INHERITANCE OF SEED QUALITY TRAITS, SEED GERMINATION AND SEED LONGEVITY IN THREE DOUBLED HAPLOID POPULATIONS OF OILSEED RAPE

Dissertation
to obtain the Ph.D. degree
in the International Ph.D. Program for Agricultural Sciences in Göttingen (IPAG)
at the Faculty of Agricultural Sciences,
Georg-August-University Göttingen, Germany

presented by

Sasanti Widiarsih

born in Tegal, Indonesia

Göttingen, September 2017

D7

1. Name of supervisor: Dr. Christian Möllers

2. Name of co-supervisor: PD Dr. Andreas Börner

Date of dissertation: 23 November 2017

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor, Dr. Christian Möllers, for his patient supervision, guidance, kindness, and encouragement throughout my Ph.D journey. I am grateful to Prof. Heiko C. Becker for giving me the opportunity to join the Plant Breeding Division. I would also like to express my gratitude to PD Dr. Andreas Börner of IPK Gatersleben for consenting to act as my co-supervisor, also to Prof. Wolfgang Link for agreeing to be the examiner in my final disputation.

Many people have been involved to help completing the research. Dr. Frank Breuer of KWS SAAT DE has provided assistance with Bulk Segregant SNP marker analysis, Prof Ivo Feuβner and Dr. Kirstin Feuβner of Plant Biochemistry Department have assisted with non-targeted metabolite analysis, and IPK Gatersleben was hosting my artificial seed aging experiment. My special thanks goes to Dr. Andreas Börner as the group leader, Dr. Manuela Nagel, for setting up the experiment, also for her generosity and patience, and the lab technicians: Sibylle Pistrick, Gabriele Matzig, Josefine Richter, and Linda Ries. In our group, I would like to thank Rosemarie Clemens for KASP genotyping and other laboratory assistance, Pitchayapa Mahasuk for helping me with candidate gene identification, and technical assistance from Dietrich Kaufmann, Gunda Asselmeyer, Julia Hartmann, Madhuri Paul, also the Ghanbari family.

My special gratitude goes to my fellow PhD students of our working group, Edy Suprianto, Mohammad Ghanbari, Eva Heinrich, Jan-Christoph Richter, and Ariana Istar Valdés Velázquez for the great teamwork and helpful discussions, and all PhD students and staffs in Plant Breeding Division for their warm friendship and great supports.

This study would not have been possible without financial support from the Indonesian Ministry of Research, Technology, and Higher Education, under RISET-PRO Ph.D scholarship program. I would also thank my work institution, the National Nuclear Research Agency (NNEA) of Indonesia, for granting me the opportunity to pursue the higher degree.

In the end, I dedicate my deepest gratitude to my dear husband, parents, brother, sister, and to all my big family in Indonesia for their help, love, prayers and encouragement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1 INTRODUCTION

Oilseed rape (*Brassica napus* L.; genome AACC, 2n = 38) is the world's second-leading source of vegetable oil for human nutrition and industrial products, after soybean. The production contributes to about 14 % of the world vegetable oil supply (Carré and Pouzet, 2014). The main consumers of oilseed rape cultivation is not only the food and fuel industries, but also the by-product of oilseed rape, the meal, is deemed to be a valuable market. In EU countries, the demand is continuously growing, and the total production is about 20.6 million tons (FEDIOL, 2016). Oilseed rape meal, as also other crop meals, like soybean, sunflower and cotton seeds, are utilized as livestock feed. The demand of protein meal will continue to rise together with the increase of global livestock production demand.

Considering the nutritional values of the meal, the meal of oilseed rape has excellent balanced composition of essential amino acids (Tan et al., 2011). For feeding purposes, the absorption of the nutritional values in oilseed rape meal can be improved by reducing limiting factors like dietary fibers and some anti-nutritional compounds, such as glucosinolates.

Yellow seed character has been becoming a subject of interest in oilseed rape for the last two decades. It has been associated with lower dietary fiber content, also higher oil and protein content (Wang et al., 2015). Yellow seed color is often accompanied by thinner and transparent seed coat (testa), which enabled the yellow cotyledons inside to be seen (Neubert et al., 2003). The testa color is determined by the type of pigment deposited in the seed coat cells. Yellow seeded genotypes were reported to have lower pigment content and smaller testa proportion than the black seeded ones (Zhang et al*.,* 2006). The pigments are mostly of flavonoid groups, normally present at high levels in most seeds. Seed flavonoids seem to play protective role against solute leakage, imbibition damage, pathogen or pest attacks, and also contribute to physiological functions, such as seed maturation, dormancy, viability and seedling vigor as well as protection against ultraviolet (UV) light (Zhang et al*.,* 2006, Neubert et al., 2003).

Unfortunately, thinner seed coat (testa) also means the seed is more prone to be damaged by various environmental factors, and also easier to be imbibed by water (Debeaujon et al., 2000). Seed viability and vigor are important aspects of seed quality, and important in determining the success of a planted crop (Nonogaki et al., 2010). The seed longevity, or seed ability to germinate after being stored for a period of time, tends to decline more easily in yellow seeded genotypes compared to the black seeded ones. Poor seed longevity can result in economic losses, due to the impossibility of

carry-over of seed lots which lost their vigor and viability, so that they are no longer marketable (Pereira-Lima et al*.,* 2017).

The objective of this work was to study the inheritance of several seed quality traits, in relation to seed germination and seed longevity traits in three different DH populations. Two of the populations were segregating for yellow seed character, as both were originated from crosses with Express 617, a black seeded winter type line cultivar. The other parent of the first population was 4042, an old yellow seeded winter type Göttingen germplasm. 4042 x Express 617 consisted of 77 genotypes, and was grown in five environments in Germany (Reinshof and Einbeck) during the years 2014 to 2016. The second DH population used was a cross between Express 617 and DH line 1372, a yellow seeded spring type oilseed rape line from Canada (Burbulis and Kott 2005). The field experiment was grown in Reinshof 2015 and 2016. The harvested seeds of both populations were subjected to germination test, twice for each genotype, before and after aging treatment. The seed germination test before aging was completed at University of Göttingen laboratory. Later, seed artificial aging treatment and seed germination observation was executed at IPK Gatersleben.

In the first population of 4042 x Express 617, bulk segregant SNP-marker analysis which was segregating for low vs high ADL content was performed, in cooperation with KWS SAAT SE, www.kws.de). The experiment was further continued with identification of candidate genes that can be responsible for ADL content. Afterwards, non-targeted metabolism fingerprinting was carried out, in cooperation with Department of Plant Biochemistry, University of Göttingen, in order to identify the differences of the compounds level between immature seed samples of low vs high ADL content genotypes. KASP genotyping assay was also carried out to verify the result of bulk segregant analysis at allele level. The study of first population is presented in Chapter 3, while Chapter 4 describes the variations and associations found within seed quality, morphology, seed germination, and seed longevity traits of the second population, DH 1372 x Express 617 from two years of field experiment at Reinshof (2015 and 2016).

Suma et al. (2014) said that two main obstacles for studying natural aging process in seeds are the time needed for natural aging to take place, and how to control the degree of seed deterioration. Artificially aged seeds are known to germinate and grow into seedlings in a normal manner which is comparable to naturally aged seeds to a certain degree (Rajjou et al., 2008, Suma et al., 2014). This allows many researchers to draw safe enough conclusions regarding the loss of seed viability and mechanism of seed deterioration under storage. However, application of different aging protocols (Rajjou et al., 2008) or even just a slight change in temperature of deterioration treatment (Nagel et al., 2011) might alter the seed germination performance after artificial aging greatly. After all, an

artificial seed aging treatment is at best only tried to imitate the actual natural seed ageing process (Rajjou et al., 2008).

The third part of the study is investigating seed longevity on naturally aged seeds, presented in Chapter 5. The old seeds of DH Sollux x Gaoyou seeds which being stored in an ambient storage room temperature of University of Göttingen for 13 years (2001 - 2014) was used in this experiment. The population consisted of 291 genotypes of DH Sollux x Gaoyou (Zhao, 2002), harvested in 2001 in four different environments (two locations in China and two others in Germany). Both parents are black seeded winter type oilseed rape. Sollux is a commercial German cultivar, and Gaoyou is a local Chinese cultivar. New plants of this population were regenerated from an old stock of self-pollinated seeds in the green house in 2016, and the newly harvested seeds were used as control treatment which representing the seed germination ability before seed natural aging. Spearman's rank correlations were also estimated between the seed germination traits of the present study and their seed quality traits previously measured by Suprianto (2014).

The last chapter presents a general discussion, covering the comparison of the findings from all these three studies. Before these results are presented, the following chapter gives a short literature review on the inheritance of seed germination and seed longevity traits, in relation to seed color and seed quality traits of *B. napus*.

2 LITERATURE REVIEW

2.1 IMPORTANCE OF OILSEED RAPE

Oilseed rape (*Brassica napus* L.; genome AACC, 2n = 38) is one of the most important members of *Brassicaceae* family. This amphidiploid species bears the A and C genomes of both progenitors, turnip rape (*Brassica rapa L., syn campestris*; genome AA, 2n = 20) and cabbage (*Brassica oleracea* L.; genome CC, 2n = 18). Created by natural interspecific hybridization, it is thought to be a 'new' species. The earliest reliable record appears only 500 years ago (Gomez-Campo et al., 1999). In the present day, *B. napus* becomes one of the most important oilseed crops. Besides serving as a source of edible oil for human consumption, oilseed rape also provides protein-rich meal for livestock feed. As feed ingredient, its meal is a good source of vitamins and minerals, high in sulfur, containing amino acids and quality protein (Sarwar et al., 2013). However, some anti-nutritional components including glucosinolates, sinapine and relatively high fiber level limit its inclusion in animal rations (Matthäus, 1998).

As potential outlook for the three major oilseed crops soybean, rapeseed, and sunflower, both the demand and production are high. The European Union market, for example, was still importing 4.8 million tons of oilseed rape in 2016 / 2017, increased by 380,000 tons from the previous year (USDA, 2017). The worldwide production of oilseed rape in 2017 / 2018 is predicted to reach a new high record of 72.6 million tons. That will be around 4.5 % increase from the five year average production (USDA, 2017). The rising demand for biofuel and industrial oils has resulted in an even stronger increase in production of this species, up to almost 70% in Europe since 2003. Out of 21.7 million tons of the total oilseed crops harvested in Europe in 2015, 68 % of it was contributed by oilseed rape. France was the largest producer with 24.5 % of the total production. Other important producers were Germany (23.1 % share), Poland (12.4 %) and the United Kingdom (11.7 %, Eurostat 2017).

2.2 IMPORTANCE OF YELLOW SEED CHARACTER IN OILSEED RAPE

Weightman et al. (2014) suggested that an improved oilseed rape genotype having characteristics of low glucosinolates, thinner seed coat with higher oil and protein and less fiber, would be highly valued financially in the feed market. Moreover, if this genotype was also of yellow seed type with reduced polyphenols in the seed coat, their taste as feed ingredients will be improved and thereby their uptake by the cattle/poultry will increase. Weightman et al. (2014) further evaluated the cost

ratio of an improved variety (containing 4 % more of oil + protein (93 % DM basis) with a thinner, yellow seed coat. In meal form, the improved (yellow) variety seems to achieve the best value in pig rather than poultry feeds. For whole seed, the improved seed type would be favored in poultry broiler diets. Within the poultry sector, the improved rape meal would appear to provide a higher value in layer diets than broiler. By industrial scale, the improved type is worth an extra € 25 to € 33 /ton of seeds, with the value of conventional oilseed rape estimated at € 378 /ton in 2014.

Yellow seed is a long sought character in oilseed rape in the last two decades. As one of the main desirable targets in oilseed breeding program, this trait proved to be very elusive. Many studies have been dedicated to this particular trait. There is no known spontaneous mutation of yellow seeded types of *B. napus* (Rahman et al., 2011), although seed color variations are common in *B. rapa* L., *B. juncea* Czern and Coss and *B. carinata* Braun (Tang et al., 1997). According to Tang et al. (1997) spontaneous mutations more likely resulted in yellow-seeded variants after the formation of these species. In *B. rapa* (AA), the evidence supports theory of seed color monogenic control, although a distorted segregation pattern was observed in some generations (Chen and Heneen, 1992). Other reports of one to three genes inheritance patterns in the same species are also available (Stringam 1980; Hawk 1982). In *B. juncea* (AABB), the black or brown seed color was regulated by two independent dominant genes (Vera and Woods, 1982). It is then assumed that the genomes of *B. rapa* and *B. nigra* (BB) each donates one of the two genes for seed color of *B. juncea.* In contrast, the inheritance of seed color in C genome is more complicated. A possibility of digenic control of seed color in the *B. alboglabra* genome (a form of *B. oleracea,* CC) was indicated by resynthesized *B. napus* (AACC) crosses (Tang et al., 1997). Pure yellow seeded lines of C genome are also hard to be obtained. An interspecific cross between black seeded *B. alboglabra* and yellow-seeded *B. carinata* resulted in only a light brown seeded *B. alboglabra* (Chen et al., 1988). Until now, no true bred yellow seeded variety *of B. napus* has been achieved (Tang et al., 1997).

There may be various pathways for seed color pigmentation of seed color in *B. rapa* and *B. oleracea,* parallel with high polymorphic variations within the two species. Hence, it is suggested that the C genome of natural *B. napus* may be also has a complex pathway for pigmentation (Tang et al., 1997). All available yellow seeded *B. napus* lines were developed from interspecific crosses with related species, for instance, *B. rapa, B. oleracea spp. alboglabra, B. juncea* and *B. carinata*. Several molecular markers associated with the seed coat color trait in *B. napus, B. juncea*, and *B. rapa* have been developed by various research groups (Badani et al., 2006, Liu et al., 2005, Yu et al., 2013).

Yellow seed color is often associated with less seed fiber and more seed oil and protein content (Badani et al., 2006). As dark color resulted from accumulated tannin, yellow seeds have thinner

seed coat or testa (Neubert et al., 2003). The yellow color of the seed itself is actually not coming from yellow pigment on the seed coat, but from transparent one, which gives way to the yellow color of the cotyledons underneath. Seed color is controlled by a complex mechanism that is still poorly understood. Beside environmental factors such as light and temperature, genetic factors also play a significant role (Liu et al., 2012).

2.3 SEED DEVELOPMENT

A seed is basically a small embryonic plant, enclosed in a hull called the seed coat, usually accompanied by some food storage (Shaban, 2013). In general, plant seeds can be divided into three major components: (1) embryo, composed of cotyledon(s), hypocotyl, and radicle; (2) endosperm, which provides nourishment for the developing embryo; and (3) seed coat, which surrounds the embryo and the endosperm (Shaban, 2013, Ohto et al., 2007). Ohto et al. (2007) further added that in mature rapeseed seeds, the endosperm degenerates and the seed coat enwraps the embryo tightly.

According to Bewley et al. (2006), during the first weeks of seed development, the seed coat expands inside the young pod (silique). At this stage, the seed is almost translucent, and the embryo develops rapidly within the seed coat, filling the space which previously was occupied by fluid. The seed weight increases and seed filling are completed in 35-45 days after pollination. During this time the cotyledons are filled with oil and protein reserves. At maturity, the embryo fills up the entire seed, bright yellow in color, and having moisture content of 10 to 20%. Seed filling is followed by seed maturation and ripening. These two processes are characterized by color changes. The seed coat alters from green to yellow to brown, and the silique changes from green to straw color. Hajduch et al. (2006) added that at 5 WAF (weeks after flowering), the seed development would enter the desiccation phase. Protein content will be increased dramatically during this period and reach about 10% of fresh seed weight at 6 WAF.

Bewley et al. (2006) further explained that *Brassica* inflorescence is formed from multiple indeterminate racemes. At this time the lower pods on the main raceme have elongated and turned green and most of the leaves have died. The green silique walls and stems become the main source of photosynthate during seed maturation. The uppermost pods at the main racemes are usually the longest and contain the largest seeds, but the pollination, seed filling and maturation will continue on lateral racemes as long as products of photosynthesis are available. At 40 to 60 days after first flowering, the seeds in lower pods have ripened fully and changed to their final seed coat color.

2.4 SEED GERMINATION

The human interest in seed germination and its influence on plant performance has started since the beginning of agriculture in around 10.000 B.C. Today, nearly 80 % of economically important crops are propagated by seeds (Marcos-Filho, 2015). The germination starts as the dry seeds begin to take up water, and ends when the embryonic axis elongates. The radicle tip protrusion through the seed envelope is the visual sign of seed germination (Debeaujon et al., 2000, Nonogaki et al., 2013). Seed viability is the ability of the embryo to germinate (Shaban, 2013). Many factors, both genetic and environment, can affect the seed viability, such as the ability of the plant to produce viable seeds, pathogen damage, and climate (Shaban, 2013).

In mature seeds, only the aleurone (outermost of endosperm) layer is physiologically active, while seed coat or testa layers are filled with dead cells. These cells expire after vigorous developmental changes during late seed maturation. The seed coat itself protects the embryo from the various detrimental environmental factors. Testa pigmentation provides better resistance against solution leakage, imbibition damage, and soil-born fungal infection; therefore it improves the seed vigor and germination (Kantar et al., 1996).

2.5 SEED LONGEVITY

The seed age also affects its germination ability, since seeds are living embryos. Over time, cells eventually die and cannot be replaced (Shaban, 2013). Maximum physiological potential is achieved close to seed maturity and just after this stage, seeds become prone to deterioration depending on harvest time, environmental conditions, and procedures adopted for seed drying, processing and storage. The most obvious sign of initial seed aging is a reduction in germination speed of viable seeds, followed by a decrease in seedling size, and an increased incidence of abnormal seedlings (Marcos-Filho, 2015).

2.5.1 Natural seed aging

Physiological aging (or deterioration) is a reduction in the ability of seeds to carry out all the physiological functions that allow them to perform. It starts before harvest and continues during harvest, processing and storage. It progressively reduces performance capabilities, for example due to changes in cell membrane integrity, enzyme activity and protein synthesis. These biochemical changes can occur very quickly (a few days) or more slowly (years), depending on genetic, production and environmental factors which are not yet fully understood (Shaban, 2013). The rate of

seed aging, as expressed by the rate constants of seed germination loss and vigor decline, increased exponentially with increasing water content. Biochemical deterioration and viability loss are rapidly increased in seeds stored under a high critical temperature (Murthy et al., 2003). Lipid rich seeds have limited longevity due to their specific chemical composition (Balešević-Tubić et al., 2010).

2.5.2 Artificial seed aging

Artificially aged seeds are known to germinate and grow into seedlings in a normal manner comparable to naturally aged seeds, making it possible to arrive at safe conclusions regarding the loss of seed viability and mechanism of seed deterioration under storage (Suma et al., 2014). Artificial aging of seeds is generally accepted to mimic extended seed storage because similar changes in the proteome, oxidation of proteins and detoxification of metabolites were observed (Rajjou et al. 2008). However, as soon as aging conditions are modified, different genomic regions are activated to deal with the stress (Nagel et al., 2011). Therefore, germination behavior after long-term storage might be different to what is observed after artificial aging. Accelerated seed aging, *i.e.*, seed lot exposure to high temperature and high relative humidity leads to a loss of vigor and eventually to a loss of viability (Balešević-Tubić et al., 2011). Accelerated aging test was considered effective to predict the length of storage life of sunflower and soybean seed (Balešević-Tubić et al., 2010).

2.6 SEED QUALITY CHARACTERS

NIRS (Near-infrared Reflectance Spectroscopy) has a light source which shines through a monochromator. Its light will be reflected by a set of samples, and the detector will measure the absorbed radiation. This equipment utilizes a developed calibration equation to relate to the samples properties. The NIR region of electromagnetic spectrum itself ranges from 780-2526 nm (Reich, 2005). NIRS has many advantages compared to chemical-based analysis. It is non-destructive, rapid, and cost-effective, not requiring labor intensive sample processing, environmentally safe, and allowing several traits to be measured simultaneously in one sample. How reliable is the estimation of compounds in routine analysis by NIRS is determined by the calibration equation quality being used (Stuth et al., 2003).

2.6.1 Seed oil, protein, and glucosinolates

Beside yield, other main objectives of oilseed rape breeding are the seed quality characters, such as oil, protein, fatty acid, glucosinolates, fiber contents etc. Increase in seed oil content is a major goal

for oilseed rape breeding. However, seed oil content is under a complex genetic determinism. As the pressed meal of oilseed rape is commonly used as animal feed, protein content is also being considered. Seed protein content is generally negatively correlated with seed oil content (Zhao et al., 2006) and improvement of the seed quality in oilseed rape has been conducted with little attention paid to the protein fraction. Many of these seed quality traits are tightly related to each other, and also to the seed germination and seed longevity (Nesi et al., 2008).

2.6.2 Seed fiber

Determination of fiber content values can be done by several methods, but the most popular one is the detergent system which was developed by Van Soest et al. (1991). This method is based on the principle that the plant cell walls can be divided into two materials: the less digestive walls, which consisted of hemicellulose, lignin, and cellulose; and the second is the easily digestive cell contents, which consisted of starch, proteins, and sugar. These two materials can be separated using neutral and acid detergent solutions. The traits of seed fiber is usually explained by three fraction/component values, based on the detergent system which was developed by Van Soest et al. (1991).

The remaining component of the meal sample after the digestion with neutral detergent solution (Na-lauryl sulfate, EDTA, pH =7.0), followed by gravimetric determination of the fiber residue is called neutral detergent fiber (NDF). It represents the "total" seed fiber, which comprises cellulose, hemicellulose and lignin, although a small amount of fiber may escape during the digestion process (Von Soest et al., 1991). NDF value is important for nutritionist when creating animal feed ration formulations, because this value reflects the amount of feed that animals can consume (Möller, 2008). In general, the intake of dry matter will be less as NDF percentage increases (Suprianto, 2014).

After meal sample was digested by acid detergent solution (5 % sulfuric acid), ADF value is determined as the residue remaining after adding an acidified solution. It is basically the NDF without the hemicelluloses, refers to meal proportion which belongs to cellulose and lignin. Cetyl trimethyl-ammonium bromide (CTAB) separates proteins from the remaining cellulose and lignin, and minerals (ash), while the acid detergents solution recovers cellulose and lignin (Zaklouta et al., 2011). This value represents how much of feed that an animal can digest. The higher the ADF value, the feed digestibility will be reduced. Combined with ADL, ADF is used as indicators of dietary energy and intake (Möller, 2008).

Animal nutritionists suggest that ADL is the most nutritionally relevant fiber fraction. Largely consists of lignin related phenolic compounds, the poor digestibility of ADL, both in ruminant and monogastric animals, reduces the energy uptake from high-ADL meals (Kracht et al., 2004). The ADL fraction was found to have a positive correlation with seed color, both in yellow and dark seeded population. Correlations of ADL to seed coat phenolic compounds suggest that low ADL content is associated with reduced seed coat thickness (Wittkop et al., 2012).

2.7 LIGNIN PATHWAY/BIOSYNTHESIS: RELATION TO SEED COLOR

Besides cellulose, lignin is the most prominent polymer on Earth (Vogt, 2010). Plant lignins are produced via another branch of the phenylpropanoid pathway. Lignin is related to proanthocyanidins (PAs) by sharing the precursor 4-coumaroyl CoA in the phenylpropanoid pathway (Yu et al., 2013). Marles and Gruber (2004) noted that lignin and PAs are deposited in different compartments. Lignin accumulation is normally within the cell wall, while PAs are accumulated in the plant vacuole. Pigments are initially deposited in the inner integument of the seed coat, which is located next to the highly lignified palisade cells. Lignin variability may influence the pigment transferability to seed coat outer layers.

2.8 FLAVONOID BIOSYNTHESIS: RELATION TO SEED COLOR

The pigments that give color to seeds, flowers, and fruits are mostly flavonoids as plant secondary metabolites (Koes et al., 2005). The genetic and biochemical study of flavonoid metabolism during seed development focuses mainly on PAs accumulation in *Arabidopsis*. PA information of *Brassica* seeds is scarce, perhaps due to the complexity of compound extractions and analyses (Yu et al., 2013). According to Li et al. (1997), their high reactivity made flavonoids chemically too toxic to be kept inside the cytoplasm. Therefore, right after synthesis they are immediately being removed from cytoplasm, either by exclusion in the central vacuole (as for anthocyanins and PAs), or by excretion into the cell wall. Vacuoles offer a larger storage space than cell walls, added Klein et al. (2000), which are important for flavonoids to reach great enough concentrations to function as attractant, protection against predators and pathogens, or as UV light sunscreens.

The pigmentation process of the seed coat in dark seeded *Arabidopsis* was explained by Debeaujon et al. (2001) in two steps. During the heart stage of embryo development, colorless PAs fill up the vacuoles. Visible brown pigments appeared on the late torpedo stage, as products of PA oxidation. These colorless phenolic compounds are prone to react with oxygen molecules inside the vacuoles,

and color is changing from colorless to brown. Lepiniec et al. (2006) later suggested, as early as 1-2 days post-fertilization, proanthocyanidins begin to accumulate inside the embryo. The accumulation spread further 5 to 6 days after fertilization. Afterwards, PAs oxidation which brings the brown pigment formation occurs 30 to 40 d after fertilization, during the seed desiccation. In the end, Debeaujon et al. (2001) reported that the pigments diffused through 3 parenchyma layers and turned the dead cells of seed coat layers dark brown as the seed matured.

2.9 TRANSPARENT TESTA GENES

Mutants deviating from normal dark seed color as the outcome of the colorless PAs oxidation at maturity, among some other names, have been termed *transparent testa* (*tt*) (Lepiniec et al., 2006). Until recently, 26 mutations in the flavonoid pathway involved in seed color have been identified, and 23 of them had been identified at molecular level. Nineteen belong to *tt* genes family (Table 1), and either correspond to enzymes (*CHS, CHI, F3H, F3'H, DFR, LDOX, FLS, GST, ANR, LACCASE*), transporters (*TT12, TT19*), or regulatory factors (*TT1, TT2, TT8, TT16*) (Baxter et al., 2005). The nature and function of *TT9, TT13*, and *TT17* is still unclear (Debeaujon et al., 2000, Nesi et al., 2002).

Debeaujon et al. (2001) said that in *tt12* seeds, a reduction of PAs accumulation may lead to limited formation of the brown flavonoid pigment. This might explain the light seed color phenotype of the *tt12* mutant. Debeaujon et al. (2000) confirmed that structural and/or pigmentation defects of the Arabidopsis seeds can affect dormancy, germination, and longevity, together with seed morphology (slight reduction in size and weight). They encountered these morphological changes in most *tt* mutants. Histological analysis of the mature *tt12* testa by Debeaujon et al. (2001) revealed a lack of phenolic compounds in the endothelium layer. A vanillin assay confirmed the defect existence, either in biosynthesis or in the PAs deposition (Debeaujon et al., 2000). In nature, PAs exist in polymeric state and they are able to bind proteins. These characteristics might explain their impermeability and cell-cementing properties, and furthermore their role in the germination-restrictive action of the testa (Debeaujon et al., 2001).

Chai et al. (2009) isolated two TT12 genes from *B. napus*, one gene from *B. oleracea,* and one gene from *B. rapa*. Southern hybridization confirmed the result, thus validated *B. napus* as an amphidiploid. *BrTT12* and *BoTT12* are the progenitors of *BnTT12-1* and *BnTT12-2,* respectively. All *Brassica* TT12 proteins displayed high levels of identity (> 99 %) to each other and also to AtTT12 (> 92 %).

Table 1 Transparent testa related mutants in *Arabidopsis thaliana* and their functions (adapted from Yu et al., 2013)

TT mutant	GenBank accession no.	Function	Seed coat color	References
tt1	At1g34790	WIP subfamily zinc finger proteins	Golden yellow	Sagasser et al. (2002)
tt2	At5g35550	R2R3MYB DNA binding domain proteins	Golden yellow	Nesi et al. (2001)
tt3	At5g42800	Dihydroflavonol 4-reductase (DFR)	Grayish yellow	Shirley et al. (1992)
tt4	At5g13930	Chalcone synthase (CHS)	Pale yellow	Shirley et al. (1995)
tt5	At3g55120	Chalcone isomerase (CHI)	Lemon yellow	Shirley et al. (1992)
tt6	At3g51240	Flavanone 3-hydroxylase (F3H)	Pale brown	Pelletier and Shirley (1996)
tt7	At5g07990	Flavonoid 3'-f-monooxygenase/ hydroxylase (F3'H)	Pale brown	Schoenbohm et al. (2000)
tt8	At4g09820	Basic helix–loop–helix (bHLH) DNA binding domain transcription factors	Yellow	Nesi et al. (2000)
tt9	(unknown)	(unknown)	Grayish beige	Shirley et al. (1995)
tt10	At5G48100	Laccase-like	Pale brown	Pourcel et al. (2005)
tt18/ tt11	At4g22880	Leucoanthocyanidin reductase (LDOX)	Yellowish brown, pale	Abrahams et al. (2003)
tt12	At3g59030	MATE (multidrug and toxin compound extrusion) transporter	Dull pale brown	Debeaujon et al. (2001)
tt13	(unknown)	(unknown)	Pale brown	Debeaujon et al. (2003)
tt14/ tt19	At5G17220.1	ATGSTF12 : glutathione S-transferase phi 12 (GST)	Pale brown	Kitamura et al. (2004)
tt15	At1g43620	UDP-glucose: sterol glycosyltransferase UGT80B1	Pale greenish brown	DeBolt et al. (2009)
tt16	At5g23260	BSISTER MADS domain	Straw-colored	Nesi et al. (2002)
tt17	(unknown)	(unknown)	Brown yellow	Bharti and Khurana (2003)

MATE is a family of proteins which function as drug/sodium or proton antiporters (Brown et al., 1999). TT12 gene encodes a membrane protein with 12 predicted transmembrane helices, and belongs to MATE (multidrug and toxic compound extrusion) transporter family, also known as multi-antimicrobial extrusion protein or multidrug and toxin extrusion. In Arabidopsis, *AtTT12* encodes a protein of MATE transporter TT12 at vacuolar membrane, and acts as a proton-dependent

antiporter, assisting vacuolar localization of proanthocyanidins in the testa (Debaujon et al., 2001, Marinova et al., 2007, Zhao and Dixon, 2009, and Chai et al., 2009).

2.10 KASP GENOTYPING

Single nucleotide polymorphism (SNP) refers to a single base change in a DNA sequence. The measurement of genetic variation caused by SNPs starts with the identification or determination of the genotypes of the particular individuals of the same species, namely "genotyping" (He *at al*., 2014). Genotyping by next-generation sequencing is an emerging method of SNP genotyping being increasingly adopted for discovery applications. One of the widely used SNP genotyping platforms is Kompetitive Allele Specific PCR (KASP^{TM}) from KBioscience or LGC Genomics (http://www.lgcgenomics.com) (Semagn et al., 2014).

KASP can deliver high levels of flexibility, handling starting from 1 SNP over at least 22 samples, until thousands of SNPs over thousands of samples, generating millions of data points in a day. This platform has been utilized by small and large laboratories in research for genetic improvement of animals and field crops (Robinson and Ganske, 2012). Comparing it to the performance of chip base Illumina 'GoldenGate' assay, Semagn et al. (2014) found that 81 % of the SNPs used in GoldenGate assay were transferable to KASP. Furthermore, the average genotyping error in positive control DNA samples by KASP was evidently lower.

According to Patterson et al. (2017), a common reverse primer paired with two forward primers can discriminate two alleles of a SNP, one specific to each allele. Each forward primer also has a nucleotide sequence that hybridizes to either the HEX or FAM fluorophore quencher. Amplification would permit forward primers to bind, only if they are perfectly complementary to the template sequence. Fluorescence is released from the quencher molecule when a forward primer is incorporated in a PCR product, and will be detected at the end of the assay by a real-time PCR machine. The proportion of fluorescence from HEX, FAM, or both, indicates the sample genotype. He et al. (2014) indicated that in case of a homozygous genotype at a given SNP, only one of the two possible fluorescent signals will be generated. However, if the individual is heterozygous, the result will be a mixed signal.

3 INHERITANCE OF SEED QUALITY TRAITS, SEED GERMINATION AND SEED LONGEVITY IN THE WINTER OILSEED RAPE DOUBLED HAPLOID POPULATION 4042 X EXPRESS 617, SEGREGATING FOR THE YELLOW SEED CHARACTER

3.1 INTRODUCTION

Oilseed rape (rapeseed; *Brassica napus* L., genome AACC, *2n* = 38) resulted from spontaneous hybridization between turnip *(Brassica rapa)* (AA, *2n* = 20) and cabbage *(Brassica oleracea)* (CC, *2n* = 18) (Nesi et al., 2008). The oilseed rape oil production contributed to about 14% of the world vegetable oil supply; made it the second largest world oilseed, after soybean (Carré and Pouzet, 2014). Except for production of high-quality edible oil, the by-product, oilseed rape meal, is also utilized as livestock feed (Shahidi, 1992).

Considering the nutritional values, the meal of oilseed rape has excellent balanced composition of essential amino acids (Tan et al., 2011). Digestibility of the meal is greatly influenced by the presence of high amounts of fiber in residual hulls. For feeding purposes, the nutrition absorption and digestibility of the meal can be improved by reducing the dietary fiber and some anti-nutritional compounds (Nesi et al., 2008).

Yellow seed color has become an important breeding objective in rapeseed (Jiang et al., 2007). Yellow seed character is often associated with lower dietary fiber content, higher oil and protein content (Meng, 1998, Rahman and McVetty, 2011). Since no yellow seed genotypes occurred naturally in *B. napus*, yellow-seeded lines have been developed through interspecific introgression of yellow seed coat color genes from related species *(B. rapa, B. carinata, B. juncea* (Nesi et al., 2008).

Despite important research efforts during the last 20 years, attempts to develop a true breeding rapeseed that consistently yields pure and bright yellow seeds under a wide range of environmental conditions have not been successful (Rahman, 2001). The dark color of oilseed rape seeds is due to the accumulation of condensed tannins or proanthocyanidins (Qu et al., 2013). According to Badani et al. (2006), the yellow seed color only occurs when one or more gene(s) which encode different enzymes are mutated in the flavonoid biosynthetic pathway. This mutation brings the failure of proanthocyanidin accumulation in the seed, and further produces a transparent seed coat which made the yellow embryo visible, resulting in yellow seed color. The recent molecular study has successfully identified 26 independent loci involved in seed coat pigmentation (the so-called *Transparent Testa [TT]* genes) (Xu et al., 2006, Yu et al., 2013). Some of the *BnTT* genes were proposed to co-localize with QTL for seed color and fiber content (Badani et al., 2006).

Most of the time, the seed coat (testa) inhibits its own seed germination by being impermeable to water and/or oxygen, or by providing mechanical resistance to radicle protrusion. In many species, these properties have been positively correlated with seed coat color, due to phenolic compounds (Debeaujon et al., 2000). In spite of all advantages of yellow seed coat, the thinner testa in yellow-seeded type also means the seed is more prone to damage by various environmental factors (Neubert et al., 2003).

Seed longevity is defined as seed viability after seed dry storage for a period of time. It describes the total seed life span (Rajjou and Debeaujon, 2008). During seed storage, seeds will slowly deteriorate, lose vigor, become more sensitive to stresses during germination, and ultimately die. The aging rate depends on the seed moisture content, temperature, initial seed quality (Walters et al., 2005), and also on genetic factors (Nagel et al., 2010). The seed longevity of yellow-seeded type also tends to drop more easily compared to the black-seeded ones (Debeaujon et al., 2003). Since materials of naturally aged seeds are not always available, artificial seed aging protocols are often utilized. Exposure of seeds to high temperature and moisture conditions had been the commonly used method for aging seeds in the laboratory (Suma et al., 2014).

Line 4042 is an old yellow-seeded oilseed rape line from Gottingen, while Express 617 was a popular black-seeded German line cultivar. The aim of this experiment was to study the inheritance of seed germination and seed longevity in a doubled haploid (DH) populations derived from the cross between line 4042 and Express 617, also to study the inheritance of seed quality traits in this populations (oil, protein, fiber content, etc.) and their correlation to seed germination and seed longevity.

3.2 MATERIALS AND METHODS

3.2.1 Plant materials

The plant material consisted of 77 genotypes; 75 double haploid lines derived from crossing between line 4042 x Express 617 and both parents. Line 4042/2002 is an old local doubled haploid line originated from the Department of Crop Sciences at the Georg-August-Universität Göttingen which has yellow seed coat. The second parent is Express 617, a popular oilseed rape line cultivar with a black seed coat. Both parents were of winter oilseed rape type.

3.2.2 Field experiment

In total, five field experiments have been conducted for DH population 4042 x Express 617; at Reinshof in 2014, at both Reinshof and Einbeck in 2015, also repeated at Reinshof and Einbeck in 2016. The field experiment was designed with no replication for all locations. One hundred seeds were spread in two rows for each genotype plot. At maturity around 5-10 main racemes of each plot was harvested randomly and bulked in one sample bag for each genotype. All harvested seeds were dried, cleaned from the pods and stems, and stored separately for every sample.

3.2.3 Analytical methods

NIRS prediction analysis was conducted using seed samples around 3 g using by Near Infrared Reflectance Spectroscopy (NIRS) monochromator model 6500 (NIRSystem Inc., Silverspring, MD, USA). WINISI software is used to predict several seed quality traits, e.g. seed oil content, protein content, glucosinolates, etc. The fiber content values (NDF, ADF, and ADL) are estimated by calibration equation fibr2013.eqa which developed by Suprianto (2014). All fiber content values are given as percentage of fiber in the defatted meal. Meanwhile, oil, protein, and glucosinolates contents were predicted separately, using commercial calibration equation of raps2012.eqa provided by VDLUFA Qualitätssicherung NIRS GmbH (Am Versuchsfeld 13, D-34128 Kassel, Germany). Three values (oil, protein and glucosinolates), were estimated at seed basis of 91% dry matter. Oil and protein are expressed as percentages, and glucosinolates are expressed as μmol/g seeds. Total seed oil and protein content (oil+P) was obtained by simply adding contents of oil content (Oil) and protein (P).

The values of oil and protein content further were used to calculate protein of defatted meal value (PDM) following Suprianto (2014), which estimated as below:

Protein of defatted meal (%) = [% protein / (100 - % oil)] x 100

Beside NIRS-predicted traits, three other seed characters were also collected: thousand seed weight (TSW), percentage of pre-harvest germination (PHG), and seed coat color (SC). Thousand seed weight (TSW) was obtained by weighing samples of 500 seed weight, times 2 to reach one thousand. Percentage of pre-harvest germination (PHG) was gained by counting the number of seeds indicating pre-harvest germination in 100 random seeds of the sample.

Seed color was scored visually, from color score 1 (uniform yellow) to 9 (uniform black, Figure 1). The color scoring code was as followed: (1) uniform yellow; (2) mix yellow and pale brown; (3) mix of yellow as predominant color and small portion of dark brown/black; (4) mix pale brown and dark

brown/black; (5) uniform brown/mix 50:50 yellow and black; (6) mix of dark brown as predominant color and small portion of yellow ; (7) dark brown; (8) reddish black/grey; and (9) uniform black. As the observed seeds were sometimes mixed of two or more colors, even though harvested from one individual plant, the scoring system is based on the predominant color, or the ratio of mixed colors.

Fig. 1 The proposed oilseed rape seed coat color scoring system

3.2.4 Germination test

Germination test were conducted two times for each seed sample. The first germination test was performed before seed aging, and the second one was completed after controlled seed deterioration treatment, or also known as artificial seed aging test. The fresh seed germination test was performed using freshly harvested seeds; at least they have been stored for 6 weeks after harvesting from the field (Table 2).

The germination test was carried out in Petri dishes (92 x 16 mm, Sarstedt, reference code 82.1473), using customized filter paper (90 mm in diameter, Macherey-Nagel, GmbH & Co. KG, reference code 400866009.1) with 50 indented holes each, to hold 50 sample seeds per genotype tested. De-ionized water was added, 12 ml each Petri dishes. The sample seeds were chosen randomly, eliminating the broken, abnormal, and pre-harvest germinated seeds. The Petri dishes containing seeds that already being watered were then placed into plastic trays. The trays afterward covered with thin cellophane to reduce evaporation. These trays were then placed into dark germination chambers for 10 days, ambient temperature 16.5 – 17.5 ^{o}C, RH 90-95%. Germination in dark condition would provide

uniform environment to all seed samples and to eliminate the light competition factor in seed germination vigor.

Table 2 Schedule for fresh seed germination test of DH population of 4042 x Express 617

Population	Harvest time	Start Germination (9-10 days)	Start Counting
Reinshof 2014	4. Jul 2014	20. Oct 2015	29. Oct 2015
Reinshof 2015	20. Jul 2015	20. Oct 2015	29. Oct 2015
Einbeck 2015	17. Jul 2015	21. Oct 2015	30. Oct 2015
Reinshof 2016	19. Jul 2016	23. Sep 2016	3. Oct 2016
Einbeck 2016	25. Jul 2016	20. Oct 2016	29. Oct 2016

Observations were carried out on day 9 - 10 of dark period, to count the radicle protrusion percent\age (RPP), full germination percentage (FGP), non-germination percentage (NGP), hypocotyl length (HL), and infected seed percentage (ISP) (Fig 2). Radicle protrusion is defined as the condition when the seed radicle has visually elongated and protruded out of seed coat, but the cotyledons were not yet swollen and still embedded within the seed coat. Full germination is defined as the condition when the radicle has fully elongated, and both cotyledons are outside of the seed coat. Hypocotyl length is measured in centimeter (cm), representing the average value of the hypocotyl length of all germinated seeds in one Petri dish. Infected seeds were identified by bacterial infection of the seeds on the filter paper.

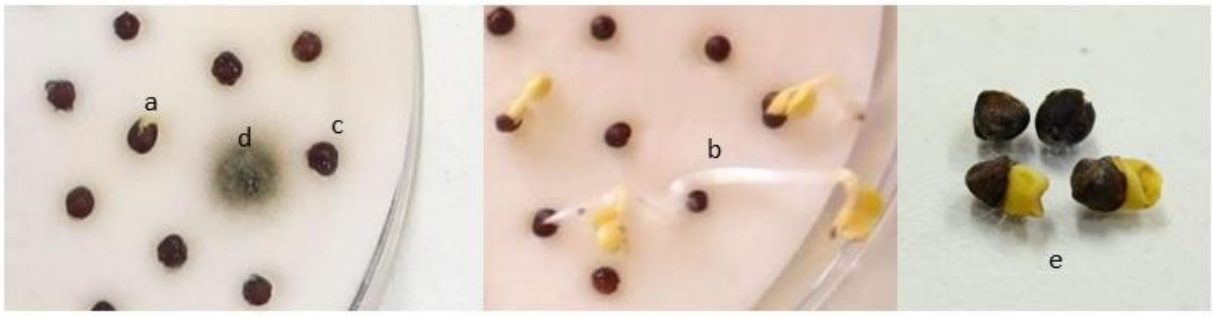


Fig. 2 Examples on visual scoring determination of seed germination-related traits

Note: a) seed with radicle protrusion, b) fully germinated seed, c) non germinated seed, d) infected seed, e) seeds without (first row) and with pre-harvest germination (second row).

The artificial seed aging or controlled deterioration tests were performed at IPK Gatersleben (Abteilung Genbank: PD Dr. Andreas Börner and Dr. Manuela Nagel), following the protocol of

Cromarty et al. (1982). It starts at the equilibration stage, in which the seeds are exposed to 47 % RH, 20^{o}C for ten days. The seed aging stage came next, in which the seeds were incubated at 45^{o}C and 60 % RH for 50 days. The last step is the germination test which takes 9 - 10 days at 16.5 - 17.5^{o}C and 90-95 % RH in dark condition. All three stages were completed at IPK Gatersleben. The seed material was divided into two working batches in different year (Table 3).

Table 3 Timetable for artificial seed aging treatment DH population of 4042 x Express 617

Seed samples	Begin equilibration (10 days)	Begin seed aging (50 days)	Begin germination test (9 days)	Begin counting
R 2014 Rep 1	03. Dec 15	13. Dec 15	01. Feb 16	9. Feb 16
R 2015 Rep 1	04. Dec 15	14. Dec 15	02. Feb 16	10. Feb 16
E 2015 Rep 1	05. Dec 15	15. De 15	03. Feb 16	11. Feb 16
R 2014 Rep 2	03. Dec 15	13. Dec 15	01. Feb 16	9. Feb 16
R 2015 Rep 2	04. Dec 15	14. Dec 15	02. Feb 16	10. Feb 16
E 2015 Rep 2	05. Dec 15	15. Dec 15	03. Feb 16	11. Feb 16
R 2016 Rep 1	15. Dec 16	25 Dec 16	13 Feb 17	22. Feb 17
E 2016 Rep 1	15. Dec 16	25 Dec 16	13 Feb 17	22. Feb 17
R 2016 Rep 2	16. Dec 16	26 Dec 16	14 Feb 17	23. Feb 17
E 2016 Rep 2	16. Dec 16	26. Dec 16	14 Feb 17	23 .Feb 17

More variations are expected to arise after seed aging. Each seed sample is consisted of 100 seeds, and divided into two Petri dishes. The first batch was being carried out for seeds from the first three environments: Reinshof 2014, Reinshof 2015 and Einbeck 2015. It started on 3-5 December 2015 and finished on 9 – 11 February 2016. The second batch was consisted of seeds of the year 2016, harvested from Reinshof and Einbeck. The harvest was completed in August 2016, followed by drying and seed cleaning and processing. The freshly harvested seed germination test was performed at University of Göttingen in October 2016. The equlibration treatment began on 15 - 16 December 2016 and the observation on germination test was performed on 22 - 23 February 2017.

The germination protocols are slightly different between the first and second batch. For the first batch, the germination test was performed on 3 - 4 layers of regular filter paper (without indented holes) and utilizing 50 seeds or sometimes more per sample. The observation data was later converted into percentage. The second batch was performed on customized filter paper (90 mm

diameter, Macherey-Nagel, GmbH & Co. KG, reference code 400866009.1), and using exactly 50 seeds per sample. Both germination batches were performed in Petri dishes of 92 x 16 mm diameter (Sarstedt, reference code 82.1473).

3.2.5 Statistical analysis

Analysis of variance and prediction of heritability values were performed by PLABSTAT software (Utz, 2011). All the experiments were conducted without replicate; therefore, the significance of the G x E factor could not be estimated. Both environment and genotype factors were considered as random variables. The general model for analysis of variance is as follow:

$$Y_{ij} = \mu + g_i + e_j + ge_{ij}$$

where Y is observation of genotype i in environment j; μ is general mean; g*i* and e*j* were the effects of genotype i and environment j; ge_{ij} is the interaction between genotype x environment of genotype i with environment j. Broad sense heritability (h^2) was calculated as follow:

$$h^2 = \frac{\delta^2 G}{\delta^2 G + \frac{\delta^2 GE}{E}}$$

where $\sigma^2 g$ was variance component for genotype, $\sigma^2 e$ are was variance component for environment, and $\sigma^2 ge$ was variance component for interaction between genotype and environment. Spearman's ranks of correlation coefficients between traits and t-test mean value comparison were predicted from mean values of the genotypes across all environments.

3.2.6 Non-targeted metabolite fingerprinting

Non-targeted metabolite finger printing was applied to detect metabolic differences between yellow and black seeded DH lines (Bruckhoff et al., 2016). First, fully mature dry seeds were used for the analysis. However, no clear differences for metabolites between the two groups could be found. For seed metabolite fingerprinting, seeds ideally should have a solid endosperm but still having a soft texture and should not have started to change the color to dark (Hajduch et al., 2006).

Fifteen genotypes were chosen as representative from each groups of high and low ADL contents. The non-targeted metabolism analysis was long, detailed, and laborious, therefore only few genotype samples can be accommodated. Two seeds per genotype were sown in small pots. Plants were grown in the green house in June 2016. Green/immature siliques were harvested 3 to 4 weeks

after self-pollination. Harvest took place between 16th of August and 2nd of September 2016 and siliques were put directly in Petri dishes on ice. In the lab about 30 green seeds per genotype were isolated and collected in Eppendorf tubes, their fresh weight was determined and they were frozen immediately in liquid nitrogen and stored in the -80 °C freezer. Selected genotypes were split into 3 bulk samples (Table 4).

Table 4 Selected genotypes of DH population of 4042 x Express 617 for non-targeted metabolite fingerprinting

Bulk	Yellow group (low ADL content)	ADL content	Bulk	Black group (high ADL content)	ADL content
1	DH 4042 x E – 14	4.55	4	DH 4042 x E - 63	9.40
	DH 4042 x E – 53	4.84		DH 4042 x E - 40	9.91
	DH 4042 x E – 29	4.98		DH 4042 x E - 8	10.01
	DH 4042 x E - 46	5.15		DH 4042 x E - 47	10.10
	DH 4042 x E - 70	5.43		DH 4042 x E - 21	10.10
2	DH 4042 x E - 4	5.58	5	DH 4042 x E - 10	10.61
	DH 4042 x E - 31	5.62		DH 4042 x E - 51	11.00
	DH 4042 x E - 30	5.71		DH 4042 x E - 64	11.48
	DH 4042 x E - 12	5.78		DH 4042 x E - 25	11.93
	DH 4042 x E - 35	5.80		DH 4042 x E - 39	12.11
3	DH 4042 x E - 72	5.82	6	DH 4042 x E - 23	12.19
	DH 4042 x E - 13	5.84		DH 4042 x E - 28	12.20
	DH 4042 x E - 15	5.85		DH 4042 x E - 3	12.41
	DH 4042 x E - 19	5.86		DH 4042 x E - 5	13.02
	DH 4042 x E - 54	5.87		DH 4042 x E - 9	13.67

Seeds of each group were sent to Göttingen Center for Molecular Biosiences (GZMB), Department of Plant Biochemistry (Prof. Ivo Feußner) for further analysis, using the same protocol as in Bruckhoff et al. (2016). The procedure in general was divided into two steps, non-targeted metabolic fingerprinting and structure determination of marker metabolites.

For the analysis seed samples were kept frozen in liquid nitrogen and homogenized. Each bulked sample then being analyzed twice by Ultra Performance Liquid Chromatography (UPLC), connected

to a photo diode array (PDA) detector and an orthogonal time-of-flight mass spectrometer (TOF-MS). Liquid chromatography was performed at 40 °C temperature, 0.2 ml/minute flow rate and with a binary gradient of solvent A (water/formic acid (100/0.1, v/v) and solvent B (acetonitrile/formic acid (100:0.1, v/v). For liquid chromatography, an ACQUITY UPLC BEH RP18 column (1 x 100 mm, 1.7 µm particle size) was used for the non-polar extraction phase samples and an ACQUITY UPLC HSS T3 (1 x 100 mm, 1.8 µm particle size) for these of the polar extraction phase. The following gradient was applied for the sample analysis of the polar extraction phase: 0 – 0.5 min for 10% solvent B, 0.5 – 3 min from 10% to 28% solvent B, 3 – 8 min from 28% up to 95% solvent B, 8 – 10 min 95% solvent B and 10 – 14 min 10% solvent B. For the sample analysis of the non-polar extraction phase: 0 – 0.5 min 46% solvent B, 0.5 – 5.5 min 46 to 99% solvent B, 5.5 – 10 min 100% solvent B and 10 – 13 min 46% solvent B. The TOF-MS was operated in W optics to ensure a mass resolution larger than 10,000 in negative as well as positive electrospray ionization (ESI) mode. The capillary and the cone voltage were kept at 2,700 V and 30 V and the temperature for desolvation and source were 350°C and 80°C, respectively.

Raw data were acquired and processed by MassLynx 4.1 software. Further data processing was carried out with the toolbox MarVis (MarkerVisualization, http://marvis.gobics.de). An ANOVA test combined with a multiple testing (Benjamini and Hochberg, 1995) was performed to filter and extract features with a false discovery rate (FDR) < 10^{-4}. Subsequently, the data from the bulk samples were matched. Selected high quality features were chosen and their masses were corrected for the negative ionization mode. The data sets were combined, used for visualization by cluster analysis and automated database search. For database search, the following databases were used: KEGG (http://www.genome.jp/kegg), LipidMaps (http://www.lipidmaps.org), Aracyc (https://www.arabidopsis.org/biocyc), Knapsack (http://kanaya.naist.jp/KNApSAcK) and Inhouse databases. The identity of marker metabolites was confirmed by UHPLC-ESI-MS/MS analysis.

The second part is the structure determination of marker metabolites. The identity of marker metabolites from metabolite fingerprinting was confirmed by UHPLC-ESI-MS/MS analysis. The samples were analyzed by LC 1290 Infinity (Agilent Technologies, USA) coupled with an 6540 UHD Accurate-Mass Q-TOF LC MS instrument with Agilent Dual Jet Stream Technology as ESI source (Agilent Technologies, USA). For liquid chromatography, an ACQUITY UPLC HSS T3 column (2.1 x 100 mm, 1.8 µm particle size, Waters Corporation, USA) was used at 40°C, flow rate 0.5 ml/min. The solvent system consists of solvent A (water/formic acid (100/0.1, v/v) and solvent B (acetonitrile/formic acid (100/0.1, v/v). The gradient was comparable as applied for UPLC TOF-MS

analysis. The Q-TOF MS instrument was operated with a detection frequency of 2 GHz in the Extended Dynamic Range and the targeted MS/MS mode. The source conditions were: gas temperature: 250°C; drying gas flow: 8 l min-1; nebulizer pressure: 35 psi; sheath gas temperature: 300°C; sheath gas flow: 8 l min-1; VCap voltage: 3 kV; nozzle voltage: 200 V; fragmentor voltage: 100 V. Samples were ionized in negative and/or positive ESI mode with collision energy 10–30 eV. Isolation of precursor ions occurred within the narrow isolation width of 1.3 m/z. Data were acquired by Mass Hunter Workstation Acquisition software B.05.01 (Agilent Technologies, USA). Mass Hunter Qualitative Analysis B.06.01 (Agilent Technologies, USA) was used for data analysis. The quantitative data of identified compounds are expressed in nmol/g fresh weight.

3.2.7 Bulk Segregant SNP-marker Analysis

From the frequency distribution of seed ADL content of DH population of 4042 x Express 617 of three first environments (Reinshof 2014, Reinshof 2015, and Einbeck 2015), a bimodal type of frequency distribution was found. It suggested a major gene controlling this particular trait. In order to investigate further, a bulk segregant analysis was performed in cooperation with KWS SAAT SE (www.kws.de; Dr. Frank Breuer). Frequency distribution of ADL content of DH 4042 x Express 617 from 3 environments is displayed on Fig. 3.

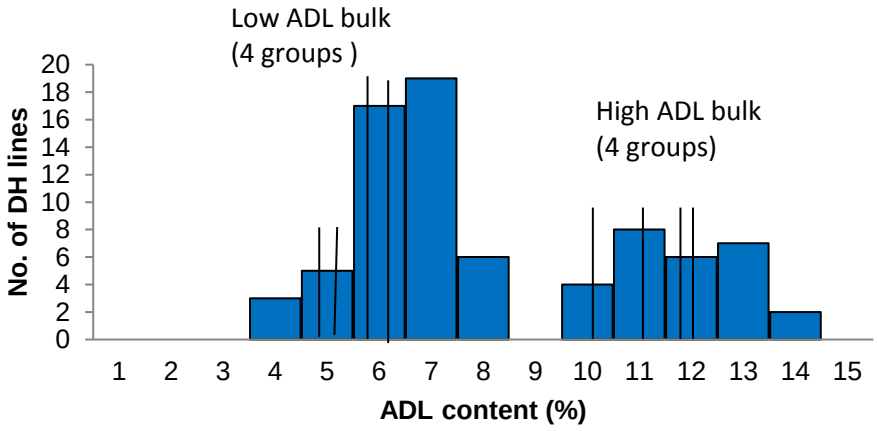


Fig. 3 Frequency distribution of ADL content of DH population of 4042 x Express 617 from 3 environments.

Note: the black lines representing the mean values of the sample bulk groups taken.

Twenty genotypes were selected from the middle of each group, which has the highest frequency. Further, the 20 genotypes were divided into 4 bulk groups, having 5 genotypes in each group. There were two groups, one with yellow seed color and one with black color, so in total we used 40

genotypes divided into 8 bulks as shown in Tab 5. Seeds of the 40 genotypes were sent to KWS SAAT SE for DNA extraction and SNP genotyping with the KWS SAAT SE proprietary Illumina 20K SNP chip.

Table 5 Eight selected bulks of DH population of 4042 x Express 617 representing the yellow and black genotypes

Bulk code	No	Genotype	ADL content (%)	Bulk code	No	Genotype	ADL content (%)
Yellow - I	1	DH 4042 x E - 12	5.78	Black -I	21	DH 4042 x E - 63	9.4
	2	DH 4042 x E - 35	5.8		22	DH 4042 x E - 40	9.91
	3	DH 4042 x E - 36	5.82		23	DH 4042 x E - 8	10.01
	4	DH 4042 x E - 72	5.82		24	DH 4042 x E - 47	10.1
	5	DH 4042 x E - 13	5.84		25	DH 4042 x E - 21	10.1
Yellow - II	6	DH 4042 x E - 15	5.85	Black - II	26	DH 4042 x E - 10	10.61
	7	DH 4042 x E - 19	5.86		27	DH 4042 x E - 7	10.65
	8	DH 4042 x E - 54	5.87		28	DH 4042 x E - 24	10.81
	9	DH 4042 x E - 37	5.88		29	DH 4042 x E – 51	11.00
	10	DH 4042 x E - 18	5.94		30	DH 4042 x E - 62	11.06
Yellow - III	11	DH 4042 x E - 68	6.03	Black - III	31	DH 4042 x E - 22	11.35
	12	DH 4042 x E - 59	6.09		32	DH 4042 x E - 64	11.48
	13	DH 4042 x E - 41	6.17		33	DH 4042 x E - 58	11.62
	14	DH 4042 x E – 15b	6.18		34	DH 4042 x E - 25	11.93
	15	DH 4042 x E - 38	6.27		35	DH 4042 x E - 52	11.96
Yellow - IV	16	DH 4042 x E - 27	6.27	Black - IV	36	DH 4042 x E - 39	12.11
	17	DH 4042 x E - 32	6.28		37	DH 4042 x E - 23	12.19
	18	DH 4042 x E - 2	6.3		38	DH 4042 x E - 28	12.2
	19	DH 4042 x E - 17	6.31		39	DH 4042 x E - 1	12.3
	20	DH 4042 x E - 66	6.31		40	DH 4042 x E - 3	12.41

3.2.8 Candidate genes identification

The bulk segregant SNP-marker analysis revealed a number of bulk-specific SNP-markers on linkage group C03. Using the SNP-marker sequence information (Clarke et al. 2016), the BRAD database of *Brassica oleracea* genome (brassicadb.org) indicated that there are 450 gene loci located within the interval marked by the SNP-markers on chromosome C03. In order to identify which genes that correspond to ADL function, each loci within the gene region was compared to NCBI database through BLAST (Basic Local Alignments Search Tool) function (https://blast.ncbi.nlm.nih.gov/) to identify its gene name and function. The Plant Ensemble genomic database (plants.ensemble.org) of three *Brassica species: B. napus, B. rapa,* and *B. oleracea* was also cross-checked for more gene function information, and in case for *B. rapa,* for investigating synteny possibilities. The most

probable candidate genes where then selected from the identified gene list, considering the gene name, position, length, and function.

Additionally, a molecular physical map was constructed for chromosome C03 (MAPCHART version 2.2; Voorrips, 2002) using the information from detected polymorphic markers. This physical map identified the assumed loci positions of the two candidate genes related to the seed ADL content within the targeted gene interval.

3.2.9 KASP genotyping

For confirming the SNP-marker results from the bulk segregant analysis, a number of KASP-markers based on the SNP sequence information (Table 6) were ordered from the LGC Group (www.lgcgroup.com/genomics). Seeds of the 77 genotypes were sown in the green house in the tray pots, one seed per genotype. Young leaf sample was taken from the germinated plants and used for DNA extraction (innuPREP DNA Mini Kit; https://www.analytik-jena.de/de).

DNA was successfully isolated from 73 genotypes of DH population of 4042 x Express 617, including both parents. The KASP genotyping was performed at University of Göttingen with Bio-rad CFX96 Touch™ Real Time PCR Detection System, C1000 Thermal Cycler (www.bio-rad.com). Three out of 6 KASP markers responded well and gave clear results (BN-SCAFF_18322_1-P1044275, BN-SCAFF_18322_1-P1238111, and BN-SCAFF_18322_1-P1655555).

The KASP genotyping assay protocol was as followed. First, DNA samples were arrayed into the 96-well reaction plate, including no-template controls (NTCs) on the same plate. The genotyping mix was prepared, with wet/dry DNA method. All reagents need to be vortexed before used. For each sample, 5 ul DNA was required, and added by 2 x KASP master mix 5 µl and KASP Assay mix 0.14 µl. After dispensing the genotyping mix into the wells, the reaction plate was sealed with clear seal to avoid evaporation. The centrifuge then arranged at 550 x.

The next step was running the thermal plate using standard thermal cycle (Table 7). After the thermal cycling was completed, and the temperature cooled down to under 40^{o}C, the plate reading was performed through software Bio-rad CFX Manager version 3.1. HEX allele was reported on Y axis, and FAM allele was reported on Y axis.

Table 6 List of primers and the sequences

Primers	Oligo sequences (5' to 3')	
BN-SCAFF_18322_1-P1044275	F	AAAATCGGGTGGAAGCAAATACCAGTCAACATATGAAGGCTACRGTTCCCCGGTAAGCTG[A/G]
	R	TATTCTTTTTCTGTGTTTGTATGTATTTATGTGGCGCTGACATGACCTGATGATTCCTTA
BN-SCAFF_18322_1-P1103558	F	CATATATTTGGTAGATTTCAAATGCTAGTCTAGCAGATTTATATTAATCTACGGATACTC[A/G]
	R	GCAGAGYGTTTACTCTACTTTYATAGCAGACTAGGAAACTGCACAACWTATGAAAGTCTA
BN-SCAFF_18322_1-P1238111	F	TTACAAGCGCTTTTAAATCAAGAAGCTGTAGTACCTCCACAATAAGGAGCTTCTTGTCCC[T/C]
	R	GTGGAAAAGCTSTGKCATGCTGTAMTGCCTACTGGTGGTTTTCTTTTTCCYTATTTCGTA
BN-SCAFF_18322_1-P1490938	F	GCAGTATTCTTTTATTGAAGAGAAWATTATAGTTAWAGTCTCATCCATGTCAATGTGACT[T/C]
	R	ATCAGGTATAATATCTATGAGAAGGATGATCAAGCATATAACAAAACTAGATAACCAGTM
BN-SCAFF_18322_1-P1655555	F	CCACATTTCCAGATGATGGACGAACAGGAACATGGGTACCATCTAAAGCACCTAGCTATGCAATTCTTAAAGTAAGGCCAATACTGCGAATTCTCTACTAGCATTTGTGAACTCATTCCTAGTCAGTTTCACAATGTCTGATGCAAGCTT[T/C]
	R	AAGACAGAACTTAAAACCTCTTCTAGCTTCCTACTCACAGTGTCAGTTGAATGCTGATAACGCTCTGCTATAACCCGTACTGTTGCGTCTTKTCCAGCTGTCTCTAGGAACATTGCAACACTCTCCTCAAGGTAAACATTAAGAGTCTCT
BN-SCAFF_18322_1-P1717349	F	GCAGCAATCAATAACTCTATATTATCTTGGAAAAGGAAAGCAACACGGATCAAATTCTGCAAGTGCTAATCACAACATTGCTCCAAAAGACTACTAAAATTCCCATTACCAGAAAAGGCATCATGCAACCAAAACTGCGTCTCAGATGGA[A/G]
	R	CAACAAAGACAAAACACTCAATTCAATTAAAATAGAACGTTGATTCATACACAAACACAACAGAAAACAGATAGATCTCGACAAACTGAAACCAAAACTATGTCTCAGAAGCAACATCAAAGACAAGAATTAAAGGCTATCTTACCAGTT

There were several genotypes giving blank results on the first reading of the plates. To improve the clarity of the reading, the plates were given additional 3 cycles and then read again. The first step was denaturation (94 ^{o}C) for 20 second, followed by annealing/elongation step (57 ^{o}C) for 60 second.

The additional thermal cycles were repeated 3 – 4 times, until the readings give clear results for almost all the genotypes.

Table 7 Thermal cycling condition for the KASP chemistry

Step	Description	Temperature	Time	No. of cycles/step
1	Activation	94°C	15 minutes	1 cycle
2	Denaturation	94°C	20 sec	10 cycles
	Annealing/elongation	61-55°C	60 sec (drop 0.6°C/cycle)	
3	Denaturation	94°C	20 sec	26 cycles
	Annealing/elongation	55°C	20 sec	

3.3 RESULTS

3.3.1 Variation among traits

Significant genotypic differences were found for all seed traits, except for the percentage of infected seeds in the DH population of 4042 x Express 617 consisting of 77 genotypes (Table 8). The effect of the environment was highly significant for all traits. The size of the variance component for the effect of environment for most traits was much larger than the effect of the genotype. Heritability values ranged from low for glucosinolates content to high for the fiber component traits.

Both genotype and environment factors were significant for the seed color, TSW (thousand seed weight), and pre-harvest germination percentage. Seed color heritability trait was the highest among observed traits (0.95), while heritabilities for TSW and pre-harvest germination percentage were 0.72 and 0.61, respectively.

The variance components for seed germination traits were very diverse. The radicle protrusion percentage has significant effects only by environment. The percentage of full germination and no germination indicated that environment factor was insignificant to these two traits. Hypocotyl length displayed significant effects for both factors. The percentage of infected seeds revealed significant effect only for environment factor. Heavy seed infection in one field experiment (Reinshof 2016) was noted. The infection level in average was nearly 40% (data not shown). Meanwhile, the mean values of the infected seed percentage in the other four environments were all under 10%, two were even under 1%. During the harvesting time at Reinshof 2016, many of the oilseed rape pods were heavily

infected by fungi in the field. Heritability ranged from 0.33 for radicle protrusion percentage to the 0.71 for full germination percentage.

Table 8 Variance components for seed traits of DH population of 4042 x Express 617 tested in five environments

Source of variance	Genotype (G)	Environment (E)	G x E	Heritability (h^2)
Oil content (%)	1.20**	4.85**	3.34	0.64
Protein content (%)	0.37**	6.81**	1.46	0.56
Oil+Protein (%)	0.48**	1.17**	1.22	0.66
Glucosinolates (umol/g seed)	14.44**	59.92**	21.01	0.26
Protein defatted meal (%)	0.44*	14.31**	2.13	0.51
NDF (%)	3.18**	10.58**	4.64	0.81
ADF (%)	2.88**	5.59**	2.98	0.85
ADL (%)	2.05**	3.81**	3.64	0.86
Seed color	2.12**	0.02**	0.62	0.95
Pre-harvest germination (%)	0.41**	0.20**	1.28	0.61
Thousand seed weight (g)	0.17**	0.89**	0.32	0.72
Radical protrusion (%)	0.34*	0.23**	3.42	0.33
Full germination (%)	4.84**	0.13	9.78	0.71
Hypocotyl length (cm)	0.92**	0.11**	0.42	0.52
Infected seeds (%)	6.12	12.12**	24.36	0.50
Radicle protrusion (%) (AA^1)	6.52*	44.34**	71.81	0.31
Full germination (%)(AA^1)	116.15**	149.92**	266.23	0.69
Hypocotyl length (cm) (AA^1)	0.06**	0.28**	0.29	0.53
Infected seeds (%)(AA^1)	35.18**	7.96**	97.75	0.64

[1] following Artificial Aging

** marked as significant at level P=0.01

After the aging treatment, the seed germination test was repeated. Analysis of variance showed significant effects for both genotype and environment. Beside hypocotyl length, all other traits showed high G x E values. Percentage of radicle protrusion has the lowest heritability of 0.31, while full germination percentage has the medium values (0.69). Hypocotyl length and infected seeds percentage scored 0.53 and 0.64 for heritability.

Line 4042, yellow in seed color, contained less oil, protein, total oil and protein than Express 617, also less fiber (NDF, ADF, ADL) contents (Table 9). Both parents' seed size was almost similar (TSW 4.53 g in 4042 compared to 5.03 g in Express 617). For seed germination traits, both before or after

artificially aged treatment, Express 617 seeds were superior to 4042 in all aspects. Full germination rate of Express 617 was 100 % in freshly harvested seeds and 68.19 % in aged seeds, whereas in line 4042 the values were only 97.20 % and 40.05 %, consecutively. The hypocotyl length was better in Express 617 for both treatments. The level of seed infection and radicle protrusion of Express 617 was constantly lower than line 4042.

Table 9 Descriptive statistics of the DH population of 4042 x Express 617 (mean values over five environments)

Traits	4042 (P1)	Express 617 (P2)	Min	Max	Means	SD	LSD 5%
Oil content (%)	40.7	44.8	39.4	46.9	44.3	1.37	2.28
Protein content (%)	18.9	16.9	18.9	20.6	17.1	0.81	1.50
Oil & protein content (%)	59.7	61.6	59.1	63.8	61.5	0.85	1.38
Glucosinolates (umol/g seed)	43.0	22.9	22.9	64.9	48.3	0.93	18.04
NDF (%)	22.6	33.0	22.6	33.4	30.4	1.99	2.42
ADF (%)	16.2	24.1	16.3	24.8	21.1	1.89	2.06
ADL (%)	4.1	10.9	4.1	13.3	8.5	2.16	2.28
Protein defatted meal (%)	31.8	30.4	29.2	34.0	30.6	0.93	1.82
Seed color (score 1-9)	3.6	8.8	3.2	9.0	6.6	1.50	0.98
Pre-harvest germination (%)	0.6	0.2	0.0	4.7	0.8	0.82	1.41
Thousand seed weight (g)	4.5	5.1	3.7	6.5	4.9	0.48	0.71
Radical protrusion (%)	2.4	0.0	0.0	14.0	0.9	1.01	2.30
Full germination (%)	97.2	100.0	81.5	100.0	98.4	2.61	3.89
Hypocotyl length (cm)	4.2	5.6	4.0	5.8	4.9	0.42	0.81
Infected seeds (%)	24.2	5.2	0.0	34.6	3.2	7.91	20.15
Radicle protrusion (%) (AA[1])	19.0	16.6	4.1	29.2	13.6	4.57	10.55
Full germination (%)(AA[1])	40.0	68.2	20.8	86.2	62.8	13.02	20.31
Hypocotyl length (cm) (AA[1])	1.3	2.6	0.9	2.7	2.1	0.35	0.67
Infected seeds (%)(AA[1])	38.7	5.8	2.1	39.7	11.0	7.40	12.31

[1] following Artificial Aging

Based on 91% seed dry matter, the average seed oil content was 44.3 %, the protein content was 17.1 %, total oil & protein was 61.5 %, and glucosinolates 48.3 umol/g seeds. For seed fiber, the range of NDF value was 22.6 – 33.4 %, ADF was 16.3 – 24.1 %, and ADL was 4.1 – 13.3 %. The seed color score for DH population of 4042 x Express 617 ranged between 2 to 9, with 6.6 as average. Pre-harvest germination was between zero to 9 %, and average value was under 1 %. TSW (Thousand Seed Weight) was quite diverse, ranging between 2.6 – 7.9 g, and the mean value was 4.9 g.

The radicle protrusion percentage for DH population of 4042 x Express 617 seeds was low, ranged between zero to 14 %, and in average was less than 1 %. After germination test without aging treatment, in average 98.40 % were fully germinated. Seed infection across five environments was very diverse, from zero to 100 % infection, but average was only 3.23 %. The hypocotyl length was ranged between 3 – 8 cm, indicating good seed vigor, and the average value was 4.94 cm. After artificial aging treatment, the value ranges were changed. Full germination percentage were becoming very diverse, ranged from zero to 98.04 %, with mean value 62.83 %. Radicle protrusion was increased, between 0 to 51.08 % (means 13.61 %). Hypocotyl length was also shortened, ranging from 0.50 – 4.50 cm, with average 2.07 cm.

3.3.2 Frequency distributions

The frequency distribution for seed fiber component (NDF, ADF, and ADL) contents and seed color, consisted of 77 genotypes of DH population of 4042 x Express 617 over 5 environments, were developed (Fig. 4). The first graph, NDF content, showed more or less a normal distribution with few outliers (including parent 4042) separated from the rest of the population. The ADF content graph started to shift from normal distribution to bimodal distribution, and exhibited two peaks at 21 and 24 %. The graph of ADL content displayed clearer bimodal distribution, segregated into two groups at 10 % value. Previously ADL frequency distribution graph has been generated (Fig. 3) from 3 earlier environments (Reinshof 2014, 2015, and Einbeck 2015). A scatterplot test (Fig. 5) confirmed that the mean values of ADL content from 3 (Fig. 3) and 5 environments (Fig. 4) has high similarity with R^2 = 0.93. The seed color frequency distribution also had two peaks (at 6 and 9), its shape was somewhere between normal and bimodal distribution type.

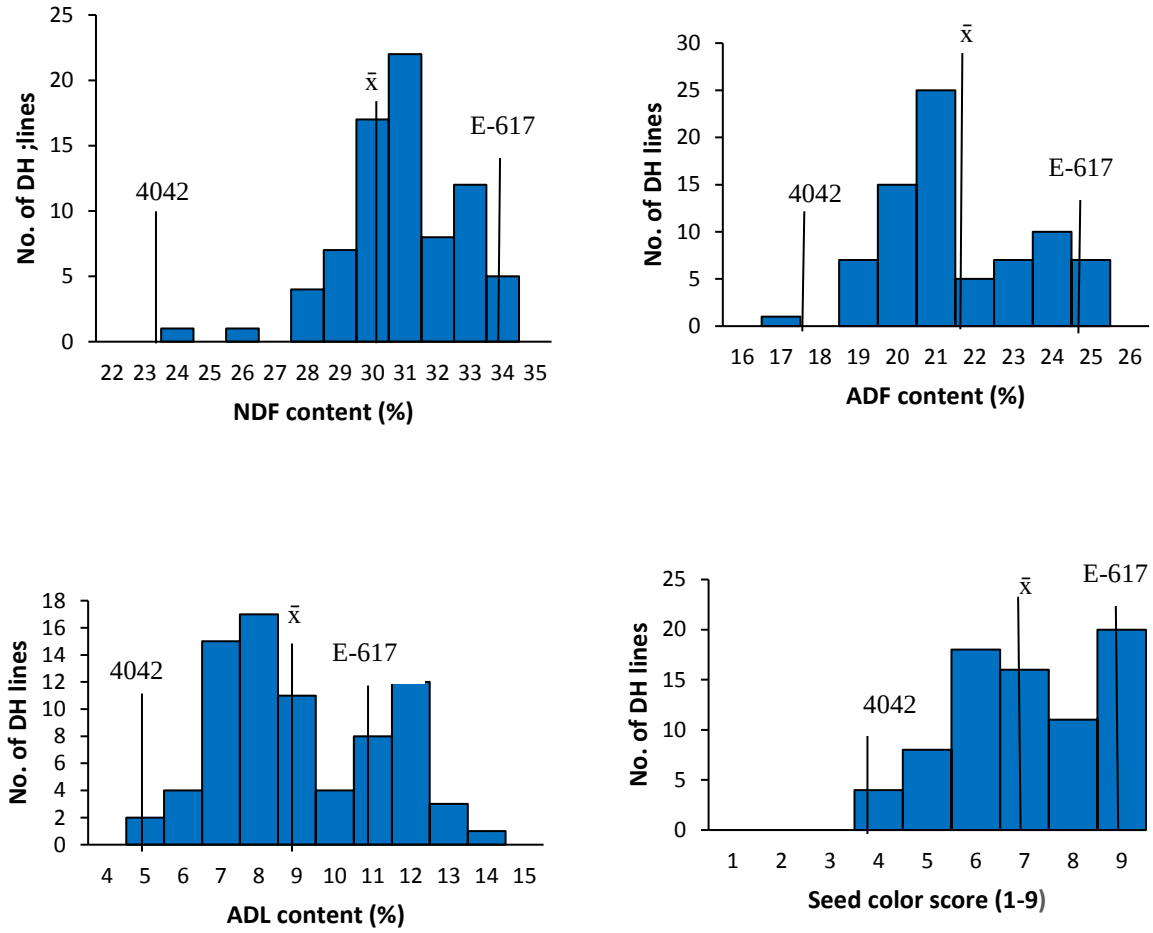


Fig. 4 Frequency distribution of seed fiber contents (NDF, ADF, and ADL) and seed coat color of DH population of 4042 x Express 617 over 5 environments

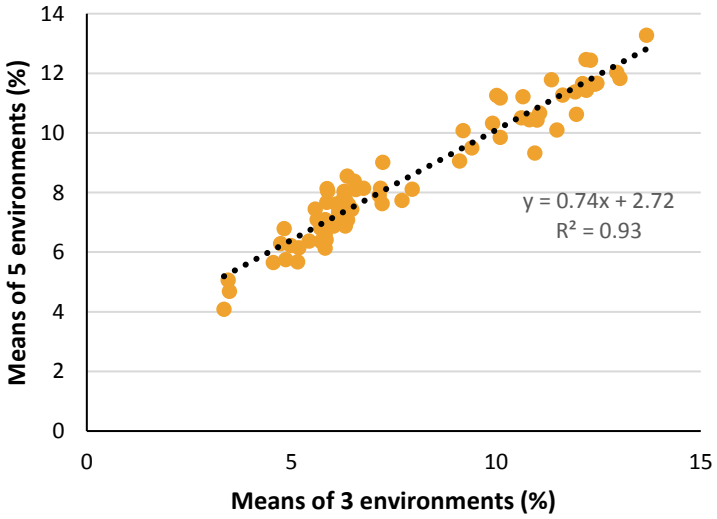
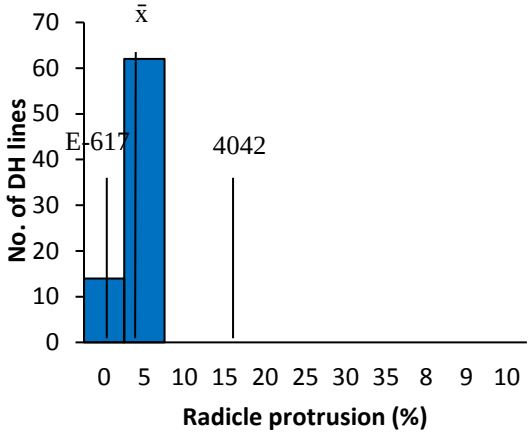
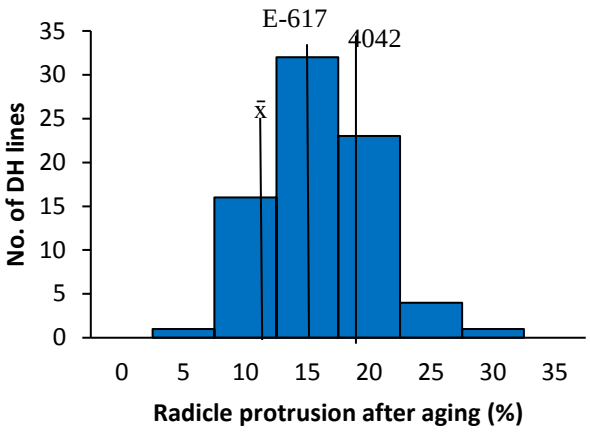
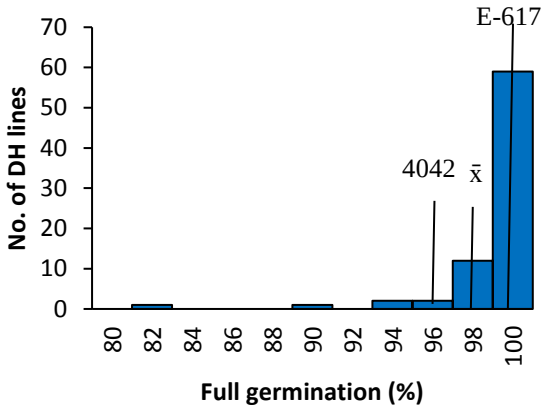
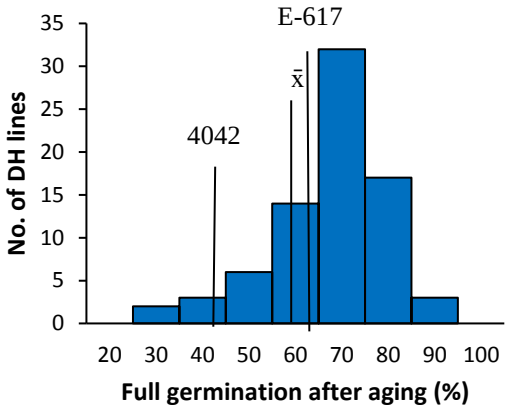


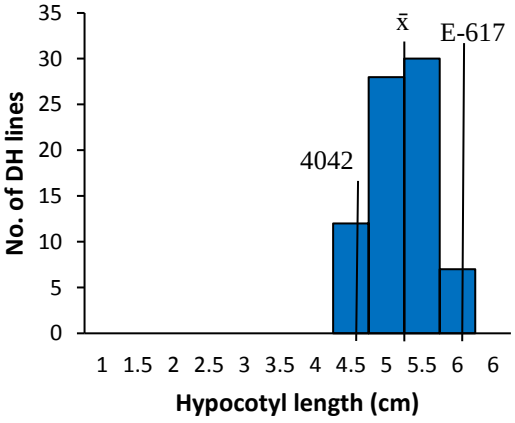
Fig. 5 Scatterplot graph of ADL mean values of DH population of 4042 x Express 617 between 3 and 5 environments

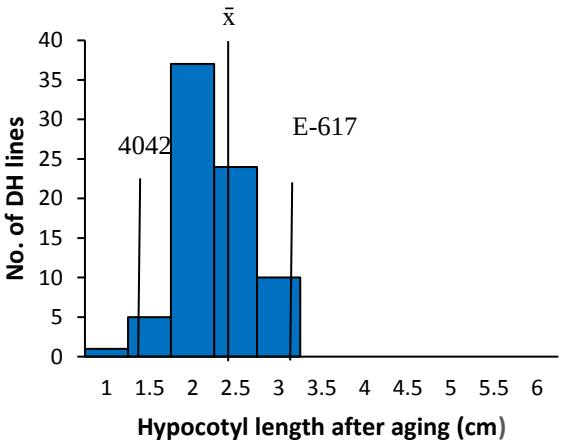

No. of DH lines
70
60
50
40
30
20
10
0
x̄
E-617
4042
0 5 10 15 20 25 30 35 8 9 10
Radicle protrusion (%)


No. of DH lines
35
30
25
20
15
10
5
0
E-617
4042
x̄
0 5 10 15 20 25 30 35
Radicle protrusion after aging (%)


No. of DH lines
70
60
50
40
30
20
10
0
E-617
4042
x̄
80 82 84 86 88 90 92 94 96 98 100
Full germination (%)


No. of DH lines
35
30
25
20
15
10
5
0
E-617
x̄
4042
20 30 40 50 60 70 80 90 100
Full germination after aging (%)


No. of DH lines
35
30
25
20
15
10
5
0
x̄
E-617
4042
1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6 6
Hypocotyl length (cm)


No. of DH lines
40
35
30
25
20
15
10
5
0
x̄
4042
E-617
1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6
Hypocotyl length after aging (cm)

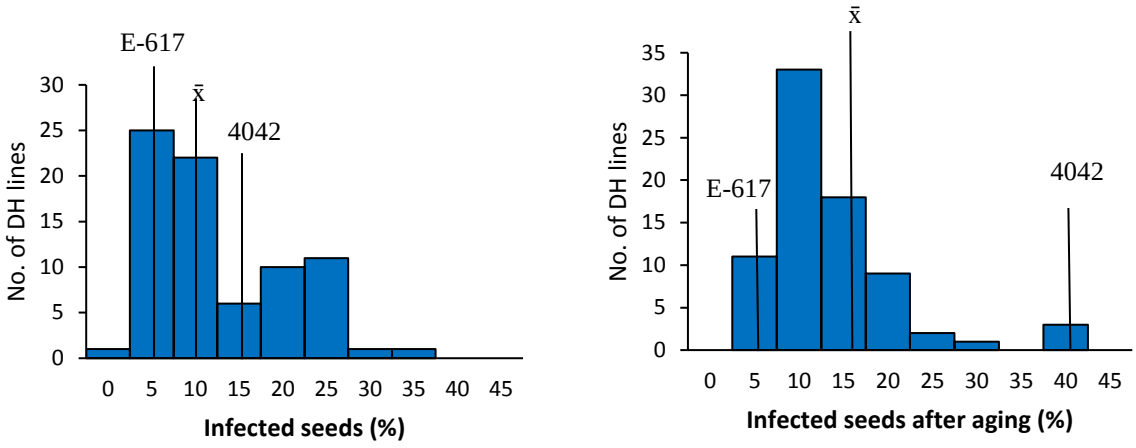


Fig. 6 Frequency distribution of seed germination and seed longevity traits of DH population of 4042 x Express 617 from five environments

The segregations of seed germination traits in comparison to both parents were more clearly displayed by frequency distribution graphs on Fig. 6. In full germination percentage and hypocotyl length traits, Express 617 was performed better than the average value and line 4042. In term of radicle protrusion, Express 617 has zero value, while the other parent (line 4042) displayed higher percentage of seeds with radicle protrusion than average. For seed infection, however, both parents scored higher compared to the mean value, although the seed infection level from the black-seeded parent of Express 617 (5.20 %) was much lower than from the yellow-seeded line of 4042 (24.20 %).

The segregation of seed longevity traits were again better displayed through frequency distribution graphs (Fig. 6), in comparison to the values of both parent materials. As previously in the seed germination traits, the full germination percentage and hypocotyl length of Express 617 was higher than both the average value and line 4042. For radicle protrusion, both parents exhibited higher percentage of seeds with radicle protrusion compared to the mean value. The black-seeded Express 617 was having slightly lower radicle protrusion (16.55%) than yellow-seeded line 4042 (19.05 %). For seed infection, while the mean value was 11.00 %, the difference between two parents was quite far. The black-seeded Express 617 infection level was low at 5.83 %, but the yellow seeds of line 4042 were more susceptible to seed infection with 38.75 %.

3.3.3 Spearman's rank of correlation coefficients

The estimation of Spearman's rank of correlation coefficients among traits were executed using mean values over five field environments. The coefficient values are shown in Table 10. Close and

significant correlations were found between seed oil, protein, total oil and protein, and protein of defatted meal. Seed oil is negatively correlated to protein, but no correlation to protein of defatted meal. Positive correlations were found between seed oil and total oil and protein, also between seed protein and protein of defatted meal. The association between seed protein and total seed and protein content is negative. Glucosinolates content has no correlations to any traits. Strong positive correlations were found among the three fiber content components: NDF, ADF, and ADL with values over 0.90. In this study, seed fiber components have strong positive association to seed color (0.84 – 0.87), but not to oil or protein content.

There were no correlations observed between TSW (Thousand Seed Weight) and pre-harvest germination percentage. Pre-harvest germination percentage was negatively correlated to seed oil and protein (-0.30 and -0.31, respectively), also to NDF value (-0.33). Seed size or TSW (Thousand Seed Weight) has no correlation to any traits except to radicle protrusion percentage before aging.

Light seed color was associated with lower full germination percentage (0.31), and higher probability of radicle protrusion (-0.41) and seed infection (-0.44). All three seed fiber components (NDF, ADF, ADL) have significant contributions to the increase of radicle protrusion percentage (-0.37 to 0.43), but only ADL was correlated to the increase of seed infection (-0.30). Obviously, high percentage of full germination would have strong negative correlation to both radicle protrusion (-0.78) and infected seed percentage (-0.31), but interestingly not to hypocotyl length. Radicle protrusion also correlated to infected seed percentage (0.37). Hypocotyl length has no significant correlations with any seed germination traits.

The seed longevity traits were measured after artificial aging. Seeds with higher total oil and protein content has better seed germination rate after aging. High seed fiber components and dark seed color were found to be positively prolonged the seed longevity. Similar associations were also observed for total oil and protein content in relation to seed longevity traits. Full germination percentage and hypocotyl length would be improved by the increase of seed fiber (NDF, ADF, ADL), dark seed color, and higher total oil and protein content, while chance of getting radicle protrusion or seed infection would become lower. TSW and pre-harvest germination have no correlations to any seed longevity traits.

Table 10 Spearman's rank of correlation among traits of DH population of 4042 x Express 617 over five environments

	XL	XP	XLP	PDM	GSL	NDF	ADF	ADL	SC	PHG	TSW	HL	RPP	FGP	ISP	RPPA	FGPA	HLA
XP	**-0.71****																	
XLP	**0.86****	**-0.31****																
PDM	-0.28*	**0.84****	0.17															
GSL	-0.21	0.17	-0.13	0.16														
NDF	0.27*	-0.23*	0.19	-0.15	-0.27*													
ADF	0.08	-0.08	0.04	-0.07	-0.27*	**0.93****												
ADL	0.06	0.07	0.08	0.06	-0.18	**0.92****	**0.97****											
SC	0.05	0.04	0.11	0.08	-0.14	**0.84****	**0.84****	**0.87****										
PHG	**-0.30****	**0.31****	-0.19	0.25*	0.01	**-0.33****	-0.25*	-0.23*	-0.28*									
TSW	-0.02	0.13	0.08	0.17	0.08	-0.12	-0.12	-0.13	0.04	0.21								
HL	-0.14	0.16	-0.09	0.06	-0.08	-0.02	0.04	0.02	0.05	0.10	0.18							
RPP	-0.06	-0.01	-0.13	-0.01	0.02	**-0.43****	**-0.37****	**-0.43****	**-0.41****	**0.31****	0.12	-0.23*						
FGP	0.03	0.04	0.11	0.05	0.08	0.24*	0.20	0.26*	**0.31****	-0.28*	-0.06	0.22	**-0.78****					
ISP	0.07	-0.21	-0.09	-0.29*	-0.08	-0.26*	-0.24*	**-0.30****	**-0.44****	0.13	0.03	-0.14	**0.38****	**-0.37****				
RPPA	0.23*	0.10	**0.41****	0.29*	-0.11	**-0.26***	**-0.22***	**-0.30****	**-0.31****	0.02	0.08	0.12	0.11	0.04	0.20			
FGPA	0.22	0.10	**0.42****	0.29*	-0.07	**0.43****	**0.40****	**0.47****	**0.49****	-0.03	0.08	0.14	-0.38**	0.20	-0.31**	**-0.54****		
HLA	0.18	0.06	**0.32****	0.20	-0.16	**0.48****	**0.46****	**0.52****	**0.51****	-0.10	0.04	0.02	-0.39**	0.25*	-0.36**	**-0.53****	**0.78****	
ISPA	-0.21	-0.11	**-0.37****	-0.25*	0.13	**-0.52****	**-0.46****	**-0.53****	**-0.54****	0.03	-0.07	-0.11	0.46**	-0.25*	0.39**	**0.30****	**-0.78****	**-0.62****

** marked as significant at level P=0.01

Abbreviation notes

XL : oil content
XP : protein content
XLP : total oil and protein content
PDM : protein of defatted meal content
GSL : glucosinolates content
NDF : neutral detergent fiber
ADF : acid detergent fiber
ADL : acid detergent lignin
SC : seed color
PHG : pre-harvest germination
TSW : thousand seed weight
RPP : radicle protrusion percentage
FGP : full germination percentage
HL : hypocotyl length
ISP : infected seed percentage
RPPA : radicle protrusion percentage after aging
FGPA : full germination percentage after aging
HLA : hypocotyl length after aging
ISPA : infected seed percentage after aging

Seed longevity traits were not correlated directly to full germination rate before aging. Instead, significant relations were found between full germination (-), hypocotyl length (-), and seed infection (+) after aging to both radicle protrusion and seed infection percentage before aging. Four observed seed longevity traits were each strongly correlated with each other. Full germination has positive association to hypocotyl length, and negative association to radicle protrusion and seed infection percentage.

3.3.4 T-test two mean values comparison

There were the two distinct groups formed by the different level of seed ADL contents based on mean value of 3 environments, separated by the missing 9 % seed ADL content (see Fig. 4). The member of the two groups was more or less the same in the frequency distribution histogram of mean value of 5 environments (Fig. 5), except for one genotype. The first group with low seed ADL content (below 10 %) consisted of 49 genotypes, while the second group with high seed ADL content (10 % and higher) has 28 genotypes. In order to confirm whether these two groups were differing also in other characters, a T-test comparison was executed (Table 11).

Between the two groups, there were no differences found in term of seed quality traits except in seed fiber components. Also, no differences were found for seed germination traits, except for infected seed percentage. The significant differences were revealed for seed fiber components (NDF, ADF, ADL), seed color, infected seed percentage, and all seed longevity traits (radicle protrusion, full germination, infected seed percentages, and hypocotyl length).

Table 11 T-test comparison between 2 groups of yellow seeded (low ADL content) and black seeded genotypes (high ADL content) (means over five environments are presented)

Traits	Low ADL Means	High ADL Means	T-test
Oil	48.9	48.2	ns
Protein	18.1	18.5	ns
Oil+Protein	67.1	66.7	ns
Glucosinolates	53.1	51.2	ns
NDF	30.8	34.7	10.0**
ADF	18.3	22.6	16.0**
ADL	5.9	11.3	21.3**
Protein of defatted meal	29.5	29.8	ns
Seed color	5.8	8.4	15.1**
Pre-harvest germination (%)	1.1	0.8	ns
Thousand seed weight (g)	5.1	5.0	ns
Radicle protrusion (%)	0.8	0.5	ns
Full germination (%)	98.6	98.2	ns
Hypocotyl length (cm)	5.0	5.1	ns
Infected seeds (%)	3.6	2.0	2.32**
Radicle protrusion (AA[1]) (%)	10.6	6.9	3.4**
Full germination (AA[1]) (%)	66.8	78.6	3.9**
Hypocotyl length (AA[1]) (cm)	2.3	2.7	4.4**
Infected seeds (AA[1]) (%)	13.4	8.0	3.2**

[1] following Artificial Aging (AA)

** marked as significant at level P=0.01

3.3.5 Seed metabolite fingerprinting

The samples used for the analysis were bulked into different groups from samples used for the KASP genotyping, since only 15 samples were chosen each from low and high ADL group. The exact compounds detected from non-targeted metabolite fingerprinting and mass spectroscopy listed in Fig. 7 & 8 are still putative at best. Purification and quantification of each compound would be needed to find out the further details. There were noticeable differences of metabolite levels

revealed between the high ADL vs low ADL content bulk groups. The seeds with high ADL content were mostly dark (score 7-9), and the seeds with low ADL content were lighter in color (score 3-5) (data not shown). The differences in compounds' quantity started from phenylpropanoid biosynthesis pathway, continued to proanthocyanidins pathway, and also influenced flavonols/flavones, amino acids, and some other compounds. The two metabolites in phenylpropanoid pathway, sinapoyl glucose and caffeoyl shikimate, was found to be abundant in black seed samples, but was nearly zero in yellow seed samples.

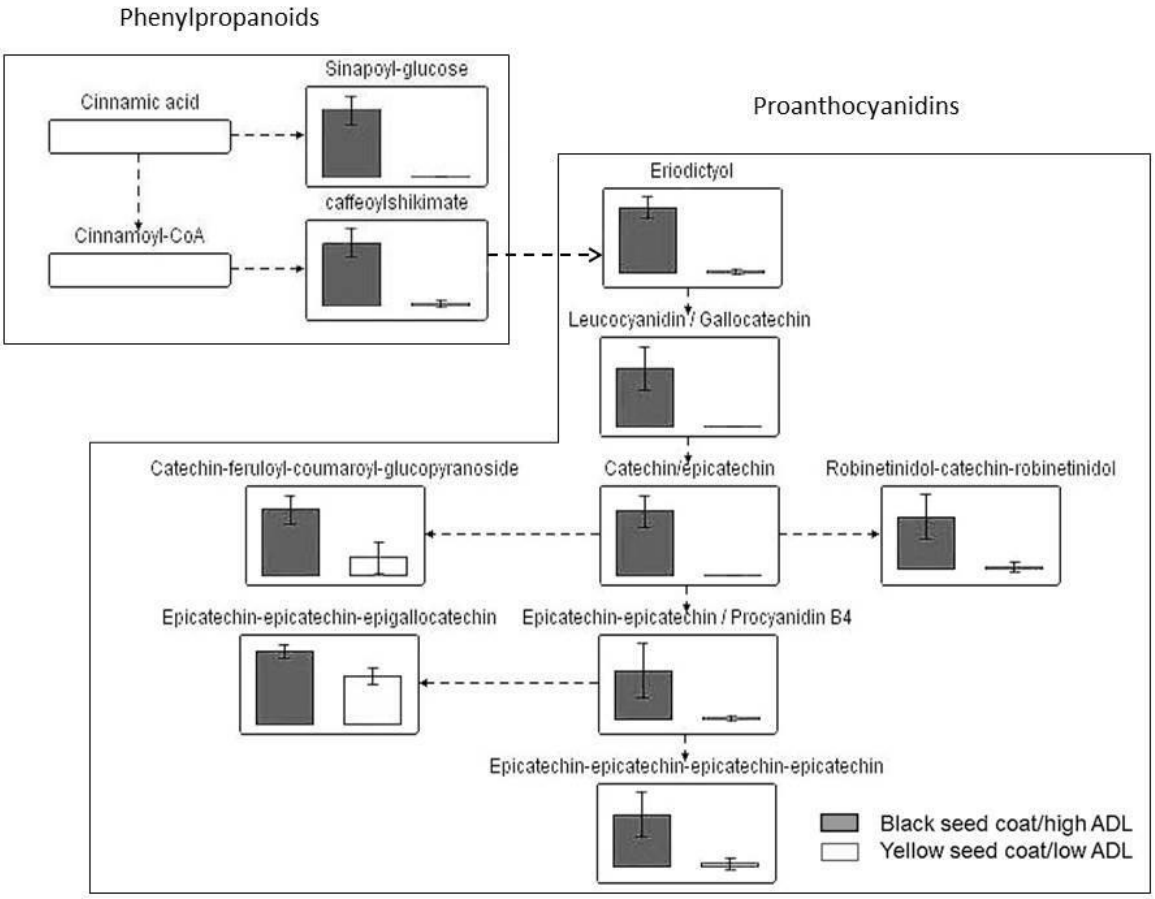


Fig. 7 Comparison of compound contents related to phenylpropanoids and proanthocyanidins pathway between yellow vs black-seeded pools of 4042 x Express 617

Note: Each data point represents the mean value of the compound quantity within seed samples (nmol/g fresh seed weight) ± SD of 3 replicates. The biosynthesis pathways were adapted from Debeaujon et al. (2000) and Debeaujon et al. (2003).

Among the different compounds of the proanthocyanidins pathway, most of them gave nearly similar results, in which black seeded samples contain higher amount of some compounds compared to the yellow seeded ones. The yellow-seeded samples produced some results for catechin-feruloyl-coumaroyl-glucopyranoside and epicathecin-epicathecin-epigallocathecin compounds, but the levels were still below the black-seeded ones.

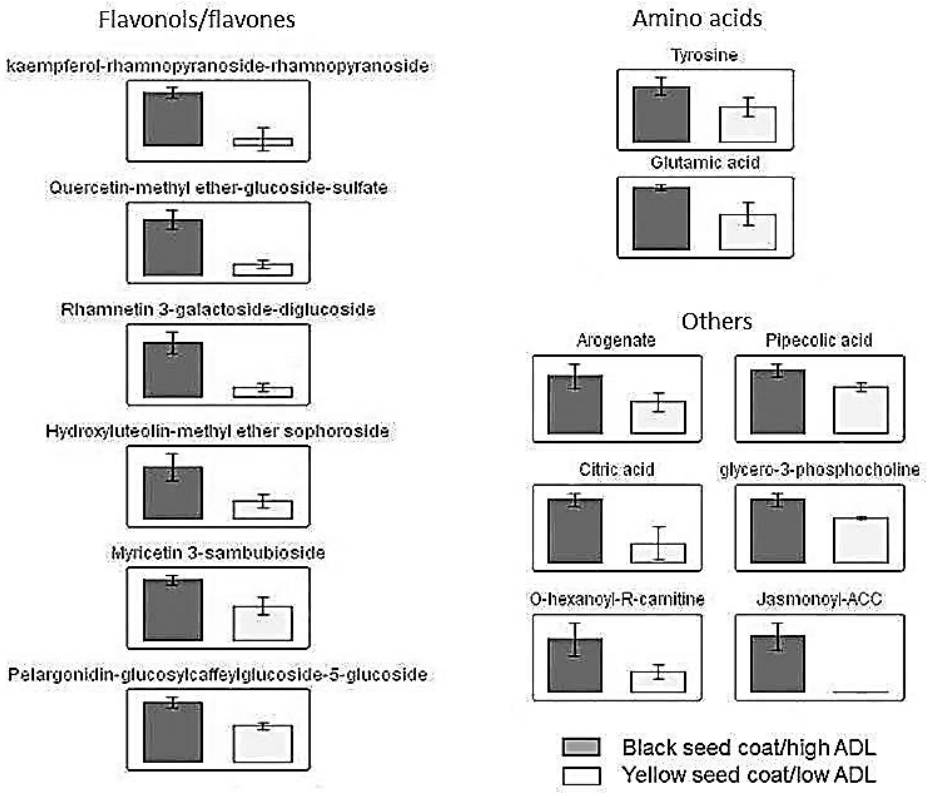


Fig. 8 The comparison of compound contents related to flavonoids, amino acids and other compounds between yellow vs black seeded pools of DH population of 4042 x Express 617 .

Note: Each data point represents the mean value of the compound quantity within seed samples (nmol/g fresh seed weight) ± SD of 3 replicates.

Some flavones or flavonols compounds were also detected (Fig. 8). Pelargonidin-glucosylcaffeylglucoside-5-glucoside and myricetin-3-sambubioside were found abundantly for both bulk samples, but their levels in the black seeded samples were about twice as much as the yellow seeded ones. Other flavones/flavonols such as kaempferol-rhamnopyranoside-rhamnopyranoside, quercetin-methyl ether-glucoside-sulfate, and rhamnetin-3-galactoside-diglucoside were only being

produced in high levels in black-seeded samples. The seeds with yellow color have very low level for these metabolites. The amount of two amino acids, tyrosine and glutamic acid, were detected in slightly lower level in yellow seeds compared to the black ones. The traces of other compounds were also detected in different amounts. Arogenate, pipecolic acid, citric acid, glycerol-3-phosphocholine, and O-hexanoyl-R-carnitine were identified in both seed sample groups, but the yellow seeds gave consistently lower results. Jasmonoyl-ACC was only spotted in black seeded group.

3.3.6 Bulk Segregant Analysis

In total, there were 40 genotypes of DH population of 4042 x Express 617 utilized for Bulk Segregant Analysis (BSA). Twenty genotypes represented the low ADL content group, and another 20 genotypes represented the high ADL content group. For seed ADL content, two peaks were observed in the frequency distribution over the first three environments (Fig. 3). This method compares two contrasting bulks on a particular trait, using two bulk groups of contrasting values. From each groups of low vs high ADL contents, four bulk groups each consisted of 5 genotypes were chosen.

Table 12 List of markers giving polymorphic results in DH population of 4042 x Express 617

Marker name (Clarke et al., 2016)	Chromo-some	Physical position (bp)
N-SCAFF_18322_1-P1655555	C3	7298559
BN-SCAFF_18322_1-P1717349	C3	7405366
BN-SCAFF_18322_1-P1612916	C3	7476656
BN-SCAFF_18322_1-P1490938	C3	7591715
BN-SCAFF_18322_1-P1412794	C3	7664631
KWS-Marker	C3	7698435
BN-SCAFF_18322_1-P1238111	C3	7800453
BN-SCAFF_18322_1-P1229371	C3	7808488
BN-SCAFF_18322_1-P1103558	C3	7959152
BN-SCAFF_18322_1-P1044275	C3	8030301
KWS-Marker	C3	8053064

From 20,000 SNP markers of 20K chip by KWS, eleven were giving polymorphic bands between the two pooled groups of low ADL and high ADL content. All of the eleven polymorphic markers were detected in chromosome C03, with physical map position between 7,298,559 and 8,053,064 bp (Table 12).

3.3.7 Identification of candidate genes

There were eleven polymorphic markers located in the chromosome C03. Altogether, those markers covered an interval of ± 760,000 base pairs. Through comparison with the Brassica database (brassicadb.org/brad), at least 450 identified genes on the C genome were detected within the mentioned interval. The sequences of these genes were compared against NCBI database through BLAST function. Later, the annotations were checked for gene function, size, and position. Similar inquiries were also performed through Ensembl Plants website (plants.ensembl.org) against three *Brassica* species genome databases: *B. napus, B. rapa*, and *B. oleracea*. From 450 genes, a large portion encoded unknown proteins. Only 70 genes were recorded to have annotations in *Brassica* species.

BoI028063 was identified as MATE (multi-antimicrobial extrusion protein) transporter, also known as *TT12* gene, which involved in seed coat pigmentation. *BoI028063 was* a short gene. Its full length was 735 bps, located in chromosome C03, and its locus position was started from 7,828,189 until 7,828,924 bps (Table 13). The second candidate gene was discovered a little beyond the flanking interval. *BoI004610* was also located on chromosome C03, and its locus position was started from 8,139,451 to 8,141,336 bps, in total 1,885 bps in size. This gene encodes for *C4H* (cinnamate 4-hydroxylase) protein, which was one of the vital precursors to lignin biosynthesis.

Table 13 Proposed candidate genes responsible for seed ADL content

	Chromosome	Start	Stop	Function
BoI028063	C03	7828189	7828924	MATE transporter (TT12)
BoI004610	C03	8139451	8141336	cinnamate 4-hydroxylase (C4H)

MATE: Multi-antimicrobial extrusion protein.

C4H: cinnamate 4-hydroxylase, one of the precursors to lignin biosynthesis.

A molecular physical map of chromosome C03 was generated by MAPCHART version 2.2, focusing on specific gene region of the targeted flanking interval (Fig 9). The exact positions of the eleven polymorphic markers and the two candidate genes were displayed in this map in order to provide better perspective of their positions. Further SNP analysis with more markers downstream of the range in the future would provide verification of the exact position of the C4H locus.

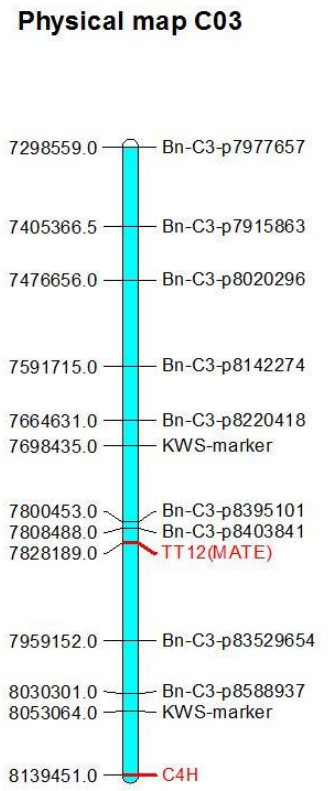


Fig. 9 The molecular physical map of chromosome C03, based on identified polymorphic marker positions on Table 12.

3.3.8 KASP genotyping

The KASP genotyping was carried out for 73 genotypes of DH population of 4042 x Express 617, including the two parental lines. Four of the original 77 genotypes failed to produce good quality DNA during DNA extraction in KWS. PCR was performed with 3 KASP primers, representing the beginning (BN-SCAFF_18322_1-P1655555), the middle (BN-SCAFF_18322_1-P1238111), and the end (BN-SCAFF_18322_1-P1044275) of the marker interval mentioned in Table 12 and Fig. 9. The outcome of KASP genotyping (Fig. 10) confirmed the result of Bulk Segregant Analysis (BSA). The genotypes of low ADL proved to have different alleles compared to genotypes of high ADL content. Aside from few blank readings, the allelic distributions from three different KASP markers were giving identical results. At the point of 10 % seed ADL content value, the allelic distributions clearly segregate and changed from allele 1 to allele 2 type. The line 4042 as parent exhibited the smallest ADL content from the whole population. Express 617, while possessing allele 2, contained the second lowest ADL content from the group of genotypes with allele 2 type.

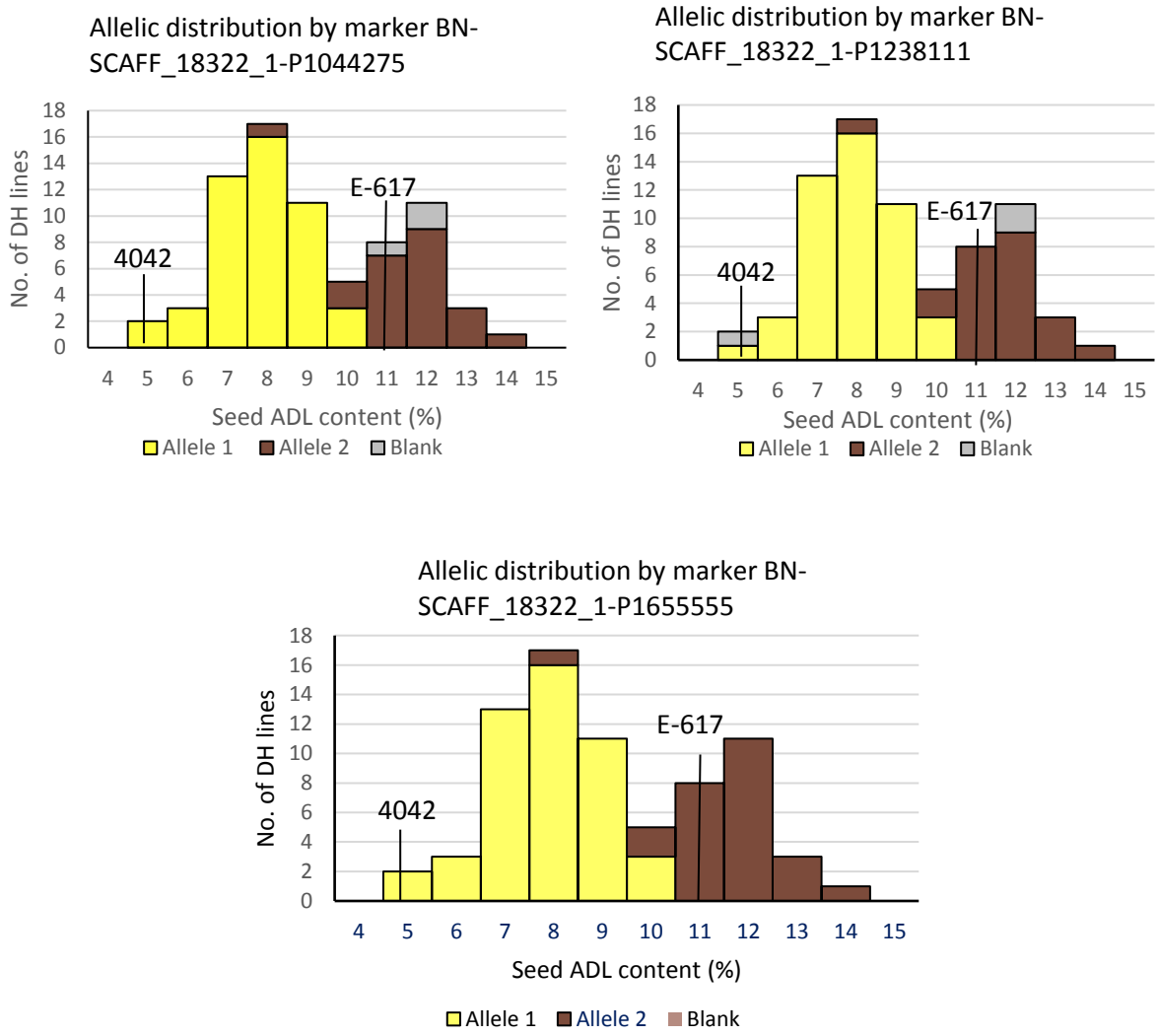


Fig. 10 The allelic distributions generated by three KASP markers of DH population of 4042 x Express 617 based on seed ADL content mean value frequency distribution over 5 environments

In general, seeds from genotypes of allele 1 type were lighter in seed color appearance, while genotypes of allele 2 type were darker. The first group, however, had wider range of seed color (mean value of 3.2 to 7.6) compared to the second group (7.8 to 8.8) (data not shown).

One genotype (#11) was suspected to be an outlier. Even after the KASP genotyping for its DNA sample was repeated, this genotype was consistently detected having allele 2 type by all three different KASP markers. However, it has relatively low ADL content (mean value 7.73) so that this genotype was isolated from the rest of allele 2 genotype group in the frequency distribution in Fig 10. The seeds of this genotype exhibited dark color with score 7.8.

3.4 DISCUSSION

3.4.1 Variation among traits

In the present study, a significant and large variation was found for nineteen characters among 77 DH lines originated from a cross between yellow seeded and black seeded winter oilseed rape cultivars evaluated in field experiments at five environments in Germany. The environment showed a dominant effect on all seed quality traits, also on seed color and pre-harvest germination percentage. One of the parent, line 4042 was an old yellow-seeded line originated from Göttingen, while Express 617 was a double low (00) modern canola cultivar, suggesting a segregation in glucosinolates level. Glucosinolates content variance for was high compared to other seed quality traits. Bushan et al. (2013) reported lower glucosinolates content for infected plants. In one of the field experiment (Reinshof 2016), the plants were heavily infected by fungi. This event could be reflected on low heritability (0.26), and the high value of both environment and G x E.

Heritabilities were high (above 0.80) for NDF, ADF, ADL and even higher for seed color (0.95). In comparison, Körber et al. (2016) discovered heritability higher than 0.80 for NDF, ADF, and ADL content traits among 405 oilseed rape accessions in winter trials, and above 0.90 at spring trials. Zhang et al. (2006) stated that seed color in *B. napus* is mostly controlled by maternal genotype, although at times can be influenced by interaction between maternal and embryonic effects.

In the present study, analysis of variance showed significant effects of both genotype and environment, for germination traits after aging treatment. The similar result was achieved by Schatzki et al. (2013) for seed longevity. They claimed that environmental factors as nutrient supply and growth conditions of the mother plant may affect the longevity of the harvested seeds, as also stated by Kochanek et al. (2011). Beside hypocotyl length trait, all other traits (radicle protrusion, full germination, no germination and infected seed percentages) showed high G x E component values. Mersal (2011) listed many factors affecting the accelerated aging test results, i.e. relative humidity, temperature, exposure period, seed size and seed chemical composition which play an important role in water absorption. All of these might induce some variations in the test result and affect the test accuracy.

Heritabilities were low (0.24 to 0.53) for seed germination and seed longevity traits. Schatzki et al. (2013) also obtained moderate heritability values (0.70 to 0.71) for seed germination rate before and after artificial aging from black-seeded materials.

3.4.2 Spearman's rank of correlations

In order to determine the associations among nineteen observed traits of DH population of 4042 x Express 617 population, the estimation of Spearman's rank of correlation coefficients were performed by PLABSTAT. Strong positive correlations were found among the three fiber components. In this study, all seed fiber components have strong positive association to seed color, but none to oil or protein content. Pre-harvest germination percentage was negatively correlated to seed oil and protein, also to NDF value.

Yellow seed color was associated with low full germination percentage (0.31), and high probability of radicle protrusion and seed infection. All seed fiber components contributed to the increase of radicle protrusion percentage, but only ADL was correlated to the increase of seed infection. In other crops like rice (Umnajkitikorn et al., 2013), lower tannin contents were associated with higher risk of the seed embryo being damaged by biotic and abiotic stress. The germinated seeds of pigmented cultivars are more robust against salinity stress, due to antioxidant capacity. Obviously, high percentage of full germination would have strong negative correlation to both radicle and infected seed percentage, but interestingly not to hypocotyl length. Radicle protrusion also correlated to infected seed percentage. Hypocotyl length has no significant correlations with any seed germination traits.

The seed longevity traits had interesting results. Although these traits have no association to seed color or seed size, but they were significantly correlated to seed fiber contents. All fiber traits (NDF, ADF, ADL) were strongly correlated with seed longevity traits. They were positively associated to percentage of both full germination and hypocotyl length, and negatively correlated to percentage of seed infection. It was possible that the thick testa, fortified by high fiber content, would help protecting the seeds from deterioration during the storage. Phenylpropanoid based polymers, like lignin or condensed tannins which accumulated only in the seed coat, provide substantial protection against mechanical or environmental damage (Vogt, 2010). It may also explain the lower infection level for seeds with darker color. In nature, condensed tannins exist in polymeric state, and able to bind proteins. These characteristics might explain their impermeability properties, also their role in the germination-restrictive action of the testa (Debeaujon et al., 2001).

Similar associations were also observed for total oil and protein content in relation to seed longevity traits. Full germination percentage and hypocotyl length were improved by the increase of seed fiber and total oil and protein contents, while chance of getting radicle protrusion or seed infection would become lower. Seeds with less oil content has better seed longevity rate. Schatzki et al. (2013) experiment revealed a weak negative relationship between oil content and seed longevity as also

observed in the present study (-0.32). Nagel and Börner (2010), also earlier found the same association between oil content and seed longevity across different species of cereals, legumes, and oilseeds. The existence of oil can be detrimental to seed viability in long term. Lipid oxidation induced an increase of free fatty acid level and free radicals (Grilli et al., 1995). Free fatty acid can act as detergent agent and harmed the lipid bilayer of the membrane. Free radicals can also destroyed membrane, enzymes, protein, DNA, and in the end the cellular repair mechanism (Booth & Bai, 1999). Pritchard & Dickie (2004) earlier confirmed that oily seeds aged more rapidly, and further suggested that not only membranes that were susceptible to lipid oxidation. Oil-rich seeds owned reserved lipids which can provide more free radicals attack throughout the seeds. However, adjustment of moisture content during equilibration process might counteract this effect.

Seed longevity traits are not correlated to full germination rate before aging. Instead, significant relations are found between full germination (-), hypocotyl length (-), and seed infection (+) after aging to both radicle protrusion and seed infection percentage before aging. Only radicle protrusion after aging trait is free from such correlations to seed longevity.

Also, there was no correlation between seed color to both oil and protein content. This was contrary to Rahman and McVetty (2011) which mentioned that yellow seeded *Brassica spp.* was often associated with high oil and protein content. Comparing yellow vs brown seeded lines of *B. napus* originated from eight different sources, Tang et al. (1997) previously discovered that the seed coat oil content of yellow seeds are superior to dark seeds. However, the oil content of the embryo and total oil content are mainly determined by their genetic background, not by seed color or seed coat thickness.

3.4.3 Frequency distribution and T-test two means comparison

The seed fiber components showed a certain degree of similarity among their frequency distribution graphs. All demonstrated segregation toward two peaks within the distribution, although the clearest segregation was exhibited in seed ADL content. Comparing the two groups of low vs high ADL contents, the mean value of the low ADL group was 5.9 %, and the high ADL group was 11.3 %. In comparison, Wittkop et al. (2009) obtained 3.2 % of ADL content of a yellow seeded doubled haploid line, and 5.9 % of a black seeded one.

In the present study, there was no significant difference found in oil or protein content between yellow and black-seeded lines. Simbaya et al. (1995) discovered that in average, the yellow-seeded types contained 2 % more protein than the brown-seeded ones, although they utilized only 33

genotypes which came from various Brassica species. Tang et al. (1997) mentioned that although most of the times yellow seeded lines have higher oil content than the black ones, it depends also on their genetic background. There are some cases where they found that the yellow seeds are inferior instead.

Dietary fiber content in yellow seeded samples is found to be significantly 6 % lower from the dark seeded types. Lower dietary fiber content in yellow seeded samples as compared to dark seeded samples was reflected in a lower content of lignin with associated polyphenols (4.3% vs 8.2%, Simbaya et al., 1995). Strong correlations between seed color and acid detergent lignin (ADL) are also noted by Liu et al. (2012), but not between ADL and cellulose or hemicellulose contents.

In this population, the seed size (TSW/Thousand Seed Weight) was also not significantly different between two groups. Tang et al. (1997) stated that TSW is not influenced by seed color, but by genetic background. Minkowski (2002) suggested that the seed size matters to determine seed oil content. Larger seeds tend to have lower seed hull proportion, with larger portion of cotyledon and less portion of seed coat. Yan et al. (2009) proved that seed hull proportion was negatively correlated to oil content. In this case, although the two groups of yellow and black seeds have different seed fiber levels, the seed size was not affected, and therefore the other seed quality traits (seed oil, protein, total oil & protein, protein of defatted meal, glucosinolates contents) were also not differ significantly.

3.4.4 Seed metabolites fingerprinting

According to Hajduch et al. (2006), the energy and metabolism-related protein groups were represented the highest in the immature or developing seed of *Brassica napus*, as much as 24.3 % and 16.8 % of the total proteins, respectively. The abundance of these amino acid metabolism expression profiles was the highest at 2 WAF (Week after Flowering), then it slowly decreased until reached midpoint of seed filling, and remained constant afterwards. The earlier transcript analysis profiles by Dong et al. (2004) also confirmed that the seeds at 10-20 DAP (Days after Pollination) has the highest active cell proliferation, which used to develop metabolic networks for further seed maturation. Therefore, to get better reading at the various metabolite levels of the seeds, the metabolite analysis was at best to be performed on immature seeds.

There were significant differences of compound content levels revealed between the high ADL vs low ADL content groups (Fig. 9). The differences can be observed starting from phenylpropanoid biosynthesis pathway, continued to flavonoid and proanthocyanidins pathway. According to Vogt

(2010), the biosynthesis pathways of lignin and flavonoids are interrelated. Phenylpropanoids, proanthocyanidins, and several flavones or flavonols are the by-products of these two pathways. Lignin itself is mostly built based on phenylpropanoid units, derived from the oxidative polymerization of hydroxycinnamoyl alcohol derivatives. Seed flavonoids were classified into several groups: flavonols, anthocyanins, phlobaphenes, isoflavones, and proanthocyanidins (Lepiniec et al. 2006). Proanthocyanidin, also known as condensed tannin, was only accumulated in the seed coat. This compound was synthesized through a phenylpropanoid pathway in the flavonoid pathway (Lepiniec et al. 2006). Seed flavonoids are involved in defense against biotic and abiotic stresses and contribute to physiological processes such as reinforcement of seed longevity and dormancy (Auger et al., 2010).

Anthocyanins and PAs were accumulated in the vacuole, where polymerization of PA precursors was followed by conversion to brown oxidation products (Lepiniec et al., 2006). Comparing mutant tt12 seeds to the wild type, Marinova et al. (2007) observed the absence of epicathecin in PA (proanthocyanidins) pathway. The metabolism fingerprinting for DH population of 4042 x Express 617 seeds obtained the same result for yellow seeded vs black seeded samples. Further, Marinova et al. (2007) also found that the quantity of quercetin-3-O-rhamnoside (Q3R) was reduced to 30% in *tt12* mutant seeds.

3.4.4 Bulk Segregant Analysis (BSA) and identification of candidate genes

According to Shoba et al. (2012), in QTL mapping, normally each plant of a large mapping population should be genotyped with numerous molecular markers. This process is considered time consuming and labor intensive. The difficulty of genotyping all the plants in a mapping population can be reduced through selective genotyping through Bulked Segregant Analysis or BSA. This molecular analysis involves selection of two extreme phenotypic outcomes (e.g. resistant vs recessive genotypes), and pooling their DNA into two bulks (Michelmore et al., 1991).

In an artificial aging study in maize by Ku et al (2014), 22 candidate genes related to seed vigor were detected. These candidate genes had functions related to responses to stress, molecular chaperones, hydrolase activity, energy, cell growth and division, protein targeting and storage, signal transduction, translation, protein metabolism, amino acid metabolism and play important roles in seed ageing and seed vigor. Previously, Wang et al. (2015) detected similar genes controlling seed ADL content on C05, A05, and A09 chromosomes in the oilseed rape genome. The constructed physical map of chromosome C03 (Fig. 10) contains the predicted positions of both candidate genes

(MATE and C4H). The loci of the two candidate genes are located close to each other (±310 kbps on physical map). It is possible that these two loci are linked and being inherited together.

TT12 (Transparent Testa 12) gene was first time identified by Debeaujon et al. (2001) in *Arabidopsis*. Its gene function encodes MATE transporter, and turns the seed coat color to dull pale brown. Yu et al. (2013) included gene *TT12* (GenBank accession number At3g59030) in the list of the transparent testa and related mutants in *Arabidopsis thaliana*. Chai et al. (2009) confirmed the existence of TT12 gene(s) in *B. napus* and its parental species (*B. oleracea and B. rapa*). According to Chai et al. (2009), all Brassica TT12 proteins displayed high levels of identity to each other (>99 %) and *to AtTT12* (>92 %).

Marinova et al. (2007) specified the importance of *TT12* or MATE transporter gene for accumulation of proanthocyanidins in the vacuoles of the *Arabidopsis* seed coat cells. Proanthoyanidins inside the vacuoles would interact with oxygen molecules, being oxidized, and give color of brown or black. As the seed matures, the outer cells of the seed gradually died, and the pigments were osmotically transported from inner layer to the seed coat cells. The higher the accumulation of oxydized proanthocyanidins, the darker the seed coat color would become.

The second candidate gene, *BnCH4*, is not found within the predicted chromosome interval, but a bit downstream of the last identified marker position. The estimated position of *BnCH4* at 8,139,451 – 8,141,336 bps on chromosome C03 is based on Qu et al. (2013) reference, and its existence in this population needs to be verified in the future. Lignin provides mechanical strength and aids in resistance to pathogen attack and water impermeability to the cell wall (Vanholme et al., 2010). Chen et al. (2007) has successfully cloned two isoform genes which encoding cinnamate 4-hydroxylase (C4H) from *B. napus*. Chen et al. (2007) further detected strong expression of these genes at both high lignin organs (hypocotyl and stem), and low lignin organs (cotyledon, flower and bud). Therefore, C4H might have additional role in other process, such as flavonoid biosynthesis.

Beside its lignification function, C4H was acknowledged as the second key enzyme to the common phenylpropanoid pathway. According to Vogt (2010), 4-coumaroyl CoA can be changed into catechin through proanthocyanidins pathway, into flavonoids, and lignin. The initial three steps of the pathway, catalyzed by 3 enzymes (phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumaroyl CoA-Ligase (4CL)) (see Fig. 11), were mandatory and provide the basis for all subsequent branches and resulting metabolites.

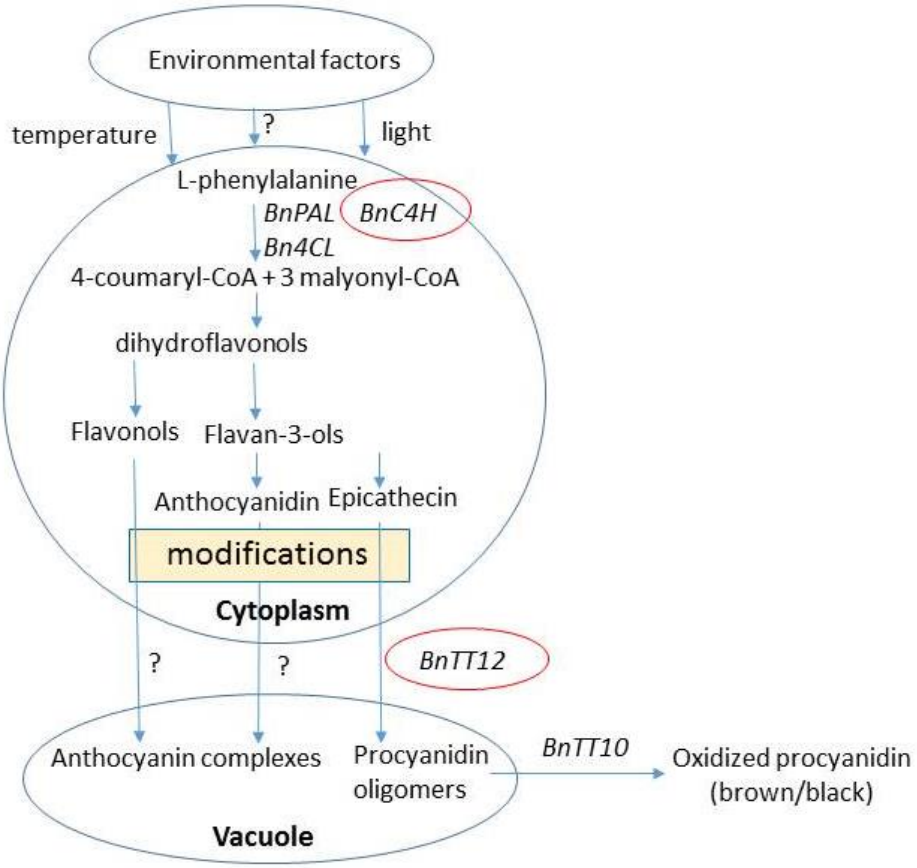


Fig. 11 Positions of *BnC4H* and *BnTT12* in modified model for molecular mechanisms controlling seed coat color in *B. napus*, based on Qu et al. (2013)

Note: *BnPAL*, l-phenylalanine ammonia-lyase; *BnC4H*, cinnamate 4-hydroxylase; *Bn4CL*, 4-coumaroyl CoA ligase.

To achieve their function/s, secondary metabolites generally accumulate to high concentrations in different tissues and/or cell types. Storage in suitable compartments suggests that this process is highly regulated, since some of the secondary metabolites are toxic to the plants themselves (Yazaki, 2005). Secondary metabolites are transported in various ways, either between tissues or within a cell. Vacuoles play a central role in the storage of secondary metabolites such as alkaloids and flavonoids in plant cells. Vacuolar membranes (tonoplasts) contain a large number of transporters, channels and pumps (Marinova et al., 2007). Polinceusz (2011) mentioned that since modified flavonoids are hydrophilic, their intra- and inter- cellular transport depends heavily on membrane bound transporters.

Debeaujon et al. (2001) proposed that MATE transporter could transport potential PA precursor(s) into the vacuole. Zhao and Dixon (2009) and Polinceusz (2011) provided further genetic and physiological evidence which implicated the importance of *TT12*, a MATE transporter, to facilitate

vacuolar uptake of epicatechin 3'-O-glucoside. Epicatechin 3'-O-glucoside is a precursor for proanthocyanidin biosynthesis. Debeaujon et al. (2001) confirmed that the blocking of flavonoid transport from the cytosol into the central vacuole might reduce anthocyanin and PA production, and multidrug and toxin extrusion (MATE) transporter proteins have been shown genetically to be involved in both anthocyanin and PA precursor transport.

3.4.5 KASP genotyping

The outcome of KASP genotyping has confirmed the result of Bulk Segregant Analysis (BSA). The genotypes of low ADL proved to have different alleles from genotypes of high ADL content. The allelic distributions from three different KASP markers were giving identical results.

In general, genotypes of allele 1 type are lighter in seed color appearance, and allele 2 type genotypes are darker. The first group, however, has wider range of seed color (mean value of 3.2 to 7.6) than the second group (7.8 to 8.8) (data not shown). It means that the seeds of genotypes of allele 1 type have wider color spectrum, can vary from yellow to dark brown, whereas the genotypes of allele 2 type only vary from dark brown to black. Comparing to the segregation in earlier sub chapter of seed metabolites fingerprinting, there the first group has more narrow seed color range (3-5), and the second group ranged from 7-9.

One genotype (#11) was consistently detected having allele 2 type by all three different KASP markers, despite located in the first peak. However, it has relatively low ADL content (mean value 7.73) so that this genotype was isolated from the rest of the group of allele 2 genotypes in the frequency distribution in Fig 10. Its seed color was dark (score 7.8), therefore if grouped based on seed color, it belonged to the second group.

The proportion of first group with low ADL/yellow seeds (n=49) are much larger than the second group with high ADL/dark seeds (n=28). It can be because there are more numbers of yellow seeded DH lines being generated successfully from F1 plants compared to the black seeded DH lines in this population.

In this study, only half of the tested KASP markers were verified. 50 % conversion rate was less than what Islam et al. (2015) achieved (66.7 %), but still higher than earlier work of Byers et al. (2012) with 35.8 %. Islam et al. (2015) assumed that the validation failure of some SNP markers in KASP genotyping might be due to the incorrect primer design near SNP, the presence of duplicate loci, the wrong identification of fake SNPs, and the less than optimal PCR condition. Higher conversion rate

from selected SNPs to functional KASP assays could probably be increased by optimization of primer design and amplification conditions.

3.5 CONCLUSIONS

In DH population of 4042 x Express 617 population, most of the seed quality traits showed strong significance for both genotype and environment factor. The environment showed a dominant effect on all seed qualitative traits, also on seed color and pre-harvest germination percentage. Heritabilities were high (above 0.80) for NDF, ADF, ADL and seed color. The seed color has strong positive correlation to seed fiber content (0.6 - 0.8), however the seed color in this population was not associated with oil and protein content. No significant difference of seed size between yellow vs black seed groups might be the reason of the absence of those correlations. Usually yellow seeds were smaller in size, and have seed coat and cotyledon ratio, which in turn improve the seed oil content.

Light seed color was associated with lower full germination percentage, and higher probability of radicle protrusion and seed infection. All three seed fiber components have significant contributions to the increase of radicle protrusion percentage, but only ADL was correlated to the increase of seed infection. Radicle protrusion also correlated to infected seed percentage. Hypocotyl length has no significant correlations with any seed germination traits.

The seed longevity traits had no association to seed color or size, but they were significantly correlated to seed fiber contents. All three fiber traits were strongly correlated with seed longevity traits, they were positive to percentage of full germination and hypocotyl length, and negative to percentage of seed infection. It was possible that the thick testa, fortified by high fiber content, would help protecting the seeds from deterioration during the storage.

Through seed metabolite fingerprinting, significant differences of compound levels were revealed betwen the high ADL vs low ADL content groups. Low ADL/yellow seed group has consistently lower levels of compounds involved in phenylpropanoid biosynthesis pathway, also flavonoid and proanthocyanidins pathway. The high content of flavonoids in dark seeds, in contrast to yellow seeded genotypes, could also help prolong the seed longevity and protect from seed infection.

We found two candidate genes that possibly controlling ADL content in chromosome C03. The constructed physical map of chromosome C03 contained the predicted positions of both candidate genes (MATE and C4H). The first candidate gene was *TT12 (Transparent Testa 12)* or MATE

transporter gene which responsible for the accumulation of proanthocyanidins in the vacuoles of the seed coat cells. The second candidate gene, C4H (trans-cinnamate 4-hydroxylase) was encoding a precursor to lignin biosynthesis, and may be further involved also in flavonoid biosynthesis. Its position needs a further verification study, by employing more KASP markers for DH population of 4042 x Express 617 to the downstream direction from the gene interval investigated in this study.

4 INHERITANCE OF SEED QUALITY TRAITS, SEED GERMINATION AND SEED LONGEVITY IN SPRING X WINTER OILSEED RAPE DOUBLED HAPLOID POPULATION DH 1372 X EXPRESS 617, SEGREGATING FOR YELLOW SEED CHARACTER

4.1 INTRODUCTION

Oilseed rape (*Brassica napus*) is the second most important oilseed crop worldwide (Nguyen et al., 2016). Yellow seed coat color is a desirable trait in many oilseed *Brassica* species. Yellow-seeded cultivars were reported to have thinner seed coat than black-seeded ones (Liu et al*.,* 2005). Furthermore, Wightman et al. (2014) suggested that the seed meal from yellow-seeded cultivars contains higher protein and lower fiber content, which improves the meal feeding value for poultry and livestock.

However, thick seed coat also provides protection for seed embryo from the harsh environment outside. Seeds of darker color would imbibe water and germinate later than yellow seeds, probably due to their thicker seed coat and phenolic compounds which are affecting the seed coat permeability (Debeaujon et al*.* 2000, Rahman et al*.* 2001, Neubert et al., 2003).

Mature seeds *of B. napus* will gradually lose their viability during long term storage; this process is defined as natural aging (Yin et al., 2015). Seed viability can be influenced by several environmental factors, such as seed maturity and physiology, but partly also determined by genetic factors (Nagel et al*.,* 2010). Seed aging is an acknowledged problem for agriculture, and the involved mechanisms which bring the loss of seed viability and vigor are worth investigating. The aging process is well displayed through delayed germination and emergence, slower growth rate, increased susceptibility to disease and environmental stress, and finally, by germination failure (Kruger-Giurizatto et al*.,* 2012).

Running the seed longevity test using natural seed aging process is not an easy task. For accurate prediction of seed response to storage time, it is mandatory to use a reliable assay (Ku et al., 2014). In ambient storage condition of 20^{o}C and 50 % relative humidity, generally it takes 7.3 years for *Brassica spp.* seeds to lose half of their viability (Nagel and Börner, 2010). However, there are several artificial seed aging techniques to achieve nearly similar effect of natural aging, with various degree of success (Suma et al., 2014; Yin et al., 2015). Some examples of these methods are hot water aging (immersion into hot water of 58^{o}C), controlled deterioration (raising the seed moisture content to 15 % at 40^{o}C), and potassium nitrate method (exposing seeds to high relative humidity of 95 % using saturated solution of potassium nitrate at 40^{o}C) . Suma et al. (2014) found that the controlled

deterioration protocol was the most reliable from the three, and Rajjou et al. (2008) said that it was able to imitate many of the seed molecular and biochemical events as if during natural seed aging.

The inheritance of seed color (seed coat pigmentation) has been studied in several *Brassica* species in which black- and yellow-seeded types occur, such as *B. rapa* (Stringam, 1980, Hawk, 1982), *B. juncea* (Vera and Woods, 1982, Negi et al., 2000), *B. oleracea* var. *alboglabra* (Heneen and Brismar, 2001), and *B. napus* (Liu et al., 2005). In earlier studies (Rahman and McVetty., 2001, Liu et al., 2005), it was assumed that one to four gene loci were involved in the seed color determination, and that yellow seed coat color was recessive trait. Until recently, 26 independent loci involved in seed coat pigmentation (the so-called *Transparent Testa [TT]* genes) have been identified (Xu et al., 2006, Yu et al., 2013). Some of the *BnTT* genes were proposed to co-localize with QTL for seed color and fiber content (Badani et al., 2006).

During two reciprocal crosses by Liu et al. (2005) in *B. napus*, the immediate F1 seeds from both crosses had the same color as the self-pollinated seeds of the respective black- and yellow-seeded female parents, giving evidence of the maternal control of seed color. Furthermore, the F1 plants produced yellow-brown seeds, suggested the partial dominance of yellow seed over black. However, Rahman *et al.* (2005) reported that when the yellow-seeded lines were used as maternal parent and black-seeded parents were used as pollen source, the F1 seed coat color turned dull yellow or yellowish brown. This indicated that pollen grains from the black seeded parent may give a xenia effect on yellow seeded maternal lines.

Digestibility of rapeseed meal is highly influenced by fiber amount in residual hulls. Meal of high seed fiber content can be partially digested by pig, but not at all by poultry (Nesi et al., 2008). Yellow-seeded *B. napus* is considered more favorable for the meal quality thanks to a thinner seed coat and higher protein content [Wittkop, 2009], along with reduced quantities of fiber (cellulose and hemicellulose) and anti-nutritional polyphenolics (acid detergent lignin: ADL; Simbaya et al., 1995).

The oilseed rape breeding for seed quality has resulted in the development of ‘canola’ type with zero or low erucic acid and low glucosinolates (Kennedy et al., 2011). DH 1372 is a yellow-seeded Canadian canola spring type, and Express 617 is a black-seeded German oilseed rape winter cultivar. The aim of this experiment was to study the inheritance of seed quality traits, seed germination and seed longevity using artificial seed aging treatment in a doubled-haploid oilseed rape population of DH 1372 x Express 617, which segregated for yellow seed character.

4.2 MATERIALS AND METHODS

4.2.1 Plant material

The plant material was a doubled haploid population generated from microspore culture of F1 plants cross between line DH1372 and Express 617. DH 1372 is a yellow seeded doubled haploid double low ("00") quality Canadian spring canola genotype, derived from a cross between two black seeded cultivars, Star and Bolero (Burbulis and Kott, 2005). Express 617 is an inbred line of the black seeded German winter oilseed rape cultivar Express. The crossing between DH line 1372-15 (an increase from line NL310-1, from Burbulis and Kott, 2005) and Express 617 was performed in 2012 in Göttingen. Express 617 served as the father plant. Reciprocal crossing was also performed but unsuccessful. The seeds of F1 obtained was brown in color (Fig. 12), in total 95 seeds were produced.

Fig. 12 Seed coat color of the Star and Bolero (parental cultivars of DH 1372), DH 1372, Express 617, and F1 DH 1372 x Express 617

In May 2013, five F1 plants of DH 1372-15 x Express 617 were grown in a phytochamber for microspore culture donor. In September 2013, microspore culture from three F1 plants (individual number 1, 3, and 5) was very successful. The embryos were incubated for 10 days at 2 ^{o}C in the dark in B5 medium, Afterwards, around 300 embryos were grown on filter paper (each filter paper

contain 50 embryos), and another 300 in NLN medium, both were kept for 30 days at 2 ^{o}C in the dark. In October 2013, 144 embryos from each media were transferred to soil and B5 medium in small tray pots. After being treated in vernalization chamber for flowering induction, in February 2014, three plant trays were transferred to individual pots, and maintained in the green house. The plants were self-pollinated with individual plastic bags and harvested per individual plants in May 2014. In total there are 224 DH lines that were successfully generated, although for some DH lines, the seeds obtained were very few (less than 10 seeds).

4.2.2 Field experiments

The self-pollinated seeds of DH 1372 x Express 617, 224 genotypes in total, was sown without replicate in small observation plots in the field at oilseed rape breeding nursery at Reinshof in September 2014. For each genotype, 100 seeds were sown in the field in two rows. Due to cold and rainy weather, the germination rate in the field of 2014 was not very high and only about two third of the DH lines survived the winter in the field. In total, 119 open-pollinated and 136 self-pollinated genotypes were harvested in August 2015. Open pollinated seeds were bulked from 5-10 main racemes of individual plants for each genotype. Self-pollinated seeds were collected from main raceme which covered by pollination bag during flowering period.

The 224 DH lines from green house experiment of 2014 was being sown again without replicate for the field experiment in Reinshof in August 2015, and harvested in July 2016. In this experiment, 204 DH lines survived and matured to produce seeds. In this year, only open pollinated seeds were harvested. In this year both parents were sown together with DH lines, but only Express 617 survived the winter.

4.2.3 Analytical methods

All harvested seeds were dried and cleaned separately for each genotype. NIRS prediction analysis was conducted using seed samples around 3 g using by Near-infrared Reflectance Spectroscopy (NIRS) monochromator model 6500 (NIRSystem Inc., Silverspring, USA). The measurements to obtain the NIRS predicted values, thousand seed weight (TSW), percentage of pre-harvest germination (PHG), and seed color scoring system was the same as explained previously in Chapter 3. Among seed quality traits, the oil, protein, total oil & protein contents (in percentage), also glucosinolates content (in µmol/g seeds) are expressed on a seed basis at 91 % dry matter content.

4.2.4 Seed germination test

Seed germination test was carried out for all harvested genotypes using bulked seeds from 5 to 10 open pollinated plants. The germination test was performed in Petri dishes (92 x 16 mm diameter, Sarstedt, reference code 82.1473), and customized filter papers (90 mm diameter, Macherey-Nagel, GmbH & Co. KG, reference code 400866009.1) with 50 indented holes to fit exactly 50 seeds on each filter paper. After placing sample seeds on filter paper with forceps, 12 ml of de-ionized water was applied to each Petri dish. The Petri dishes filled with sample seeds furthermore being placed into plastic trays, and the trays were covered with thin cellophane film to reduce evaporation. Next, these trays were kept in a dark germination chamber with ambient temperature 16.5 – 17.5 ^{o}C, RH 90-95 % for 10 days period. The observation was performed on the tenth day. Observed traits for germination test were radicle protrusion percentage (RPP), full germination percentage (FGP), hypocotyl length in cm (HL), and infected seed percentage (ISP).

Germination test was performed twice for each seed sample. The first test was using seeds without any aging treatment. After being harvested, these seeds were dried, processed and cleaned from stems and pods. There should be at least six weeks period after harvesting before the fresh seed germination test can be started, to break the seed dormancy. The aim of this first test is to predict the original germination viability of the genotype, before the seed aging treatment. The schedule for the first seed germination test is shown in Table 14.

Table 14 Schedules of seed germination test for DH 1372 x Express 617

Location/Harvest year	Seed Harvest	Start Germination	Start Counting
Reinshof 2015	20. Jul 2015	9. Nov 2015	29. Nov 2015
Reinshof 2016*	19. Jul 2016	14./24. Oct 2016	24. Oct/4. Nov 2016

*(germination testing was performed in 2 batches)

The second test is seed germination following artificial aging treatment, or controlled deterioration test. The test was performed at IPK Gatersleben laboratory, following the protocol of Cromarty et al. (1982), as explained in Chapter 1. The first stage is called equilibration, in which the seeds are exposed to 47% RH, 20 ^{o}C for ten days. The treatment was followed by seed aging stage for 50 days at 60% RH and 45 ^{o}C. The last step is the germination test which takes 9-10 days, under dark condition of 90-95 % RH, 16-17 ^{o}C. The total number of genotypes was 140 of each location, each

genotype has two sample replications, and each seed sample has 50 seeds. The same traits as in the seed germination test were observed and recorded for this artificial seed aging treatment (Table 15).

Table 15 Timetable for artificial seed aging treatment DH 1372 x Express 617 in 2017

Population	Begin equilibration (14 days)	Begin seed aging (50 days)	Begin germination test	Begin counting
Reinshof 2015 Rep 1	02. Jan	15. Jan	06. Mar	15. Mar
Reinshof 2015 Rep 2	03. Jan	16. Jan	07. Mar	16. Mar
Reinshof 2016 Rep 1	04. Jan	17. Jan	08. Mar	17. Mar
Reinshof 2016 Rep 2	05. Jan	18. Jan	09. Mar	18. Mar

For seeds harvested in 2015, it was expected that the result of germination might underwent slight seed deterioration and have lower viability compared to the 2016 samples prior to longer storage period. The first one has been stored for 18 months, while the second was only 4 months old from harvest time. Throughout the time interval, the seeds were stored in the cold seed storage chamber (temperature 4 ^{o}C) to preserve the seed viability.

4.2.5 Statistical analysis

There are 3 data sets available for the statistical analysis: the OP Reinshof 2015 population (119 DH lines), the self-pollinated Reinshof 2015 population (139 DH lines), and the OP Reinshof 2016 (204 DH lines). For combined data analysis, only 109 genotypes were consistently present in all three data sets. With 145 genotypes, minimal 2 out of 3 data sets were represented for each genotype. These 145 genotypes were used for further analysis, except for seed germination related traits which only have 140 genotypes.

The analysis of variance and estimation of heritability values was completed by PLABSTAT software (Utz, 2011). The experiments have been conducted with no replicate. Therefore, the significance of the G x E interactions could not be tested. Environment and genotype were considered as random variables. The general model for analysis of variance is as follow:

$$Y_{ij} = \mu + g_i + e_j + ge_{ij}$$

Where Y is observation of genotype i in environment j; μ is general mean; g*i* and e*j* are the effects of genotype i and environment j; ge_{ij} is the interaction between genotype i with environment j. Heritability (h^2) of the mean values over environment was calculated from components of variance according to Hill et al. (1988):

$$h^2 = \frac{\delta^2 G}{\delta^2 G + \frac{\delta^2 GE}{E}}$$

where $\sigma^2 g$ is variance component for genotype, $\sigma^2 e$ is variance component for environment and $\sigma^2 ge$ is variance component for interaction between genotype and environment. Spearman's ranks of correlation coefficients between traits were predicted from mean values of the genotypes across all environments.

4.3 RESULTS

4.3.1 Phenotypic variation and heritabilities

Considerable variations were revealed for the nineteen traits observed in 145 genotypes of DH 1372 x Express 617 over three environments: OP seeds Reinshof 2015, self-pollinated seeds Reinshof 2015, and OP seeds Reinshof 2016. The traits can be broken down into of seed quality traits (seed oil, protein, total oil and protein, protein of defatted meal, glucosinolates, and NDF, ADF, ADL contents), seed characteristics (seed color, TSW (Thousand Seed Weight), and pre-harvest germination percentage), seed germination and seed longevity traits (both covered % radicle protrusion, % full germination, % infected seeds, and hypocotyl length). The values of variance of components are displayed in Table 16.

There are significant effects found both in genotypes and the environment factors for seed quality traits from NIRS prediction for the population of DH 1372 x Express 617. The genotype influence is higher than environment for protein, glucosinolates, and NDF content traits. For protein of defatted meal, both genotype and environment effects are equally significant. For oil, total oil & protein, ADF and ADL content traits, the environment factor has stronger influence. The heritability values for seed quality traits are ranged from low to high. Protein and protein of defatted meal content was similarly low with 0.55 and 0.54. Oil, total oil & protein, and glucosinolates contents have medium score of 0.73 and 0.70, and 0.75, respectively. Among the fiber components, NDF kept medium heritability (0.72), while ADF and ADL scored higher at 0.88 and 0.89, respectively.

Table 16 Variance component of DH 1372 x Express 617 over 3 environments (n=145)

Source of variance	Genotype (G)	Environment (E)	GxE	Heritability (h^2)
Oil content (%)	0.52**	1.75**	1.95	0.73
Protein content (%)	0.36**	0.08**	0.89	0.55
Oil & Protein (%)	1.36**	1.43**	1.18	0.70
Glucosinolates (µmol/g seeds)	23.01**	3.63**	22.97	0.75
NDF (%)	3.42**	2.38**	3.97	0.72
ADF (%)	3.34**	9.12**	1.37	0.88
ADL (%)	4.42**	5.67**	1.63	0.89
Protein defatted meal (%)	0.50**	0.49**	1.28	0.54
Seed color	1.66**	0.08**	0.95	0.84
Pre-harvest germination (%)	1.47**	0.01	2.98	0.60
Thousand seed weight (g)	0.17**	0.06**	0.21	0.70
Radical protrusion (%)	0.16	0.03	10.83	0.03
Full germination (%)	0.07	0.07	21.50	0.06
Hypocotyl length (cm)	0.14**	0.02*	0.50	0.37
Infected seeds (%)	72.00**	15.48**	261.04	0.36
Radicle protrusion (%) (AA^1)	26.00**	6.02**	68.37	0.43
Full germination (%)(AA^1)	182.18**	0.00	305.36	0.54
Hypocotyl length (cm) (AA^1)	0.05*	0.00	0.24	0.31
Infected seeds (%)(AA^1)	55.58**	0.00	137.65	0.45

[1] following Artificial Aging

* marked as significant at P= 0.05, ** as significant at P=0.01

Note: All the seed germination traits (before and after AA) were analyzed using 140 genotypes and 2 environments (self-pollinated seeds Reinshof 2015 and OP seeds of Reinshof 2016)

The seed characteristics are also significantly influenced by both genotype and environment factors, except for pre-harvest germination percentage which only influenced by genotype. In all three traits (seed color, TSW, and pre-harvest germination), genotype factor has the higher influence to the population variance compared to environment. Heritability of seed color trait is high (0.84), while TSW and pre-harvest germination has medium heritability of 0.60 and 0.70, respectively.

In seed germination traits, the radicle protrusion and full germination percentage were not affected by either by genotype or environment factors. The infected seed percentage are influenced by both genotype and environment factors. For hypocotyl length, only genotype factor was significant. Percentage of infected seeds has the highest variance values among all germination traits (Table 16). The seed germination traits in general are very low in heritability. The radicle protrusion and full germination percentage are each 0.03 and 0.06, respectively. For other traits, the heritability values are also still low, 0.37 for hypocotyl length, and 0.36 for seed infection percentage. After aging, the variance among seed germination traits was drastically increased. Most of the seed longevity traits have significant effects on genotype factor, except hypocotyl length. Environment effect is only significant for radicle protrusion. The heritability values for seed longevity traits are low, the lowest is hypocotyl length (0.31), followed by radicle protrusion and infected seed percentages (0.43 and 0.45), then full germination (0.54).

The minimum, maximum, and mean values, standard deviation and LSD 5 % of DH 1372 x Express 617 over 3 environments are listed in Table 17. Only the values of one parent material (Express 617) are available. The seed quality traits are also diverse. The average oil content at 91 % of seed dry matter is 43.6 %, protein 18.87 %, total oil & protein content 62.67 %, and protein of defatted meal 33.6 %. Since both parents are from canola (00) type, the glucosinolates values are relatively low. The mean of glucosinolates content is 20.2 umol/g seeds. The fiber components, start from the smallest to the largest value is ADL (average 28.9 %), ADF (20.9 %), and NDF (8.6 %). There is no genotype which has average seed color of score 1 or uniform yellow across the environments, therefore the range for seed color is start from 2 (mix yellow and pale brown) to 9 (uniform black). Pre-harvest germination percentage is quite low, between 0 to 14 % occurrences in seed samples. The seed size, represented by TSW is diverse, ranged between 3.46 – 7.48 g.

Radicle protrusion percentage is ranged between 0 to 20 % maximum, although the mean value is very low at 1.71 %. Full germination rate is relatively high with average value of 97.51 %. The seeds of both environments (2015 and 2016) of DH 1372 x Express 617 showed a very good germination and vigor before seed aging (data not shown), in spite of the different storage time. Seed infection rate has full range from no infection to 100 % infection, and the average value is only 7.81 %. The hypocotyl length is also high, ranged from 3 – 7 cm, with 4 cm as average. After aging treatment, the percentage of seeds with radicle protrusion ranged between 0 – 64 %, with average 17.85 %. Full germination has reduced drastically for some genotypes, although some managed to maintain the viability. The range became very diverse from zero to 95 %, and average 57.55 %. Infected seed

percentage is a bit reduced by the value range (0 – 90 %), but increased by mean value (10.11 %). Hypocotyl length is severely reduced to average value of 1.50 cm.

Table 17 Descriptive statistics of DH 1372 x Express 617 over three environments (n=145)

Traits	DH 1372 (P1)	Express 617 (P2)	Min	Max	Mean	SD	LSD 5%
Oil content (%)	-	45.4	36.1	49.0	43.6	2.34	3.26
Protein content (%)	-	17.6	15.3	23.1	18.9	0.66	2.29
Oil & protein (%)	-	63.0	59.4	64.7	62.7	1.15	2.15
Glucosinolates (umol/g seed)	-	17.3	10.7	42.3	20.2	5.53	10.61
NDF (%)	-	30.9	17.0	31.96	28.9	2.30	3.21
ADF(%)	-	24.7	16.6	25.5	20.9	1.80	1.88
ADL (%)	-	12.2	3.0	13.7	8. 6	2.13	2.05
Protein defatted meal (%)	-	32.2	31.1	36.4	33.6	1.02	1.82
Seed color	-	8.7	2.0	9.00	6.3	1.36	1.56
Pre-harvest germination (%)	-	0.01	0.0	14.0	1.3	1.46	2.78
Thousand seed weight (g)	-	5.2	3.5	7.5	5.5	1.50	0.75
Radical protrusion (%)	-	0.0	0.00	20.0	1.7	2.36	6.52
Full germination (%)	-	100.0	87.0	100.0	97.5	3.38	9.19
Hypocotyl length (cm)	-	4.0	3.0	7.0	4.6	0.63	1.39
Infected seeds (%)	-	5.4	0.0	100.0	7.8	14.22	32.01
Radicle protrusion (%) (AA[1])	-	29.5	0.0	64.0	17.9	7.85	16.35
Full germination (%)(AA[1])	-	60.5	0.0	88.5	57.6	18.65	34.56
Hypocotyl length (cm) (AA[1])	-	2.0	0.0	3.3	1.5	0.46	0.96
Infected seeds (%)(AA[1])	-	6.5	0.0	90.0	10.1	11.27	23.20

[1] following Artificial Aging

Note: All the seed germination traits (before and after AA) were analyzed using only 140 genotypes and 2 environments (self-pollinated seeds Reinshof 2015 and OP seeds of Reinshof 2016)

4.3.2 Frequency distributions

Frequency distributions would display the number of observations occurred within a given interval or range. In this study, the graphs have exhibited diverse frequency distributions among 75

genotypes of DH 1372 x Express 617. This sub chapter is focusing on seed fiber components, seed color, and seed germination traits before and after seed aging.

The frequency distribution graph for NDF mean values (Fig. 13) is heavily skewed to the right. For ADF mean values, the frequency distribution is starting to show bimodal distribution. The bimodal shape is even more pronounced in the similar graph of ADL mean values, indicating that one major gene may control the particular trait. The first peak is larger than the second peak, which perhaps caused by skewed segregation. It is likely that within the DH 1372 x Express 617 population, there are more genotypes which regenerated from the low ADL genotypes than from the high ADL.

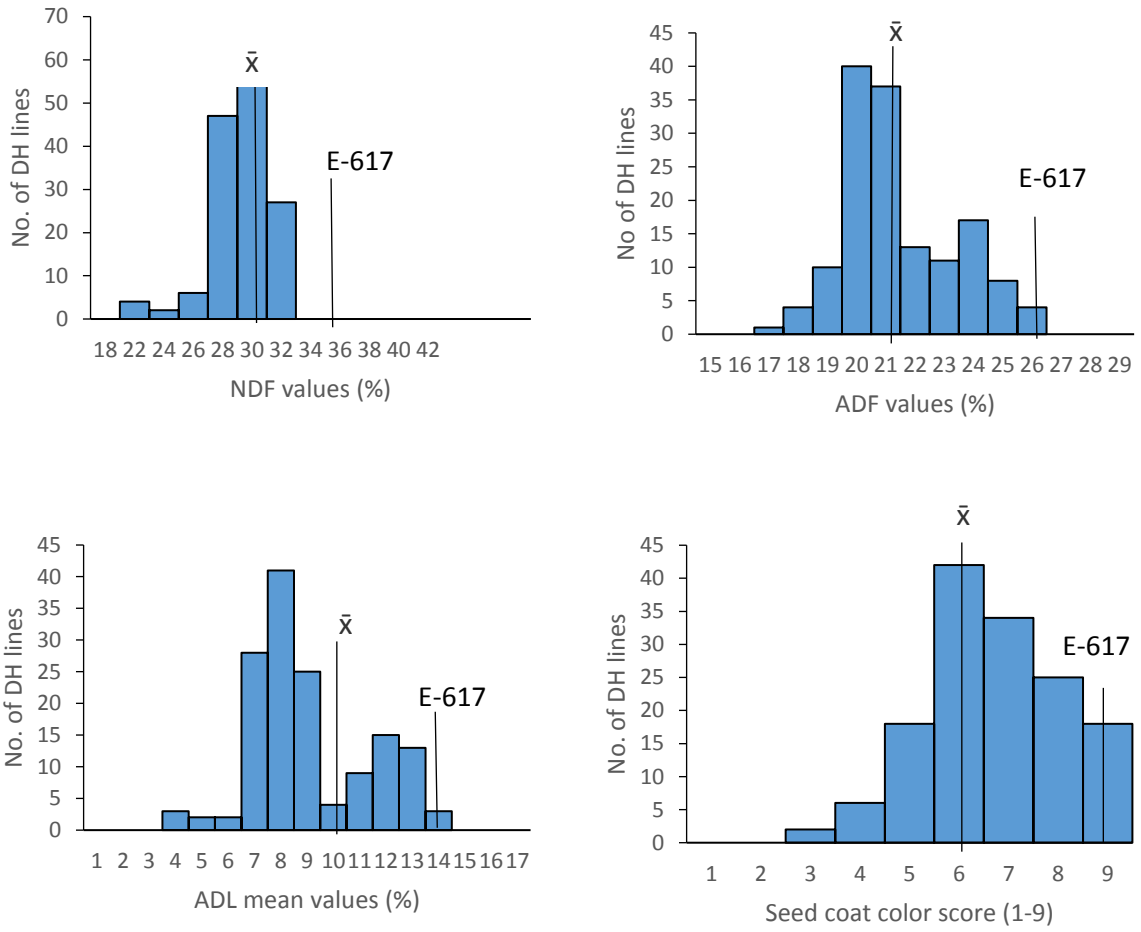


Fig. 13 Frequency distributions of mean values of seed fiber components and seed coat color of DH 1372 x Express 617 in 3 environments (n=145)

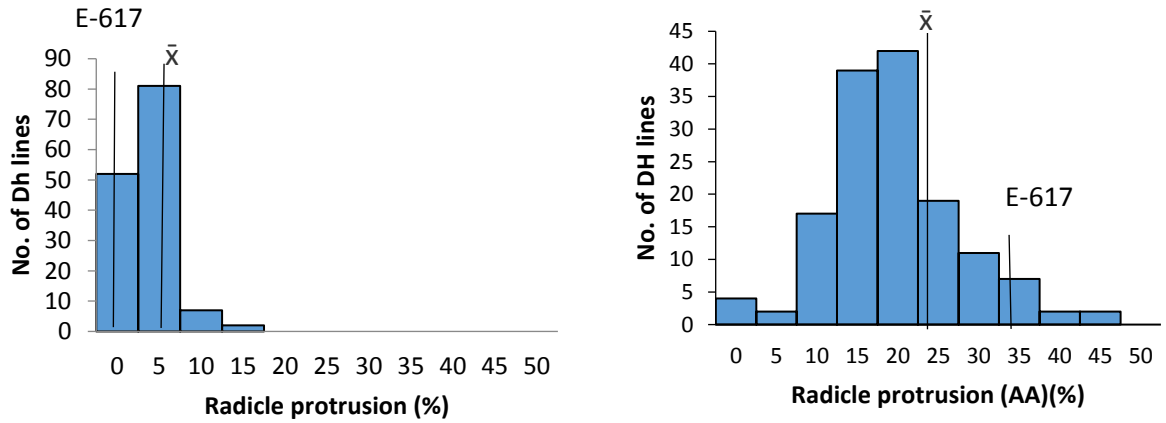
For seed coat color frequency distribution, the graph resembles a normal distribution which skewed to the right. The seed coat color with highest frequency is category 6, which is dark brown mixed

with yellow. From the observation, the seeds of population of DH 1372 x Express 617 exhibit a high occurrence of mixed seed color (Fig. 14).

Fig. 14 Some examples of genotypes having mixed color seeds of DH 1372 x Express 617

Note: left: score 5 (#305), middle: score 6 (#153), right: score 8 (#119). The close-up photo of the seeds was taken from the middle genotype.

The comparison of frequency distributions of seed germination traits from DH 1372 x Express 617, measured from 140 genotypes, before and after seed aging treatment are shown in Fig. 15. Some of the histograms are skewed and not following normal distribution, such as in radicle protrusion and full germination percentage before aging. After applying few types of data transformation, however, the resulted histograms remain more or less the same. Therefore, in this study, the original values are used to produce the following graphs.



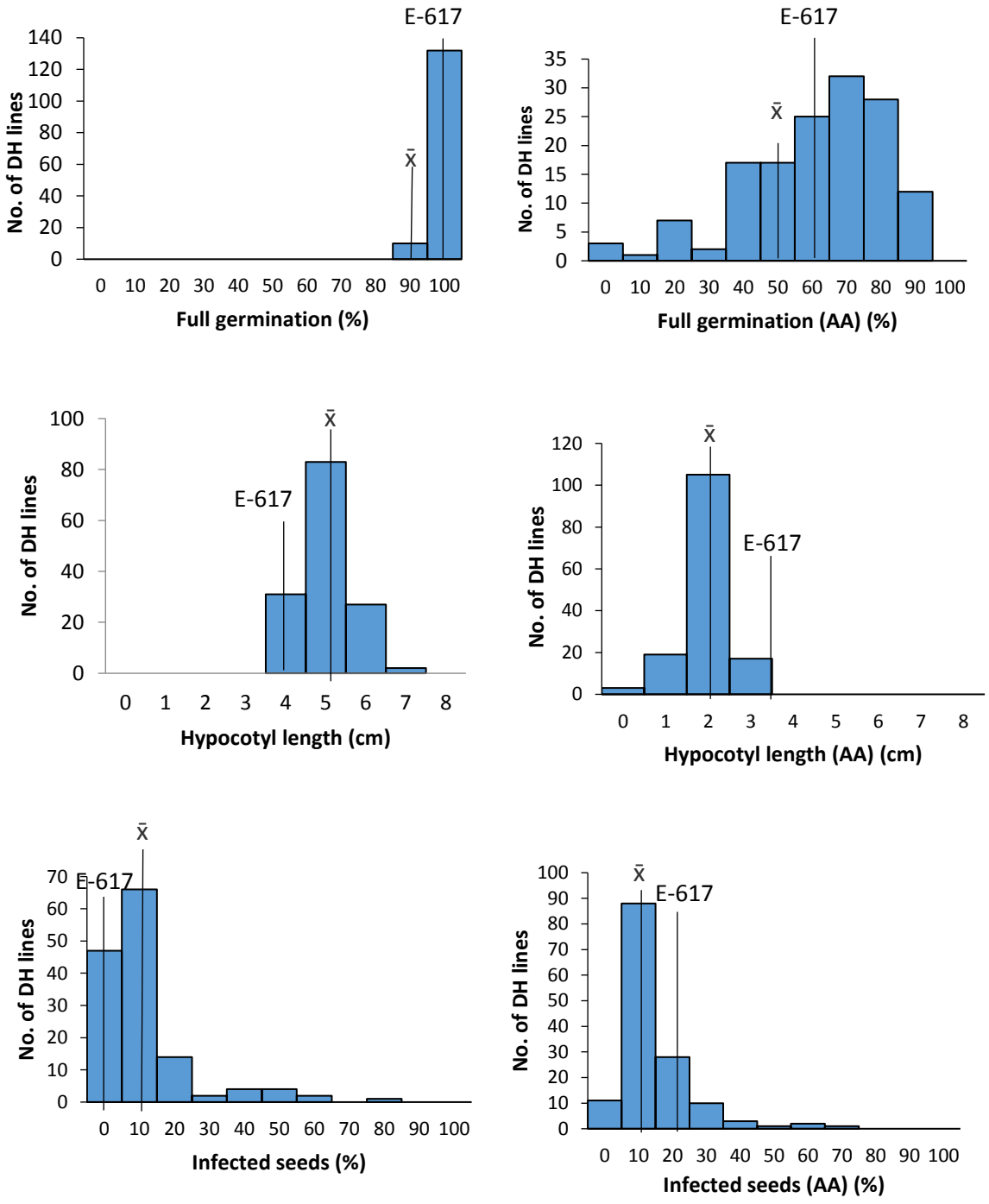


Fig. 15 Frequency distributions of mean values of seed germination traits of DH 1372 x Express 617 before and after aging for Reinshof 2015 & 2016 (n=140)

The percentage of seeds with radicle protrusion is very low. More than 50 % of the population has zero radicle protrusion percentage, and none of them exceeded 11 %. For full germination, 97 % of the DH population has 100% germination rate, and the lowest germination score is 87.5 %. The hypocotyl length ranged from 3.5 to 6.5 cm, with the highest frequency at 4.5 cm. The majority of

the population has zero to 20 % seed infection, and for the rest of the population, only very few genotypes has seed infection rate above 30 to 80 %. The value range of infected seed percentage is a bit reduced (maximum 90 %) but the average is increased from 7.8 to 10.1 %.

The percentage of seeds with radicle protrusion increased drastically after artificial aging treatment. Before the treatment, among the DH 1372 x Express 617 population, none of the genotypes has the percentage of radicle protrusion over 11 % (Fig 5). After seed aging treatment, the maximum value was raised to 45 %, with the most common (highest frequency) value is 20 % of radicle protrusion. Full germination percentage after aging has a very large variation, ranged from zero to 90%. Only three genotypes had failed to germinate at all, while the rest of the population managed to fully germinate with various degrees of success. It can be seen from the frequency distribution graph which skewed to the right that the majority of the population can retain their germination ability. The mean value for full germination percentage is 57.6 %, while the highest frequency is at 70 %.

In comparison to the range values of each trait, in this segregating population for the yellow seed color character, the Express 617 parent is found near to maximum value for oil, protein of defatted meal, fiber content, seed color, hypocotyl length after aging, and full germination percentage for both treatments, and near to the minimum value for protein, pre-harvest germination, seed germination traits (except full germination), and seed longevity traits (except full germination and hypocotyl length).

4.3.3 Spearman's rank correlations

The Spearman's ranks of correlation coefficients are given in Table 18. Naturally there are strong correlations among oil, protein, total oil & protein, and protein of defatted meal contents, since they are related to each other. From all three fiber component traits, only NDF value has positive correlation value, which is related to oil (0.33). Both NDF and ADF exhibit negative correlation to protein content (-0.41 and -0.26), and protein of defatted meal content (-0.22 and -0.29). It is interesting that ADL has no correlation at all to oil and protein traits, although the correlation values among the fiber components are strongly positive (0.78 to 0.96). However, there are no correlations between fiber component traits and total oil and protein content.

Table 18 Spearman's rank of correlations of DH 1372 x Express 617 traits (Reinshof 2015 & 2016)

	XL	XP	XLP	GSL	PDM	NDF	ADF	ADL	TSW	SC	PHG	HL	RPP	FGP	ISP	RPPA	FGPA	HLA
XP	**-0.74****																	
XLP	**0.82****	**-0.27****																
GSL	-0.18*	0.18	-0.12															
PDM	-0.18	**0.77****	-0.17	0.07														
NDF	**0.36****	**-0.41****	0.21*	0.00	**-0.27****													
ADF	-0.10	**-0.26****	-0.05	0.09	**-0.29****	**0.77****												
ADL	-0.04	-0.10	0.02	0.11	-0.12	**0.82****	**0.96****											
TSW	-0.03	0.04	-0.02	0.00	0.04	-0.07	-0.02	0.01										
SC	-0.09	0.10	0.12	0.13	0.17	**0.70****	**0.78****	**0.85****	0.03									
PHG	**-0.44****	**0.29****	**-0.44****	0.01	0.02	**-0.38****	-0.22*	**-0.25****	0.10	**-0.23****								
HL	-0.02	0.12	0.12	0.21*	0.03	0.06	0.08	0.10	0.05	0.10	0.01							
RPP	-0.14	0.04	-0.19*	-0.12	-0.05	**-0.34****	**-0.27****	**-0.32****	0.17	**-0.33****	**0.43****	0.08						
FGP	0.17	-0.05	0.23*	0.13	0.08	**0.37****	**0.29****	**0.35****	-0.12	**0.39****	**-0.46****	-0.04	**-0.88****					
ISP	-0.15	0.07	-0.18	-0.14	0.00	**-0.42****	**-0.42****	**-0.46****	0.04	**-0.46****	**0.29****	-0.13	**0.45****	**-0.55****				
RPPA	0.05	-0.06	0.01	-0.08	-0.01	0.06	-0.02	-0.02	-0.05	0.01	-0.01	-0.03	-0.01	-0.02	0.10			
FGPA	0.01	-0.11	-0.04	0.04	-0.15	-0.04	0.11	0.08	-0.01	0.01	0.14	0.08	**0.27****	**-0.30****	0.06	**-0.27****		
HLA	0.02	-0.06	-0.02	-0.04	-0.09	-0.15	-0.01	-0.04	0.09	-0.11	0.12	0.05	**0.30****	**-0.25****	0.01	**-0.26****	**0.54****	
ISPA	0.09	-0.03	0.08	-0.07	0.01	0.06	-0.04	-0.02	-0.02	0.00	-0.12	-0.13	-0.14	0.10	0.04	0.17	**-0.40****	**-0.33****

Abbreviation note

XL : oil content
XP : protein content
XLP : total oil and protein content
PDM : protein of defatted meal content
GSL : glucosinolate content
NDF : neutral detergent fiber
ADF : acid detergent fiber
ADL : acid detergent lignin
SC : seed color
PHG : pre-harvest germination
TSW : thousand seed weight
RPP : radicle protrusion percentage
FGP : full germination percentage
HL : hypocotyl length
ISP : infected seed percentage
RPPA : radicle protrusion percentage after aging
FGPA : full germination percentage after aging
HLA : hypocotyl length after aging
ISPA : infected seed percentage after aging

Seed color has weak but significant negative correlation to pre-harvest germination (-0.23). Therefore, yellow seeds would have higher percentage of pre-harvest germinated seeds in comparison to seeds of darker color. Black seeds are rarely containing pre-harvest germinated seeds. Other trait that significantly correlated to pre-harvest germination percentage is oil, protein, and total oil and protein content. The correlation is positive for protein content (0.29), and negative for oil and total oil and protein content (both -0.44). It implies that the seeds with high oil or total oil and protein content will have less percentage of pre-harvest germinated. But seeds with high protein content will have higher chance to contain pre-harvest germinated seeds.

It is interesting that the hypocotyl length trait has no significant correlation with four other germination traits (percentage of radicle protrusion, full germination, non-germination, seed infection). However, among these four traits, the correlations are all strongly significant. Full germination percentage is negatively correlated to radicle protrusion (-0.88) and infected seed percentage (-0.55). Meanwhile, radicle protrusion and infected seed percentages are positively correlated to each other (r= 0.45 - 0.49). Pre-harvest germination also significantly correlated to almost all seed germination traits, except hypocotyl length, but also no correlation to seed longevity. The correlation is negative for full germination percentage (-0.46), and positive for radicle protrusion and infected seed percentage.

There are significant correlations among most of seed longevity traits. In contrast to before aging results, hypocotyl length after aging is significantly correlated to all seed longevity traits, except radicle protrusion percentage. Radicle protrusion percentage only has weak but significant negative correlations with both full germination (-0.27) and hypocotyl length (-0.26). A strong positive correlation is found between full germination and hypocotyl length (0.54), and negative ones to infected seeds (-0.40).

Seed color very has strong positive correlation with all three fiber component traits (NDF, ADF, and ADL) (range of 0.70 to 0.85). Another trait, pre-harvest germination percentage, displayed negative significant correlations with these three traits, although much weaker (-0.22 to -0.38). Thousand Seed Weight (TSW), has no significant correlation with any other measured traits. In this population, the seed size has no influence either on seed quality traits or seed germination traits of both treatments (with and without aging). Seed color is also has no influence on seed size in this population.

There are no significant correlation between seed quality traits and seed germination traits, except for the fiber components. NDF, ADF, and ADL are significantly correlated to most seed germination

traits, including percentage of radicle protrusion, full germination, and seed infection. The exception is only for hypocotyl length, which has no correlations with any fiber components. The absence of correlation for hypocotyl length is perhaps because little variations were found within this trait (most seeds germinated well with shoot length range 3 - 7 cm). Positive correlations are found between full germination percentage and NDF, ADF, ADL (0.29 to 0.37), while for all other germination traits (percentages of radicle protrusion and infected seeds) the results are significantly negative but weak (-0.12 to -0.46). The seeds which contain high fiber inclined to have better germination rate. The similar seeds would also have lower percentage of seeds with radicle protrusion, and infected seeds.

Seed color also proved to affect the seed germination performance before aging treatment. Percentage of full germinated seeds is positively correlated (0.39) to seed color, while all other seed germination traits (percentages of seeds with radicle protrusion and infected seeds) are showing negative correlation values. Hypocotyl length is again has no significant correlation to seed color. It means that the darker seeds performed better in seed germination, although there is no significant relation found between seed color and the hypocotyl length.

There are no significant correlations between seed longevity traits and any seed quality traits, or with seed phenotypic traits. Seed color, especially, have no influence on seed longevity for DH 1372 x Express 617. However, between seed germination and seed longevity traits, there are weak but interesting significant correlations. Full germination percentage before aging is negatively correlated to both full germination (-0.30) and hypocotyl length after aging (-0.25). Radicle protrusion percentage before aging is positively correlated to full germination (0.27) and hypocotyl length (0.30) after aging.

4.4 DISCUSSION

4.4.1 Variation among traits

In this study, a doubled haploid population developed from a cross between a yellow seeded spring type DH 1372 of Canada origin and a black seeded winter type German cultivar of Express 617 was tested in Reinshof field experiment in the year 2015 and 2016. The population sizes were inconsistent for each data set due to several environmental factors, especially by their winter survival in the field. One of the parent material (DH 1372) is an oilseed rape spring type, thus some members of the offspring may have inherited its lack of winter hardiness. Winter types such as Express 617 cultivar are mostly grown in Western Europe, where winters are quite mild. They are sown in late summer and require a period of cold to set flowers (Nesi et al., 2008). On the contrary,

spring cultivars, such as DH 1372, are usually sown at the end of winter, predominate in northern latitudes (e.g., Eastern Europe, Canada, Asia) and Australia. In our field experiment, none of plants of the parent line DH 1372 survived in the field. However, there was no specific problem of the germination detected under controlled laboratory condition for the seed germination before aging treatment.

The variance revealed in this doubled haploid population was mostly significant for genotype and environment factors. The exceptions to this observation were for seed germination and seed longevity traits. Low heritability in seed germination traits may arose from narrow variation within the measured traits. Most seed samples from all locations and genotypes performed well in germination test. Almost all genotypes reached 100% germination. Regardless of the genotypes, these traits have no or little variation. However, genotype factor has some significant influence over the variability in hypocotyl length and the percentage of infected seed.

Infected seed percentage after aging is reduced by the value range (maximum 90 % instead of 100 %), but increased by mean value (10.1 % instead of 7.8 %). Perhaps the aging treatment of exposing seeds to higher temperature (45^{o}C) had killed some of the seed borne pathogens, but not by much. Higher humidity of 65 % RH may also somehow supported microbial growth.

The seed color trait is partly influenced by environmental factor, such as temperature (Van Deynze et al., 1993). Burbulis and Kott (2005) indicated that this was the case for DH line 1372. Together with some other sister lines, DH 1372 grown in Canada increased its seeds yellowness with high temperatures, and turned darker with cooler temperatures. The present study showed that both genotype and environment factors are significant for variance in seed color of DH 1372 x Express 617. The genotype effect on seed color trait is very strong with heritability 0.84, but a high occurrence of mixed seed color, even mottled seeds were observed. Rahman and McVetty (2011) mentioned that the seed color was often affected by the environment changes, thus sometimes resulting in darker seeds and/or black spots on the seed coat. Similar phenomenon of seeds exhibited half-black half-yellow color was also reported by Chen and Heneen (1992). Van Deynze et al. (1993) suggested that the high ambient temperatures may inhibit biochemical processes which lead to pigments' production and the thickening of secondary wall in rapeseed. In the case of the mottled seeds, it can be that the dark pigments were already accumulated in the seeds, since the pigment accumulation was started from seed formation. But on the last ripening month, the heat drastically goes up, diminishing the pigment input thus creating lighter spots on the dark seed coat. The opposite situation might lead to yellow seeds with dark spots. In this study, some genotypes,

especially which fall under score 5, 6 and 7 has mottled seeds, in which one single seed has mosaic color of both dark and yellow spots.

Seeds are living embryos, and over time cells die and cannot be replaced (Shaban, 2013). Therefore, the seed age may affect its germination ability. The seeds of both harvest years (2015 and 2016) showed a very good germination and vigor (SD 3.39 for full germination and 0.63 for hypocotyl length), despite of the storage time difference. The seeds harvested in 2015 were stored for longer period, more than a year (18 months old), while the ones harvested in 2016 were only 4 months old. However, the seeds of both populations were kept in a cold storage room which maintained at 4 ^{o}C. According to Ramiro et al. (1995) in the cold storage of 5 ^{o}C and 8 % RH, seeds of *B. montana* and *B. cretica* can maintain their viability up to 10-12 years. For long term seed preservation, FAO/IPGRI (1994) recommends the combination of storage temperature below 8 ^{o}C and 3 - 7 % moisture content.

4.4.2 Spearman's rank of correlation coefficients

Yellow seeds are generally believed to be related to high oil content in rapeseed (Jiang et al., 2007). This study did not find any association between seed color and oil or protein content. Many publications which showed higher oil content for yellow seeds compared to the black/brown seeds, suggesting the seed size difference as the main reason for increased oil content. Tang et al. (1997), for example, revealed 3 % differences between seed oil content of yellow vs dark seeds of same genetic background, and the seed coat ratio of yellow seeds was 4.2 % lower. Hu et al. (2013) observed a significant positive correlation (0.43) between the cotyledon ratio and seed size or TSW (Thousand Seed Weight). Neubert et al. (2013) mentioned that yellow-seeded oilseed rape has smaller seed size, and therefore the improved proportion between seed hull and endosperm will bring better seed oil and protein contents. Hu *et al*. (2013) stressed the importance of cotyledon ratio in relation to seed oil content, since around 80 -90 % seed oil is accumulated in cotyledon cells. They also confirmed that not all high oil content lines are yellow in color. Jiang et al. (2007) implied that the low oil content plants were belonging to the yellow-seeded group, and high oil content plants belonged to the brown-seeded one. Further, they recommended that the yellow seeded trait should not be considered as the sole character for high oil content in breeding.

Significant genotype variance was found for seed size trait in DH 1372 x Express 617 population, but no significant correlation was found between seed size and seed color. In fact, the seed size, represented by Thousand Seed Weight (TSW), was not correlated with any other measured traits. In this study, the seed size has no influence either on seed quality traits (oil, protein, glucosinolates

etc.), seed color, or seed germination traits of both treatments (seeds with and without aging). Bettey et al. (2000) similarly failed to find correlation between seed weight or size to seed germination percentage in *B. oleracea*.

From all three fiber component traits, the correlation values among the fiber components (NDF, ADF, and ADL) are strongly positive. NDF is positively correlated to seed oil content (0.36) and negatively correlated to protein content (0.41), while ADF is only negatively correlated to protein content (-0.26). Simbaya et al. (1995) found strong negative correlation (-0.71) of dietary seed fiber (unspecified of the type, most probably NDF) with protein content. Badani et al. (2006) also found significant negative correlations (-0.55 and -0.56) between ADF and protein content in two crosses of yellow vs black seeded lines.

NDF can be divided into hemicellulose, cellulose, and lignin; ADF into cellulose and lignin; and ADL into undigestible lignin (Von Soest et al., 1991). Simbaya et al. (1995) found identical non starch polysaccharide (NSP) profiles after comparing meal quality from yellow vs brown seeded oilseed rape cultivars. NSP (mostly cellulose and hemicellulose, largest element of NDF and ADF) is the biggest constituents of dietary fibers. Major differences were found for non-NSP fractions (such as lignin and polyphenols and cell wall protein). The different compositions of seed fiber constituents of selected parents may affect also the relationship of seed fiber and other seed quality characters. Therefore, research utilizing different kinds of parent cultivars may lead to slightly different result. Badani et al. (2006) was employing two segregating populations of yellow seed character as main objective, regardless of both parents' oil/protein contents. Tang et al. (1997) also revealed that the content of cellulose in yellow seed testa is significantly and consistently less than in the dark seeds, regardless of their genetic background (average differences are 17.74 %).

Despite significant correlations between NDF to oil (+) and both NDF and ADF to protein (-), these two fiber traits have no relation with total oil and protein content. Hu et al. (2013) mentioned that cotyledon cells have major contribution to seed oil content. In mature seeds, the cytoplasm of cotyledon cells was completely filled with oil and protein bodies. The more the oil bodies in the cytoplasm, the less the protein bodies were found. The capacity of the cytoplasm itself is relatively constant.

In this study, high correlations were revealed between seed color and seed fiber components (NDF, ADF, ADL). The seeds with high fiber contents are darker in color, and the seeds with low fiber contents have lighter seed color. Similar results were obtained by Badani et al. (2006), Burbulis and Kott (2005), and Wang et al. (2017). Yellow seed color and low fiber are coincided together, perhaps

because the biochemical pathways to pigment and lignin synthesis have common precursors (Nesi et al., 2008).

The seeds with high fiber contents tend to have lower percentage of pre-harvest germination, and vice versa, the seeds with less fiber contents are more likely to have higher pre-harvest seed germination percentage. Bewley et al. (2013) mentioned that some seeds may initiate the germination process or complete the emergence of the radicle out of the seed coat barrier, but fail on the next step to develop into normal seedlings and judged to be abnormal. Further, the seeds that failed to exhibit any visible signs of germination are considered to be dead. Another trait that significantly correlated to pre-harvest germination percentage is oil, protein, and total oil and protein content. The seeds with high oil or total oil and protein content will have less percentage of pre-harvest germinated. But seeds with high protein content will have higher chance to contain pre-harvest germinated seeds. Ruan et al. (2000) found reduced seed oil content in seeds contain high percentage of pre-emergence sprouting (also known as vivipary seeds) in hybrid oilseed rape, but it has no significant effect on seed protein content.

Pre-harvest germination in this study is significantly correlated to almost all seed germination traits, except hypocotyl length. It also has no correlation to seed longevity. This result is supported by Ruan et al. (2006) who also observed reduction in seed germination percentage and hypocotyl length in vivipary seeds. Ren and Bewley (1998) suggested that the testa structure of vivipary mutant seeds is altered, with thinner testa as a result of less secondary cell wall materials. Seeds of thinner testa are more prone to mechanical damage, which lead to higher ratio of abnormal or dead seeds and less viability, and also less protection against fungal/bacterial infections.

The only seed quality traits influencing seed germination are the fiber components. The seeds containing high fiber are inclined to have better full germination percentage. The similar seeds would also have lower percentage of seeds with radicle protrusion, non-germinating seeds, and infected seeds. The fiber component (NDF, ADF, ADL) contents constructing the largest part of seed coat or testa (Von Soest at al., 1997). The thicker the testa, the better will be its impermeability against mechanical damage which reduces seed viability (Neubert et al., 2003). Hypocotyl length, unlike other seed germination traits, has no correlations with any fiber components.

Seed color also proved to affect the seed germination performance. The darker seeds performed better in seed germination in comparison to yellow seed. This result is similar to Neubert et al. (2006), which also added that yellow oilseed rape seeds have reduced seed vigor (hypocotyl length)

and field emergence in breeding nurseries. In contrary, this study found no significant relation between seed color and the hypocotyl length.

In DH 1372 x Express 617, seed longevity traits are not associated with observed seed quality or seed morphology traits. In comparison, after 10 days of artificial seed aging test by Zhang et al. (2006), the yellow seeds almost lost their germination ability to zero, while the black seeds retained higher germination percentage of 32 %, and vigor index by 21 %. Seed longevity traits in this study were only correlated to the initial seed germination, before aging treatment. This is supported by Walters et al. (2010) who proposed that the seed aging rate depend on the seed moisture content, temperature, and initial seed quality. There might be other factors besides seed color and seed fiber contents which control seed longevity. The genetic basis of seed longevity is still unclear (Nguyen et al., 2012).

The seeds could accumulate some internal protection against desiccation: heat shock proteins, sugars, proteins (Mach, 2015), and enzymes (Wagner et al. 2012). Some of the aging harmful effects are associated with deterioration at membrane, nucleic acids and protein levels (Fujikura and Karssen, 1995). Protein age-damaged repairs mechanism could be responsible also for seed longevity. Eleven thermal-stable proteins were identified in high concentration in 1,300 years old viable sacred lotus seed (*Nelumbo nucifera*) (Shen-Miller et al., 2013). Ogé et al. (2008) confirmed that PIMT1 (protein l-isoaspartyl methyltransferase) overexpression would improve both seed longevity and germination vigor in *Arabidopsis*. In addition, accumulation of enzymes involves in free radical ROS (reactive oxygen species) scavenging in oilseed rape could also prolonged their seed storage potential (Wagner et al., 2012).

4.5 CONCLUSIONS

Yellow seed character is an important trait in oilseed rape breeding program. It was often represented higher seed oil and lower seed fiber contents, which are all desirable traits in oilseed rape. In the segregating population for the yellow seed color character of DH 1372 x Express 617 over 3 environments, there was no association of seed color to seed oil, protein, total oil and protein, and protein of defatted meal contents. The reason was perhaps because there was also no correlation between seed color and seed size or TSW (Thousand Seed Weight). Yellow seeds have better oil contents since they have thinner seed coat, which lead to better cotyledon ratio. Since there was no significant seed size differences between yellow and black seeds, the seed oil content of both seed color groups.

There is strong correlation between seed color and all three fiber content values (ADL, ADF, and ADL), but only NDF and ADF has significant correlations to seed oil and protein contents. Seed color is sometimes influenced by temperatures, resulted in occasional mottled seeds, but in general the seed color score is stable in different environments (h^2 = 0.94). Pre-harvest germination rate is associated many other traits, including seed fiber contents, seed color, and seed germination traits, except for hypocotyl length.

Before artificial seed aging treatment, yellow seeds has better germination rate compared to the black seeded genotypes. Similar condition also applied to seed fiber contents (NDF, ADF, ADL). Before artificial seed aging treatment, the seeds containing high fiber would show significant higher germination ability, has less radicle protrusion and seed infection compared to low fiber seeds. But after subjected to artificial seed aging, the association between seed longevity traits and seed fiber contents is gone.

In DH 1372 x Express 617, seed longevity traits were only correlated to the initial seed germination. These traits are not associated with any observed seed quality traits, such as seed oil, protein, total oil and protein, glucosinolates, protein of defatted meal contents) or seed morphology traits, such as seed color, TSW (Thousand Seed Weight), or pre-harvest germination percentage. Seed longevity traits in this study are perhaps controlled by other mechanisms other than seed color, which become the focus of this study.

It is necessary to continue this study with DNA extraction of the existing populations, KASP marker analysis utilizing BSA (Bulk Segregant Analysis) of two contrasting groups of high and low ADL content. For further investigation, an identification of candidate genes controlling the ADL content within this population will be essential.

5 INHERITANCE OF SEED QUALITATIVE CHARACTERS IN RELATION TO SEED GERMINATION AND SEED LONGEVITY IN NATURALLY AGED SEEDS OF DOUBLED HAPLOID WINTER OILSEED RAPE DH SOLLUX X GAOYOU

5.1 INTRODUCTION

Oilseed rape (*Brassica napus* L.) is an important agricultural crop in the last thirty years and has now become one of the world's largest sources of vegetable oil (Kennedy et al., 2011). Seed viability is one of the important issues which can potentially influence crop yield by disrupting seedling establishment, particularly under adverse environmental conditions. Some seeds can remain viable under optimal conditions for many years, and others for only a season cycle (Ghassemi-Golezani et al., 2010). Lutman and López-Granados (1998) mentioned that in Europe, the seeds of oilseed rape could re-emerge in the field as voluntary plants until 10 years or more.

Over the time, seeds would slowly deteriorate and gradually lose its viability. This process is defined as natural seed aging (Yin et al., 2015). In general, for any member of *Brassica* family, half-viability period is estimated to be 7.3 years under ambient storage conditions (20°C, 50% RH; Nagel and Börner, 2010). Artificial aging methods can be applied to mimic seed behavior in storage, since data of longer term stored seeds are rarely available. Therefore, more studies on seed longevity were based on artificial seed aging (Zhang et al*.,* 2006, Ku et al*,.* 2014, Yin et al*.,* 2015), which subjected the seeds to heat and high humidity to simulate the natural seed aging.

Seed longevity relates to how well seeds can retain its germination capability after being stored for certain time period. It is a complex trait (Bentsink et al., 2000) which genetic basis is still not well understood (Nguyen et al., 2012). Bentsink et al. (2000) and Clerkx et al. (2004) showed in *Arabidopsis* that seed longevity is controlled by several genetic factors, which act either through cell structure protection or damage recovery system. Clerkx et al. (2004) identified several quantitative trait loci affecting seed viability, which were located on different chromosomes. This finding suggested that seed longevity is a multigenic trait including various seed traits. Other molecular studies also support the idea of the complex genetic basis of seed longevity, as diverse mechanisms were documented to play a role. For example, *Arabidopsis* mutants affected in either flavonoid (Debeaujon et al., 2000) or tocopherol (Sattler et al., 2004) biosynthetic pathways, both mutant seeds displayed reduced longevity. Protection against reactive oxygen species (ROS) production is also said to be vital for *Arabidopsis* seed longevity (Clerkx et al., 2004). Other than genetic factors, environmental constraints during seed development may also affect seed germination and seedling emergence (Zhang et al., 2008).

Chinese landraces and European cultivars of oilseed rape belong to two distinct gene pools (Zhao et al., 2005). Combination of both gene pools could bring improvements for both regions. In this study, we utilized oilseed rape seeds of a doubled haploid population, originated from a cross between Sollux and Gaoyou, both are black-seeded oilseed rape cultivars. Sollux is an old German winter cultivar, and Gaoyou is a semi-winter Chinese cultivar from the Zhejiang Province. The open-pollinated seeds of DH Sollux x Gaoyou were harvested in both Germany and China in 2001 and have been kept for 13 years under ambient storage conditions in Germany. This study investigated the germination rate and the germination vigor of seeds of the DH population of Sollux x Gaoyou after thirteen years of storage in a normal seed storage room.

The aim of this research is to study the inheritance of seed longevity in the naturally aged seeds of a DH population and its correlation to seed quality traits which have been analyzed before (Zhao et al. 2005, 2012; Suprianto, 2014).

5.2 MATERIALS AND METHODS

5.2.1 Plant materials

The plant material used consisted of 291 genotypes of a doubled-haploid population developed from F1 cross of Sollux x Gaoyou cultivars. Sollux is an old winter oilseed cultivar from Germany, while Gaoyou is a semi-winter cultivar from China (Zhejiang province). Both cultivars are of black-seeded type. Previously in 2001, Zhao (2002) has grown and harvested the DH Sollux x Gaoyou population in field trials in four different locations. Two field experiments were located in Germany, i.e. Weende/Dragoner-Anger and Reinshof, and two others were located in China (Xian and Hangzhou). According to Zhao et al. (2005), Gaoyou cultivar is normally sown in autumn in China. However, it needs no vernalization and its winter hardiness is poor. In Germany, Gaoyou behaved more like a spring type. Both cultivars have high erucic acid, glucosinolate, and seed oil contents. The field experiment design used was randomized complete block design with two replicates for every genotype in each location. Zhao et al. (2005) also mentioned that the seeds were sown in double rows for each plot, with rows of 2.5-m length and a spacing of 0.33 m between rows. The plant distance within rows in Germany was 0.12 m and in China was 0.15 m. The seeds used in the present study were collected and bulked from 5 - 10 main racemes of open-pollinated plants.

5.2.2 Seed germination test after aging

After the drying, processing and cleaning, the harvested seeds from open-pollinated plants were ever since stored at the University of Göttingen in a normal seed storage room. The ambient storage temperatures varied from 7 to 25°C depending on the season.

Starting from July 2014 (Table 19), these naturally aged seeds were germinated. The seed germination protocol and data collection were as mentioned earlier in Chapter 3 and 4. Observations were carried out on day 10 of dark period, to count the radicle protrusion percentage (RPP), full germination percentage (FGP), hypocotyl length (HL), and infected seeds percentage (ISP), exactly as in Chapter 3. Fifty seeds per seed sample were germinated on customized filter paper in a Petri dish.

Table 19 Timetable for germination test of naturally aged seeds of DH Sollux x Gaoyou (harvested in four locations in 2001)

Replication	Location	No. of Genotype	Start Germination (9-10 days)	Start Counting
I	Hangzhou	1 (#1-90)	11. Jul 2014	21. Jul 2014
		2 (#191-185)	15. Jul 2014	25. Jul 2014
		3 (#186-291)	16. Jul 2014	26. Jul 2014
	Xian	1 (#1-140)	25. Jul 2014	4. Aug 2014
		2 (#141-291)	27. Jul 2014	7. Jul 2014
	Reinshof	1 (#1-100)	15. Aug 2014	25. Aug 2014
		2 (#101-200)	18. Aug 2014	28. Aug 2014
		3 (#201-291)	19. Aug 2014	29. Aug 2014
	Weende	1 (#1-140)	9. Sep 2014	19. Sep 2014
		2 (#141-291)	10. Sep 2014	20. Sep 2014
II (40 selected genotypes)	Hangzhou + Xian	80 (40 + 40)	24. Nov 2014	3. Dec 2014
	Reinshof + Weende	80 (40 + 40)	1. Dec 2014	11. Dec 2014

There are 291 genotypes of DH Sollux x Gaoyou that were tested for each location. The observation was split out in many batches, in average 100-150 samples per batch. After samples of the first replicate from all four locations was completed their germination tests and scored, the best and worst 20 genotypes were chosen. From the second replication seed batch, these 40 genotypes from each four locations were selected to undergo the second replicates of the seed germination test. The

results of both replicates (replicate 1 (n=291) and replicate 2 (n=40) was combined together, and the mean values of both replicates in four locations was then used in further analysis.

5.2.3 Seed germination test before aging

Beside naturally aged seeds, newly harvested seed material was also needed as a control treatment to represent initial seed viability before seed aging. Therefore, the self-pollinated seeds of DH Sollux x Gaoyou were sown again in the University of Göttingen green house to produce new seeds. Since there was no new seed stock available for this DH population, the old stock of self-pollinated seeds (harvested from green house in 2000) was sown in the green house at 23-24 November 2015. For each genotype, 2 – 3 seeds were sown on soil in small tray pots.

The germination rate was low, less than 50% of the total genotypes were successfully germinated on the first sowing. Re-sowing the non-germinating seeds was performed twice, 1 December and 9-10 December 2015. The germination of old seeds was at much slower rate than normal ones. For many genotypes, the prolonged radicle finally emerged from the soil in 3-4 weeks after sowing. For about 30% of the population, it was necessary to conduct germination in Petri dishes in controlled laboratory condition on January 2016, then after 1-2 weeks the seeds were transplanted to soil in tray pots.

The established plants were transferred to cold chamber for vernalization treatment in January and February 2016, and transferred back to the green house and moved to individual pots in April 2016. In the end, 258 out of 291 original genotypes were able to be maintained in the green house and harvested in July 2016. In regards of the parent materials, only Sollux survived and produced new seeds, while Gaoyou seeds by chance failed to germinate.

The new harvested seeds of DH Sollux x Gaoyou from the green house were then subjected to another seed germination test. Two replicates were performed according to the timetable of Table 20, and each replicates were divided into two batches. Most genotypes were represented by two or three plants, which harvested individually. Replicates for seed germination test were taken from seeds of the same genotypes but coming from different plants.

Table 20 Schedules of seed germination test before aging for DH Sollux x Gaoyou

Replication	Harvest period	Batch	Start Germination	Start Counting
1	7-11. Jul 2016	I	19. Aug 2016	29. Aug 2016
		II	23. Aug 2016	1. Sep 2016
2	7-11. Jul 2016	I	3. Sep 2016	12. Sep 2016
		II	6. Sep 2016	16. Sep 2016

5.2.4 Analytical analysis of seed quality traits

The analytical analysis of seed quality traits by NIRS (Near Infrared Spectroscopy) of DH Sollux Gaoyou seed samples were completed previously by Suprianto (2014), only for the seeds from Reinshof and Weende in 2001. The method was similar as explained in Chapter 3 and 4. The traits measured were oil, protein, total oil and protein, glucosinolates (all by 91 % dry matter base), protein of defatted meal, and fiber contents (NDF, ADF, ADL). Since both parent cultivars were of black seeded type, no seed coat color score was performed.

5.2.5 Statistical analysis

The analysis of variance and estimation of heritability for both germination experiments before and after natural aging was completed by PLABSTAT software (Utz, 2011). The natural aging germination has been conducted with two replicates of unequal sizes. The first replicate was using the whole population (n=291), and the second one was only of 40 selected genotypes. Both replicates were involving seeds from 4 environments (Xian, Hangzhou, Reinshof, and Weende). After conducting natural aging germination test for all 291 genotypes of DH Sollux x Gaoyou from four environments, the mean value of the observed traits were calculated. The 291 genotypes were sorted according to their full germination percentage. Twenty genotypes of the highest mean value and twenty of the lowest one was selected, and the seeds of the chosen genotypes from replication 2 of all four locations were subjected to the second replicates of seed germination test.

Analysis of variance and heritability estimations was performed involving the total 291 genotypes without replicate. The observation values for 40 genotypes with replicates were represented by its mean value across 2 replicates in ANOVA calculation. Therefore, the significance of the G x E interactions could not be tested. Environment was considered as the random variable. The ANOVA for natural aging germination test data across 4 environments were performed by using this model:

$$Y_{ij} = \mu + g_i + e_j + ge_{ij}$$

where Y is observation of genotype i in environment j; μ is general mean; gi and ej are the effects of genotype i and environment j; ge_{ij} is the interaction between genotype i with environment j. Heritability (h^2) was calculated as follow:

$$h^2 = \frac{\delta^2 G}{\delta^2 G + \frac{\delta^2 GE}{E}}$$

where $\sigma^2 g$ is variance component for genotype, $\sigma^2 e$ is variance component for environment and $\sigma^2 ge$ is variance component for interaction between genotype and environment.

The observation values for 40 replicated genotypes were analyzed separately by ANOVA model as followed:

$$Y_{ijk} = \mu + g_i + e_j + r_k + ge_{ij} + gr_{ik} + er_{jk} + ger_{ijk}$$

where Y is observation of genotype *i* in environment *j* and replicate *k*; μ is general mean; g_i, e_j and r_k are the effects of genotype *i*, environment *j* and replicate *k*; ge_{ij}, gr_{ik}, er_{jk} and ger_{ijk} are the interactions between genotype *i,* environment *j,* and replicate *k*. Heritability (h^2) was estimated by this formula:

$$h^2 = \frac{\delta^2 G}{\delta^2 G + \frac{\delta^2 GE}{E} + \frac{\delta^2 E}{ER}}$$

where $\sigma^2 g$ is variance component for genotype, $\sigma^2 e$ is variance component for environment, $\sigma^2 r$ is variance component for replication, $\sigma^2 ge$ is variance component for interaction between genotype and environment, E is number of environments and R is number of replicates.

The third analysis of variance and heritability estimations was performed for seed germination test of 258 genotypes harvested from the green house experiment. Two replicates were performed for every genotype. The significance of the G x R interactions could not be tested. Both genotype and replicate were considered as the random variable. The ANOVA was performed by using this model:

$$Y_{ik} = \mu + g_i + r_k + gr_{ik}$$

where Y is observation of genotype i in replicate k; μ is general mean; g_i and r_k are the effects of genotype i and replicate k; gr_{ik} is the interaction between genotype i with replicate k. Heritability (h^2) was calculated as this formula:

$$h^2 = \frac{\delta^2 G}{\delta^2 G + \frac{\delta^2 GR}{R}}$$

where $\sigma^2 g$ is variance component for genotype, $\sigma^2 r$ is variance component for replicate and $\sigma^2 gr$ is variance component for interaction between genotype and replicate, R is number of replicates.

In all the first and third ANOVA analyses, the genotype and/or environment and/or replicate are treated as random effect. The second ANOVA analysis treats the genotype as fixed effect, since the genotypes involved were intentionally selected previously, while environment and replicate were used as random effects.

Suprianto (2014) in an earlier study has measured the values of seed quality traits (seed oil, protein, total oil and protein, glucosinolates, protein of defatted meal, NDF, ADF, and ADL contents) of the same seeds of DH Sollux x Gaoyou by NIRS analysis. Seed samples and genotype identities were available for only 233 genotypes, and only for the two locations in Germany. Data from these 233 genotypes were used for estimating the Spearman's rank of correlation coefficients among traits, combining 3 data sets (seed quality, natural aging germination and fresh seed germination). The mean values of the genotypes across two German environments (Reinshof and Weende in 2001) of the first replicate were used to represent naturally aged seed germination and seed quality traits. Meanwhile, the values representing seed germination traits before aging were obtained from mean values of 2 replicates of seeds harvested from green house experiment in 2016.

5.3 RESULTS

5.3.1 Variation among traits

5.3.1.1 Seed germination traits after natural aging (n=291)

There were large and highly significant differences in the seed germination traits of the DH population after 13 years of storage (Table 21). High significant effects of the locations are observed, in all four measured traits for natural seed aging treatment. The variance components are all showing significant contributions of both genotype and location factor in the four germination traits.

Table 21 Variance components of seed germination traits in DH Sollux x Gaoyou (2001) from four environments

Source of variance	Genotype (G)	Environment (E)	G x E	Heritability (h^2)
Radical protrusion (%)	2.52**	2.24**	23.80	0.30
Full germination (%)	115.20**	17.10**	203.14	0.68
Hypocotyl length (cm)	0.24**	0.06**	0.57	0.63
Infected seeds (%)	4.85**	5.20**	34.47	0.36

** marked as significant at level P=0.01

Genotype plays dominant role for full germination and hypocotyl length, but percentage of infected seeds is influenced more by environment. Heritabilities are low for radicle protrusion (0.30) and infected seeds percentage (0.36), but relatively high for full germination percentage (0.68) and hypocotyl length (0.63).

Table 22 presents the descriptive statistics of the observed germination traits from naturally aged seeds of DH Sollux x Gaoyou harvested in four different environments. For radicle protrusion rate, the values of both parents are not much different. Radicle protrusion of Sollux seeds is 2.50 % of, and of Gaoyou is 3.00 %.

Table 22 Descriptive statistics of seed germination traits in DH Sollux x Gaoyou (2001) from four environments (no replicates)

Observed traits	Sollux (P1)	Gaoyou (P2)	Min	Max	Mean	SD	LSD5%
Radical protrusion (%)	2.50	3.00	0.00	16.00	5.12	0.03	6.77
Full germination (%)	1.75	21.50	0.00	57.25	18.34	0.13	20.26
Hypocotyl length (cm)	3.00	0.50	0.00	3.10	0.95	0.62	1.05
Infected seeds (%)	0.13	0.65	0.00	29.50	3.53	0.04	8.17

Full germination percentage is the most diverse among observed traits. After being stored in ambient storage condition for 13 years (2001 – 2014), the full germination rate varied between 0 - 57.25 %. However, in average, only 18.34 % of the oilseed rape seeds managed to attain full germination. The performance of both parents was displaying a contrasting result (Table 4). Sollux has very low germination (1.75 %) but high seed vigor (hypocotyl length 3.00 cm), while Gaoyou has better germination (21.50 %), but low vigor (0.50 cm). Seed infection rate in both parents are low, both under 1 %. The value varied between 0 to 29.50 %. However, the average values were much lower (3.53 %).

Seeds harvested from different environments might behave differently during germination test, after being kept for long period. Table 23 displayed the mean values of seed germination traits from seeds taken from four environments. The average full germination percentage is slightly higher in the China grown seeds (19.94 %) compared to the German ones (16.85 %). However, it was not always the case in each growing locations. The full germination rate of Weende seeds was 18.85 %, a little higher than of Xian seeds which were 15.78 %. Seed longevity of Hangzhou seeds is the highest at 24.09 %, while of Reinshof seeds were the lowest at 14.85 %. The seeds harvested in China in general contained very little seeds with radicle protrusion percentage (1.85 %) compared to the ones from Germany (5.92 %).

Hypocotyl length from germinated seeds from China is better compared to the ones harvested in Germany. In germination test of seeds from both locations in China, the mean values are either 1.00

cm (Hangzhou) or higher (1.26 cm of Xian), while the germinated seeds from both German locations were under 1 cm of length (0.69 cm of Reinshof and 0.87 cm of Weende).

Table 23 Mean values of DH Sollux x Gaoyou seed germination traits after natural aging (from each environment)

Mean value	Xian	Hangzhou	Reinshof	Weende	China	Germany	LSD 5%
Radical Protrusion (%)	3.16	0.54	6.85	4.98	1.85	5.92	6.77
Full germination (%)	15.78	24.09	14.85	18.85	19.94	16.85	20.26
Hypocotyl Length (cm)	1.26	1.00	0.69	0.87	1.13	0.78	1.05
Infected seeds (%)	1.63	6.70	3.79	2.03	4.17	2.91	8.17

The infection level varied from none to 29.50 %, but the average is quite low (3.53 %). Seeds from China has heavier infection rate (4.17%) than Germany (2.91 %), although the heaviest infection were only exhibited by seeds from Hangzhou with 6.70 %.

The performance of naturally aged seeds harvested in four different environments in germination test can be exemplified in Fig. 16. Genotype #40 and #74 are representatives of genotypes of low germination rate, while #111 and #206 represent the genotypes of good germination ability. Seed infection is formed by fungi or bacteria infestation. In some cases both pathogens, or even from few different strains at once were present on one Petri dish.

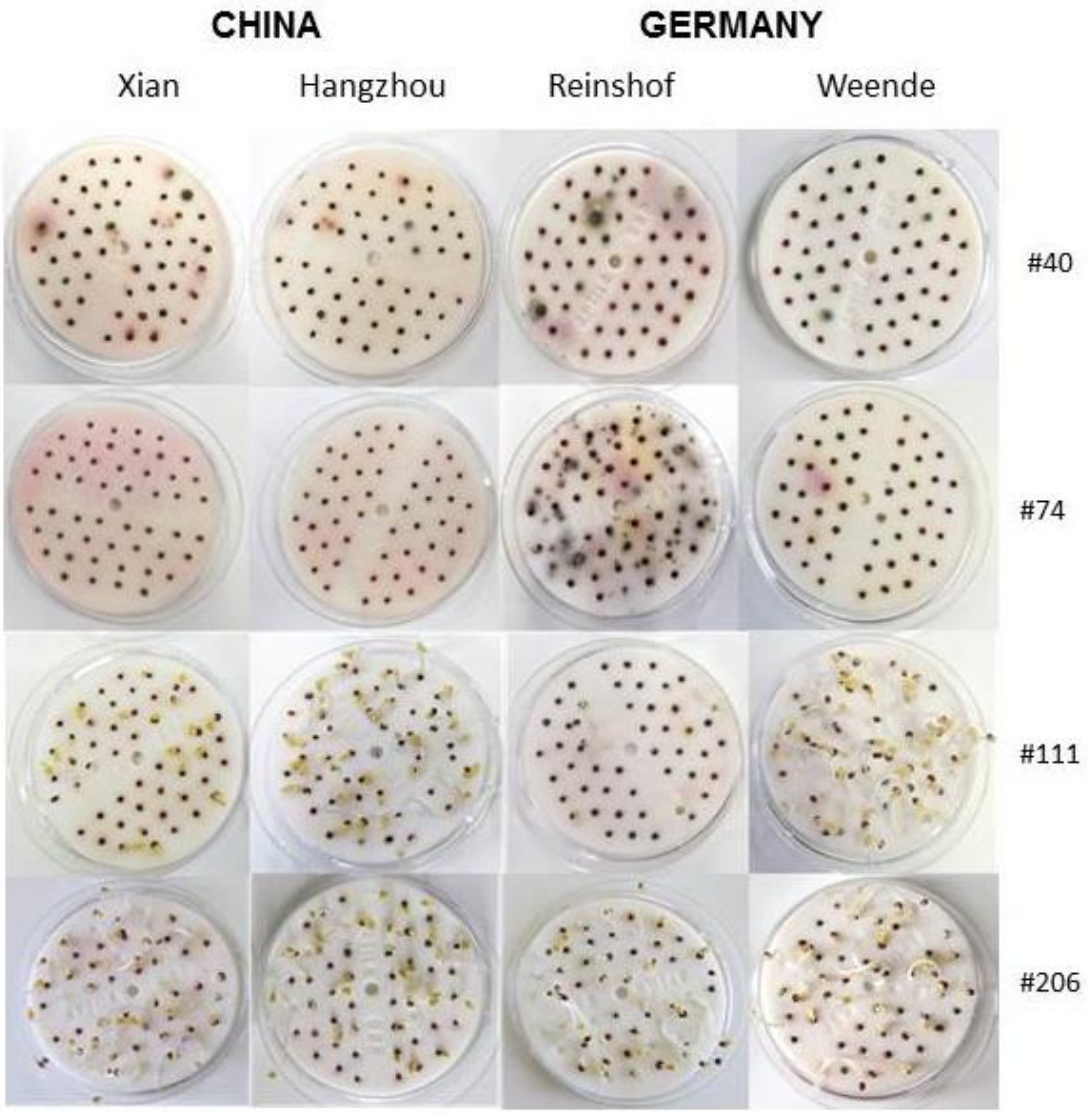


Fig. 16 Germination test of naturally aged seeds of DH Sollux x Gaoyou (harvested in 2001 in 4 locations)

Note: The two upper rows (#40 and #74) are representatives of genotypes with low germination percentage, and the two lower rows (#111 and #206) are representatives of genotypes with high germination percentage.

Gaoyou seeds from all four environments are in general having better germination rate than Sollux (Fig. 17) but the seedlings were mostly very short. Although the maximum length of hypocotyl length is 5 cm, 80 % of the population has less than 2 cm hypocotyl length (Table 22).

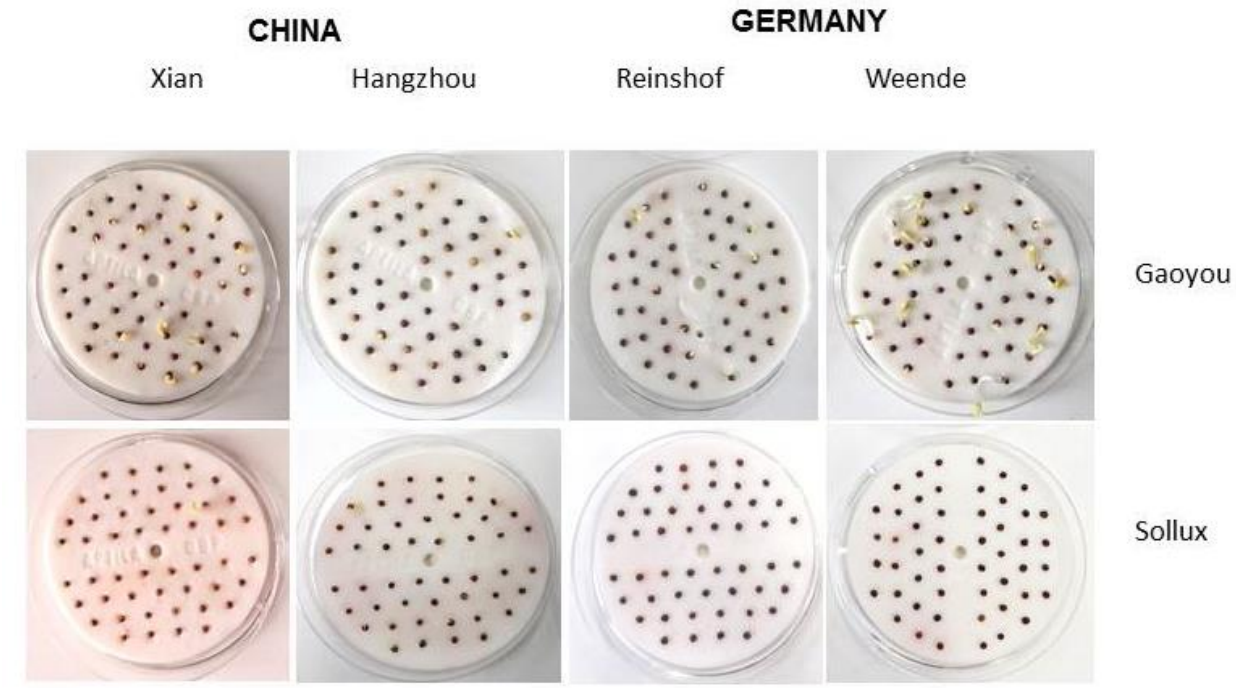


Fig. 17 The germination performance of 2 parental cultivars (Sollux and Gaoyou)

5.3.1.2 Seed germination traits after natural aging (n=40)

Twenty genotypes of the highest mean value and twenty of the lowest one from the first replicate of the previous population of total 291 genotypes was chosen to be included in the second replicate of germination test of naturally aged seeds. The low germination genotype group was ranged from zero to 3 % full germination, and the high germination group, consisted of genotypes with 40 – 70 % full germination percentage. Another analysis of variance component was performed separately. The ANOVA and descriptive statistics of the sub population of DH Sollux x Gaoyou (n=40) is displayed in Table 24 and 25 below.

Table 24 Variance components of seed germination traits of DH Sollux x Gaoyou (n=40)

Source of variance	Genotype (G)	Environment (E)	GE	Replicate (R)	Heritability (h^2)
Radical protrusion (%)	3.27**	0.25*	10.87**	0.66	0.70
Full germination (%)	447.90**	36.96**	263.07**	80.6	0.93
Hypocotyl length (cm)	1.04**	0.07**	0.75**	0.11	0.92
Infected seeds (%)	0.60	2.42**	1.56*	0.33	0.21

** marked as significant at level P=0.01

The variabilities within each trait were mostly higher than the initial population. All seed longevity traits showed dominant effects of genotype, except for seed infection rate, which was only influenced by environment factor. Variations among G x E in seed germination test were significant, except for seed infection rate. Heritabilities were high for full germination rate and hypocotyl length (0.93 and 0.92), moderate for radicle protrusion (0.70), but low for seed infection rate (0.21).

The value ranges across four environments for 40 genotypes were narrower than the total population of 291 genotypes in term of radicle protrusion and infected seeds percentages (Table 25). For full germination rate and hypocotyl length, the value ranges remained the same. Radicle protrusion percentage was ranged from none to 7.50 %, and for infected seeds was from none to 8.25 %. The average values, however, were mostly increased in this sub population of 40 genotypes, except for hypocotyl length which was reduced from initially 0.95 cm to 0.85 cm.

Table 25 Descriptive statistics of seed germination traits after natural aging of DH Sollux x Gaoyou over 4 environments (n=40, r=2)

Traits	Min	Max	Means	LSD 5%
Radical protrusion (%)	0.00	7.50	3.15	3.25
Full germination (%)	0.00	57.25	22.65	15.97
Hypocotyl length (cm)	0.00	3.10	1.13	0.85
Infected seeds (%)	0.00	8.25	2.60	4.24

Correlation coefficients between the values of selected 40 genotypes from the first and second replicates are shown in Table 26 below. The full germination percentage has the strongest correlation value (0.71) among all 4 measured germination traits. The value of hypocotyl length is also strongly correlated (0.59). On the other hand, weak and no correlations are subsequently found between 2 replicates of radicle protrusion (0.26) and infected seed rate (-0.05).

Table 26 Correlation coefficients between the first and second replicates (n=40) of DH Sollux x Gaoyou

RPP	FGP	ISP	HL
0.26	0.71	-0.05	0.59

5.3.1.3 Seed germination traits before natural aging

Among 258 genotypes of DH Sollux x Gaoyou, generated from the green house experiment, some variation in seed germination traits was detected. Full germination, hypocotyl length and infected seeds percentage showed significant genotype effects in the analysis of variance (Table 27). Meanwhile, significant replicate effects were displayed only by hypocotyl length and percentage of infected seeds.

Table 27 Variance component of seed germination traits before aging of DH Sollux x Gaoyou (n=258)

Source of variance	Genotype (G)	Replicate (R)	G x R	Heritability (h^2)
Radical protrusion (%)	0.13*	0.00	0.85	0.24
Full germination (%)	17.63**	0.16	43.30	0.45
Hypocotyl length (cm)	0.40**	0.85**	1.16	0.41
Infected seeds (%)	30.37**	3.60**	62.92	0.49

** marked as significant at level P=0.01

Genotype was a dominant factor for radicle protrusion, full germination and infected seeds percentage, but hypocotyl length was more determined by replicate factor. G x R interaction values were high for both full germination and infected seeds percentages. Heritability estimations were low for all traits. Heritability of radicle protrusion was 0.24, full germination was 0.45, hypocotyl length was 0.41, and infected seeds percentage was 0.49.

The values of the parent cultivar, minimum, maximum, average, and LSD 5 % of the seed germination traits measured for DH Sollux x Gaoyou population of 258 genotypes before aging treatment is included in Table 28. Unfortunately, Gaoyou cultivar has no seeds available from seed regeneration experiment in the green house.

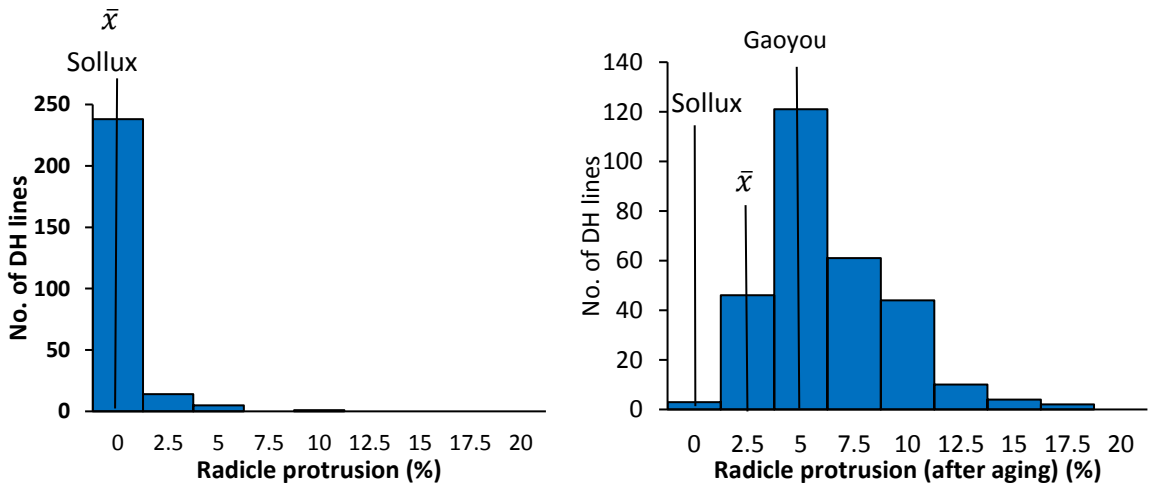
Sollux seeds showed perfect germination (100 % germination rate). The germinated seeds of Sollux cultivar had average hypocotyl length of 5.5 cm, and the seed infection level of 4.0 %. Among 258 genotypes utilized in this experiment, most of them were germinating well. Although the radicle protrusion rate were between zero to 6.0 %, but the mean value was nearly zero (0.2 %). The average for full germination percentage was 98.7 % (range 52.0 – 100 %), for hypocotyl length was 5.5 cm (range 2.4 - 8.3 cm), and infected seeds percentage was 5.2 % (range 0 - 72.6 %).

Table 28 Descriptive statistics of seed germination traits before aging of DH Sollux x Gaoyou (n=258)

Traits	Sollux (P1)	Gaoyou (P2)	Min	Max	Means	LSD 5%
Radical protrusion (%)	0.0	-	0.0	6.0	0.2	1.81
Full germination (%)	100.0	-	52.0	100.0	98.7	12.97
Hypocotyl length (cm)	5.5	-	2.4	8.3	5.5	2.12
Infected seeds (%)	4.0	-	0.0	72.6	5.2	15.63

5.3.2 Frequency distribution

Frequency distribution graphs were generated in order to get better perspective of the variations of seed germination traits of DH Sollux x Gaoyou (Fig. 18). The graphs represented the mean values of radicle protrusion, full germination, hypocotyl length and infected seeds percentage of DH Sollux x Gaoyou from seed germination test before and after natural seed aging. The seed germination test before aging was involving 258 genotypes and 2 replicates. Meanwhile, the seed germination test after aging was using 291 genotypes, average from 4 environments and 2 replicates. Some of the traits presented here are not displaying normal frequency distribution. Some data transformation efforts have been performed, but the frequency distribution remained more or less the same. Therefore, these frequency distribution graphs are generated by original data.



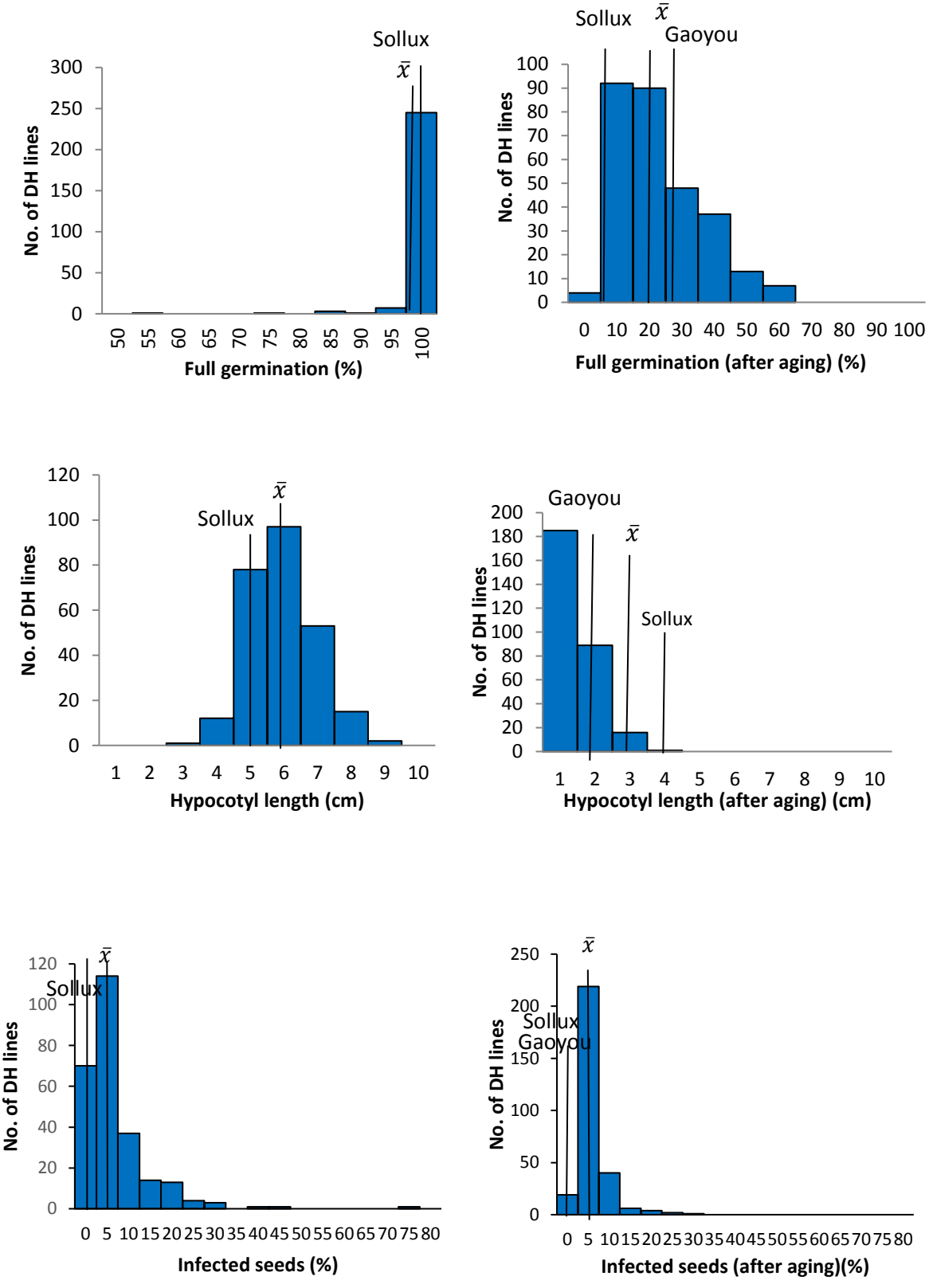


Fig. 18 Frequency distribution of DH Sollux x Gaoyou mean values of seed germination traits before and after natural aging (n = 258).

The radicle protrusion percentage before aging was very low. More than 90 % of the genotypes were exhibited under 1 % radicle protrusion. Its highest value was less than 5 %. After natural aging of 13 years, the rate increased significantly. Almost none of the genotypes have zero radicle protrusion rates anymore. This trait made a normal frequency distribution that skewed to the left, with the highest frequency at 5 %, and none above 17.5 %.

In seed germination test before aging, 13 genotypes showed less than 98 % germination rate from total 258 genotypes tested, or 5 % of total population. For these genotypes, another replication of germination test was performed to test whether the result is persistent. The mean values of these two replicates were then used as final result. Among 258 genotypes, only one has 55 % germination rate, one has 75 %, and three has 80 %. Two percent of the total population has less than 95 % germination rate.

Before aging treatment, the full germination percentage was mostly near 100 %. But after 13 years of storage, the seeds deteriorated gradually and lost their viability. Although very few genotypes showed zero germination, the highest frequency distribution was under 20 %.

The hypocotyl length frequency distribution graph showed a balanced normal distribution before aging, and more skewed to the left after aging. The values were also reduced from 3 – 9 cm to below 4 cm. There were some outliers in seed infection trait, but most of the population before aging treatment has less than 30 % infection rate. After aging, again there were some outliers, but the majority of the population has less infection level, all below 12.5 %.

5.3.3 Correlation between seed quality traits and seed germination traits

Only 233 genotypes were available in all three data observations: seed quality (mean value of 2 German environments), natural aging seed germination (mean value of 2 German environments), and seed germination before aging (mean value of 2 replicates). The data set of 233 genotypes of DH Sollux x Gaoyou were then used to generate the Spearman's rank of correlation coefficients, in order to find the associations among seed quality traits, seed germination traits, and seed longevity traits. The seed quality traits were measured by Suprianto (2014) only for seeds harvested from German locations. Table 29 displayed the correlation values of the observed traits from 233 genotypes of DH Sollux x Gaoyou, grown in two German environments (Reinshof and Weende).

Seed fiber components were all associated positively with seed oil content (0.20 – 0.27), negatively with seed protein (-0.26 to -0.39) and protein of defatted meal content (-0.25 to -0.43), but there

were no correlations to total oil and protein content. Among seed fiber content (NDF, ADF, ADL), interestingly only ADF and ADL are correlated (0.68). Glucosinolates content was only correlated to NDF content (-0.43).

There are four traits measured for seed germination traits before aging: radicle protrusion, full germination, hypocotyl length, and infected seeds percentage. Among these four traits, the only association found was between full germination and radicle protrusion percentages (0.25). Radicle protrusion rate was also had weak but significant correlation to NDF content (-0.18).

The same four traits were measured in seed germination test for naturally aged seeds, also called as seed longevity traits. Among these traits, radicle protrusion has positive association to hypocotyl length (0.35) and full germination rate (0.50). Full germination rate also have strong positive correlation to hypocotyl length (0.75). Percentage of seed infection after aging was only found to be correlated to full germination percentage after aging (-0.18) and to NDF content (0.20).

Table 29 Spearman's rank of correlation of DH Sollux x Gaoyou (n=233)

ADF	0.12														
ADL	0.09	**0.68****													
XL	**0.24****	**0.20****	**0.27****												
XP	**-0.39****	**-0.26****	**-0.39****	**-0.84****											
XLP	-0.01	0.04	0.05	**0.77****	**-0.35****										
GSL	**-0.38****	0.11	0.06	-0.06	0.12	0.03									
PDM	**-0.43****	**-0.25****	**-0.39****	**-0.51****	**0.88****	0.09	0.15*								
HLA	-0.06	0.09	0.12	-0.07	0.07	-0.07	0.04	0.04							
RPPA	0.03	-0.01	0.08	-0.04	0.03	-0.06	0.06	0.02	**0.35****						
FGPA	-0.05	0.00	0.10	-0.05	0.07	-0.02	0.02	0.07	**0.75****	**0.50****					
ISPA	**0.20****	0.01	0.05	-0.02	-0.04	-0.04	-0.07	-0.10	-0.12	-0.07	**-0.18****				
RPP	**-0.18****	-0.11	-0.05	0.04	0.03	0.10	0.04	0.07	-0.01	-0.13*	-0.05	-0.06			
FGP	0.02	0.03	0.02	0.07	-0.08	0.04	0.04	-0.06	0.04	-0.02	0.02	-0.08	**0.25****		
ISP	-0.15*	-0.01	-0.03	0.11	-0.05	0.15*	-0.04	0.01	-0.04	-0.13*	-0.08	-0.09	0.14*	0.08	
HL	0.00	-0.03	0.05	0.14*	-0.14*	0.09	-0.06	-0.12	0.09	-0.05	0.12	-0.05	0.07	-0.04	0.04
	NDF	**ADF**	**ADL**	**XL**	**XP**	**XLP**	**GSL**	**PDM**	**HLA**	**RPPA**	**FGPA**	**ISPA**	**RPP**	**FGP**	**ISP**

** marked as significant at level P=0.01

Abbreviation notes

XL : oil content
XP : protein content
XLP : total oil and protein content
PDM : protein of defatted meal content
GSL : glucosinolate content
NDF : neutral detergent fiber
ADF : acid detergent fiber
ADL : acid detergent lignin
RPP : radicle protrusion percentage
FGP : full germination percentage
HL : hypocotyl length
ISP : infected seed percentage
RPPA : radicle protrusion percentage after aging
FGPA : full germination percentage after aging
HLA : hypocotyl length after aging
ISPA : infected seed percentage after aging

5.4 DISCUSSION

5.4.1 Variation among traits

Seed longevity is a quantitative trait in which variations among accessions are commonly raised (Nguyen et al., 2012). The variance components of 291 genotypes of DH Sollux x Gaoyou harvested from 4 environments after 13 years of storage were showing significant contributions of both genotype and environment. Nagel et al. (2011) agreed that seed germination rate was in part genetically determined. The second group of selected 40 genotypes and two replicates mostly has higher variabilities within each trait than the initial population. For example, in the first ANOVA (n=291), for radicle protrusion heritability is low (0.30), but improves to moderate (0.70) in the second ANOVA. The sub population of 40 genotypes was consisted of selected genotypes of 2 extremes, eliminated the intermediates, and then added with another replicate. Identical with the result of the original population, in the second ANOVA all seed longevity traits showed dominant effects of genotype, except for seed infection rate, which was only influenced by environment. In the second ANOVA, it was found that variations among replicates were significant, and their effects were larger than environment, except for seed infection rate.

Leimu et al. (2006) stated that there are generally positive close relationships between population size, genetic variation, and fitness. Therefore, population size should always be taken into account in multi-population studies of genetic variation. Small populations would suffer negative consequences of reduced genetic variation due to loss of rare alleles through genetic drift. In comparison to this study, Sorensen and Gill (1984) practiced disruptive selection (selection of two extremes) in *Drosophila* for 3 generations, and the heritability rose from 0.37 to 0.68. The heritability was declined after a random mating event.

The seed infection rate trait has interesting result. In the first ANOVA, utilizing 291 genotypes, both genotype and environment factor are significant, and environment played a bigger role. The second ANOVA showed that this trait was influenced only by environment. Both analyses resulted in low heritability values. Although at times resistant genotypes may have less seed infection, in this case seed infection rate depend a lot on growing environment conditions: climate, humidity, temperature, and severity of bacterial or fungi infection on the field. Both parent cultivars (Sollux and Gaoyou) exhibited very low (less than 1 %) seed infection rate after aging. Water splash in wet, windy conditions favors the bacteria dispersal from droplets and the rapid disease spread among field crops (Kocks et al., 1999). Roberts et al. (1999) added that seeds that already infected from the field may carry the initial inoculum and expressed during germination. It becomes a critical factor which determines the infection severity, and will vary within and between seed lots.

Sollux has very low germination rate (1.75 %) but high seed vigor (hypocotyl length 3.00 cm), while Gaoyou has better result germination rate (21.50 %), but low vigor (0.50 cm). In all four environments, Sollux seeds in general had better germination rate than Gaoyou but the seedlings were mostly very short. Although some of the genotypes showed better performance than the parents, but in general the germination rate and hypocotyl length of this population is low. By comparison, the average *B. napus* seed viability gradually dropped to 92.9, 79.1 and 65.7 % after 7, 10 and 26 years of storage in IPK Gatesleben, respectively (Nagel et al., 2011). However, they were kept in controlled storage room of 7 ± 3 ^{o}C and 6 ± 2 % RH.

Strong correlations are found for full germination rate and hypocotyl length between the measurement results of replicate 1 and 2 (n=40) over 4 different environments. On the other hand, radicle protrusion and infected seed rate have subsequently weak and no correlations at all. These somehow correspond to the second ANOVA result, which shows highest heritability (above 0.90) for both full germination rate and hypocotyl length. Among 4 traits, infected seed percentage is the only one with no significant genotype influence. Radicle protrusion rate (R=0.26) has strong heritability (0.70) and significant genotype role, but the performance among different environments are not significantly different. Since the correlation value was estimated from individual data of each one of 4 environments, the lack of environment role for radicle protrusion rate made the correlation between the two replicates weak.

The seed longevity performance found in this study after 13 years of storage was better than what Nguyen et al. (2012) observed in Arabidopsis seeds of different natural aging period. The eleven years old seeds of Arabidopsis failed to germinate at all, even after water imbibition treatment of 30 days. Two other naturally aged seed samples in Nguyen et al. (2012) experiment were 7 and 8 years old, and presented maximum germination of about 45 % and 23 %, respectively.

In soybean, germination capacity was obtained early during seed filling, but seed longevity reached maximum at later stage during maturation, and progressively doubled until the seeds reached the dry state (Pereira Lima et al., 2017). Walters et al. (2010) mentioned that seed longevity can be determined by seed moisture, storage temperature and seed traits that are influenced by genetic and environmental interactions during seed maturation and harvest. Interactions among these factors are believed to contribute to the wide variation observed within and among seed lots and species. Zhao et al. (2005) explained that during growing period in 2000 - 2001, the climate at the Germany locations was 1 – 4 ^{o}C lower than at both China locations. The average total growth periods in Germany were 84 days longer, and the plants grown at Xian was 8 days longer than at Hangzhou. The growth periods from flowering to maturity were 72, 58, and 55 days at Germany, Xian, and Hangzhou respectively. On average, the Chinese parent, Gaoyou, was 25 and 15 days earlier in flowering and maturity, respectively, than Sollux.

The highest seed germination rate was found in Hangzhou grown seeds, which has the shortest growing period. However, seeds from Xian, which also matured at shorter growth period, have lower germination rate than German grown seeds. Pereira Lima et al. (2017) discovered 18 % reduction of P50 (half-life viability) on the soybean seeds harvested from hotter year with 3.7^{o}C temperature increase. Zanakis et al. (1994) suggested that delaying harvest could increase the risk of rapid deterioration of mature seeds in the field due to high humidity and temperature. Through physiological, sugar and transcriptome analysis, Pereira Lima et al. (2017) also found that seed maturation has not fully stopped at physiological maturity, and an extra 14 days period after physiological maturity would be beneficial to achieve maximum longevity.

By definition, germination of a seed begins with the water uptake, and is completed with the embryo emergence, in most species it is the radicle first, through the surrounding structure. Thereafter, the seed is considered as having germinated (sometimes termed 'visible germination', Nonogaki et al. 2010). Only 258 genotypes of the old seeds of 2001 were able to germinate in the green house and grown into mature plants which produced seeds. Full germination, hypocotyl length and infected seeds percentage showed significant genotype effects in the analysis of variance. Genotype was a dominant factor for radicle protrusion, full germination and infected seeds percentage, but hypocotyl length was more determined by replicate factor. Heritability estimations were low for all traits.

It was obvious that most of the 258 genotypes utilized in the fresh seed germination test were germinating well. The germination rate was approaching 100 %. The radicle protrusion and infected seeds percentages were very low, and the germinated seeds showed vigorous hypocotyl length. Nevertheless, the G x R interaction was high for full germination and infected seeds percentages. High G x R interaction indicates that some genotypes performed differently in another replicate. The seed were harvested gradually throughout 5 days period (7-11 July 2016) (Table 20). The first batch of germination test was executed on 19 August 2016, nearly 6 weeks after the last day of harvesting. According to post harvest ripening theory (Adolphe, 1979), freshly harvested seeds were still metabolically active, so their heat production and respiration were high. This period of active respiration may continue up to six weeks after harvest. Within this period, the seeds were largely dormant. It was possible that some genotypes on the first batch of germination test were harvested on later days. The seed dormancy might inhibit their seed germination performance on the first replicate, but on the second replicate (2 weeks after the first) these genotypes displayed optimal seed germination.

5.4.2 Spearman's rank of correlation coefficients among traits

This study found a strong positive association for radicle protrusion and full germination rate (0.50) after natural aging. It was also positively correlated to hypocotyl length after aging (0.35). It was

worth noted that after natural aging, both the mean values of seed germination rate and hypocotyl length were very low (18.34 % and 0.95 cm, respectively). The majority of the seeds were not germinated at all. A seed with radicle protrusion stands for incomplete germination, in which the radicle has elongated and pierced the seed coat barrier, but cotyledons were still within the seed coat. The presence of seeds with radicle protrusion in the germinated seed samples was still a sign that the seeds were somehow viable, even if not able to fully complete the germination process.

None of seed longevity traits had significant associations with any of seed quality traits, except percentage of seed infection after aging to NDF content. Likewise, Nagel *et al.* (2015) did not find any correlations between seed deterioration and seed oil, protein, and tocopherol-related compound contents in an artificial seed aging test in barley.

NDF (Neutral Detergent Fiber) is the only seed fiber components in this study that associated with any seed germination or longevity traits. What made NDF different from both ADF and ADL was that NDF contained the hemicellulose content (Von Soest, 1991). Pereira-Lima et al., (2017) added that seed longevity was also associated with the presence of raffinose family oligosaccharides (RFO), which possibly involved in protection against oxidative damage during storage. RFOs are known as protectant agent against seed desiccation (Sengupta et al., 2015). Galactinol, the precursor of RFO, was also found to be a marker for seed longevity in Arabidopsis, cabbage and tomato (de Souza Vidigal et al., 2016). In this study, NDF was only associated with radicle protrusion rate before aging and infected seeds rate after aging, which might be connected to the seed coat protection against adverse environment or pathogen infection.

There are several reasons why it has been so difficult to identify the key events to the completion of germination. Some reasons relate to the nature of the seed itself. It is a multi-cellular organism in which the major cell mass is storage tissue. There is limited mobilization of reserves during germination, perhaps to provide a source of sugars and amino acids, but this is very small compared to reserve utilization following germination (Nonogaki et al., 2010).

Many further works can still be done following this study. QTLs analysis regarding seed longevity traits of this population is in progress. The new seeds of DH Sollux x Gaoyou from the green house experiment were sown in Reinshof field experiment and will be harvested in August 2017. The acquired seeds could be subjected to artificial seed aging like similar experiments of Chapter 3 and 4. Seed companies in general rely more on artificial aging to predict seed storability. However, Nagel et al. (2011) proved that even a small change in controlled deterioration protocols, such as 2 ^{o}C temperature increase, can give major impacts to the expression of relevant genes. Therefore, a comparison study to investigate the physiological and molecular mechanisms of both natural and artificial seed aging would be appreciated.

Seed longevity study utilizing long term (more than 10 years) naturally aged seeds, especially in oilseed rape, from ambient room temperature storage condition, are still rare. Nagel et al. (2011) provide *B. napus* seed longevity data from naturally aged seeds of up to 26 years old, but those seeds were kept in cold storage room under 10^{o}C. More studies were available in other crops, such as Arabidopsis of four year storage (Debeaujon et al., 2000 and Bentsink et al., 2000), several years in 5^{o}C refrigerator (Rajjou et al., 2008), 20 months (Clerkx et al., 2016), also in sunflower and soybean, 6 and 12 months (Balesevic-Tubic et al. 2010), and soybean, 120 days (Kruger-Giurizzato et al., 2012).

5.5 CONCLUSIONS

Diverse variations were found among seed germination traits before and after natural seed aging in the population of DH Sollux x Gaoyou. In average, the seed germination rate and hypocotyl length after aging were low. Radicle protrusion percentage was positively correlated to full germination percentage and hypocotyl length, perhaps due to this low germination issue. In contrast, the mean value of seed germination rate of DH Sollux x Gaoyou population before natural aging treatment is nearly 100 %.

The first ANOVA for the original population (n=291 genotypes, no replicates) over 4 environments exhibits significant variabilities for both genotype and environmental effects, but low and moderate heritability. The second ANOVA for second population (40 genotypes, 2 replicates) over the same 4 environments resulted in higher variability in genotype, environment, and replicate, also higher heritability for radicle protrusion, full germination percentage, and hypocotyl length.

After natural seed aging, the seedlings from the China grown seeds have better hypocotyl length than the German ones. The seed physiological maturity may play some role in this matter, since China seeds were grown in warmer temperatures and have shorter growing period than the Germany seeds.

Seed infection in naturally aged seeds of DH Sollux x Gaoyou was most probably controlled only by environmental or replication effect. Genotype effects were significant for full germination percentage, hypocotyl length, and infected seeds percentage. The heritabilities found were very low, due to narrow variabilities within traits.

No correlation was found among any measured seed quality traits with seed germination or seed longevity traits, except weak but significant association to NDF content. NDF was found to be correlated to radicle protrusion percentage before aging, and infected seeds percentage after aging. NDF might be connected to the seed coat characteristics as the seed main protection mechanism against adverse environment and pathogen infection.

6 GENERAL DISCUSSIONS

6.1 Variations among traits

The first two doubled haploid populations (DH population of 4042 x Express 617 and DH 1372 x Express 617) were originated from a cross between yellow and black seeded types. They both have higher proportion of yellow seeded genotypes than the black ones. The first population, DH population of 4042 x Express 617 contains 63.6 % yellow seeded genotypes from the total 77 genotypes. DH 1372 x Express 617, by comparison, have higher ratio of 72.4 % yellow seeded genotypes (105 yellow and 40 black seeded genotypes). These findings are in the contrary to Van Deynze and Pauls (1994) report which said that black seed was dominant over yellow. Controlled by 3 independent genes, the yellow seeds were produced only when all three loci were in the recessive homozygosity. In both of our populations, the higher ratio of yellow seeded genotypes can be just coincidence. During the development of doubled haploid population, there might be more of the yellow seeded genotypes over the black ones that survived from the plantlets generated from F1 microspore culture. Temperature has been reported to also affect the seed color of parent DH 1372 (Burbulis and Kott, 2010). This perhaps explains why there are more mixed color seeds or mottled seeds in DH 1372 x Express 617. Both populations of DH population of 4042 x Express 617 and DH 1372 x Express 617 in general exhibited stable seed color in different environments (h^2 = 0.94 and 0.84, respectively).

The inheritance of yellow seed color in *B. napus* is complex. It shows both dominant and recessive effects, and influenced by intra-and inter-genomic actions (Zhang et al., 2011). Mumtaz et al. (2015) came to the same conclusions in a review comparing previous studies in several Brassica species. Conflicted theories exist of either epistasis, partial dominance, or total dominance, controlled by 1 - 3 genes. Zhang et al. (2009), so far gave the most comprehensive QTLs study for *B. napus* seed color inheritance. They suggested that yellow seed color (Y gene) is partially dominant over black (controlled by B and C genes), with two or three dominance epistasis ratio (also supported by Liu et al., 2005 and Badani et al., 2006). In some other crosses, however, black is dominant over yellow seeds. A new dominant D gene was revealed, which inhibit the action of a previously dominant yellow gene, resulting in black seed color. They concluded that the inheritance of seed color in *B. napus* is controlled by at least 4 genes, an intricate combination of B, C, Y and inhibitor D genes.

In both populations of Chapter 3 and 4, the correlations between seed color and ADL content is strongly positive. Light color seeds have less ADL content, and vice versa. DH population of 4042 x Express 617 is more loyal to this association with only one out of 77 genotypes deviates from this trend (1.3 %). In comparison, in total there are 17.9 % of the DH 1372 x Express 617 genotypes not

following the trend. It is interesting to see that in the first case, ADL showed less heritability than seed color (0.86 and 0.95, respectively). ADL heritability was higher than seed color (0.89 compared to 0.84) in the second case. Beside the environment factor which slightly influence the second population, it is possible that gene(s) interaction mentioned in Zhang et al. (2009) also played some role. Further molecular works on this population are still needed to make this issue clearer.

6.2 Correlations among traits

There is a strong positive correlation between seed coat color and seed fiber contents (ADL, ADF, and ADL), but no correlation is found between seed color and other seed quality traits (oil, protein, total oil & protein, protein of defatted meal, and glucosinolate contents). These are applied to both populations of yellow x black-seeded types, 4042 x Express 617 and DH 1372 x Express 617.

In both populations, seed color is also significantly correlated to some seed germination traits, such as radicle protrusion, infected seed, and full germination percentages. Before artificial aging, in both populations seed color has strong positive correlation to full germination percentage. It means that black seeded lines has better germination rate compared to the yellow seeded ones. After aging treatment, there is no correlation exist between seed color and seed longevity traits in both populations. Black seeds have better germination ability than the yellow ones, but seed color is not very reliable to identify whether the seeds have good longevity or not.

Seed size or Thousand Seed Weight (TSW) has no influence on any observed traits in both populations. The pre-harvest germination percentage, on the other hand, seems to be significantly correlated to many traits. In short, in both populations, seeds with high percentage of pre-emergence sprouts would have less oil content, higher protein content, lower NDF content and germination rate, also higher percentage of radicle protrusion. Especially for DH 1372 x Express 617 population, the genotypes exhibiting this trait will be also mostly yellow in color, contain lower total oil & protein content, lower seed fiber components (NDF, ADF, ADL), and higher seed infection rate.

In DH 617 population of 4042 x Express, the correlations between fiber contents (NDF, ADF, and ADL) and seed germination traits are only exist for radicle protrusion (-0.35 to -0.42) and infected seed (-0.37 to -0.42) percentages. Therefore, the high content of seed fiber components has no influence to full germination rate or seed vigor, but only effective against seed infection or occurrence of incomplete germination, which represented by percentage of radicle protrusion. After aging, the correlations between fiber contents (NDF, ADF, ADL) and seed longevity traits (percentages of radicle protrusion, full germination, infected seeds, and hypocotyl length) are significant. Seeds with low fiber contents tend to deteriorate more than high fiber seeds, indicated by lower full germination rate and hypocotyl length, and higher rate of radicle protrusion and seed infection. In DH 1372 x

Express 617, seed fiber components would display significant correlations to most seed germination traits, except hypocotyl length. The seeds containing high fiber would be inclined to have higher full germination, less radicle protrusion and seed infection percentages compared to low fiber content seeds. But such associations ceased to exist between seed fiber and seed longevity traits. Some of the seed fiber components in DH 1372 x Express 617 population is also associated to higher seed oil content (only NDF), and higher of seed protein and protein of defatted meal contents (both only for NDF and ADF).

Seed longevity in the second populations did not have any correlations with seed quality traits, including seed fiber components. It is also true for most seed phenotypic traits. There are significant correlations exist between seed germination and seed longevity traits, but only in DH 1372 x Express 617. The relations are found between radicle protrusion and full germination rate (before aging) and full germination, non-germination, and hypocotyl length after aging. There are no such correlations found in DH population of 4042 x Express 617.

6.3 Natural vs artificial aging test

After seed aging, the mean value of germination rate is reduced from nearly 100 % to 62.83 % for DH population of 4042 x Express 617 and 57.55 % for DH 1372 x Express 617. The DH Sollux x Gaoyou germination rate for natural aging seeds was ranged from zero to 89%, and the China-grown seeds displayed better germination percentage compared to the German ones. The average value of seed germination rate is 95 %, and after natural aging of 13 years was reduced to 18 %.

Clerkx et al. (2004) compared seed longevity in Arabidopsis mutants by CDT (controlled deterioration test) and natural aging of 4 year storage. The *ats (abberant tests shape*) mutants with seed coat alterations showed stronger germination rate reduction after storage. Nguyen et al. (2012) mentioned that CDT (controlled deterioration test) is not completely imitating natural aging. A QTL analysis detects a particular QTL (*GAAS5)*, which appears to be specific for natural aging. This QTL is not found after controlled deterioration in the same populations. On the other side, Bentsink et al. (2000) favored the artificial seed aging over natural aging, since the major QTLs controlling storability were detected in both seed aging assays. The CDT effect that they found on seed viability was much stronger than natural aging, resulting in more accurate mapping. TeKrony (2005) added that artificial seed aging gives more accurate imitation of seed emergence on the field under stress environment, better than standard germination tests. The results from Chapter 3 and 4 indicates that the artificial seed aging treatment using CDT is effective enough to differentiate genotypes of low vs high seed longevity.

The ADL content in the first population exhibits a bimodal frequency distribution, an indication that this trait is controlled by a major gene. Through the bulk segregant SNP marker analysis, two candidate genes that possibly control the ADL content were found. Both are located in chromosome C03. The first one is MATE transporter, related to expression of TT12 gene, which encodes transparent testa lead to yellow seed coat trait. The second one is cinnamate 4-hydroxylase (C4H), encodes one of the precursors to lignin biosynthesis. DNA and marker analysis for the second population (DH 1372 x Express 617) will be essential for further investigation.

These are more or less true if comparing the traits to the discussion of correlation values (see Table 6 and sub chapter 2.4), except for seed longevity values, which have no significant correlations with seed color. If we check closely for seed longevity traits for Express 617, the values are not exactly at the minimum or maximum value range, so there can be other factors influencing the phenotypic segregation. Also, although there are no direct correlations between seed color and seed longevity traits, there are significant correlations between seed germination and seed longevity traits, which might give indirect effect to the seed longevity.

Diverse variations were found among seed germination traits before and after natural seed aging in the population of DH Sollux x Gaoyou. In average, the seed germination rate and hypocotyl length after aging were low (18.34 and 0.95 cm, respectively). Radicle protrusion percentage was positively correlated to full germination percentage and hypocotyl length, perhaps due to this low germination issue. In contrast, the mean value of seed germination rate of DH Sollux x Gaoyou population before natural aging treatment is nearly 100 %.

Seed infection in natural aging seeds of DH Sollux x Gaoyou was most probably controlled only by environmental or replication effect. Genotype effects were significant for full germination percentage, hypocotyl length, and infected seeds percentage. The heritabilities found were very low, due to narrow variabilities within traits. Spearman's rank of correlation was not found among any measured seed quality traits with seed germination or seed longevity traits, except weak but significant association to NDF content. NDF was found to be correlated to radicle protrusion percentage before aging, and infected seeds percentage after aging. NDF might be connected to the seed coat characteristics as the seed main protection mechanism against adverse environment and pathogen infection.

This is the first study to find ADL related gene on the chromosome C03 of *Brassica napus*. Qu et al. (2013) provided a list of the loci distribution of various TT genes (including the lignin biosynthesis related PAL and C4H) in the genome of *B. rapa* and *B. oleracea*. Wang et al. (2015) previously found some genes controlling ADL content on the chromosomes C05, A05, and A09. Liu et al. (2012) reported a single, dominant, major locus which brings a substantial reduction in ADL, which they

identified as a key gene in lignin biosynthesis, *Bna.CCR1* (*CINNAMOYL CO-A REDUCTASE 1),* in the chromosome C08. Verification of the candidate genes of this research can be done in further study through gene cloning or developing transgenic plant of the particular gene. The identified mutants could provide a better understanding on the gene mechanism of controlling seed fiber content, in particular on ADL content in the oilseed rape breeding.

7 REFERENCES

Abrahams S, Lee E, Walker AR, Tanner GJ, Larkin PJ, Ashton AR (2003) The Arabidopsis TDS4 gene encodes leucoanthocyanidin dioxygenase (LDOX) and is essential for proanthocyanidin synthesis and vacuole development. Plant J 35:624–636

Adolphe D (1979) Canola: Canada's rapeseed crop. Publication No. 56. Rapeseed Association of Canada. Winnipeg, MB: Shaw Printing

Badani AG, Snowdon RJ, Wittkop B, Lipsa FD, Baetzel R, Horn R, De Haro A, Font R, Lühs W, Friedt W (2006) Colocalization of a partially dominant gene for yellow seed colour with a major QTL influencing acid detergent fibre (ADF) content in different crosses of oilseed rape *(Brassica napus),* Genome 49:1499–1509

Balešević-Tubić S, Tatić M, Đorđević V, Nikolić Z, Đukić V (2010) Seed viability of oil crops depending on storage conditions. *Helia*, 33(52):153-160. Retrieved 18 Jul. 2017, doi:10.2298/hel1052153b

Balešević-Tubić S, Tatić M, Dorđević D, Nikolić Z, Subić J, Ducic V (2011) Changes in soybean seeds as affected by accelerated and natural aging. Rom Biotechnological Letters 16 (6): 6740-6747

Baxter IR, Young JC, Armstrong G, Foster N, Bogenschutz N, Cordova T, Peer WA, Hazen SP, Murphy AS, Harper JF (2005) A plasma membrane H+-ATPase is required for the formation of proanthocyanidins in the seed coat endothelium of Arabidopsis thaliana. PNAS 102 (7): 2649–2654

Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate: A practical and powerful approach to multiple testing. J Royal Statistical Society. Series B (Methodological), 57(1): 289-300. Retrieved from http://www.jstor.org/stable/2346101

Bentsink L, Alonso-Blanco C, Vreugdenhil D, Tesnier K, Groot SPC, Koornneef M (2000) Genetic analysis of seed-soluble oligosaccharides in relation to seed storability of *Arabidopsis*. Plant Physiol 124(4): 1595–1604.

Bettey M, Finch-Savage WE, King GJ, Lynn JR (2000) Quantitative genetic analysis of seed vigour and pre-emergence seedling growth traits in *Brassica oleracea*. New Phytologist, 148: 277–286. doi:10.1046/j.1469-8137.2000.00760.x

Bewley JD, Black M, Halmer P (2006) The encyclopedia of seeds: science, technology and uses. CAB International. ISBN-13:978-0-85199-723-0. 769p

Bewley JD, Bradford KJ, Hilhorst HWM, Nonogaki H (2013) Seeds: Physiology of development, germination and dormancy. 3rd Ed. Springer. ISBN 978-1-4614-4692-7. DOI: 10.1007/978-1-4614-4693-4

Bharti AK, Khurana JP (2003) Molecular characterization of transparent testa (tt) mutants of *Arabidopsis thaliana* (ecotype Estland) impaired in flavonoid biosynthetic pathway. Plant Sci 165:1321–1332

Bhushan G, Mishra VK, Iquebal MA, Singh YP (2013) Effect of genotypes, reproductive developmental stages, and environments on glucosinolates content in rapeseed mustard. Asian J Plant Sci. & Res. 3(1):75-82

Booth DT, Bai Y (1999) Imbibition temperature effects on seedling vigor in crops and shrubs. J. Range Manag. 52: 534-538

Bruckhoff V, Haroth S, Feussner K, König S, Brodhun F, Feussner I (2016) Functional characterization of CYP94-genes and identification of a novel jasmonate catabolite in flowers. PLoS ONE 11(7): e0159875. https://doi.org/10.1371/journal.pone.0159875

Byers RL, Harker DB, Yourstone SM, Maughan PJ, Udall JA (2012) Development and mapping of SNP assays in allotetraploid cotton. Theor Appl Genet 124:1201. https://doi.org/10.1007/s00122-011-1780-8

Burbulis N, Kott LS (2005) A new yellow-seeded canola genotype originating from double low black-seeded *Brassica napus* cultivars. Can. J. Plant Sci. 85: 109–114

Carré P, Pouzet A (2014) Rapeseed market, worldwide and in Europe. *OCL* 21 (1), pp. D102. DOI: 10.1051/ocl/2013054

Chai YR, Lei B, Huang HL, Li JN, Yin JM, Tang ZL, Wang R, Chen L (2009) TRANSPARENT TESTA 12 genes from *Brassica napus* and parental species: cloning, evolution, and differential involvement in yellow seed trait. Molecular genetics and genomics: MGG 281 (1):109–123. DOI: 10.1007/s00438-008-0399-1

Chen BY, Heneen WK, Jönsson R (1988) Resynthesis of *Brassica napus* L. through interspecific hybridization between *B. alboglabra* Bailey and *B. campestris* L. with special emphasis on seed colour. Plant Breeding, 101: 52–59. doi:10.1111/j.1439-0523.1988.tb00266.x

Chen BY, Heneen WK (1992) Inheritance of seed colour in *Brassica campestris* L. and breeding for yellow-seeded *B. napus* L. Euphytica 59 (2-3): 157-163. https://doi.org/10.1007/BF00041268

Chen AH, Chai YR, Li JN, Chen L (2007) Molecular cloning of two genes encoding *Cinnamate 4-Hydroxylase* (C4H) from oilseed rape (*Brassica napus*) BMB Reports 40 (2):247-260. DOI: 10.5483/BMBRep.2007.40.2.247

Clarke WE, Higgins EE, Plieske J, Wieseke R, Sidebottom C, Khedikar Y, Batley J, Edwards D, Li R, Lawley CT, Pauquet J, Laga B, Cheung W, Iniquez-Luy F, Dyrszka E, Rae S, Stich B, Snowdon RJ, Sharpe AG, Ganal MW, Parkin IAP (2016) A high-density SNP genotyping array for *Brassica napus* and its ancestral diploid species based on optimised selection of single-locus markers in the allotetraploid genome. Theoretical and applied genetics. 129 (10): 1887–1899. DOI: 10.1007/s00122-016-2746-7

Clerkx EJ, El-Lithy ME, Vierling E, Ruys GJ, Blankestijn-De Vries H, Groot SP, Vreugdenhil D, Koornneef M (2004) Analysis of natural allelic variation of Arabidopsis seed germination and seed longevity traits between the accessions Landsberg erecta and Shakdara, using a new recombinant inbred line population. Plant Physiology, 135 (1): 432-443. DOI: 10.1104/pp.103.036814

Cromarty A, Ellis RH, Roberts EH (1982) The design of seed storage facilities for genetic conservation. Revised 1985. International Board for Plant Genetic Resources, Rome. 109p

Debeaujon I, Léon-Kloosterziel KM, Koornneef M (2000) Influence of the testa on seed dormancy, germination, and longevity in Arabidopsis. Plant Physiol 122: 403–414

Debeaujon I, Peeters AJM, Léon-Kloosterziel KM (2001) The TRANSPARENT TESTA12 gene of *Arabidopsis* encodes a multidrug secondary transporter-like protein required for flavonoid sequestration in vacuoles of the seed coat endothelium. Plant Cell 13 (4): 853–872. DOI: 10.1105/tpc.13.4.853

Debeaujon I, Nesi N, Perez P, Devic M, Grandjean O, Caboche M, Lepiniec L (2003) Proanthocyanidin-accumulating cells in *Arabidopsis* testa: regulation of differentiation and role in seed development. Plant Cell 15 (11): 2514–2531. DOI: 10.1105/tpc.014043

DeBolt S, Scheible WR, Schrick K, Auer M, Beisson F, Bischoff V, Bouvier-Navé P, Carroll A, Hematy K, Li Y, Milne J, Nair M, Schaller H, Zemla M, Somerville C (2009) Mutations in UDP-Glucosesterol glucosyltransferase in Arabidopsis cause transparent testa phenotype and suberization defect in seeds. Plant Physiol 151:78–87

de Souza Vidigal D, Willems L, van Arkel J, Dekkers BJ, Hilhorst HW, Bentsink L (2016) Galactinol as marker for seed longevity. Plant Sci. 246: 112-8

Dong J, Keller W, Yan W, Georges F (2004) Gene expression at early stages of *Brassica napus* seed development as revealed by transcript profiling of seed-abundant cDNAs. *Planta, 218* (3), 483-491. Retrieved from http://www.jstor.org/stable/23388277

Eurostat (2017) Main annual crop statistics. Eurostat: Statistics explained. Last updated January 2017. http://ec.europa.eu/eurostat/statistics-explained/index.php/Main_annual_crop_statistics. ISSN 2443-8219. Accessed on 17 August 2017

FAO/IPGRI (1994) Genebank Standards. Food and Agriculture Organization of the United Nations, and International Plant Genetic Resources Institute, Rome, 13pp

FEDIOL (2016) Oil Production, Imports, Exports and Consumption http://www.fediol.be/data/1503559867Stat%20seeds%202016.pdf (Site visited 04.09.2017)

Fujikura Y, Karssen CM (1995) Molecular studies on osmoprimed seeds of cauliflower, a partial amino acid sequence of a vigour-related protein and osmopriming-enhanced expression of putative aspartic protease. Seed Sci. Res. 5, 177-181

Jiang J, Niu Y, Qing Y, Guo S (2007) Variation in seed color and relationship to oil content in a yellow-seeded landraces in *Brassica rapa* L. Genetic and Breeding: Breeding for Quality, pp. 163–166.

Kruger-Giurizatto MI, Ferrarese-Filho O, Lucio Ferrarese MdL, Dias Robaina A, Gonçalves MC, Lima Cardoso CA (2012) a-Tocopherol levels in natural and artificial aging of soybean seeds. Acta Scientiarum. Agronomy, 34 (Jul-Sep) 339-343.

Ghassemi-Golezani K, Bakhshy J, Raey Y, Hossainzadeh-Mahootchy A (2010) Seed vigor and field performance of winter oilseed rape (*Brassica napus* L.) cultivars. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 3 (38), pp. 146–150.

Gómez-Campo C, Prakash S (1999) Origin and domestication. *In* Biology of Brassica Coenospecies. Gomez-Campo C, editor. Elsevier, Amsterdam pp. 33–58. eBook ISBN: 9780080528021

Grilli I, Bacci E, Lombardi I, Spano C, Floris C (1995) Natural aging: poly (A) polymerase in germination embryos of *Triticum* durum wheat. Ann. Bot. 76:15-21

Hajduch M, Casteel JE, Hurrelmeyer KE, Zhao S, Agrawal GK, Thelen JJ (2006) Proteomic analysis of seed filling in *Brassica napus*. Developmental characterization of metabolic isozymes using high-resolution two-dimensional gel electrophoresis. Plant Physiology 141 (1) 32-46; DOI: 10.1104/pp.105.075390

Hawk JA (1982) Single gene control of seed colour and hypocotyl colour in turnip rape. Can. J. Plant Sci. 62, 331—334

He C, Holme J, Anthony J (2014) SNP genotyping: the KASP assay. In: Fleury D., Whitford R. (eds.) Crop Breeding. Methods in Molecular Biology (Methods and Protocols), vol 1145. Humana Press, New York, NY. DOI: https://doi.org/10.1007/978-1-4939-0446-4_7

Heneen, WK, Brismar K (2001) Maternal and embryonal control of seed colour by different *Brassica alboglabra* chromosomes. Plant Breeding 120, 325—329

Hu ZY, Hua W, Zhang L, Deng LB, Wang XF, Liu GH, Hao WJ, Wang HZ (2013) Seed structure characteristics to form ultrahigh oil content in rapeseed. *PLoS ONE* 8 (4): e62099. DOI: 10.1371/journal.pone.0062099

Islam MS, GN Thyssen, JN Jenkins, and DD Fang (2015) Detection, validation, and application of genotyping-by-sequencing based Single Nucleotide Polymorphisms in upland cotton. Plant Genome 8 (1):1-10. doi:10.3835/plantgenome2014.07.0034

Kantar F, Pilbeam CJ, Hebblethwaite PD (1996) Effect of tannin content of faba bean *(Vicia faba)* seed on seed vigour, germination and field emergence. Ann Appl Biol 128: 85–93

Kennedy Y, Yokoi S, Sato T, Daimon H, Nishida I, Takahata Y (2011) Genetic variation of storage compounds and seed weight in rapeseed (*Brassica napus* L.) germplasms. Breeding Sci. 61 (3): 311-315. DOI: http://doi.org/10.1270/jsbbs.61.311

Kitamura S, Shikazono N, Tanaka A (2004) TRANSPARENT TESTA 19 is involved in the accumulation of both anthocyanins and proanthocyanidins in Arabidopsis. Plant J 37:104–114

Klein M, Martinoia E, Hoffmann-Thoma G, Weissenböck G (2000) A membrane-potential dependent ABC-like transporter mediates the vacuolar uptake of rye flavone glucuronides: Regulation of glucuronide uptake by glutathione and its conjugates. Plant J. 21, 289–304

Kochanek J, Steadman KJ, Probert RJ, Adkins JW (2011) Parental effects modulate seed longevity: exploring parental and offspring phenotypes to elucidate pre-zygotic environmental influences. New Phytol. 191, 223—233

Kocks CG, Zadoks JC, Ruissen MA (1999) Spatio-temporal development of black rot (*X. campestris pv. campestris*) in relation to initial inoculum levels in field plots in the Netherlands. Plant Path. 48, 176–88

Körber N, Bus A, Li J, Parkin IAP, Wittkop B, Snowdon RJ, Stich B (2016) Agronomic and seed quality traits dissected by genome-wide association mapping in *Brassica napus*. Frontiers in Plant Sci, 7, 386. http://doi.org/10.3389/fpls.2016.00386

Kracht W, Danicke S, Kluge H, Keller K, Matzke W, Henning U, Schumann W (2004) Effect of dehulling of rapeseed on feed value and nutrient digestibility of rape products in pigs. Arch. Anim. Nutr. 58: 389–404

Ku L, Cui X, Cheng F, Guo S, Qi J, Tian Z, Han T, Ren Z, Zhang L, Su H, Chen Y (2014) Genetic dissection of seed vigour under artificial ageing conditions using two joined maize recombinant inbred line populations. Plant Breed 133 (6): 728–737. DOI: 10.1111/pbr.12221

Leimu R, Mutakainen P, Koricheva J, Fischer M (2006) How general are positive relationships between plant population size, fitness and genetic variation? Journal of Ecology 94 (5):942-952. DOI: 10.1111/j.1365-2745.2006.01150.x

Lepiniec L, Debeaujon I, Routaboul J, Baudry A, Pourcel L, Nesi N, Caboche M (2006) Genetics and biochemistry of seed flavonoids. Annu. Rev Plant Biol. 57: 405-430

Li ZS, Alfenito M, Rea PA, Walbot V, and Dixon RA (1997). Vacuolar uptake of the phytoalexin medicarpin by the glutathione conjugate pump. Phytochemistry 45, 689–693

Liu XP, Tu JX, Chen BY and Fu T D (2005) Identification and inheritance of a partially dominant gene for yellow seed color in *Brassica napus*. Plant Breed. 124:9-12

Liu L, Stein A, Wittkop B, Sarvari P, Li J (2012) A knockout mutation in the lignin biosynthesis gene CCR1 explains a major QTL for acid detergent lignin content in Brassica napus seeds. Theor Appl Genet 124: 1573 - 1586

Lutman PJW, López-Granados F (1998) The persistence of seeds of oilseed rape (*Brassica napus*). Aspects Appl. Biol. 51 (2): 147-152

Mach J (2015) A sleep like death: identification of genes related to seed longevity in *Medicago truncatula* and *Arabidopsis*. The Plant cell 27 (10), p. 2671. DOI: 10.1105/tpc.15.00833

Marcos-Filho, J (2015) Seed vigor testing: an overview of the past, present and future perspective. Scientia Agricola, 72(4), 363-374. https://dx.doi.org/10.1590/0103-9016-2015-0007

Marinova K, Pourcel L, Weder B, Schwarz M, Barron D, Routaboul JM, Debeaujon I, Klein M (2007) The Arabidopsis MATE transporter TT12 acts as a vacuolar flavonoid/H+ -antiporter active in proanthocyanidin-accumulating cells of the seed coat. Plant cell 19 (6): 2023–2038. DOI: 10.1105/tpc.106.046029

Marles MS, Gruber MY (2004) Histochemical characterisation of unextractable seed coat pigments and quantification of extractable lignin in the Brassicaceae. J Sci Food Agric 84:251-262

Matthaüs B (1998) Effect of dehulling on the composition of antinutritive compounds in various cultivars of rapeseed. Fett/Lipid 100: 295−301

Meng J, Shi S, Gan L, Li, Z., and Qu, X. (1998). The production of yellow-seeded *Brassica napus* (AACC) through crossing interspecific hybrids of *B. campestris* (AA) and *B. carinata* (BBCC) with *B. napus*. Euphytica 103, 329–333. DOI: 10.1023/A: 1018646223643

Mersal IF (2011) The accuracy of accelerated aging test for evaluating the physiological quality of seeds. J. Plant Production, Mansoura Uni 2 (9):1249–1258. http://www.eulc.edu.eg/eulc_v5/Libraries/UploadFiles/DownLoadFile.aspx?RelatedBibID=MmYzMjhmZTEtZDI2Zi00MjgzLWJkMTQtMjE0NjI2MTFhN2E1X2l0ZW1zXzEyMTIwMDgwXzM1Mjg5MV9f&filename=429.doc

Michelmore RW, Paranand I, Kessele RV (1991) Identification of markers linked to disease resistance genes by bulk segregant analysis: a rapid method to detect markers in specific genome using segregant population. Proc Natl Acad Sci USA 88:9828–9832

Minkowski K (2002) Influence of dehulling of rape seeds on chemical composition of meal. Anim Feed Sci Technol 96:237-244

Moller J (2008) Animal feeding stuff: global standard for the determination of acid detergent fibre (ADF) and lignin. Focus FOSS Gr. J Technol Food, Dairy Agric Anal 32: 22 - 24

Murthy UMN, Kumar PP, Sun WQ (2003) Mechanisms of seed ageing under different storage conditions for *Vigna radiata* (L.) Wilczek: lipid peroxidation, sugar hydrolysis, Maillard reactions and their relationship to glass state transition. J Exp Bot 54 (384): 1057-1067. https://doi.org/10.1093/jxb/erg092

Nagel M, Börner A (2010) The longevity of crop seeds stored under ambient conditions. Seed Sci. Res. 20 : 1-12

Nagel M, Arif MAR, Rosenhauer M, Börner A (2010) Longevity of seeds -intraspecific differences in the Gatersleben genebank collections. Lehr- und Forschungszentrum für Landwirtschaft Raumberg-Gumpenstein. 60 Tagung der Vereinigung der Pflfl anzenzüchter und Saatgutkauflfl eute Österreichs 2009, 179 – 182

Nagel M, Rosenhauer M, Willner E, Snowdon RJ, Friedt W, Börner A (2011) Seed longevity in oilseed rape (Brassica napus L.) – genetic variation and QTL mapping. Plant Genet. Resour. 9 (02), pp. 260–263. DOI: 10.1017/S1479262111000372

Negi MS, Devic M, Delseny M (2000) Identification of AFLP fragments linked to seed coat colour in Brassica juncea and conversion to a SCAR marker for rapid selection. Theor. Appl. Genet. 101, 146—152

Nesi N, Jond C, Debeaujon I, Caboche M, Lepiniec L (2001) The Arabidopsis TT2 gene encodes an R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulation in developing seed. Plant Cell 13:2099–2114

Nesi N, Debeaujon I, Jond C, Stewart AJ, Jenkins GI, Caboche M, Lepiniec L (2002) The TRANSPARENT TESTA16 locus encodes the ARABIDOPSIS BSISTER MADS domain protein and is required for proper development and pigmentation of the seed coat. Plant Cell 14:2463–2479

Nesi N, Delourme R, Bregeon M, Falentin C, Renard M (2008) Genetic and molecular approaches to improve nutritional value of *Brassica napus* L. seed. *Comptes rendus biologies* 331 (10), pp. 763–771. DOI: 10.1016/j.crvi.2008.07.018

Neubert AB, Lühs W, Baetzel R, and Friedt W (2003) Influence of seed colour on seed vigour in *Brassica napus.* In Proceedings of the 11th International Rapeseed Congress. 6-10 July 2003. Edited by H. Sorensen. Dept of Chemistry, Royal Veterinary and Agricultural University, Frederiksberg, Denmark 1, pp. 253–256, checked on 12/20/2016

Nguyen TP, Keizer P, van Eeuwijk F, Smeekens S, Bentsink L. (2012) Natural variation for seed longevity and seed dormancy are negatively correlated in Arabidopsis. Plant Physiol. (160): 2083 - 2092. DOI: https://doi.org/10.1104/pp.112.206649

Nguyen TCT, Obermeier C, Friedt W, Abrams SR, Snowdon RJ (2016) Disruption of germination and seedling development in *Brassica napus* by mutations causing severe seed hormonal imbalance. Frontiers in Plant Sci. 7: 1-14 DOI: 10.3389/fpls.2016.00322

Nonogaki H, Bassel GW, Bewley JD (2010) Germination—Still a mystery. In Plant Science 179 (6), pp. 574–581. DOI: 10.1016/j.plantsci.2010.02.010

Ogé L, Bourdais G, Bove J, Collet B, Godin B, Granier F, Boutin JP, Job D, Jullien M, Grappin P (2008) Protein repair l-Isoaspartyl methyltransferase1 is involved in both seed longevity and

germination vigor in Arabidopsis. The Plant Cell Online 20 (11) 3022-3037; DOI: 10.1105/tpc.108.058479

Ohto M, Stone SL, Harada JJ (2007) Genetic control of seed development and seed mass. In: Bradford KJ, Nonogaki H, eds. Seed development, dormancy and germination. Annu Plant Rev 27. Wiley-Blackwell, Oxford, 1–24

Patterson EL, Fleming MB, Kessler KC, Nissen SJ, Gaines TA (2017) A KASP genotyping method to identify northern watermilfoil, Eurasian watermilfoil, and their interspecific hybrids. Front. Plant Sci. 8:752. doi: 10.3389/fpls.2017.00752

Pereira Lima JJ, Buitink J, Lalanne D, Rossi RF, Pelletier S, da Silva EAA, Leprince O (2017) Molecular characterization of the acquisition of longevity during seed maturation in soybean. PLoS ONE, 12(7), e0180282. http://doi.org/10.1371/journal.pone.0180282

Pelletier MK, Shirley BW (1996) Analysis of flavanone 3-hydroxylase in Arabidopsis seedlings. Coordinate regulation with chalcone synthase and chalcone isomerase. Plant Physiol 111:339–345

Pritchard HW, Dickie JB (2004) Predicting seed longevity: the use and abuse of seed viability equations. pp. 653–722 in Smith RD, Dickie JB, Linington SH, Pritchard HW, Probert RJ (Eds) Seed conservation: turning science into practice. Kew, London, Royal Botanic Gardens Kew.

Polinceusz AI (2011) Molecular characterisation of the Arabidopsis TT12 MATE protein and functional analysis of other plant MATE transporters. Ph.D Dissertation. University of Zurich, Faculty of Science. http://doi.org/10.5167/uzh-59369.

Pourcel L, Routaboul JM, Kerhoas L, Caboche M, Lepiniec L, Debeaujon I (2005) TRANSPARENT TESTA10 encodes a laccase-like enzyme involved in oxidative polymerization of flavonoids in Arabidopsis seed coat. Plant Cell 17:2966–2980

Qu C, Fu F, Lu K, Zhang K, Wang R, Xu X, Wang M, Lu J, Wan H, Zhanglin T, and Li J (2013) Differential accumulation of phenolic compounds and expression of related genes in black- and yellow-seeded Brassica napus. *J of Exp. Bot* 64 (10), pp. 2885–2898. DOI: 10.1093/jxb/ert148.

Ramiro MC, Peréz-García F, Aguinagalde I (1995) Effect of different seed storage conditions on germination and izozyme activity in some *Brassica* species. *Annals of botany* 75: 579–585

Rajjou L, Debeaujon I (2008) Seed longevity: survival and maintenance of high germination ability of dry seeds. C R Biol 331: 796–805

Rajjou L, Lovigny Y, Groot SPC, Belghazi M, Job C, Job D (2008) Proteome-wide characterization of seed aging in Arabidopsis: A comparison between artificial and natural aging protocols. Plant Physiology 148: 620–641, www.plantphysiol.org/cgi/doi/10.1104/pp.108.123141.

Rahman MH (2001) Production of yellow-seeded *Brassica napus* through interspecific crosses. Plant Breeding 120, 463—467

Rahman MH, McVetty PBE, Li GY (2005) Inheritance of seed coat color genes of *Brassica napus* (L) and tagging the genes using SRAP molecular markers. Bulletin GCIRC. B-22 edition. Techn. Meeting, Winnipeg, Manitoba, Canada, June 2005. http://gcirc.org/fileadmin/documents/Bulletins/B22/Inheritance_of_Seed_Coat_Color_Genes_of.pdf. Downloaded on 11 July 2017.

Rahman M, McVetty P (2011) A review of Brassica seed color. Can. J. Plant Sci. 91 (3): 437–446. DOI: 10.4141/CJPS10124

Reich G (2005) Near-infrared spectroscopy and imaging: Basic principles and pharmaceutical applications. Advanced Drug Delivery Reviews 57: 1109– 1143. DOI: 10.1016/j.addr.2005.01.020

Ren C, Bewley JD (1998) Seed development, testa structure and precocious germination of Chinese cabbage *(Brassica rapa* subsp. *pekinensis).* Seed Sci Res **8:** 385–397

Roberts SJ, Hiltunen LH, Hunter PJ, Brough J (1999) Transmission from seed to seedling and secondary spread of *Xanthomonas campestris pv. campestris*. Brassica transplants: effects of dose and watering regime. European Journal of Plant Pathology 105, 879–89

Robinson P, Ganske F (2012) High speed FRET based SNP Genotyping Measurement on the PHERAstar, http://www.bmglabtech.com/application-notes/fret/snp-genotyping-fret-Kbioscience-160.cfm. Accessed on 18 August 2017.

Ruan SL, Duan XM, Hu WM (2000) Occurrence of seed vivipary in hybrid rape (*Brassica napus* L.) and its effect on seed quality. J. of Zhejiang University. Vol.26 No.5 pp.573-578. ISSN : 1008-9209

Sagasser M, Lu GH, Hahlbrock K, Weisshaar B (2002) *A. thaliana* TRANSPARENT TESTA 1 is involved in seed coat development and defines the WIP subfamily of plant zinc finger proteins. Genes Dev 16: 138–149

Sattler SE, Gilliland LU, Magallanes-Lundback M, Pollard M, Della Penna D (2004) Vitamin E Is essential for seed longevity and for preventing lipid peroxidation during germination. Plant Cell 16 (6) 1419-1432; DOI: https://doi.org/10.1105/tpc.021360

Schoenbohm C, Martens S, Eder C, Forkmann G, Weisshaar B (2000) Identification of the Arabidopsis thaliana flavonoid 3'-hydroxylase gene and functional expression of the encoded P450 enzyme. Biol Chem 381:749–753

Semagn K, Babu R, Hearne S, Olsen M (2014) Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): overview of the technology and its application in crop improvement. Mol Breeding 33 (1): 1-14. https://doi.org/10.1007/s11032-013-9917-x

Sengupta S, Mukherjee S, Basak P, Majumder AL (2015) Significance of galactinol and raffinose family oligosaccharide synthesis in plants. Frontiers in Plant Sci, 6, 656. http://doi.org/10.3389/fpls.2015.00656

Shaban, M (2013) Study on some aspects of seed viability and vigor. IJABBR) 1 (12): 1692-1697. www.ijabbr.com/ article_ 7964_e758718f310d754c9455a352 feea57ff.pdf

Shahidi F, Naczk M. (1992) An overview of the phenolics of canola and rapeseed: chemical, sensory and nutritional significance, J. Am. Oil Chem. Soc. 69: 917–924

Shoba D, Manivannan M, Vindhiyavarman P, Nigam SN (2012) SSR markers associated for late leaf spot disease resistance by bulked segregant analysis in groundnut (*Arachis hypogaea* L.). Euphytica 188:265–272. DOI 10.1007/s10681-012-0718-9

Shen-Miller J, Lindner P, Xie Y, Villa S, Wooding K, Clarke SG, Loo RRO, Loo JA (2013) Thermal-stable proteins of fruit of long-living Sacred Lotus *Nelumbo nucifera* Gaertn var. China Antique. Tropical plant bio 6 (2-3). DOI: 10.1007/s12042-013-9124-2

Shirley BW, Hanley S, Goodman HM (1992) Effects of ionizing radiation on a plant genome: analysis of two Arabidopsis transparent testa mutations. Plant Cell 4:333–347

Shirley BW, Kubasek WL, Storz G, Bruggemann E, Koornneef M, Ausubel FM, Goodman HM (1995) Analysis of Arabidopsis mutants deficient in flavonoid biosynthesis. Plant J 8:659–671

Simbaya J, Slominski BA, Rakow G, Campbell LD, Downey RK, Bell JM (1995) Quality characteristics of yellow-seeded *Brassica* seed meals: Protein, carbohydrates, and dietary fiber components. J Agr Food Chem 43: 2062–2066

Sorensen DA, Hill WG (1983) Effects of disruptive selection on genetic variance. Theoret. Appl. Genetics 65 (2): 173-180. DOI: https://doi.org/10.1007/BF00264888

Stringam GR (1980). Inheritance of seed colour in turnip rape. Can. J. Plant Sci. 60, 331—335

Stuth J, Jama A, Tolleson D (2003) Direct and indirect means of predicting forage quality through near infrared reflectance spectroscopy. F Crop Res 84: 45-56

Schatzki J, Allam M, Klöppel C, Nagel M, Börner A, Möllers C, Chevre AM (2013) Genetic variation for secondary seed dormancy and seed longevity in a set of black-seeded European winter oilseed rape cultivars. *Plant Breed* 132 (2), pp. 174–179. DOI: 10.1111/pbr.12023.

Suma A, Sreenivasan K, Radhamani J (2014): Evaluating different artificial ageing techniques and Variability in tolerance to ageing of seeds of *Brassica spp* and *Eruca sativa*. J Oilseed Brassica 5 (1): 1–12

Suprianto E (2014) Genetic variation and inheritance of seed fibre in winter oilseed rape (*Brassica napus* L.). Doctoral Dissertation. University of Göttingen

Tang ZL, Li JN, Zhang XK, Chen L, Wang R (1997) Genetic variation of yellow-seeded rapeseed lines (*Brassica napus* L.) from different genetic sources. Plant Breeding 116: 471–474

Tan SH, Mailer RJ, Blanchard CL, Agboola SO (2011), Canola proteins for human consumption: extraction, profile, and functional properties. J Food Sci 76: R16–R28. doi:10.1111/j.1750-3841.2010.01930.x

TeKrony DM (2005). Accelerated aging test: principles and procedures. Seed Technology, 27(1), 135-146. http://www.jstor.org/stable/23433228

Umnajkitikorn K, Faiyue B, Saengnil K (2013) Enhancing antioxidant properties of germinated Thai rice (*Oryza sativa* L.) cv. Kum Doi Saket with salinity. J Rice Res 1:103. doi: 10.4172/jrr.1000103.

USDA (2017) Oilseeds: world markets and trade. USDA-FAS July 2017. https://apps.fas.usda.gov/psdonline/circulars/oilseeds.pdf (accessed 17 July 2017).

Utz HF (2011) PLABSTAT: A computer program for statistical analysis of plant breeding experiments. Version 3A of 2011-06-14. http://www.uni-hohenheim.de/ ipspwww/ soft.html

Vanholme R, B Demedts, K Morreel, J Ralph, W Boerjan (2010) Lignin biosynthesis and structure. Plant Physiol. 153 (3): 895–905. DOI: 10.1104/pp.110.155119

Van Deynze AE, Beversdorf WD, Pauls KP (1993) Temperature effects on seed color in black- and yellow-seeded rapeseed. Can. J. Plant Sci. 73: 383-387

Vera CL, Woods DL (1982) Isolation of independent gene pairs at two loci for seed coat in *Brassica juncea*. Can. J. Plant Sci. 62, 47—50.

Vogt T (2010) Phenylpropanoid biosynthesis. Molecular Plant 3 (1): 2–20. DOI: 10.1093/mp/ssp106.

Van Soest PJ, Robertson JB, Lewis BA (1991) Methods for dietary fiber, Neutral Detergent Fiber, and Nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74 (10): 3583-3597. DOI: https://doi.org/10.3168/jds.S0022-0302(91)78551-2

Voorrips, R.E., 2002. MapChart: Software for the graphical presentation of linkage maps and QTLs. J Heredity 93 (1): 77-78.

Wagner, MH, Ducournau S, Luciani A, Léchappé J (2012) From knowledge-based research towards accurate and rapid testing of seed quality in winter rape. Seed Sci. Res. 22 (S1): S80-S85. DOI: 10.1017/S0960258511000316

Walters C, Hill LM, Wheeler LJ (2005) Dying while dry: kinetics and mechanisms of deterioration in desiccated organisms. Integr Comp Biol 45: 751–758

Walters C, Ballesteros D, Vertucci VA (2010) Structural mechanics of seed deterioration: standing the test of time. Plant Sci 179 (6): 565–573. DOI: 10.1016/j.plantsci.2010.06.016

Wang J, Jian H, Wei L, Qu C, Xu X, Lu K, Qian W, Li J, Li M, Liu L (2015) Genome-wide analysis of seed Acid Detergent Lignin (ADL) and hull content in rapeseed (*Brassica napus* L.). PLoS ONE 10 (12): e0145045. DOI: 10.1371/journal.pone.0145045

Wang J, Xian X, Xu X, Qu C, Lu K, Li J, Liu L (2017) Genome-Wide Association Mapping of seed coat color in *Brassica napus*. J. Agr. & Food Chem. 65 (26): 5229–5237. DOI: 10.1021/acs.jafc.7b01226

Weightman R, Garland P, Phelps E, Clarke S, Hazzledine M, Berry P (2014) Nutritional value of oilseed rape and its co-products for pig and poultry feed: potential improvements and implications for plant breeders. HGCA Research Review 80. AHDB Cereals & Seeds. https://cereals.ahdb.org.uk/publications/ 2014/july/09/nutritional-value-of-oilseed-rape-and-its-co-products-for-pig-and-poultry-feed-potential-improvements-and-implications-for-plant-breeders.aspx

Wittkop B, Snowdon RJ, Friedt W (2009) Status and perspectives of breeding for enhanced yield and quality of oilseed crops for Europe. Euphytica 170: 131–140

Xu BB, Li JN, Zhang XK, Wang R, Xie LL, Chai YR (2006) Cloning and molecular characterization of a functional flavonoid 3-hydroxylase gene from *Brassica napus.* J. Plant Physiol. 164 : 350–363

Yan XY, Li JN, Fu FY, Jin MY, Chen L, Liu LZ (2009) Co-location of seed oil content, seed hull content and seed coat color QTL in three different environments in *Brassica napus* L. Euphytica 170:355-364

Yazaki K (2005) Transporters of secondary metabolites. Current Opinion in Plant Biology 8: 301-307

Yin X, He D, Gupta R, Yang P (2015) Physiological and proteomic analyses on artificially aged *Brassica napus* seed. Frontiers in Plant Sci 6, 112. DOI: 10.3389/fpls.2015.00112

Yu CY (2013) Molecular mechanism of manipulating seed coat coloration in oilseed Brassica species. J Applied Genetics 54 (2):135–145. DOI: 10.1007/s13353-012-0132-y

Zanakis GN, Ellis RH, Summerfield RJ (1994) A comparison of changes in vigour among three genotypes of soyabean (*Glycine max*) during seed development and maturation in three temperature regimes. Expl. Agric. 30:157–70

Zaklouta M, Hilali M, Nefzaoui A, Haylani M (2011) Animal nutrition and product quality laboratory manual. ICARDA, Aleppo, Syria. viii + 92 pp.

Zhang, XK, GT Yang, L Chen, JM Yin, ZL Tang, JN Li (2006) Physiological differences between yellow-seeded and black-seeded rapeseed (*Brassica napus* L.) with different testa characteristics during artificial ageing. Seed Sci. & Technology 34: 373–381

Zhang H, Hu LY, Hu KD, He YD, Wang SH, Luo JP (2008) Hydrogen sulfide promotes wheat seed germination and alleviates oxidative damage against copper stress. J Integr Plant Biol. 2008 Dec;50(12):1518-29. doi: 10.1111/j.1744-7909.2008.00769.x

Zhang Y, Li X, Ma C, Shen J, Chen B, Tu J, Fu T (2009) The inheritance of seed color in a resynthesized Brassica napus line No. 2127-17 including a new epistatic locus. Genes & Genomics 31: 413-419

Zhao J (2002) QTLs for oil content and their relationships to other agronomic traits in an European x Chinese oilseed rape population. Doctoral Dissertation. University of Göttingen

Zhao J, Becker HC, Zhang D, Zhang Y, Ecke W (2006) Conditional QTL mapping of oil content in rapeseed with respect to protein content and traits related to plant development and grain yield, Theor. Appl. Genet. 113 : 33–38

Zhao J, Becker HC, Zhang D, Zhang Y, Ecke W (2005) Oil content in a European x Chinese rapeseed population: QTL with additive and epistatic effects and their genotype-environment interactions. Crop Sci 45:51–59

Zhao J, Dixon RA (2009) MATE transporters facilitate vacuolar uptake of epicatechin 3'-O-glucoside for proanthocyanidin biosynthesis in *Medicago truncatula* and *Arabidopsis*. Plant Cell 21 (8): 2323–2340. DOI: 10.1105/tpc.109.067819

8 SUMMARY

Oilseed rape (*Brassica napus* L.) is the world's third largest source of vegetable oil for human nutrition; also the meal is regularly used as animal feed. Yellow seed is one of the target characters in the breeding program, as it associated to lower dietary fiber content and higher oil and protein content. Mature seeds *of B. napus* will gradually lose their viability during long term storage; this process is defined as natural aging. Seed viability is influenced by several environmental factors, but partly it is also determined by genetic factors. Seed aging is an acknowledged problem in agriculture, because it is involved in mechanisms leading to loss of viability and vigor. Yellow-seeded genotypes tend to deteriorate faster compared to the black-seeded ones. They show a reduced seed longevity, i.e. the ability to germinate after being stored for a longer period. Since material of naturally aged seeds are not always available, artificial seed aging protocols are often utilized to imitate the natural aging. Exposing seeds to high temperature and moisture have been commonly used for aging seeds artificially in the laboratory. The research aimed to study the inheritance of seed germination and seed longevity traits in two DH populations which segregate for yellow seed coat color, also in relation with several seed quality traits. A further objective was to investigate the inheritance of seed germination and seed longevity employing natural seed aging of the DH Sollux x Gaoyou population. This population has been stored in an ambient storage conditions at the University of Göttingen for 13 years.

The first two doubled haploid populations were derived from crosses between the two yellow-seeded type 4042 (winter type) and DH 1372 (spring type) with the black seeded winter oilseed rape cultivar Express, inbred line 617. The 4042 x Express 617 population was grown in the five environments Reinshof 2014, Reinshof 2015, Einbeck 2015, Reinshof 2016, and Einbeck 2016. The DH 1372 x Express 617 population was grown at Reinshof 2015 and 2016. The third DH population was derived from the cross of the old German cultivar Sollux and the Chinese semi-winter cultivar Gaoyou; both were black seeded. Field experiments have been performed at two locations each in China and in Germany in the year 2001.

Seed quality traits (e.g. oil, protein, glucosinolates, and fiber components (NDF, ADF, ADL) were predicted by NIRS (Near Infrared Reflectance Spectroscopy) estimation of seed samples. Other seed phenotypic traits were also observed, such as seed color, TSW (Thousand Seed Weight), and pre-harvest germination percentage. Comparison of seed germination before and after artificial aging treatment was carried out by determining percentages of radicle protrusion, complete germination, and of infected seeds. Hypocotyl length (cm) was also measured as an indicator for vigor. Statistical analysis was performed by PLABSTAT software for analysis of variance components, heritabilities and Spearman's rank of correlation coefficients.

In both populations of yellow x black seeded types (4042 x Express 617 and DH 1372 x Express 617), a large genetic variation was found for seed oil, protein, glucosinolates, fiber contents (NDF, ADF, ADL), seed coat color, and full germination rate and hypocotyl length after aging. There is a strong positive correlation between seed coat color and fiber contents (ADL, ADF, and ADL), but no correlation is found between seed color and seed oil and protein content. Heritability is high (above 0.80) for both populations for seed color and seed fiber contents.

The pre-harvest germination percentage in the present study is associated with many traits. In both populations, seeds containing high percentage of pre-emergence sprouts are also exhibit less seed oil content, higher protein content, lower NDF content and germination rate, also higher percentage of radicle protrusion. The yellow seeds of DH 1372 x Express 617 are exhibiting more of this trait, but there is no such association existed in DH population of 4042 x Express 617. Especially in DH 1372 x Express 617 population, these type of seeds are also associated with lower total oil & protein content, lower seed fiber components (NDF, ADF, ADL), and higher seed infection rate.

In both populations, seed color is highly correlated to all seed fiber contents. Before seed aging treatment, seed color is positively correlated to full germination rate, negatively correlated to radicle protrusion and seed infection rate, and no correlation to hypocotyl length. It means that black-seeded lines has better germination rate compared to the yellow-seeded ones. After artificial aging, seed color has no influence in seed longevity traits in both populations. The mean value of germination rate is dropped after aging treatment from nearly 100 % to 62.83 % for DH population of 4042 x Express 617 and to 57.55 % for DH 1372 x Express 617.

A different story was observed for seed fiber contents (NDF, ADF, ADL). In DH 1372 x Express 617, the seeds containing high fiber have significant higher germination rate, less radicle protrusion and seed infection compared to low fiber seeds. But after artificial seed aging, these correlations did not exist anymore. Before aging, in DH population of 4042 x Express 617, seed fiber contents only limit the radicle protrusion rate. Seeds with high fiber contents will have better full germination rate and hypocotyl length, and less radicle protrusion and seed infection rate. Less full germinated rate after seed aging are exhibited by seeds of high oil content and total oil and protein.

The ADL content in the first two populations exhibits a bimodal 1:1 frequency distribution, an indication that this trait is controlled by a major gene. For 4042 x Express 617 population, a bulk specific SNP-markers was performed at KWS SAAT SE in Einbeck with an Illumina Infinium 20K SNP chip for low and high ADL content bulks. The result was later confirmed with individual genotypes through KASP genotyping. In addition, non-targeted metabolite fingerprinting analysis was executed on green seeds of this population to measure the content of metabolite compounds related to the ADL content. Eleven polymorphic loci was detected in 4042 x Express 617, all are located in

chromosome C03. Two candidate genes that possibly control ADL content were identified. The first one is MATE transporter related to expression of *TT12* gene, encodes transparent testa which lead to yellow seed color. The second one is trans-cinnamate 4-hydroxylase (*C4H*), encodes one of the precursors to lignin biosynthesis. DNA and marker analysis for the second population (DH 1372 x Express 617) will be essential for further investigation.

The seeds of the DH population Sollux x Gaoyou were naturally aged by storing them in ambient room temperature for 13 years (2001 - 2014). Aged seeds were germinated and grown in the green house in 2016 to obtain fresh seeds (before aging treatment). The same germination method as described above was employed for both naturally-aged seeds and fresh harvested seeds. Data of seed quality traits was available only for German-grown locations from a previous study.

The DH Sollux x Gaoyou germination rate for natural aging seeds was ranged from zero to 89 %. The China-grown seeds displayed better germination percentage than the German ones. The average of seed germination rate is 95 %, and after 13 years of natural aging it was drastically reduced to 18 %. Although there are some significant correlations among seed quality traits, there are no significant correlations between seed quality traits and seed germination or seed longevity traits worth noted, perhaps due to both parents are black-seeded cultivars, therefore having more narrow variability.

For future outlook, further verification study is necessary to confirm the position of second candidate gene, C4H (trans-cinnamate 4-hydroxylase), by employing more KASP markers for DH population of 4042 x Express 617 to the downstream direction from the gene interval investigated in this study. The second population, DH 1372 x Express 617 was grown again in 2017 in Reinshof. Similar observations will be completed after the harvest in August 2017 and added to the first two environment data. The extracted DNA has been sent for KASP marker analysis, and later will be followed by candidate gene identification. DH Sollux x Gaoyou has completed the natural aging treatment, and will have newly harvested seeds from Reinshof in August 2017. Subjecting these seeds to artificial seed aging treatment, then comparing the results with previous data will provide us with better understanding of the effects of both seed aging treatments (natural and artificial) to seed longevity.

9 CURRICULUM VITAE

PERSONAL DATA

Name : Sasanti Widiarsih

Date and place of birth : 17.07.1978, Tegal (Indonesian)

Nationality : Indonesian

Address : Plant Mutation Breeding Division, National Nuclear Research Agency, Jl. Lebakbulus Raya No. 49, South Jakarta 12440, Indonesia

EDUCATION

PhD Candidate in Plant Breeding, 01/2014 – 11/2017

University of Göttingen, Germany

MS major in Plant Breeding, minor in Plant genetic resource management and conservation, Grade: 1.39/5.00, 07**/**2007 – 11/2009

University of the Philippines Los Baños, the Philippines

BSc in Plant Breeding, Grade: 3.41/4.00, 08/1996 – 09/2000

Bogor Agricultural University, Indonesia

EXPERIENCE

Researcher, Plant Mutation Breeding Division, 12/2003 – 11/2017

National Nuclear Research Agency, Indonesia

FUNDING

PhD Scholarship, RisetPro program, Ministry of Research & Technology of Indonesia, Nov 2013 – Oct 2017

University of Göttingen, Germany

MS Scholarship, SEARCA - SEAMEO, 07/2007 – 11/2009

University of the Philippines Los Banos (UPLB), the Philippines