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Seed storage under the ultra-dry condition

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Wageningen University and Research
2018

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Course: MSc Thesis Plant Breeding(PBR-80436)
for partial fulfillment of Master of Sciences in Plant Sciences Specialization Plant Breeding and Genetic Resources,

Plant Developmental System Group
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Wageningen University & Research
October 2018

Acknowledgment

This is my first individual thesis and paper, doing more than a year since September 2017. I have experienced different difficulties in the experiment and essay writing, and I feel the sense of accomplishment and frustration of scientific research. When I start this thesis, my goal is to gain more knowledge and practical value in the seed storage. During the process, I fully understand the relevant experiments and further expand my horizons. Thanks to Dr. S.P.C. Groot from plant developmental system group and Dr.ir. AB (Guusje) Bonnema from Plant Breeding group in Wageningen UR, gave me the chance to start a cooperated thesis. And Thanks for their kindly help and patience in the past time, they give me a lot of encouragement like a parent. I feel warm and thankful. Meanwhile, much thanks to Jan Kodde and Manjunath from PDS group helped me a lot during the experiment period. I also want to thank my parents, grandma, and my best friends (Beta,Li,Shuang,Si, Yiyuan,Zihan) for supporting me move forward during the last few months and also the whole PDS groups. Last but not least, thanks for Bejo company provided the cabbage seed for such great experiment.

I will cherish everything in this period, the good and the bad are extremely precious to me.

Hope you enjoy the short journey and catch some ideas about seed storage.

Abstract

Seeds are the one of the most important production on the planet providing the sustainable food and energy for lifes. The storage methods of the seeds are the essential to the seed longevity which will influence the seed production and protection. Through the years, scientists are working on the optimal methods to store the seeds for a long term with less damage. Many literatures reveal that the species of the seeds, environmental factors like temperature, humidity, and oxygen level, as well as the pest and mould, etc. all have the effect on the seed longevity. Commercial seeds stores in the warehouse with a certain temperature(15-20°C) and moisture content(30%) to ensure that crops seeds have a slower aging speed and well preserved for the further use. To explore the aging speed and test the seed longevity, this kind of warehouse storage consumes too much time. Thus, higher temperature and humidity level of storage named accelerated aging (AA) and controlled deterioration (CD) are designed to test the seed vigor and speculate the seed longevity.

Along with the development of physiology, this two kinds of test are questioned that whether AA and CD can represent the same condition when the seeds stored in a dry and cold environment. The elevated partial pressure of oxygen (EPPO) is later designed to use the high-pressure oxygen to aging the seeds under the controlled temperature and humidity. EPPO methods is a success but with a side-effect that the pressure of gas also causes damages to the seeds. So, can we step further on the seed aging test to obtain a better method? According to the experiment result we have, we design an ultra-dry(0-2%RH) environment and the high pure oxygen level to give a extreme aging condition on the other side. We found that the ultra-dry system can age the seed faster and significant. During the aging method test, we also applied the different maturity of the cabbage seeds and different color of arabidopsis seed to see whether they have the predicted interaction with ultra-dry storage. The results indicate that mutant arabidopsis(different color) have no significant difference with the wildtype after eight weeks of storage, but some have differences between the mutants. Due to the maturity levels of the cabbages are not different from itself, we don't have much conclusion about that relationship with ultra-dry storage. Meanwhile, the comparision among the ambient storage, EPPO/EPPN, CD and ultra-dry with high oxygen will discuss in this report.

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Abbreviation

UD/UDHO: Ultra-dry/Ultra-Dry with High Oxygen
EPPO/EPPN: Elevated Partial Pressure of Oxygen/Nitrogen
AA: Accelerated Aging
CD: Controlled Deterioration
CF: Chlorophyll Fluorescence
tt: *transparent testa* (mutants of Arabidopsis)

1.Introduction

1.1 Importance of seed quality

Seeds are important agricultural materials for crop production, which affect not only the yield of the crops but also the quality of the crops. Along with the rapid development of agricultural production technologies, high seeds quality accompanied by appropriate cultivation techniques, have become essential to obtain a high yield and a high-quality agricultural product. Because of this, the better-quality seed has a higher demand on the market. Seed quality includes many aspects, such as seed purity, seed vigor, seed longevity etc.

1.2 Seed longevity and shelf life- essential to the seed quality

Seed longevity refers to the longest period that a seed population maintains viability under certain environmental conditions, in another word, that the time at which the seed can survive (Ellis 1991). Shelf lives are closely related to the seed longevity, given the length of time that can store the seed for sowing (Dictionary 2008). The longevity of seeds can differ among families, genera and even within species. Seeds can be divided into three categories: orthodox (withstanding drying up around 5% moisture content and storage under freezing condition, e.g., maize, apple), recalcitrant (intolerant to dry storage condition, e.g., avocado, mango), and intermediate storage (seeds can be tolerant to some degree of drying storage condition but are sensitive to storage at low temperatures, e.g., papaya) (Ellis, Hong et al. 1990, Bewley, Bradford et al. 2012). The difference in lifespan between seeds can also vary a lot, such as the lotus seed (*Nelumbo nucifera*) sleeping in the ancient tomb can live for one thousand years (Ming, VanBuren et al. 2013), while the lifespan of the willow tree seeds (*Salix caroliniana*) is only a few dozen hours (Castro-Morales, Quintana-Ascencio et al. 2014).

Seed Longevity is affected by many factors. Firstly, it is regulated by the seed's composition and genes related to lifespan, so that different kinds of seeds have their inherent storage potential. For example, starch-storing seeds like rice are store much longer than the oil-contained seeds like beans. The variation between and within species indicates that many genes are involved in regulating seed longevity. E.g., functional compositions like heat shock proteins (HSP) are translated to protect the seed from stress, the different expression amount of that gene may

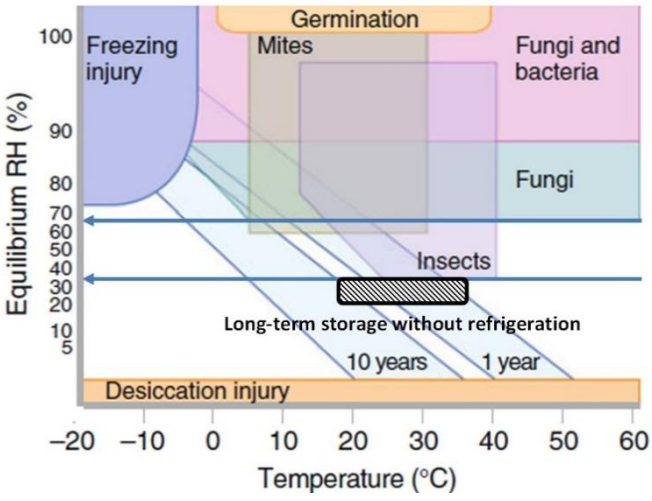


Figure 1.*Different abiotic and biotic factors that influent the seed storage. Cited from (Bewley, Bradford et al. 2012). Two light blue area indicate the 1-year or 10-year storage ability for different species.*

differ the longevity of each kind of seeds. Secondly, environmental conditions influence the survival rate of the seed referred to the Ellis(Ellis 1991). During the growth and development of seeds, changes in climatic conditions(temperature, moisture content), damage caused by pests and diseases, pesticides, and mechanical damage during harvesting, transportation, will directly affect seed quality or seed integrity. These damages may disable seed function and change the structure of the seeds and result in shorter seed longevity. In addition, after harvesting, the seeds are affected by environmental stress during storage, resulting in accumulation of damage to biological macromolecules, such as proteins, lipids, and nucleic acids. This damage will result in a decreasing quality and a shorter lifespan. (Sano, Rajjou, et al. 2015)

1.3 Effects of external factors on seed longevity

The main external factors affecting seed longevity are surrounding humidity, storage temperature, and the effects of biotic stress like pests and diseases (McDonald 1999). The recent article also reveals that environment oxygen content also influences the seed longevity(Schwember and Bradford 2011),. To further improve the level of seed storage, it is necessary to grasp the mechanism of various factors affecting seed senescence and adopt appropriate methods to prolong seed longevity.

1.3.1 Effect of water content on seed longevity

The water content in the seed is one of the most important factors affecting the longevity of the seed, and it determines the chemical reaction rate. If seeds store in an environment with higher air humidity, the proportion of free water and bound water will increase, and respiratory rate increases. This situation will produce toxic metabolites to seeds, and heat to accelerate protein denaturation and also the moulds will start to grow during the storage. Thereby decrease the seed longevity. (Robertson, Lute et al. 1939). After moderately drying (20~30%eRH), the seed metabolism rate is stopped due to the inactivity of the enzyme and no respiration (Figure2). Meanwhile, antioxidant will defense as the main protection way in the seed because of the repair process terminated by the lack of enzyme and energy (Ellis, Hong et al. 1988).

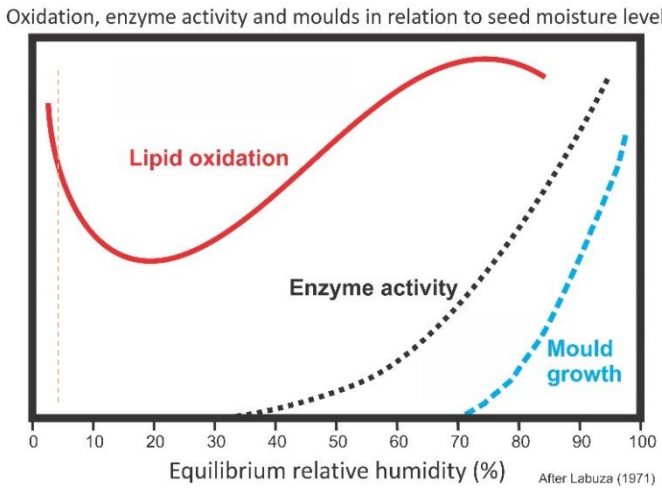


Figure 2.*Relative reaction rate in the seeds under different water activity. Adapted from (Labuza and Dugan Jr 1971). The orange dashed lines indicate the high lipid oxidation, low enzyme activity in the seeds during the extreme dry(0-3%)*

Furthermore, given the excessive low moisture treatment (<10%) also has a negative effect on the seed storability. At present, most scholars believe that the cause of seed deterioration is lipid peroxidation, which begins with the production of free radicals inside the seeds (McDonald 1999). Excessive free radicals in cells

have a strong oxidation effect, which can change the structure of the cell membrane (Figure.3) and cause the oxidation of biological macromolecules such as proteins, DNA and RNA (Apel and Hirt 2004). In the overdried seeds, extreme dehydration also destroys the continuous interface of the water film covering the cell membrane. It also exposes macromolecules at the sensitive sites of the membrane, making them more vulnerable to reactive oxygen species. This progress accelerates the chain reaction of membrane lipid peroxidation, eventually leading to faster aging of cells and the decline of seed vigor. In the study of Labuza (Figure 1), the article shows that the food will have extremely high lipid oxidation activity when the water content comes to a very low level (Figure.2) (Labuza and Dugan Jr 1971).

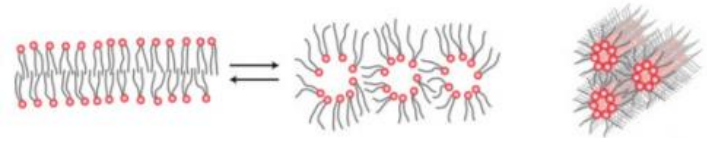


Figure 3. *The two hydrophobic fatty acid will change the structure inwards towards each other under the extreme dry condition. After further drying, due to the lack of surrounding water and energy, the micelles form a hexagonal state (Bewley, Bradford et al. 2012)*

1.3.2 Effect of temperature on seed longevity

In general, temperature and water content play a role in the process of affecting seed life. Temperature affects seed longevity primarily by altering the activity of the enzymes in the seed. The intensity of the respiration primarily determines the consumption of the internal nutrition. (Ellis, Hong et al. 1991) Within a certain range, the higher the temperature and water content, the stronger the seed respiration. The study revealed that the temperature has a positive linear relationship with the germination rate in the different legume crops from the base temperature(germination rate = 0) to the optimum temperature (maximum germination rate) (Covell, Ellis et al. 1986). Therefore, reasonable control of the ambient temperature will reduce the rate of physiological and biochemical reactions in the seed, which can prolong the seed longevity properly (Ellis and Roberts 1980).

1.3.3 Effect of oxygen level on seed longevity

Reactive oxygen species (ROS) can oxidize or per-oxidize many macromolecules (lipids, nucleic acids, and proteins), causing changes in cell structure during cell division and organism damage. Studies have shown that 02 in the air was involved in the deterioration of seeds (Abdalla and Roberts 1968), and storing under low oxygen (0~2%) conditions is beneficial to prolong seed longevity (Schwember and Bradford 2011).

1.3.4 "Ultra-dry" long-term storage with different oxygen level

As mentioned above, in the case of extreme drying, the storage of seeds causes a change in the cell membrane structure of the species to initiate a possible oxidation reaction which reduces the longevity of the seed. We call this extreme moisture condition "ultra-dry." However, there have been many studies in history that have revealed that seeds that have been stored for many years under "ultra-dry" conditions still have high germination rates. (Shejbal 1979, Steiner and Ruckenbauer 1995, Ellis, Hong et al. 1996, Hong, Ellis et al. 2005, Pérez-García, González-Benito et al. 2007, Singh, Dash et al. 2016), However, there are few research that has shown seeds after

ultra-dry treatment are not as good as the ambient control (Demir and Ozcoban 2007, Schwember and Bradford 2011). Is the ultra-dry storage environment conducive to seed storage? By reading and comparing, Shejbal 's team store the seeds under the nitrogen atmosphere (Shejbal 1979); in Pérez-García experiment, the team seal the seeds in the anoxia (CO_2) environment (Pérez-García, González-Benito et al. 2007), and also in Hong's experiment they store the seed in the vacuum laminated aluminum foil packets (Hong, Ellis et al. 2005). These results lead us to speculate that oxygen content plays an important role in seed deterioration during ultra-dry storage, which is one of the bases of our hypothesis.

1.4 Methods to study the influence of factors that influence seed longevity

Vegetable seed company most often use controlled condition to store the seeds, such like the warehouse with the equilibrated relative humidity of 30%, and a constant temperature of 15–20°C with the normal oxygen level. However, these conditions are all for long-term storage, which may store the seeds for years with slow aging speed. Experiments under those conditions take too long time, so experimental aging conditions, which give faster results, are needed. That shortens the time for scientist to study what happens during this storage and how different protection mechanisms function.

1.4.1 Accelerated aging and controlled deterioration

High temperature and high humidity level can decrease the seed longevity, which means seeds could aging fast by given such extreme condition for the storage. So the scientists use 75-100%RH and 40-50°C as a standard method to test the seed longevity, this method called accelerated aging (AA). Controlled deterioration (CD) (Powell and Matthews 2005) is another fast aging method based on the AA in a high moisture level (65-80% RH) to detect seed vigor validated by the International Seed Test Association (ISTA) for some crops. The CD methods give the seeds a relative higher humidity and temperature to store for a quick deterioration in the seeds. Since aging is the main cause of the seed vigor, the decline of the germination rate and survival rate indicate the seed different seed quality. But with the development of physiology under the extreme condition, whether the seed viability level in high temperature and humidity can reveal the same under the dry and frozen condition is widely discussed. The degree of lipids changes under different extreme conditions is also questioned by (Powell and Harman 1985).

1.4.2 EPPO&EPPN - one step further with oxygen

The elevated partial pressure of oxygen (EPPO) and elevated partial pressure of nitrogen (EPPN) are the pairs of the new experimental aging methods invented Steven's seed lab team in Wageningen UR (Groot, Surki et al. 2012). This method is based on the oxygen level act an important role in seed aging under dry storage conditions. EPPO method using the steel tanks under the pure high-pressure air, oxygen (~200bars) with the appropriate relative humidity(created by silica gel). EPPN, where seeds are stored under the same pressure but with nitrogen gas, is always seen as the control to the EPPO storage. The EPPN results can shows if the gas pressure induces seed damage during the process (Groot, Surki et al. 2012). Recently, the EPPO methods have been confirmed that can quickly age the barley seed (Nagel, Kodde et al. 2016), and also help to accelerate the seed dormancy release related to the (Domancy of

germination) DOG gene loci (Buijs, Kodde et al. 2018). We use this method in our aging test to compare with the ultra-dry high oxygen storage and AA and CD method in Arabidopsis and Brassica seeds.

1.4.3 Ultra-dry with high oxygen – better aging method?

From the former introduction, we indicate that seeds storage under the ultra-dry condition without oxygen can achieve long-term storage with low seed deterioration, while ultra dry in the presence of oxygen accelerated the aging compared to storage at, e.g. 30% relative humidity. We hypothesize that ultra-dry storage in combination with the 100% oxygen can accelerate the oxidation activity inside the seed faster. The ultra-dry condition may process as an alternative experimental fast-aging method that reveals the real seed longevity in the dry condition, and high oxygen level may deteriorate the seeds in the maximum speed. For making the environment an ultra-dry condition to equilibrate the seeds, we choose the dry beads®(Hay, Thavong et al. 2012) - a kind of zeolite clay bead that can absorb the moisture from the air, to achieve the 1%-2% relative humidity "Ultra-dry" level. We seal the seeds in the glass bottle surrounding by dry beads® fulfilling with pure oxygen for the storage. We hope to use this as a new standard aging method to real estimate the seed longevity without the potential inaccurate relations in AA/CD or the side effects caused by the high pressure in EPPO/EPPN.

1.5 Mechanisms of seeds internal protection under dry storage

1.5.1 Antioxidant protection system

The seeds also have the antioxidant system to protect them self from oxidation in dry condition. This system is classified into enzymatic antioxidant systems and non-enzymatic antioxidant systems. Whether or not the enzymatic anti-oxidant system can work normally is crucial for the ability of ultra-dried seeds to function as a scavenger of reactive oxygen species during engorgement (Bailly 2004). However, the enzymatic reaction requires water as the reaction medium and lacks sufficient moisture in the dry seeds, so the enzymatic reaction has taken place and may only begin to work upon the imbibition. Labuza also revealed the relationship between enzyme activity and lipid oxidation during storage of food (Figure 2) (Labuza and Dugan Jr 1971),

Thus, non-enzymatic antioxidant systems play an important role in the protection of dried seeds, including beta-carotene, ascorbic acid(vitamin C), alpha-tocopherol, glutathione, flavonoids etc., may be the main component of the elimination of reactive oxygen species (Bailly 2004). Among them, flavonoids are a large class of secondary metabolites produced by plants during long-term ecological adaptation to resist harsh ecological conditions, animals, and microorganisms. They are carbon-based skeleton compounds that are widely distributed in the plant kingdom. Currently found more than 4,000 kinds of flavonoids can be divided into the following categories: flavonols, flavones, isoflavones, and anthocyanins (Taylor and Grotewold 2005).

1.5.2 Arabidopsis *transparent testa* mutants – antioxidants difference

The seed testa is developed from the integument, accompanied by the specialization of some

cells and the deposition of pigments, which constitute a barrier to protect the seeds (Moïse, Han et al. 2005). The seed coat acts as an important channel for the exchange of substance and energy between the internal matter of the seed and the external environment, and its structural composition determines the life of the seed to a certain extent (Mohamed-Yasseen, Barringer et al. 1994).Scientists have produced a large number of mutants with different testa color, based on Columbia and Landsberg accessions in Arabidopsis. The *transparent testa* mutants have significant difference in flavonoid content accumulation (Routaboul, Kerhoas et al. 2006, Appelhagen, Thiedig et al. 2014), This flavonoid content includes flavonol, anthocyanin, etc. – the antioxidant we mentioned above which could help enhance the seed storability during the seed storage (Debeaujon, Léon-Kloosterziel et al. 2000). So, it is meaningful to use these seeds to test the relationship between seed longevity and seed coat color.

1.5.3 Seed maturity on the longevity under dry stress

Seed maturity is critical to seed desiccation tolerance and quality (Ekpong 2009). Seed lots from lesser maturity are more sensitive to a controlled deterioration treatment (Groot, Birnbaum et al. 2006). Chlorophyll levels can be measured based on it fluorescent properties, and studies show the chlorophyll content decreases during the seed maturation. We can use the chlorophyll fluorescence levels to distinguish the different maturity in the seed lots. (Jalink, van der Schoor et al. 1998). Chlorophyll fluorescence sorted seeds of lower maturity are also more sensitive towards physical sanitation treatments as hot water or aerated steam (Groot, Birnbaum et al. 2006). So, it is good to have research on the relationship between seed maturity and ultra-dry storage, to see that whether the higher maturity has better storability against dry storing environment.

2. Experiment Objectives

Test the sensitivity under the ultra-dry aging method to the pure oxygen: Compare different aging methods using cabbage and arabidopsis seeds. Does the ultra-dry condition under pure oxygen aging faster than the AA/CD/EPPO conditions? What's the relationship between the aging method? Can we use ultra-dry plus pure oxygen as a quick experimental aging method?

Test the relationship between seed coat color and aging speed under the ultra-dry condition: Is the transparent Arabidopsis seed aging faster than the wild-type and dark mutant? Does the different color of the testa make sense to the longevity?

Test the relationship between seed maturity and aging speed under the ultra-dry condition: Do different maturities of seeds have different aging speed? Aging speed get faster when the seed has lower maturity?

3. Material and Methods

3.1 Cabbage seeds and Arabidopsis seeds

The experiment chose two kinds of seeds as our research target: *Brassica oleracea* (Kale cabbage and red cabbage)and *Arabidopsis thaliana*. The seed company Bejo was requested to support the research by providing *B.Oleracea* seeds with different maturity levels, using chlorophyll fluorescence (CF) sorting. They provided CF sorted seeds from Kale and red cabbage seed lot. The seed lots were labeled as marked as CF1 to CF3. The bioscience group produced eight lots of *Arabidopsis thaliana* seeds initially obtained from Wageningen University, in which one seed lot is wild-type and the others are all GMO mutants. All the materials are listed below.

Table 1 *List of all the materials used in the project. Cabbage seeds are all from Bejo company,(CF1-3 represent the maturity from low to high) and Arabidopsis have the seed colour (First column) cited from the (Appelhagen, Thiedig et al. 2014).*

Cabbage	Name	Seed Nr.	Remark	Replication
bejo	Kale CF1	4921	Seed lots for Bejo™ company	Around 60 seeds one replication, each treatment each accession has 3 replications
	Kale CF2	4922		
	Kale CF3	4923		
	Rode CF1	4924		
	Rode CF2	4925		
	Rode CF3	4926		
Arabidopsis	**Name**	**Seed Nr.**	**Remark AGI Nr.**	**Replication**
	Col-0	4799	Wildtype	Around 100 seeds one replication, each treatment each accession has 3 replications
	ban5	4801	At1g61720	
	tt2	4802	At4g09820	
	tt4-13	4803	At5g13930	
	tt6-3	4805	At3g51240	
	tt7-5	4806	At5g07990	
	tt8	4807	At4g09820	
	tt10-4	4810	At5g48100	

3.2 Four methods for equilibrating the seeds

We tested four storage methods – tubes (with holes), seed paper bags and two innovate new storage methods – tea bags and plastic bags for bread. Tubes for the barley are used the 15ml plastic test tubes with holes made by the heated needle. The arabidopsis seeds are used PCR tubes with the holes on the caps. For each method each kind of seeds we have four replicates.

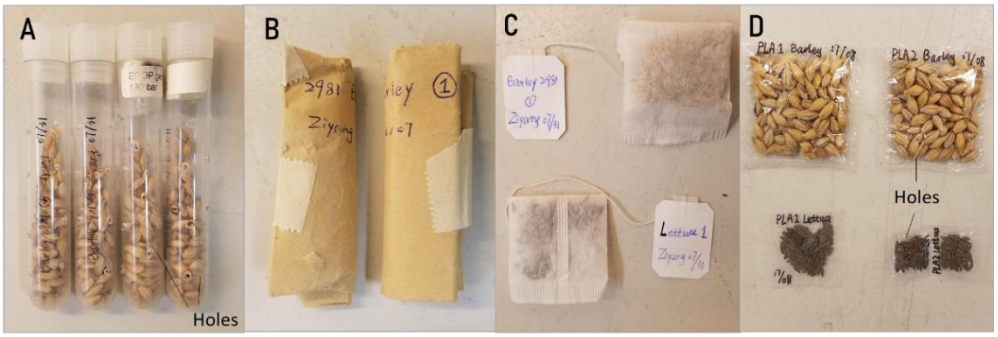


Figure 4.*Four methods to contain seeds for quick equilibrium. (A) Tubes with holes; (B) Paper bags; (C) Tea bags; (D) Plastic bag with holes (original use for bread)*

3.3 Water activity meter

We used four sets of Water activity meter from Rotronic Hygrolab to test the water activity of the seeds, which can be transformed to the equilibrium relative humidity from the seeds (Rotronic 2011). First, we choose the right height block and put it inside the container, then sample all the seed above the block. Close the cap, using the mechanical structure presses the lid tightly. This process will isolate the interior container from the outside and thus obtain the moisture content of the seed. After about 45 mins, statistics become stable can be read from the reader(Figure5).

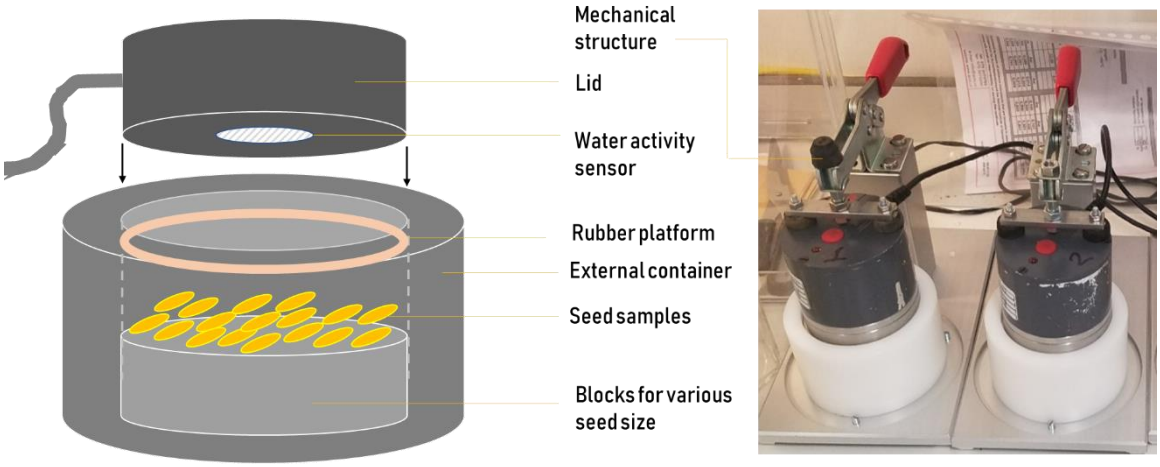


Figure 5. *Lateral perspective of the water activity meter(L);Sets of water activity meter for testing seeds relative humidity(R).*

3.4 Five methods for seed aging

In total, we prepared five aging methods to store the seeds. Except the EPPO and EPPN use 1.5L diving-use tank to contain the seeds, all the other methods use a glass bottle with a red dot (which can use as a detector to the oxygen level) as a container. The details of all the methods are listed in table 2:

Table 2. Details of five different ageing methods in temperature, oxygen level, relative humidity.

Methods	Tm	Oxygen Level	eRH%	Equilibrum Medium	Experiment Period
Ambient	30°C	21% (1.01 bar)	40%	Silica Gel	40 Weeks (test every 4 wks)
Ultra-dry/High O2	30°C	99% (1.01 bar)	1%	Dry Beads®	40 Weeks (test every 4 wks)
CDT	30°C	21% (1.01 bar)	75%	Silica Gel	10 Weeks (test every 2 wks)
EPPO	30°C	21% (200 bar)	40%	Silica Gel	6 Weeks (test every 2 wks)
EPPN	30°C	1% (200 bar)	40%	Silica Gel	6 Weeks (test every 2 wks)

3.5 Pre-humidification before sowing

Equilibrating the seeds again in the 4°C (dark) in the cabinet overnight before the germination test was essential for the seeds, especially the seeds stored under the dry condition. Because of the huge difference in the water potential, once put the dried seeds on the water, it will crash over and damage the membrane(FANG and ZHOU 2007). Pre-humidification can avoid such rapid water uptake during the imbibition to get a more accurate result from the storage aging.

3.6 Seed germination test

For the germination test, cabbage seeds were spread on the white germinating paper sowing on the Copenhagen table at 20°C with 12hrs light and 12hr dark in the open air. 96 wells seed dispenser from LabTIE (www.labtie.com) was used to help better organize the seeds sowing and confirm the number of the seeds. Each group of seeds have 3 technical replicates with all the position of the samples randomized on the table. Cabbage germination data were counted twice a day manually, mostly around 9:30 a.m and 17:00 p.m, counting the number of germinated seeds. One germination test with cabbage seeds lasted about ten days.

Figure 6.*60 cabbage seed sowed on the paper.*

For all the Arabidopsis seeds will be sowed on the blue germination paper in the trays, sealed in the plastic bag in the22°C all light cabinet. Every day takes the two-time-point a photo to collect the germination data. Next step after collected all the data, we used the Germinator system developed by the lab (Joosen, Kodde et al. 2010). One germination test with Arabidopsis seeds lasts about six days.

.Results

4.1 Testing four different containers to equilibrate the seeds

This pre-experiment was designed to test the optimal methods to make samples ultra-dry quickly. We stored the seeds in four storage methods – tubes (with holes), seed paper bags and two innovate new storage methods – tea bags and plastic bags for bread-use. These four methods have a different way in air/water-contact with the surrounding atmosphere, and the best method could be used for the later experiments. The main experiment is performed on the arabidopsis(small seed) and cabbage seeds(large seed). Because of limitations in available seeds from those species, we used the lettuces and barley seeds in this pre-experiment.

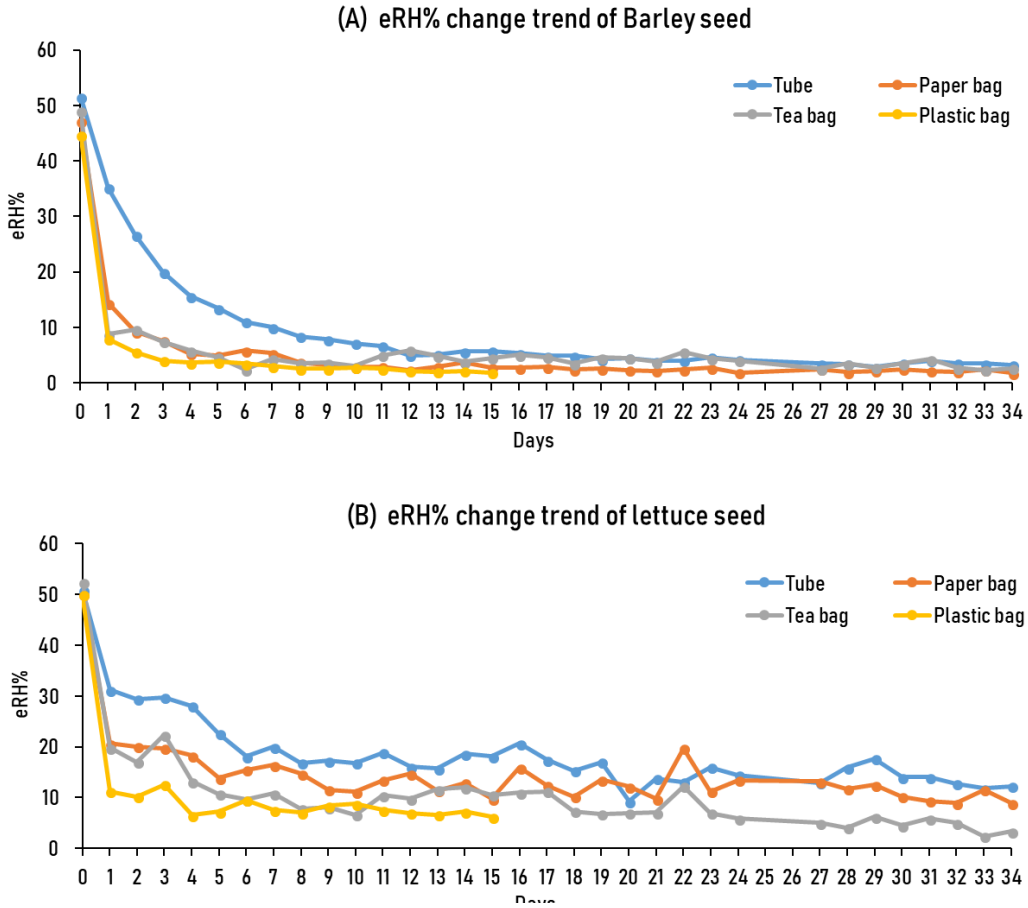


Figure 7.*Development of seed moisture level, measured as equilibrium relative humidity, during drying of barley (A) or lettuce (B) seeds in four different types of containers mixed with drying beads.*

From the figure 7A above, we can see that for the barley seeds, the humidity of the seeds gets down gently in a relatively smooth curve. Compared to the other three methods, the plastic bag only has 15 days of data available, due to the late start of the testing. The curves indicate that all the methods lead the seeds to a same related humidity after 30 days storage, all the difference of the dry speed mainly in the first 15 days. The eRH of seeds stored in the plastic bags have a sharp decrease after storing one day, and they decreased faster than the other three methods in the first 15 days. On the contrary, seeds in the tube with holes have the slowest speed.

The measurement data of the lettuces shows more rocking point during the days. We assume it is possible because of the small volume of the lettuce seeds are not fit with the blocks in the water activity meter. Thus there is a large space lag between the seeds and the sensor which may make seeds absorb more moisture during the test(see figure5). Moreover, the sensor may absorb moisture from the room RH in the space lag, causing the rise of the detected value in a high room humidity day(e.g. raining or foggy days). So, measurement is not exactly accurate when the using the water activity meter to test small size seeds with a relatively large probe size. The chart (figure7B) indicated that until the third week, a tube with holes, paper bags, and tea bags performed closely the same. Tea bags have the fastest decreasing trend, which equilibrates the seeds from 55% to 10% within the first week. The plastic bag for bread shows a sharper decrease in the first weeks.

Due to the overall consideration, we chose the plastic bag as the latter method for the cabbage seed storage. For the arabidopsis seeds,considering the unstable data during the tests, our final decision was using small tubes (PCR tubes) with tiny holes on the lid to store the small arabidopsis in the following experiments.

4.2 Effect of ultra-dry storage on aging speed

The cabbage and arabidopsis seeds were packaged in corresponding storage containers and placed in different environments to begin seed aging and seed longevity testing. Figure 8 shows that the maximum germination rate of the extremely dry seeds decreased significantly from the 8th week to the 12th week compared with the ambient storage. From the chart, we can see that the germination rate of the kale cabbage decreased from ~100%to the 80% and red cabbage seed after 12 weeks have been reduced to around 60%. The result indicated that two kinds of cabbage had lost some of the longevity during the 12 weeks storage, that is to say, ultra-dry with high oxygen storage method has the function aging the seeds fast. Additionally, two different genotype cabbage showed a significant different after the storage, which reveals a diverse inherent storability between the seeds.

All the Arabidopsis mutant have no significant difference at maximum germination with the Col-0 (WT) after eight weeks of ultradry storage(Figure 9). But among all the mutant arabidopsis seeds did have some significant difference. This result indicated that during the eight-week period of the arabidopsis experiment, different kinds of mutant arabidopsis seeds had different degrees of aging. E.g., some of which aging faster than 4806 (tt7-5), and the germination rate was only about 50% after eight weeks. This is also the next step to study the differences in seed storage properties of different seed colors for seeds under adverse conditions.

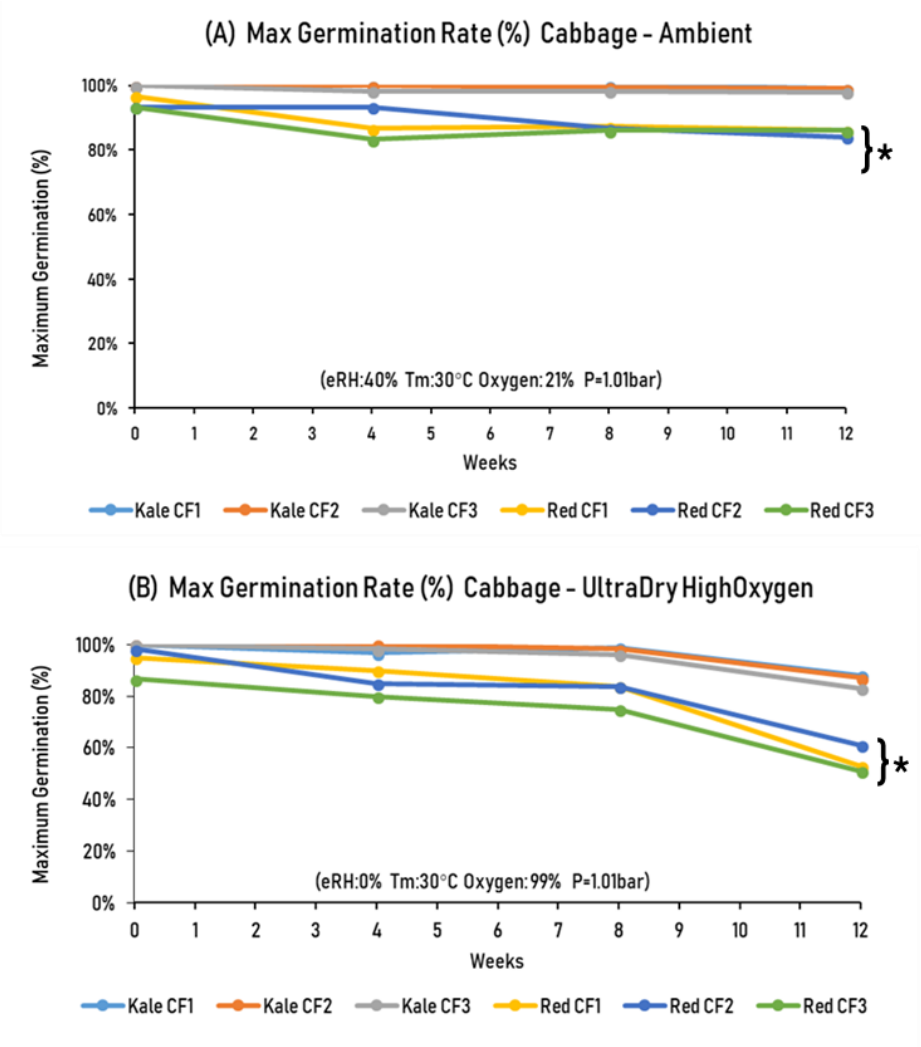


Figure 8. *Germination rate of cabbage seeds with different CF level under ambient (A) and ultra-dry with high oxygen level (B) treatments (0-12 weeks). The asterisk indicates a significant difference($p<0.05$) between the genotypes.*

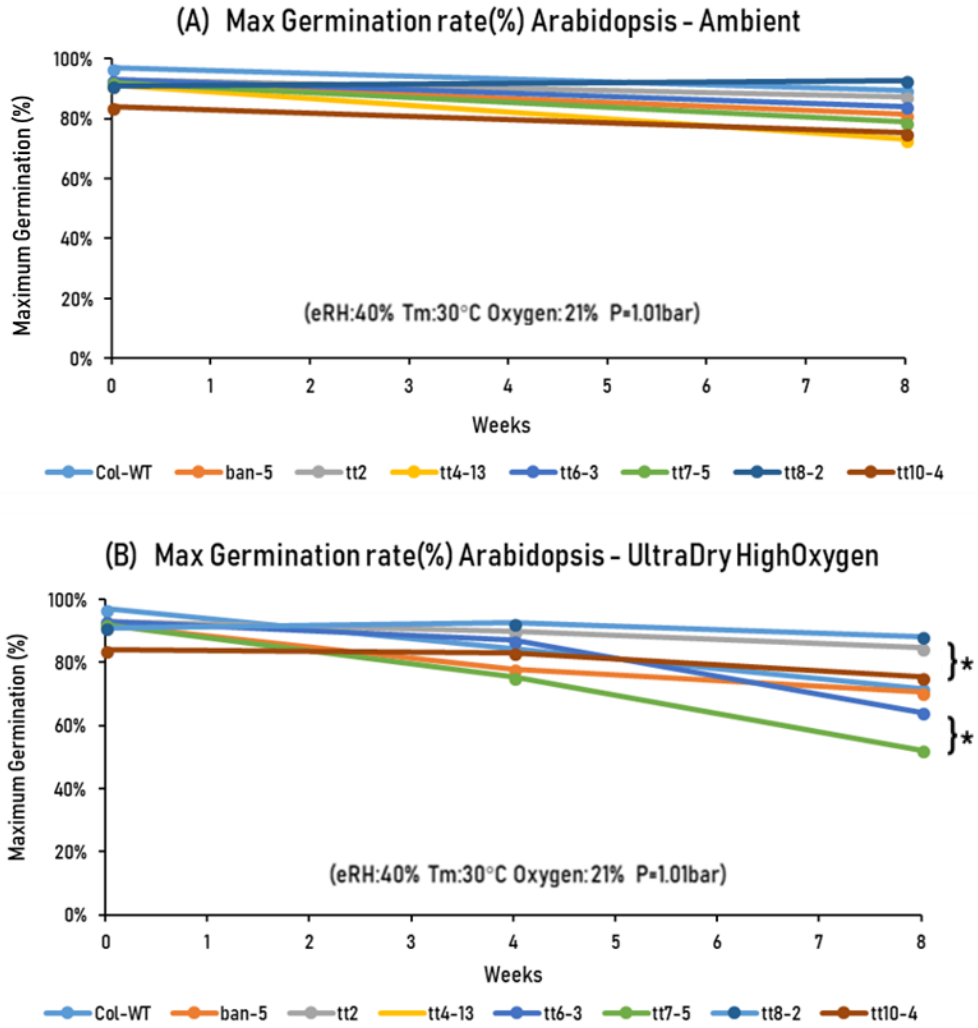


Figure 9. *Germination rate of Arabidopsis seeds with different seed coat colour under Ambient (A) and Ultra-dry with high oxygen level (B) treatments (0-8 weeks). The asterisk indicates a significant difference($p<0.05$) only between the different mutants*

4.3 Effect of different Arabidopsis seed coat color on the seed storability

For the different aging speeds of different categories of arabidopsis seeds seen in the experiment of germination, we aimed to further explore the primary potential correlation of seed coat colour to seed storage for further exploration and research. We used the eighth week of Arabidopsis seeds to carry out the germination experiment together with the untreated (week 0) seeds, and based on the experimental results, the results are shown in the following figure10. From the figure10, we can draw the following two conclusions: 1. The mutant seeds did not differ significantly from the wild-type seeds in their sensitivity to eight weeks storage under ultra-dry conditions with 100% oxygen 2. there was a certain difference between different mutants, which reveals a different sensitivity to the storage environment. So, we could expect that different seed coat color may serve as the indicator of the antioxidant that influences the seed longevity during the ultra-dry storage. However, with relative light-colored seeds, the germination rate is higher after the eighth week compared to the darker seeds, which conflicts with our hypothesis.

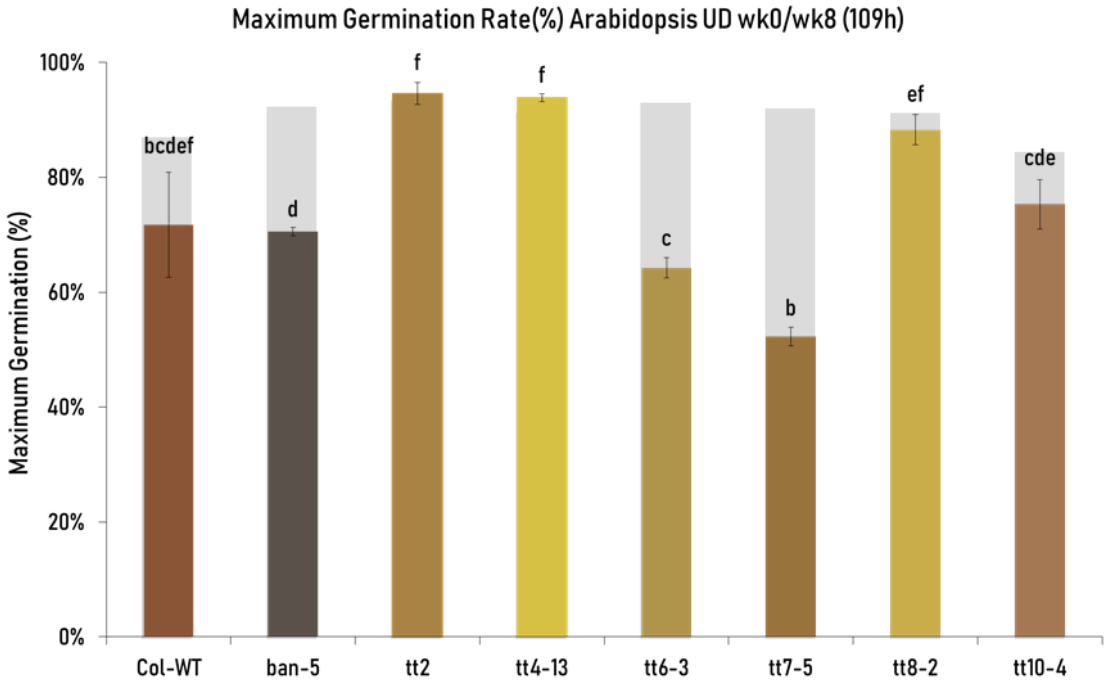


Figure 10.*Different Arabidopsis seeds maximum germination rate difference after eight weeks ultra-dry storage (The colour of the columns represents the same seed coat colour of the different seed categories. Grey background shows the decreased germination rate after the ultra-dry with high oxygen treatment)*

4.4 Effect of different cabbage seed maturities on seed storability

From the experimental results 8, we can see that the seeds from different CF fractions from the kale and red cabbage seed lot did not differ in their sensitivity to ultra-dry storage, which deviates from our expectations. So, we measured the chlorophyll content of the seeds obtained from the Company Bejo™ ourselves. Through experimental data analysis, the Figure.11 reveals that with both varieties, except for kale CF 3, there was no significant difference in the CF levels among the three original seed lots., which indirectly leads to the deviation between the expected experimental results and the actual results.

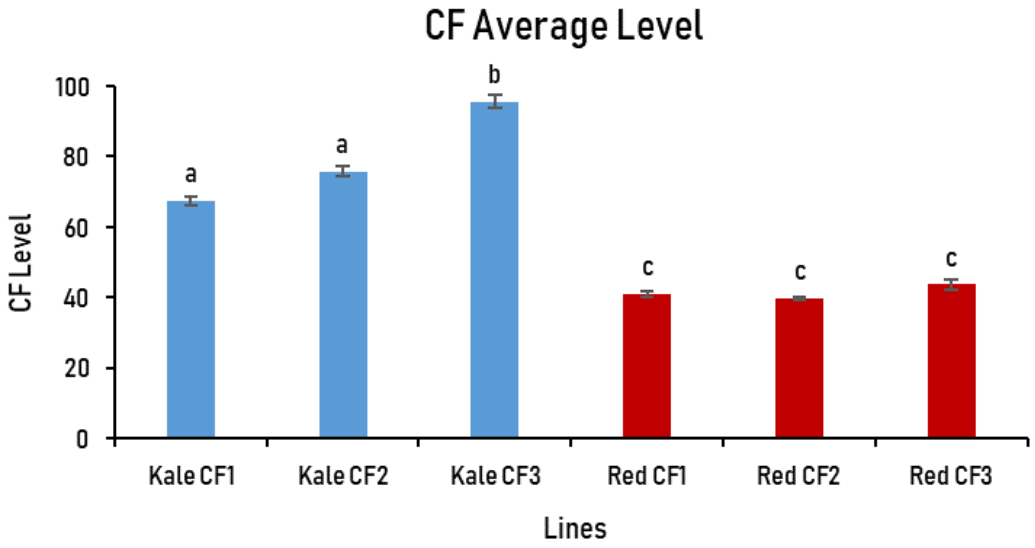


Figure 11. *Chlorophyll fluorescence levels for the different seed lot fractions provided by Bejo. Blue columns represent three Kale cabbages seed fractions and red columns refers to the fractions from the red cabbage seed lot.*

4.5 Effects of EPPO and EPPN storage on different seeds

In previous experiments, scientists had demonstrated that EPPO could be quickly used in seed aging tests. This experiment also carried out the great aging effect of the seed under high oxygen pressure.

Compared with the control treatment EPPN (Figure 14A), the germination rate of two genotype cabbage seeds has decreased during the six weeks under EPPO storage. This result confirmed that high-pressure oxygen level could age the seeds fast without high temperature and humidity level. Additionally, Kale cabbage remain a high germination rate in the EPPN situation (Figure 14B), contrarily, for red cabbage, the chart showed the high pressure of the nitrogen also brought slight damage to the seeds after storage. The conclusion again verified the side-effect of EPPO system that high pressure may cause damage during the storage in Groot's article (Groot, Surki et al. 2012). Form the Figure 15A; The EPPO treatment shows effect within six weeks, the wild-type along with all the mutants got lower than 30% maximum germination rate. However, both two elevated partial pressure methods don't have a significant difference within all the mutant and the wildtype.

From the comparison of Figure 8 and Figure 14 we can see that during the simultaneous aging of the cabbage seeds, two kinds of cabbage seed have a significant difference in all the treatments. The maximum germination rate of the variety Kale Cabbage after a period remains relatively stable and relatively high. Especially under the EPPO method - the germination rate of about 80% can still be maintained. In comparison, red cabbage seeds are more susceptible to environmental humidity, oxygen level, and air pressure influence, demonstrating more sensitive seed longevity and significant changes during the aging process. Therefore, Kale Cabbage has better storage ability and seed quality for the company's two seed varieties we used.

In our experiments, we also found that when the high-pressure treated cabbage seeds are placed on the germinated paper, the cabbage seed coat will burst when the seeds imbibed the water, and white spots will appear on the embryos. After the germination test, it turned out that seeds with bubbles on the seed coat can germinate and grow, but the cracked seed will not germinate anymore. The phenomenon will be discussed later next part. In the Arabidopsis lines, this phenomenon is not observed but still needs more experiment to confirm (Figure 12).

We collected the same maturity Kale seeds from the different timepoint of EPPO and compared with the ambient week0 which is untreated seeds. These seeds were taken from the -20 centigrade and then treated on the infiltrated germination paper. The figure clear shown that kale cabbage seeds stored after two weeks already had the white bubble pattern (Figure 13) and the seed coat already cracked. And after six weeks under the treatment of EPPO, Kale cabbage seeds had much more bubble pattern than week 2. This kind of seed cracks also observed in mung beans under the much higher pressure 475 MPa(~4750bar) (Peñas, Gómez et al. 2010).

The comparison also conducted between the EPPO and EPPN treatments, and from the figure13 it shows that the EPPN seeds have the same bubble pattern as EPPO which indicate that the high gas pressure or its release is the main cause of the seed coat damage. Whatsmore, we also collected the cracked seeds from EPPN, it shows the damage was not only happened in the seed coat level but also have the cracks at the hypocotyl, which may cause lethal damage to the seeds.

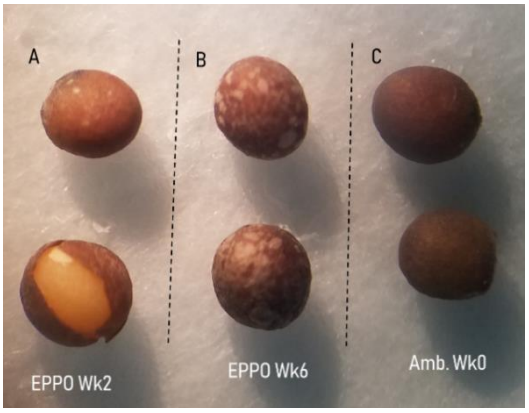


Figure 12. *Kale cabbage seeds after treatment in EPPO system compare with control*

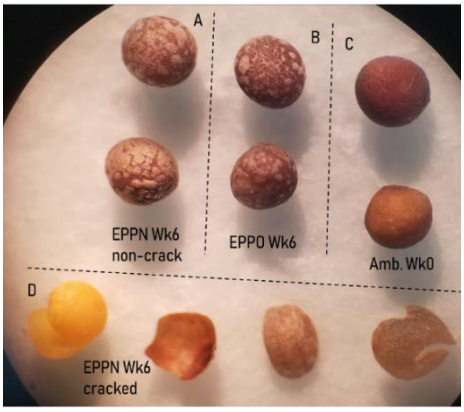
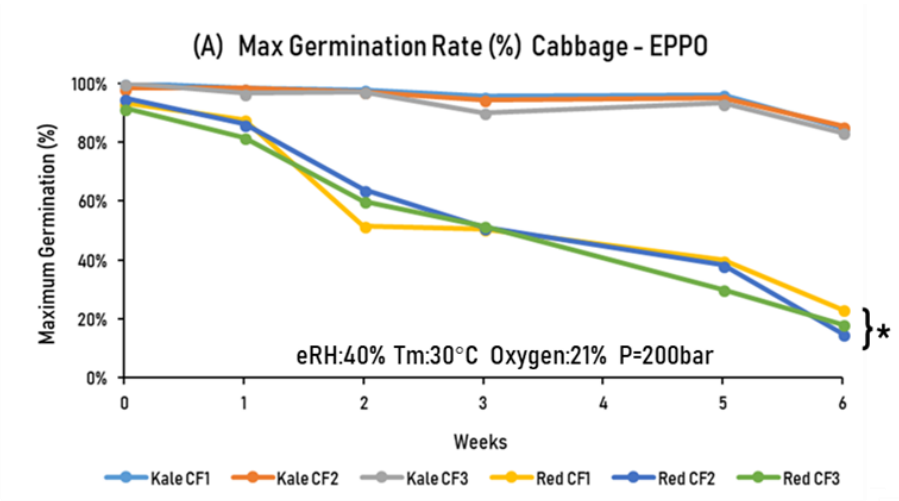


Figure 13. *Non-crack and cracked Kale cabbage seeds after treatment in EPPO and EPPN ageing system*



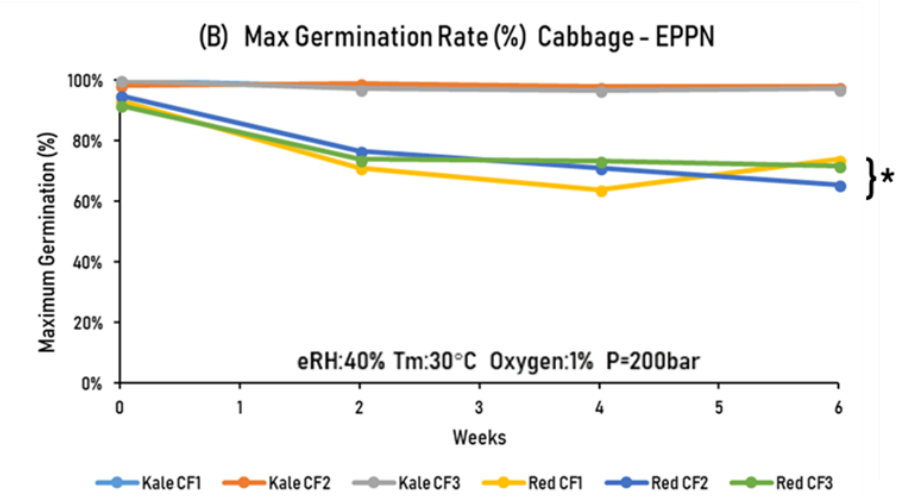
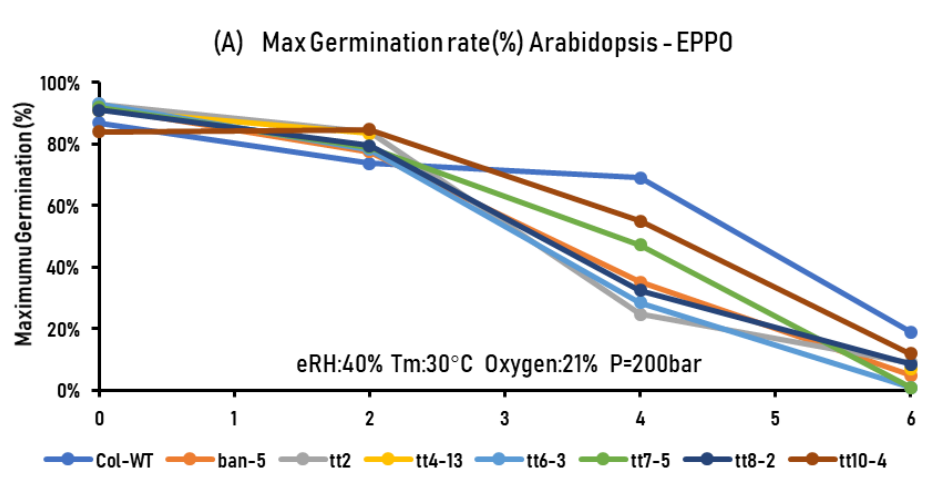


Figure 14. *Germination rate of cabbage seeds with different maturities under EPPO (A) and EPPN (B) treatments (0-6 weeks). The asterisk indicates a significant difference ($p<0.05$) between two kinds of cabbage.*



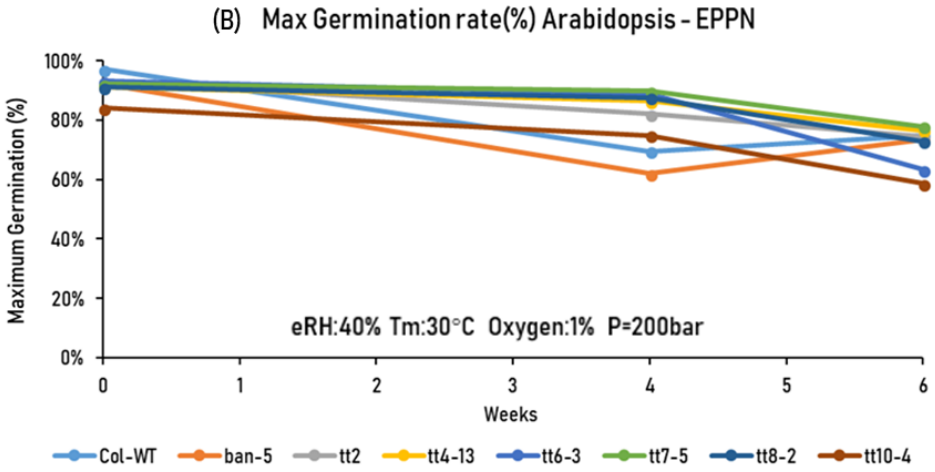


Figure 15 *Germination rate of Arabidopsis seeds with different seed coat colour under EPPO (A) and EPPN (B) treatments (0-6 weeks).*

5. Discussion

5.1 Gas pressure and Seeds burst

In the first introduction of the EPPO system(Groot, Surki et al. 2012), gas pressure had been aroused as a possible negative influence that will cause damage to the stored seeds to expect from the methods we apply. So they applied a pressure releasing system to recover this problem. But from our research, we still found that from some timepoint, EPPO and EPPN Kale brassica seeds had the significant burst phenomenon when starting water imbibition, and red cabbage was not significant.

Why the kale cabbage seeds are more easily cracked than the red cabbage seeds under the high gas pressure is not clear, According to the new research in the soybeans, scientists have the evidence that the pigmented seeds or unperfect-black seeds have more possibilities to crack after the high-pressure storage(Senda, Yamaguchi et al. 2017). And that buff-pigmented linked with the accumulation of the proanthocyanidins(PAs) and lignin deposition in the seed coat that may change the physical properties. Compare with the red cabbage; kale cabbage has lighter seed coat color and larger seed size. But in total, the cabbage seeds are different from the yellow soybean, so it is good to test the PAs level in the kale/rode cabbage to make a contrary. What's more, in the article by (Liang, Davis et al. 2006, Zhang, Lu et al. 2013), they find that in Arabidopsis AtLAC15/tt-10 and *Brassica napus* TT10 gene can regulate the accumulation of PAs and lignin. We also used tt-10 mutant in our experiment, but we didn't give specific notice about that. So, it is possible to have those tt10 knockdown mutant lines in both Arabidopsis and Brassica to correlate the relationship between the seed crack and accumulation of PAs and lignin.

For this experiment, we didn't observe the seed integrity of arabidopsis seeds after the EPPO and EPPN storage, which is worth for the further exploration. In figure 15, two kinds of mutants have even lower germination rate at week four compare to the week 6. Seed burst may be a reason that makes the diverse color seeds differ from each other in the high-pressure storage and make the germination rate lower than normal condition.

5.2 Antioxidant function during ultra-dry storage

From the figure 10, We can see that there is still a difference in germination rate between different seed mutants, especially between dark and light seeds. However, regarding results, the maximum germination rate of light-colored seeds is higher than that of dark seeds. That is to say; the dried darker seed has a relatively faster aging rate than the lighter seed. In some crops, light-colored soy seeds are indeed more shelf-storing than darker soybeans(Liu, Qin et al. 2017). However, according to our assumption, for Arabidopsis, dark seeds means normal antioxidant flavonoid components in the seed coat, which should be beneficial to resist the internal oxidation reaction of the seeds during the drying process, but the experimental results are opposite. About the antioxidant function during the seed storage especially the dry condition, there still have some controversy, In 5% eRH seed aging of four Australian species over 18 months, the group showed the insignificant difference between the antioxidant activity and seed viability after the

storage. (Merritt, Senaratna et al. 2003). On the other hand, Chen's team reveal that the peroxiredoxin antioxidant can enhance the Arabidopsis seed longevity in the CDT/abiotic aging experiment (Chen, Chu et al. 2016). So it is also good to have more detailed research on the antioxidant content analysis in each mutant against the viability after ultra-dry aging method, to one more step confirm the real function induced by the antioxidant on the seed coat.

5.3 Seeds under controlled deterioration

In this experiment, we also aged the two kinds of seeds in the controlled deterioration. Finally, the germination rate of Arabidopsis seeds in the eighth week was close to 0, and the red cabbage seed was only 50% of the maximum germination rate (Kale cabbage still with high germination rate which shows high tolerant to EPPO and CD). However, we did not see a significant difference with the wildtype in the Arabidopsis mutant, which is also no tt mutant was observed in the CDT environment and wild-type in the Tesnier's experiment (Tesnier, Strookman-Donkers et al. 2002). These are different from Chen's experimental results(Chen, Chu et al. 2016) work on the NnPER1 mutant. So it is possible to re-select the Arabidopsis mutant accessions to re-do the experiment, maybe the transparent testa lines are not relatively linked to the antioxidant activity value during the stress storage. At the same time, due to the small difference in the maturity(CF value) of the cabbage itself, we have not been able to observe the difference between different maturity levels in the CDT environment. It's better to have the opportunity to use the seeds with large differences in maturity for the related experiment.

6. References

Abdalla, F. and E. Roberts (1968). "Effects of temperature, moisture, and oxygen on the induction of chromosome damage in seeds of barley, broad beans, and peas during storage." Annals of Botany **32**(1): 119-136.

Appelhagen, I., et al. (2014). "Update on transparent testa mutants from Arabidopsis thaliana: characterisation of new alleles from an isogenic collection." Planta **240**(5): 955-970.

Bailly, C. (2004). "Active oxygen species and antioxidants in seed biology." Seed Science Research **14**(2): 93-107.

Bewley, J. D., et al. (2012). Seeds: physiology of development, germination and dormancy, Springer Science & Business Media.

Buijs, G., et al. (2018). "Seed dormancy release accelerated by elevated partial pressure of oxygen is associated with DOG loci." Journal of experimental botany: ery156.

Castro-Morales, L. M., et al. (2014). "Environmental factors affecting germination and seedling survival of Carolina willow (Salix caroliniana)." Wetlands **34**(3): 469-478.

Chen, H. h., et al. (2016). "Ectopic expression of NnPER1, a Nelumbo nucifera 1 - cysteine peroxiredoxin antioxidant, enhances seed longevity and stress tolerance in Arabidopsis." The Plant Journal **88**(4): 608-619.

Covell, S., et al. (1986). "The influence of temperature on seed germination rate in grain legumes: I. A comparison of chickpea, lentil, soyabean and cowpea at constant temperatures." Journal of experimental botany **37**(5): 705-715.

Debeaujon, I., et al. (2000). "Influence of the testa on seed dormancy, germination, and longevity in Arabidopsis." Plant physiology **122**(2): 403-414.

Demir, I. and M. Ozcoban (2007). "Dry and ultra-dry storage of pepper, aubergine, winter squash, summer squash, bean, cowpea, okra, onion, leek, cabbage, radish, lettuce and melon seeds at− 20° C and 20° C over five years." Seed Science and Technology **35**(1): 165-175.

Dictionary, O. E. (2008). "Oxford english dictionary." Retrieved May **30**: 2008.

Ekpong, B. (2009). "Effects of seed maturity, seed storage and pre-germination treatments on seed germination of cleome (Cleome gynandra L.)." Scientia Horticulturae **119**(3): 236-240.

Ellis, R., et al. (1996). "Survival of dry and ultra-dry seeds of carrot, groundnut, lettuce, oilseed rape, and onion during five years' hermetic storage at low temperatures." Seed Science and Technology (Switzerland). v. 24 (2).

Ellis, R., et al. (1990). "An intermediate category of seed storage behaviour? I. Coffee." Journal of experimental botany **41**(9): 1167-1174.

Ellis, R., et al. (1991). "Effect of storage temperature and moisture on the germination of papaya seeds." Seed Science Research **1**(1): 69-72.

Ellis, R. and E. Roberts (1980). "Improved equations for the prediction of seed longevity." Annals of Botany **45**(1): 13-30.

Ellis, R. H. (1991). "The longevity of seeds." HortScience **26**(9): 1119-1125.

FANG, Y. and J. ZHOU (2007). "Study on Pre-Humidification Treatment for Ultra-dried Betula

luminifera Seeds [J]." Seed **2**: 010.

Groot, S., et al. (2006). "Effect of seed maturity on sensitivity of seeds towards physical sanitation treatments." Seed Science and Technology **34**(2): 403-413.

Groot, S., et al. (2012). "Seed storage at elevated partial pressure of oxygen, a fast method for analysing seed ageing under dry conditions." Annals of Botany **110**(6): 1149-1159.

Hong, T., et al. (2005). "Survival and vigour of ultra-dry seeds after ten years of hermetic storage." Seed Science and Technology **33**(2): 449-460.

Jalink, H., et al. (1998). "Chlorophyll fluorescence of Brassica oleracea seeds as a non-destructive marker for seed maturity and seed performance." Seed Science Research **8**(4): 437-443.

Joosen, R. V., et al. (2010). "GERMINATOR: a software package for high - throughput scoring and curve fitting of Arabidopsis seed germination." The Plant Journal **62**(1): 148-159.

Labuza, T. P. and L. Dugan Jr (1971). "Kinetics of lipid oxidation in foods." Critical Reviews in Food Science & Nutrition **2**(3): 355-405.

Liang, M., et al. (2006). "Involvement of AtLAC15 in lignin synthesis in seeds and in root elongation of Arabidopsis." Planta **224**(5): 1185.

Liu, J., et al. (2017). "Metabolism variation and better storability of dark-versus light-coloured soybean (Glycine max L. Merr.) seeds." Food chemistry **223**: 104-113.

McDonald, M. (1999). "Seed deterioration: physiology, repair and assessment." Seed Sci. Technol. **27**: 177-237.

Merritt, D., et al. (2003). "Seed ageing of four Western Australian species in relation to storage environment and seed antioxidant activity." Seed Science Research **13**(2): 155-165.

Ming, R., et al. (2013). "Genome of the long-living sacred lotus (Nelumbo nucifera Gaertn.)." Genome Biol **14**(5): R41.

Mohamed-Yasseen, Y., et al. (1994). "The role of seed coats in seed viability." The Botanical Review **60**(4): 426-439.

Moïse, J. A., et al. (2005). "Seed coats: structure, development, composition, and biotechnology." In Vitro Cellular & Developmental Biology-Plant **41**(5): 620-644.

Nagel, M., et al. (2016). "Barley seed aging: genetics behind the dry elevated pressure of oxygen aging and moist controlled deterioration." Frontiers in plant science **7**: 388.

Peñas, E., et al. (2010). "Effects of combined treatments of high pressure, temperature and antimicrobial products on germination of mung bean seeds and microbial quality of sprouts." Food control **21**(1): 82-88.

Pérez-García, F., et al. (2007). "High viability recorded in ultra-dry seeds of 37 species of Brassicaceae after almost 40 years of storage." Seed Science and Technology **35**(1): 143-153.

Powell, A. and G. Harman (1985). "Absence of a consistent association of changes in membranal lipids with the ageing of pea seeds." Seed Science and Technology (Netherlands).

Powell, A. A. and S. Matthews (2005). "Towards the validation of the controlled deterioration vigour test for small seeded vegetables." Seed Testing International **129**: 21-24.

Rotronic, A. G. (2011). "HygroLab C1 Bench-Top Indicator User Guide."

Routaboul, J.-M., et al. (2006). "Flavonoid diversity and biosynthesis in seed of Arabidopsis thaliana." Planta **224**(1): 96-107.

Sano, N., et al. (2015). "Staying alive: molecular aspects of seed longevity." Plant and Cell Physiology **57**(4): 660-674.

Schwember, A. R. and K. J. Bradford (2011). "Oxygen interacts with priming, moisture content and temperature to affect the longevity of lettuce and onion seeds." Seed Science Research **21**(3): 175-185.

Senda, M., et al. (2017). "Accumulation of proanthocyanidins and/or lignin deposition in buff-pigmented soybean seed coats may lead to frequent defective cracking." Planta **245**(3): 659-670.

Shejbal, J. (1979). "Preservation of cereal grains in nitrogen atmospheres." Resource Recovery and Conservation **4**(1): 13-29.

Singh, N., et al. (2016). "Survival of chickpea, sesame, niger, castor and safflower seeds stored at low and ultra low moisture contents for 16-18 years." Seed Science and Technology **44**(3): 542-555.

Steiner, A. and P. Ruckenbauer (1995). "Germination of 110-year-old cereal and weed seeds, the Vienna Sample of 1877. Verification of effective ultra-dry storage at ambient temperature." Seed Science Research **5**(4): 195-199.

Taylor, L. P. and E. Grotewold (2005). "Flavonoids as developmental regulators." Current opinion in plant biology **8**(3): 317-323.

Tesnier, K., et al. (2002). "A controlled deterioration test for Arabidopsis thaliana reveals genetic variation in seed quality." Seed Science and Technology **30**(1): 149-166.

Zhang, K., et al. (2013). "Gene silencing of BnTT10 family genes causes retarded pigmentation and lignin reduction in the seed coat of Brassica napus." PloS one **8**(4): e61247.