishes in a photoperiod of 16 hours light at 20 °C. For both species, the germinated seeds were scored daily by counting seeds having an emerged radicle with length ≥ 1 mm.

4.2.2.2 Stratification

To release dormancy prior to germination tests, scarified seeds of *M. truncatula* were imbibed at 20 °C for 4 h in Petri dishes onto filter papers then transferred at 4 °C for three days in the dark. For *A. thaliana,* seeds were imbibed for 3 days directly at 4°C in the dark.

4.2.2.3 Longevity assay

Scarified seeds of Medicago and mature seeds of Arabidopsis were placed over a saturated solution of NaCl (75% RH) at 35°C in hermetically sealed boxes for different time spans, for *M. truncatula* the seeds were scarified before. Thereafter, they were tested for germination to assess their viability as described above. Longevity was expressed as P50, defined as the time (days) at which the stored seed lost 50% of viability during storage. For *M. truncatula* after storage, seeds were imbibed at 20°C for 5h then transferred to 4°C for 3 days to release dormancy. The 5h warm imbibition was performed to avoid imbibitional damage that could otherwise occur.

4.2.2.4 Fluridone sensitivity

Mature seeds of *M. truncatula* were imbibed in a 10 µM fluridone solution freshly prepared from a 1 mM stock solution where fluridone was dissolved in ethanol and Tween 20 solution (2/1 vol/vol).

4.2.2.5 Abscisic acid sensitivity

Mature seeds were imbibed on moist filter paper on a range of mixed isomers of ABA (Sigma-Aldrich) at concentrations of 1, 5 and 10 µM prepared from a stock solution of 500 µM dissolved in methanol The control condition consists of germinating the seeds in a methanol solution of equivalent concentration.

4.2.2.6 Soluble sugar assay

Three replicates of 30 seeds were lyophilized and dry weight was determined gravimetrically. Seeds were ground in a mortar to a powder, weighted and used for the extraction in the presence of 1 ml 80% methanol containing melizitose as the internal sugar standard. After heating at 76°C for 15 min, the liquid was evaporated under vacuum. The residue was dissolved in 1 ml distilled water and centrifuged for 1 min at 13 000 g. Sugars were analyzed by HPLC on a Carbopac PA-1 column (Dionex Corp.) as described by Hoekstra et al. (1994).

4.2.2.7 Extraction of genomic DNA

Leaves were placed in 2 ml eppendorf tubes with 2 glass beads and milled with a Tissue Lyzer II ball mill (Qiagen). The obtained frozen powder was homogenized with 600 µL of extraction buffer (100 mM tris-HCl, 20 mM EDTA, 20 mM NaCl, 2% CTAB, pH 8) supplemented with 1% sodium bisulfite and heated to 65 °C immediately prior to use. The lysate was then heated at 65°C for 10-15 minutes then mixed with 600 µL chloroform. After mixing, the homogenate was centrifuged for 15 min at 10000g and the aqueous layer was taken to a new tube. DNA was precipitated with 1/10 volume of 3M sodium acetate and 1 volume of isopropanol. After centrifugation (15000g for 30 min at 4 °C), the pellets were washed with 70% cold ETOH, centrifuged 15000g for 10 min at 4 °C and then the dry pellets were re-suspended in 50 µL milliQ H_2O.

Table 2. PCR Mix and details of programs used for PCR.

Reaction components	[ ] Initial	[ ] Final	Volume per reaction (µL)	Thermocycler:	
H_2O			12.8		
Buffer DreamTaq	10 X	1 X	2	Program:	
DNTPs	2.5 mM	125 mM	1	95 °C	2 min
Primer- Foward	10 µM	0.5 µM	1	95 °C	30 sec
Primer- Reverse	10 µM	0.5µM	1	x °C	sec
DreamTaq Polymerase	(5 u) / µL	0.05 u /µL	0.2	72 °C	sec
				35 X	
DNA			2	72 °C	5 min
				10 °C ∞	

4.2.2.8 PCR of genotyping

For the genotyping of the different mutants, PCRs were performed in a final volume of 20 µl, according to the parameters described in table 2, PCR products were observed after 20 min migration at 135 Volts in a 1% agarose, 0.5% TAE supplemented with SYBR Safe (Invitrogen) DNA intercalating agent.

4.2.2.9 RNA extraction

RNA extraction was performed on 3 replicates of 30 seeds that were ground in liquid nitrogen using mortar and pestle. Total RNA was extracted using the NucleoSpin RNA Plant Kit (Macherey Nagel) according to the manufacturer instruction. The amount of RNA extracted was verified by Nanodrop ND1000 spectrometer.

4.2.2.10 Quantitative real time RT-qPCR

For sample preparation for RT-qPCR were used seeds of *Medicago truncatula* that were freshly harvested (dormant), and after 3 months of post maturation (non-dormant), both in mature dry seeds and after 6h of imbibition.

The cDNAs synthesis was carried out with the kit iScriptTM Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, Inc) according to the manufacturer instruction. Briefly, 1 µg of total RNA was mixed with 4 µl of iScript RT Supermix and supplemented with nuclease-free water up to 20 µl. The reverse

transcription was performed using Biorad thermocycler with a cycle of 5 minutes at 25 °C, then 20 min at 46 ° and a final of 1min at 95 °C cycle to inactivate the enzyme. For the qRT-PCR, the cDNA obtained was diluted 6-times. qPCR was performed with SsoAdvanced ™ Universal SYBR® Green Supermix (Bio-Rad Laboratories). Before gene expression analysis, primers efficiencies were determined with a dilution range of 1, 1/5, 1/10, 1/20, 1/40 and 1/80 of the diluted cDNA solution obtained from wild type seeds. Primers with and efficiency of 100% +/- 15% were retained for further analysis. The real-time apparatus was a CFX96 Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA). The reference genes were *MSC27* (Medtr2g436620 - Translationally-controlled tumor protein (TCTP)) and *ACTIN11* (Medtr2g008050 - Actin-related protein 4A) commonly used in the laboratory for *M. truncatula* qRT-PCR analysis (ZINSMEISTER et al., 2016). The relative expression (RE) was normalized with the geometric mean of the 2 reference genes previously mentioned and was calculated according to the following formula: $RE= 2^{\wedge \Delta Ct}$ (where ΔCt = geometric mean of the Ct reference genes - Ct target gene) with Ct is the value of the detection cycle of the transcript. Each point represents the average of three independent biological replicates of 30 seeds each.

4.2.3 Statistical analysis

An ANOVA test was performed for seed quality experiments and gene expression data at the level of 5% ($p \leq 0.05$). The average was compared by post-hoc Student-Newman-Keuls.

4.3 RESULTS

4.3.1 Identification of *MtHSFA2.2* in *M. truncatula* as the ortholog of HSFA9

In order to identify the ortholog of Ha*HSFA9* in *M. truncatula*, we performed a phylogeny reconstruction using homologous HSFA protein sequences that were retrieved from *M. truncatula, Glycine max* and *Lotus japonica* databases using a blast of the HSFA9 from sunflower and *A. thaliana* (ALMOGUERA et al., 2002; KOTAK et al., 2007). Using ClustalOmega, we aligned a curated list of amino acid

sequences including Medtr4g126070 described in VERDIER et al., 2013, several members of class A HSF from *Lotus japonicas, M. truncatula,* soybean (that were found associated with longevity, LIMA et al., 2017) and two monocot HSFA2 proteins, TaHSFA2 and OsHSFA2, also strongly expressed in seeds (CHAUHAN et al., 2011, 2013). The phylogeny reconstruction performed using maximum parsimony shows that Medtr4g126070, annotated in the database as MtHSFA2.2, is part of a subgroup containing AtHSFA9, HaHSFA9 and GmHSFA9.1 and GmHSFA9.2 (Figure 1, red box). We therefore focused further investigation on the role of MtHSFA2.2.

Figure 1. Maximum parsimony analysis of HSFA proteins involving 47 amino acid sequences and showing orthologs of HaHSFA9 (delineated in red) from legumes (*Glycine max, Medicago truncatula, Lotus japonica* and *Arabidopsis thaliana*). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates (1000) are collapsed. The tree was obtained using the Subtree-Pruning-Regrafting algorithm using MEGA7. For soybean the two paralogs are shown when appropriate.

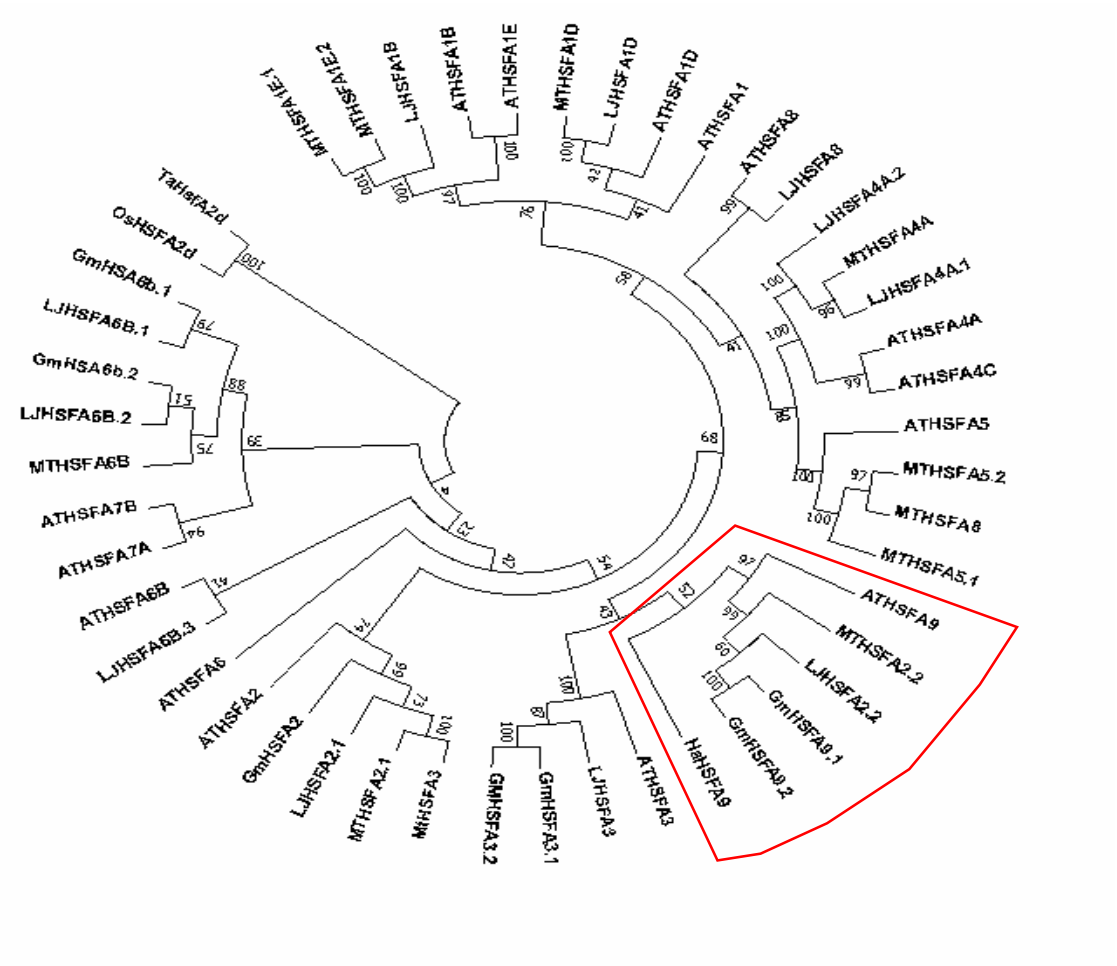
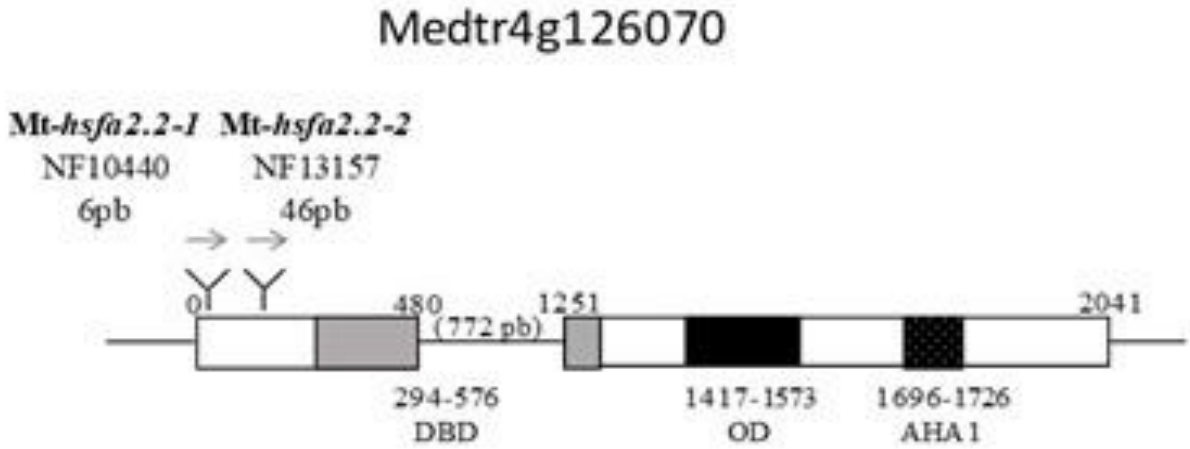


Table 3. Two-way ANOVA of longevity in *Mthsfa2.2* mutants using P50 values calculated from 3 survival curves for each treatment. Survival curves during storage were fitted with sigmoidal equations using 5 data points implicating 30 seeds.

Treatment	F value	P value
Genotype	21.563	< 0.001
Temperature during development	226.254	<0.001
Genotype X Temp. interaction	5.275	0.01

Figure 2. Schematic representation of the MtHSFA2.2 gene (Medtr4g126070). The coding sequence is indicated in rectangle, the intron in line. The positions of the Tnt1 insertions are indicates as well the DBD, DNA binding domain (DBD); the oligomerization domain OD and the AHA1 domain.



Source: Zinsmeister et al., unpublished data.

Table 4. Average number of days after flowering to reach pod abscission and seed dry weight at maturation in the different genotypes of *M. truncatula* cultivated at 20 and 26 °C. DW values are the average of 5 replicates of 50 seeds. DW, dry weight; DAF, days after flowering; aWT, associated wild type.

Genotype	20°C		26°C	
	DAF	DW (mg/seed)	DAF	DW (mg/seed)
WT	43	3.74 ± 0.14	24	3.32 ± 0.08
Awt	43	3.56 ± 0.10	24	3.22 ± 0.20
Mt-hsfa 2.2-1	43	3.75 ± 0.12	25	3.14 ± 0.12
Mt-hsfa 2.2-2	44	3.69 ± 0.18	25	3.03 ± 0.04

4.3.2 Isolation of two independent lines of *Mthsfa2.2*

Two segregating T0 lines for *Mt-hsfa2.2* identified in the *Tnt1* insertion mutant bank of he Samuel Noble Foundation were ordered and homozygous mutant lines were obtained by PCR characterization. The two alleles *Mthsfa2.2-1* and

Mthsfa2.2-2 have respectively *Tnt1* insertions at 6 and 46 base pairs of the start codon corresponding to the first exon (figure 2). In both cases, insertions were located before the DBD motif and the AHA1 motif that is essential for the transcriptional activity of class A HSFs (KOTAK et al., 2004) (Figure 2). The mutant allele *Mthsfa2.2-1* had 38 additional insertions whereas 27 other insertions were reported in the *Mthsfa2.2-2* allele (data not shown). Backcrossing of the *Mthsfa2.2-1* allele was carried out with the wild-type pollen in order to reduce the number of off-target insertions.

4.3.3 Physiological characterization *Mthsfa2.2* mutants

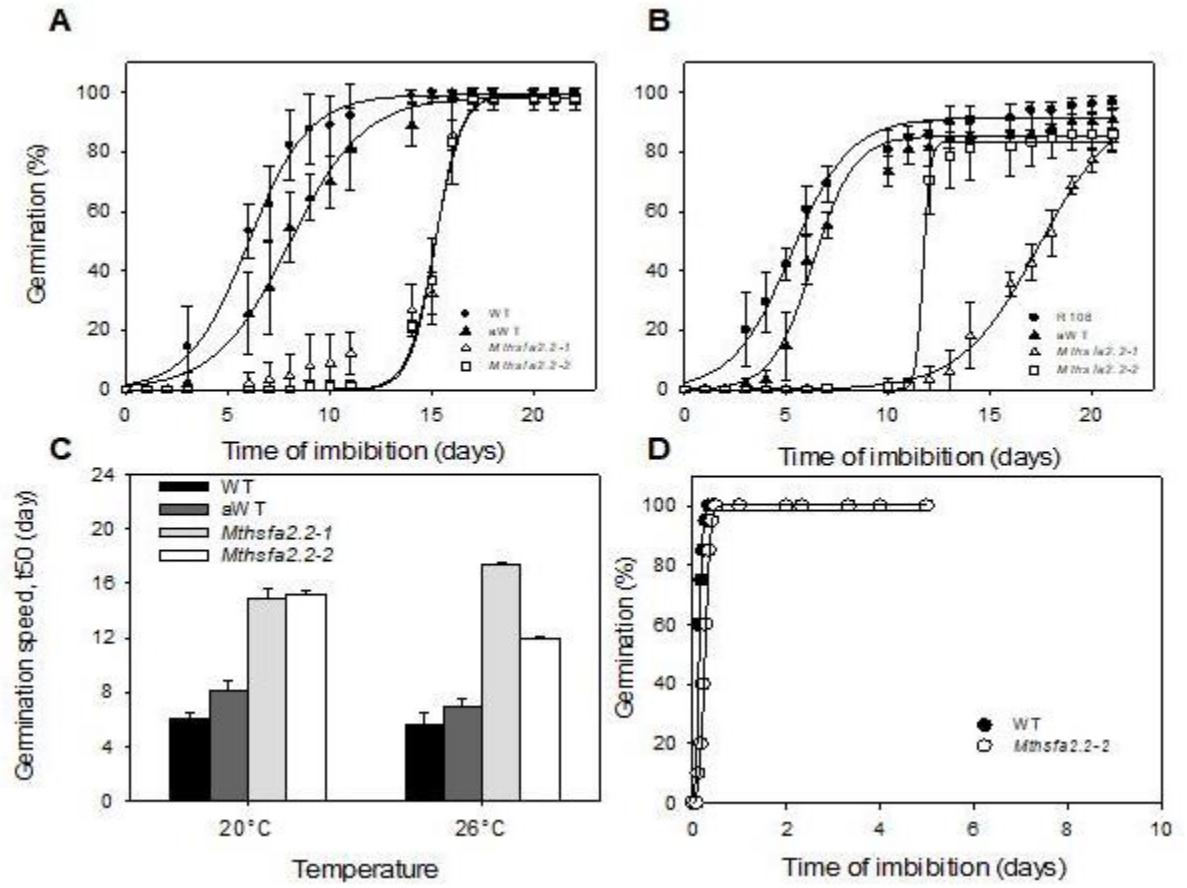
Expression levels of *HSFA2.2* increase during the acquisition of seed longevity in developing seeds (VERDIER et al., 2013). In *M. truncatula*, seed longevity is negatively impacted when developing seeds are exposed to a heat stress (RIGHETTI et al., 2015). We investigated if *MtHSFA2.2,* being developmentally regulated, could also contribute to the response to heat during development. Seeds of the two *MtHhsfa2.2* alleles and the associated wild type (aWT) and the wild type (R108) were grown simultaneously at 20°C (standard condition) and 26°C (high temperature condition). Flowers were tagged at the pollination stage to assess the length of seed development until abscission. Table 4 shows that seed development was shortened at 26°C compared to 20°C for all genotypes. Pod abscission at 20°C and 26°C occurred respectively at 731-748°days and 552-575°days. Likewise, seed filling was significantly impacted because heat-treated seeds were lighter than those grown at 20°C (Table 4). Total number of seeds/plant was also affected by the transfer to 26°C, indicating that this temperature is stressful for the fecundation.

A physiological characterization of the WT and *Mthsfa2.2 mutant* seeds was performed on seeds harvested at the pod abscission stage. First, we examined the germination of freshly harvested seeds obtained at 20°C and 26°C that were imbibed under optimal conditions (Figure 3). WT seeds germinated to 100%. As found previously, scarified wild type seeds take approximately 10 d to germinate, reflexing physiological dormancy of the seeds as found previously (BOLINGUE et al., 2010). No significant difference in the final percentage of germination was found between the *Mthsfa2.2* and wild type seeds, regardless of the temperature. In contrast, the

Mthsfa2.2 mutant seeds germinated slower than the wild type seeds, reaching final percentage of germination at around 17 days after imbibition (Figure 3A-B).

To highlight differences in germination behavior, Figure 3C shows the germination speed measured as t50 (time required to obtain 50% germination in days). Wild type seed lots exhibited a t50 around 6 and 8 days, for 20 and 26°C, respectively. For the *Mthsfa2.2* seeds, t50 was 15 days from lots coming from plants cultivated at 20°C for both *hsfa2.2* alleles and 12 days (*Mthsfa2.2-1*) or 17 days (*Mthsfa2.2-2*) from plants grown at 26°C. Considering that Medicago seeds can germinate within 1.5 days (BOLINGUE et al., 2010), this suggests that *Mthsfa2.2* seeds are more dormant than wild type and this effect was not dependent on the growth temperature. To verify this, seeds were stratified to release dormancy (Figure 3D). When imbibed at 4°C for 3 days prior to the germination assay, both wild-type and the *Mthsfa*2.2 mutant seeds fully germinated after one day, although slight differences still existed between the wild type seeds and *hsfa2.2* mutants (Figure 3D). The increase in germination speed from a t50 of 12 days to <1days after stratification demonstrates that the mutant seeds exhibit a much more profound physiological dormancy phenotype compared to wild type seeds.

Figure 3. Germination curves of freshly harvested mature seeds grown at 20°C (A) and 26°C (B). Seeds were incubated at 20°C in the dark. Data points were fitted with sigmoidal curves. C) germination speed (t50) calculated from individual sigmoidal curves obtained for each replication. D) Germination curves of stratified seeds. Stratification was carried out during imbibition for 3d at 4°C prior to incubation at 20°C. Data are the mean of 3 replicates of 30 seeds each. Data represents the mean of 3 replicates of 30 seeds and error bars represent the standard error. Statistical analysis was carried out using the t-test and differences were considered as significant at $p < 0.05$.



4.3.4 Role of HSFA2.2 in seed longevity

To determine whether MtHSFA2.2 is involved in seed longevity, we performed an aging test by storing mature seeds at 75% RH and 35°C. Longevity was then determined by assessing the P50 (the storage time needed to lose 50% of viability) at different storage interval from 0 to 38 days. There is no significant difference between P50 of *Mthsfa* 2.2 seeds and wild-type seeds coming from plants grown at 20°C (Figure 4). The mean P50 was 21.3 days for the wild type (R108), 26.7 for aWT, and 22.3 and 24.5 days for the *Mthsfa2.2-1* and *Mt-hsfa2.2-2* respectively. Seed lots that were produced at 26°C displayed significantly higher P50 for all

genotypes compared to 20°C, reaching 27.1 and 33.1 days for the WT and aWT, respectively, and 33.6 and 32.4 days for the *Mthsfa2.2-1* and *Mthsfa2.2-2* mutants, respectively (Figure 4).

Altogether these results show that high growth temperature slightly improves seed longevity but that HSFA2.2 does not play a role in the acquisition of longevity.

Figure 4. Longevity of WT and *Mt-hsaf2.2* seeds harvest from plants grown at 20°C and 26°C. Longevity was expressed as P50, corresponding to days of storage at which the seed lost 50% viability. Data are the mean of 3 survival curves established with 5 data points during storage involving 30 seeds each. Bars represent the standard error. Different letters indicate significant differences between genotypes for each temperature treatment using ANOVA and post-hoc Student-Newman-Keuls comparisons ($p<0.05$).

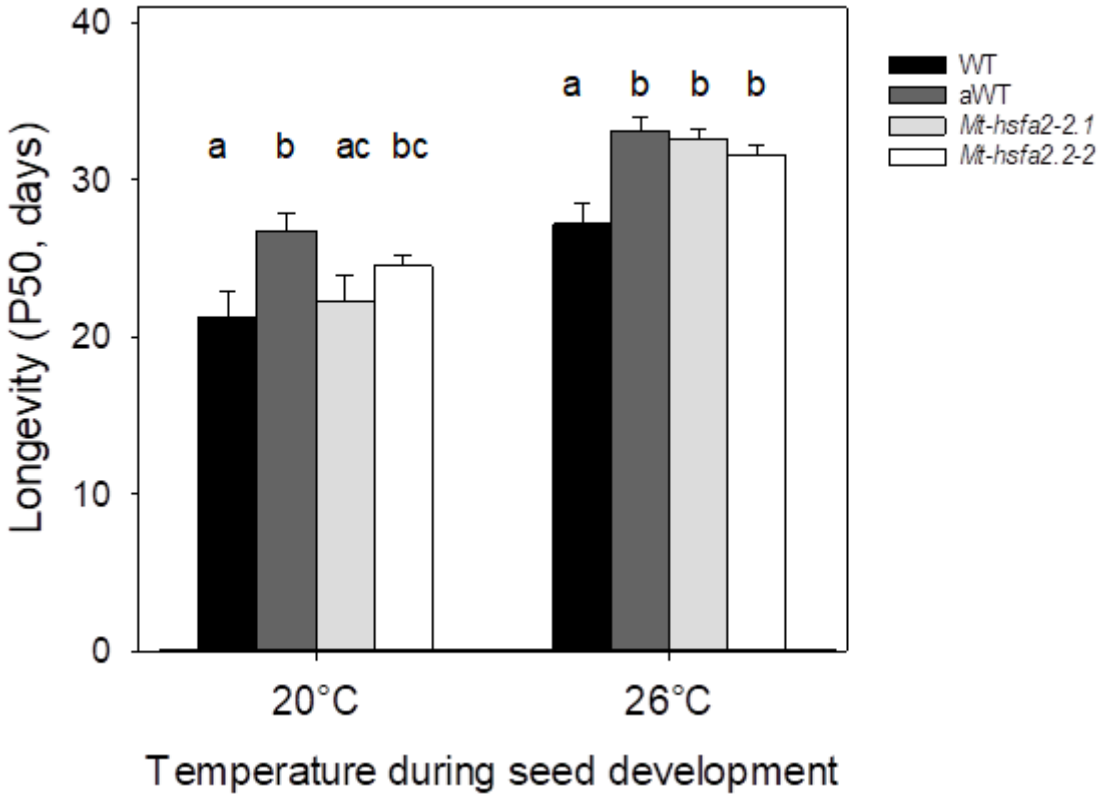
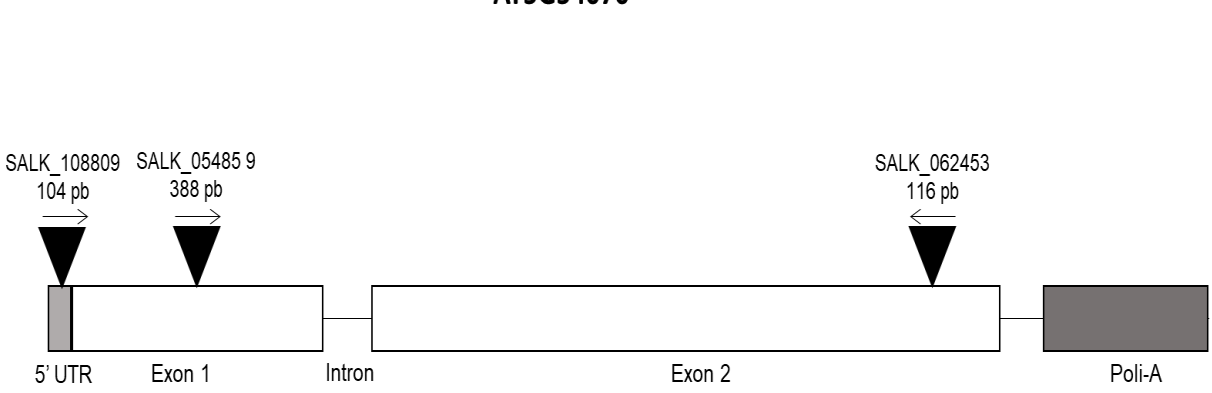


Figure 5. Schematic representation of the AtHSFA9 gene (At5g54070). The coding sequence is indicated in rectangle, the introns in line. The arrows represent the positions of the t-DNA insertions.



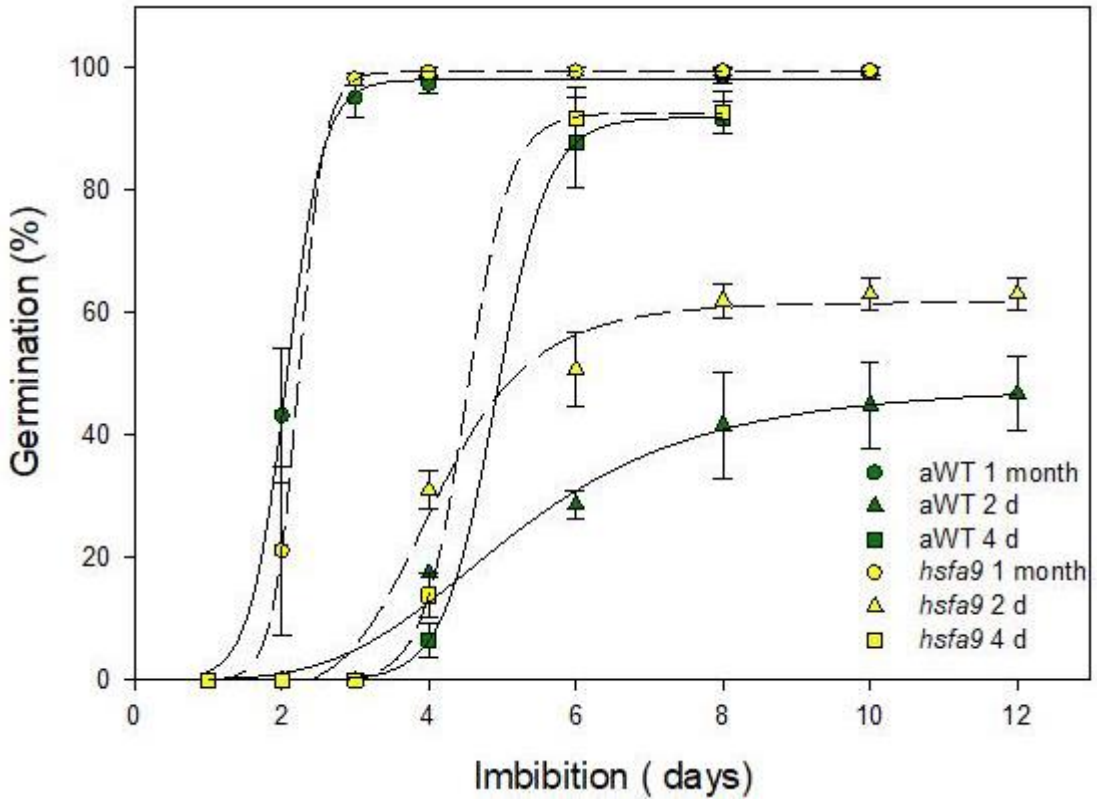
4.3.5 Identification of *hsfa9* mutants of Arabidopsis and characterization of their physiological quality

In the literature, HaHSFA9 has been shown to improve longevity when overexpressed in tobacco seeds (ALMOGUERA et al., 2015) whereas our data using deficient mutants of *M. truncatula* does not support this hypothesis. In order to verify if the orthologs of HSFA2.2 play the same function in other species we evaluated the role of *HSFA9* from Arabidopsis. Three t-DNA insertion mutants were ordered from NASC (Figure 5). For one of the alleles, we could only confirm the insertion at the end of the last exon (SALK_062453). The results presented here are therefore obtained from a single allele and should be considered preliminary. *Hsfa9* mutant seeds harvested at abscission from plants grown at 20°C were used to perform the dormancy and longevity tests.

In order to assess whether *hsfa9* has a dormancy phenotype like *Mthsfa2.2*, freshly harvested seeds were tested for germination at different times of after-ripening (2d, 4d and 1 month). The seed lot of the *hsfa9* mutant that was after-ripened for 2 days germinated faster than the associated wild type seeds (Figure 6), but final percentages of germination were comparable. After-ripening led to faster germination, but the aWT and mutant seeds showed similar germination kinetics (Figure 6). These results suggest that the dormancy phenotype of the mutants is the opposite of what was found for the Medicago ortholog, with faster germination for the *hsfa9* seeds instead of slower germination (Figure 3A),

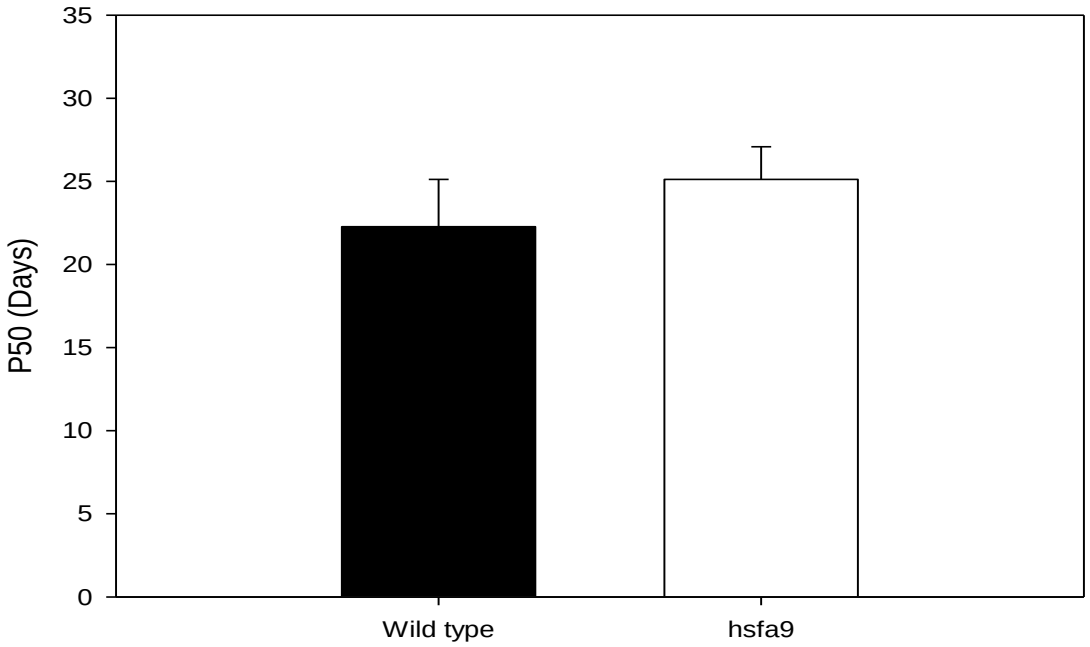
although the differences are quite small, arguing for the absence of a dormancy phenotype.

Figure 6. Germination curves of mature seeds of associated wild type (aWT) and hsfa9 seeds of Arabidopsis at different times of after-ripening. Data represent the mean of 3 biological replicates of 200 seeds each. Error bars represent the standard error.



Next, we determined if *hsfa9* seeds were affected in longevity. Seeds were stored at 75% RH and 35°C and P50 was calculated using the loss of germination curve (Figure 7). No significant difference in P50 could be detected between WT or *hsfa9* seeds, suggesting that the HSFA9 in Arabidopsis does not critically influence longevity.

Figure 7. Longevity of associated WT and hsa9 seeds of Arabidopsis. Longevity was expressed as P50, corresponding to days of storage at which the seed lost 50% viability. Data are the mean (± SE) of 3 survival curves established with 5 data points during storage involving 30 seeds each.

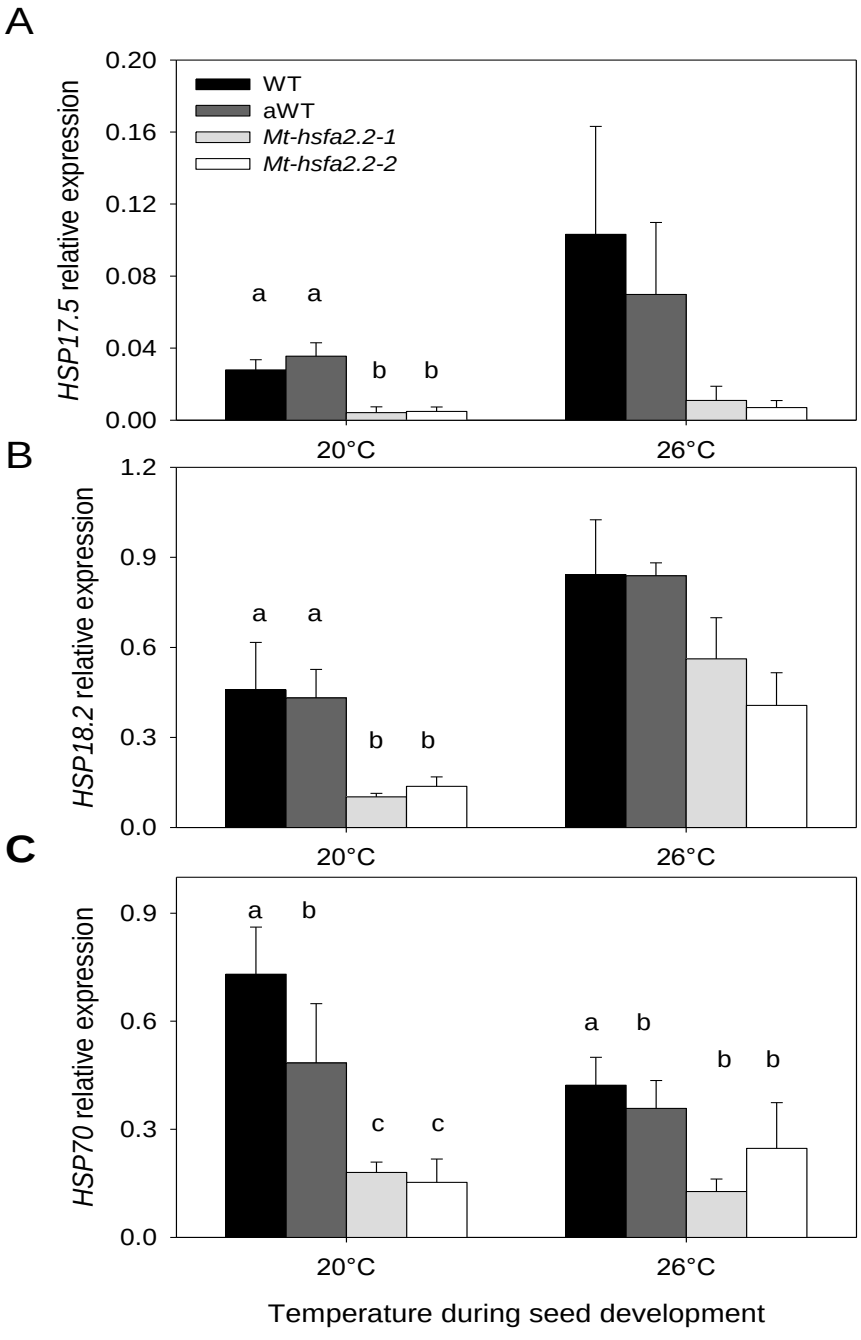


4.3.6 *Mt-hsfa2.2* seeds show deregulated expression of seed specific *HSPs*

Next, we investigated whether the mutation in MtHSFA2.2 led to the downregulation of its targets. Previously, an ectopic system using a GFP on hairy roots (VERDIER et al., 2013) was used to overexpress *MtHSFA2.2* in *M. truncatula* and a transcriptome analysis was performed on this (VERDIER et al., 2013; ZINSMEISTER et al., unpublished results). This analysis revealed 133 transcripts that were significantly differentially expressed in the transgenic roots expressing *35S::MtHSFA2.2:GFP* relative to the control expressing 35S::GUS:GFP (p-value <0.05 and 1> log2 ratio> 1). Several small Heat Shock Proteins (sHSP) and HSPs known to be regulated by the HSFA9 homologue from Arabidopsis (KOTAK et al. 2007) and sunflower (PRIETO-DAPENA et al., 2006) were found to be overexpressed by HSFA2.2 (ZINMEISTER et al., unpublished results). Here, we validated these results using a RT-qPCR study on Mt*HSP*70, Mt*HSP*18.2 and Mt*HSP*17.5 (Figure 8). All three genes were significantly repressed in the two *Mthsfa2.2* alleles compared to the wild types, indicating that MtHSFA2.2 positively regulates the expression of these HSP at the end of the maturation stage. The transcript levels of the 3 HSPs were not completely absent in *Mthsfa2.2*, which

could be due to their regulation by other HSF transcription factor from the same family. Next, transcript levels were determined in mature seeds that were subjected to high temperature during seed development (Figure 8, 26°C). No significant increase could be detected in these samples.

Figure 8. Relative transcript abundance of *HSP17.5* (A), *HSP18.2* (B), *HSP70* (C) in mature seeds of *Medicago truncatula* after 3 months of storage. Data represents a mean of 3 replicates (±SD) of 30 seeds. Different letters indicate significant differences between genotypes using ANOVA and post-hoc Student-Newman-Keuls comparisons ($p<0.05$). Absence of letters means no significant differences between genotypes.



4.3.7 Regulation of galactinol synthase (GOLS2) in Mt*hsfa2.2* of Medicago

A galactinol synthase gene *GOLS2* was also found among the up-regulated genes in the transcriptome of *M. truncatula* roots over expressing *MtHSFA2.2* (ZINSMEISTER et al., unpublished results). GOLS2 is a key enzyme in the metabolic pathway leading to RFO accumulation. To investigate if RFO metabolism was related to MtHSFA2.2, we examined whether the accumulation of *MtGOLS2* transcript and sugar composition was affected in *Mthsfa 2.2* seeds. The glucose content was significantly high in mature *Mthsfa2.2* mutant seeds than WT and aWT (Figure 9A). However, the sucrose content *Mthsfa2.2* mutant seeds were similar with associated Wild type (aWT, Figure 9 B), and only different from WT. Whilst stachyose, the major RFO in the seeds, accumulated to the same level in wild type and mutant seeds (Figure 9C), verbascose content was 4-fold lower in *Mthsfa2.2* mutants compared to both wild types (aWT and WT) (Figure 9D). A RT-qPCR study of the Mt*GOLS2* transcripts showed similar levels in mature wild type seeds (aWT) and in the two mutant alleles *Mthsfa2.2-1* and *Mthsfa2.2-2* (Figure 10). Transcript levels of *MtGOLS2* in WT were significantly lower than the other three genotypes. This might be explained by a random *TNT1* insertion segregating with the TNT1 at the *HSF2.2* locus and affecting the *MtGOLS2* expression independently of *HSFA2.2.* Possibly, Mt*GOLS2* might be regulated earlier during seed development.

Figure 9. Sugar contents in WT and *M-thsfa2.2* mature seeds: A) glucose; B) sucrose; C) raffinose; D) stachyose and E) verbascose. Data represents the mean of 3 replicates of 30 seeds (± SD). When indicated, different letters show significant differences between genotypes as assessed by ANOVA ($p<0.05$).

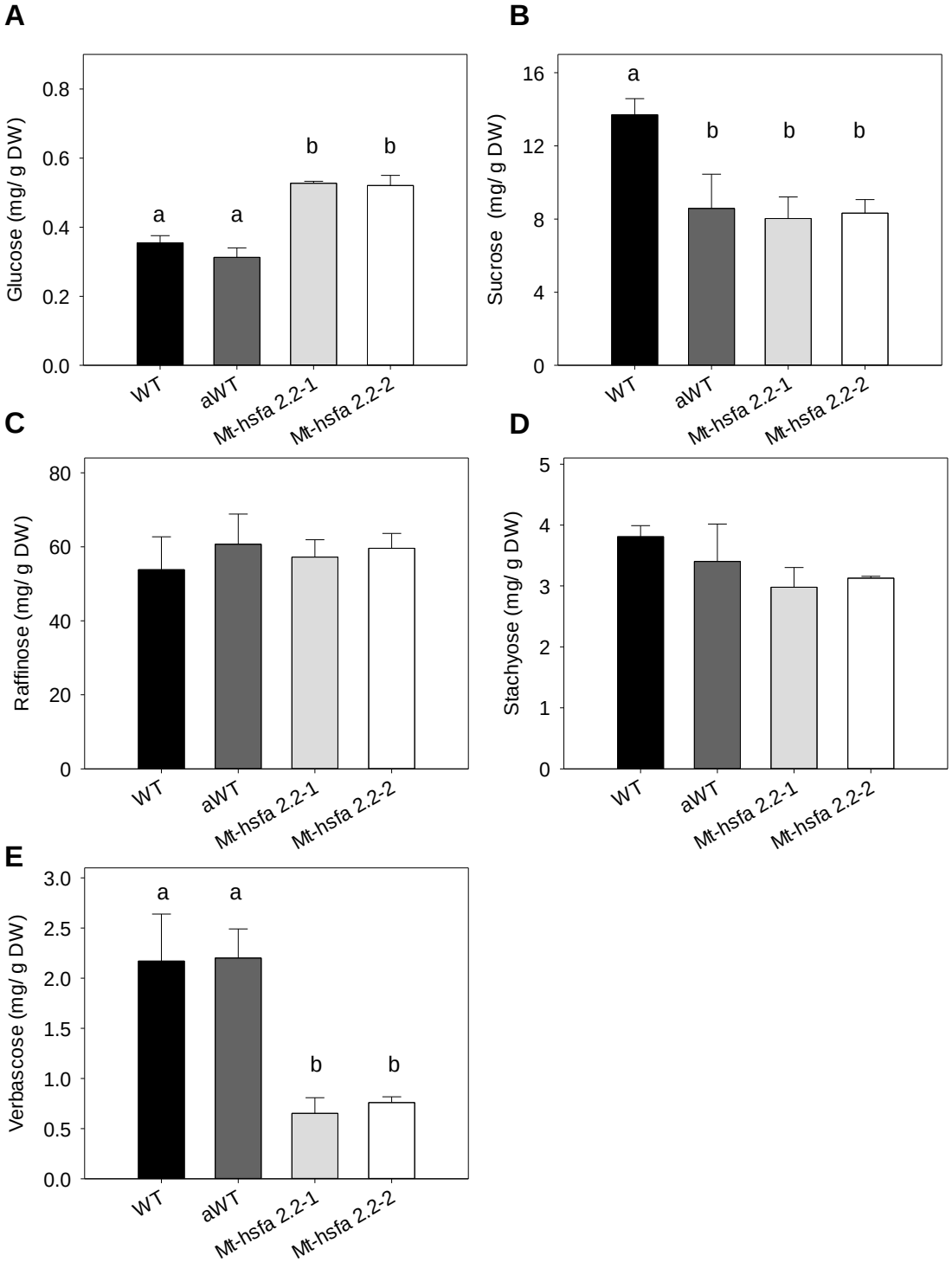
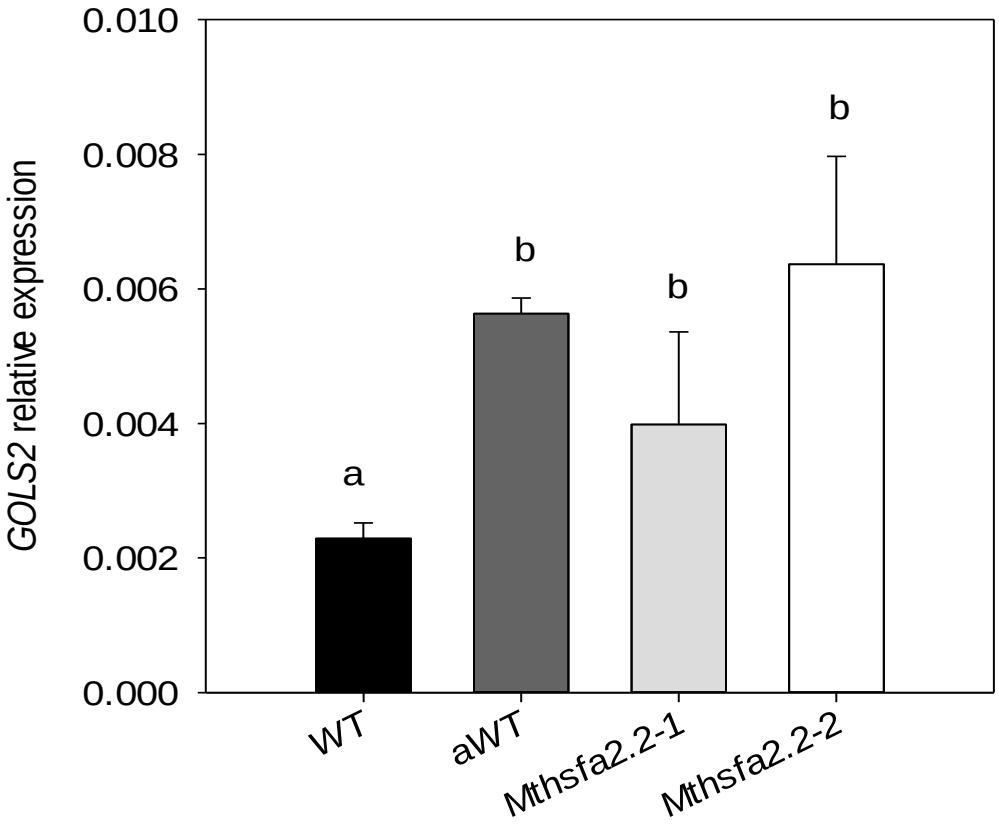


Figure 10. Relative transcript abundance of *GolS2* in mature seeds after 3 months of storage. All seeds were from plants grown at 20°C. Expression was normalized using *MSC27* and ACTIN115 (*Medtr2g008050*) as reference genes. Data represent the mean of 3 replicates of 30 seeds each. Bars represent the standard error. No significant difference was found as assessed by ANOVA ($p<0.05$).

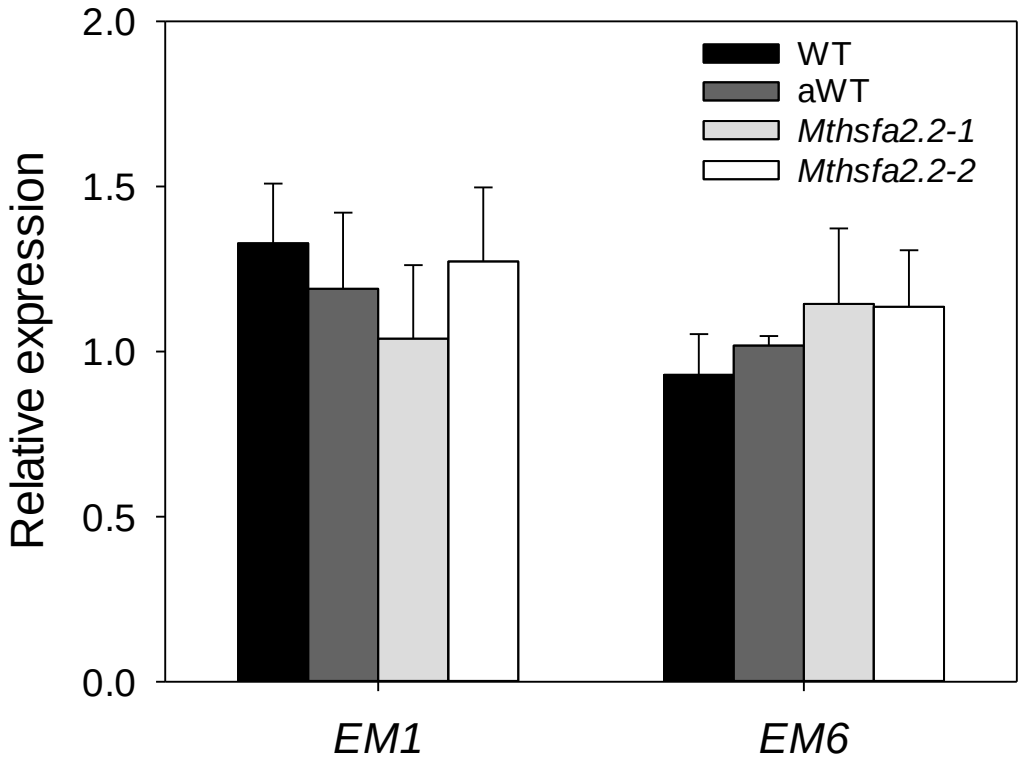


4.3.8 Regulation of LEA in *Mthsfa2.2* of Medicago

Late embryogenesis abundant (LEA) proteins are family of hydrophilic proteins that confer cellular protection upon stress like chaperones (HSPs proteins). Many LEA proteins accumulate during seed maturation and have been proposed to play a role in seed longevity (HUNDERTMARK et al., 2011b). In *M. truncatula*, four most abundant seed LEA proteins correlated with the increase in longevity during maturation; including members of group LEA5 (MtEM and MtEM6) (CHATELAIN et al., 2012). Like chaperone and HSPs, LEA proteins have a protective role during seed maturation drying, where they have been proposed to protect the cells from the water potential decrease by stabilizing proteins and preventing their aggregation (CHAKRABORTEE et al., 2007; LI et al., 2012). Moreover, LEA protein was found to be connected to HSF transcription factor (particularly HSFA9

homologue) in the desiccation tolerance module in the maturation gene co-expression network constructed by Verdier et al., (2013). Since *Mthsfa2.2* mutant did not exhibit a longevity phenotype, we made the hypothesis of a compensation mechanism through accumulation of LEA proteins. To test this, we investigated whether seed specific LEA genes were miss-regulated in *Mthsfa2.2* mutant seeds at abscission stage. The results showed no significant differences in the relative transcript accumulation of the 2 LEA genes MtEM1 and MtEM6 between MtHsfa2.2 and WT (Figure 11).

Figure 11. Relative transcript abundance of EM1 and EM6 in mature seeds after 3 months of storage. All seeds were from plants grown at 20°C.Expression was normalized using MSC27 and ACTIN115 (Medtr2g008050) as reference genes. Data represent the mean of 3 replicates of 30 seeds each. Bars represent the standard error. No significant difference between treatments was found as assessed by ANOVA ($p<0.05$).



4.3.9 Role of ABA in the dormancy phenotype of *hsfa2.2* mutant seeds

It is well established that seed dormancy is under the control of ABA signaling. ABA produced by the embryo and the albumen is known to induce physiological dormancy during seed development (GRAEBER et al., 2012). We investigated

whether the enhanced dormancy phenotype of *Mthsfa2.2* was due to an increased ABA content during seed maturation or an altered synthesis during seed imbibition. For this purpose, seeds of *Mthsfa2.2-1* and *Mthsfa2.2-2* mutants were imbibed in the presence of fluridone, an inhibitor of ABA biosynthesis (Figure 12). Fluridone strongly increased the germination speed in wild type and in a less extent in *Mthsfa2.2* seeds. However, a two-fold difference was still observable in the T50 between these lines (Figure 12A). Similar results were found seeds harvested from plants grown at 26°C (Figure 12B). Nevertheless, the comparison between panels A and B shows that *Mthsfa2.*2 seeds from 26°C had a deeper dormancy than those grown at 20°C, whereas no difference was visible between the respective wild type seeds (Figure 12B). Thus, blocking ABA synthesis during imbibition decreases the dormancy phenotype, suggesting that *de novo* ABA synthesis is needed for dormancy maintenance. However, in the light of the data obtained from seeds at 26°C, this cannot entirely explain the dormancy phenotype of the *Mthsf2.2a* mutants suggesting that de novo ABA biosynthesis was playing a key role in maintaining dormancy in WT, while the mutant was hypersensitive to small amounts of ABA presumably remaining in fluoridone-treated seeds.

Next, we tested whether ABA sensitivity is altered in *Mthsfa2.2* mutant seeds. Mature seeds of mutants and wild type, after 3 months of storage, were imbibed in the presence of different concentrations of ABA. At 1µM ABA, *Mthsfa2.2* seeds reached a lower final percentage of germination compared to aWT and WT (Figure 13). In addition, the T50 of *Mthsfa2.2* germination on 1µM ABA compared to wild type seeds increased in time to mean germination in the presence of ABA compared to the control seed batches and to wild type seeds, where t50 was 4.9 and 15.2 days in *Mthasfa2.2-1* and *Mthasfa2.2-2* respectively as opposed to 2.1 days in aWT. These data suggest that *Mthsfa*2.2 seeds are hypersensitive to ABA.

The data shown above indicate that *Mthsfa2.2* dormancy phenotype is related to an altered ABA sensitivity. In the transcriptome of *M. truncatula* hairy root transiently overexpressing *MtHSFA2.2* (ZINSMEISTER et al., unpublished data), *CYP707A4*, an ABA 8′- hydroxylase known to play a predominant role in ABA catabolism was upregulated. In Arabidopsis, seeds of *cyp707a2* mutant defective in ABA deactivation exhibited a hyper dormancy phenotype (KUSHIRO et al., 2004). Therefore, expression of *MtCYP707A4,* was determined in *Mthsfa2.2* seeds that were freshly harvested (dormant), and after 3 months of post maturation (non-

dormant), both in mature dry seeds and after 6h of imbibition. Expression analysis did not reveal significant difference in *MtCYP707A4* transcript accumulation between dormant and non-dormant *Mthsfa2.2* and wild type seeds (Figure 14).

Figure 12. Effect of fluridone on seed germination of Mt-hsfa2.2 seeds. A Germination curves in the dark at 20°C in 10 µM fluridone using seeds obtained from plants grown at 20°C (A) and 26°C (B). Data represent the mean (± SE) of 3 replicates of 30 seeds.

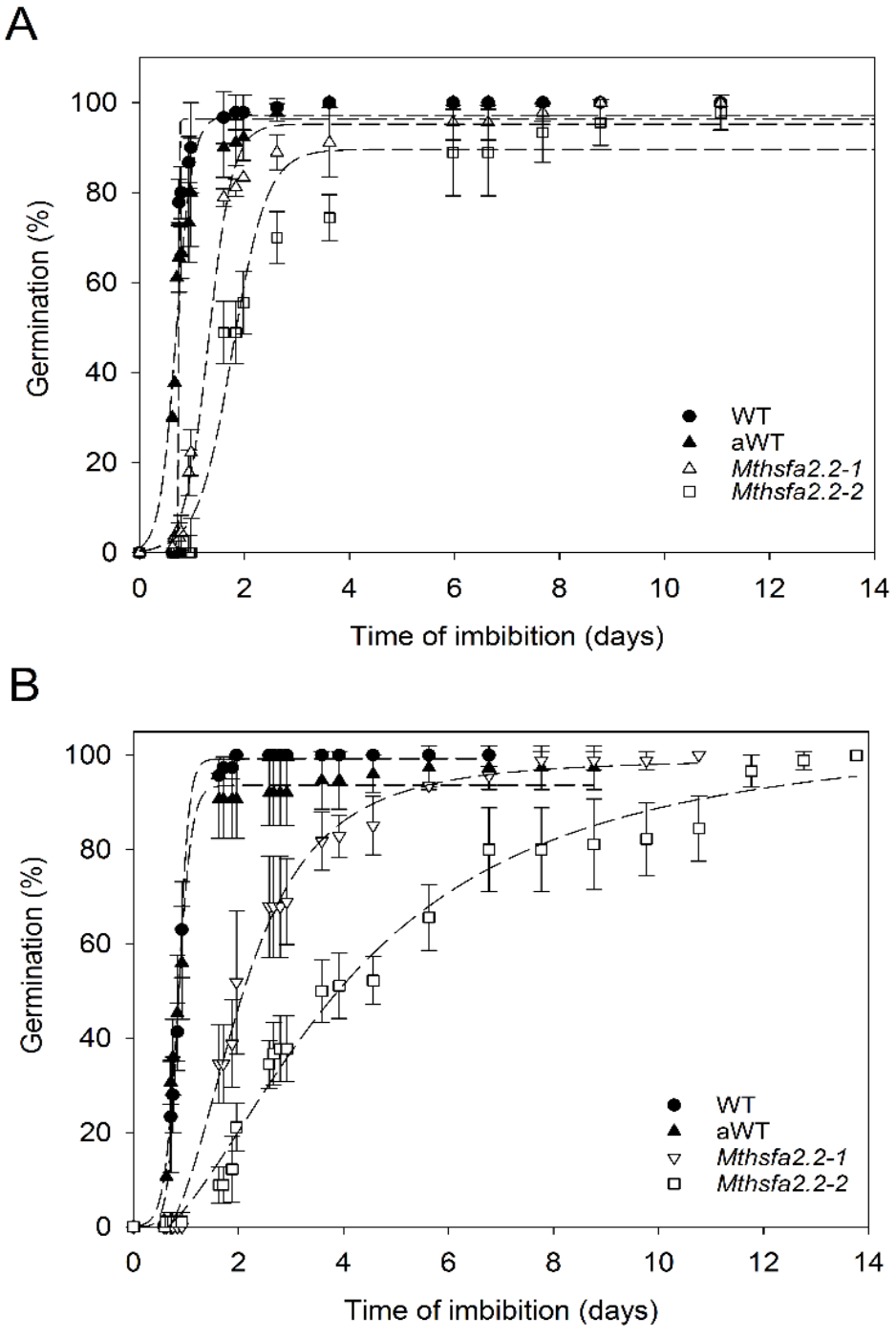


Figure 13. Impact of ABA on germination of mature seeds of *Mt-hsfa2.2* compared to wild-type seeds. Seeds stored for 3 months then imbibed in water and 1 µM ABA. Data represent a batch of 20 seeds from plant grown at 20°C.

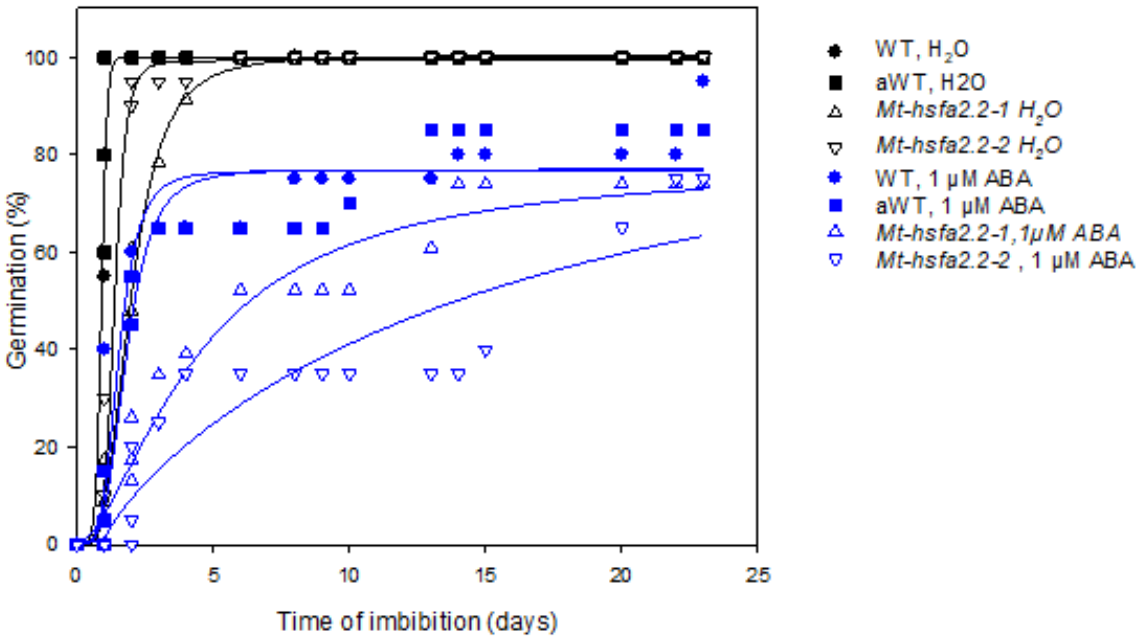
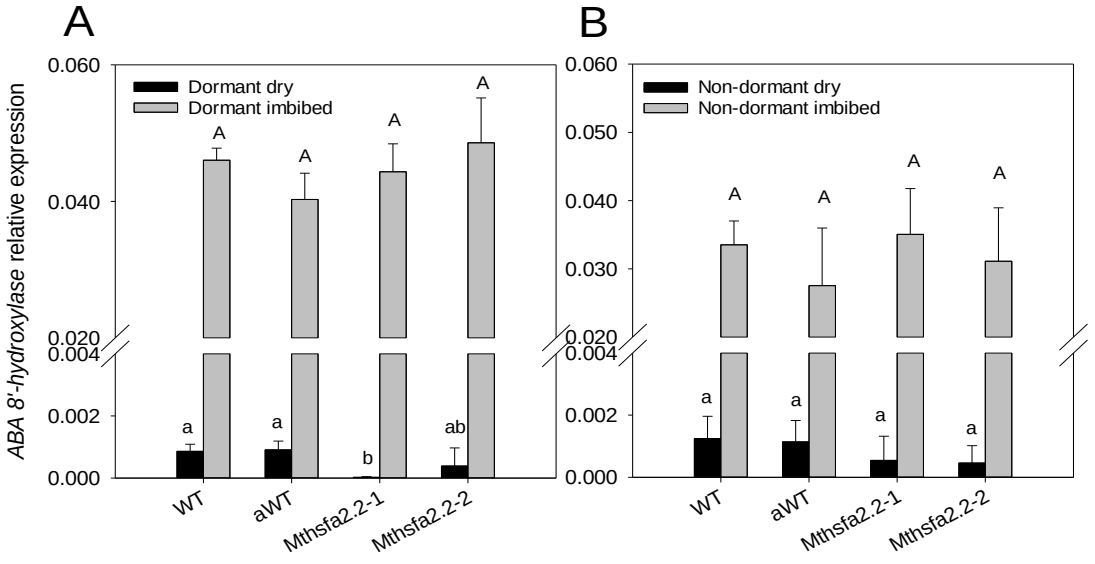
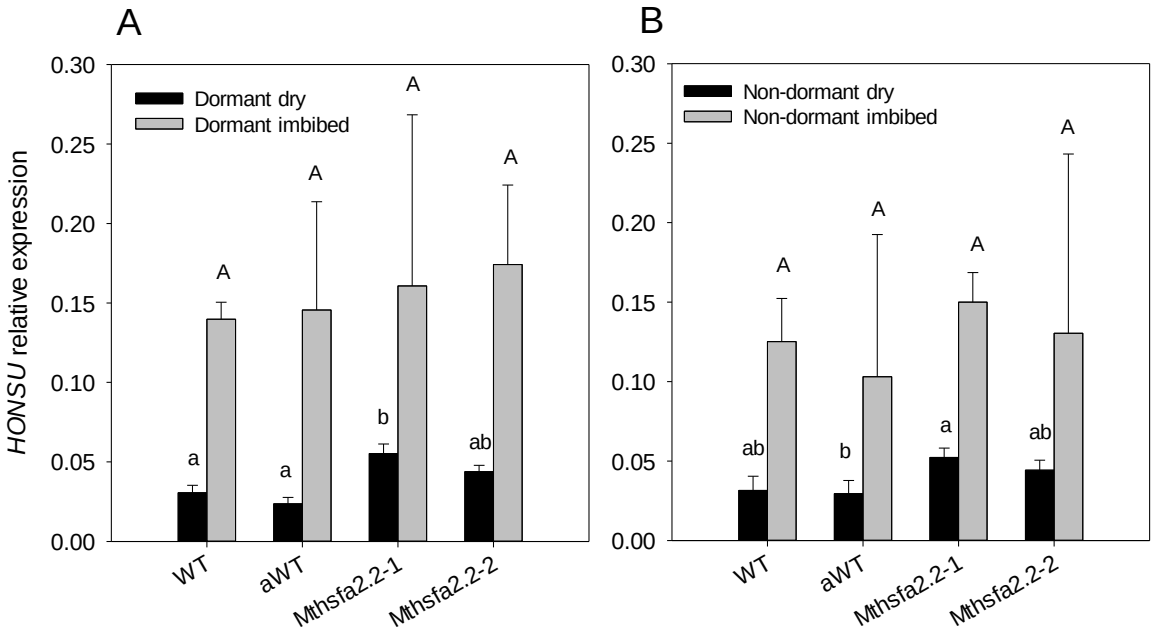


Figure 14. Relative abundance of *ABA 8'-hydroxylase* (*CYP707A4*, Medtr8g072260) transcripts in mature seeds and after 6 h of imbibition in the dark. A. dormant seeds. B) after-ripened seeds for 3 months. Expression was normalized using *MSC27* and *ACTIN115* (Medtr2g008050) as reference genes. Data represents the mean of 3 replicates of 30 seeds each. Different letters indicate significant differences between genotypes with small letter for the comparison between non-imbibed seeds, and capitals for 6h-imbibed seeds, determined by a one-way ANOVA (P<0.05) following by Tukey's test for multiple comparisons.



In the hairy root transcriptome of *35S::HSFA2.2*, transcript levels of *HONSU* were also significantly upregulated. HONSU (HAI2) is a PP2C family member A, a seed-specific negative regulator of ABA signaling (KIM et al., 2013). In this study, *MtHONSU* transcript accumulation was assessed in dormant (freshly harvested) and non-dormant (3-month post-harvest) seeds before and during imbibition. Compared to wild-type seeds, *MtHONSU* transcript level was significant higher in dry seeds of the *hsfa2.2-1* allele but not in *hsfa2.2-2* (Figure 15). During imbibition, there was an increase in transcript level, regardless of the genotype and the level of dormancy but this result need to be confirmed, owing the large standard errors.

Figure 15. Relative abundance of *HONSU* (Medtr3g068200) transcripts in mature seeds and after 6 h of imbibition in the dark. A. Dormant seeds. B) after-ripened seeds for 3 months. Expression was normalized using *MSC27* and *ACTIN115* (Medtr2g008050) as reference genes. Data represents the mean of 3 replicates of 30 seeds each. Different letters indicate significant differences between genotypes with small letter for the comparison between non-imbibed seeds, and capitals for 6h-imbibed seeds, determined by a one-way ANOVA ($p<0.05$).

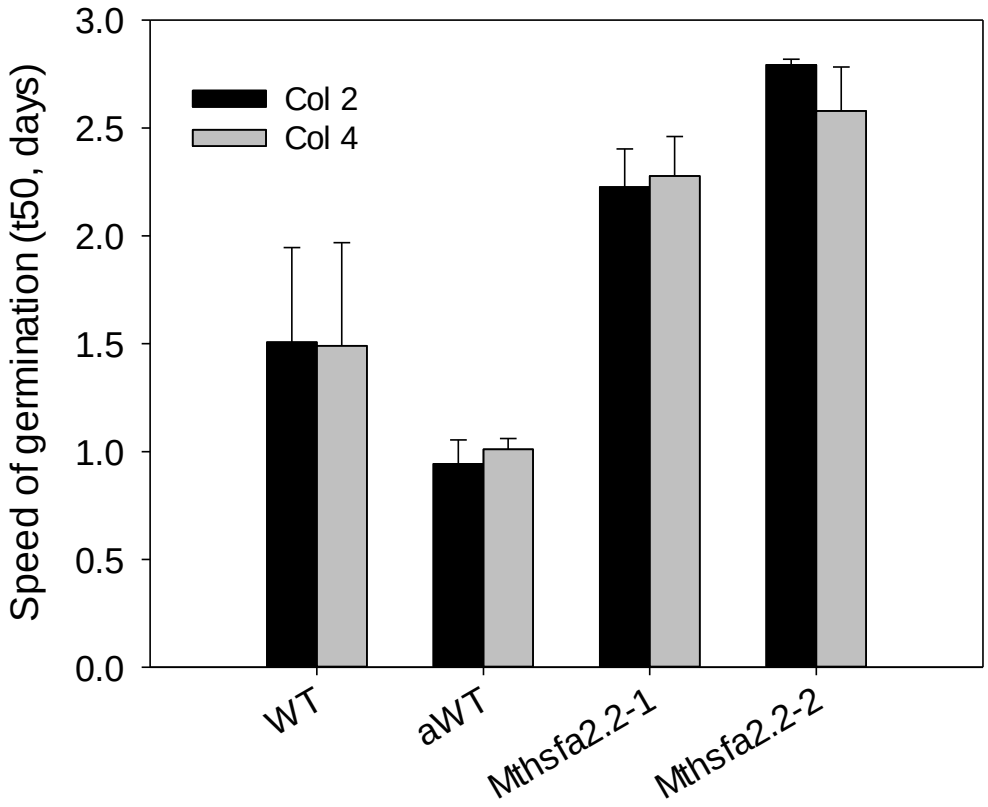


4.3.10 Role of Gibberellic Acid in the dormancy phenotype of *hsfa2.2* mutant seeds

Dormancy is regulated via the dynamic balance between gibberellic acid (GA) and ABA (FINCH-SAVAGE and LEUBER-METZER, 2006; GRAEBER et al.,

2012). In order to further characterize the role of MtHSFA2.2 in dormancy, we studied the germination response of freshly harvested seeds to the presence of GA in the imbibition medium. The germination speed in the presence of 10µM GA_{4+7} did not change in the *Mthsfa2.2* mutants (Figure 16). As observed previously (BOLINGUE et al., 2010), even in the wild type seed (WT and aWT) GA_{4+7} did not improve the germination, suggesting that either GA is less important in releasing dormancy in *Medicago truncatula* or that there is a problem with penetration of the molecular into the seed tissue.

Figure 16. Germination speed, expressed as T50, of mature seeds of wild type seeds (WT and aWT) and Mt-hsfa2.2 in 10µM gibberellic acid (GA 4+7). Scarified were imbibed in the dark at 25 °C in the presence of 10 µM GA4+7 (black bars) or water containing 1% (v/v) ethanol (grey bars).



Since the pharmacological study did not provide a definite answer, expression of genes involved in GA synthesis and degradation were investigated. Three genes were chosen: GA_2-oxidase1 (*MtGA2Ox1* and *MtGA2Ox2*) that catalyze the deactivation of active GAs to the inactive forms GA_{34} and GA_8, and *GA3Ox1* that encodes a 3-β-hydroxylase adding hydroxyl group to C-3 to form the active

gibberellins GA_1. No significant difference was found for GA2ox1 transcript levels between wild type and *Mthsfa2.2* seeds (Figure 17). In contrast, in dry seeds, *GA2Ox2* transcript levels were significantly higher (5-fold) in mature seeds of the mutants compared to the wild types (Figure 18A, B). Upon imbibition, transcript levels decreased in *Mthsfa2.2* seeds, both in dormant and non-dormant seeds. These data suggest that the dormancy phenotype of *Mthsfa2.2* could be related to an increased GA degradation at the end of maturation. Transcript levels of *GA3Ox1* measured in *Mthsfa2.2* seeds did not reveal any differences between dormant and non-dormant seeds and even during imbibition (Figure 19).

Figure 17. Relative abundance of *GA2Ox1* transcripts in mature seeds and after 6 h of imbibition in the dark. A. Dormant seeds. B) after-ripened seeds for 3 months. Expression was normalized using *MSC27* and *ACTIN115* (Medtr2g008050) as reference genes. Expression was normalized using *MSC27* and *ACTIN115* (Medtr2g008050) as reference genes. Data represents the mean of 3 replicates of 30 seeds each. Different letters indicate significant differences between genotypes with small letter for the comparison between non-imbibed seeds, and capitals for 6h-imbibed seeds, determined by a one-way ANOVA ($p<0.05$).

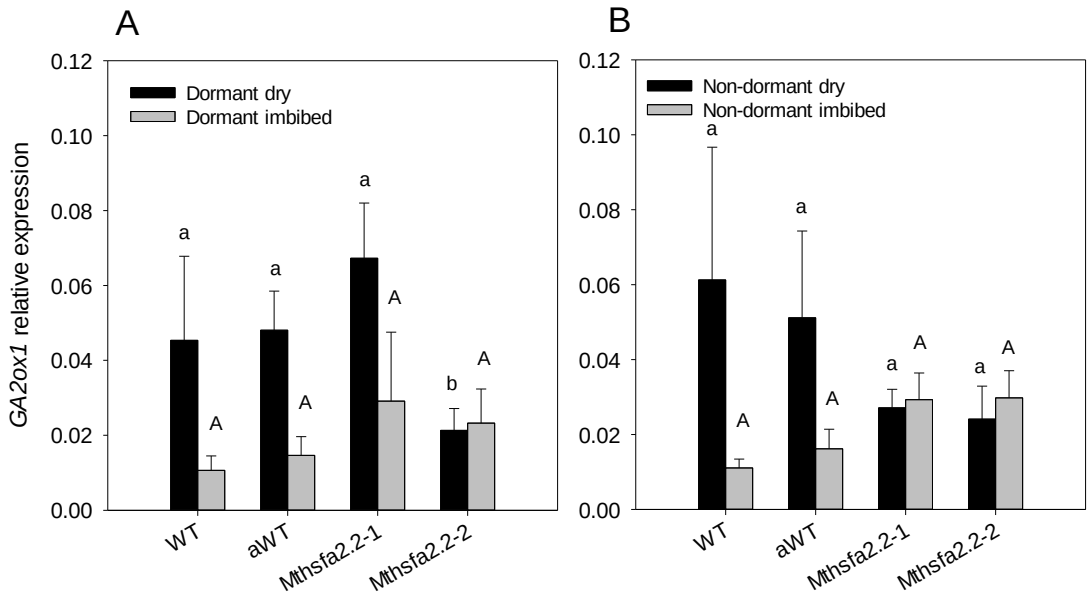


Figure 18. Relative abundance of GA2Ox2 transcripts in mature seeds and after 6 h of imbibition in the dark. A. Dormant seeds. B) after-ripened seeds for 3 months. Expression was normalized using MSC27 and ACTIN115 (Medtr2g008050) as reference genes. Data represents the mean of 3 replicates of 30 seeds each. Different letters indicate significant differences between genotypes with small letter for the comparison between non-imbibed seeds, and capitals for 6h-imbibed seeds, determined by a one-way ANOVA ($p<0.05$).

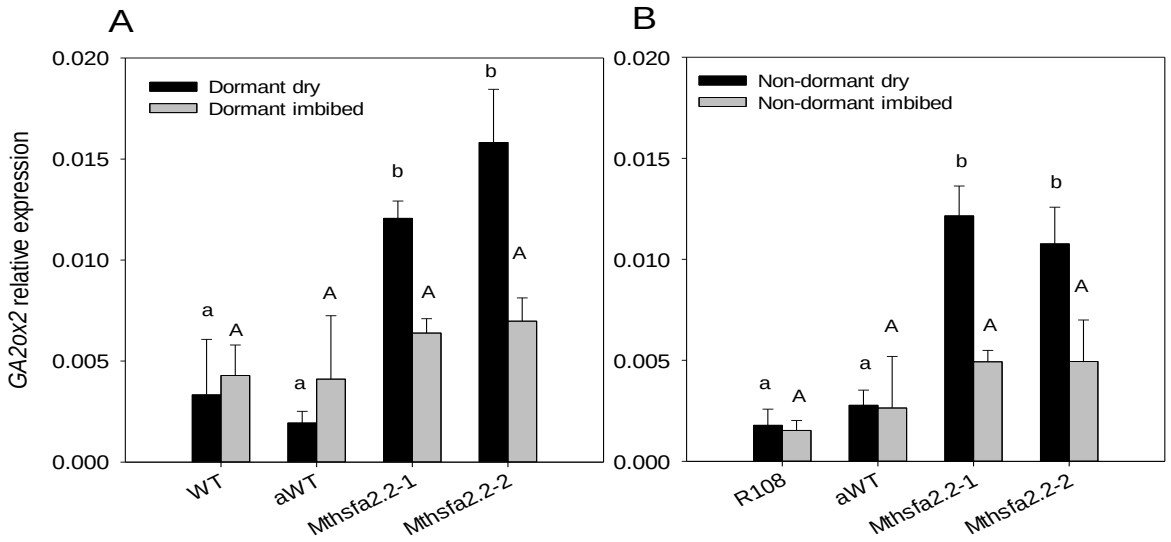
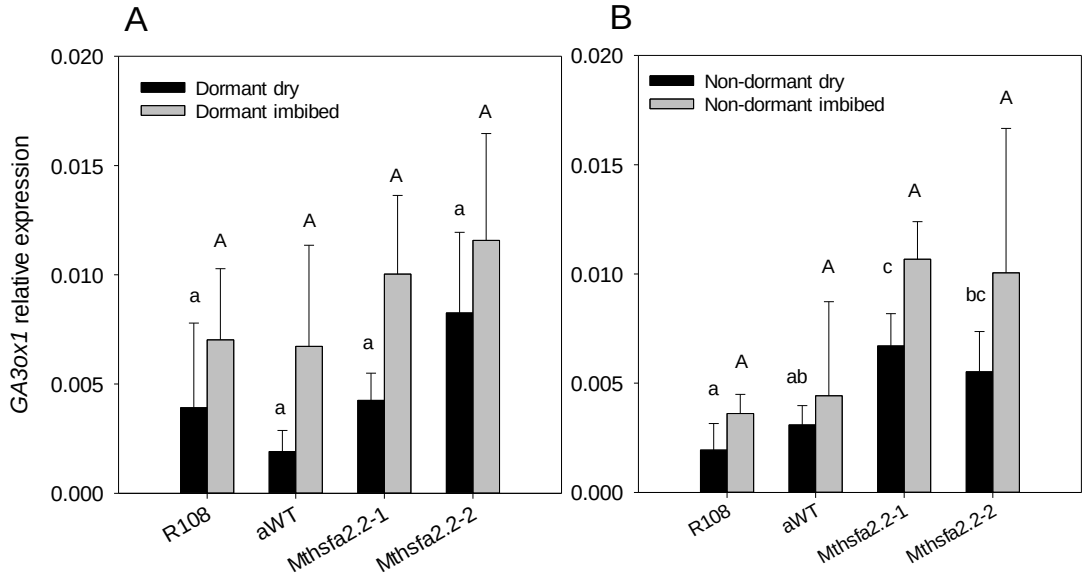


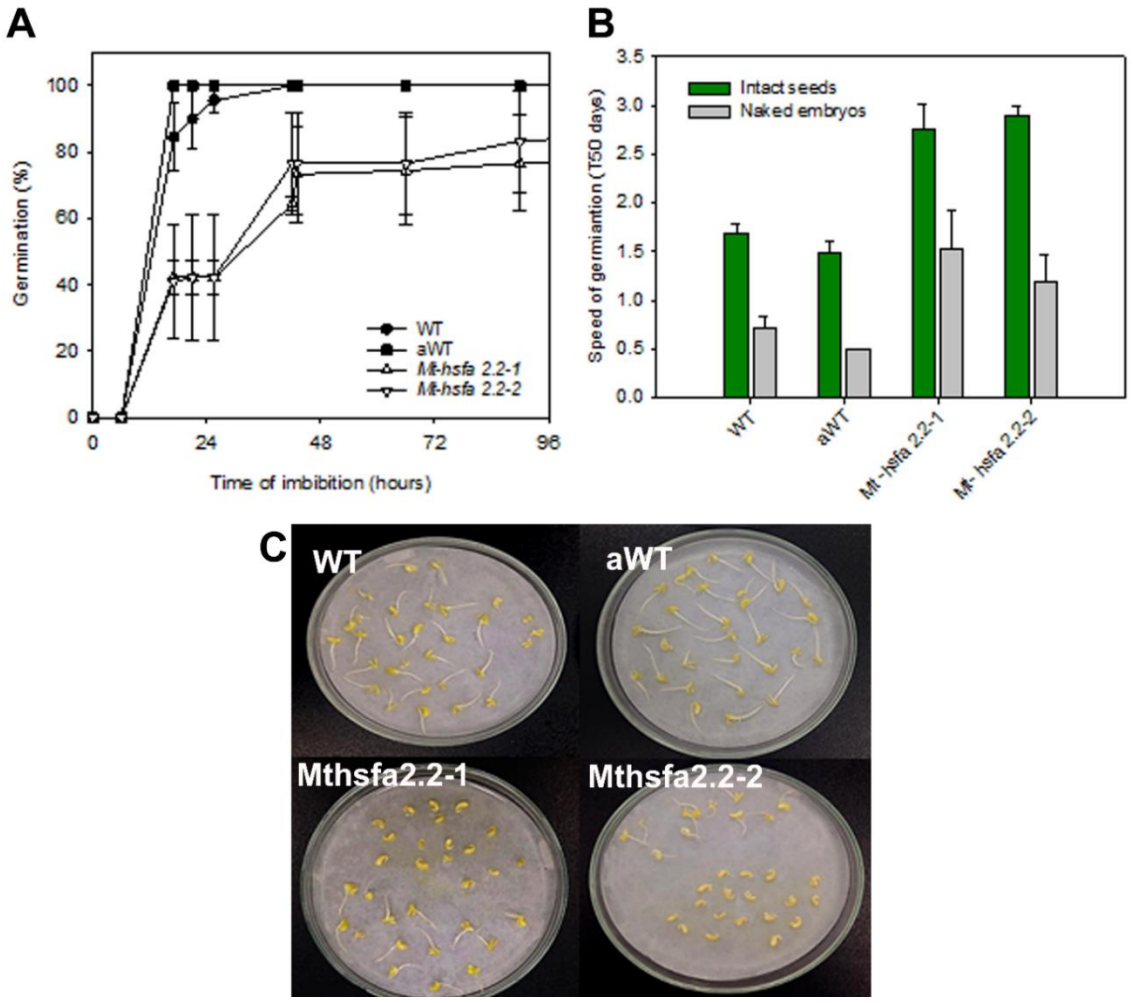
Figure 19. Relative abundance of *GA3Ox1* transcripts in mature seeds and after 6 h of imbibition in the dark. A. Dormant seeds. B) after-ripened seeds for 3 months. Expression was normalized using *MSC27* and *ACTIN115* (Medtr2g008050) as reference genes. Data represents the mean of 3 replicates of 30 seeds each. Different letters indicate significant differences between genotypes with small letter for the comparison between non-imbibed seeds, and capitals for 6h-imbibed seeds, determined by a one-way ANOVA ($p<0.05$).



4.3.11 The dormancy phenotype of *Mthsfa2.2* seeds has an embryonic origin

Physiological dormancy can be imposed by the embryo or the surrounding tissues (testa, endosperm) or combinations of these tissues, and their sum and interaction determine the degree of dormancy (FINCH-SAVAGE and LEUBNER-METZGER., 2006). To evaluate whether the dormancy phenotype of the Mt*hsfa*2.2 mutants comes from the embryo or the covering structures, seeds were imbibed for 5 hours in the dark at 20°C, after which the endosperm and seed coat were carefully removed taking care of not damaging the embryos. Intact seeds and naked embryos were incubated in water at 25°C and radicle growth was monitored. For all genotypes, embryo radicles grew faster when the seed coat was removed, as was observed previously (BOLINGUE et al., 2010). However, the time for the radicle to grow took longer for the naked *Mthsfa2.2* embryos and the final percentage of seeds with radicles that had grown was smaller in *Mthsfa2.2* compared to wild type (Figure 20). Thus, the difference in germination/growth in the mutants is independent of the surrounding layers and appears to reside in the embryo.

Figure 20. (A) Germination of intact embryos naked embryos of wild type and *Mthsfa2.2* mutants during imbibition. (B) Germination of naked embryos naked embryos of wild type and *Mthsfa2.2* mutants during imbibition. (C) photo of naked embryos after 5 days of imbibition. Data are the average (±SE) of 3 replicates of 30 seeds. Whole seeds from plants grown at 20°C and stored for 7 months were imbibed for 5 hours in the dark at 20 °C then were dissected to remove the seed coat and endosperm. Naked embryos were then transferred to a new moist filter paper at 25°C.



4.4 DISCUSSION

Dormancy and longevity, two complementary seed quality traits are important for agriculture and conservation of biodiversity and also key adaptive traits that contribute to seed lifespan and dispersal. The impact of the maternal environment such as temperature on seed dormancy has received wide attention (FINCH-SAVAGE and LEUBER-METZER, 2006) in contrast to longevity. Likewise, efforts were targeted to identify and understand the molecular mechanisms regulating dormancy rather than longevity. In seeds, HSF are versatile transcription factors that

regulate gene expression in response to thermal stress but also to developmental cues. Several HSF such as HSFA9 homologues in Arabidopsis (KOTAK et al., 2007), sunflower (ALMOGUERA et al., 2009) and soybean (LIMA et al., 2017) are particular among the HSF family in that they are exclusively expressed in the late stage of maturation, when seed dormancy and longevity are acquired. During seed maturation, expression of several HSPs are regulated by the seed-specific transcription factor, HSFA9 also shown to be involved in the control of a genetic program that regulates desiccation tolerance and longevity (PRIETO-DAPENA et al., 2006; TEJEDOR CANO et al., 2010; ALMOGUERA et al., 2015). This work tested that whether a putative homologue of HSFA9 and hub gene involved in seed maturation (VERDIER et al., 2013) play a role in seed longevity in *M. truncatula*. While this hypothesis was not validated, our results using deficient mutants grown in contrasting environments showed that MtHSFA2.2 is a negative regulator of seed dormancy, acting independently of the maternal environment and likely controlling GA catabolism and ABA sensitivity. This role appears to be specific to *Medicago truncatula* as no phenotype was found in Arabidopsis. This is analogous to ABI5 that regulates seed dormancy and longevity in developing seeds of grain legumes but not in Arabidopsis (ZINSMEISTER et al., 2016), reinforcing the idea that the regulatory mechanisms controlling seed vigor differ between the Brassicacea and Fabaceae family.

HSF regulates the expression of HSPs by recognizing conserved heat shock element (HSE)-binding sequences in the promoter regions upstream of heat shock responsive genes. Many genes encoding MtHSPs were found in *M. truncatula* to be correlated with the acquisition of desiccation tolerance like MtHSP70, small MtHSP18.2, MtHSP17.4, MtHSP17.6 among other (VERDIER et al., 2013). In this work, we clearly show that HSFA2.2 controls the expression of small HSPs such as HSP70, HSP18.2, HSP17.5 as their expression was significantly impaired in *MtHsfa2.2 m*utant seeds (Fig). This data confirms that MtHSFA2.2 is a functional HSF in Medicago and that it specifically regulates the expression of seed specific-developmentally regulated HSPs.

The ectopic expression of HaHSFA9 in tobacco seeds was shown to confer a better resistance to controlled deterioration (PRIETO-DAPENA et al., 2006). Suppression of HaHSFA9 expression resulted in an impaired resistance to artificial aging treatment (TEJEDOR CANO et al., 2010). In contrast, our work shows that

the seeds of *Mthsfa2.2* mutants were not sensitive to aging compared to wild-type seeds (Figure 4). These results were found for both temperature conditions. Several possibilities might explain this discrepancy. In our system, seeds were exposed to mild storage conditions whereas transgenic tobacco seeds were exposed to 42°C, 100 % RH (PRIETO- DAPENA et al., 2006). In these conditions, they would imbibe and resume metabolism while tolerating the heat stress. Consistent with this, OsHSP18.2 gene expression increased 60 fold when rice seeds were stored for 6 days at 45°C, 100%RH (KAUR et al. 2015). These conditions are far from ours where the seed water content was still low enough to prevent resumption of metabolism during storage (BUITINK and LEPRINCE, 2004).

Another plausible explanation for the discrepancy could be the existence of functional redundancy with other seed-specific HSFs in *M. truncatula*. The longevity module of gene regulatory network contained two HSF encoding genes, which could act redundantly to induce longevity (VERDIER et al., 2013). For further clarification, they expression should be analysed in *Mthsfa2.2* mutants. The expression of other members of the A group of HSFs should also be considered, although they might not be seed-specific. In Arabidopsis seeds, HSFs such AtHSFA1a and AtHSFA1b appear to have redundant functions in early heat shock responses and vegetative thermotolerance (LOHMANN et al., 2004). So, it is possible that the mutation of MtHSFA2.2 is offset by other HSFs that accumulate during seed maturation. In developing soybean seeds, the expression of several HSF from different groups (HSFA6B, HSFA3, C1 e AD1) was correlated with the acquisition of seed longevity (LIMA et al., 2017). In *M. truncatula* the class A HSFs includes 12 members.

Another possible type of functional redundancy could act downstream of HSF by acting on LEA proteins. HSPs and LEA proteins share similar functions in terms of their protective roles in the survival to dry state (BASHA et al. 2012). In *M. truncatula*, the accumulation of the four most abundant seed LEA proteins (EM, D-113.I/II, D-34.III et CapLEA.I/II) was correlated with the increase in longevity during maturation (CHATELAIN et al., 2012). Moreover, in Arabidopsis Hundertmark et al. (2011) showed that in RNAi lines where the seed specific LEA14 expression was reduced, seed survival during storage dramatically decreased.

In our study LEA proteins EM1 and EM6 transcript accumulation was not affected in the *Mthsfa2.2* mutants, even in seeds harvested from plants grown at 26°C, which correlates with the absence of a longevity phenotype. This data is in accordance with the observation that transgenic tobacco plants overexpressing HaHSFA9, displayed increased seed HSPs expression but did not affect the level of dehydrins (PRIETO-DAPENA et al., 2006).

Galactinol synthase1 (GolS1), an enzyme involved in catalysing the synthesis of galactinol the precursor of RFO is one of the genes that is heat-inducible (PANIKULANGARA et al., 2004). In Arabidopsis; the expression of several genes encoding galactinol synthase (GolS) are indeed induced by HSFA2 and HSFA3 in responses to abiotic stress like tolerance to drought, high salinity, cold and oxidative stresses (TAJI et al., 2002; ZHUO et al., 2013; SONG et al., 2016). However, our analysis of soluble sugars in *Mthsfa2.2* seeds showed that their contents were similar to wild type, except for verbascose content that was slightly significantly lower in the mutants. Also, we did not observe any differences in GolS transcript level in *Mthsfa2.2* seeds compared to wild type. This suggests that *MtHSF2.2* does not alter sugar metabolism. Assays of galactinol content needs to be performed in the mutant seeds to validate this hypothesis. Perhaps the induction of GolS is specific to the HSAF2 and HSFA3 and/or to vegetative tissues.

4.4.1 MtHSFA2.2 is a negative regulator of seed dormancy

Our results show that dormancy is deeper in the *Mthsfa2.2* mutants compared to the control. Dormancy was confirmed by the fact that stratification released dormancy in a similar way in wild type and mutant seeds (Figure 3). To our knowledge, there is no data showing a role for HSF in seed dormancy.

Therefore, this dormancy phenotype in *Mthsfa2.2* seeds is likely to be related to an increased ABA synthesis during imbibition since we observed that the ABA biosynthesis inhibitor fluridone reversed the delayed-germination phenotype. Fluridone (an inhibitor of ABA biosynthesis) did not reversed the delayed-germination phenotype in HSFA2.2 mutant, but rather abolished dormancy in WT, and in a less extent in MtHSFA2.2 mutant, suggesting that de novo ABA biosynthesis was playing a key role in maintaining dormancy in WT while the mutant was hypersensitive to small amounts of ABA presumably remaining in

fluoridone-treated seeds. The transcriptome analysis of hairy roots ectopically overexpressing MtHSFA2.2. revealed that the CYP707A1 gene transcripts encoding an ABA 8 'hydroxylase enzyme was less abundant that wild type roots (ZINSMEISTER et al., unpublished). This was not confirmed in this study. The transcript levels of CYP707A1 in *Mthsfa2.2* seeds were similar to those of wild type seeds in dormant and non-dormant state and after imbibition (Figure 14). Further work is necessary to assay the ABA content to assess whether *Mthsaf2.2* regulates gene expression of the ABA synthesis pathway.

We found that *Mthsfa2.2* seeds were hypersensitive to ABA, which could indicate that they do not necessarily accumulate such a high level of ABA but have rather an increased sensitivity at the end of maturation. Consistent with this, the transcriptome of ZINSMEISTER et al. (unpublished data) revealed a slight increase in the amount of transcripts encoding HONSU, an ortholog (HAI2) a PP2C family member. In *A. thaliana*, HONSU is required for dormancy, the level of the transcripts being inversely correlated with dormancy. Its role is to negatively regulate ABA signalling and induce ABA degradation (KIM et al., 2013). However, our expression analysis of MtHONSU could not entirely explain the dormancy phenotype found in *Mthsfa2.2* seeds. The observation that transcript levels of this gene was higher in *Mthsfa*2.2-1 deserves further investigation.

Gibberellins stimulate germination by antagonistically suppressing ABA-triggered seed dormancy (GRAEBER et al., 2012). We provide evidence that the function of HSFA2.2 in Medicago seeds is in agreement with this model. Our data suggest that MtHSFA2.2 could act on GA metabolism to regulate dormancy. The fact that the dormancy phenotype lies in the embryo supports this hypothesis (Figure 20). Also, our qPCR analysis showed that the deactivating GA2OX2 transcripts accumulated to higher level in dry mutant seeds (Figure 18) whereas those of biosynthesis gene GA3Ox1 were not significantly affected (Figure 19) This suggests that GA catabolism rather than synthesis is perturbed in *Mthsfa2.2.* Furthermore, the difference in GA2OX2 transcript abundance between dry mutant and wild type seeds disappeared during imbibition. This would indicate that such perturbation in GA deactivation in *Mthsfa2.2* mutants occurred during development at the end of maturation stage. In Arabidopsis, a function of GA2Ox2 in seed dormancy was also reported (YAMAUCHI et al., 2007). We cannot yet conclude on a putative altered GA sensitivity in the *Mthsfa2.2* seeds. We could not show

changes in GA sensitivity between dormant and non-dormant seeds of wild type, very likely due to a problem of GA penetration in the seeds. This result corroborates with the work of Bolingue et al. (2010), who showed that there was also no influence of GA_3 on speed of germination in several genotypes of *M. truncatula*.

While this work suggests that MtHSA2.2 acts as a negative regulator of dormancy controlling the ABA/GA antagonism, future work is needed to decipher whether HSF acts directly on genes regulating hormone metabolism and sensitivity or whether it acts indirectly through the action of specific HSP under the control of MtHSFA 2.2. The increased dormancy in *Mthsfa22* seeds was associated with a decreased level of small HSP and *HSP70.* Overexpression of small HSP in tobacco seeds overcame the inhibitory effect of light on germination but had no effect in the dark (KOO et al., 2015). In the literature, there is increasing evidence that the chaperone activity of HSP goes beyond a repair function after heat stress, such as (BASHA et al., 2012).

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GENERAL DISCUSSION AND PERSPECTIVES

With the prospects of climate changes, the need to produce high vigor seeds that withstand the negative impact of environmental conditions during production and processing and are adapted during subsequent sowing has become a challenge for researchers and the seed industry. This is partly because the impact of the maternal environment on the acquisition of seed vigor remains poorly understood. Therefore, this work contributes to the knowledge of the main events related to seed quality acquisition that occur during seed maturation in legumes when the developing seeds prepare for the dry stage. Seed maturation is a very important developmental stage as desiccation tolerance, vigor and longevity are acquired.

We confirmed that longevity in soybean is progressively acquired during seed maturation after seed filling (Figure 7, chapter 2). Further work is necessary to understand the mechanisms triggering the embryo into a seed longevity program and which protective factors are implicated. There is increasing evidence that synthesis of protective mechanisms that allows the acquisition of longevity are induced sequentially and increases 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). Therefore, it is likely that developing seeds of soybean harvested at different maturity stages are endowed with different amounts of protective compounds. 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). The question whether heat shock proteins are required for seed longevity is debatable since in Medicago, seeds of *hsfa2.2* mutants had a longevity similar to wild type (Figure 4-chapter 4). Whether there is a redundant mechanism that allows the synthesis of key HSP during maturation remains to be assessed. In contrast, dormancy was increased in *Mthsfa2.2* seeds, suggesting a role of HSP during seed imbibition (Figure 8 -chapter 4). Using different drying rates, we suggest that the acquisition of longevity is an autonomous embryonic

program that does not depend entirely on the connections with the vascular tissues of the mother plant (Figure 4 -chapter 2). In this respect, it would be interesting to examine how the developing seeds resort on their own metabolism to synthesize the necessary protective compounds such as LEA proteins and oligosaccharides.

Our analysis also revealed a complex relationship between heat and longevity suggesting that high temperatures can be beneficial for the longevity program whereas they are detrimental to seed filling. This relationship warrants confirmation by using additional climate data and determine the breakpoint temperature at which heat negatively impacts longevity, since the temperature during this study was not very high. Further analysis of the impact of the temperature in relation with the photoperiod is necessary. Developing seeds and mother plants remain sensitive to photoperiod during most of the post-flowering period, generating a quantitative and continuous response to day length (KANTOLIC and SAFFLER, 2007). Perhaps, nigh temperature might be less beneficial then day temperature. The impact of temperature could be also dependent on the plant water status. In our experiments, soybean plants were irrigated which could have influenced the beneficial of effect of heat. Our study needs also to be expanded with different genetic material and maturity groups to establish a cause-effect relationship between heat (day or night) during seed development and acquisition of longevity.

Our data reinforces the argument that late seed maturation is a distinct and necessary phase in the production of high quality soybean. The resistance of elongation capacity to ageing was similar to longevity assessed by germination (Figure 9 - chapter 3). Further work is necessary to understand why the pattern of acquisition of organ elongation is so different between years. The growth capacity of the radicle and hypocotyl was acquired progressively during maturation in 2015 but not in 2016. Considering that modifications were made between the experimental set up between years, we suspect that elongation capacity is more sensitive to the environment after germination than to the developmental stage at which seeds are harvested. This could be tested by measuring elongation capacity of immature seeds under different deleterious conditions.

During seed maturation, expression of several HSPs are regulated by the seed-specific transcription factor, HSFA2.2, a homologue of HSA9 also shown to be involved in the control of a genetic program that regulates desiccation tolerance and longevity in sunflower (PRIETO DAPENA et al., 2006; KOTAK et al., 2007;

ALMOGUERA et al., 2009;TEJEDOR-CANO et al., 2010). We tested whether MtHSFA2.2, a homologue of HSFA9 and hub gene involved in seed maturation plays a role in seed longevity in *M. truncatula*. Our data confirmed that MtHSFA2.2 is a functional HSF in Medicago that specifically regulates the expression of seed specific-developmentally regulated HSPs (Figure 8- chapter 4). Our results using deficient mutants showed that MtHSFA2.2 is a negative regulator of seed dormancy, acting independently of the maternal environment and likely controlling GA catabolism and ABA sensitivity. This role appears to be specific to *Medicago truncatula* as no phenotype was found in Arabidopsis. A similar observation was made of ABI5, a major regulator of seed desiccation tolerance and maturation where mutations in this gene induced a range of pleiotrophic effects in Medicago and pea, but not in Arabidopsis (ZINSMEISTER et al., 2016). Altogether, this reinforces the idea that the regulatory mechanisms controlling seed vigor differ between the Brassicacea and Fabaceae family. In addition, we did not find that growing mutant seeds in optimal and heat conditions did not induce the dormancy phenotype compared to wild-type. Therefore, HSFA2.2 is unlikely to be a key gene involved in the seed adaptation to its environment a functional HSF in Medicago and that it specifically regulates the expression of seed specific-developmentally regulated HSPs.

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