WAGENINGEN UNIVERSITY & RESEARCH

MASTER THESIS

Rice Seed Longevity

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A thesis submitted in fulfilment of the requirements for the degree of Master of Science

in the

Bioscience Institute
Wageningen Plant Research

2019-05-20

Declaration of Authorship

I, SMA (Stefan) VROOM, declare that this thesis titled, "Rice Seed Longevity" and the work presented in it are my own. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University.
- Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated.
- Where I have consulted the published work of others, this is always clearly attributed.
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work.
- I have acknowledged all main sources of help.
- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself.

Signed: 

Date: 2019-05-20

"*Variety is the very spice of life, That gives it all its flavor.*"

William Cowper

"*Of all things, I liked books best.*"

Nikola Tesla

"*Food is the moral right of all who are born into this world.*"

Norman Borlaug

"*If we crave some cosmic purpose, then let us find ourselves a worthy goal.*"

Carl Sagan

"*No man is an island, entire of itself; every man is a piece of the continent, a part of the main.*"

John Donne

"Science is not only a disciple of reason but, also, one of romance and passion."

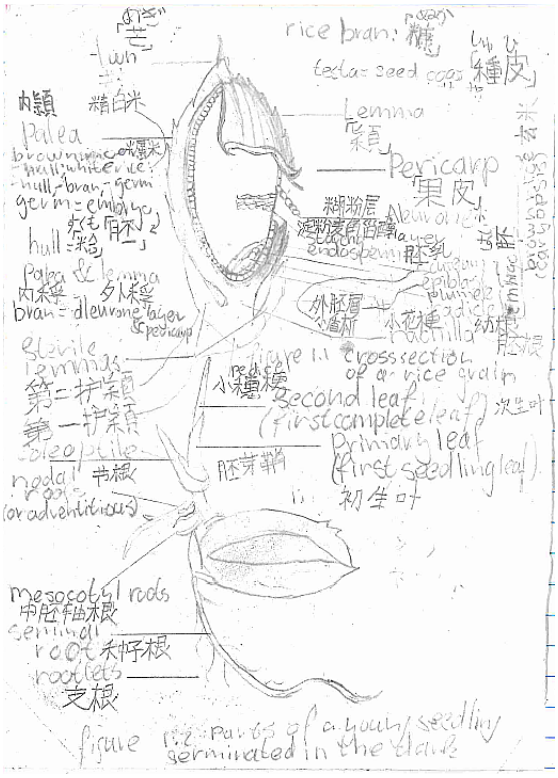
Steven Hawking

"*Failure is success in progress.*"

Albert Einstein

"*Practice must always be founded on sound theory, and to this Perspective is the guide and the gateway; and without this nothing can be done well in the matter of drawing.*"

Leonardo Da Vinci



Figures redrawn from Maclean, Hardy, and Hettel, 2013.

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Abstract

Plant Sciences
Wageningen Plant Research

Master of Science

Rice Seed Longevity

by SMA (Stefan) VROOM

Seed longevity, defined as seed vigour after drying; is an important trait in the future of plant breeding, as food security becomes evermore important. Increase in seed longevity gives seedlings a head start and thus can result in better yields. Genes involved in seed longevity are numerous. In this study focus was put on rice, the crop that feeds near half of the world's population and, more specifically the Rc gene in rice and its ortholog gene TT8 among other genes involved in the flavonoid biosynthesis pathway in thale cress. The flavonoid biosynthesis pathway is known for its production of antioxidant components, such as proanthocyanidin. Studies by others have elucidated that the Rc is encoding for a bHLH transcription factor and is involved in the production of proanthocyanidin. Furthermore, others found out that under dry conditions elevated partial pressure of oxygen decreases seed longevity for many different plant species. This study illustrates the effect on seed longevity by proanthocyandin accumulation of the pericarp of rice under elevated partial pressure of oxygen under different humidity levels. For this, two nearly-isogenic lines were used that either had a functional or non-functional Rc gene. This study showed that in rice it was shown that the Rc gene increases seed longevity under EPPO conditions under various humidities and also decreases the permeability of the pericarp of the seed. It does this by encoding for bHLB transcription factor which is involved in the transcription of Rd, which indirectly gives the rice a red colour as the condensed tannins colourise under oxidation. The increase in resistance against oxygen is most likely caused by the accumulation of proanthocyanidins, since they have show decent antioxidant activity. In thale cress results were contradicting with the Rc ortholog i.e. TT8, perhaps this is because of different accumulation of flavonoids altering the total antioxidant capacity in opposite fashion. Furthermore, research in to other thale cress genes showed large differences in seed longevity; one of these mutations resulting in an increased seed longevity which could be readily explained was the ban5 gene. The ban5 mutation was accumulating more anthocyanidins than proanthocyanidin in the pericarp; this results in a higher total antioxidant activity. Exciting opportunities await in the realm of seed longevity research and enough information is available already to incorporate in to a breeding program. Genes that are worth researching in rice seed in terms of longevity include but are not limited to: PIMT1, Cht-2, Cht-3 and Kala4.

Acknowledgements

Thanks to all the plants and fungi for the inspiration.
Thanks to all the neurons that fired their action potentials.
Thanks to all the electric circuitry for on and off outputting output when inputting input.
Thanks to Joost and Gerco H. for the help during my neurophysiological Bachelor's thesis.
Thanks to Manjunath, Jan, Steven and Gerco A. for the help during my Master's thesis.
Thanks to family and friends for the social and financial support.
Thanks to all the teachers that paved our roads with fungi and plants.
Thanks to professor Gu for providing the genetic material.
Thanks to Vel and Johannes Böttcher for the template in LaTeX.
Thanks to the people of Overleaf for speeding up writing in LaTeX.
Thanks to 宮本武蔵 (*Miyamoto Musashi*) for the 二鑷子一 (two forceps as one) technique.
Thanks to 村岡仁 (*Muraoka Jin*) for the 抹茶入り玄米茶 (*matcha-iri genmaicha*).

Contents

List of Figures

List of Tables

List of Abbreviations

NIL	**N**early **I**sogenic **L**ine
WT	**W**ild **T**ype
GA	**G**ibberellic **A**cid
ABA	**AB**scisic **A**cid
EPPO	**E**levated **P**artial **P**ressure **O**xygen
EPPN	**E**levated **P**artial **P**ressure **N**itrogen
bHLH	**b**asic **H**elix-**L**oop-**H**elix
QTL	**Q**uantitative **T**rait **L**ocus
TF	**T**ranscription **F**actor
GR	**G**ermination **R**ate
CDT	**C**ontrolled **D**etoriation **T**reatment
AAT	**A**ccelerated **A**ging **T**reatment
TEAC	**T**rolox **E**quivalent **A**ntioxidant **A**ctivity
DSR	**D**irect- **S**eeded **R**ice
GMO	**G**enetically **M**odified **O**rganism
Q-Organism	**Q**uarantined-Organism

Dedicated to all the fungi and plantæ…

Chapter 1 Introduction

This chapter introduces the aspects of rice seed longevity.

1.1 Rice Seed

This section focuses on the physiological and genetic aspects of rice seed longevity and why it demands improvement.

1.1.1 Cultivation and Culture

The start of *Oryza spp.* i.e. rice, cultivation, dates back either to 6500 BCE near the Yangtze (Traditional Chinese: 長江 [Long river]), which is based on archaeological discoveries (Normile, 1997; Deng et al., 2015) or near the middle of the Zhujiang (Traditional Chinese:珠江 [Pearl River]) located in the Guanxi province, based on genomic mapping (X. Huang et al., 2012). As of today, for low and low-middle income countries, rice is the dominant food crop (Maclean, Hardy, and Hettel, 2013). Especially for these countries, it will be beneficial to have improved seed storage.

Rice is grown on all continents except on crop-less Antarctica. Next to that rice is integrated in many cultures across the world, where it pays a strong contribution to culture and is celebrated through festivals and religious events (Chauhan, Jabran, and Mahajan, 2017). Furthermore, rice straw can be used to make useful things such as hats (菅笠) and sandals (草鞋). Moreover, rice straw, as well as brown rice can be used as mushroom substrate.

Two species of rice are currently being cultivated; African rice: *O. glaberrima* and Asian rice: *O. sativa*, the latter feeds nearly half of the *Homo sapiens* population. Asian rice, knows two subspecies that have been distinguished for over 2000 years, namely *O. sativa ssp. indica* (Chinese/Japanese: 籼) and *O. sativa ssp. japonica* (Chinese/Japanese: 粳) (Maclean, Hardy, and Hettel, 2013; 3,000 Rice Genomes Project, 2014; W. Wang et al., 2018).

1.1.2 Motivation

Where seed vigour increases, germination time shortens, allowing for beneficial factors, e.g. faster rooting, increased stress tolerance, such as to competing weeds, resulting in better yields (Bewley, Bradford, Hilhorst, et al., 2012; Stewart and Bewley, 1980).

1.1.3 Development

After a fusion of male and female gametes, a zygote is formed. Through embryogenesis the zygote develops into an embryo, storage organs such as the endosperm are formed and the maternal integument layers morph into testa (seed coat). Nutrition, gene expression and hormones play an important role in further development, quality and size of seeds (Bewley, Bradford, Hilhorst, et al., 2012).

1.1.4 Shattering and Dormancy

Spreading of seeds in space and time, succeeds through seed shattering and seed dormancy, respectively. ***Shattering***, is the physical dispersal of seeds. ***Dormancy***, is state of rest of the seed, where the seed is not triggered to germinate under the species specific germination conditions. In general dormancy inducing/breaking conditions are: water, temperature, light and chemicals e.g. smoke. Dormancy can arrive from seed development, as primary dormancy or dormancy can be induced during a state of non-dormancy, as secondary dormancy. Furthermore, there are species other than rice, where seeds require periods of warm, followed by cold temperatures to break dormancy, i.e. warm stratification (WS) and cold stratification (CS), respectively. Finally, similar conditions can trigger germination in non-dormant seeds, as shown in figure 1.1 (Bewley, Bradford, Hilhorst, et al., 2012).

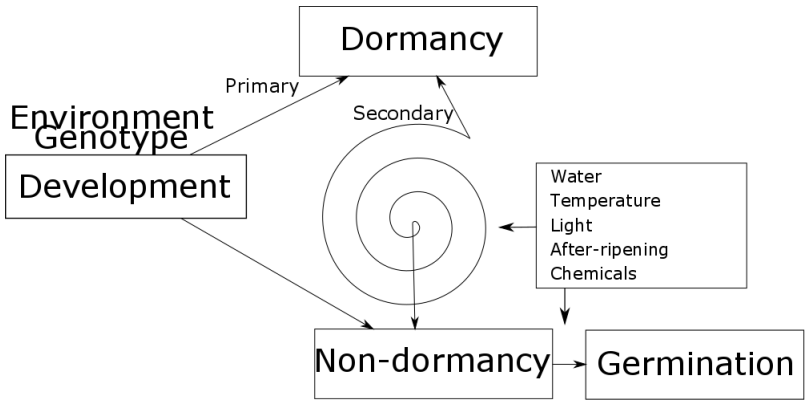


Figure 1.1: Dormancy cycle, image modified from Bewley, Bradford, Hilhorst, et al., 2012

Phytohormones

Two hormones are primarily involved with dormancy. *Abscisic acid (ABA)*, ABA is relevant to seed quality that in can induce and maintain the seed dormancy and is assosciated with reaction to environmental stresses. *Gibberellins (GAs)*, GAs are relevant to seed longevity in that it can break dormancy. In plants GA_1 and GA_4 can be found. While in bacteria and fungi other GAs can be found (Bewley, Bradford, Hilhorst, et al., 2012). Not to mention GAs play an

important role of rice internode elongnation; e.g. under complete submergence (flooding) by products of SNORKEL 1 and 2 genes. These genes can aid in a breeding program to prevent plants from drowning (Hattori et al., 2009). Perhaps these genes can also aid in underwater germination. Nottomention, stem elongnation can help young seedlings thrive in a competitive weedy environment by increasing the odds of receiving direct sunlight.

1.1.5 Germination

Germination sensu stricto is observed upon until radicle emergence as result of expansion of cells. Germination, may be dependent on several factors such as light, oxygen, water availability and temperature (Bewley, Bradford, Hilhorst, et al., 2012).

1.1.6 Longevity

The seed viability after the drying of seeds (storability) defines seed longevity (Rajjou and Debeaujon, 2008). Seed longevity is ensured by ideal genetic material and optimal storage of seeds. Optimal storage can be defined as having the right temperature, moisture conditions and measurements against pathogens and pests (Bewley, Bradford, Hilhorst, et al., 2012). Deterioration of seeds is usually associated with factors such as increased moisture content, temperature, oxygen and fungal,bacterial and insect activity. With increased moisture content and temperature; enzyme activity can go up. Enzymes such as lipoxygenase can decrease seed longevity (J. Huang et al., 2014; Ma et al., 2015; H. Xu et al., 2015). While, embryonic repair enzymes can improve seed longevity (Wei et al., 2015).

Ageing

F. Wang et al., 2012 note that regarding the mechanisms of seed ageing several mechanisms that have been proposed include but are not limited to: *oxidative stress* and *lipid peroxidation and degradation* (McDonald, 1999; Shin, Kim, and An, 2009; Wilson, 1986; Zou et al., 2002).

Viability

Viability, the ability, for a seed, to live succesfully can be described using the mathematical probit (**prob**ability + uni**t**) model:

$$v = K_i - p/\sigma \tag{1.1}$$

Where v probit of percent viability, K_i the seed initial quality indicator, p is the time-dependent expected seed loss and σ the environmental conditions (Bewley, Bradford, Hilhorst, et al., 2012). The probit function can be transformed to a germination curve using the error function (Alu, 2011), as shown in equation 1.2.

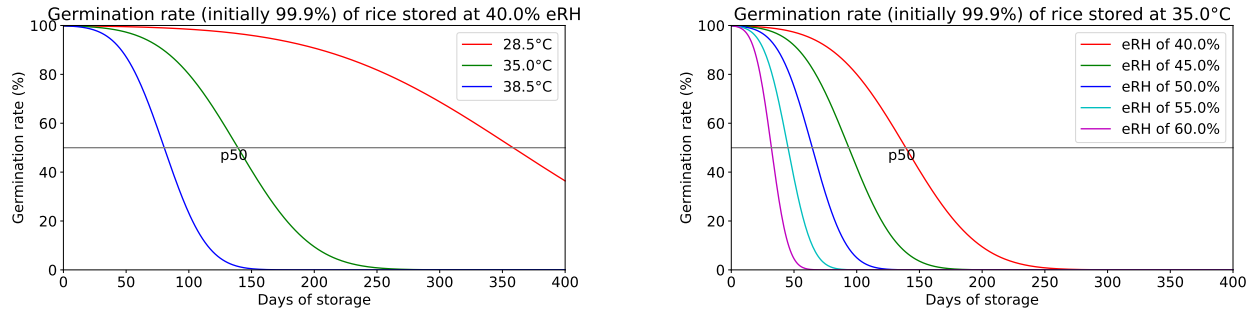
$$\Phi(v) = \frac{1}{2}\left[1 + erf\left(\frac{v}{\sqrt{2}}\right)\right] \tag{1.2}$$

Temperature and seed moisture content

Further expansion on R. Ellis and E. Roberts, 1981 Seed Viability Equation includes the effect of temperature and seed moisture content:

$$\log \sigma = K_e - C_w \log m - C_h T - C_q T^2 \tag{1.3}$$

Where: K_e is a species constant and equals 8.668 for O. sativa, m the seed moisture as fresh weight basis, T is the temperature, C_h and C_q are emperically derived species specific temperature constants from experiments with varying temperature and moisture contents, C_w is the moisture constant. For O. sativa C_w is estimated at 5.03. Whereas, temperature constants C_h and C_q are universally estimated at 0.0329 and 0.000478, respectively (Flynn and Turner, 2004; Bewley, Bradford, Hilhorst, et al., 2012), as shown in figure 1.2.



(a) Effect of temperature

(b) Effect of humidity

Figure 1.2: Theoretical rice seed viability with initial germination rate of 99.9% and the effect of temperature and varying relative humidities (calculated from seed moisture content), p50 indicates 50% of the seeds fail to germinate (R. Ellis and E. Roberts, 1980; Cromarty, R. H. Ellis, E. H. Roberts, et al., 1982). With viability constants derived from a single seed lot (Flynn and Turner, 2004).

As seed moisture content (and thus water activity) increases when seeds move from dry (30-40% RH) to humid (60%) storage, factors such as enzymatic activity, non-enzymatic browning and lipid oxidation increase (Labuza and Dugan Jr, 1971), as shown in figure 1.3.

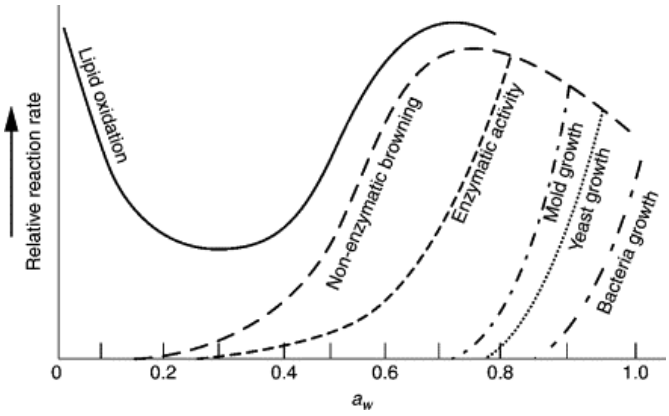


Figure 1.3: Effect of water activity (a_w) on seed lipid oxidation, non-enzymatic browning, enzyme activity, bacteria growth, mold growth and yeast growth; illustration by Labuza and Dugan Jr, 1971.

Fungi et al.

Fungi can be classified as *(1)*, field fungi and *(2)*, storage fungi. Field fungi, invest the seeds during developmental stages and before harvest periods of the plants. Whereas, storage fungi invest the seeds during the storage period of the seeds. Nonetheless, they both require high levels of relative humidity for reproduction, growth and enzymatic activity (Bewley, Bradford, Hilhorst, et al., 2012). An example of a devastating fungus, is primarily seedborne Gibberella fujikuroi, causing the foolish rice disease (translated from Japanese: 馬鹿苗病).This fungus produces an accessive amount of gibberellins that damages the plant (Sparks, Castilla, and Vera Cruz, n.d.). Research indicates, the symbiosis of other fungi with plants. Suggestions arise that symbiotic fungi ward off against pathogens in rice (Baby and Manibhushanrao, 1996), or in barley as well improve abiotic stress tolerance and increase yields of plants (Waller et al., 2005). Use of fungicide can then lead to reduced friendly fungal activity (Kough, Gianinazzi-Pearson, and Gianinazzi, 1987). However, studying genetic interactions between beneficial/harmful fungi and plants and, perform breeding may help solely fight pathogenic fungi.

In contrast to fungi, bacteria barely affect seed deterioration under normal seed storage, as they require water content levels that are not obtained under normal seed storage (Bewley, Bradford, Hilhorst, et al., 2012).

Arthropods

Next to microorganisms, arthropods can negatively affect seed longevity. Insects such as weevils, flour beatles or borers, thrive in warm and humid climates, above 20°C and RH 35%. While mites thrive below 60% RH and can tolerate near frosty temperatures (Bewley, Bradford, Hilhorst, et al., 2012; Maclean, Hardy, and Hettel, 2013).

Acceleration

Several methods of seed aging have been developped. Such methods are controlled detoriation treatment (CDT), accellerated aging treatment (AAT) (Delouche and Baskin, 2016) and elevation of partial pressure of oxygen [O_2] (EPPO) devellopped by Groot et al.. CDT and AAT are characterised by the use of elevated moisture and temperature levels. In research by others CDT the temperature levels range from 35-45°C and the humidity levels are around 70-80% for AAT temperatures are similar, the humidity levels however have been increased to 85-100%. The accelleration by AAT is thus much greater than CDT, seeds die within days instead of weeks. Research by Groot et al. shows that accelerated decrease in seed longevity, can be achieved by means of EPPO under dry conditions. As negative control, elevated partial pressure of nitrogen [N_2] (EPPN) was used (Groot et al., 2012).

1.1.7 Colouration

In rice, purple and red coloured seeds have been linked with the production of anthocyanidin and proanthocyanidin respectively, through a flavonols biosynthesis pathway (T. Furukawa et al., 2007; Maeda et al., 2014), as shown in figure 1.4. The light reflected by anthocyanidin and proanthocyanidin appear on opposite sides of the visible light spectrum. The reflection caused by anthocyanidin may provide protection to solar UV-B and prevent degradation of DNA (Mori et al., 2005; Silván, Reguero, and Pascual-Teresa, 2016). The anthocyanidin and proanthocyanidin content in rice has been linked with antioxidant activity. Antioxidant activity in turn, shows positive correlation with increased health benefits (Goufo and Trindade, 2014; Shao et al., 2018; M.-H. Chen, McClung, and Bergman, 2017). Maeda et al., 2014 have shown that through introgression breeding of black (purple) rice genetic material. In this study, a taste panel proofed that superior eating quality can be maintained.

1.1.8 Genetics

The red pericarp colour of *O. rufipogon*, is phenotypically associated with weedy traits such as seed shattering and dormancy (Gu, Kianian, et al., 2005).

A study by Oikawa et al., 2015 has shown that purple pericarp colour in rice is caused by a rearrangement in the promoter region of the basic helix-loop-helix (bHLH) transcription factor (TF) Kala4 gene (Os04g0557500) on chromosome 4.

In weedy rice, seed shattering and dormancy quantitative trait loci (QTLs) surround the Rc (Os07g0211500) gene on the pericentromeric region of chromosome 7. Using Ensembl genome viewer, one can find that Rc is a paralog of

Kala4 (www.gramene.org/ accessed on 23/11/2018). The functional pleiotropic Rc gene, codes for a bHLH TF and two myeloblastosis (MYB) TFs, it indirectly increase seed dormancy by promoting expression of ABA-biosynthesis genes (Gu, Foley, et al., 2011). The Rc gene together with the Rd gene encoding dihydroflavonol-4-reductase (DFR), as shown in figure 1.4 alters the colour of the pericarp (Sweeney et al., 2006; Gu, Foley, et al., 2011; T. Xu et al., 2017). If both genes are non-functional *rcrd* or, the Rc gene is non-functional *rcRd*, the seed coat is white; if the Rc gene is functional and the Rd gene is not *Rcrd* the pericarp colour turns brown; if both the Rc gene and Rd gene are functional *RcRd*, the pericarp colour turns red as proanthocyanidins oxidise (T. Furukawa et al., 2007). Finally, research by Asem et al., 2015; Gianinetti et al., 2018 suggests that purple and red rice respectively, have increased resistance towards pathogenic fungi, in comparison with white rice.

In thale (mouse-ear) cress there are additional genes available for studying seed longevity and the flavonol pathway, as shown in figure 1.4. Genes of interest are: TT2 (transparent testa) encoding for a R2R3 MYB domain, which together with TT8 (similar to Rc) that encodes for a bHLH, is regulating leucoanthocyanidin reductase (LAR), DFR and is involved in the transcription of BANULYS which translates to anthocyanidin reductase (ANR) (Baudry et al., 2004; Zimmermann et al., 2004; Bowerman et al., 2012; Nesi et al., 2001; Rosso et al., 2003), TT4 is responsible for chalcone synthase (CHS) (Buer, Sukumar, and Muday, 2006; Saslowsky, Dana, and Winkel-Shirley, 2000), TT5 for chalcone isomerase (CHI) (Rosso et al., 2003), TT6 for Flavanone 3-hydroxylase (F3H) (Rosso et al., 2003), TT9 is required for the accumulation of flavonoids in the seed coat (Ichino et al., 2014), TT10 encoding for a Laccase-like 15 (LAC15), is required for seed coat browning by the mediation of polymerisation of proanthocyanidins from the monomeric precursor epicatechin and is known to slightly increase dormancy (M Koornneef, 1990; Debeaujon, Léon-Kloosterziel, and Maarten Koornneef, 2000; Pourcel et al., 2005; X. Cai et al., 2006; Liang et al., 2006).

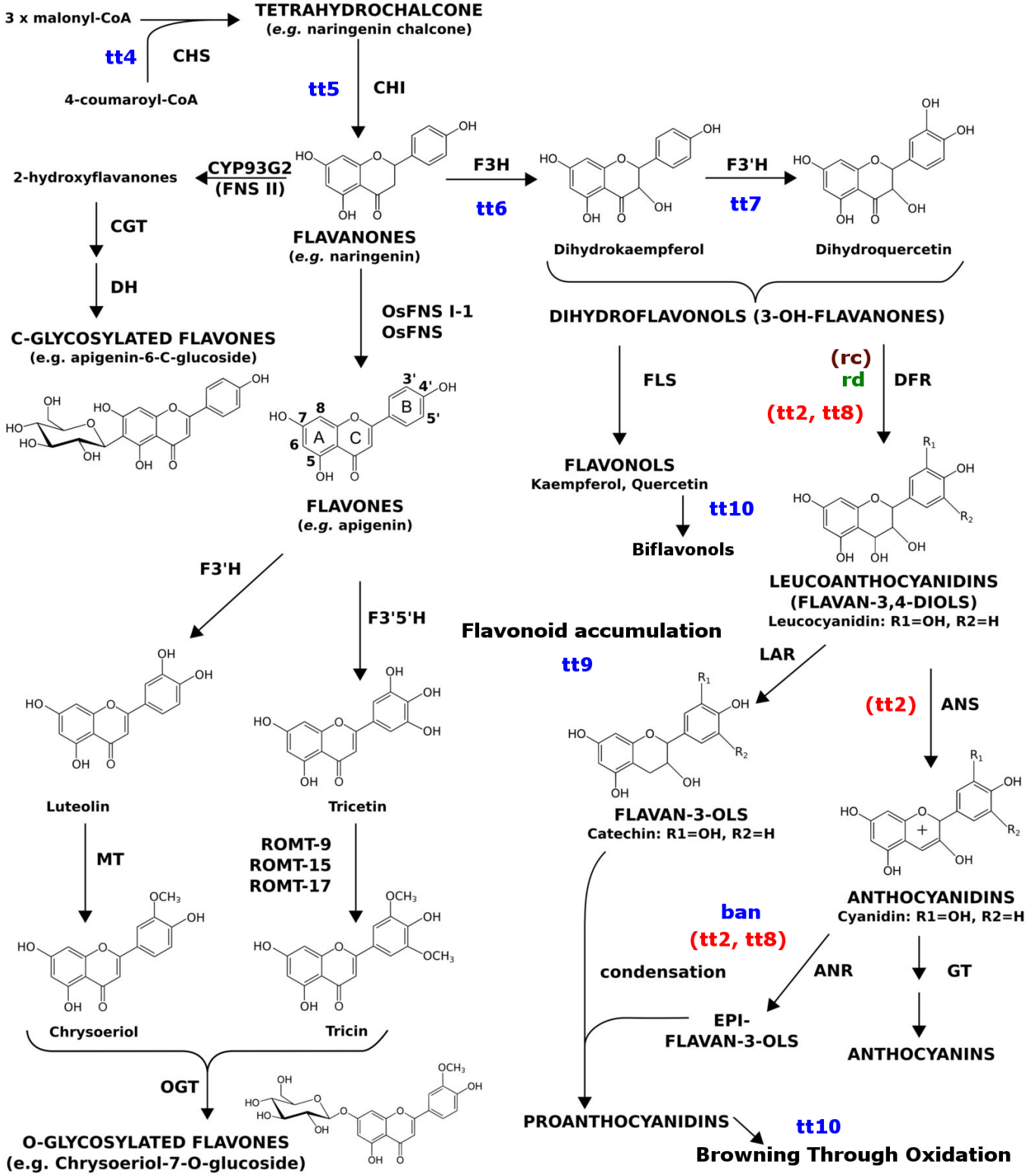


Figure 1.4: Flavonoid biosynthesis pathway; image modified from Galland et al., 2014. Text in brackets indicates the functional gene product is a transcription factor, text with no brackets indicates that the functional gene product is an enzyme. Red and blue text is used for thale cress, brown and green text is used for rice.

1.1.9 Flavonoid Antioxidant Activity

The previous section discussed the genetic background of the flavanoids and introduced its pathway, this section illustrates the fact that flavonoids, such as flavanols, show different total antioxidant capacities (Rice-evans et al., 1995; Rice-Evans, Miller, and Paganga, 1996). In these studies by Rice-evans et al., 1995; Rice-Evans, Miller, and Paganga, 1996, the total antioxidant capacities have been measured using the Trolox equivalent antioxidant activity (TEAC) assay, which measures the scavenging ability of the antioxidant compound on the compound ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate)] , a stable radical cation (Zhong and Shahidi, 2015). Results of these studies are summarised and shown in figure 1.5. While observing this figure, note that proanthocyanidins may consists of up to 17 flavanols (Guyot et al., 1997).

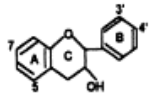
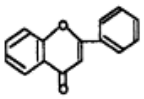
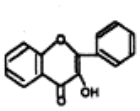
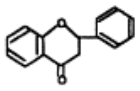
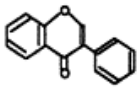
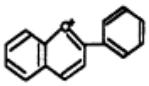
Class	General structure	Flavonoid	Substitution Pattern	Dietary Sources	TEAC (mM)
Flavanol		(+)-catechin	3,5,7,3′,4′-OH	Tea (camellia sinensis)[6]	2.4
		(-)-epicatechin	3,5,7,3′,4′-OH	Tea[6]	2.5
		Epigallocatechin gallate	3,5,7,3′,4′,5′-OH,3-gallate	Tea[6]	4.75
Flavone		chrysin	5,7-OH	Fruit skins	1.43
		apigenin	5,7,4′-OH	Parsley, celery	1.45
		rutin	5,7,3′,4′-OH, 3-rutinose	Red wine[5] , buckwheat[7] citrus, tomato skin[8]	2.4
		luteolin	5,7,3′,4′-OH	Red pepper[11]	2.1
		luteolin glucosides	5,7,3′-OH, 4′-glucose		1.74
			5,4′-OH, 4′,7-glucose		0.79
Flavonol		kaempferol	3,5,7,4′-OH	Leek, broccoli, endives grapefruit, black tea	1.34
		quercetin	3,5,7,3′,4′-OH	Onion, lettuce, broccoli tomato, tea, red wine berries, olive oil, appleskin	4.7
		myricetin	3,5,7,3′,4′,5′-OH	Cranberry grapes, red wine	3.1
		tamarixetin	3,5,7,3′-OH,4′-OMe		
Flavanone (dihydroflavon		naringin	5,4′-OH,7-rhamnoglucose	Citrus, grapefruit	0.24
		naringenin	5,7,4′-OH	Citrus fruits	1.53
		taxifolin	3,5,7,3′,4′-OH	Citrus fruits	1.9
		eriodictyol	5,7,3′,4′-OH	Lemons[64]	1.8
		hesperidin	3,5,3′-OH,4′-OMe, 7-rutinose	Oranges[9]	1.08
Isoflavone		genistin	5,4′-OH, 7-glucose	Soybean[10]	1.24
		genistein	5,7,4′-OH	Soybean[10]	2.9
		daidzin	4′-OH, 7-glucose	Soybean[10]	1.15
		daidzein	7,4′-OH	Soybean[10]	1.25
Anthocyanidin		apigenidin	5,7,4′-OH	Colored fruits	2.35
		cyanidin	3,5,7,4′-OH,3,5-OMe	Cherry, raspberry, strawberry	4.42

Figure 1.5: Trolox equivalent antioxidant activities (TEAC) of flavonoids image by Heim, Tagliaferro, and Bobilya, 2002 with data from Rice-evans et al., 1995; Rice-Evans, Miller, and Paganga, 1996. Proanthocyanidins may consists of up to 17 flavanols (Guyot et al., 1997).

1.2 Objective

At the Bioscience seed research team genetic variation for seed longevity is being studied, using rice as one of the model species. The team has been using EPPO as a way to assay seed longevity. Using genome wide association studies (GWAS) for tolerance against EPPO, the Rc gene has been identified. Following an analysis of near-isogenic lines for the Rc gene, the lines possessing Rc show higher tolerance to ageing than those without. From, aforementioned research by others, it is known that Rc which is involved in the production of proanthocyanidins, is indirectly involved with ABA biosynthesis. Next to that, it is thought that the functional Rc gene limits the diffusion of oxygen through the pericarp.

Thus, the research objective is to figure out how different flavonoid composition affects seed longevity and whether the Rc gene limits the diffusion of oxygen through the pericarp. This is studied on seed material with various genetically induced disruptions in the flavanoid biosynthesis pathway.

Chapter 2 Materials & Methods

This chapter describes the materials and methods used in the thesis' project.

2.1 Genetic material

For rice and thale cress genetically various non-dormant seeds were used. As the rice seeds were imported from the US and, they were treated as quarantined organisms (Q-organisms), even though the seeds that were used were from offspring plants. As the thale cress seeds were treated as GMO, as non-GMO seeds and genetically modified organism (GMO) seeds were used together in all thale cress experiments.

To adhere to the national laws and regulations regarding Q-organisms and GMOs. Special measurements were taken to handle these organisms. Colored labelling was used to identify the plant material: green for non-Q-organism and non-GMO, blue for Q-organisms and red for GMO. Each label contained the experiment number and, in case of GMO also contained the GMO license number. Next to providing for labels, when handling Q-organisms, a blue lab coat was used that remained in the lab room; next to that for both Q-organisms and GMOs the working bench was cleaned with 70% ethanol; hands were rinsed with ethanol.

2.1.1 Rice

For rice two nearly-isogenic lines were used (NILs), as shown in subfigures 2.1a and 2.1b, material was obtained from Gu, Foley, et al., 2011 and grown in UniFarm Wageningen by Manjunath Prassad. The NILs were of the line EM93-1 and did have either a functional or non-functional Rc gene.

(a) Functional Rc gene

(b) Non-functional Rc gene

Figure 2.1: Pericarp phenotype in rice, caused by (non-)functional Rc gene in an EM93-1 NIL.

2.1.2 Thale cress

Seeds used were wild-type (WT) or mutants of Columbia-o (Col-o) and Landsberg *erecta* (Ler), as shown in subfigures 2.2a-2.2j. Certain seeds were genetically engineered for one specific gene, resulting in a loss of function for the gene: banyuls 5 (ban5) and transparent testa (tt) genes: tt2, tt4-13, tt5-3, tt6-3, tt7-5, tt8, tt8-1, tt10-4 in a Col-o background and, tt9-1 in a Ler background.

(a) Col-o **(b)** tt2 **(c)** tt4-13 **(d)** tt5-3 **(e)** tt6-3 **(f)** tt7-5 **(g)** tt8 **(h)** tt8-1 **(i)** tt10-4 **(j)** ban5 **(k)** Ler **(l)** tt9-1

Figure 2.2: Pericarp phenotype in thale cress, from left to right: Colombia-o (Col-o) with genetically engineered mutants: transparent testa (tt)2, tt4-13, tt5-3, tt6-3, tt7-5, tt8, tt8-1, tt10-4 and banyuls (ban)5 and, Landsberg *erecta* (Ler) with the mutant: tt9-1.

2.2 Storage methods

The storage experiments shared the following components: use of 100 g sillica gel in a nylon stocking which was brought at the relevant equilibrium by the use of a humidity cabinet at 20°C. Settings for eRH were **dry**: 40% eRH for rice, 30% for thale cress and, **humid**: 60% for both rice and thale cress. The sillica gel eRH was confirmed by the use of a water activity meter (Rotronics Inc.) at 20±1°C, as shown in subfigure 2.3e. Calibration of the water activity meter was carried out by Jan Kodde, by using salt solutions of which the humidity was known to be stable at 20°C. Seed sample packets; which were either stored in rolled up and paper tape sealed paper bags (63 x 93 mm packet size, 60g/m2 Bleached Kraft Paper, Baumann Saatzuchtbedarf GmbH, Germany) for rice or, for thale cress in 1.5 mL microtubes (Greiner Bio-One, Austria) topped with a double punctured wipe paper inlayed screw cap (Sarstedt Aktiengesellschaft & Co., Germany), as shown in subfigure 2.3a. Seeds were equilibrated at 20°C for the right RH in either a humidity cabinet or, in 1 L Kilner jars (Le Parfait, France) filled with six 100 g sillica gel containing nylon stockings, as shown in subfigure 2.3d. After equilibriation for thale cress, every pair of microtubes except for those of the initial germination experiment, were placed in a capped reaction tube perforated with holes and placed in the pressure tank or ambient jar, as shown in subfigure 2.3b. In a similar fashion for rice, the enveloppes were put in the tanks and jars, as shown in subfigure 2.3c. Thereafter, one nylon stocking was put in each jar or tank. Tanks were opened and closed by using a screw in bar and rubber hammer, as shown in subfigure 2.3o. Jars and tanks were incubated at either 28.5, 35 and 38.5°C for rice or, 20°C for thale cress, as shown in subfigure 2.3n. The incubation of rice at 38.5°C was accidental, this incubator used only for the dry storage of rice experiment, was operating 3.5°C higher than the intended storage temperature of 35°C thus, further use of this incubator was ceased. Similarly, CDT and AAT experiments were supposed to be set at 35°C to mimic the temperature conditions used in the other experiments however, the incubator was in reality set at 28.5°C.

2.2.1 Ambient storage

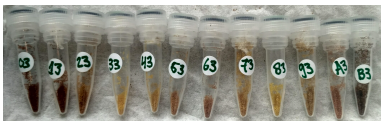
Ambient storage took place in 777 mL hexagonal jars for rice or, in 1 L round Kilner jars (Le Parfait Super, France) for thale cress. Two data RH & Temperature loggers were placed in the largest time interval of the ambient jars for each experiment.

2.2.2 EPP(N/O) storage

A modified methodology Groot et al., 2012 was used. EPP(N/O) storage took place in 1.5 L tanks brought up to 200 bar pressure by the use of a 10 L diving tank for EPPO or, a large industrial nitrogen tank for EPPN, as shown in subfigures 2.3j and 2.3k. Pressure was elevated at a rate of 1 bar per 10 seconds in the tanks simultanously using a pressure powered pump and interconnecting pressure resistant rubber pipes with T-connections while tanks were placed in a tub filled with water to prevent overheating. Deflation of pressure happened at a 0.5% bar per minute with the aid of a dedicated computer system, to avoid damage to the seeds, as shown in subfigure 2.3l.

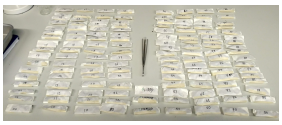
2.2.3 CDT and AAT storage

For this 194 mL hexagonal jars were used; seeds were placed in perforated plastic bread bags, which facilitated airflow, as shown in subfigure 2.3m. Salts were used to keep high humidities; for 20-28.5°C theoretically: $NaCl$ 75% and K_2SO_4 97%. A plastic framed container was put in the jars to keep the seeds out of direct contact with the solution. Equilibration was done at 20°C.



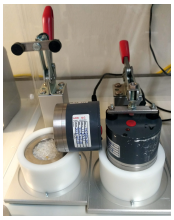
(a) Thale cress microtubes with wipe paper inlayed perforated caps

(b) Reaction tube that can hold two microtubes



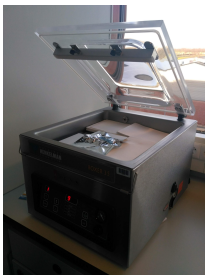
(c) Rice samples in enveloppes

(d) Sillica stocking equilibriation jars



(e) Water activity meter

(f) Dryer



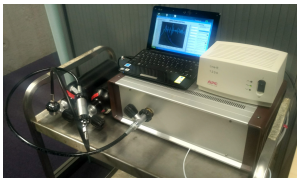
(g) Vacuumer

(h) Freezer

(i) Dried vacuumed frozen rice samples

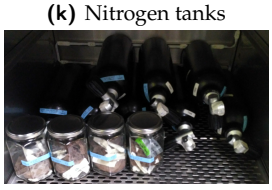
(j) Building up oxygen pressure

(k) Nitrogen tanks



(l) Decrease of pressure

(m) Salt humidity jars



(n) Jars and tanks in the incubator

(o) Opening and closing of tank

Figure 2.3: Storage equipment used for experiments.

2.3 Germination

After each of the treatments' time interval seeds were either directly set up for germination testing or stored at 35%RH 20°C with air flow for drying for two days, as shown in subfigure 2.3f, thereafter these seeds were packed in an aluminum bag, vacuumed and sealed with the Boxer 35 (Henkelman, The Netherlands) set to program 1 and, as shown in subfigures 2.3g and 2.3i, stored in the freezer, as shown in subfigure 2.3h. The latter method allowed for less variation in the germination data. For the the germination procedure and image analysis a modified methodology of Joosen et al., 2010 was used.

2.3.1 Germination procedure

Seed germination happened in plastic trays (15×21cm, DBP Plastics), as shown in subfigure 2.4a, seeds were placed on a double layer of blue filter paper (5.6'×8' Blue Blotter Paper; Anchor Paper Company). Seed distrubution was guided by using a transparent plastic mask, as shown in subfigure 2.4b, with two large rectangular seed slots for rice and, six round seed slots for thale cress. Both masks had an extra rectangular slot in the top left corner to provide for a tray identification label (binary code). A Microsoft Excel script, provided with The Germinator package (Joosen et al., 2010), created randomization for sample packet distribution over the trays, for rice sample packets were put in the right tray, in the right mask slot first before distribution. Seed samples were distributed in the seed slots by swinging an upside down sample enveloppe for rice or, by dipping a brush into the tube filled with seeds for thale cress. Upside down petridishes were used to protect seeds from jumping to adjecent sample slots when, using the brush. Adjustments to seed placement were made either by foreceps for rice or, by brush for thale cress. Germination trays contained either around two times fortyfive seeds for rice or, six times a hundred to two hundred seeds for thale cress. After placement, the filtration paper was sprinkled with 48 mL of water for thale cress and, 50 mL of water for Rice. Trays were stacked on top of each other. Top and bottom trays had one sheet of lightly blue filtration paper inserted, sprinkled with 48 or 50 mL water (species dependent, as shown in above) and were free of seeds. Trays were surrounded by a large plastic bag which were sealed by a knot near the top of the trays. Incubation took place in an incubator set at 25°C without light for rice or, 22°C continous light for thale cress. Filtration paper was kept at a constant water retaining level by adding 2 mL to 6 mL per tray every other day, judgement on water requirement was made by the colouration of the paper, the lighter average hue of the paper the more water was added. In most cases adding 2 mL was considered sufficient. In a few occurances however, the paper formed light spots because the paper started to show waviness, up to 6 mL of water was added to compensate for this by waviness caused irregularity in local absorption.

After finishing the experiments, germination trays with seeds were emptied and the waste was put in a Q-organism labelled bin, with a bag inserted that later was put in the autoclave. Then trays would be washed with dish soap and water and rinsed with demineralised water.

2.3.2 Image analysis

Seeds were evaluated up to two weeks or less if all seeds had shown germination. Evaluation took place twice a day during weekdays, early morning and late afternoon or once in the morning during weekends. For analysis, a digital camera (Nikon D80 with Nikkor AF-S 60mm f/2.8G Micro ED; Nikon) attached to a repro stand was controlled by a computer using software (Nikon camera control pro software version 2.0), as shown in subfigure 2.4c. Two fluorescent tl-tubes (40cm) were vertically placed; one left and one right from the stand, one fluorescent tl-tube (150cm) was horizontally placed in a container above the stand, beaming away from the stand. Reflection was minimized by using white plastic that allowed diffusion of the light from the vertically placed tl-tubes.

The full manual control settings were: ISO400, F/7.1, 1/2 sec, manual focus. File names for the images contained the following format: exxxxxx (experiment number) mmddyy-hhmm and were stored as JPEG. Seeds were positioned for analysis using the above described transparent plastic mask.

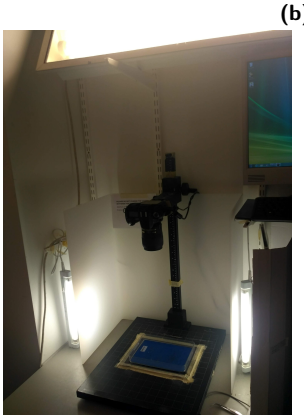
For rice, before taking each image, germinated seeds were removed from the trays. After imaging the pictures were analysed using The Germinator package (Joosen et al., 2010) or, for thale cress using a self-made python script, i.e. *The Thaleminator*™, in combination with manual scoring, as shown in appendix C for *The Thaleminator*™ script. An example images for analysis for thale cress and rice respectively, can be seen in subfigures 2.4d and 2.4e. As with germination rate, seed count for the T_{50} of max germination in thale cress was judged manually. A modified equation from Coolbear, FRANCIS, and Grierson, 1984 by FAROOQ et al., 2005 was used to calculate T50, as shown in equation 2.1.

$$t_{50} = T_i + \frac{(\frac{N}{2} - Ni) * (Tj - Ti)}{Nj - Ni} \tag{2.1}$$

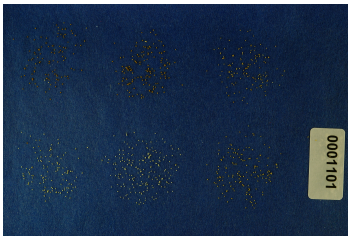
With t_{50} being median 50% germination time, N is maximum seeds germinated, Ni seeds germinated at time point i, Nj seeds germinated at time point j, Ti time at time point i and Tj time at time point j.

(a) Experiment preparation

(b) Mask, samples and forceps



(c) Camera setup



(d) Image analysis material for thale cress

(e) Image analysis material for rice

Figure 2.4: Germination equipment used for experiments.

2.4 Oxygen diffusion and pericarp permeability

To following subsections describe methods for testing pericarp characteristics.

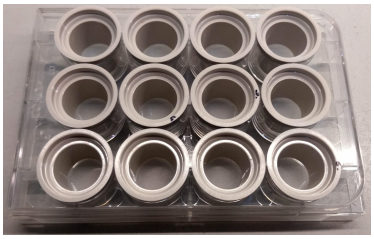
2.4.1 Oxygen diffusion

Half of the seeds were cut topped off at 2 to 3 fourths of the seeds length by a transverse cut, using a scalpel and forceps leaving the embryo in tact.

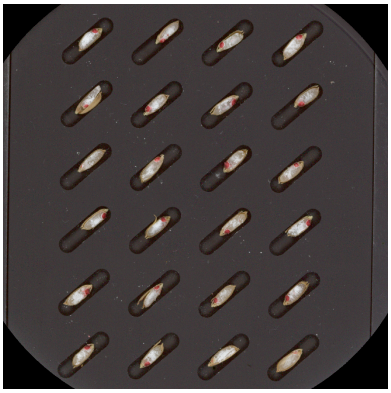
2.4.2 Pericarp permeability

This experiment was inspired by Debeaujon, Léon-Kloosterziel, and Maarten Koornneef, 2000, which showed that there is a difference in tetrazolium permeability between thale cress testa mutant and WT.

Following the rules from International Seed Testing Association et al., 2019: imbition of rice seeds took place 18 hours before incubation at 20°C in trays with double filtration paper; in a similar fashion as described in section 2.3.1. All seeds that received a tetrazolium treatment were either cut with scalpel and forceps, longitudal radially through the embryo for 2/3rd of the seed length or, were left uncut. Incubation with 1% tetrazolium at different time intervals was applied to the seeds at 30°C in a plastic bag in a 12-wells plate with sieve inlays, as shown in subfigure 2.5a, to remove seeds from the solution with ease. Seeds that were incubated and seeds left untreated, the latter used for reference, were cut longitudal radially in two halves, and one half of each seed was analysed using a Videometer with VideometerLab software version 1.8 (Videometer A/S, Denmark) to observe a possible difference in tetrazolium conversion activity, as shown in subfigure 2.5b. Callibration of the Videometer took place using the VideometerLab software and callibration disks right before the start of the measurements. The tetrazolium solution was prepared using distilled water, measurement beaker, a precision balance and a magnetic stirrer. Python scripts was used to analyse the data, as shown in appendix F.



(a) Well used for incubation of seeds.



(b) Sample to be analysed by VideometerLab software.

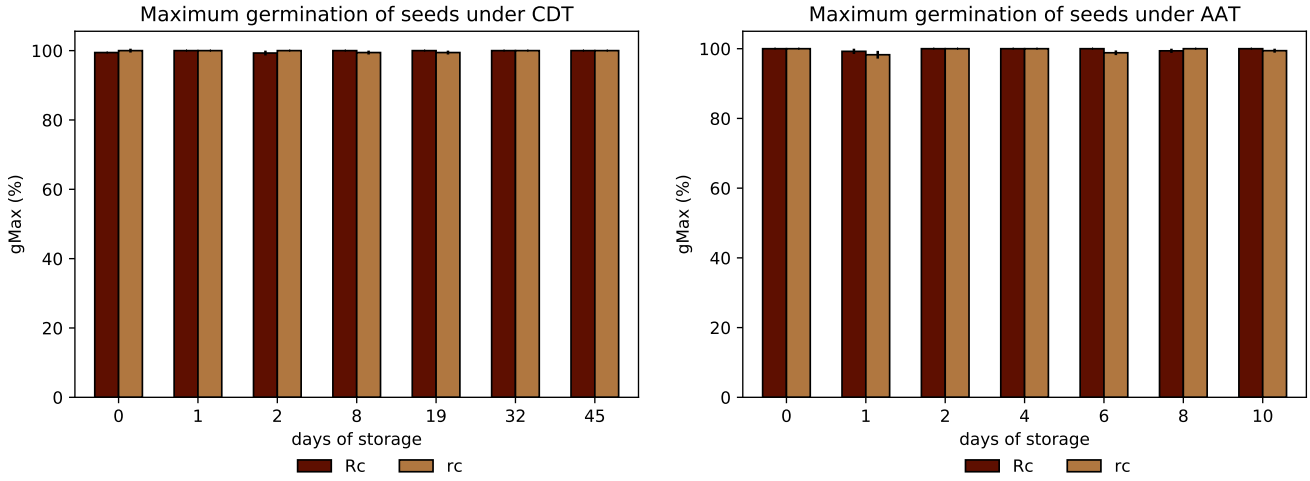
Figure 2.5: Tetrazolium permeability equipment.

Chapter 3 Results

This chapter describes the result of rice and thale cress experiments on seed longevity.

3.1 O. sativa controlled deterioration and accelerated ageing treatments

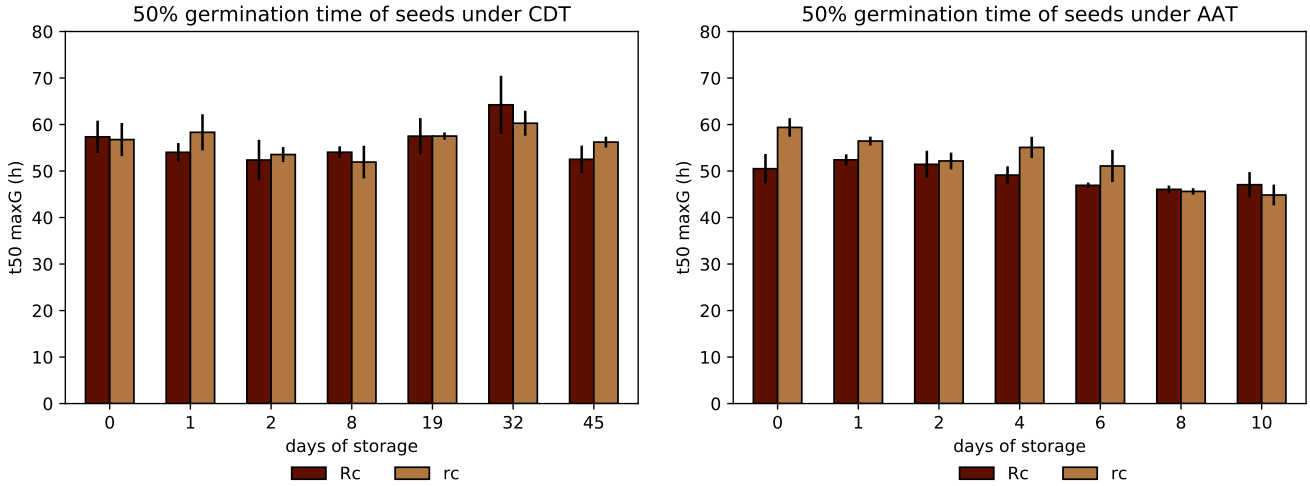
These experiments test other methods of ageing and aim to as shown in whether there is a difference to be observed between the lines. The results show that neither germination rate nor time to 50% germination was affected for both treatments, as shown in figures 3.1 and 3.2 and no difference between the lines was observed.



(a) Controlled deterioration treatment

(b) Accelerated ageing treatment

Figure 3.1: Germination test results of rice seed in controlled deterioration (75% eRH) and accelerated ageing (97% eRH) treatments, both at 28.5°C. Seeds were of genotypes EM93-1 with functional Rc (Rc) and EM93-1 without functional Rc (rc). Treatments each consisted of four biological replicates and each biological replicate contained forty five seeds. Error bars represent the standard error.



(a) Controlled deterioration treatment

(b) Accelerated ageing treatment

Figure 3.2: Time to 50% germination results of rice seed in controlled deterioration (75% eRH) and accelerated ageing (97% eRH) treatments, both at 28.5°C. Seeds were of genotypes EM93-1 with functional Rc (Rc) and EM93-1 without functional Rc (rc). Treatments each consisted of four biological replicates and each biological replicate contained forty five seeds. Error bars represent the standard error.

3.2 O. sativa pericarp permeability

This experiment aims to find the difference in the permeability of tetrazolium through the pericarp between the lines. In the negative control, the absence of tetrazolium incubation, none of the seeds had a stained embryo for both genotypes, whereas the positive control by ISTA rules (International Seed Testing Association et al., 2019), showed staining of all embryos for both genotypes, indicating that all seeds where viable. In intact seeds, the rate of stained embryos by the reduction of tetrazolium seemed to be higher in non-functional rc rice, compared to functional Rc rice, as shown in figure 3.3.

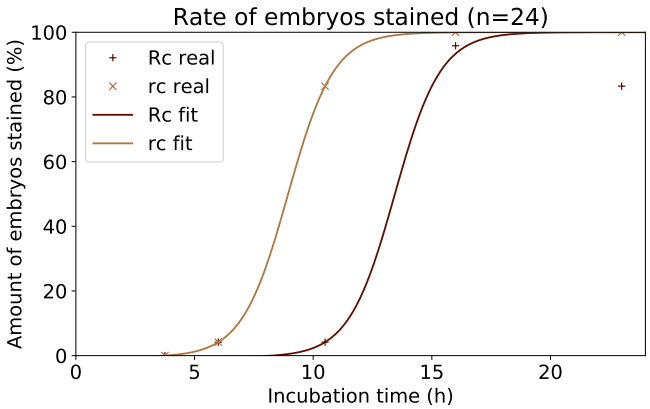
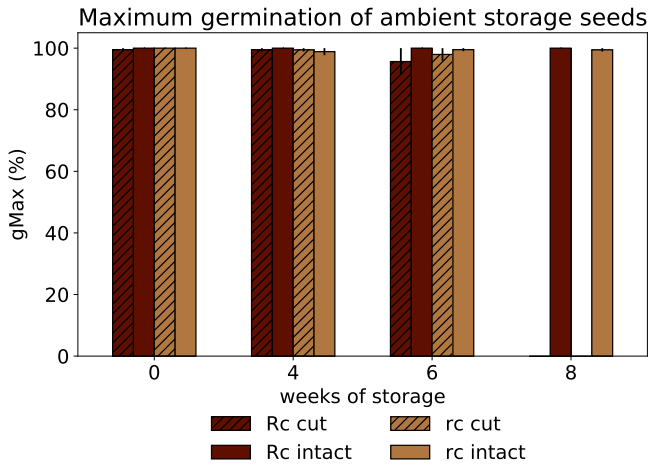


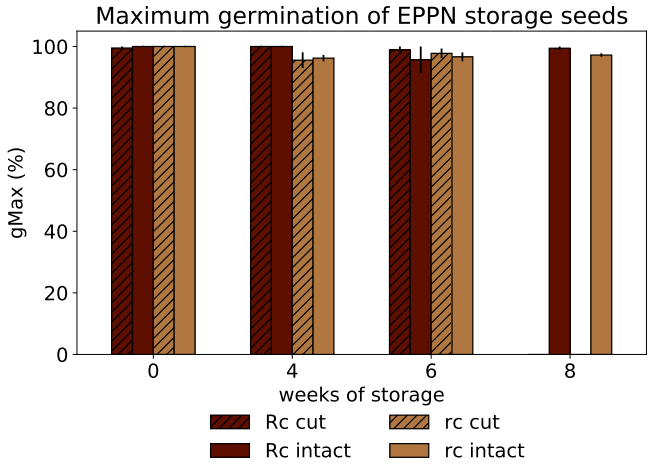
Figure 3.3: Difference in the permeability of tetrazolium through the pericarp of intact seeds between functional Rc and non-functional EM93-1 NIL lines. Graphs shows the rate of embryos that were stained by tetrazolium.

3.3 O. sativa dry storage and oxygen diffusion germination

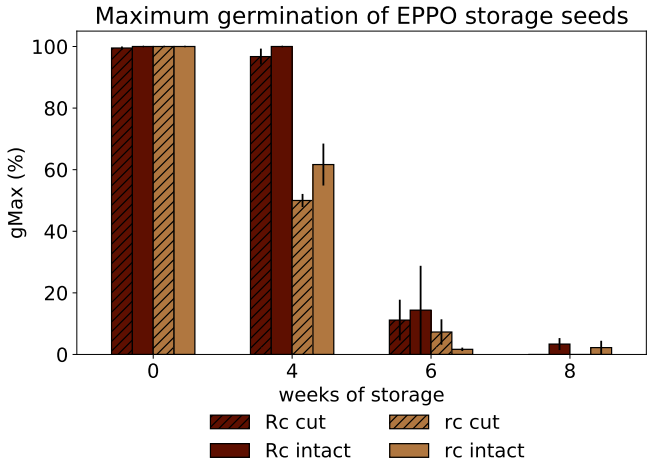
This experiment aims to as shown in whether there is a difference between the lines' seed longevity for dry storage, where enzymatic activity is reportedly minimal or absent (Labuza and Dugan Jr, 1971). Next to that the experiment also tries to investigate whether the aforementioned possible difference is due to oxygen diffusion differences of the pericarp between the lines, by having either the tip of the seed cut or intact. In this experiment, seeds were barely germinating after 8 weeks EPPO treatment, as shown in subfigure 3.4c. What is visible however, is the consistancy in the ambient storage, no decline or difference between genotypes can be observed. In contrast, the EPPO treatment shows stark difference between genotypes' germination for intact seeds after four weeks of treatment, the line with non-functional Rc gene is performing less than the line with the functional Rc-gene. The intact seeds of the non-functional Rc line perform better after four weeks than the cut ones, as well. The cut seeds in general also showed greater numbers of fungal activity, as judged by the researcher from image analysis pictures. Finally, time to reach 50% germination had shown great significant delay, in seeds under four week EPPO treatment, as shown in subfigure 3.5c.



(a) Ambient storage

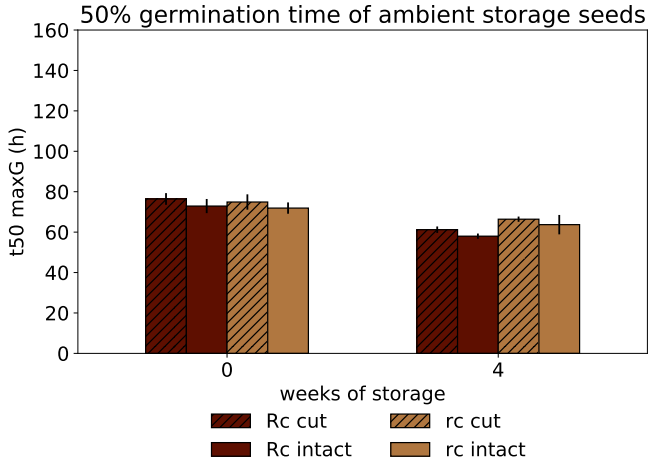


(b) EPPN storage

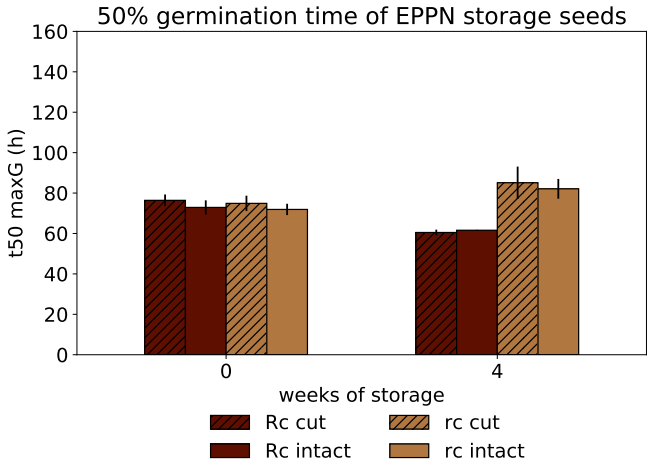


(c) EPPO storage

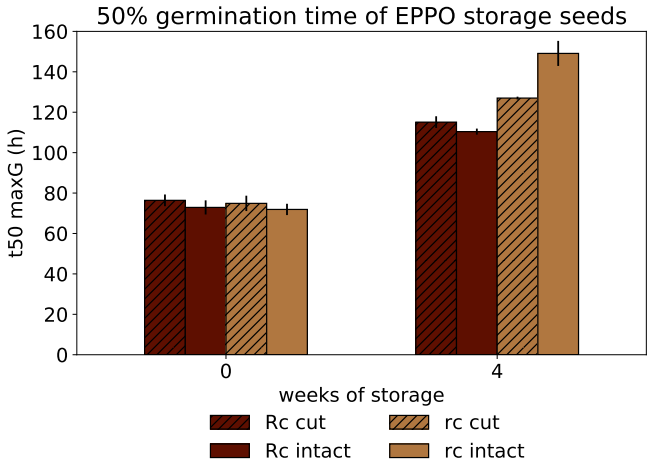
Figure 3.4: Germination test results of 0, 4, 6 and 8 week storage at 38.5°C ambient or 200 bar elevated partial pressure of nitrogen/oxygen (EPPO/EPPN) storage of rice seeds at an equilibrium RH of 40%. Seeds were of genotypes EM93-1 with functional Rc (Rc) and EM93-1 without functional Rc (rc) and were left uncut or were cut transversely at about half to two thirds of initial length. Treatments each consisted of four biological replicates and each biological replicate contained forty five seeds. Error bars represent the standard error.



(a) Ambient storage



(b) EPPN storage

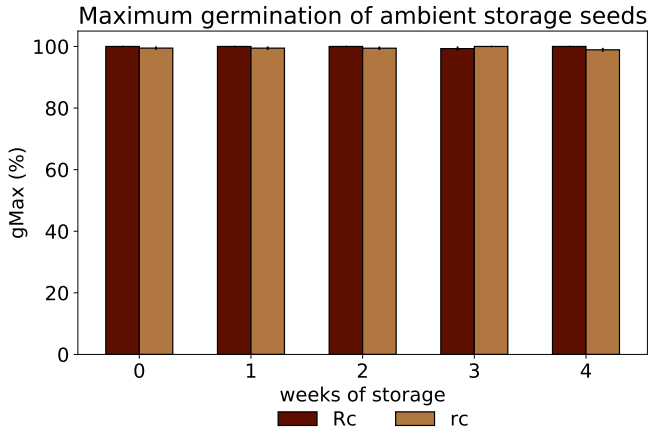


(c) EPPO storage

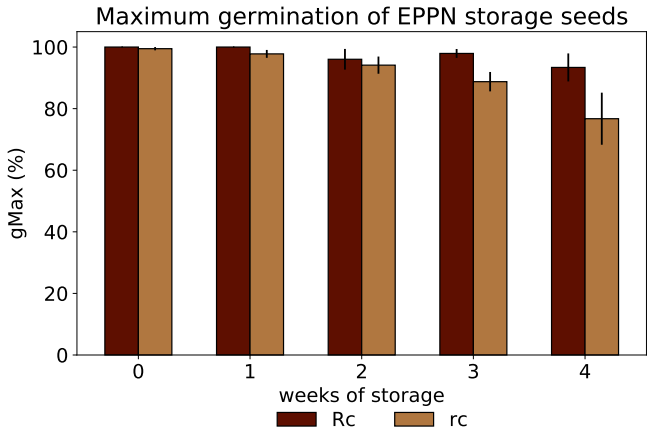
Figure 3.5: Time to 50% germination results of 0, 4, 6 and 8 week storage at 38.5°C ambient or 200 bar elevated partial pressure of nitrogen/oxygen (EPPO/EPPN) storage of rice seeds at an equilibrium RH of 40%. Seeds were of genotypes EM93-1 with functional Rc (Rc) and EM93-1 without functional Rc (rc) and were left uncut or were cut transversely at about half to two thirds of initial length. Treatments each consisted of four biological replicates and each biological replicate contained forty five seeds. Error bars represent the standard error.

3.4 O. sativa humid storage germination

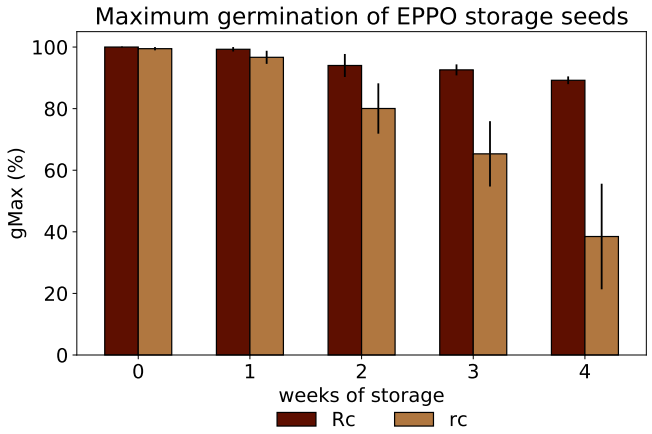
This experiment aims to as shown in whether there is a difference between the lines' seed longevity for humid storage, where enzymatic activity is reportedly present (Labuza and Dugan Jr, 1971). Converting this RH to moisture content with the equation of Cromarty, R. H. Ellis, E. H. Roberts, et al., 1982 and comparing it to dry storage as used in the previously mentioned experiment: the moisture content on dry weight basis is 13% for the humid condition and 10% in the earlier described dry condition. As seen from figures 3.6 and 3.7, the difference between the genotypes in germination rate can be observed for both EPPN and EPPO treatments and solely for the time to 50% germination in EPPO. The NIL without functional Rc shows to have decreased germination rates and increased time to 50% germination.



(a) Ambient storage

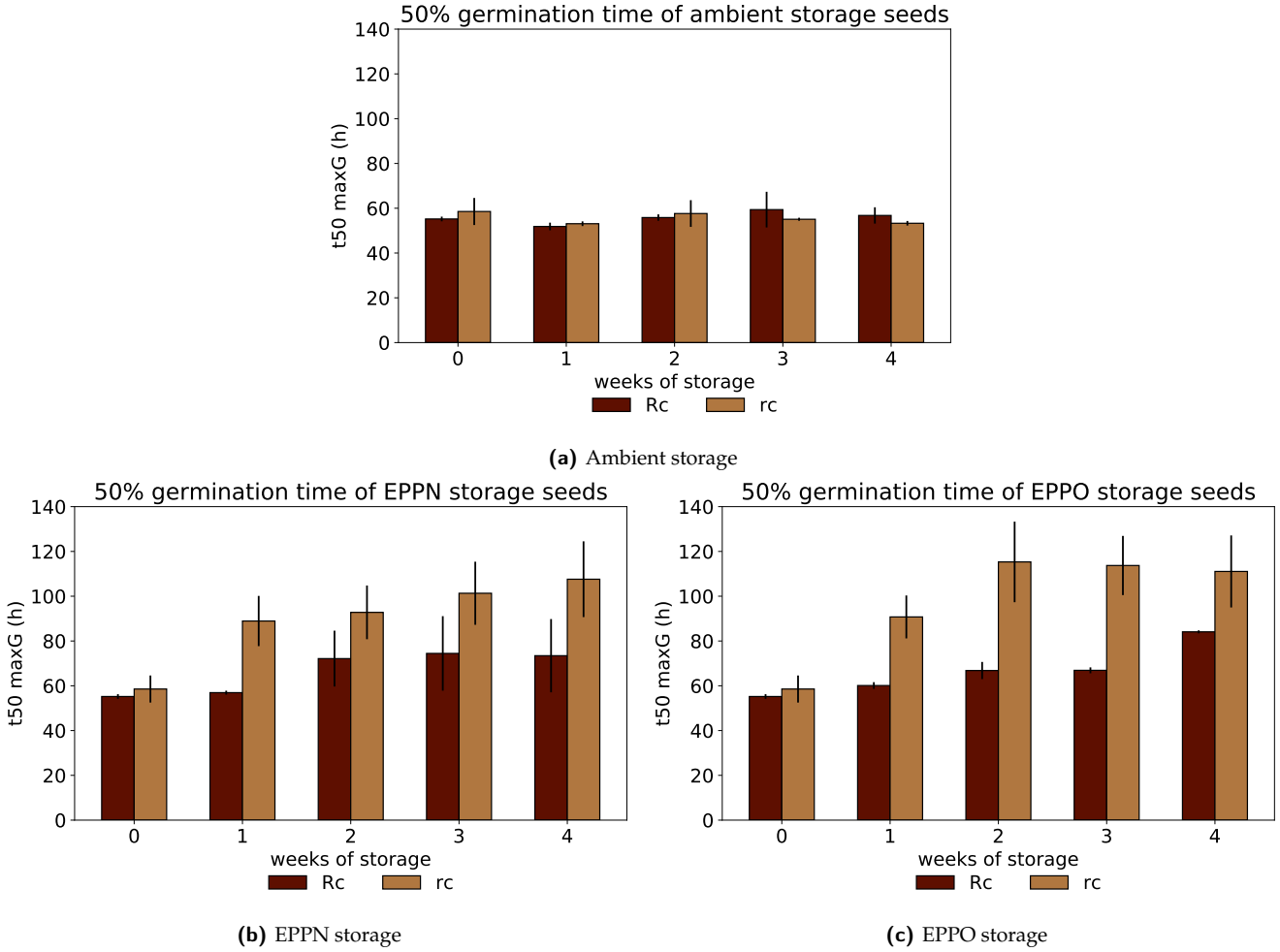


(b) EPPN storage



(c) EPPO storage

Figure 3.6: Germination test results of 0, 1, 2, 3 and 4 week storage at 35°C ambient or 200 bar elevated partial pressure of nitrogen/oxygen (EPPO/EPPN) storage of rice seeds at an equilibrium RH of 60%. Seeds were of genotypes EM93-1 with functional Rc (Rc) and EM93-1 without functional Rc (rc). Treatments each consisted of four biological replicates and each biological replicate contained forty five seeds. Error bars represent the standard error.



(a) Ambient storage

(b) EPPN storage

(c) EPPO storage

Figure 3.7: Time to 50% germination results of 0, 1, 2, 3 and 4 week storage at 35°C ambient or 200 bar elevated partial pressure of nitrogen/oxygen (EPPO/EPPN) storage of rice seeds at an equilibrium RH of 60%. Seeds were of genotypes EM93-1 with functional Rc (Rc) and EM93-1 without functional Rc (rc). Treatments each consisted of four biological replicates and each biological replicate contained forty five seeds. Error bars represent the standard error.

3.5 A. thaliana dry storage germination

This experiments illustrates the effect of EPPO under dryer conditions. The aim is to find whether there is a difference to be observed in seed longevity between the genotypes in the (near) absence of enzymatic activity (Labuza and Dugan Jr, 1971) plays a role in that. From 3.8 it can be seen that no highly significant differences can be observed after 16 weeks EPPO treatment, the same holds true for 32 week EPPO treatment. However, after 32 week EPPO treatment all the seeds have stopped germinating.

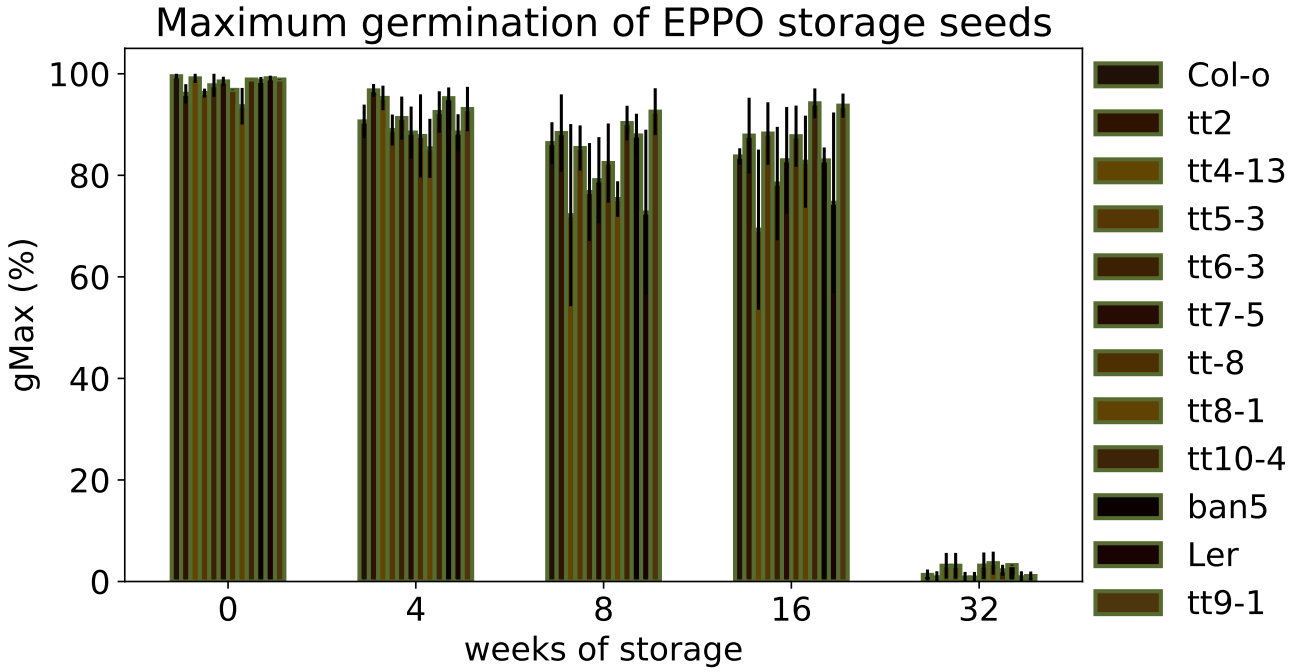
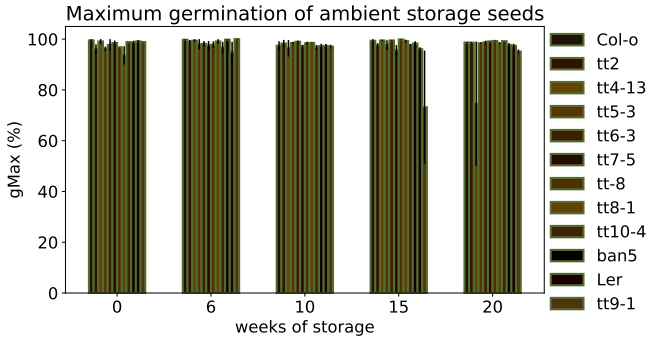


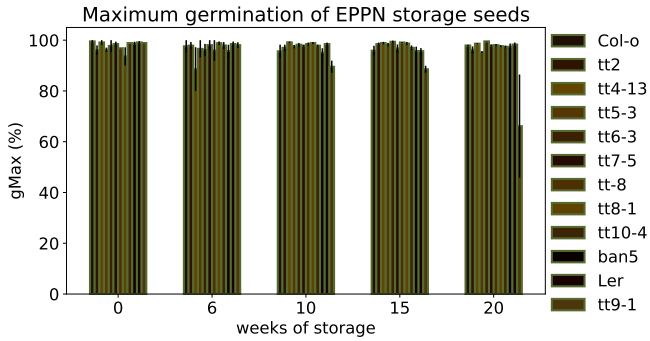
Figure 3.8: Germination test results of 0, 4, 8, 16 and 32 week EPPO storage at 20°C ambient or 200 bar elevated partial pressure of nitrogen/oxygen (EPPO/EPPN) storage of thale cress seeds at an equilibrium RH of 30%. Seeds used were WT Col-o and Ler and genetically engineered transparent testa mutants tt2, tt4-13, tt5-3, tt6-3, tt7-5, tt-8, tt8-1, tt10-4 and ban5 with a Col-o background and tt9-1 with a Ler background. For the 0 week storage, the seed genotypes were replicated two times. For all the other storage treatments the seed genotypes were replicated two times. Error bars represent the standard error. Bars appear from left to right in the descending order of the legend.

3.6 A. thaliana humid storage germination

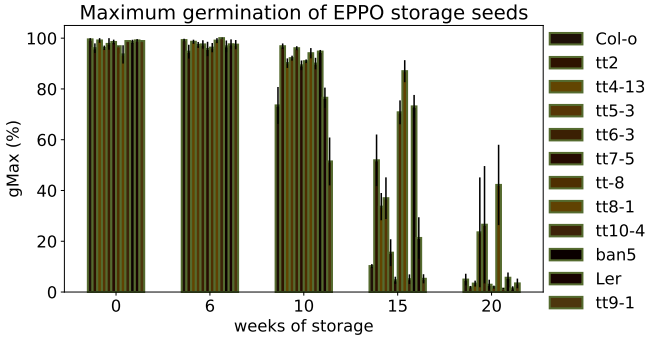
Effects of EPPO have been well researched under dry conditions, this experiments aims to as shown in the effects of EPPO under more moist conditions, as under moist conditions enzymatic activity is prevalent (Labuza and Dugan Jr, 1971). After 10 weeks EPPO, as shown in subfigure 3.9c it can be seen that the Col, Ler and t9-1 are having decreased performance in germination rate, also the genotype average time to reach 50% germination rate under EPPO conditions shows an increase, as shown in subfigure 3.10c. After 15 weeks EPPO, all the genotypes have decreased germination rates: Col-0, Ler, tt7-5, tt9-1 and tt10-4 being *severely affected* ($0\% \leq GR \leq 30\%$), tt2, tt4-13 and tt5-3 being *moderately affected* ($30\% \leq GR \leq 60\%$), tt8 and tt8-1 being *slightly affected* ($60\% \leq GR \leq 90\%$). Furthermore, after 15 weeks, all genotypes have increased time to 50% germination: Col-o, Ler, tt2, tt4-13, tt5-3, tt6-3, ban5 are *moderately affected* ($t50maxG[h] \geq 100$) and tt7-5, tt-8, tt8-1, tt9-1 and tt10-4 *slightly affected* ($50 \leq t50maxG[h] \leq 100$). From the 20 weeks humid EPPO treatment it can be seen that the tt8-1 allele performs still better than wild type while, the tt8 allele does not not.



(a) Ambient storage

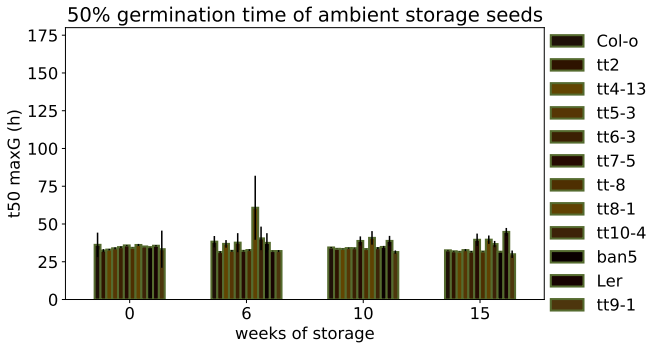


(b) EPPN storage

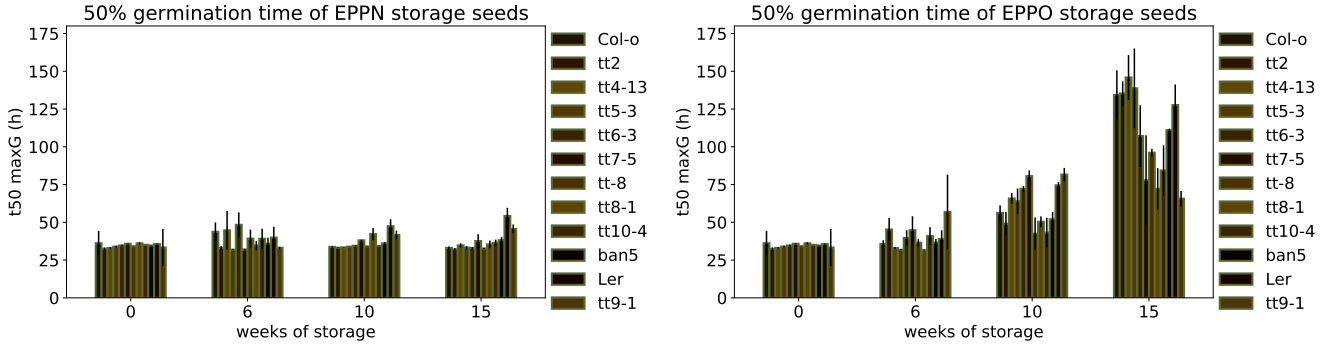


(c) EPPO storage

Figure 3.9: Germination test results of 0, 6, 10, 15 and 20 week storage at 20°C ambient or 200 bar elevated partial pressure of nitrogen/oxygen (EPPO/EPPN) storage of thale cress seeds at an equilibrium RH of 60%. Seeds used were WT Col-o and Ler and genetically engineered transparent testa mutants tt2, tt4-13, tt5-3, tt6-3, tt7-5, tt-8, tt8-1, tt10-4 and ban5 with a Col-o background and tt9-1 with a Ler background. For the 0 week storage, the seed genotypes were replicated two times. For all the other storage treatments the seed genotypes were replicated four times. Error bars represent the standard error. Bars appear from left to right in the descending order of the legend.



(a) Ambient storage



(b) EPPN storage

(c) EPPO storage

Figure 3.10: Time to 50% germination results of 0, 6, 10, 15 and 20 week storage at 20°C ambient or 200 bar elevated partial pressure of nitrogen/oxygen (EPPO/EPPN) storage of thale cress seeds at an equilibrium RH of 60%. Seeds used were WT Col-o and Ler and genetically engineered transparent testa mutants tt2, tt4-13, tt5-3, tt6-3, tt7-5, tt-8, tt8-1, tt10-4 and ban5 with a Col-o background and tt9-1 with a Ler background. For the 0 week storage, the seed genotypes were replicated two times. For all the other storage treatments the seed genotypes were replicated four times. Error bars represent the standard error. Bars appear from left to right in the descending order of the legend.

3.7 Synopsis

As mentioned in previous sections: the functional Rc gene had an increased seed longevity over the non functional Rc gene in rice after both dry and humid EPPO treatments. In Arabidopsis however, the functional TT8 (ortholog of Rc), in the Col-o line, had decreased seed longevity as compared to the tt8 and tt8-1 mutants after humid EPPO treatment. Furthermore, humid EPPO treatment had varying effects on seed longevity: mutants of tt2,tt4,tt5,tt6,tt10 and ban having increased seed longevity over wild type while, mutants tt7 and tt9 had decreased seed longevity, as shown in figure 3.11.

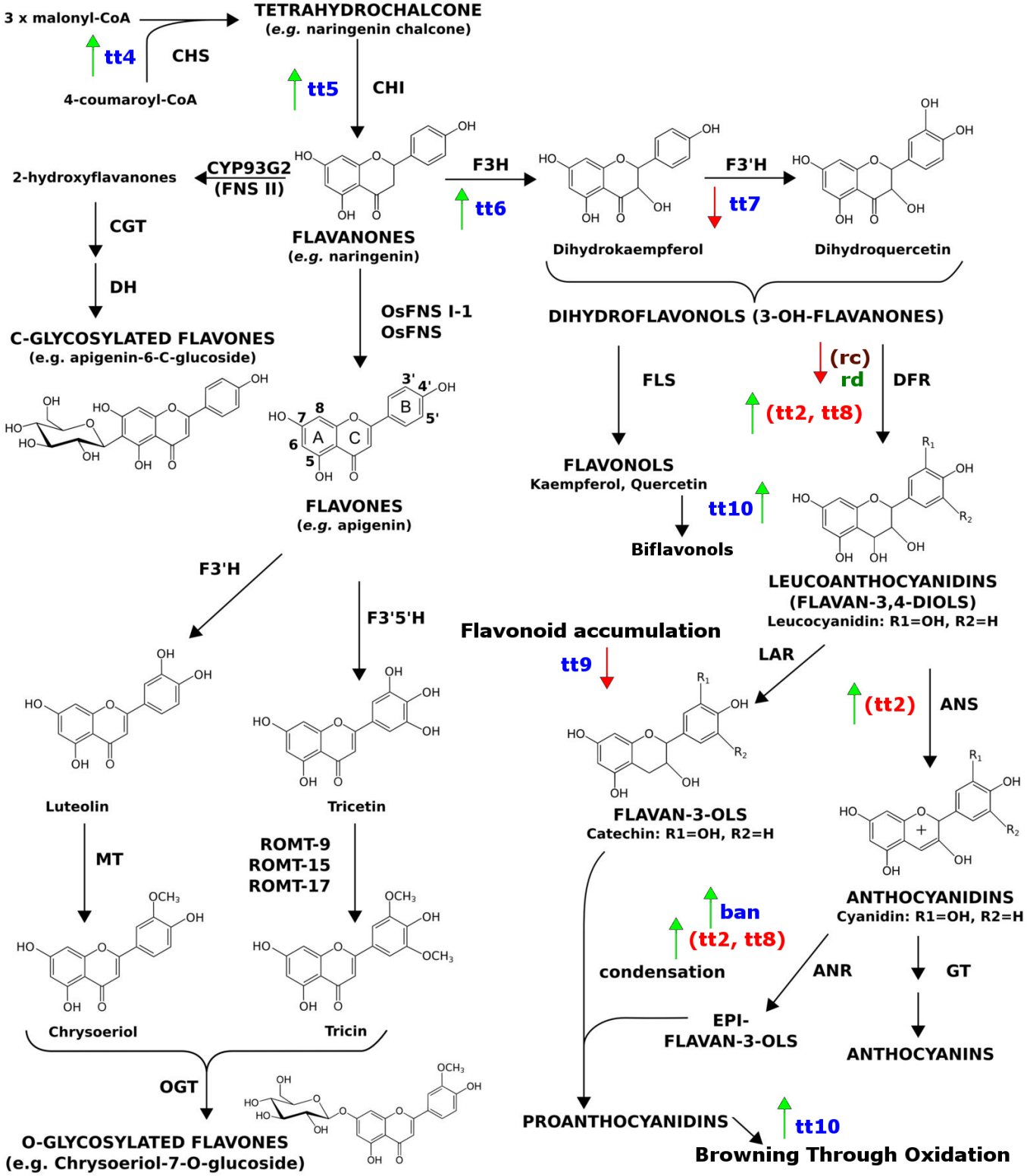


Figure 3.11: Flavonoid biosynthesis pathway disruptions by non-functional or mutant genes and the effect on seed longevity of seeds receiving 15 week humid (60% eRH) EPPO treatment; image modified from Galland et al., 2014. Text in brackets indicates the functional gene product is a transcription factor, text with no brackets indicates that the functional gene product is an enzyme. Red and blue text is used for thale cress, brown and green text is used for rice. Arrows indicate the effect on seed longevity of the non-functional Rc gene in a EM93-1 background in rice and mutations in a Col-o background (all but tt9) and Ler (tt9) in thale cress. A green arrow indicates an increase and a red arrow a decrease of seed longevity when the mutation is present. Results from thale cress are extrapolated over the biosynthesis flavonoid pathway in rice.

Chapter 4 Discussion

It was thought that the functional Rc gene limited the diffusion of oxygen through the pericarp, compared to the non-functional Rc line however, in rice it was not shown that the pericarp had any direct effect on the diffusion of oxygen. This was tested by cutting the tip of the seed off, this in turn caused increased fungal activity, which in turn may cause decreased longevity, such was the case after a four week treatment. Fungal activity may also increase as seed ageing increase, due to an increase in leaky metabolites. These metabolite leakage may be explained by the seed having an increased amount of damaged cells as seed ageing continues. Next to harm, fungi may also assist the seeds in germination by breaking dormancy through GA production. Perhaps specific beneficial fungal spores can be used in the future to increase seed quality factors such as longevity.

Regarding germination it could be seen that the functional Rc gene line had higher germination rates than the non-functional Rc gene line. This indicates that the activity of the Rc gene causes higher tolerance to EPPO ageing.

Accidentally, the effect of increased temperature was observed when an incubator set at 35°C was operating at 38.5°C, an elevated temperature of 3.5°C may have drastic effects as it reduces longevity by around 50%, based on aforementioned theory by R. Ellis and E. Roberts, 1980 and, practical experience in the rice experiments. In this study at 38.5°C there was hardly any germination after 8 weeks whereas, in a similar study using identical genetic material by Manjunath Prassad, unpublished data, it was shown at 35°C that at 8 weeks EPPO a large fraction of seeds was still germinating.

Seeds under EPPO storage showed an increased time needed to reach 50% germination. This may be explained by the fact that lipid peroxidation causes all sorts of damage, including damage to mitochondria. Mitochondrial damage which, results in less energy being available to the seeds in combination with other forms of destruction, result in slower to absent germination.

It was not shown that Rc gene had an effect in CDT and AAT, because the seeds were stored at lower temperatures than intended. Unfortunately, the seeds had depleted and the experiments could not be repeated at the time. It will be interesting for another researcher to repeat the experiments with a new batch of of seeds in the near future.

The functional Rc gene also seems to influence the permeability of the pericarp. Permeability seems to be slowed down by Rc, as seeds with the Rc gene show slower rates of embryo staining than seeds with the rc gene. Lignin content in soy bean is correlating with mechanical damage resistance and seed permeability as shown by Capeleti et al., 2005. The study of T. Furukawa et al., 2007 showed with variation in functionality of Rc and Rd, that white rice was not able to produce any proanthocyanidins while red rice is able to produce proanthocyanidins. Thus, it seems logical that the pericarp permeability is correlating with the proanthocyanidin content.

Various other genes may affect seed longevity. For example, genes encoding for lipoxygenase, this enzyme seem to decrease seed longevity, e.g. LOX2 and LOX3 recessives can thus be used to increase longevity (J. Huang et al., 2014; Ma et al., 2015; H. Xu et al., 2015). Seed doramancy and longevity in thale cress, seem to have a negative correlation as was found by Nguyen et al., 2012; Buijs, 2019. The study by Nguyen et al., 2012 based this on the co-location the genes: Delay Of Dormancy (DOG) and Germination Ability After Dry Storage (GAAS). Genes involved in repair such as the gene encoding for protein repair L-isoaspartyl methyltransferase 1 (PIMT1) (Wei et al., 2015). Genes encoding for chitinase, e.g. Cht-2 and Cht-3, this enzyme can catalyse chitin the cell wall component of fungi and, hence fence off fungal pathogen attack. Chitinase activity has been found in rice seeds, as active defense mechanism against devastating fungi such as rice blast (Inui et al., 1991; Nishizawa et al., 1999). Finally, the Kala4 gene may be interesting to test for increased longevity. As it is a paralog of Rc and gives rice to a purple pericarp, indicating presence of anthocyanidin, studying Kala4 may help further elucidate the physiological mechanisms behind increased seed longevity. Different metabolic activity may also play a role in seed ageing. As Labuza and Dugan Jr, 1971 mentions metabolic activity; at metabolic activity and repair mechanisms are much less at 40% RH than 60% RH. This indicates that fluctuating RH may have a benificial effect. Thus, perhaps having a brief period of high RH may actually increase seed longevity.

Testa flavonoids play a major role in seed longevity, flavonols for example protect the seed from UV-B radiation in Brassica rapa as Griffen, Wilczek, and Bazzaz, 2004 found out. However, other polyphenolic compounds exist within the seed coat i.e. lignins, which are a polymer of monolignol and lignans. Lignans are also thought to have antioxidant capacity however, their role on seed longevity remains to be investigated.

Results in this study deviated from the dry storage and respectively CDT results in the studies of Debeaujon, Léon-Kloosterziel, and Maarten Koornneef, 2000; Clerkx et al., 2004. Henceforth proceed reasonable explanations. In the first study dry storage was used, the results presented follow from humid EPPO storage. In humid storage enzyme activity is present Labuza and Dugan Jr, 1971 found. The difference is thus the result of the effect of oxygen and enzymatic activity which is present in humid EPPO, absent in the study by Debeaujon, Léon-Kloosterziel, and Maarten Koornneef, 2000. For the second study CDT was used for assessing the seeds, the CDT from Clerkx et al., 2004 which in turn follows the CDT methods of Tesnier et al., 2002, follows higher temperatures (40°C) and humidity (85%) at this humidity Labuza and Dugan Jr, 1971 shows that enzymatic activity is present. However, this CDT maintains normal pressures of oxygen, while humid EPPO treatment does not. The difference thus mainly lies in the 20°C difference in temperature and 25% difference in RH and 41 bar difference in oxygen pressure. Since temperature has a strong effect on therotical (R. Ellis and E. Roberts, 1980) seed longevity, it may be that these results are overshadowing the effects of

EPPO. Furthermore, research by Routaboul et al., 2006 shows that there is a various profile in flavonoid composition over the different thale cress wild type and mutant seeds. This may explain the results shown in the 15 week EPPO treatment. From this it seems evident that different flavonoid biochemical and, perhaps lignin and lignan composition of seed coat and embryo may result in different seed longevity under EPPO treatment. This, together with the information from figures 1.4 and 1.5 based on research by Galland et al., 2014; Rice-evans et al., 1995; Rice-Evans, Miller, and Paganga, 1996; Guyot et al., 1997, may explain for the results that were found in the thale cress and rice experiments.

Mutants of tt2, tt8 and tt8-1 performs better than wild type, this may be explained by an increase in high TEAC compounds flavonols called querecetin in thale cress, which has higher mean TEAC compared to proanthocyanidin and anthocyanidin producing wild type. In rice rc had decreased seed longevity, perhaps more of the flavonol kaempferol is created which has reduced TEAC compared to proanthocyanidin and anthocyanidin producing Rc rice. Next to that, after 20 weeks humid EPPO treatment, it was seen that tt8-1 remained having an increased seed longevity over wild type, while tt8 did not. A study by M Koornneef, 1990 found that tt8-1 mutants still transcribe TT8. In seedlings with the tt8-1 allele Shirley et al., 1995; Pelletier and Shirley, 1996; Pelletier, Murrell, and Shirley, 1997 have found no difference in expression for CHS, CHI, F3H, FLS, and LDOX compared to wildtype, while the amount of DFR mRNA's was modified. It may be the case that this allele provides for different antioxidant compound composition than wildtype elsewhere as well in the pathway, perhaps the tt8-1 modifies the amount of F3'H compared to its wildtype and thus results in a higher amount of dihydroquercetin, which increases the antioxidant activity.

The tt4-13 and tt5-3 mutations are performing better than wild type. The tt4-13 does not catalyse malonyl-CoA nor 4-coumaryol to tetrahydrochalcone, perhaps these compounds start to accumulate in high quantities increasing the antioxidant activity and seed longevity. Similarily tt5-3 does not convert tetrahydrochalcone to flavones, while tetrahydrochalcone is low in TEAC, if the concentration of these compounds far exceeds the wild type it may have a higher relative antioxidant activity and thus longer seed longevity.

Mutant tt-6-3 performs similar to wild type, suggesting that the TEAC of the compounds is similar in both mutant and wild type.

Mutation tt7-5 performs worse than wild type, this can be explained by that the catalyzation of dihydrokaempferol into dihydroquercetin is interrupted in the biosynthesis pathway, the product of dihydrokampferol, kaempferol has a lower antioxidant activity than quercetin the product of dihydroquercetin.

As TT10 is involved in browning by oxidation it is perhaps logical that mutations in this gene increase seed longevity, confirmed by the results of this study, as the proanthocyanidins presumably have greater antioxidant capacity, without the activity of TT10. TT10 is also involved in seed coat biosynthesis of lignin as studied by Liang et al., 2006; K. Zhang et al., 2013.

indent Mutation in ban5 performs better than wild type due to anthocyanidins not being catalysed into proanthocyanidins, resulting in a higher TEAC, this idea is reinforced by the following study of Albert, Delseny, and Devic, 1997.

It was also shown that Ler performs better than its mutant, as tt9 is necessary for accumulating of flavonoids in the pericarp tissue.

Chapter 5 Conclusion

This study showed that in rice it was shown that the Rc gene increases seed longevity under EPPO conditions under various humidities and also decreases the permeability of the pericarp of the seed. It does this by encoding for bHLB transcription factor which is involved in the transcription of Rd, which indirectly gives the rice a red colour as the condensed tannins colourise under oxidation. The increase in resistance against oxygen is most likely caused by the accumulation of proanthocyanidins, since they have show decent antioxidant activity. In thale cress results were contradicting with the Rc ortholog that is TT8, perhaps this is because of different accumulation of flavonoids altering the total antioxidant capacity in opposite fashion. Furthermore, research in to other thale cress genes showed large differences in seed longevity; one of these mutations resulting in an increased seed longevity which could be readily explained was the ban5 gene. The ban5 mutation was accumulating more anthocyanidins than proanthocyanidin in the pericarp; this results in a higher total antioxidant activity. Exciting opportunities await in the realm of seed longevity research and enough information is available already to incorporate in to a breeding program. Genes that are worth researching in rice seed in terms of longevity include but are not limited to: PIMT1, Cht-2, Cht-3 and Kala4.

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Appendix A Seed Viability Python Script

```
# -*- coding: utf-8 -*-
"""
Created on Tue Nov  6 20:49:24 2018

@author: stefan
"""

import matplotlib.pyplot as plt
import numpy as np
from scipy.special import erf#,erfinv

#Sources: Ellis and Roberts, 1980; http://data.kew.org/sid/viability/
#A.S. Cromarty; Design of Seed Storage Facilities for Genetic Conservation 1982
#For rice the following species specific oil content can be used:
D_0 = 0.018
#For rice the following species specific constant can be used:
Ke=8.668
# The following moisture content constant corresponds with O. sativa:
Cw=5.03
# The universal temperature constants can be used:
Ch=0.0329
Cq=0.000478
# Initial viability of the seed:
Ki=3
#days of storage
dos=250
#Equilibrium Temperature
eT = 20
#Storage temperature
sT = 35.0


#Equilibrium Relative Humidity (eRH)
R1 = 0.4
#Seed moisture content
m1 = ((1-D_0)*np.sqrt(-440*np.log(1-R1))/(1.1+(eT/90)))
sigma=10**(Ke-Cw*np.log10(m1)-Ch*sT-Cq*sT**2)
#Time it takes when 50% of the seeds fail to germinate
p50=Ki*sigma
v1 = []
#Storage in days
for x in range (dos+1):
    #p is equal to the days of storage
    p = x
    probit=Ki-p/sigma
    probability=1/2*(1+erf(probit/np.sqrt(2)))
    v1.append(probability*100)

#Relative humidity
R2 = 0.45
#Seed moisture content
m2 = ((1-D_0)*np.sqrt(-440*np.log(1-R2))/(1.1+(eT/90)))
sigma=10**(Ke-Cw*np.log10(m2)-Ch*sT-Cq*sT**2)
#Time it takes when 50% of the seeds fail to germinate
p50=Ki*sigma
v2 = []
#Storage in days
for x in range (dos+1):
    #p is equal to the days of storage
    p = x
    probit=Ki-p/sigma
    probability=1/2*(1+erf(probit/np.sqrt(2)))
    v2.append(probability*100)

#Relative humidity
R3 = 0.50
#Seed moisture content
m3 = ((1-D_0)*np.sqrt(-440*np.log(1-R3))/(1.1+(eT/90)))
sigma=10**(Ke-Cw*np.log10(m3)-Ch*sT-Cq*sT**2)
#Time it takes when 50% of the seeds fail to germinate
p50=Ki*sigma
v3 = []
#Storage in days
for x in range (dos+1):
    #p is equal to the days of storage
    p = x
    probit=Ki-p/sigma
    probability=1/2*(1+erf(probit/np.sqrt(2)))
    v3.append(probability*100)


#Relative humidity
R4 = 0.55
#Seed moisture content
m4 = ((1-D_0)*np.sqrt(-440*np.log(1-R4))/(1.1+(eT/90)))
sigma=10**(Ke-Cw*np.log10(m4)-Ch*sT-Cq*sT**2)
#Time it takes when 50% of the seeds fail to germinate
p50=Ki*sigma
v4 = []
#Storage in days
for x in range (dos+1):
    #p is equal to the days of storage
    p = x
    probit=Ki-p/sigma
    probability=1/2*(1+erf(probit/np.sqrt(2)))
    v4.append(probability*100)

#Relative humidity
R5 = 0.60
#Seed moisture content
m5 = ((1-D_0)*np.sqrt(-440*np.log(1-R5))/(1.1+(eT/90)))
```

```
sigma=10**(Ke-Cw*np.log10(m5)-Ch*sT-Cq*sT**2)
#Time it takes when 50% of the seeds fail to germinate
p50=Ki*sigma
v5 = []
#Storage in days
for x in range (dos+1):
    #p is equal to the days of storage
    p = x
    probit=Ki-p/sigma
    probability=1/2*(1+erf(probit/np.sqrt(2)))
    v5.append(probability*100)


#The following article describes going from probits to probabilities and vice versa:
#https://dc.etsu.edu/cgi/viewcontent.cgi?article=2497&context=etd
#Where the probability can be calculated as:
probability=1/2*(1+erf(Ki/np.sqrt(2)))
#The probit function may be expressed in terms of the inverse error function as:
#probit=np.sqrt(2)*erfinv(2*probability-1)

plt.show()
[p1,p2,p3,p4,p5] = plt.plot(v1,'r-',
         v2,'g-',
         v3,'b-',
         v4,'c-',
         v5, 'm-')
plt.ylim(0,100)
plt.xlim(0,dos)
plt.rcParams['font.size']=10
plt.rcParams["figure.figsize"]=[6*1.5,4*1.5]
plt.xlabel('Days of storage')
plt.ylabel('Germination rate (%)')
plt.title('Germination rate of rice with ' + str(round(probability*100, 1)) +
          '% initial germination rate stored at ' + str(sT) + '°C' )
plt.axhline(50,color='gray')
plt.text(125,45,'p50',rotation=0)
plt.legend([p1,p2,p3,p4,p5],['eRH of '+ str(round(R1*100, 0)) + '%',
           'eRH of '+ str(round(R2*100, 0)) + '%',
           'eRH of '+ str(round(R3*100, 0)) + '%',
           'eRH of '+ str(round(R4*100, 0)) + '%',
           'eRH of '+ str(round(R5*100, 0)) + '%'],
            loc='upper right'), #bbox_to_anchor=(0.5, 0., 0.5, 0.5))

plt.show()
```

Appendix B Labelling

Labels were created using a two-digit number; the first digit indicated the genotype and the second digit the treatment, e.g. 'A0', see the code columns in the following tables.

Arabidopsis thaliana

ID	Testa	BG	Code	ID	Testa	BG	Code
5072	WT	Col-o	0	5084	WT	Col-o	0
5073	WT	Ler	1	5085	WT	Ler	1
5074	tt2	Col-o	2	5086	tt2	Col-o	2
5075	tt4-13	Col-o	3	5087	tt4-13	Col-o	3
5076	tt5-3	Col-o	4	5088	tt5-3	Col-o	4
5077	tt6-3	Col-o	5	5089	tt6-3	Col-o	5
5078	tt7-5	Col-o	6	5090	tt7-5	Col-o	6
5079	tt8	Col-o	7	5091	tt-8	Col-o	7
5080	tt8-1	Col-o	8	5092	tt8-1	Col-o	8
5081	tt9-1	Ler	9	5093	tt9-1	Ler	9
5082	tt10-4	Col-o	A	5094	tt10-4	Col-o	A
5083	ban5	Col-o	B	5095	ban5	Col-o	B

Table B.1: Arabidopsis thaliana seeds used in the experiment

Oryza sativa

ID	Rc/rc	Code
6041	Rc	0
6042	Rc	1
6043	Rc	2
6044	Rc	3
6045	Rc	4
6046	rc	5
6047	rc	6
6048	rc	7
6049	rc	8
6050	rc	9

Table B.2: Rice seeds used in the experiment, harvested around 40 days postanthesis

Storage	(Un)cut	Code
Initial	Unut	0
Initial	Cut	1
Ambient (A)	Uncut	2
Ambient (A)	Cut	3
Ambient (B)	Uncut	4
Ambient (B)	Cut	5
Ambient (C)	Uncut	6
EPPO (A)	Uncut	7
EPPO (A)	Cut	8
EPPO (B)	Uncut	9
EPPO (B)	Cut	A
EPPO (C)	Uncut	B
EPPN (A)	Uncut	C
EPPN (A)	Cut	D
EPPN (B)	Uncut	E
EPPN (B)	Cut	F
EPPN (C)	Uncut	G

(a)
Rice 40%eRH 38.5°C

Storage	Code
Initial	0
Ambient (A)	2
Ambient (B)	1
Ambient (C)	3
Ambient (D)	4
EPPO (A)	5
EPPO (B)	6
EPPO (C)	7
EPPO (D)	8
EPPN (A)	9
EPPN (B)	A
EPPN (C)	B
EPPN (D)	C

(b)
Rice 60%eRH 35°C
&
Thale cress 60%eRH 20°C

Storage	Code
Initial CD	0
Initial AA	1
CD (A)	2
CD (B)	3
CD (C)	4
CD (D)	5
CD (E)	6
CD (F)	7
AA (A)	8
AA (B)	9
AA (C)	A
AA (D)	B
AA (E)	C
AA (F)	D

(c)
Rice
CDT (75%eRH)
&
AAT (97%eRH)
both 28.5°C

Table B.3: Experiments treatment labels

Appendix C The Thaleminator™

C.1 Python Program

```
# -*- coding: utf-8 -*-
"""
Created on Thu Jan 24 16:10:44 2019

@author: vroom016
"""

#OnceaPunAtalianaintheWest
#This Thaleminator script counts Thale cress seeds and uses a model to correct for touching seeds.
#Together with manual germination scoring this script can help the user with calculating the germination rate.
#import matplotlib.pyplot as plt
import numpy as np
from skimage import (filters, color, exposure,
                     segmentation, morphology)
from skimage.measure import label,regionprops
from skimage.color import label2rgb
from skimage import restoration, img_as_float
#from skimage.exposure import histogram
from skimage.util import invert
from skimage.io import imread_collection, imsave
#make sure the working folder is in an experiment folder and named after the storage duration or other criterium
#for instance eXXXXXX/6w
from os import path, getcwd
#unrotated images are used for analysis (label_position='bottom right')
#in case rotated images are used replace "6-(3*j+k)" with "1+(3*j+k)"
seq = imread_collection("*.jpg", conserve_memory=True)
#read the table from which sample data can be extracted
sample_table=np.genfromtxt("sample_table.csv", delimiter=",", dtype='str')
#number of trays used in the experiment
ntrays = sample_table.shape[0]
#the order of trays may be shuffled thus ther results will be saved in a different array
result_table=np.empty((sample_table.shape[0]*2,sample_table.shape[1]), dtype=sample_table.dtype)
object_table=np.empty((sample_table.shape[0]*2,sample_table.shape[1]), dtype=sample_table.dtype)
object_area_table=np.empty((sample_table.shape[0]*2,sample_table.shape[1]), dtype=sample_table.dtype)
#result_log=np.empty(0, dtype=str)
for i in range(len(seq)):
    full_image = seq[i]
    #create a dark image with the shape of full_image
    image_scan=np.zeros(full_image.shape)
    #Analysis of the binary number:
#    binnum = full_image[1800:,3560:]
    binnum = full_image[1400:1400+1000,3500:3500+400]
    binnum = invert(binnum)
#    fig, ax = plt.subplots()
#    ax.imshow(binnum);
    # change to greyscale for image analysis
    binnum=color.rgb2gray(binnum)

    eqim=exposure.equalize_adapthist(binnum)
    thrs=filters.threshold_yen(eqim)

    binnum_thrs= eqim>thrs+0.2
    crs=segmentation.clear_border(binnum_thrs)
    # look for objects
    mrs=morphology.closing(crs,morphology.square(3))
    # label objects
    lrs=label(mrs)
    rrs=regionprops(lrs)
    # remove small objects around the seeds
    lbrs=morphology.remove_small_objects(lrs)
    binnum=label2rgb(lbrs,binnum)
    binnum=restoration.denoise_nl_means(binnum,multichannel=True)
    #analyse whether the number is 0 or 1 based on its area:
    objectarea=[]
    for l in range(len(np.unique(lbrs)[1:])):
        objectarea.append(np.sum(lbrs==np.unique(lbrs)[1:][l]))
    number=[]
    for j in range(len(np.unique(lbrs)[1:])):
        if np.sum(lbrs==np.unique(lbrs)[1:][j]) >= np.amax(objectarea)-np.amax(objectarea)/10:
            number.append(0)
        elif np.sum(lbrs==np.unique(lbrs)[1:][j]) >= np.amax(objectarea)/2-np.amax(objectarea)/10:
            number.append(1)
    #this format works for Experiment[space]Date[space]Time.jpg
    print('Experiment: ',seq.files[i].split()[0],
          " Tray: ", ''.join(map(str, number)),
          ' Date: ', seq.files[i].split()[1],
          ' Time: ',  seq.files[i].split()[2][0:2], ':',
                    seq.files[i].split()[2][2:4], ':',
                    seq.files[i].split()[2][4:6], sep='')
    #include the binary number for reference
    image_scan[1400:1400+1000,3500:3500+400]=img_as_float(binnum)
    #When the first germination batch is analysed:
    if i < ntrays:
        # there is a match with the binary number recognised and
        if len(np.where(sample_table==''.join(map(str, number)))[0])==1:
            result_table[2*i][0]= \
                str(sample_table[np.where(sample_table==''.join(map(str, number)))][0])
            object_table[2*i][0]=\
                str(sample_table[np.where(sample_table==''.join(map(str, number)))][0])
            object_area_table[2*i][0]=\
                str(sample_table[np.where(sample_table==''.join(map(str, number)))][0])
        else:
            result_table[2*i][0]= 'pic_'+str(i+1)
            object_table[2*i][0]= 'pic_'+str(i+1)
            object_area_table[2*i][0]= 'pic_'+str(i+1)
        result_table[2*i+1][0]='scount'
        object_table[2*i+1][0]= 'ocount'
        object_area_table[2*i+1][0]= 'oarea'
```

```
        for j in range(2):
            for k in range(3):
                #for this consider each spot's centre and move x,y away from there
                image = img_as_float(
                        full_image[150+1200*j:1150+1200*j, 150+1100*k:1150+1100*k])
                image_scan[150+1100*j:1150+1100*j, 150+1100*k:1150+1100*k] = image
                # change to greyscale for image analysis
                image=color.rgb2gray(image)
#                hist, hist_centers = histogram(image)

                # equalise the lightning levels
                eqim=exposure.equalize_adapthist(image)
                thrs=filters.threshold_yen(eqim)
                image_thrs= eqim>thrs
                crs=segmentation.clear_border(image_thrs)
                # look for objects
                mrs=morphology.closing(crs,morphology.square(3))
                # label objects
                lrs=label(mrs)
                rrs=regionprops(lrs)
                # remove small objects around the seeds
                lbrs=morphology.remove_small_objects(lrs)
                image=label2rgb(lbrs,image)
                image=restoration.denoise_nl_means(image,multichannel=True)
#                fig, ax = plt.subplots()
#                ax.imshow(image);
                #For each seed calculate the number of pixels and return that value
#                germinated=[]
                #objects may be one or several seeds
                objectarea=[]
                objectcount=len(np.unique(lbrs)[1:])
                #the first photo session should have the most accurate seed count
                for l in range(len(np.unique(lbrs)[1:])):
                    objectarea.append(np.sum(lbrs==np.unique(lbrs)[1:][l]))
                #based on 6w fit model: 1.2818*x - 11.8046
                seedcount=int(1.2818*objectcount - 11.8046)
                if len(np.where(sample_table==''.join(map(str, number)))[0])==1:
                    print("Result of sample",
                         sample_table[
                            int(np.where(sample_table==
                                ''.join(map(str, number)))[0])][6-(3*j+k)],
                        ":", seedcount,
                         "seed(s) counted based on an object count of",
                         objectcount)
                    result_table[2*i][6-(3*j+k)]=sample_table[
                            int(np.where(sample_table==
                            ''.join(map(str, number)))[0])][6-(3*j+k)]
                    object_table[2*i][6-(3*j+k)]=sample_table[
                            int(np.where(sample_table==
                            ''.join(map(str, number)))[0])][6-(3*j+k)]
                    object_area_table[2*i][6-(3*j+k)]=sample_table[
                            int(np.where(sample_table==
                            ''.join(map(str, number)))[0])][6-(3*j+k)]
                else:
                    print("Result of sample",
                         6-(3*j+k),
                        ":", seedcount,
                         "seed(s) counted based on an object count of",
                         objectcount)
                    result_table[2*i][6-(3*j+k)]=str(6-(3*j+k))
                    object_table[2*i][6-(3*j+k)]=str(6-(3*j+k))
                    object_area_table[2*i][6-(3*j+k)]=str(6-(3*j+k))
                result_table[2*i+1][6-(3*j+k)] = str(seedcount)
                object_table[2*i+1][6-(3*j+k)] = str(objectcount)
                object_area_table[2*i+1][6-(3*j+k)] = str(np.mean(objectarea))
    #for the last batch of pictures they are only saved
    elif len(seq) -i <= ntrays:
        for j in range(2):
            for k in range(3):
                #for this consider each spot's centre and move x,y away from there
                image = img_as_float(
                        full_image[150+1200*j:1150+1200*j, 150+1100*k:1150+1100*k])
                image_scan[150+1100*j:1150+1100*j, 150+1100*k:1150+1100*k] = image
    #for the first and last set of photo's save; remember i starts at 0 and ends at len(seq)-1
    if i < ntrays or len(seq) -i <= ntrays:
        #save man_ass_pic MANual ASSessment PICtures each with the i
#        image_name=seq.files[i][:-22] + str(i) + '.png'
        image_name=seq.files[i][:-22] + '_' + str(path.basename(getcwd())) + '_man_ass_pic_' + str(i+1) + '.png'
        imsave(image_name,image_scan)
np.savetxt("automatic_result_"+seq.files[i][:-22] + '_' + str(path.basename(getcwd()))+ ".csv", result_table, delimiter=",",fmt='%s')
np.savetxt("automatic_object_"+seq.files[i][:-22] + '_' + str(path.basename(getcwd()))+ ".csv", object_table, delimiter=",",fmt='%s')
np.savetxt("automatic_object_area_"+seq.files[i][:-22] + '_' + str(path.basename(getcwd()))+ ".csv", object_area_table, delimiter=",",fmt='%s')
```

C.2 Linear Model

This is the linear model used in the script which compensates for touching seeds. For the analysis 24 trays were used each containing six samples. Two of those trays were taken out of the analysis as the trays were not properly alligned with the camera, so that the sample size equals 132. Touching seeds were seen by the computer as one object, object count with the computer was compared with human count. Output from StatsModel OLS in text below and NumPy and Matplotlib, see figure C.1.

```
                            OLS Regression Results
==============================================================================
Dep. Variable:                      a   R-squared:                       0.849
Model:                            OLS   Adj. R-squared:                  0.847
Method:                 Least Squares   F-statistic:                     728.8
Date:                Mon, 18 Mar 2019   Prob (F-statistic):           3.83e-55
Time:                        13:56:35   Log-Likelihood:                -599.64
No. Observations:                 132   AIC:                             1203.
Df Residuals:                     130   BIC:                             1209.
Df Model:                           1
Covariance Type:            nonrobust
==============================================================================
                 coef    std err          t      P>|t|      [0.025      0.975]
------------------------------------------------------------------------------
Intercept    -11.8046      6.250     -1.889      0.061     -24.170       0.561
```

```
b              1.2818      0.047     26.996      0.000       1.188       1.376
==============================================================================
Omnibus:                       13.760   Durbin-Watson:                   1.597
Prob(Omnibus):                  0.001   Jarque-Bera (JB):               18.221
Skew:                           0.590   Prob(JB):                     0.000111
Kurtosis:                       4.385   Cond. No.                         413.
==============================================================================

Warnings:
[1] Standard Errors assume that the covariance matrix of the errors is correctly specified.
```

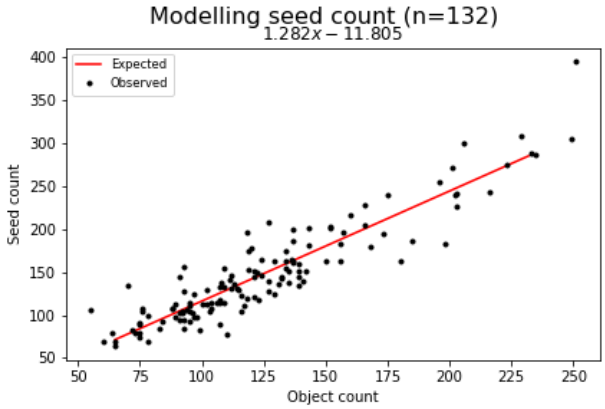


Figure C.1: Model of seed count using numpy.polyfit

Appendix D Python Statistics

D.1 Rice germination

```
# -*- coding: utf-8 -*-
"""
Created on Tue Feb 19 23:50:34 2019

@author: stefan
"""
import pandas as pd
stats=pd.read_csv("e347500.csv")
stats.loc[:,'gMAX (%) avg'] *= 100
stats.loc[:,'gMAX (%) SE'] *= 100
color_list= ['#5E0F00', '#5E0F00','#B07740','#B07740']*3
color_list.append('#5E0F00')
color_list.append('#B07740')

init_df=stats[stats['storage condition']=='initial']
storage_df=stats[stats['storage condition']=='ambient']
result = pd.concat([init_df,storage_df])

ax=result.pivot(index='weeks of storage', columns='sample', values='gMAX (%) avg')\
    .plot(legend=False,kind='bar',color=color_list,title='Maximum germination of seeds under ambient storage',
          edgecolor='black', width=0.6,
          yerr=result.pivot(index='weeks of storage', columns='sample', values='gMAX (%) SE'))
ax.set_ylabel("gMax (%)")
bars = ax.patches
patterns =('///', ' ', '///', ' ')
hatches = [p for p in patterns for i in
           range(len(result.pivot(index='weeks of storage', columns='sample', values='gMAX (%) avg')))]
for bar, hatch in zip(bars, hatches):
    bar.set_hatch(hatch)
lgd=ax.legend(bbox_to_anchor= (1, -0.15), ncol=4,
            borderaxespad=0, frameon=False)
for tick in ax.get_xticklabels():
    tick.set_rotation(0)
ax.figure.savefig('ambient.pdf',format='pdf',bbox_extra_artists=(lgd,), bbox_inches='tight')

storage_df=stats[stats['storage condition']=='EPPN']
result = pd.concat([init_df,storage_df])

ax=result.pivot(index='weeks of storage', columns='sample', values='gMAX (%) avg')\
    .plot(legend=False,kind='bar',color=color_list,title='Maximum germination of seeds under EPPN storage',
          edgecolor='black', width=0.6,
          yerr=result.pivot(index='weeks of storage', columns='sample', values='gMAX (%) SE'))
ax.set_ylabel("gMax (%)")
bars = ax.patches
patterns =('///', ' ', '///', ' ')
hatches = [p for p in patterns for i in
           range(len(result.pivot(index='weeks of storage', columns='sample', values='gMAX (%) avg')))]
for bar, hatch in zip(bars, hatches):
    bar.set_hatch(hatch)
lgd=ax.legend(bbox_to_anchor= (1, -0.15), ncol=4,
            borderaxespad=0, frameon=False)
for tick in ax.get_xticklabels():
    tick.set_rotation(0)
ax.figure.savefig('EPPN.pdf',format='pdf',bbox_extra_artists=(lgd,), bbox_inches='tight')

storage_df=stats[stats['storage condition']=='EPPO']
result = pd.concat([init_df,storage_df])
ax=result.pivot(index='weeks of storage', columns='sample', values='gMAX (%) avg')\
    .plot(legend=False,kind='bar',color=color_list,title='Maximum germination of seeds under EPPO storage',
          edgecolor='black', width=0.6,
          yerr=result.pivot(index='weeks of storage', columns='sample', values='gMAX (%) SE'))
ax.set_ylabel("gMax (%)")
bars = ax.patches
patterns =('///', ' ', '///', ' ')
hatches = [p for p in patterns for i in
           range(len(result.pivot(index='weeks of storage', columns='sample', values='gMAX (%) avg')))]
for bar, hatch in zip(bars, hatches):
    bar.set_hatch(hatch)
lgd=ax.legend(bbox_to_anchor= (1, -0.15), ncol=4,
            borderaxespad=0, frameon=False)
for tick in ax.get_xticklabels():
    tick.set_rotation(0)
ax.figure.savefig('EPPO.pdf',format='pdf',bbox_extra_artists=(lgd,), bbox_inches='tight')
```

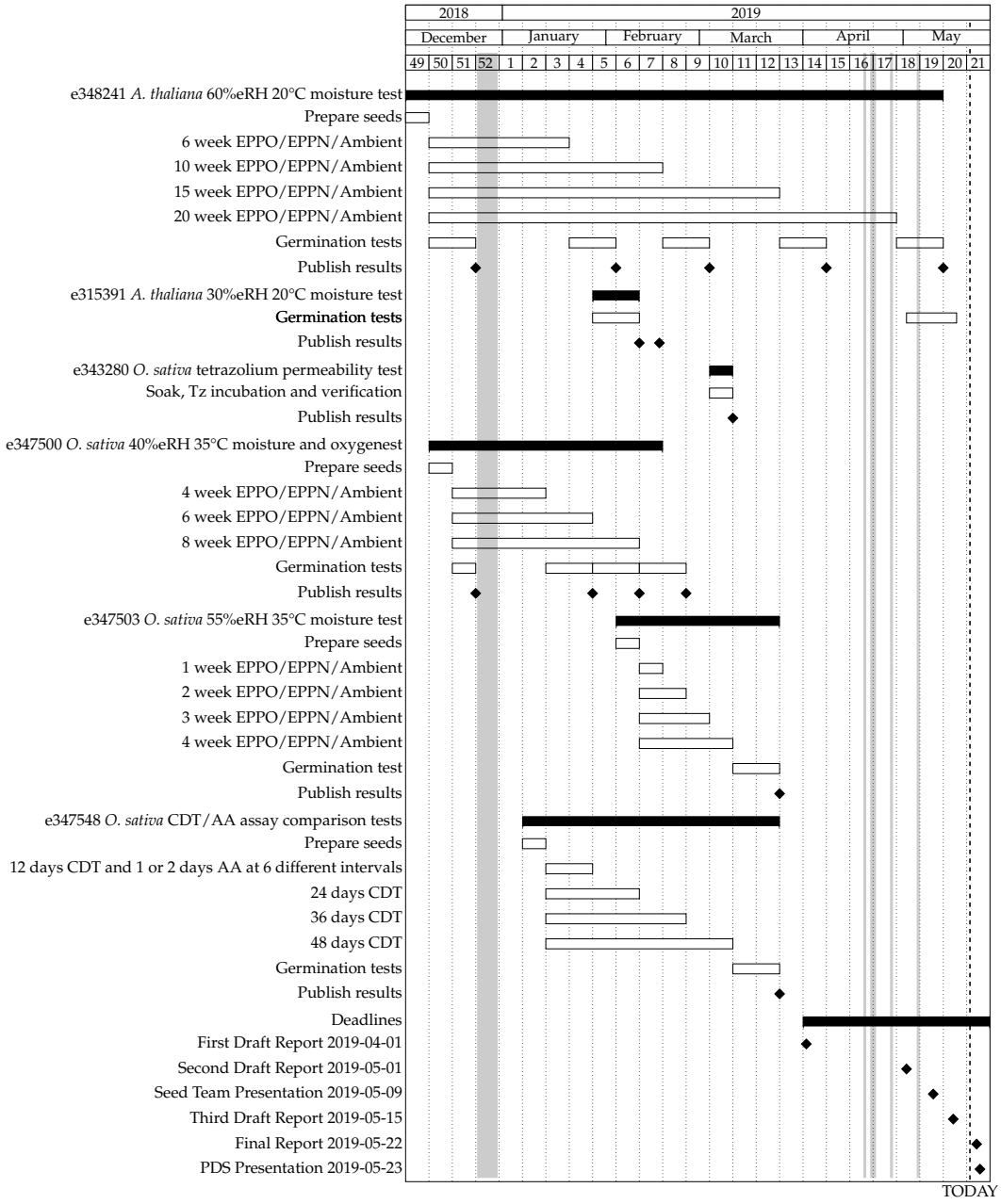
D.2 Thale cress germination

```
# -*- coding: utf-8 -*-
"""
Created on Tue Feb 19 23:50:34 2019

@author: stefan
"""
import pandas as pd
stats=pd.read_csv("e348241.csv")
stats.loc[:,'gMAX (%) avg'] *= 100
stats.loc[:,'gMAX (%) SE'] *= 100
color_list= ['#1f1108', '#180302', '#2e1300','#624501','#563605','#3b2003',
             '#260b02','#4e2f01','#604202','#4d360d', '#3d2308', '#0a0000']
column_order = ["Col-o","Ler","tt2","tt4-13","tt5-3","tt6-3","tt7-5","tt-8","tt8-1","tt9-1","tt10-4","ban5"]
init_df=stats[stats['storage condition']=='initial']
storage_df=stats[stats['storage condition']=='ambient']
result = pd.concat([init_df,storage_df])
ax=result.pivot(index='weeks of storage', columns='sample', values='gMAX (%) avg')\
    .reindex_axis(column_order, axis=1).plot(legend=False,kind='bar',
                color=color_list,title='Maximum germination of seeds under ambient storage',
          edgecolor='darkolivegreen', linewidth=2, width=0.6,
          yerr=result.pivot(index='weeks of storage', columns='sample',
```

```
                          values='gMAX (%) SE').reindex_axis(column_order, axis=1))
ax.set_ylabel("gMax (%)")
lgd=ax.legend(bbox_to_anchor= (1, 1), ncol=1,
           borderaxespad=0, frameon=False)
for tick in ax.get_xticklabels():
    tick.set_rotation(0)
ax.figure.savefig('ambient.pdf',format='pdf',bbox_extra_artists=(lgd,), bbox_inches='tight')
storage_df=stats[stats['storage condition']=='EPPN']
result = pd.concat([init_df,storage_df])
ax=result.pivot(index='weeks of storage', columns='sample', values='gMAX (%) avg')\
    .reindex_axis(column_order, axis=1).plot(legend=False,kind='bar',
                color=color_list,title='Maximum germination of seeds under EPPN storage',
          edgecolor='darkolivegreen', linewidth=2, width=0.6,
          yerr=result.pivot(index='weeks of storage', columns='sample',
                          values='gMAX (%) SE').reindex_axis(column_order, axis=1))
ax.set_ylabel("gMax (%)")
lgd=ax.legend(bbox_to_anchor= (1, 1), ncol=1,
           borderaxespad=0, frameon=False)
for tick in ax.get_xticklabels():
    tick.set_rotation(0)
ax.figure.savefig('EPPN.pdf',format='pdf',bbox_extra_artists=(lgd,), bbox_inches='tight')
storage_df=stats[stats['storage condition']=='EPPO']
result = pd.concat([init_df,storage_df])
ax=result.pivot(index='weeks of storage', columns='sample', values='gMAX (%) avg')\
    .reindex_axis(column_order, axis=1).plot(legend=False,kind='bar',
                color=color_list,title='Maximum germination of seeds under EPPO storage',
          edgecolor='darkolivegreen', linewidth=2, width=0.6,
          yerr=result.pivot(index='weeks of storage', columns='sample',
                          values='gMAX (%) SE').reindex_axis(column_order, axis=1))
ax.set_ylabel("gMax (%)")
lgd=ax.legend(bbox_to_anchor= (1, 1), ncol=1,
           borderaxespad=0, frameon=False)
for tick in ax.get_xticklabels():
    tick.set_rotation(0)
ax.figure.savefig('EPPO.pdf',format='pdf',bbox_extra_artists=(lgd,), bbox_inches='tight')
```

Appendix E Project Planning



Appendix F Tetrazolium

```
# -*- coding: utf-8 -*-
"""
Created on Thu May  2 15:25:07 2019

@author: vroom016
"""
import numpy as np
import pandas as pd
import matplotlib.pyplot as plt
from scipy.optimize import curve_fit
stats = pd.read_csv("count_summary.csv")

def sigmoid(x, L ,x0, k, b):
    y = L / (1 + np.exp(-k*(x-x0)))+b
    return (y)

fig=plt.figure()
ax=fig.add_subplot(111)
stats = stats[stats['incubation'] != -2]
stats = stats[stats['incubation'] != -1]
stats = stats[stats['genotype'] == 'Rc']
xdata=stats.iloc[:,1].as_matrix()
ydata=stats.iloc[:,4].as_matrix()
plt.plot(xdata,ydata*100,'+',label='Rc real',color='#5E0F00')
stats = pd.read_csv("count_summary.csv")
stats = stats[stats['incubation'] != -2]
stats = stats[stats['incubation'] != -1]
stats = stats[stats['genotype'] == 'rc']
xdata=stats.iloc[:,1].as_matrix()
ydata=stats.iloc[:,4].as_matrix()
p0 = [max(ydata), np.median(xdata),1,min(ydata)] # this is a mandatory initial guess
popt, pcov = curve_fit(sigmoid, xdata, ydata,p0, method='dogbox')
x = np.linspace(-5, 25, 1000)
y = sigmoid(x, *popt)*100
plt.plot(xdata,ydata*100,'x',label='rc real',color='#B07740')
#this value was guessed since curve fitting for the second set of data proved to be difficult to automate
plt.plot(x+4.5,y,label="Rc fit",color='#5E0F00')
plt.plot(x,y,label="rc fit",color='#B07740')
ax.set_xbound(0,24)
ax.set_ybound(0,100)
lgd=plt.legend()
plt.title("Rate of embryos stained (n=24)")
plt.ylabel('Amount of embryos stained (%)')
plt.xlabel('Incubation time (h)')
plt.savefig('tzr.pdf',format='pdf',bbox_extra_artists=(lgd,), bbox_inches='tight')
plt.show()
```