



T.R.

EGE UNIVERSITY

Graduate School of Applied and Natural Science



# **INHERITANCE OF SEED FIBER TRAITS IN OILSEED RAPE (*Brassica napus* L.)**

**MSc THESIS**

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Department of Seed Science and Technology

İzmir

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OILSEED RAPE (*Brassica napus* L.)**

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İzmir

2020



Here we certify that this thesis entitled “**Inheritance of Seed Fiber Traits in Oilseed Rape (*Brassica napus* L.)**” conducted, prepared and submitted for the degree of Master of Science by Kefas Luka BAIYI has been recommended for acceptance and approval for oral defence as it complies both with the “Ege University Graduate School policies” and “Ege University Graduate School of Natural and Applied Sciences' rules and regulations”. This study is fully adequate, in scope and quality, as a thesis for the degree of Master of Science and the Candidate was found successful and the thesis was approved by the thesis committee unanimity/majority voting on 18 / 09 / 2020.

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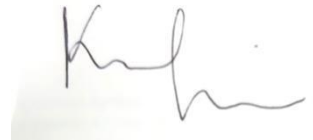
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18 / 09 / 2020



Kefas Luka BAIYI



**ÖZET****KOLZADA (*Brassica napus* L.) TOHUM LİF ÖZELLİKLERİNİN  
KALITIMI**

BAIYI, Kefas Luka

Yüksek Lisans Tezi, Tohumluk Bilimi ve Teknolojisi Anabilim Dalı

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İkinci Danışman: Dr. Christian MÖLLERS

Eylül 2020, 100 sayfa

Kolza küspesi, iyi dengelenmiş aminoasit bileşimi nedeniyle özellikle geviş getiren hayvanlar ve tek mideli hayvanların beslenmesinde önemli bir protein kaynağıdır. Ayrıca bitkisel proteinlere yönelik artan bir talep bulunmaktadır ve kolza tohumu proteini insan beslenmesi için de kullanılabilir. Bununla birlikte, kolza küspesinin yüksek lif içeriği, proteinin içeriğini ve gıda ve yemdeki bulunabilirliğini sınırlar. Bu çalışmanın amacı, tohumda daha düşük lignin içeriğine sahip beş farklı genetik kaynak arasındaki melezlerden türetilen dört farklı F<sub>2</sub> popülasyonunda lifle ilgili özelliklerin kalıtımını analiz etmektir. Ebeveynler ve F<sub>2</sub> popülasyonları, Almanya Göttingen Üniversitesi Bitki Genetiği Bölümü serasında yetiştirilmiştir. Lif içeriği, ANKOM 200 filtre torba sistemi uygulanarak yağı alınmış kolza tohumu küspesinin kimyasal analizi ile analiz edilmiştir. Ek olarak, lif içeriğini tahmin etmek için bir NIRS kalibrasyonu kullanılmıştır. C03 ve C05 genomuna bağlı iki ana QTL, kolza çeşitlerinde azaltılmış lif içeriği ile ilişkilendirilmiş, bu nedenle SNP bazlı bir genotipleme yöntemi - Rekabetçi Allele Özgü PCR (KASP) markörü, ebeveynler arasındaki allelik farklılığı ve F<sub>2</sub> popülasyonundaki açılma oranını gözlemlemek için kullanılmıştır. Bu prosedür için, C03 ve C05 ile ilişkili farklı markörler test edilmiş ve allelik farklılıkları gözlemlemek için polimorfik markörler kullanılmıştır. Ayrıca, lignin içeriği için aday genin genomu - fenilalanin amonyak-liyaz (PAL) geni, allelik diziyi incelemek ve ebeveynler arasındaki farklılıkları gözlemlemek için PCR-primerleri geliştirmek için kullanılmıştır. Hem PCR ve hem de KASP'den elde edilen sonuçlar, ebeveynler arasındaki farklılıkları ve F<sub>2</sub> popülasyonlarında lignin içeriğinin açılmasını doğrulamaktadır.

**Anahtar Kelimeler:** Kolza, Brassica, Lif, NDF, ADF, ADL, PAL geni.



**ABSTRACT****INHERITANCE OF SEED FIBER TRAIT IN OILSEED RAPE****(*Brassica napus* L.)**

BAIYI, Kefas Luka

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Supervisor: Prof. Dr. Muzaffer TOSUN

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Rapeseed meal is an important protein source for animal nutrition especially for ruminants and monogastric animal species due to its well-balanced amino acid composition. There is also an increasing demand for vegetable proteins and the rapeseed protein could be used for human alimentation. However, high fiber content of the rapeseed meal limits protein content and availability in food and feed. The objective of this study was to analyze the inheritance of fiber related traits in four different F<sub>2</sub> populations derived from crosses between five different genetic resources with lower lignin content in the seed. Parents and F<sub>2</sub> populations were grown in the greenhouse of the Division of Crop Plant Genetics of the University of Göttingen, Germany. Fiber content was analyzed by wet chemistry analysis of defatted rapeseed meal by applying the ANKOM 200 filter bag. In addition, an NIRS calibration was used to predict fiber content. Two major QTLs linked to C03 and C05 genome were associated with reduced fiber content in rapeseed cultivars, thus a SNP based genotyping method- Kompetitive Allele Specific PCR (KASP) marker was used to observe allelic difference between parents and segregation ratio in F<sub>2</sub> population. For this procedure, different markers associated to C03 and C05 were tested and polymorphic markers were used to observe allelic differences. Also, the genome of a candidate gene for lignin content – phenylalanine ammonia-lyase (PAL) gene was used to develop PCR-primers to study allelic sequence and observe differences among parental lines. Results from both PCR and KASP confirm differences between parents and segregation of lignin content in the F<sub>2</sub>-populations.

**Keywords:** Oilseed rape, Brassica, Fiber, NDF, ADF, ADL, PAL gene.



## PREFACE

Due to the growing demand for vegetable protein in recent years, rapeseed production has increased tremendously. Oil production in rapeseed has the most economical value, followed by rapeseed meal. The meal, which is the residue after oil extraction, is largely utilized as a protein source in animal nutrition and is the most important protein source after soybean meal. The presence of anti-nutritive factors such as glucosinolates, erucic acid, sinapine, high fiber contents impairs with protein utilization in ruminants, monogastrics or poultry species. Despite its rich amino acid composition, rapeseed meal is far behind soybean meal. Recent studies have shown that a candidate gene- phenylalanine ammonia-lyase (PAL) is associated with reduced fiber contents in crop plant species. In addition, QTLs have also been mapped on C03 and C05 genome of rapeseed and their association with fiber content was clearly outlined. Several studies have also supported the negative correlation between fiber content and protein content in rapeseed.

With this understanding, the goal of this research was to investigate the nature of inheritance of seed fiber traits in rapeseed cultivars. An understanding of the mode of inheritance of fiber related traits such as neutral detergent fiber, acid detergent fiber and acid detergent lignin will be important in selection and subsequent step in breeding programs targeted at reducing fiber content. The reduction of the fiber content most especially lignin, which is the indigestible fiber, will result in a better protein utilization of rapeseed meal in animal nutrition and offer a platform for subsequent genetic studies in protein or fiber related associations.

IZMIR

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Kefas Luka BAIYI



## TABLE OF CONTENTS

|                                                         | <u>Page</u> |
|---------------------------------------------------------|-------------|
| ÖZET .....                                              | vii         |
| ABSTRACT .....                                          | ix          |
| PREFACE .....                                           | xi          |
| TABLE OF CONTENTS .....                                 | xiii        |
| LIST OF FIGURES .....                                   | xvii        |
| LIST OF TABLES.....                                     | xx          |
| LIST OF ABBREVIATIONS .....                             | xxii        |
| 1. INTRODUCTION .....                                   | 1           |
| 2. LITERATURE REVIEW .....                              | 4           |
| 2.1 History and Domestication .....                     | 4           |
| 2.2 Plant Description .....                             | 4           |
| 2.3 Market of Rapeseed .....                            | 7           |
| 2.3.1 Rapeseed oil.....                                 | 7           |
| 2.3.2 Rapeseed meal/cake.....                           | 7           |
| 2.3.3 Rapeseed for biodiesel.....                       | 9           |
| 2.4 Introduction to Rapeseed Breeding and Genetics..... | 9           |

## TABLE OF CONTENTS (continued)

|                                                           | <u>Page</u> |
|-----------------------------------------------------------|-------------|
| 2.4.1 Breeding and genetics .....                         | 11          |
| 2.4.2 Other important Brassica species .....              | 12          |
| 2.5 Fiber .....                                           | 14          |
| 2.5.1 Importance of fiber to diet .....                   | 14          |
| 2.5.2 Measurement of fiber .....                          | 14          |
| 2.5.3 Fiber composition .....                             | 17          |
| 2.5.4 Fiber fractions .....                               | 19          |
| 2.5.4 Improving or breeding for low Fiber .....           | 21          |
| 2.6 Anti-nutritional compounds .....                      | 24          |
| 2.6.1 Glucosinolates .....                                | 25          |
| 2.6.2 Erucic acid .....                                   | 25          |
| 2.6.3 Sinapine .....                                      | 26          |
| 2.7 Genetic Studies .....                                 | 27          |
| 2.7.1 QTL mapping .....                                   | 27          |
| 2.7.2 Polymerase Chain Reaction (PCR) .....               | 29          |
| 2.7.3 Near infrared Reflectance Spectroscopy (NIRS) ..... | 31          |

## TABLE OF CONTENTS (continued)

|                                                    | <u>Page</u> |
|----------------------------------------------------|-------------|
| 2.7.4 Kompetitive Allele Specific PCR (KASP) ..... | 34          |
| 2.8 Inheritance .....                              | 36          |
| 3. MATERIAL AND METHODS.....                       | 38          |
| 3.1 Plant Material.....                            | 38          |
| 3.2 Experimental Setup.....                        | 38          |
| 3.3 Laboratory Procedures.....                     | 39          |
| 3.3.1 DNA extraction.....                          | 39          |
| 3.3.2 KASP genotyping .....                        | 40          |
| 3.3.3 PCR procedure.....                           | 44          |
| 3.4 ANKOM Method.....                              | 46          |
| 3.4.1 NDF Step .....                               | 46          |
| 3.4.2 ADF step .....                               | 48          |
| 3.4.3 ADL step.....                                | 50          |
| 3.4.4 DM content .....                             | 50          |
| 3.5 Analysis of Seed Quality Traits.....           | 51          |
| 4. RESULTS.....                                    | 53          |

**TABLE OF CONTENTS (continued)**

|                                                                      | <u>Page</u> |
|----------------------------------------------------------------------|-------------|
| 4.1 PCR Amplification.....                                           | 53          |
| 4.2 ANKOM Fiber Analyzer results .....                               | 54          |
| 4.3 Population I L239 x Lord.....                                    | 56          |
| 4.4 Population II 4042xExpDH14 x Lord.....                           | 60          |
| 4.5 Population III R19 x Lord .....                                  | 64          |
| 4.6 Population IV SGD14 x 4042xExpDH14 .....                         | 68          |
| 4.7 KASP marker analysis of Population IV SGD14 x 4042xExpDH14 ..... | 72          |
| 5. DISCUSSION .....                                                  | 74          |
| 5.1 Variation Among Traits .....                                     | 74          |
| 5.2 Spearman Rank Correlation .....                                  | 75          |
| 5.3 Molecular Data.....                                              | 76          |
| 6. CONCLUSIONS.....                                                  | 78          |
| REFERENCES.....                                                      | 79          |
| ACKNOWLEDGEMENTS .....                                               | 98          |
| CURRICULUM VITAE .....                                               | 100         |

## LIST OF FIGURES

| <u>Figure</u>                                                                                                           | <u>Page</u> |
|-------------------------------------------------------------------------------------------------------------------------|-------------|
| 2.1. Flowers of <i>B. Napus</i> .....                                                                                   | 5           |
| 2.2. Triangle of U, showing the 6 important species of the Brassica<br>genus .....                                      | 10          |
| 2.3. Genome duplication in <i>B. Napus</i> .....                                                                        | 10          |
| 2.4. An overview of the most important members of the Cruciferae<br>family .....                                        | 13          |
| 2.5. Crude fiber analysis based on Weende method .....                                                                  | 15          |
| 2.6. Weende method .....                                                                                                | 16          |
| 2.7. Detergent Fiber based on Van Soest method.....                                                                     | 17          |
| 2.8. Modified Weende method by Van Soest .....                                                                          | 17          |
| 2.9. Separation of NDF, ADF and ADL .....                                                                               | 20          |
| 2.10. Conversion of PAL and TAL showing the product and substrate .....                                                 | 23          |
| 2.11. Mechanism underlying seed color in <i>B. napus</i> .....                                                          | 24          |
| 2.12. Overlapping or Linkage relationship among Seed ADL content<br>QTLs on chromosome A09 from different studies ..... | 29          |
| 2.13. PCR components and Steps. ....                                                                                    | 31          |
| 2.14. Development of calibration equation for nirs. ....                                                                | 33          |

## LIST OF FIGURES (continued)

| <u>Figure</u>                                                                                                                                                                                                                                    | <u>Page</u> |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|
| 2.15. Overall mechanism of KASP genotyping .....                                                                                                                                                                                                 | 35          |
| 3.1. Component of KASP genotyping.....                                                                                                                                                                                                           | 41          |
| 4.1. PCR amplification of PAL gene on parental genotype Lord, L239,<br>R19 and 4042xExpDH15. ....                                                                                                                                                | 53          |
| 4.2. Scatterplot of NDF, ADF and ADL content on dry matter<br>(ANKOM) and predicted values (NIRS). ....                                                                                                                                          | 55          |
| 4.3. Seed color of parents and F2 from population I. only two of the<br>most yellow and two of the most seed samples are shown in the<br>bottom row. the light-yellow seeds are scored 1, while the dark<br>ones are scored 9. ....              | 57          |
| 4.4. Frequency distribution of Fiber fractions in Population I. ....                                                                                                                                                                             | 58          |
| 4.5. Relationship between seed color and lignin in Population I. ....                                                                                                                                                                            | 59          |
| 4.6. Seed color of parents and F2 from Population II. only two of the<br>most yellow and two of the black seed samples are shown in the<br>bottom row, the yellow seeds are scored 3(far left) and 4 while the<br>black seeds are scored 9. .... | 61          |
| 4.7. Frequency distribution of Fiber fractions in Population I. ....                                                                                                                                                                             | 62          |
| 4.8. Relationship between seed color and lignin content in Population<br>II. ....                                                                                                                                                                | 63          |

**LIST OF FIGURES (continued)**

| <u>Figure</u>                                                                                                                                                                                                              | <u>Page</u> |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------|
| 4.9. Seed color of parents and F2 from Population III. only two the most yellow and the black seeds samples are shown in the bottom row, the most yellow seeds are scored 2 while the black seeds are scored 9. ....       | 65          |
| 4.10. Frequency distribution of fiber fractions in Population III. ....                                                                                                                                                    | 66          |
| 4.11. Relationship between seed color and lignin content in Population III. ....                                                                                                                                           | 67          |
| 4.12. Seed color of parents and F2 from Population IV. only the two most yellow and the two most black seed samples are shown in the bottom. The most yellow seeds were scored 2 while the black seeds were scored 9. .... | 69          |
| 4.13. Frequency distribution of fiber fractions in Population IV. ....                                                                                                                                                     | 70          |
| 4.14. Relationship between seed color and lignin content in Population IV. ....                                                                                                                                            | 71          |
| 4.15. Allelic segregation of marker Bn-Scaff_17441_3-P28950 linked to C05. ....                                                                                                                                            | 73          |

## LIST OF TABLES

| <u>Tables</u>                                                        | <u>Page</u> |
|----------------------------------------------------------------------|-------------|
| 2.1. Nutrient composition of rapeseed cake and meal (%).....         | 8           |
| 2.2. Amino acid composition of rapeseed cake and meal (%) .....      | 8           |
| 3.1. Composition and volume of KASP reaction .....                   | 41          |
| 3.2. Thermal cycling conditions for KASP chemistry .....             | 42          |
| 3.3. Thermal recycling conditions for KASP chemistry .....           | 42          |
| 3.4. Chromosome and physical location of polymorphic markers.....    | 43          |
| 3.5. Master Mix solution per sample.....                             | 44          |
| 3.6. PCR Steps.....                                                  | 44          |
| 3.7. Primer pairs used for PCR.....                                  | 46          |
| 3.8. Description of color scoring system.....                        | 52          |
| 4.1. Statistics of quality traits in Population I .....              | 56          |
| 4.2. Spearman's Rank Correlation Coefficient of Population I .....   | 59          |
| 4.3. Statistics of quality traits in Population II .....             | 60          |
| 4.4. Spearman's Rank Correlation Coefficient of Population II .....  | 63          |
| 4.5. Statistics of quality traits in Population III.....             | 64          |
| 4.6. Spearman's Rank Correlation Coefficient of Population III ..... | 67          |

**LIST OF TABLES (continued)**

| <u>Table</u>                                                        | <u>Page</u> |
|---------------------------------------------------------------------|-------------|
| 4.7. Statistics of TKW and TSW of Population IV .....               | 68          |
| 4.8. Spearman's Rank Correlation Coefficient of Population IV ..... | 71          |
| 4.9. The ADL contents and genotype of F2 plants from KASP .....     | 72          |



**LIST OF ABBREVIATIONS**

| <u>Abbreviation</u> | <u>Definition</u>                      |
|---------------------|----------------------------------------|
| A09                 | Chromosome 9 in genome A               |
| ADF                 | Acid Detergent Fiber                   |
| ADL                 | Acid Detergent Lignin                  |
| BOF                 | Beginning Of Flowering                 |
| C03                 | Chromosome 3 in genome C               |
| C05                 | Chromosome 5 in genome C               |
| C08                 | Chromosome 8 in genome C               |
| CellU               | Cellulose Content                      |
| DH                  | Double Haploid                         |
| DM                  | Dry Matter                             |
| DNA                 | Deoxyribonucleic Acid                  |
| EST                 | Expressed Sequence Tag                 |
| EU                  | European Union                         |
| FAO                 | Food and Agricultural Organization     |
| FRET                | Fluorescence Resonance Energy Transfer |
| GC(%)               | Guanine-Cytosine content               |

**LIST OF ABBREVIATIONS (continued)**

| <u>Abbreviation</u> | <u>Definition</u>                      |
|---------------------|----------------------------------------|
| GC                  | Gas Chromatography                     |
| HEAR                | High Erucic Acid Rapeseed              |
| HemiC               | Hemicellulose Content                  |
| KASP                | Kompetitive Allele Specific PCR        |
| LD                  | Linkage Disequilibrium                 |
| LEAR                | Low Erucic Acid Rapeseed               |
| MARS                | Marker Assisted Recurrent Selection    |
| Mb                  | Megabase (= 100 000 basepairs)         |
| Mins                | Minutes                                |
| ml                  | Milliliter                             |
| MT                  | Million Tons                           |
| NDF                 | Neutral Detergent Fiber                |
| NIRS                | Near Infrared Reflectance Spectroscopy |
| NTC                 | No Template Control                    |
| ORM                 | Oilseed Rape Meal                      |
| PAL                 | Phenylalanine Ammonia Lyase            |

**LIST OF ABBREVIATIONS (continued)**

| <u>Abbreviation</u> | <u>Definition</u>               |
|---------------------|---------------------------------|
| PCR                 | Polymerase Chain Reaction       |
| QC                  | Quality Control                 |
| QTL                 | Quantitative Trait Loci         |
| RIL                 | Recombinant Inbred Line         |
| RPM                 | Revolutions Per Minutes         |
| SD                  | Standard Deviation              |
| SHEAR               | Super High Erucic Acid Rapeseed |
| TAL                 | Tyrosine Ammonia Lyase          |
| VLCFA               | Very Long Chain Fatty Acids     |
| XL                  | Oil Content                     |
| XP                  | Protein Content                 |

## 1. INTRODUCTION

Oilseed rape or *Brassica napus* L ( $2n=4x=38$ ) is an important member of the *Brassicaceae* family. *B. napus* is an amphidiploid species resulting from a spontaneous interspecific hybridization between *Brassica oleracea* L. ( $n=9$ ) and *Brassica rapa* ( $n=10$ ) (Downey and Rimmer, 1993). Oilseed rape (also known as rapeseed) is cultivated mainly for its oil, which is useful in human nutrition, notwithstanding, it can also be used as a biodiesel among many other industrial applications and its meal and cake are used for animal nutrition. Global production of oilseed rape stands at 70 MT with Canada being the largest producer (24% of global production) followed by China (14% of global production) while India, France, Germany, and Australia are also important producers. Canada is also the first exporter (8 MT in 2011) while Germany is the highest importer of oilseed rape importing 3 MT in 2011 (Mailer, 2016). Total area of cultivation in the EU is estimated to be around 6.9 million hectares (Popescu et al., 2019).

The economic value of oilseed rape is its oil, and this oil is considered the 2<sup>nd</sup> most important vegetable oil in the world after soybean (*Glycine max*) (Carré and Pouzet, 2014; Woźniak et al., 2019). In Europe, oilseed rape stands as the highest produced vegetable oil followed by sunflower (*Helianthus annuus*) (Martínez-Force et al., 2015). Aside its use as oil source, oilseed rape contribute up to 66% of the biodiesel energy in Europe, while soybean and palm oil (*Elaeis guineensis*) accounts for only 13% and 12%, respectively (Junginger et al., 2016).

The seed oil content of oilseed rape is between 31 – 48% while the protein content is estimated at 15% (Agrawal et al., 2008), whereas fiber content accounts for 26 – 33% (Kaczmarek et al., 2016). The oil of oilseed rape is composed of fatty acid such as oleic acid (56.80 – 64.92%), linoleic acid (17.11 – 20.92%) and palmitic acid (4.18 – 5.01%) (Matthaus et al., 2016). These fatty acids are important for cell development and signaling of important molecules needed for growth and development in humans and animals (de Carvalho and Caramujo, 2018). However unsaturated fatty acids (palmitic and stearic acid) are unhealthy to human health as they have been shown to be associated with some

inflammation related diseases (van Dijk et al., 2009). Around 30 – 50% of the fatty acids found in oilseed rape is erucic acid (McVetty and Duncan, 2016).

Currently, most of the protein demand in animal diets has been met by soybean meal, the protein content of soybean meal is between 36 – 56 % DM, making it an important protein source with oilseed rape meal considered in second place (Carré and Pouzet, 2014). The protein contents of oilseed rape meal is around 36 – 38% (Woźniak et al., 2019) and this high amount has been used to meet protein demands in ruminants, monogastric and poultry species. Industrial processing of oilseed rape produces cake or meal, and these are often characterized with high protein content. However, the use of oilseed rape meal is limited by glucosinolate content, high fiber content (> 16%), presence of sinapine among other antinutritional factors. The presence of these compounds impairs with protein absorption and utilization in animal nutrition (Woźniak et al., 2019). Breeding for different traits in oilseed rape is still underway, these traits include yield, quality such as high oleic acid, meal among others (Abbadi and Leckband, 2011).

Considering the importance of fiber in relation to other quality traits such as oil and protein content, it is pertinent to understand the inheritance of fiber contents in rapeseed.

Therefore, the objectives of this study are

1. To compare laboratory reference analysis results for fiber content with NIRS predicted values
2. To analyze segregation of fiber content in four different F<sub>2</sub> populations.
3. To study relationship between fiber content and other quality related traits such as seed color, oil, protein, total seed weight, and thousand-kernel weight
4. To identify F<sub>2</sub> plants with reduced fiber contents for further field testing and selection in breeding programs

5. To observe amplification of phenylalanine ammonia-lyase (PAL) gene in parental genotypes in a PCR reaction
6. To observe allelic differences for a major QTL in DH parents and segregation ratio in the F2 using Kompetitive Allele Specific PCR (KASP)



## 2. LITERATURE REVIEW

### 2.1 History and Domestication

*Brassica napus* (AACC) is derived from a spontaneous crossing of *Brassica rapa* (AA) and *Brassica oleracea* (CC) that occurred 7500 years ago (Chalhoub et al., 2014). Further, using morphological data, Metzger (1833) correctly categorized the important Brassicas and highlighted that *B. napus* is originating from the cross between *B. rapa* and *B. oleracea* (Metzger, 1833). Earliest report of oilseed rape cultivation dated back to Chinese almanac around 3000 BC, Assyrian cuneiform documents around 1800 BC and Indian Aryan literature in 1500 BC (Prakash et al., 2011). Seeds of *B. rapa* were also found in the stomach of a mummified corpse in Scandinavians in the 4<sup>th</sup> century (Shyam et al., 2012). In Europe and the Mediterranean, *B. nigra* (black mustard), *B. napus* (rapeseed) and *B. rapa* (turnip) were known in Greece around 370 – 285 BC and in Rome around 25 BC largely as weed or vegetable crop (Bell, 1982; Livarda and Van der Veen, 2008). The name rape originates from the latin word “*rapum*” which means turnip. In 13<sup>th</sup> century in Europe, oilseed rape was largely used as a lamp oil (Colton and Sykes, 1992). In Canada, it was used as a lubricant for war ships due to its ability to adhere to metal parts in water as it makes an excellent lubricant (Snowdon et al., 2007).

### 2.2 Plant Description

Oilseed rape has a bright yellow blossoming flower, and is cultivated as an annual or biennial crop in the temperate regions of the world and can reach 170 cm in height (Gulden et al., 2008). Due to its ability to be grown in cold regions, it is considered as a winter crop in Europe, however, there are also spring cultivars grown in Canada and Australia. Plants species in this family are often associated with a secondary metabolite as part of their defense mechanism called glucosinolates and this is found in high amount in the meal after oil extraction while their seed oil is often characterized with having a very long chain fatty acid (Booth and Gunstone, 2004).

The leaves are waxy dark bluish green in color, leaves are attached to each node, there can be around 15 – 20 internodes per plant with a node distance of 5 – 10 mm.

The stem is erect with some branching, the flowers are bisexual and have terminal racemes. There are four petals and four sepals, and the stamens are tetradynamous with four long and another two short stamens. There are also two carpels with a superior ovary located above the receptacle, the flowers are entomophilous. The diagonally opposite yellow petals tend to narrow at the base forming a cross, hence the family name Cruciferae.

Oilseed rape is reproduced via sexual reproduction and can have both cross and self-pollination with 12 – 47% outcrossing rate (Williams et al., 1986; Becker et al., 1992). The degree of outcrossing is dependent on the availability of insect pollinators, weather, and the variety. There is no asexual form of propagation in oilseed rape under field conditions (Andersson and De Vicente, 2010).



Figure 2.1. Flowers of *B. Napus*.

The fruiting bodies of the Brassicaceae family is called siliques, commonly referred to pods (Lovett and Lazenby, 1979). The siliques are linear cylindrical in shape with little constrictions and a dehiscent valve located at the lower 4 – 10 cm segment of the silique. Seeds are arranged in a single row in the silique while the thick 3.5 – 5.0 cm upper segment of the silique is seedless (Callihan et al., 2000; Gulden et al., 2008).

Seed development is first observed in the basal end of the branch of the main stem. At full seed size, the seeds are translucent and later turn green before becoming hard and dark or brown (Gulden et al., 2008). Earlier research has shown that the color of the immature seeds is controlled by flavonoids, polyphenols, anthocyanidin while melanin controls the color of the matured seeds (Hanzhong and Houli, 1996).

Around 15 – 20 seeds develop in each 6 – 9 cm pod. The average weight of a single seed is 5 mg with 1 – 2 mm in diameter (Lovett and Lazenby, 1979) and there can be between 280 000 – 300 000 seeds per kg. At maturity, the seeds are dark brown or black in color and can be released from individual plant when the siliques dry out and open. One of the challenges of rapeseed growers is pod shattering during harvest. Around 70% of yield losses can occur due to pod shattering, this is not due to genotype alone but can be because of high temperature and strong winds (Colton and Sykes, 1992), the shattered seeds then become volunteer crop in the next growing season. The pollen of rapeseed was found to cause allergic reaction in some people, but no allergic reaction has been reported in humans through oil consumption of rapeseed.

Oilseed rape can be differentiated from turnip rape by some morphological features such as the leaves, the leaves of oilseed rape are hairless, fleshy, smooth, and bluish green in color while those of turnip rape are yellowish green. Further, the flowers of turnip rape are smaller and dark yellow compared to oilseed rape (Gulden et al., 2008).

## **2.3 Market of Rapeseed**

### **2.3.1 Rapeseed oil**

As of 2018, the Food and Agricultural Organization (FAO) of the United Nations (UN) estimated the global production of oilseeds crops is around 479 million tons (MT) with consumption at 492 MT (Woźniak et al., 2019). Soybean had the biggest share of all oil crops produced in 2017 (73%) followed by rapeseed (16%). In terms of production in 2017, the European Union was the largest producer with 22 MT, Canada and China followed with 21 MT and 13 MT respectively, India (7.9 MT), Australia (4.3 MT) and Ukraine (2.1 MT) were also among the top producers (Woźniak et al., 2019). The oil of oilseed rape is used for culinary purposes. Most edible fats and oil consist of triacylglycerols which is around 94 – 99% of the total lipids (Shahidi, 1990).

### **2.3.2 Rapeseed meal/cake**

After extraction of oil from oilseed rape, the residue is called oilseed rape meal (ORM) and has a very high protein content of 30.2 – 36.9% (Behnke et al., 2018). ORM represent 20 – 25% of the total seed value of oilseed rape and is often considered an excellent meal due to its well-balanced amino acid composition (Tan et al., 2011) as shown in Table 2.2. Energy components is the highest priority in animal diet composition followed by protein supply (Burton et al., 2014). Protein demand is therefore met through ORM or cake (Table 2.1). When compared to soybean meal, ORM has a lower metabolizable energy (Bell, 1993) and the presence of glucosinolates limits its intake by animals. The difference between the meal and cake is largely due to their fat content and the method of extraction. Cake is produced by pressing oilseed rape under high pressure while meal is obtained through solvent extraction. Double pressing (used to produce cake) can reduce fat content to 8 – 12% while hexane extraction (used to produce meal) can give around 1 – 3% fat. An inclusion rate of 2.5 kg/day of rapeseed cake/meal is recommended for dairy cows, however, for broilers it is around 5% of their total diet, for monogastric animals such as pigs, a 10% inclusion rate is recommended. Rapeseed cake is rich in omega-3 fatty acid as

observed in animal milks fed with rapeseed cake. Yellow seeded rapeseed was shown to have higher protein content and lower fiber content in rapeseed meal compared to black seeded genotypes (Weightman and Garland, 2014). The biggest exporters of ORM are Canada and India, exporting 3 MT and 1 MT respectively in 2012.

Another anti-nutritive compound found in ORM beside glucosinolate is the presence of sinapine which when fed to hens, produces a fishy taste of their eggs (Burton et al., 2014). Oilseed rape can also be used as a cover crop to reduce soil erosion, suppress weed growth and improving soil health (Haramoto and Gallandt, 2004).

Table 2.1. Nutrient composition of rapeseed cake and meal (%)

| <b>Nutrients</b> | <b>Rapeseed cake</b> | <b>Rapeseed meal</b> |
|------------------|----------------------|----------------------|
| Dry matter       | 88.00                | 88.00                |
| Crude Protein    | 37.50                | 38.60                |
| Crude Fiber      | 11.40                | 11.80                |
| NDF              | 33.30                | 20.70                |
| ADF              | 26.00                | 16.80                |
| Calcium          | 0.59                 | 0.65                 |
| Total Phosphorus | 0.96                 | 1.02                 |
| Ash              | 7.20                 | 7.30                 |

Source: (Feng and Zuo, 2007)

Table 2.2. Amino acid composition of rapeseed cake and meal (%)

| <b>Amino acid</b> | <b>Rapeseed cake</b> | <b>Rapeseed meal</b> |
|-------------------|----------------------|----------------------|
| Arginine          | 1.82                 | 1.83                 |
| Histidine         | 0.83                 | 0.86                 |
| Isoleucine        | 1.24                 | 1.29                 |
| Leucine           | 2.26                 | 2.34                 |
| Lysine            | 1.33                 | 1.30                 |
| Methionine        | 0.60                 | 0.63                 |
| Cysteine          | 0.82                 | 0.87                 |
| Phenylalanine     | 1.35                 | 1.45                 |
| Tyrosine          | 0.92                 | 0.97                 |
| Threonine         | 1.40                 | 1.49                 |
| Tryptophan        | 0.42                 | 0.43                 |
| Valine            | 1.62                 | 1.74                 |

Source: (Feng and Zuo, 2007)

### 2.3.3 Rapeseed for biodiesel

Global production of biofuel is far ahead of biodiesel, however, biodiesel accounts for the largest share of biofuel production in the EU (Burton et al., 2014). The FAO reported that, the production of biodiesel derived from vegetable oil crops is around 10% of total vegetable oil produced in the world. Germany and France top the European countries in terms of both the production and consumption of biodiesel. The European Union produced around 12.4 MT for biodiesel amounting to 37.5% of global production in 2016. Furthermore, oilseed rape stands as the major feedstock of biodiesel in the EU representing 52.3% of total production in 2015.

### 2.4 Introduction to Rapeseed Breeding and Genetics

The *Brassicaceae* family also known as *Cruciferae* has 375 genera including 3200 species. Oilseed rape is an amphidiploid specie and an important member of this family. This can further be explained by the triangle of U as shown in Fig 2.2 (McVetty and Duncan, 2016) showing 6 important species under the genus *Brassica*, three are diploid species or can be referred to as parents, while the other three are amphidiploid, obtained from the interspecific crosses between the parents. Figure 2.3 shows a genome alignment of *Amborella trichopoda* with *Vitis vinifera* and *Arabidopsis thaliana* and *B. rapa*, *B. oleracea* and *B. napus*. A well-defined ancestral region in *A. trichopoda* is expected to match 72 regions in *B. napus* however, 69 regions were outlined in the Fig 2.3. The gray regions at the bottom shows the conserved synteny blocks with more than ten gene pairs.

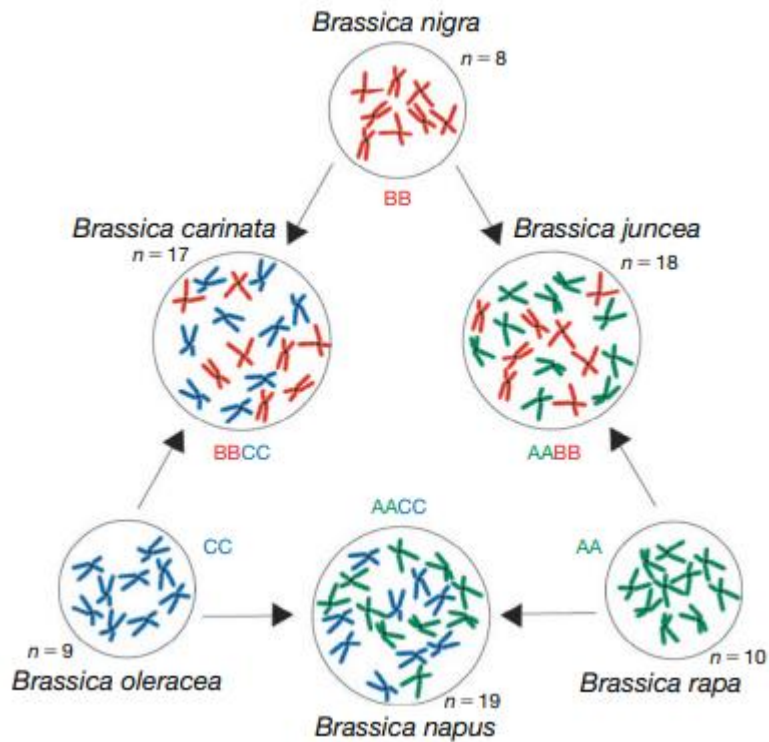


Figure 2.2. Triangle of U, showing the 6 important species of the Brassica genus Source: (McVetty and Duncan, 2016).

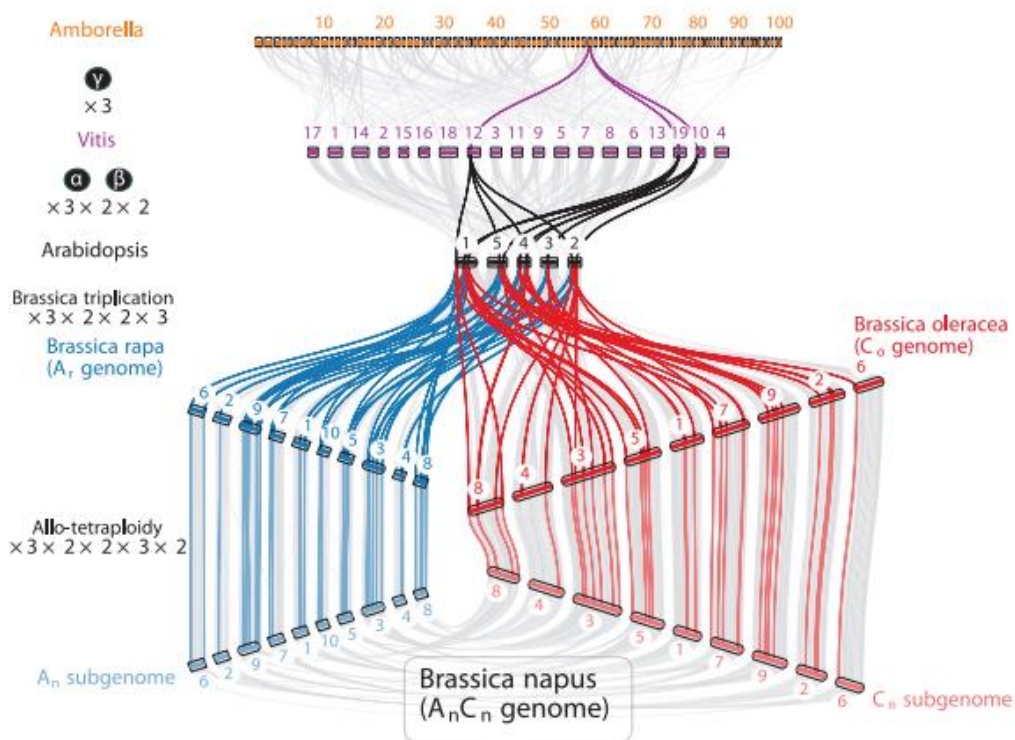


Figure 2.3. Genome duplication in B. Napus Source: (Chalhoub et al., 2014).

### 2.4.1 Breeding and genetics

The parents *B. rapa*, *B. nigra*, *B. oleracea* are said to show one-gene, two allele's mode of inheritance for most of their traits (McVetty and Duncan, 2016). This is important for various genetic studies such as whole genome sequencing, and gene cloning. The amphidiploid species *B. napus*, *B. juncea*, *B. carinata* showed a two-gene, four allele's mode of inheritance in general. However, some traits such as glucosinolates contents are controlled by multigenic inheritance. This makes the amphidiploids cumbersome to work with in terms of genetic studies (McVetty and Duncan, 2016). All members of the triangle of U are considered oilseeds crops except for *B. oleracea* which is an important vegetable. Cabbage, Broccoli, Cauliflower, Kale, Brussels sprout all fall under *B. oleracea*.

Traditional Brassica seed is composed of >40% erucic fatty acid and >100  $\mu\text{mol}$  glucosinolates per gram of dry matter (Rahman, 2013). Erucic acid is undesirable in human diet as it affect normal heart function while glucosinolates impairs with protein absorption in animal diets (Bell, 1993). Therefore, in Europe and Canada, intensive breeding programs began in 1960s and were targeted to reduce these undesirable compounds in order to make oilseed rape more beneficial for human and animal consumption. By 1978, a new cultivar of oilseed rape was successful bred in Canada called “**Canadian and oil low acid**” – **Canola**, which was international accepted because it contains < 2% erucic acid and 30  $\mu\text{mol}$  glucosinolates per gram of air-dried seed (Wanasundara et al., 2017) a value that is not detrimental to either human or animal health. Nutrient composition of canola seed include 6% of saturated fatty acid (0.3% behenic C22:0, 0.6% arachidic C20:0, 1.5% stearic C18:0, and 3.5% palmitic C16:0), 30% of polyunsaturated fatty acids (9.6% linolenic C18:3, and 20.1% linoleic C18:2,) and 62% of monounsaturated fatty acids (0.2% erucic C22:1, 1.4% gadoleic C20:1, and 60.1% oleic C18:1) (Przybylski, 2001). Canola is often referred to as double-low quality rapeseed in many European countries and can yield 3.3 – 4.5 tons per hectare (Obermeier and Friedt, 2015). In Canada and China, the yield per hectare is even below 2.1 t/ha, this can partly be attributed to the use of spring cultivars.

On the contrary, another cultivar was also bred having >50% erucic acid (McVetty and Duncan, 2016) and low glucosinolate content called HEAR (high erucic acid rapeseed). However, HEAR is mostly used for industrial purposes e.g. it can be used as a slip-promoting agent in many plastic manufacturing industries or as a green lubricant (McVetty et al., 2016). The average yield of winter-type HEAR is 2.5 – 3.0 t/ha while it springs cultivars yields 1.0 – 1.5 t/ha (Röbbelen, 1991) and was cultivated widely in Canada and the European Union. Also, an improved cultivar was subsequently released few years later called SHEAR (super high erucic acid rapeseed).

Prior to the release of both HEAR and canola, a cultivar was released in 1968 in Canada called LEAR (low erucic acid rapeseed) which contains less than 5% of erucic acid, however, this cultivar did not stay long enough in the market due to its low oil contents and yield compared to traditional cultivars (Eskin and Przybylski, 2003). Also, improvement has been made to reduce linolenic acid contents from 10% to <2% and subsequently increase oleic acid content above 70% (McVetty and Duncan, 2016) in order to encourage stability of oil under high temperature. Other breeding objectives carried out on the years that followed the released of canola include breeding for resistance against pest and diseases, increased yield, higher protein content, balanced meal quality and reducing undesirable compounds such as tannins, phytic acids, phenolics and now fiber content.

## 2.4.2 Other important Brassica species

**Cabbage - *Brassica oleracea*:** This is one of the most important species under the genus *Brassica*, subspecies of *Brassica oleracea* includes *B. oleracea* L. var. *acephala* – kale, *B. oleracea* L. var. *botrytis* L. – cauliflower, *B. oleracea* L. var. *capitata* – cabbage, *B. oleracea* L. var. *costata* DC. – tronchuda cabbage, *B. oleracea* L. var. *gemmifera* DC. – Brussels sprouts, *B. oleracea* L. var. *gongylodes* L. – kohlrabi, *B. oleracea* L. var. *italica* Plenck – sprouting broccoli. They are generally consumed as vegetables due to their phytochemical composition and other anti-oxidant properties (Butu and Rodino, 2019). The total glucosinolates in cabbage is between 1.05 – 70.56  $\mu\text{mol/g}$  DM, whereas, in

broccoli, the glucosinolate content can exceed 105  $\mu\text{mol/g}$  fresh weight with a 12.1g/100g of erucic acid (Rochfort and Jones, 2011).

**Turnips - *Brassica rapa*:** This is an annual and biennial crop found in all continents but predominantly in temperate regions. The leaves are often characterized by high pungent and bitter taste that is due to glucosinolate contents (van Doorn and van der Kruk, 1998). Furthermore, glucosinolates are found in the roots and predominantly in the seeds and can disrupt the normal digestion of livestock if consumed in high amount (Young-Mathews, 2012). On the other hand, the leaves can be eaten by humans either fresh or cooked as it provides a spicy flavor to food.

**Indian Mustard - *Brassica juncea*:** This is a natural hybrid of *B. nigra* and *B. rapa* L. The seeds of *B. juncea* are brown in color and are used as a spices in Indian cuisine (Kapoor, 1990), they also have medicinal uses and are consumed as vegetables in China. The oil content is around 30 – 43% (Thomas et al., 2012).

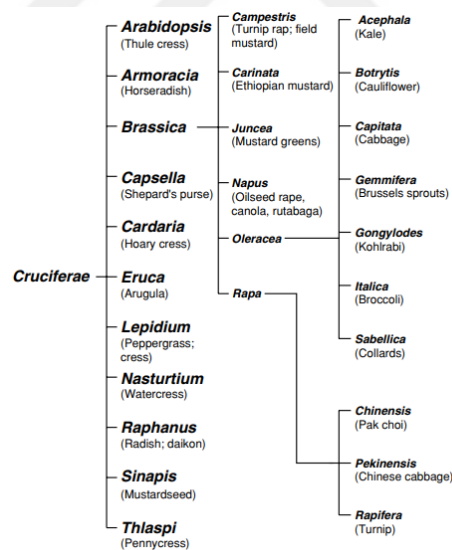


Figure 2.4. An overview of the most important members of the Cruciferae family Source: (Fahey, 2003).

## 2.5 Fiber

### 2.5.1 Importance of fiber to diet

In human nutrition, fiber has many health benefits among which is reduction of constipation and diarrhea (DeVries, 2003; Slavin and Jacobs, 2010). Earlier studies has shown that humans who consumed diets low in fiber suffered from a different disease compared to those who consumed high fiber diet (Ensminger et al., 1995). Fiber plays a key role in animal diets as well, minimum requirement is therefore set in the diet composition of monogastric, ruminants and poultry species for their maintenance and improved digestive growth (Wenk, 2001). On the other hand, the maximum fiber threshold should not be exceeded when formulating animal ration, as this will greatly result in a decline in nutrient utilization most especially protein digestibility (Lindberg, 2014). Fiber included in diet is said to increase satiety, improve gut development as well as overall animal performance (De Lange et al., 2010; De Leeuw et al., 2008; Knudsen et al., 2012). As crop species mature, their fiber contents based on DM increases while protein contents decrease resulting in low quality feed (Horrocks and Vallentine, 1999).

### 2.5.2 Measurement of fiber

Plant fiber is structural elements found in all higher plants, their main function is to offer strength and regulate the movement of other metabolites. They can offer mechanical strength to plants due to their well-designed cell wall structure which is mainly composed of polysaccharides, glycoproteins and phenolic compounds (van Dam and Gorshkova, 2003). The three main classes of fiber are dietary fiber, crude fiber, and detergent fiber.

**Dietary Fiber:** According to Kay (1982), fiber refers to the ubiquitous compounds found in food plants including their wide chemical as well as morphological characteristics which makes them resistant to enzymatic action in the human alimentary system. It can also be referred to as the residue of plant material that were not hydrolyzed by enzymes during digestion in the human system (Trowell et al., 1985). These plant materials include hemicellulose,

cellulose, lignin, oligosaccharides, pectin, gums, and waxes. There are soluble and insoluble dietary fiber, the solubility here refers to their reaction in water. Hemicellulose, cellulose and lignin are non-soluble while pectin, gums, mucilage are soluble in water forming a gummy substance (Dhingra et al., 2012).

**Crude Fiber:** Crude fiber measurement is used to estimate the quality of feeds by considering the least digestible fraction of the feed (Van Soest and McQueen, 1973). A method assigned to this measurement is called Weende method. This method was developed by Heinrich Einhof in 1806 in the German city Möglin and not in Weende. The method employs the use of a solvent, diluted aqueous acid and diluted alkali to extract fibrous plant fractions. Einhof referred to the left over after extraction as the “indigestible matter”. The acid used is 1.25%  $\text{H}_2\text{SO}_4$  and is useful in removing starch and sugars, for the removal of protein and other fractions, 1.25%  $\text{NaOH}$  is used for the alkaline hydrolysis (Möller, 2014). Weende method had many inaccuracies such that, around 80% of hemicellulose and 50 – 90% of lignin are washed away by acid or alkali during the sequential extraction (Van Soest and McQueen, 1973). These washed hemicellulose and lignin now appear in the Nitrogen Free Extract (NFE) and are calculated as part of the available carbohydrate. NFE represent the sugar and starch proportions in animal diet. The difference between the carbohydrates and crude fiber gives the NFE amount.

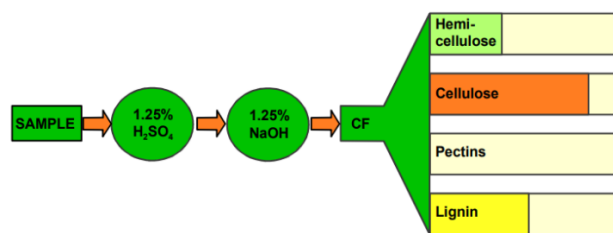


Figure 2.5. Crude fiber analysis based on Weende method

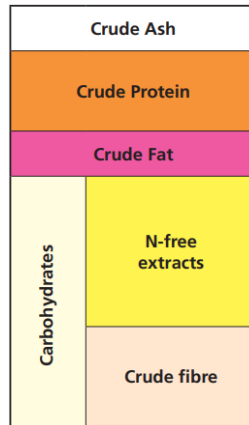


Figure 2.6. Weende method Source: (Möller, 2014).

New and better methods were then proposed in order to reduce the inaccuracy of the Weende method, however, questions were raised regarding the proposed methods such as what really are the constituents of fiber, what is the definition of hemicellulose, cellulose and lignin, the shortcomings of hemicellulose, cellulose and lignin to be biologically and chemically similar in different plant material (Van Soest and McQueen, 1973). The most practical and less error approach was the Van Soest method using detergent fiber.

**Detergent Fiber:** The method of using detergents developed by Van Soest and McQueen (1973) offered a better estimation of fiber fractions compared to Weende.

The first step involves using a neutral detergent solution (NDS) to treat samples, after treatment, the samples are rinsed using a heat stable amylase to ensure that the sugars, starch, and pectin become soluble. The residue is now composed of hemicellulose, cellulose, and lignin.

The second step involves using an acid detergent solution (ADS) to dissolve the hemicellulose while cellulose and lignin remain as residue.

The last step involves dissolving the cellulose using a concentrated sulfuric acid (72%  $\text{H}_2\text{SO}_4$ ) leaving only the lignin as residue (Möller, 2014). The fiber fractions NDF, ADF and ADL are estimated using this method.

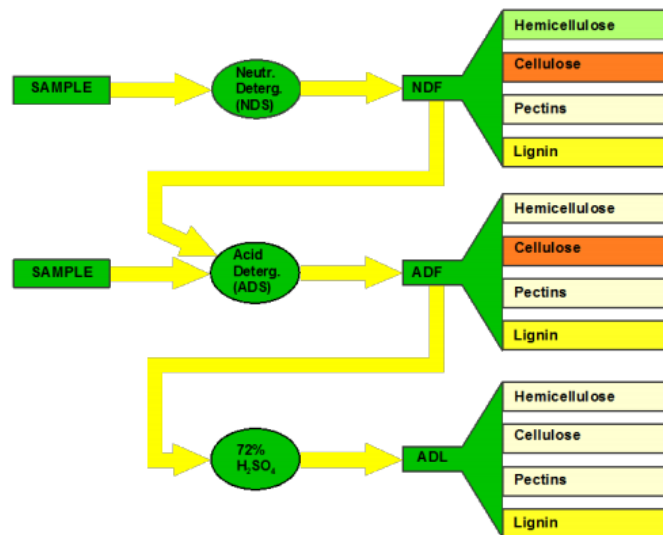


Figure 2.7. Detergent Fiber based on Van Soest method

|              |                |     |     |     |
|--------------|----------------|-----|-----|-----|
|              | Crude Ash      |     |     |     |
| Cell content | Crude Protein  |     |     |     |
|              | Crude Fat      |     |     |     |
|              | Sugar          | NSC |     |     |
|              | Starch         |     |     |     |
|              | Pectin         |     |     |     |
| Cell wall    | Organic Rest   |     |     |     |
|              | Hemi-cellulose | NDF |     |     |
|              | Cellulose      |     | ADF |     |
|              | Lignin         |     |     | ADL |

Figure 2.8. Modified Weende method by Van Soest Source: (Möller, 2014)

### 2.5.3 Fiber composition

Fiber is found in plant cell walls and it is further characterized into different compositions such as cellulose, hemicellulose, lignin, and pectin (Sorieul et al., 2016)

**Cellulose:** Cellulose makes up the largest cell wall components in plant having linear and unbranched repeating units (Dhingra et al., 2012). In other words, it is a key component of cell wall with a long chain polysaccharide structure of glucose repeating units. Each glucose unit has three free -OH available for hydrogen bonding, these bonds are important because they determine the crystal structure of cellulose (Lee et al., 2015). This strong hydrogen bonding with microfibrils makes cellulose resistant to microbial degradation, resistant to acid hydrolysis, and promote mechanical stress (Dhingra et al., 2012). In animal diet, cellulose provides an extra source of energy especially to ruminants as their rumen microbial enzymes can degrade cellulose whereas non-ruminants cannot naturally degrade cellulose. The cellulose content found in rapeseed meal is around 4.6 - 5.7% (Slominski et al., 2012).

**Hemicellulose:** Hemicellulose is found in the primary and secondary cell wall of plants and consists of wide range of different polysaccharides such as xyloglucan, xylan, arabinogalactan, mixed linkage glucans, galactomannan etc. (Sorieul et al., 2016). Most of these polysaccharides especially glucomannans, xyloglucan and xylan have a strong affinity for cellulose (Iwata et al., 1998; Hannuksela et al., 2002). However, galactomannans cannot bind with cellulose (Buchanan et al., 2015). Hemicellulose helps in the regulation of tension between the primary and secondary cell walls, strengthening the interface of cellulose and lignin, and may even play a role in lignification (Sorieul et al., 2016). Hemicellulose can be differentiated from cellulose based on their smaller size, with a branched and diverse sugar molecules (Kay, 1982). Synthesis of hemicellulose occurs in the Golgi apparatus of the cell. In animal diet, ruminants degrade hemicellulose partially, while degradation in non-ruminants is very poor (Van Soest, 1967). The hemicellulose contents included in rapeseed meal is around 13.8 – 14.3% (Slominski et al., 2012).

**Lignin:** The most abundant polymeric organic compound after cellulose in plant cell wall is lignin (Sorieul et al., 2016). However, it is important to note that lignin is not a polysaccharide but a complex random polymer. This polymer is made up of phenylpropanoid units such as sinapyl, p-coumaryl alcohol and coniferyl (Dhingra et al., 2012). Its aromatic molecule forms a three-dimensional

structure consisting of phenyl groups. Due to its strong carbon to carbon linkage, lignin is considered the most resistant naturally occurring polymer (Dhingra et al., 2012). Lignin can serve as an adhesive for cellulose microfibrils, it also plays a role in water permeability of plant cell walls and can offer protection against pathogens (Fagerstedt et al., 2010). In animal diets, lignin can serve as a physical barrier to the microbial degradation enzymes of cellulose and hemicellulose, thereby decreasing nutritional availability through direct effect on digestible energy of feeds (Jung and Allen, 1995; Moore and Jung, 2001). The lignin content found in rapeseed meal is around 10.4% (Slominski et al., 2012).

**Pectin:** They are complex polysaccharides rich in galacturonic acid and are negatively charged. They serve as adhesives in binding intercellular substances. The primary cell wall of dicots has three types of pectin structure namely, homogalacturonan, rhamnogalacturonan-I and rhamnogalacturonan-II. Pectin is a water-soluble polysaccharide and is fully metabolized by colonic bacteria (found in the large intestine of humans). Due to their solubility in water, pectin decreases the water mobility in plant cell wall and forms gels (White et al., 2014). This gelling behavior explains its hypoglycemic properties. The pectin content found in rapeseed meal is estimated to be between 1.8 - 2.8% (Slominski et al., 2012).

#### 2.5.4 Fiber fractions

Fiber is said to be made up of three important fractions based on the system proposed by Van Soest et al. (1991) namely Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF) and Acid Detergent Lignin (ADL). This system employs the use of sequential acid and neutral detergent digestion in order to distinguish feed samples in either digestible or indigestible components in comparison with the cell wall of the plant (Van Soest, 1994). All the fiber fraction can be used as an indicator for forage quality.

**Neutral Detergent Fiber:** NDF consists mostly of cellulose, hemicellulose, and lignin: This is a residue from the neutral detergent digestion and is insoluble in water. The cell wall of plant thickens with maturity, this also resulted in a corresponding increase in NDF content (Van Saun and Herdt, 2014). An increase

in NDF content increases rumen activity in ruminants resulting to a corresponding decrease in dry matter intake. Therefore, NDF can be used as a measure of feed quality and an indicator of plant maturity (Van Saun and Herdt, 2014). The NDF value can assist nutritionist in formulating feed composition with better intake ratio needed for animal consumption (Van Saun, 2006; Möller, 2008).

**Acid Detergent Fiber:** ADF consist mainly of cellulose and lignin because its acid detergent solubilizes hemicellulose. They are residuals of neutral detergent and made up of slowly fermentable as well as indigestible feed portions. Hence, ADF can be associated with feed digestibility (Van Saun and Herdt, 2014). Like NDF, ADF can also be an indicator of measuring feed quality in that high values in ADF means low quality of feeds

**Acid Detergent Lignin:** ADL consist of the non-digestible lignin. This is extracted from ADF by isolating the lignin using either hydrolysis (72%  $H_2SO_4$ ) or oxidative ( $KMnO_4$ ) method (Jung et al., 1997) with the former being the most widely used. Another method of measuring lignin content is by Klason procedure, the lignin value obtained under Klason are 2 - 5 times higher than that of ADL by sulfuric acid (Raffrenato and Van Amburgh, 2011). (Wittkop et al., 2009) reported a positive correlation between ADL and seed coat color in both yellow and black seeded cultivars. Both ADF and ADL can be used as indicator of feed digestibility (Van Saun, 2006).

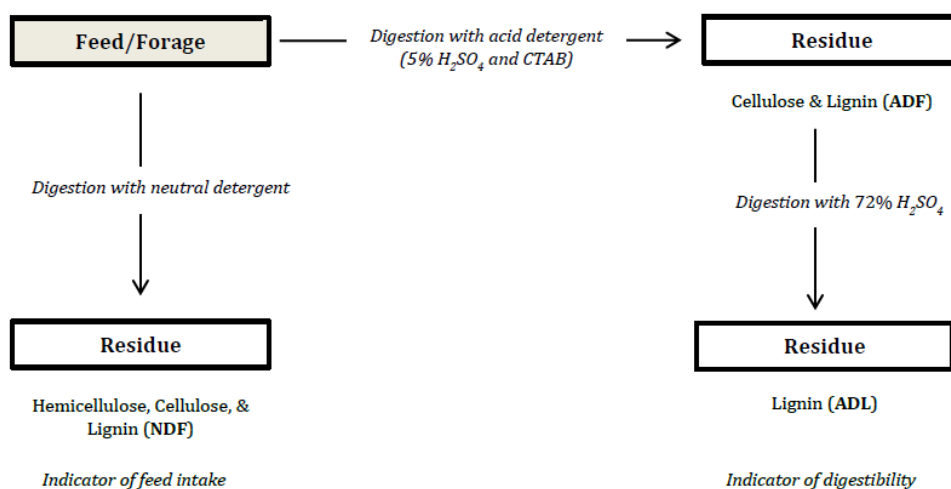


Figure 2.9. Separation of NDF, ADF and ADL Source:(Suprianto, 2014).

#### 2.5.4 Improving or breeding for low Fiber

Aside from its great benefit as a rich source of oil, rapeseed is also used as a protein source in animal feed. However, due to its lower protein content and its high fiber content when compared to soybean meal, the inclusion of rapeseed meal as protein source presented a setback to nutritionist. High fiber content is said to reduce protein digestibility in ruminant's diets, monogastric and poultry species. Classical approaches were first introduced such as removal of seed hull before oil extraction, using fungal and microbial enzymes to improve nutrient utilization in monogastric, protecting protein against rumen degradation, and breeding yellow seeded genotypes (Slominski, 1997, 1993). It is imperative to note that the color of the yellow seeded rapeseed is not due to seed coat pigmentation alone but is because of a transparent seed coat (*TT* gene), the underlying yellow cotyledon makes it appear yellow. There is still no natural yellow seeded *B. napus*. Nevertheless, natural occurring yellow seeded genotypes are found in *B. juncea*, *B. carinata*, and *B. rapa* (Rahman and McVetty, 2011; Slominski et al., 2012). These yellow seeded genotypes are reported to have high oil and protein content, low fiber content (especially lignin), lower husk proportion, and thinner seed coat (Meng et al., 1998; Wang et al., 2015). On the other hand, black color of the black seeded genotypes is as a result of high accumulation of flavonols and higher content of procyanidin (Akhov et al., 2009). Therefore, breeders started to breed for yellow genotypes to make rapeseed meal a rich source of protein in animal diet composition. Various breeding programs in Canada and Europe began to use yellow seeded cultivars to transfer target traits into the widely cultivated black seeded varieties. These new varieties (brown seeded) were found to contain lower fiber (26.6%) compared to traditional black seeded varieties (30.8%) and higher protein content 46.2% (in dry matter) in brown seeded against 45.2% in black seeded varieties (Slominski et al., 2007). The low fiber content of yellow-brown seeded varieties was due to their low polyphenols and lignin content when compared to black seeded varieties (Simbaya et al., 1995). Rakow et al. (2007) also reported similar result when they showed that the lignin content yellow seeded is 4.1% lower than black seeded. In another study, the fiber content from an interspecific cross of yellow seeded *B. napus* was 55% less than black seed varieties even as they were both coming from

the same genetic background (Rahman, 2001). Earlier studies showed that black seeded rapeseed is dominant over yellow seeded and is presumed to be controlled by three independent gene loci (Deynze et al., 1993). A few years later, further studies revealed that dominant or semi dominance of yellow seeded rapeseed over black seeded is attainable and that different loci can be involved (Tang et al., 1997; Jiana et al., 1998).

(Liu et al., 2012) crossed yellow seeded with black seeded Chinese rapeseed and revealed that a phenylpropanoid biosynthesis gene *CINNAMOYL CO-A REDUCTASE 1 (CCR1)* served as a positional and functional candidate gene for a major QTL located on the A09 chromosome explaining a larger variation of the ADL content. *CCR1* is the initial specific step involved in the biosynthesis of monolignols in the xylem and later assembled to form cell wall lignin (Piquemal et al., 1998). From this study it was revealed that *Bna.CCR1* homologue has the potential to interact with the surrounding of the gene family *CINNAMYL ALCOHOL DEHYDROGENASE (CAD)*. In *B. napus*, this *CAD*-gene family regulate seed coat in the phenylpropanoid biosynthesis pathway. These two genes (*CCR1* and *CAD*) are potential target in breeding for low ADL content in rapeseed (Stein et al., 2013).

Breeding for pure yellow seeded rapeseed variety has not been so successful partly due to the complexity of seed coat pigment biosynthesis which is strongly affected by light intensity, temperature and other environmental factors (Boesewinkel and Bouman, 1995; Burbulis and Kott, 2005). Another factor associated with low success in breeding for yellow seeded rapeseed is due to the complex inheritance of the seed color. High temperature results in a corresponding increase in yellowness of seeds (Deynze et al., 1993; Burbulis and Kott, 2005).

Furthermore, variations in fiber fractions such as NDF, ADF, ADL is possible even among black seeded European winter rapeseed (Wittkop et al., 2012). Selection is still underway in order to take advantage of this natural genetic variations, however, at the moment, there is little knowledge about the inheritance

of low fiber contents among different populations of black seeded genotypes, hence the need for this experiment.

**PAL gene:** Phenylpropanoids consist of secondary metabolites such as anthocyanins, phytoalexins, terpenoids, condensed tannin and most importantly lignin. These metabolites are all biosynthesized by plants from phenylalanine via the phenylpropanoid pathway. The regulation of the phenylpropanoid pathway is made possible by environmental factors like plant injury, exposure to UV-light, nutrient depletion, extreme temperatures, and biotic factors such as pathogen infection (Dixon and Paiva, 1995; Wanner et al., 1995). Phenylalanine ammonia lyase (*PAL*) is the initial key enzyme in the phenylpropanoid biosynthesis and helps to convert L-phenylalanine to cinnamic acid, this forms an important role in linking the primary metabolism with the secondary metabolism (Li et al., 2019). Also, PAL, can catalyze L- tyrosin to form *p*-coumaric acid in monocotyledonous plants. PAL was first found in Barley (*Hordeum vulgare*) and is now reported in many plants (Li et al., 2019)

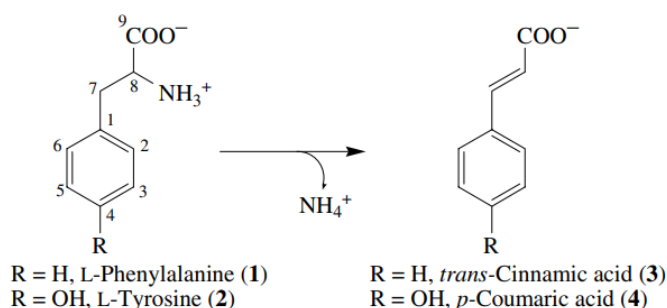


Figure 2.10. Conversion of PAL and TAL showing the product and substrate  
Source: (Cochrane et al., 2004).

There are multi-gene family of PAL expressed in different tissues of diverse plants with the number of isozymes depending on the plant species, for instance, there are eight (8) isozymes in *Brachypodium distachyon*, nine (9) in *Oryza sativa*, eleven (11) in *Zea mays*, five (5) in *Populus trichocarpa*, over 40 in *Solanum tuberosum* and four (4) in *Arabidopsis thaliana*. A recent study in *A. thaliana* showed that, there are four different PAL families namely *Atpal1*, *Atpal2*, *Atpal3*, and *Atpal4* and play a role in the defense mechanism and overall

development in *A. thaliana* (Cochrane et al., 2004). *Atpal1* is proposed to be associated with lignification process and studies using EST data showed that *Atpal2* also support lignification (Rookes and Cahill, 2003; Cochrane et al., 2004). *Atpal3* is expressed at low amounts in the leaves and roots (Raes et al., 2003) while *Atpal4* is abundant in developing seeds (Costa et al., 2003). Using *A. thaliana* as experimental plant, a research revealed that a double mutant *pal1 pal2* plant produced yellow seed due to the absence of tannin in the seed coats and most importantly, the mutant displayed a significant reduction in lignin accumulation (Huang et al., 2010).

Other genes associated with seed color include *TT* (*transparent testa* gene), *CAD* (*cinnamyl alcohol dehydrogenase*), and *TDS* (*tannin deficient seeds*) as shown in Fig 2.11.

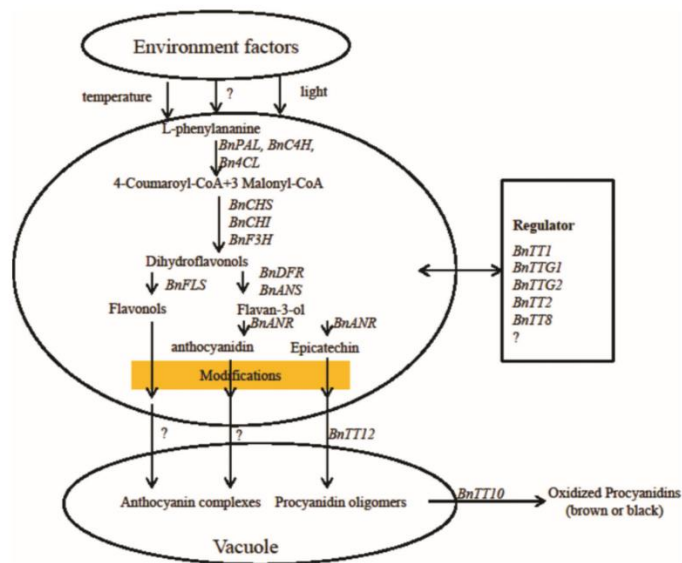


Figure 2.11. Mechanism underlying seed color in *B. napus* Source: (Qu et al., 2013).

## 2.6 Anti-nutritional compounds

Initially, the role of most of the anti-nutritional compound was to protect the plant from predators such as insects, rodents, monogastric and ruminants. One of the physiological effects of these compounds is the reduced palatability of meal in animal nutrition.

### 2.6.1 Glucosinolates

Glucosinolates are found in the seed meal following oil extraction from members of the Brassicaceae. They are secondary metabolites of sulfur and nitrogen containing compounds and general consist a sugar molecule- $\beta$ -D-thioglucose that is bounded to an aglycone. Glucosinolate are present in plant tissues at the same time with the enzyme '*myrosinase*' which is a thioglucosidase. In an intact plant, the enzyme (*myrosinase*) and the substrate (glucosinolate) are found in different part of the plant. However, if plant tissue become disrupted through chewing, pressing, or grinding to extract oil, these two compounds can come together. Therefore, the *myrosinase* cleaves the thioglucosidase bond of the glucosinolates resulting in the production of glucose and an intermediate product called aglycone (Hill, 2003). Further breakdown of aglycone yields isothiocyanate, thiocyanate and nitrile. These compounds are responsible for the bitter and hot taste in meals. When included in higher amount in diet composition, isothiocyanate and thiocyanate impaired with the thyroid activities (causing goiter) while nitriles can cause liver and kidney lesions (Hill, 2003). When lactating sows are fed with meal containing high amount of glucosinolates, it resulted in the abortion of piglets or subsequent death of piglets after birth (Hill, 2003). Through breeding, the glucosinolate content in oilseed rape cultivars has decreased from  $>100 \mu\text{mol/g DM}$  to less than  $30 \mu\text{mol/g DM}$ . This reduced amount ( $30 \mu\text{mol/g DM}$ ) is also considered safe and acceptable in animal nutrition. The concentration of glucosinolates in some plants can reach up to 3% of seed weight (Fahey, 2003).

### 2.6.2 Erucic acid

Erucic acid is also referred to as cis-13-docosenoic acid and is a long chain, monounsaturated omega-9 fatty acid of the seeds of members of the Brassicaceae family, it is also found in ornamental plants such as nasturtiums (Hebard, 2016). *In vivo*, erucic acid can be metabolized to form oleic acid. Dietary erucic acid is currently used to manage adrenoleukodystrophy (VLCFAs) as a glyceryl trierucate form commonly referred to as Lorenzo's oil (Barmaki Pour et al., 2000). Erucic acid found in the oil of oilseed rape if consumed is linked to the

accumulation of fat in the heart muscle of model laboratory rats thereby causing cardio pathogenic effects.

The average human adult can consume up to 500mg/day of erucic acid, this amount is set as the provisional tolerable daily intake (PTDI). A <2% erucic acid rapeseed (canola) is internationally accepted for use as oil for direct consumption by humans (Wanasundara et al., 2017).

The industrial application of erucic acid include its use as a raw material for hydraulic fluids, additives, lubricants, oleochemicals as well as a starting material in resins, plastics, and lacquers. (Carlsson et al., 2011). Earlier Brassica species contain more than 40% of erucic acids and above 100  $\mu\text{mol}$  of glucosinolates in their matured seeds (Rahman, 2013). Through target breeding, the erucic acid content was increased to more than 50% as found in the cultivar HEAR. Furthermore, Crambe (*Crambe abyssinica* Hochst) an annual crop from the Brassicaceae family was found to contain higher erucic acid (>60) compared to HEAR serving as an important candidate for various industries (Zhu, 2016).

### 2.6.3 Sinapine

Sinapine is a small molecular alkaloid found in the seeds of Brassicaceae. It is among the many compounds contributing to bitter taste in Brassica species and it's said to be responsible for the fishy egg taint when a meal containing sinapine is fed to brown egg laying hens (Burton et al., 2014). It is only found in seeds and as such it is easily hydrolyzed during germination to form sinapic acid and choline (Tzagoloff, 1963). High consumption of sinapine in meal can bring about reproductive problems (Pearson et al., 1980).

In *B. napus*, the sinapine content is around 7.4 – 16.2 g/kg. For other Brassica species, the sinapine content ranges from 8.5 – 14.5 g/kg in *B. carinata*; 7.3 – 14.3 g/kg in *B. juncea*; 6.7 – 12.2 g/kg in *B. nigra*, and 5.2 – 14.7 g/kg in *B. rapa* (Mailer et al., 2008). The marketable Canola seeds has a sinapine content between 0.7 – 4% with majority (>90%) of this found in the embryo (Blair and Reichert, 1984).

Sinapine is also beneficial owing to its protective property against UV, and play a contributing role in the synthesis of phosphatidylcholine-structural lipids associated with membrane binding of proteins (Strack, 1981). It also has industrial application especially in the pharmaceuticals due to its anti-inflammatory properties (Bhinu et al., 2009), anti-angiogenic effects (He, 2008), and as an antioxidant (Dubie et al., 2013).

## 2.7 Genetic Studies

*Brassica napus* (AACC,  $2n = 38$ ; genome size  $\sim 849.7$  Mb) is made up of two different genomes because of the origin of its individual parents; AA genome (*B. rapa* L., *syn campestris*;  $n = 10$ ; genome size  $\sim 312$  Mb) and CC genome (*Brassica oleracea* L,  $n = 9$ ; genome size  $\sim 540$  Mb). Due to this interspecific cross, rapeseed becomes an interesting plant species for various genomic studies. The diversity of the Brassicaceae family is also important, as the model plant used for various genetic studies *Arabidopsis thaliana* is also a species of this unique family.

### 2.7.1 QTL mapping

Plant breeding is centered on identifying interesting traits and incorporating them into a new cultivar to be released as a variety (Collard and Mackill, 2008). Variation is the key in genetic advance for any traits in breeding program. Many of these traits include yield, resistance to biotic (pest and pathogen infections) and abiotic stress (drought, salinity, etc.), quality improvement (taste, aroma, etc.) are all controlled by many genes thus referred to as polygenic or even complex, quantitative traits (Collard et al., 2005). A quantitative trait locus (pl. loci) -QTL is any region of the genome where genes controlling quantitative traits are found. The selection for these quantitative traits is not easy owing to environmental influences and their polygenic nature thus, molecular markers were developed to assist breeders in making selection on these traits (Rahman and McVetty, 2011).

A research to identify possible QTL associated to fiber and seed color was performed by Badani et al. (2006), at chromosome N18 ( $\sim C08$ ), they observed a

major QTL having wide effect on both seed color and ADF. Data from seed color and ADF content segregation coupled with QTL data revealed that a *B. napus* gene partially dominant for seed color situated on chromosome N18 is associated with reduced fiber contents in yellow seeded oilseed rape cultivars.

To detect QTL or construct genetic map, two approaches are recommended

1. Linkage Analysis: This uses a bi-parental population meaning that there is sufficient information within families. The underlying principle here lies on the associations of quantitative traits and markers that are segregated (Kearsey and Farquhar, 1998). The role of the markers is to assist and divide the mapping population into different genotypic groups due to a specific marker locus and to also determine the significant differences between groups based on the traits of interest being measured (Collard et al., 2005). The specific marker locus is said to be linked to a QTL controlling a trait if there is a significant difference between the phenotypic means in the different groups of the partitioned population.
2. Association Analysis: This considers individuals coming from diverse wild populations, landraces, germplasm collection etc. It is based on the linkage disequilibrium (LD) to analyze the relationship between phenotypic variations as well as genetic polymorphism (Flint-Garcia et al., 2003). Under this analysis, individuals of a mapping population do not necessarily have to be related. The detection of QTL under LD is based on the association between QTL and linked markers, this association is said to be heavily dependent on both the structure and extend of LD in the analyzed population (Honsdorf et al., 2010). Association analysis has been used in rapeseed to analyze phenotypic and morphological traits (Honsdorf et al., 2010), traits related to yield (Cai et al., 2014), and seed quality traits like glucosinolates (Hasan et al., 2008), seed oil (Zou et al., 2010), condensed tannin content (Rezaeizad et al., 2011), seed color and sinapine (Snowdon et al., 2010).

In a study using association analysis of 49 inbred lines of *B. napus* genetically different winter-type resulted in the identification of a major QTL affecting not only fiber content and seed color but also phenolic compounds located on chromosome A09. A bi-parental mapping population was obtained from the cross between black and yellow seeded *B. napus* Express 617 x 1012-98 and they found the same major QTL (in their prev. experiment with inbred lines) was located on linkage group A09 (Snowdon et al., 2010).

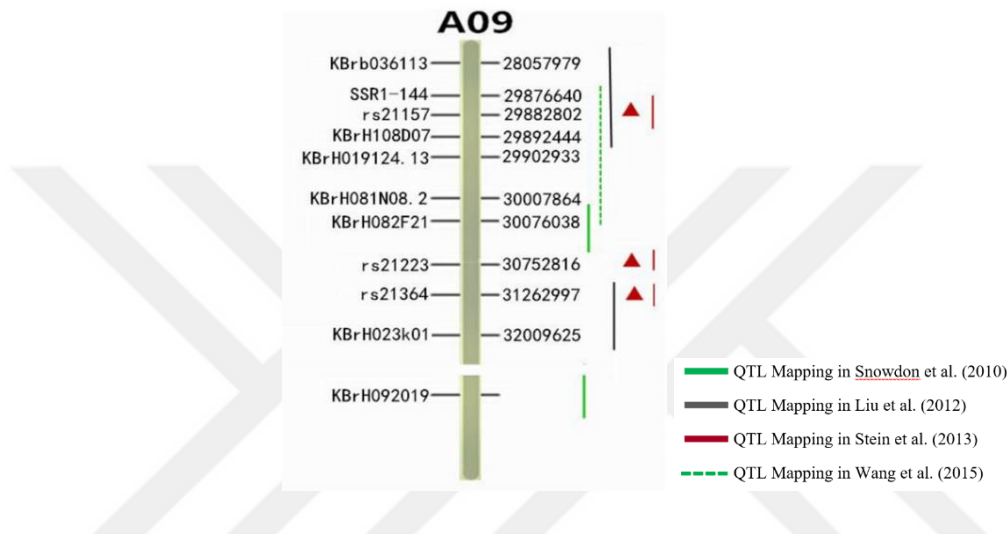


Figure 2.12. Overlapping or Linkage relationship among Seed ADL content QTLs on chromosome A09 from different studies Source: (Wang et al., 2015).

In a different research, 12 QTLs associated with seed hull content and another four QTLs associated with seed color were reported in a recombinant inbred line (RIL) obtained from the cross of yellow and black seeded parents (Yan et al., 2009).

### 2.7.2 Polymerase Chain Reaction (PCR)

This is an important technique used to make copies of a targeted DNA region in the genome of an organism. PCR is used to amplify a specific region of the DNA, thousands of copies (amplicons) of the targeted DNA is obtained at the end of PCR reaction.

There are five important components included in every PCR reaction

**DNA template:** This is the sample DNA containing the region you wish to amplify, and this can be a small tissue, genomic DNA, plasmid DNA

**Primers:** These are short oligonucleotides usually between 15 – 25 nucleotides long and anneal to the DNA templates by complementary base pairing when running the PCR reaction. There are two different primers in each reaction mix, a forward and a reverse primer. Primer design is an important step for a successful PCR experiment. High specificity (attributed to >25 bp) make primers difficult to anneal to the DNA templates. Furthermore, high concentration of primer results in low specificity and high efficiency of the reaction (Najafov and Hoxhaj, 2017). The primers provide the binding site for the DNA polymerase.

**DNA Polymerase:** The most widely used enzyme is called ‘Taq’ polymerase, isolated from a hot spring bacterium *Thermus aquaticus*, this is owing to its ability to survive in higher temperatures (>75°C). The enzyme with the aid of dNTPs synthesizes new strands of DNA that is in complementarity with the targeted region. Taq polymerase does not have the ability to proofread (3’–5’ exonuclease) newly formed sequences as such the frequency of error or mutation obtain at the end of PCR reaction is 1 in 200 000 per nucleotide per duplication of DNA (Najafov and Hoxhaj, 2017). However, Pfu Polymerase (from *Pyrococcus furiosus*) can be used due to its high fidelity and proofreading ability (3’–5’ exonuclease), Pfu polymerase offers ten times higher fidelity compared to Taq polymerase.

**Deoxynucleotide triphosphate:** This refers to the building blocks of the DNA, they are often referred to as nucleotides. There are four types of nucleotides in a PCR reaction namely ATP, CTP, GTP, TTP collectively known as dNTPs (DeLong and Zhou, 2015). These building blocks are joined at the 3’ end of a primer by the help of the Taq polymerase and are continually added in complementarity with the base pair of the template DNA.

**PCR Buffer:** This is important to maintain an optimum pH during the reaction cycles and they also provide the necessary ions needed by the enzyme. The volume of the buffer is always 10x that of the polymerase enzyme.

The basic steps involved in PCR reaction can be summed up into 3 basic steps

**Denaturation:** Here, the double stranded DNA molecule is subjected to high temperature of 95 °C, this high temperature causes the double strand to break and form single stranded DNA.

**Annealing:** The temperature for annealing is around 50 – 66 °C, this low temperature is sufficient for the primers to anneal to individual single stranded DNA.

**Extension:** In the extension phase, the Taq polymerase attaches to the primers and the dNTPs are added progressively to each strand as the enzyme moves.

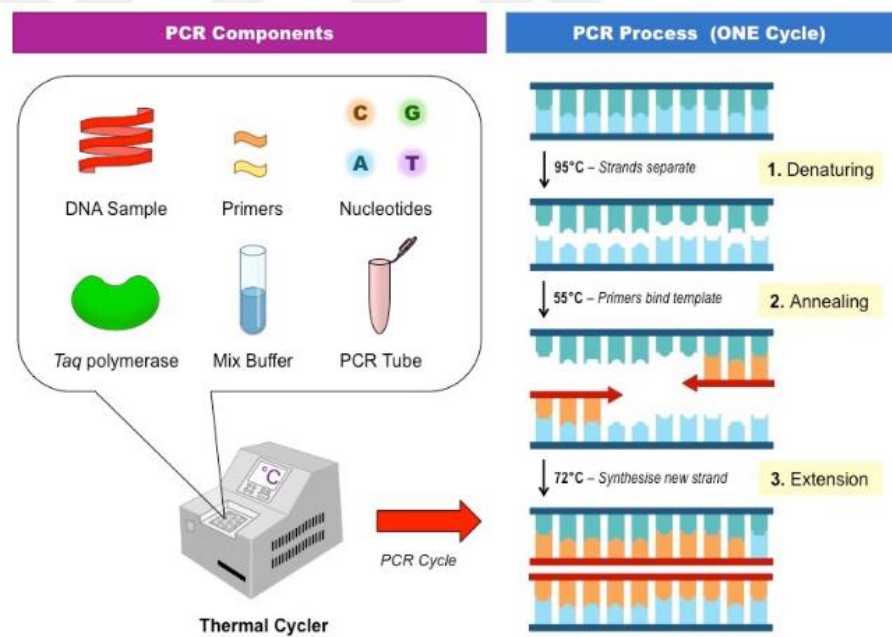


Figure 2.13. PCR components and Steps Source: (Mahajan et al., 2019).

### 2.7.3 Near infrared Reflectance Spectroscopy (NIRS)

NIRS uses the near infrared region of the electromagnetic spectrum ranging from 780 – 2526 nm to design calibration equation relevant to sample properties

(Reich, 2005). A light source (lamp) is cast on samples, their absorbed radiation caused by the reflection will be measured by a detector. The quality of the calibration equation used in NIRS is the major determinant on the reliability of the measured component (Stuth et al., 2003). To develop a robust calibration equation, important factors such as the type and origin of breeding materials should be considered. Also, a comprehensive list of samples covering the entire population to be analyzed should be considered. Origin of breeding material here means the cultivation environment, and it could play a role in the final error value of NIRS analysis. Accurate calibration equations were reported for erucic acid, oleic, linoleic, and linolenic acid because a wide range of samples was available for these traits (Leonardo Velasco and Becker, 1998). Furthermore, the existence of a large variation on the calibration data set inferred a close association between NIRS and gas chromatography (GC) results.

The accuracy of NIRS can be evaluated using some statistical values such as

- i. Coefficient of determination ( $R^2$ ) of calibration and validation: This explains the proportion of the variance in response to the calibration or validation data set.
- ii. Standard error for performance, adjusted and corrected for bias [SEP(C)]
- iii. Ratio performance deviation [RPD,  $SD/SEP(C)$ ]: A numerical value of 3 represent and excellent prediction, a value of 2.5 means a good prediction. Values between 2 – 2.5 means the prediction model is sufficient while values between 1.5 - 1.9 needs improvement (Fearn, 2002).
- iv. Range-to-error ration [RER,  $Range/SEP(C)$ ]: To be considered a good calibration, the minimum value of RER should be between 9 – 10 (Williams and Sobering, 1996).

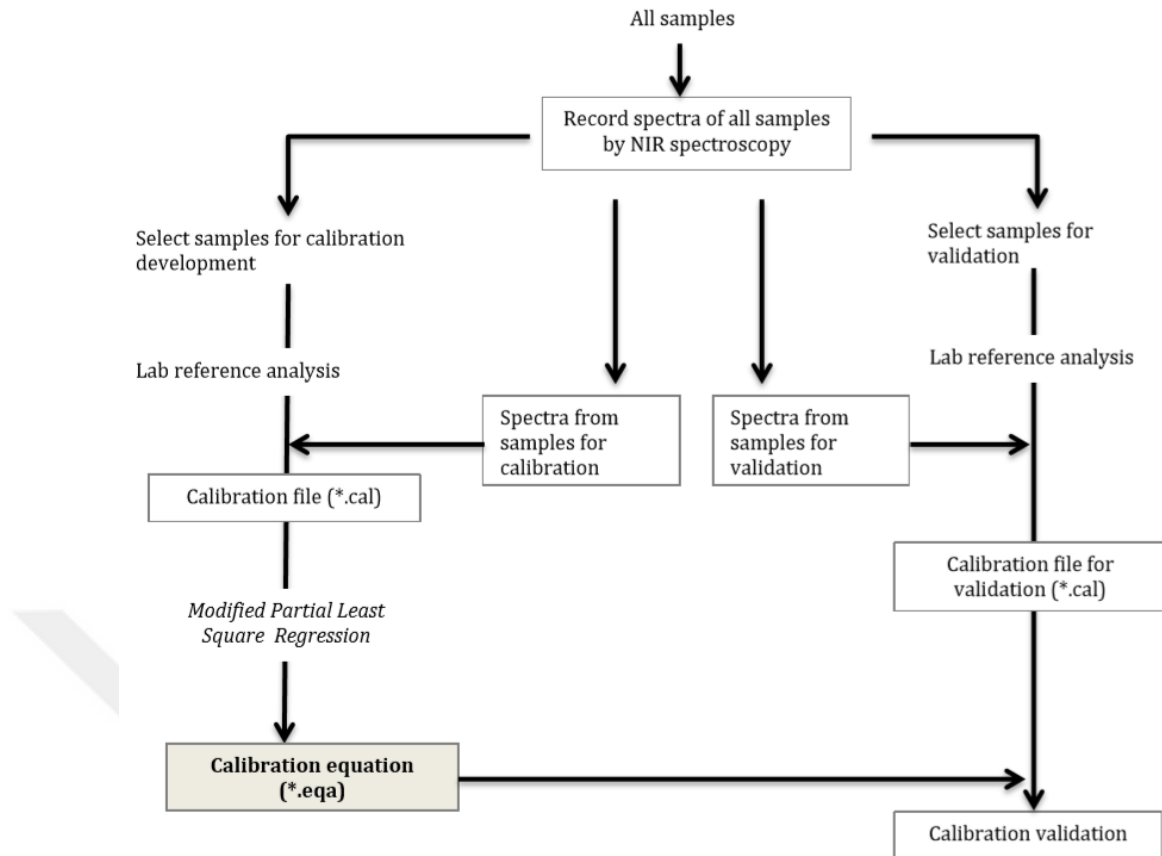


Figure 2.14. Development of calibration equation for nirs Source: (Suprianto, 2014).

NIRS has been used in oilseed rape to measure various traits such as oil content (Velasco et al., 1999), protein content (Velasco and Möllers, 2002), glucosinolate content (L. Velasco and Becker, 1998), erucic acid content (Sasongko and Möllers, 2005), sinapate esters content (Zum Felde et al., 2007), phytosterol content (Amar et al., 2009) and fatty acids such as oleic, linoleic and linolenic (Leonardo Velasco and Becker, 1998). In a recent experiment, 338 diverse winter-type rapeseed were used to develop NIRS calibration to estimate NDF, ADF and ADL, their developed calibration was propose to be use in breeding programs for both young and advanced breeding materials (Wittkop et al., 2012). To study the potential of using NIRS to evaluate the ADF in Brassica seeds, (Font et al., 2003) used *B. napus*, *B. carinata* and *B. juncea* in their experiment and concluded that NIRS can be used as a screening tool in breeding programs due to its sufficient accuracy in measure ADF contents in Brassica seeds.

NIRS is not only used in measurement of seed related traits but can be used to measure moisture content and grain hardness in forages (Batten, 1998). It was also successful in predicting fiber contents in many cereals including maize (Cozzolino et al., 2001), rice (Kong et al., 2005), wheat (Stubbs et al., 2010), and barley (Gous et al., 2012), as well as in legumes such as alfalfa (Brogha et al., 2009).

Near infrared reflectance spectroscopy is superior to classical chemical-based methods such that it is a non-destructive technique, it is very fast, relatively cheap, requires less labor and permits the analysis of various traits from one sample and it is environmentally friendly (Stuth et al., 2003).

#### **2.7.4 Kompetitive Allele Specific PCR (KASP)**

The use of molecular markers has greatly assisted and improved selection in plant breeding programs. One of the latest markers is the single nucleotide polymorphism (SNP). The advantages of the SNP over other markers includes its precision and ability to detect variation in base pairs of the genome, in addition to its reliability. There are many SNP genotyping platforms including BeadXpress<sup>TM</sup>, GoldenGate<sup>TM</sup> and Infinium<sup>®</sup> from Illumina (<http://www.illumina.com>); SNaPshot<sup>TM</sup> and TaqMan<sup>TM</sup> from the Applied Biosystems (<http://www.appliedbiosystems.com>); SNPWave<sup>TM</sup> from KeyGene (<http://www.keygene.com>); Pyrosequencing from Royal Institute of Technology (Sweden); GeneChip<sup>TM</sup> and GenFlex<sup>TM</sup> Tag array from Affimetrix (<http://www.affymetrix.com>); SNPstream<sup>TM</sup> from Beckman Coulter (<http://www.labwrench.com>); iPLEX Gold<sup>TM</sup> Assay and MassARRAY<sup>TM</sup> from Sequenom (<http://www.sequenom.com/iplex>); molecular inversion probes from ParAllele Biosciences; and Kompetitive Allele Specific PCR or KASP<sup>TM</sup> from KBioscience or LGC Genomics (<http://www.lgcgenomics.com>). KASP is a homogenous, fluorescence-based technology of genotyping. The principle is based on allele-specific oligo extension coupled with fluorescence resonance energy transfer (FRET) useful for signal generation and can be carried out on 96, 384 or 1536 well plates format (Kumpatla et al., 2012). KASP can be used to discriminate between two alleles of SNP marker using a common reverse primer paired with two forward primers where one is specific to each allele. The forward primers each has a nucleotide sequence that can hybridize to either FAM or HEX

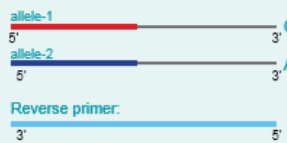
fluorophore quencher. The quencher can release fluorophore when the forward primer is integrated into a PCR product, this will cause the fluorophore to fluoresce (Patterson et al., 2017). This fluorescence can then be detected at the end of the assay using a real time PCR machine, the proportion of fluorescence from FAM, HEX or both represents the genotype of the samples. If the genotype is homozygous, only one of FAM or HEX will give fluorescence signal, but if the genotype is heterozygous, a mixed signal can be generated (He et al., 2014). The principle of KASP genotyping is shown in Fig 2.15.

### 1) Assay components:

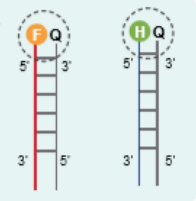
KASP uses three components: test DNA with the SNP of interest; KASP Assay mix containing two different, allele-specific, competing forward primers with unique tail sequences and one reverse primer; the KASP Master mix containing FRET cassette plus Taq polymerase in an optimised buffer solution.

#### A) KASP Assay mix

Allele-specific forward primers:



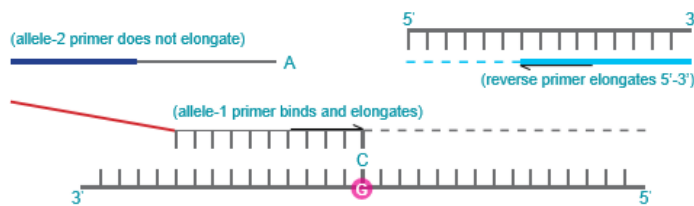
#### B) KASP Master mix



#### C) DNA template (sample)



### 2) Denatured template and annealing components – PCR round 1:



In the first round of PCR, one of the allele-specific primers matches the target SNP and, with the common reverse primer, amplifies the target region.

### 3) Complement of allele-specific tail sequence generated – PCR round 2:

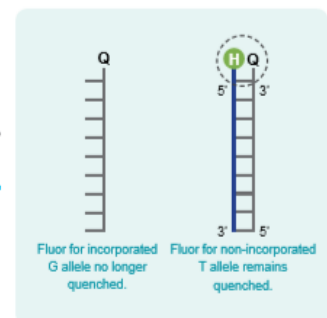


(Reverse primer binds, elongates and makes a complementary copy of the allele-1 tail.)

### 4) Signal generation – PCR round 3:



In further rounds of PCR, levels of allele-specific tail increase. The fluor labelled part of the FRET cassette is complementary to new tail sequences and binds, releasing the fluor from the quencher to generate a fluorescent signal.



Fluor for incorporated G allele no longer quenched. Fluor for non-incorporated T allele remains quenched.

Figure 2.15. Overall mechanism of KASP genotyping Source: He et al., 2014.

The advantage of KASP over other SNP genotyping tools is that, it is a faster, flexible and cost-effective method. They can be easily adjusted to fit statistical analysis like assigning probability to genotyping calls because KASP assay are quantitative (Patterson et al., 2017). KASP is used in quality control (QC), marker assisted recurrent selection (MARS), QTL mapping, large scale allele mining (Semagn et al., 2014).

## 2.8 Inheritance

One of the important traits associated with low fiber content in rapeseed is seed color. It was recently revealed that, the heritability for seed color ( $H^2 = 0.51$ ) was lower compared to fiber components ( $H^2 = 0.79$  for ADL) and that genotypes showing similar seed color can have higher variation in ADL content, this means that seed color alone is not a reliable criteria of selection for reduced fiber in breeding programs (Snowdon et al., 2010; Liu et al., 2012). The inheritance of seed color in *B. rapa* (AA) is said to be under monogenic control. Nevertheless, distorted segregation pattern can be seen in some generations (Chen and Heneen, 1992). Further, reports of one to three gene inheritance for seed color were also available (Stringam, 1980; Hawk, 1982). On the other hand, black or brown seed color was said to be regulated by two independent dominant genes in *B. juncea* (AABB) (Vera and Woods, 1982). This then let to an assumption that the genomes of *B. rapa* (AA) and *B. nigra* (BB) each contributes one of the two genes linked to seed color in *B. juncea* (AABB). On the contrary, the inheritance of seed color in many C genome cultivars is complicated. (Chen et al., 1988) made an interspecific cross between black seeded *B. alboglabra* (a subspecies of *B. oleracea* CC) and yellow seeded *B. carinata* (BBCC) and obtained light brown *B. alboglabra*. Currently, no true yellow seeded *B. napus* has been achieved through breeding (Tang et al., 1997).

The heritability of NDF, ADF and ADL content was found to exceed 0.80 among 405 rapeseed accessions in winter trials and higher than 0.90 in spring trials (Körber et al., 2016).

The heritability of oil content is reliable and has been used to improved oil seed contents from 30 to 50% (Mailer, 2016).

The ADL content is associated to the seed hull of rapeseed, and the seed hull is a maternal trait, therefore the inheritance of lignin is maternal (Widiarsih, 2017).



### 3. MATERIAL AND METHODS

#### 3.1 Plant Material

Six different parental genotypes were used to carry out this experiment, namely (4042xExpress DH15), (4042xExpress DH14), Lord, R19, L239 and SGDH14. All parental genotypes were obtained from the Division of Crop Plant Genetics, Department of Crop Sciences of the University of Göttingen Germany. Crossing was carried out to obtain four populations namely Population I- **L239 x Lord**, Population II- **[(4042xExpress DH15) x Lord]**, Population III- **R19 x Lord** and Population IV- **[SGDH14 x (4042xExpress DH14)]**. The F1 seeds from these crosses were grown to obtain F2 populations. The 4042 line is an old yellow seeded winter-type (generally having low ADL content) from Göttingen germplasm, whereas Express is a black seeded winter-type line cultivar (associated with high ADL content), a cross was made between these two lines (4042 x Express) to obtain DH population, DH15 was selected and found to have a major QTL on C03 for low ADL content. From the same DH population, DH14 was selected and then crossed with another double haploid population SGDH14 (to yield SGDH14 x 4042xExpDH14). An old winter-type cultivar from Germany called Sollux was crossed with a Chinese semi-winter cultivar Gaoyou from the Zhejiang province of China. These two cultivars are black seeded, and a double haploid was obtained from this cross where DH14 (now SGDH14) was selected for oil and other seed quality traits. Lord is an open pollinated cultivar with low ADL. Both R19 and L239 are resynthesized lines and have low ADL content.

#### 3.2 Experimental Setup

For Population I – III, seeds of the parents, F1 and F2 were sown in November 2019. A week after sowing, all plants were transferred to a cold chamber (at 8 °C) in small plates (3.5cm x 7cm x 2cm) under 8 hours light. Following nine weeks in the cold chamber (vernalization period), the plants were transferred to a bigger pot (10.5cm x 11.5cm x 7cm) and placed on a bench in the greenhouse. Population I and III were placed on two benches in Greenhouse 2, parental genotype and F1 were randomly distributed among the F2 at 20cm apart.

At the onset of flowering, a selfing bag was placed on each flowering plant. Population II was placed in Greenhouse 1, also the parental genotype and F1 were randomly distributed at 20 cm from each other on the bench, a selfing bag was also placed on plants at the beginning of flowering. Population IV was grown in Greenhouse 3, since there are no F1 genotypes here, only the parental genotypes were randomly distributed among the F2 population at 30 cm over three platforms to encourage self-pollination

In Population I, there were 96 F2 plants, parental line L239 and Lord with three replicates each and an F1 with 7 replicates grown in Greenhouse 2.

Population II has 96 F2 plants, the parental lines (4042xExpress DH15), Lord and Express617 each had 7 replicates and an F1 with 7 replicate and placed in Greenhouse 1.

In Population III, there were 96 F2 plants, the parental line R19 and Lord had 3 replicates each while the F1 had 7 replicates also grown in Greenhouse 2.

Population IV has 161 F2 plants with 7 replicates of each of the parental lines SGD14 and 4042xExpDH14. There were no F1 plants in Population IV, parents and F2 were grown in Greenhouse 3.

### **3.3 Laboratory Procedures**

#### **3.3.1 DNA extraction**

DNA sample was taken from fresh leaf material of two weeks old (after transferring them to small pots in the Greenhouse 1, 2 and 3) F<sub>2</sub>, F<sub>1</sub> and parental lines of all four populations. Three leaves samples were taken from each parental line, while F1 and F2 leaves material were also taken weighing on average 50mg and placed into a 2 ml Eppendorf tubes and frozen in liquid nitrogen, all frozen samples were shortly stored at -20 °C. Extraction of genomic DNA was performed using innuPREP Plant DNA Kit (Analytik Jena, Germany) in accordance with manufacturer's user manual. After successful DNA extraction,

DNA concentration was therefore measured with Bio-Rad Versa Fluor™ Fluorometer (<http://www.bio-rad.com/en-us/product/versafluorometer>) using Bio-Rad Fluorescent DNA Quantification Kit (BioRad Laboratories CA, USA).

### 3.3.2 KASP genotyping

DNA sample was obtained from fresh leaf material of all F<sub>2</sub> and parental genotype of Population IV. Approximately 50 mg of leaf material from each genotype was taken and inserted into 2 ml Eppendorf tubes and placed in a kyrobox and later stored at -20 °C. Extraction of genomic DNA was done in accordance with Maria von Korff Schmising method.

This method involves grinding leaf material in a milling machine, followed by adding 400µl of an isolation buffer and centrifuge at 13000rpm for 5 mins. Subsequently, 300 µl of the supernatant is transferred into a new 1.5 ml Eppendorf tube where 300 µl of isopropanol is added to aid DNA coagulation and allowed to incubate at room temperature for 3 mins. Another centrifugation for 5 mins at 14000 rpm is carried out and afterwards the supernatant is discarded. A 500 µl of 70% ethanol is added and allowed to incubate for 3mins at room temperature. The ethanol aid to remove plasma membrane layer and other fats sources to yield pure DNA. The solution was then vortexed thoroughly and a final centrifugation was carried out at 14000 rpm for another 5 mins. The supernatant was discarded and while the residue at the bottom of the top could dry for 30mins at room temperature. The final step in this process involves the addition of 50 µl of HPLC grade water into the Eppendorf tube containing the pellet, DNA solution was then stored at -20 °C.

Upon the successful extraction of the genomic DNA, a Bio-Rad Versa Fluor™ Fluorometer (<http://www.bio-rad.com/en-us/product/versafluorometer>) using Bio-Rad Fluorescent DNA Quantification Kit (BioRad Laboratories CA, USA) was used to measure the concentration of DNA.

KASP genotyping was carried out at the University of Göttingen using the Bio-rad CFX96 Touch™ Real Time PCR Detection System, C1000 Thermal

Cycler ([www.bio-rad.com](http://www.bio-rad.com)). All reagents were properly vortex before used. DNA samples were first arrayed on a 96 well reaction plates including no template control (NTC) on the same plate.

Table 3.1. Composition and volume of KASP reaction

|                 | $\mu\text{l/sample}$ |
|-----------------|----------------------|
| KASP Master mix | 5.0                  |
| KASP Assay mix  | 0.14                 |
| DNA sample      | 5.0                  |

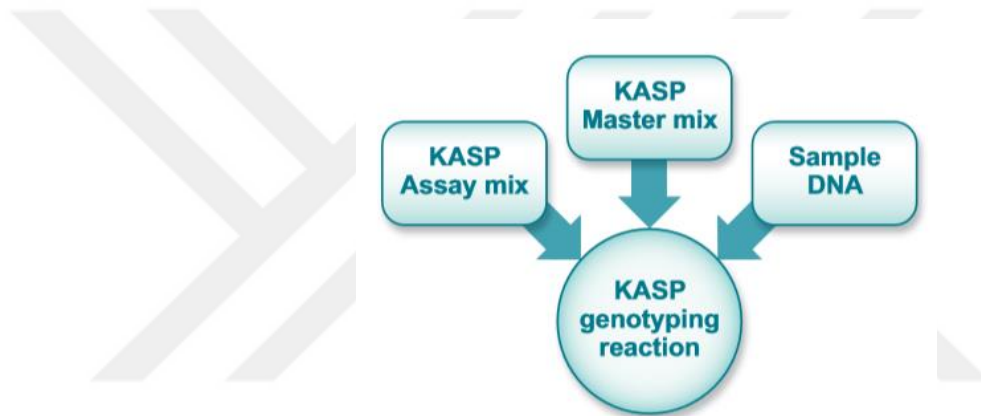


Figure 3.1. Component of KASP genotyping.

The constituent of the KASP assay mix include:

- Two allele specific forward primers (one primer for each of the SNP allele). Each primer carries a unique unlabeled tail sequence at the 5' end.
- One common reverse primer.

On the other hand, the KASP master mix is composed of:

- FAM<sup>TM</sup> and HEX<sup>TM</sup> specific FRET cassette
- Taq polymerase enzyme specifically modified for allelic specific PCR
- An optimization buffers

To prevent evaporation of samples, the reaction plate was sealed using a transparent adhesive foil. Samples were then centrifuged at 550rpm. Thermal plate was ran using standard thermal cycle, the temperature reduced to  $< 40^{\circ}\text{C}$  after the completion of the thermal cycle. To read the samples, the software Bio-rad CFX Manager version 3.1 was used where FAM allele was reported on x-axis while HEX allele was reported on y-axis. FAM and HEX are universal FRET cassette (dyes). Some genotypes may fail to give clear reading, to improve the quality of such reading, the samples were subjected to additional three cycles. The first step of the “recycling” is denaturation at  $94^{\circ}\text{C}$  for 20 seconds, followed by annealing/elongation step at  $57^{\circ}\text{C}$  for 60 seconds. This recycling process can be repeated up to three times until there are clear results of samples.

Table 3.2. Thermal cycling conditions for KASP chemistry

| Steps | Activity             | Temperature               | Time                                              | No. of cycles/step |
|-------|----------------------|---------------------------|---------------------------------------------------|--------------------|
| 1     | Activation           | $94^{\circ}\text{C}$      | 15 mins                                           | 1 cycle            |
| 2     | Denaturation         | $94^{\circ}\text{C}$      | 20 sec                                            | 10                 |
|       | Annealing/Elongation | $61 - 55^{\circ}\text{C}$ | 60 sec (drop $0.6^{\circ}\text{C}/\text{cycle}$ ) |                    |
| 3     | Denaturation         | $94^{\circ}\text{C}$      | 20 sec                                            | 26 cycles          |
|       | Annealing/Elongation | $55^{\circ}\text{C}$      | 20 sec                                            |                    |

Table 3.3. Thermal recycling conditions for KASP chemistry

| Steps | Activity             | Temperature          | Time   | No. of cycles/step |
|-------|----------------------|----------------------|--------|--------------------|
| 1     | Denaturation         | $94^{\circ}\text{C}$ | 20 sec | 3                  |
| 2     | Annealing/Elongation | $57^{\circ}\text{C}$ | 60 sec |                    |

**KASP Marker Design:** A major QTL was found in C05 and reported to be associated with reduced lignin content in a DH population (SGDH14) (Behnke et al., 2018). In a separate study, another QTL was found on C03 associated with reduced lignin content in the DH population (4042xExpDH14) (Widiarsih, 2017).

These two different DH lines were crossed SGD14 x 4042xExpDH14 and their F2 progenies analyzed to observe allelic differences.

In the SGD14 population, Behnke et al. (2018) used an Illumina Infinium Brassica 60K SNP marker. In general, 58464 SNP markers were tested for different quality traits in oilseed rape ranging from oil, protein, erucic acid, glucosinolate, fiber components etc. From these markers 15 474 markers were polymorphic for different traits. Six markers are related to lignin content in C05 genome. These markers were further tested in the population SGD14 x 4042xExpDH14 to observe polymorphism between the parents.

Using 20 000 SNP markers 20k chip from KWS to test for polymorphism in the parental genotype 4042xExpDH14, only 11 markers were polymorphic, and all were found on the C03 genome. These 11 markers were then tested in on SGD14 x 4042xExpDH14 to observe differences in the parents

For the F2 population, 54 samples were selected based on high or low ADL content as predicted by NIRS measurement. These 54 F2 were then analyzed using KASP marker related to both C05 and C03 genome to observe allelic differences.

Out of the 11 SNP markers associated with C03, four markers showed polymorphism between the parental genotype SGD14 x 4042xExpDH14. In addition, two out of the six markers linked to C05 were polymorphic between the parents. The polymorphic markers are shown in Table 3.4.

Table 3.4. Chromosome and physical location of polymorphic markers

| Marker name               | Chromosome | Physical position (Reference genome) * |
|---------------------------|------------|----------------------------------------|
| BN-SCAFF_18322_1-P1655555 | C03        | 7298559                                |
| Bn-Scaff_183_22_1_p826786 | C03        | 8204328                                |
| Bn-Scaff_18322_1_p2299816 | C03        | 6906899                                |
| Bn-Scaff_217_78_1_p215559 | C03        | 5012004                                |
| Bn-scaff_17441_3-p37334   | C05        | 40336042                               |
| Bn-scaff_17441_3-p28950   | C05        | 40322584                               |

\*Reference genome used was *B. napus* Darmor-bzh

### 3.3.3 PCR procedure

The purpose of a PCR procedure was to observe amplification of the PAL gene in the parental genotypes of Population I, II and III.

Genomic DNA was extracted from all parental genotypes of the four different population, the high DNA concentration (average 300 ng/μl) was diluted to around 50 ng/μl. For this reaction, four *B. napus* were used namely Lord, L239, R19 and 4042xExpDH15, also two *B. rapa* (Yellow Sarson, Storkin), and *B. oleracea* (Azur, BRA165) were used as a check to observe amplification. Three different primers were designed for the PAL gene using *B. napus* Darmor-*bzh* as reference genome (Chalhoub et al., 2014). The three primers pairs used for the PCR protocol include 134/135, 136/137, and 138/139.

Table 3.5. Master Mix solution per sample

| Stock solution              | μl for 1 sample |
|-----------------------------|-----------------|
| HPLC grade H <sub>2</sub> O | 17.25           |
| Buffer 10x                  | 2.5             |
| MgCl <sub>2</sub> 25mM      | 1.5             |
| dNTPs 10mM                  | 0.5             |
| F Primer 10pmol/μl          | 1               |
| R Primer 10pmol/μl          | 1               |
| Taq polymerase 5U/μl        | 0.25            |
| DNA 100nM                   | 1               |

Each sample containing 25 μl solution was placed in a Thermocycler and allowed to run for 90 minutes.

Table 3.6. PCR Steps

| Steps        | Temperature (°C) | Time (min) | No.of Cycles |
|--------------|------------------|------------|--------------|
| Denaturation | 94               | 3          | 1            |
| Denaturation | 94               | 0.5        | 30           |
| Annealing    | 59               | 0.5        |              |
| Extension    | 72               | 1*         |              |
| Elongation   | 72               | 5          | 1            |

\*up to 1000bp

Following successful PCR reaction obtained from the thermocycler, 5 µl of a loading buffer was added to each sample. This buffer is important to observe the visual PCR products at the end of the gel electrophoresis.

**Gel Preparation:** The gel was prepared using agarose powder. 2 g of agarose at a concentration of 1% was measured and placed into a beaker. 200 ml of buffer was then added into the same beaker and heated in a microwave. Following 2 mins of heating, the solution could cool down while stirring gently for 20 mins. After obtaining a clear solution, 2.5 µl of ethidium bromide was added and stirred again for few minutes. The Ethidium bromide is important in the detection and visualization of DNA fragment under UV light. The solution is then transferred to a gel chamber and allowed to gradual cool and solidify. The gel is now ready and completely submerged in a buffer solution for gel electrophoresis.

**Gel Electrophoresis:** By pipette mixing, 30 µl of each sample was transferred into the wells of the gel, a ladder was added twice in different wells, and 6 µl of ladder each was used. The samples on the gel were then allowed to run at 120 V for an initial 10 mins and afterwards 150 V for another 60 mins on the gel electrophoresis system. Subsequently, the gel was transferred to a Bio-rad analyzer to visualize PCR products.

**Primer Design:** Wang et al. (2015) reported some candidate genes (PAL4 gene) responsible for low ADL in *B. napus*, among these genes is *BnaA05g28490D* which is located on A05. The full sequence of this gene was found on Genoscope (<http://www.genoscope.cns.fr/brassicanapus/>), multiple sequence alignment was carried out using Clustal Omega (1.2.4) (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) to compare candidate gene sequence with the reference sequence of *Brassica napus* Darmor-bzh and finally the three primer pairs were designed using Primer3web version 4.1.0 (<http://primer3.ut.ee/>). Table 3.7 shows the information obtained from Primer3web about each primer.

Table 3.7. Primer pairs used for PCR

| Primer number | Primer name | Start | Len(bp) | Tm(°C) | GC(%) | Sequence 5' to 3'    | Pl  |
|---------------|-------------|-------|---------|--------|-------|----------------------|-----|
| 134           | PAL4_1_fw   | 84    | 20      | 58.92  | 50    | CCAATCGTCTTGTAAGCGCA | 724 |
| 135           | PAL4_1_rev  | 808   | 20      | 58.98  | 55    | GTCCGGCGATGTAAGAGAGA |     |
| 136           | PAL4_2_fw   | 609   | 20      | 58.91  | 55    | ACTCACGCTACCTAAGTCGG | 796 |
| 137           | PAL4_2_rev  | 1405  | 20      | 58.91  | 55    | CTAAGCGGGTGTGTCCATG  |     |
| 138           | PAL4_3_fw   | 1273  | 20      | 58.97  | 50    | ACCAAGATGATAGAGCGCGA | 823 |
| 139           | PAL4_3_rev  | 2096  | 20      | 58.88  | 60    | CACCCTCCCTCATACTCACC |     |

Len= Length, Tm= melting temperature, Pl = product length

### 3.4 ANKOM Method

To confirm the NDF, ADF, ADL results obtained earlier by the NIRS machine, the ANKOM method was employed. 22 Samples from Populations I, II and III were selected for high and low NDF, ADF and ADL contents, these samples include F2, F1 and the two parents from each population. These samples comprise of six samples from Population I, 8 samples from both Population II and III. 22 samples were first crushed and placed in small cellulose thimbles. Each thimble and sample weight were measured differently for all samples. Using Soxhlet apparatus containing hexane as extraction fuel, oil was extracted from all samples. Following 8 hours of oil extraction procedure, the samples were dried overnight in a fume hood to remove the hexane. After overnight drying, the defatted meal was dried in an oven at 100 °C for 4 hours.

#### 3.4.1 NDF Step

To estimate the NDF contents of defatted meal, ANKOM<sup>200</sup> Fiber Analyzer (ANKOM Technology, Macedon, NY, USA, <https://www.ankom.com/>) was employed and positioned in a fume hood. The apparatus used for this procedure include filter bags which contains the defatted meal, a solvent resistant pen to withstand washing by detergent, a bag holder to hold in position the filter bags, laboratory kettle, heat sealer to seal defatted meal in filter bags, and desiccant pouches and weighing balance to further dry and weight samples respectively in filter bags. Reagents includes neutral detergent solution, alpha-amylase, and sodium sulfite Na<sub>2</sub>SO<sub>4</sub>.

The procedure for this step was followed based on the manufacturer's manual (ANKOM, 2014).

Defatted meal from the thimbles was first transferred into small filter bags; the weight of each empty filter bag was measured ( $W_1$ ) and around 400 – 500 mg of each sample was transferred into each filter bag ( $W_2$ ). Each filter bag was assigned a unique identifier using a solvent resistant pen. The filter bags containing each sample were sealed and placed in on bag suspender tray with 3 samples per each tray and the weight of two blanks were recorded ( $C_1$ ) and placed at the top and bottom. The blanks are used to collect sample residues which might escape out of the filter bag. It is pertinent to know that two checks (standard) were included in the total sample. The first is “Visby” a *B. napus* cultivar, and the second is Alfalfa (*Medicago sativa*) making the total number of samples to be 24. The bag suspender was placed into the fiber analyzer.

Around 1900 – 2000 ml of neutral detergent solution was further added into the fiber analyzer in addition to 4 ml of alpha amylase and 20 g of sodium sulfite. The valve lid was tightly closed and the agitate and heat button was turned on. The agitate moves the bag suspender up and down to ensure homogenous mixture with the detergent. The temperature of the analyzer increases gradually, upon reaching 100 °C, a timer is set for 75 mins.

After running the fiber analyzer for 75 mins, the drain valve was opened gently to ease pressure from the vessel and drain the detergent. Next is the rinsing to wash away the remaining detergents. For this, distilled water was preheated using laboratory kettle to up to 70 – 90 °C and added into the vessel of the analyzer. This procedure is repeated three times and 4 ml of alpha amylase was added in each round except for the 3<sup>rd</sup> round. The rinsed samples are now removed from the vessel of the analyzer and placed into a 500 ml beaker, acetone is poured into the beaker till all samples are completely submerged and incubate at room temperature for 5 mins. Samples are then placed on paper and allowed to dry in a fume hood to evaporate acetone residue. Acetone free samples are then dried at 105 °C in an oven for 4 hours. Following this drying, samples are then

placed in a desiccant pouch to prevent moisture on filter paper during cooling. Subsequently, the weight of each filter bag was calculated in grams ( $W_3$ ).

**Estimation of NDF content:** Based on the ANKOM Technology manual, the equation for estimating the NDF content is given below

$$\%NDF \text{ content (on received basis)} = \frac{100 * (W_3 - (W_1 * C_1))}{W_2}$$

Where

$W_1$  = Bag tare weight

$W_2$  = Sample weight

$W_3$  = Dried weight of bag with fiber after extraction process

$C_1$  = Blank bag correction (running average of final oven-dried weight divided by original blank bag weight)

Furthermore, another improved method of estimating NDF other than on received basis is using the dry matter content.

### 3.4.2 ADF step

The ADF content was also measured in accordance to the ANKOM protocol for ADF estimation (ANKOM, 2014). The materials used for this procedure include ANKOM Fiber Analyzer, weighing balance to measure samples weight, filter bags, filter bag holder, heat sealer, laboratory kettle, desiccant pouch, and an acid resistant marker. The reagents used include 1900 – 2000 ml of acid detergent solution, heated distilled water.

Defatted meal from the thimbles was first transferred into small filter bags; the weight of each empty filter bag was measured ( $W_1$ ) and around 400 – 500 mg of each sample was transferred into filter bag ( $W_2$ ), also the weight of two filter bags without sample was marked as blank ( $C_1$ ) and the weight recorded. The blanks are used to collect sample residues which might escape out of the filter bag. Each filter bag was assigned a unique identifier using an acid resistant

marker. The filter bags containing each sample were sealed using a heat sealer and placed on bag suspender. Three samples were placed on each tray of the bag suspender, and two blanks were added at the bottom and top tray of the bag suspender and subsequently placed into the analyzer. Next, acid detergent solution was poured into the valve, the lid of the valve is then closed tightly and the agitate and heat button switched on. Temperature in the fiber analyzer could reach 100 °C and a countdown was initiated for 60 mins.

After the 60 mins of running, the heat and agitate button are turned off, the drain was gently opened to ease pressure before the lid was open. Next is rinsing the samples using 1700 ml heated distilled water, this step is repeated three times. In the 3<sup>rd</sup> rinse, a pH test indicator paper was used to confirm the pH of water in the vessel. If the pH is still acidic (5 – 6), a fourth rinse is carried out to ensure acid is completely washed away.

Samples are removed from the bag suspender and placed on filter paper to absorb the remaining moisture, and subsequently transferred into a 500 ml beaker. Acetone was added into the beaker and incubated at room temperature for 5 mins. Samples are then removed and placed in the fume hood to dry for 15 – 18 hours.

Finally, samples are oven dried at 105 °C for 4 hours and subsequently their weight was recorded in grams

$$\%ADF \text{ content (on received basis)} = \frac{100 * (W3 - (W1 * C1))}{W2}$$

Where

W1 = Bag tare weight

W2 = Sample weight

W3 = Dried weight of bag with fiber after extraction process

C1 = Blank bag correction (running average of final oven-dried weight divided by original blank bag weight)

### 3.4.3 ADL step

Determination of the lignin content was carried out in accordance to the ANKOM manual (ANKOM, 2014). The materials used include 2 liter (L) and 3 L beakers, desiccant pouch, pH paper, filter bags containing extracted meal (coming from ADF step above), reagents used are 72% H<sub>2</sub>SO<sub>4</sub>, acetone and distilled water.

Samples in filter bags are placed in 3 L beaker, 250 ml of 72% H<sub>2</sub>SO<sub>4</sub> was then added. A 2 L beaker is placed into the 3L beaker and ensured that all samples are submerged in the sulfuric acid. The samples were manually agitated using the 2 L beaker by moving up and down 30 times. The agitation was done every 30 mins for a total period of 3 hours. At the end of the 3 hours, the sulfuric acid is removed, and samples are rinsed using distilled water. Each rinsing required a short period of agitation to further remove the acid from the samples and rinsing is done until the samples reached neutral pH as measured by the aid of the pH paper. Around 250ml of acetone solution was placed into the beaker containing samples. This step is done for 3mins to ensure that acetone removes all the remaining water in the samples. The samples are removed and placed in a fume hood to dry overnight (18 hours). Samples are then dried in an incubator at 105 °C for 3 – 4 hours. Using a weighing balance, the final weight of sample was recorded (W3)

### 3.4.4 DM content

According to ANKOM manual (ANKOM, 2014), the NDF, ADF and ADL contents are calculated as received basis. The received basis refers to the mass of crushed seed samples after oil extraction and oven drying at 100°C. However, a more accurate approach will be to measure the fiber fractions based on dry matter content, this will reduce some errors caused possibly by moisture content after oven drying.

To determine the dry matter content, crucibles are used. Each crucible was assigned a unique number using a heat resistant pen. Crucibles are washed, clean and then dried at 105 °C for 18 hours. Following oven drying, the crucibles are

placed in drying box at 27 °C, the drying box contains silica gel, this is important to absorb any moisture content as the temperature drops.

The equation for calculating the DM is given below

$$DM = \frac{\text{mass of sample before drying} - \text{mass of sample after drying}}{\text{mass of sample before drying}} * 100$$

To calculate the NDF, ADF and ADL content on a DM basis, the equation below was used

$$\%NDF \text{ on dry matter basis} = \frac{\%NDF \text{ (received basis)} * 100}{\%dry \text{ matter}}$$

$$\%ADF \text{ on dry matter basis} = \frac{\%ADF \text{ (received basis)} * 100}{\%dry \text{ matter}}$$

$$\%ADL \text{ on dry matter basis} = \frac{\%ADL \text{ (received basis)} * 100}{\%dry \text{ matter}}$$

### 3.5 Analysis of Seed Quality Traits

**Total Seed Weight (TSW):** After harvesting of seeds, all seed samples were dried at 30 °C for 16 hours. The total weight of each seed sample from the three populations were obtained using Sartorius gold weighing scale and the values recorded in grams.

**Thousand Kernel Weight (TKW):** TKW was measured manually using a Rapeseed counting paddle. The counting paddle can contain 100 seeds at a time, five counts was performed and kernel weight was measured using a Sartorius gold weighing scale (<https://www.sartorius.com/en>). All values are expressed in grams

**Pre-harvest Germination Rate (PGR):** Pre-harvest Germination rate was estimated using 100 seeds from each genotype and manually counted and recorded as a percentage. A counting paddle was used to randomly choose the 100

seeds, with the aid of a tissue forceps and a Petri dish, the germination rate was thus determined as a percentage.

**Color Scoring:** Using a scale ranging from 1 – 9, each seed sample is assigned a value based on the brightness or darkness of the seeds with 1 been light yellow, while 9 represents the dark black seeds.

Table 3.8. Description of color scoring system

| Score value | Color                    | Phenotype    | Genotype |
|-------------|--------------------------|--------------|----------|
| 1           | Light-yellow (uniformly) | Bright       | Yellow   |
| 2           | Yellow brown (Uniformly) |              |          |
| 3           | Yellow brown             | Intermediary | Brown    |
| 4           | Brown yellow             |              |          |
| 5           | Brown                    |              |          |
| 6           | Brown black              | Dark         | Black    |
| 7           | Black brown              |              |          |
| 8           | Black red                |              |          |
| 9           | Pure Black               |              |          |

**NIRS:** Around 3g of seed samples were weight and analyzed using NIRS machine to determine the fiber fractions (NDF, ADF, ADL) as well as oil content, protein content, glucosinolates, erucic acid etc. Seeds were then scanned at 400 – 2498 nm using NIRS Monochromator (FOSS NIR Systems model 6500, NIRSystems, Inc., Silver Springs, MD, USA). The hemicellulose and Cellulose contents were then calculated from the result of the NIRS data and expressed as a percentage.

**Statistical Analysis:** All statistical analysis such as plotting of frequency distribution, correlations etc. were performed using statistical software R-programming version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org/>)

## 4. RESULTS

### 4.1 PCR Amplification

There was amplification of PAL gene on the parents of population I, II, and III (Fig 4.1). Of the three different primers designed for this gene, two primers showed clear amplification namely 136/137 and 138/139 (Table 3.7). The expected product length for primer 136/137 was 796 bp, however, from Fig 4.1, the PCR products in all four parents were around 1500 kb. On the other hand, Primer 138/139 had amplification of products as expected (823 bp).

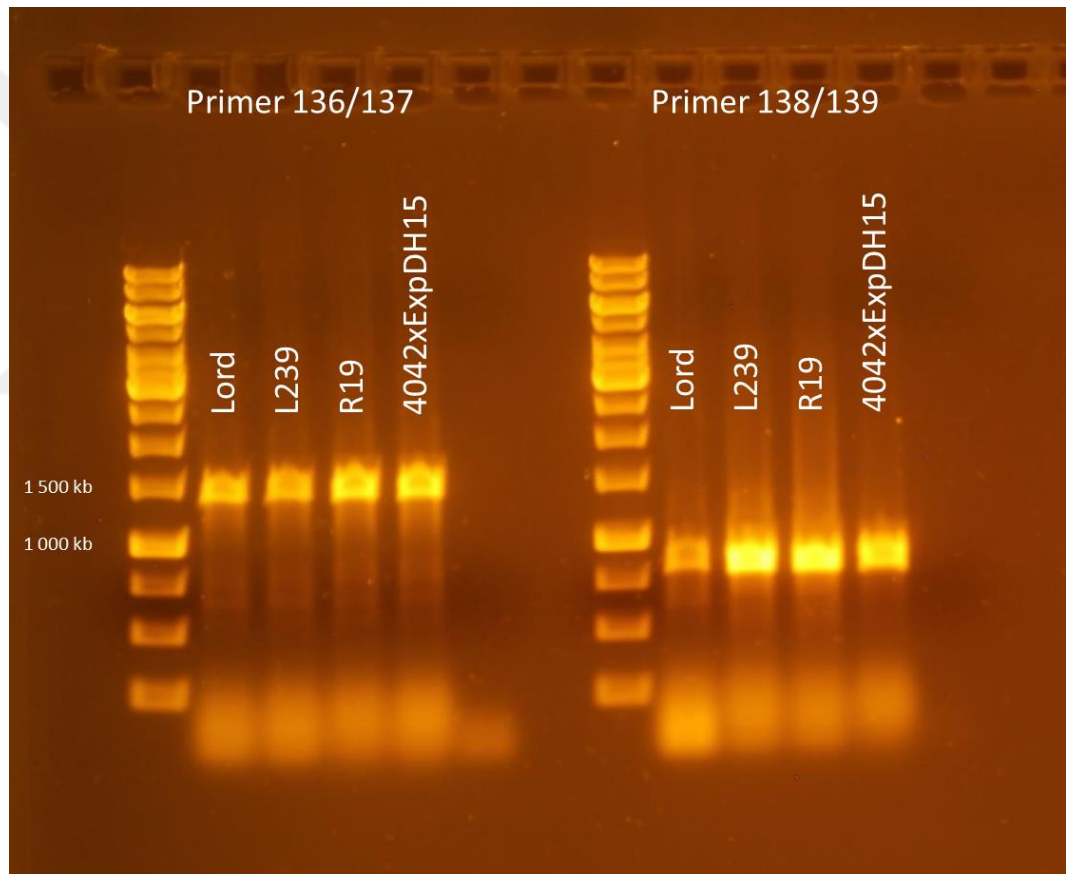


Figure 4.1. PCR amplification of PAL gene on parental genotype Lord, L239, R19 and 4042xExpDH15.

## 4.2 ANKOM Fiber Analyzer results

The large number of F3-seed samples harvested from the first three different F2-populations (Population I – III) in the greenhouse could only be analyzed by NIRS for different seed quality traits. In order to check the reliability and accuracy of the NIRS predicted results for NDF, ADF, and ADL content, seed samples with diverse ADL, ADF and NDF contents were selected and analyzed by the ANKOM filter bag method. The scatter plots in Fig. 4.2 shows the Spearman rank correlations between the laboratory reference method and the NIRS predicted results. The Spearman rank correlation was close for ADL content ( $r_s=0.65$ ), than for NDF ( $r_s=0.55$ ) and ADF ( $r_s=0.44$ )



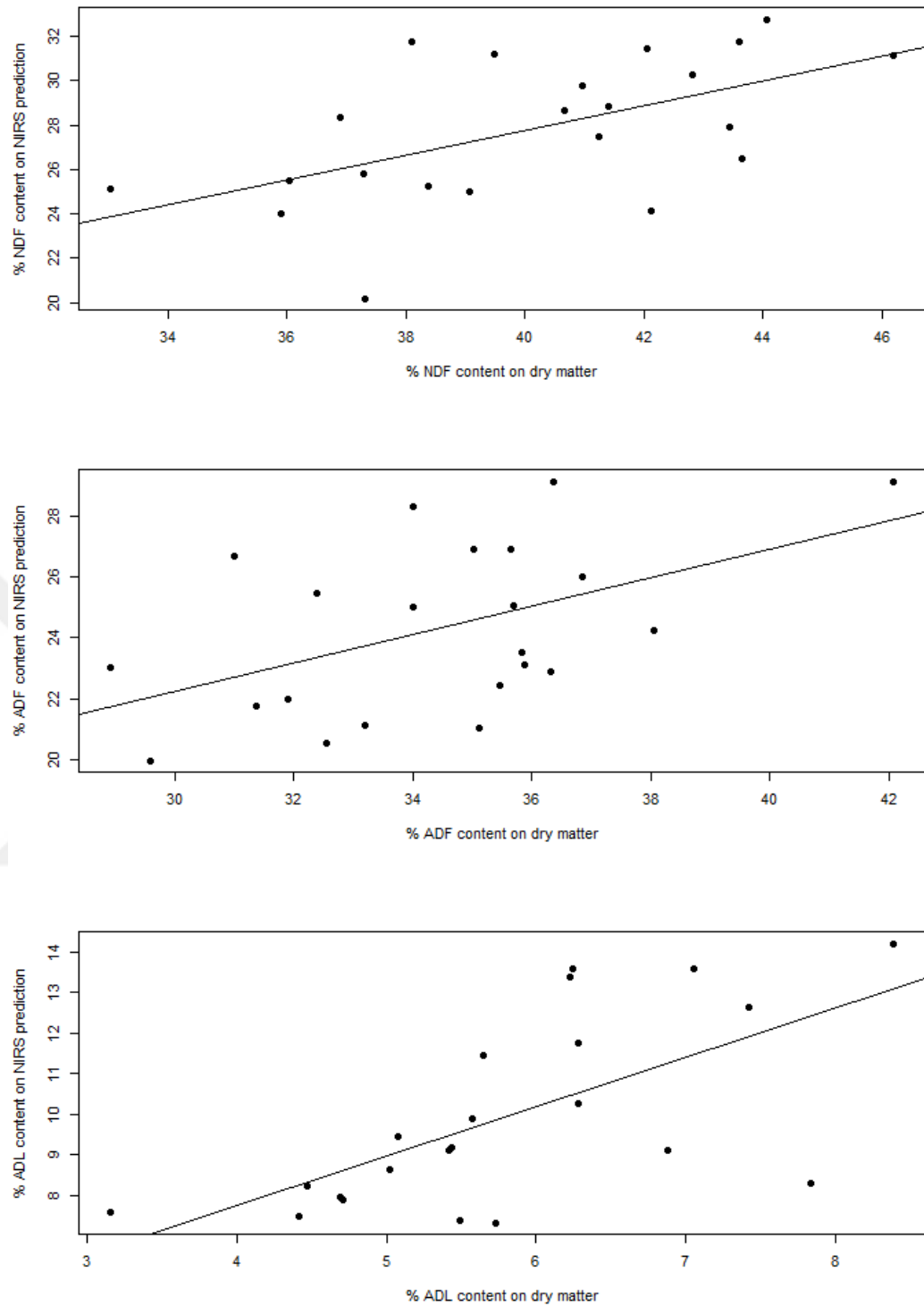


Figure 4.2. Scatterplot of NDF, ADF and ADL content on dry matter (ANKOM) and predicted values (NIRS).

### 4.3 Population I L239 x Lord

Out of the 96 F<sub>2</sub> plants sown in Greenhouse, 77 were harvested and analyzed by NIRS for different quality traits such as protein, oil, NDF, ADF, ADL, hemicellulose, and cellulose content. Furthermore, total seed weight (TSW) and thousand kernel weight (TKW) were measured and seed color was scored. The results of the F<sub>2</sub>-population (F<sub>3</sub>-seeds) is shown in Table 4.1 Individual genotypes of the F<sub>2</sub>-population showed much lower NDF, ADF and ADL contents than both parents. Also, there was a much larger variation in seed color compared to both parents which had quite similar seed color scorings. Noteworthy, the extreme genotypes with low ADL content also showed a yellowish appearance (Fig. 4.3). Large differences in the F<sub>2</sub>-population were found for thousand kernel weight (TKW), total seed weight (TSW) and for oil and protein content,

Table 4.1. Statistics of quality traits in Population I

|            | F2 (n=77) |       |       |      | L239 (n=5) | Lord (n= 4) | F1 (n=7) |
|------------|-----------|-------|-------|------|------------|-------------|----------|
| Traits     | Min       | Max   | Mean  | SD   | Mean       | Mean        | Mean     |
| TSW        | 1.68      | 7.51  | 4.25  | 1.20 | 3.83       | 5.13        | 5.43     |
| TKW        | 2.38      | 5.15  | 3.71  | 0.51 | 4.43       | 3.00        | 4.00     |
| Seed color | 1.00      | 9.00  | 7.14  | 1.97 | 8.00       | 6.50        | 8.00     |
| Oil        | 40.36     | 49.23 | 44.94 | 1.67 | 42.80      | 48.77       | 45.38    |
| Protein    | 18.23     | 24.78 | 21.24 | 1.12 | 21.85      | 19.02       | 20.65    |
| NDF        | 18.55     | 32.39 | 27.43 | 3.11 | 25.49      | 29.06       | 27.43    |
| ADF        | 16.82     | 28.70 | 24.68 | 2.59 | 23.82      | 23.69       | 24.68    |
| ADL        | 6.34      | 15.56 | 11.13 | 2.38 | 11.54      | 8.26        | 11.13    |
| HemiC      | -2.16     | 6.02  | 2.75  | 1.52 | 1.67       | 5.37        | 2.75     |
| CellU      | 10.48     | 16.08 | 13.54 | 0.88 | 12.29      | 15.43       | 13.54    |

The frequency distribution of TKW, oil, protein, hemicellulose, and cellulose content (Fig. 4.4) resembled a normal distribution. Shapiro-Wilk normality test proved normal distribution ( $p < 0.05$ ). However, NDF, ADF and ADL were not normally distributed. The frequency distribution for ADL content indicated a bimodal distribution which, however, was not observed for ADF and

NDF content. Interestingly, Lord had a lower ADL content but much higher hemicellulose and cellulose contents. The means of the F2 population was similar to the mean value of the F1 plants. L239 had a relatively high ADL content but comparatively low hemicellulose and cellulose contents.

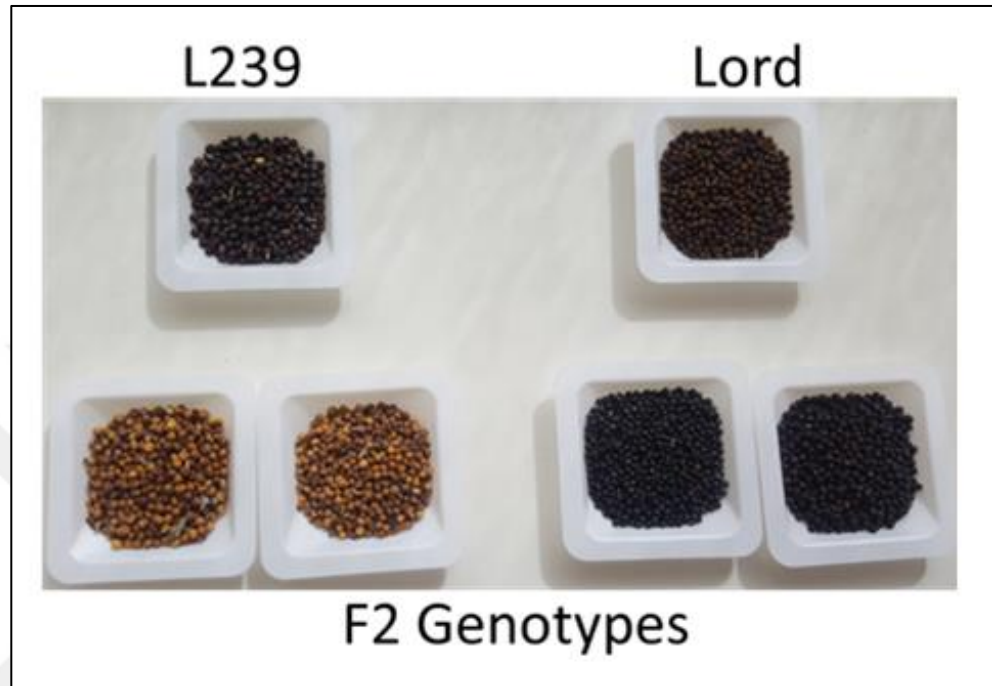


Figure 4.3. Seed color of parents and F2 from population I. only two of the most yellow and two of the most seed samples are shown in the bottom row. the light-yellow seeds are scored 1, while the dark ones are scored 9.

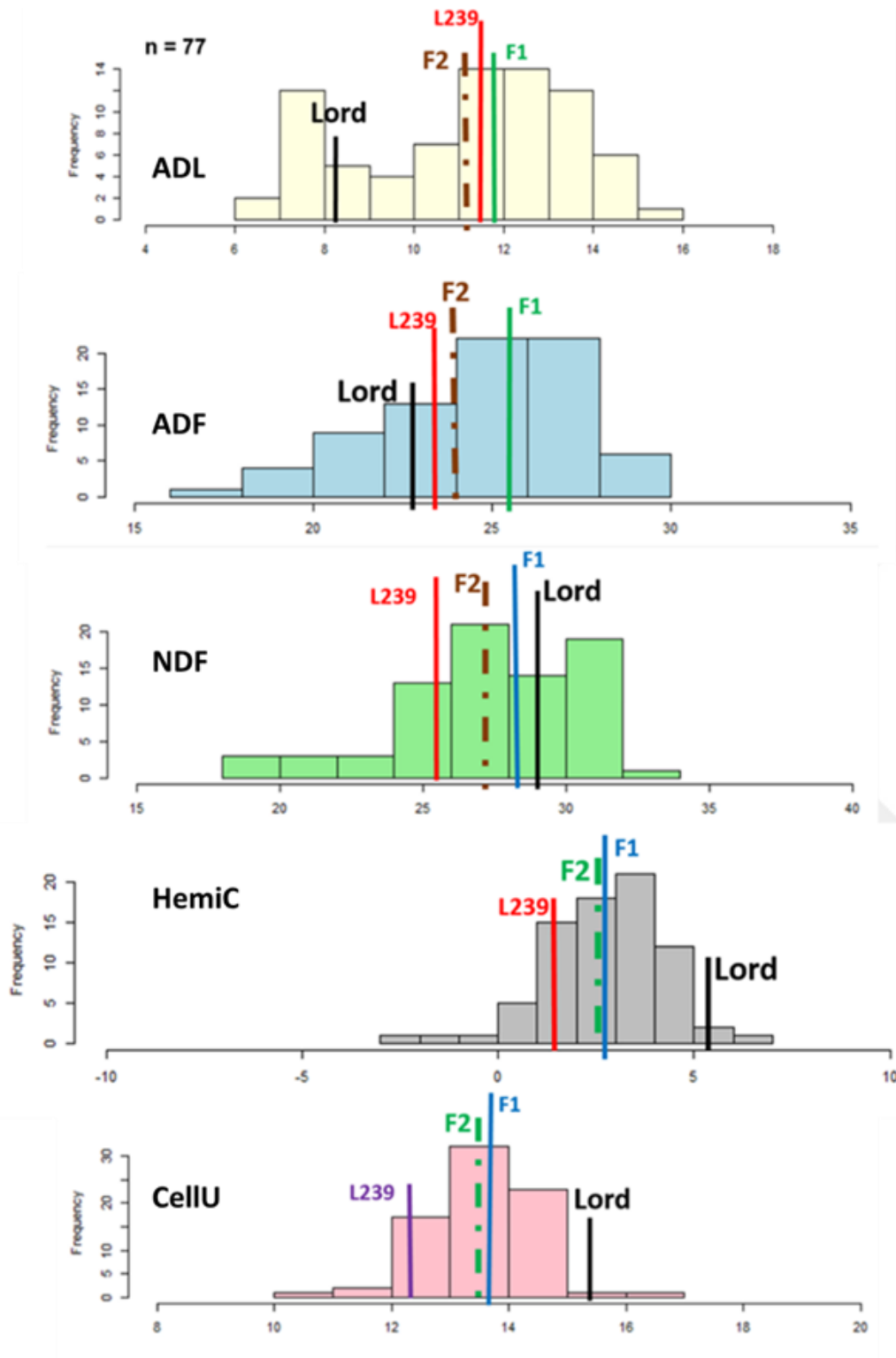


Figure 4.4. Frequency distribution of Fiber fractions in Population I.

Close correlations between NDF, ADF and ADL contents were found in the F2 population (Table 4.2). Correlations of hemicellulose and cellulose to NDF and ADF contents were somewhat lower and they were nil to ADL content. All fiber related traits were significantly negative correlated with protein content. Except for ADL content all fiber related traits were positively correlated with oil content. Protein and oil content were closely negative correlated.

Table 4.2. Spearman's Rank Correlation Coefficient of Population I

|       | NDF             | ADF             | ADL           | HemiC           | CellU           | XP              |
|-------|-----------------|-----------------|---------------|-----------------|-----------------|-----------------|
| ADF   | <b>0.89***</b>  |                 |               |                 |                 |                 |
| ADL   | <b>0.81***</b>  | <b>0.93***</b>  |               |                 |                 |                 |
| HemiC | <b>0.51***</b>  | 0.11            | 0.00          |                 |                 |                 |
| CellU | <b>0.41 ***</b> | <b>0.30 **</b>  | -0.01         | <b>0.53***</b>  |                 |                 |
| XP    | <b>-0.69***</b> | <b>-0.42***</b> | <b>-0.24*</b> | <b>-0.83***</b> | <b>-0.72***</b> |                 |
| XL    | <b>0.57***</b>  | <b>0.26***</b>  | 0.10          | <b>0.87***</b>  | <b>0.69***</b>  | <b>-0.88***</b> |

\*\*\* significance at  $p < 0.001$ , \*\* significance at  $p < 0.01$ , \* significance at  $p < 0.05$

A positive correlation was also observed between seed color and ADL content (Fig. 4.5).

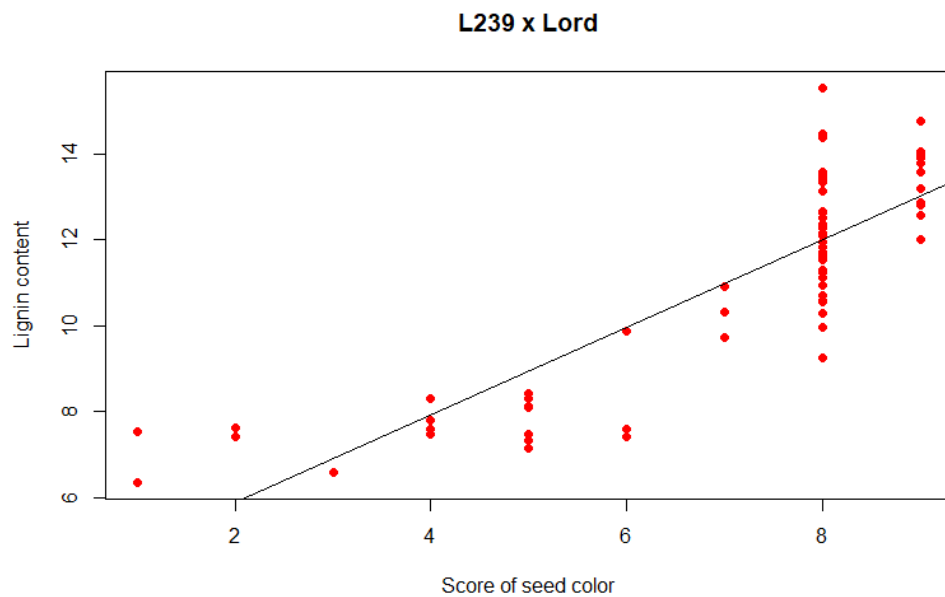


Figure 4.5. Relationship between seed color and lignin in Population I.

#### 4.4 Population II 4042xExpDH14 x Lord

Of the 96 F<sub>2</sub> plants sown, 89 were harvested and analyzed by NIRS for different quality traits mainly protein content, oil content, NDF, ADF, ADL, hemicellulose, and cellulose content. In addition, total seed weight (TSW) and thousand kernel weight (TKW) were measured and seed color was scored. The results of the F<sub>2</sub>-population (F<sub>3</sub>-seeds) is shown in Table 4.3 Individual genotypes of the F<sub>2</sub>-population showed much lower NDF, ADF and ADL contents than both parents. Also, there was a much larger variation in seed color compared to both parents which had quite similar seed color scorings. Noteworthy, the extreme genotypes with low ADL content also showed a yellowish appearance (Fig. 4.6). Large differences in the F<sub>2</sub>-population were found for thousand kernel weight (TKW), total seed weight (TSW) and for oil and protein content,

Table 4.3. Statistics of quality traits in Population II

|            | F2 (n=89) |       |       |      | 4042xExpDH15 (n=8) | Lord (n=8) | F1 (n=8) |
|------------|-----------|-------|-------|------|--------------------|------------|----------|
| Traits     | Min       | Max   | Mean  | SD   | Mean               | Mean       | Mean     |
| TSW        | 2.72      | 9.20  | 5.85  | 1.49 | 5.64               | 5.97       | 6.34     |
| TKW        | 2.66      | 4.78  | 3.47  | 0.20 | 4.43               | 3.33       | 3.23     |
| Seed color | 3.00      | 9.00  | 7.74  | 0.40 | 6.75               | 7.88       | 8.00     |
| Oil        | 44.07     | 52.03 | 49.52 | 1.42 | 47.91              | 49.65      | 50.76    |
| Protein    | 16.32     | 20.28 | 17.79 | 1.60 | 18.29              | 17.96      | 17.14    |
| NDF        | 20.34     | 35.84 | 31.15 | 0.76 | 27.32              | 32.09      | 32.92    |
| ADF        | 20.26     | 27.66 | 25.14 | 2.73 | 23.34              | 25.35      | 25.63    |
| ADL        | 6.14      | 10.75 | 9.31  | 1.39 | 8.49               | 8.99       | 9.47     |
| HemiC      | 0.08      | 8.74  | 6.01  | 0.75 | 3.99               | 6.73       | 7.29     |
| CellU      | 13.78     | 17.50 | 15.83 | 1.56 | 14.84              | 16.37      | 16.16    |

The frequency distribution of TSW, and cellulose content (Fig 4.7) resembled a normal distribution. Shapiro-Wilk normality test proved normal distribution ( $p < 0.05$ ). However, NDF, ADF and ADL, oil content, protein content and TKW were not normally distributed. Lord had a higher seed color score when compared to 4042xExpDH15 (Fig 4.6), which could explain the higher fiber contents of Lord in comparison with the F<sub>2</sub> and 4042xExpDH15. The means of

the F2 population was similar to the mean value of Lord and F1 plants for all traits measured.

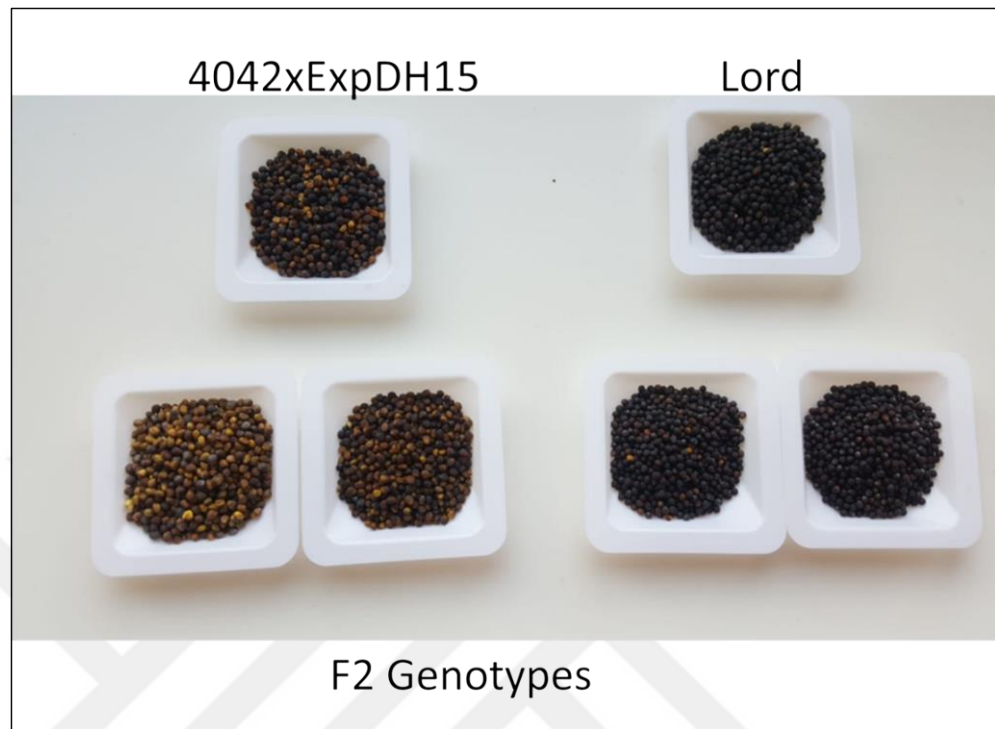


Figure 4.6. Seed color of parents and F2 from Population II. only two of the most yellow and two of the black seed samples are shown in the bottom row, the yellow seeds are scored 3(far left) and 4 while the black seeds are scored 9.

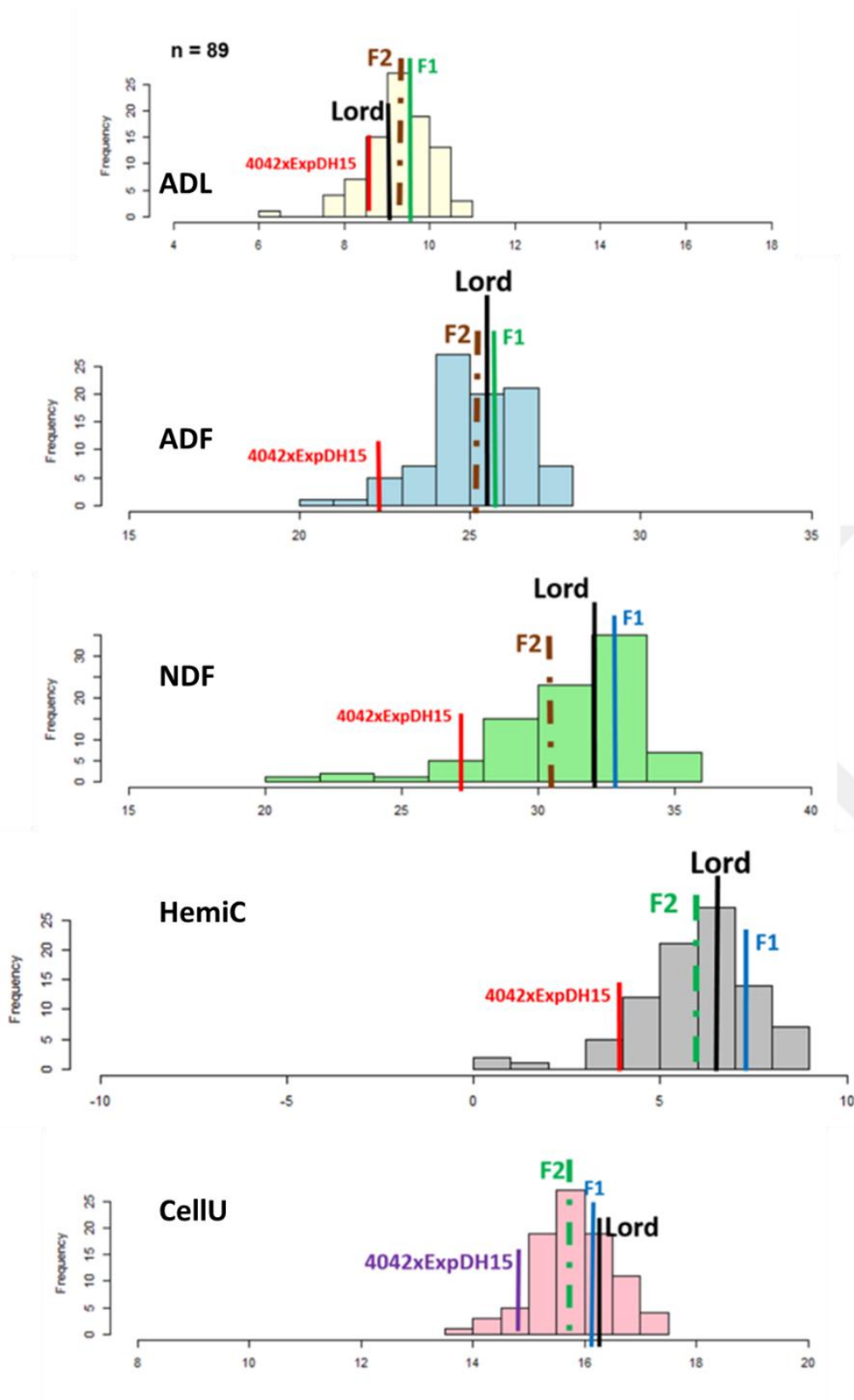


Figure 4.7. Frequency distribution of Fiber fractions in Population I.

Close correlations between NDF, ADF and ADL contents were found in the F2 population (Table 4.4). Hemicellulose and cellulose content were highly correlated with NDF, ADF and ADL. As expected, protein content showed a strong negative correlation with oil content. In addition, all other traits were significantly negative correlated with protein content.

Table 4.4. Spearman's Rank Correlation Coefficient of Population II

|       | NDF             | ADF             | ADL             | HemiC           | CellU           | XP              |
|-------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| ADF   | <b>0.89***</b>  |                 |                 |                 |                 |                 |
| ADL   | <b>0.81***</b>  | <b>0.93***</b>  |                 |                 |                 |                 |
| HemiC | <b>0.87***</b>  | <b>0.60***</b>  | <b>0.53***</b>  |                 |                 |                 |
| CellU | <b>0.87***</b>  | <b>0.94***</b>  | <b>0.77***</b>  | <b>0.61***</b>  |                 |                 |
| XP    | <b>-0.86***</b> | <b>-0.73***</b> | <b>-0.66***</b> | <b>-0.79***</b> | <b>-0.73***</b> |                 |
| XL    | <b>0.74***</b>  | <b>0.61***</b>  | <b>0.58***</b>  | <b>0.73***</b>  | <b>0.58***</b>  | <b>-0.82***</b> |

Fig 4.8 showed a positive correlation between seed color and ADL content.

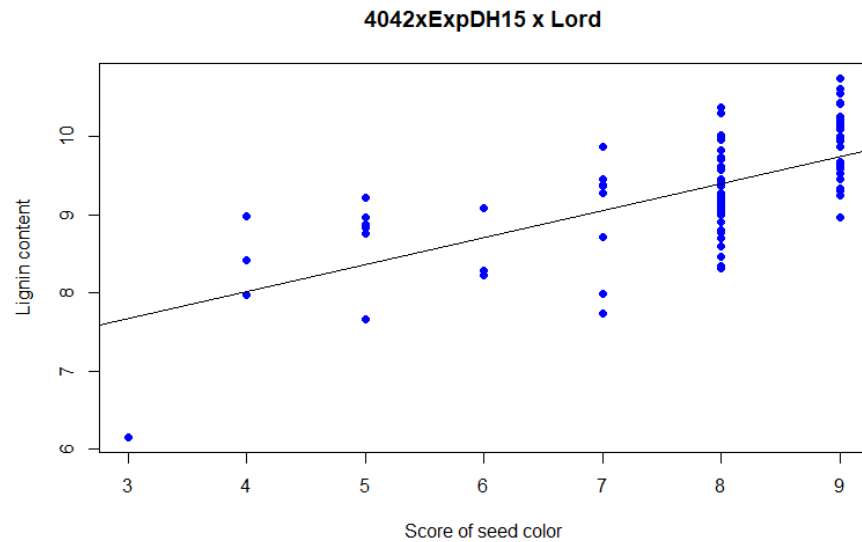


Figure 4.8. Relationship between seed color and lignin content in Population II.

#### 4.5 Population III R19 x Lord

The number of harvested F2 seeds is 77, these seeds were analyzed by NIRS for different quality traits such as protein, oil, NDF, ADF, ADL, hemicellulose, and cellulose content. Furthermore, total seed weight (TSW) and thousand kernel weight (TKW) were measured and seed color was scored. The results of the F2-population (F3-seeds) is shown in Table 4.5. Individual genotypes of the F2-population showed much lower NDF, ADF and ADL contents than both parents. Also, there was a much larger variation in seed color compared to both parents which had quite similar seed color scorings. Noteworthy, the extreme genotypes with low ADL content also showed a yellowish appearance (Fig. 4.9). Large differences in the F2-population were found for thousand kernel weight (TKW), total seed weight (TSW) and for oil and protein content,

Table 4.5 Statistics of quality traits in Population III

|                   | <b>F2 (n=77)</b> |            |             |           | <b>R19 (n=7)</b> | <b>Lord (n=4)</b> | <b>F1 (n=7)</b> |
|-------------------|------------------|------------|-------------|-----------|------------------|-------------------|-----------------|
| <b>Traits</b>     | <b>Min</b>       | <b>Max</b> | <b>Mean</b> | <b>SD</b> | <b>Mean</b>      | <b>Mean</b>       | <b>Mean</b>     |
| <b>TSW</b>        | 2.07             | 7.04       | 4.21        | 1.07      | 3.53             | 5.10              | 4.73            |
| <b>TKW</b>        | 2.36             | 5.75       | 3.74        | 0.62      | 4.50             | 2.87              | 3.44            |
| <b>Seed color</b> | 2.00             | 9.00       | 6.49        | 1.91      | 7.71             | 6.25              | 7.71            |
| <b>Oil</b>        | 42.66            | 50.62      | 46.85       | 1.86      | 46.39            | 47.86             | 49.33           |
| <b>Protein</b>    | 17.47            | 23.72      | 20.67       | 1.09      | 21.39            | 19.52             | 19.40           |
| <b>NDF</b>        | 19.36            | 32.13      | 26.30       | 2.91      | 24.54            | 27.72             | 27.90           |
| <b>ADF</b>        | 19.36            | 29.13      | 23.53       | 2.44      | 22.24            | 22.98             | 23.59           |
| <b>ADL</b>        | 6.72             | 14.20      | 9.21        | 1.88      | 9.08             | 7.95              | 8.69            |
| <b>HemiC</b>      | -1.81            | 6.56       | 2.77        | 1.52      | 2.30             | 4.74              | 4.31            |
| <b>CellU</b>      | 12.18            | 16.16      | 14.32       | 0.81      | 13.17            | 15.03             | 14.90           |

The frequency distribution of TSW, oil, protein, hemicellulose, cellulose and NDF content (Fig. 4.10) resembled a normal distribution. Shapiro-Wilk normality test proved normal distribution ( $p < 0.05$ ). However, only ADF, ADL and TKW were not normally distributed. Lord had a lower ADL content but much higher hemicellulose and cellulose contents. The NDF, ADF and ADL mean values of F2 population was similar to the mean value of the F1 plants. R19 had a relatively high ADL content but comparatively low hemicellulose and cellulose contents.

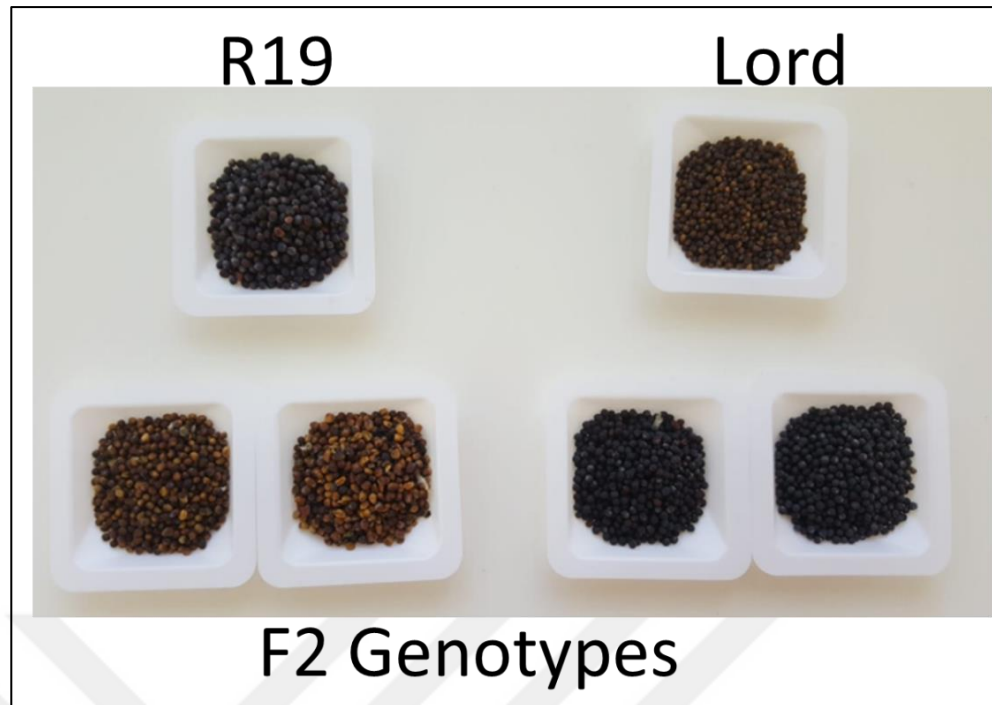


Figure 4.9. Seed color of parents and F2 from Population III. only two the most yellow and the black seeds samples are shown in the bottom row, the most yellow seeds are scored 2 while the black seeds are scored 9.

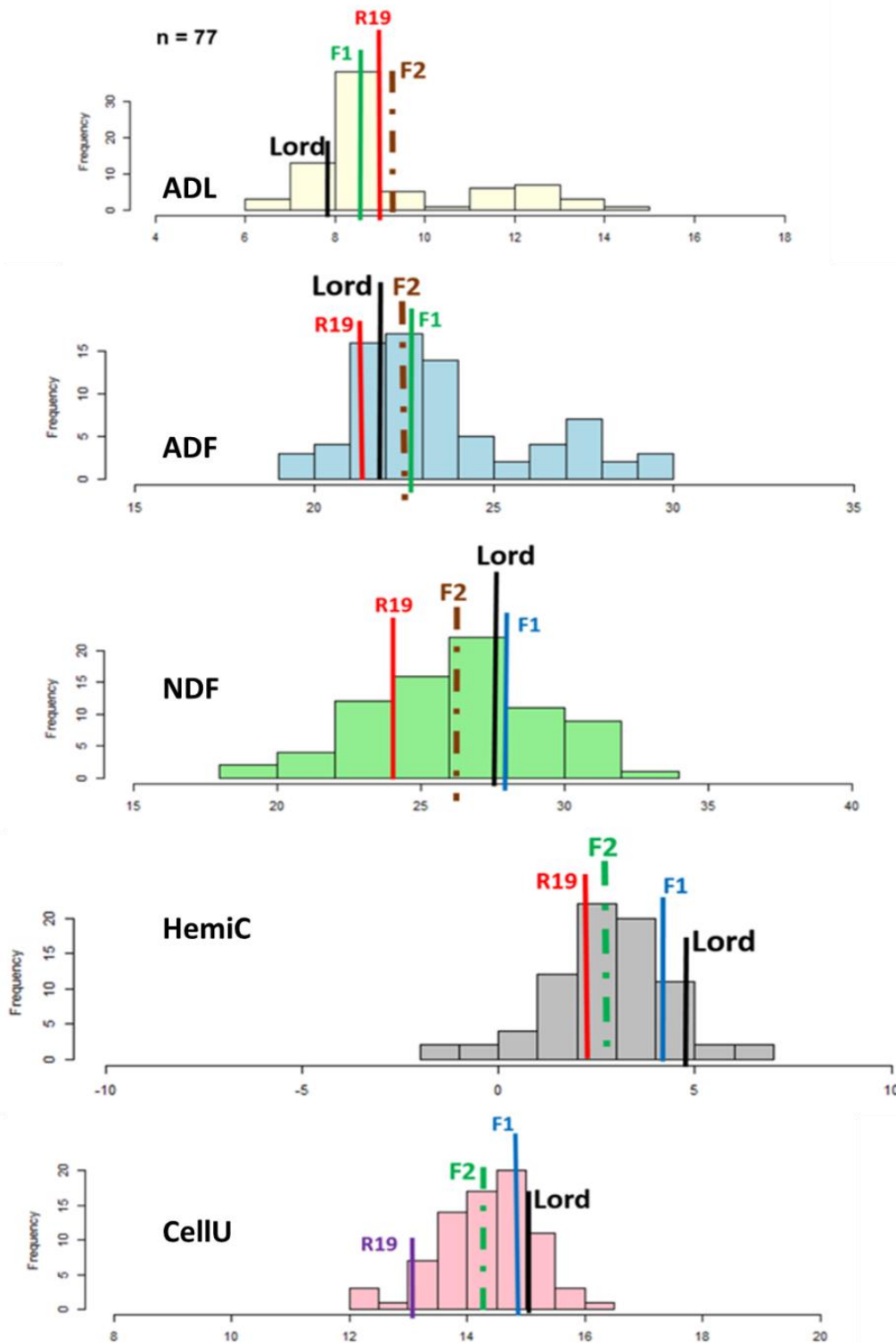


Figure 4.10. Frequency distribution of fiber fractions in Population III.

A strong correlation between NDF, ADF and ADL contents were found in the F2 population (Table 4.6). Correlations of hemicellulose to NDF and ADF contents were somewhat lower and they were almost nil to ADL content. Except for NDF, ADF, and cellulose, ADL was weakly correlated with all traits analyzed. On the other hand, cellulose was strongly correlated with NDF, ADF, and ADL with high significance. All fiber related traits were significantly negative correlated with protein content. With the exclusion of ADL content all fiber related traits were positively correlated with oil content. Protein and oil content showed a high significance and were negatively correlated.

Table 4.6 Spearman's Rank Correlation Coefficient of Population III

|       | NDF             | ADF             | ADL            | HemiC           | CellU           | XP              |
|-------|-----------------|-----------------|----------------|-----------------|-----------------|-----------------|
| ADF   | <b>0.89***</b>  |                 |                |                 |                 |                 |
| ADL   | <b>0.79***</b>  | <b>0.92***</b>  |                |                 |                 |                 |
| HemiC | <b>0.47***</b>  | 0.13            | 0.02           |                 |                 |                 |
| CellU | <b>0.80 ***</b> | <b>0.88***</b>  | <b>0.69***</b> | <b>0.24*</b>    |                 |                 |
| XP    | <b>-0.61***</b> | <b>-0.41***</b> | <b>-0.27*</b>  | <b>-0.75***</b> | <b>-0.54***</b> |                 |
| XL    | <b>0.50***</b>  | <b>0.28*</b>    | 0.16           | <b>0.74***</b>  | <b>0.44***</b>  | <b>-0.75***</b> |

Seed color and ADL content were positively correlated as show in Fig 4.11.

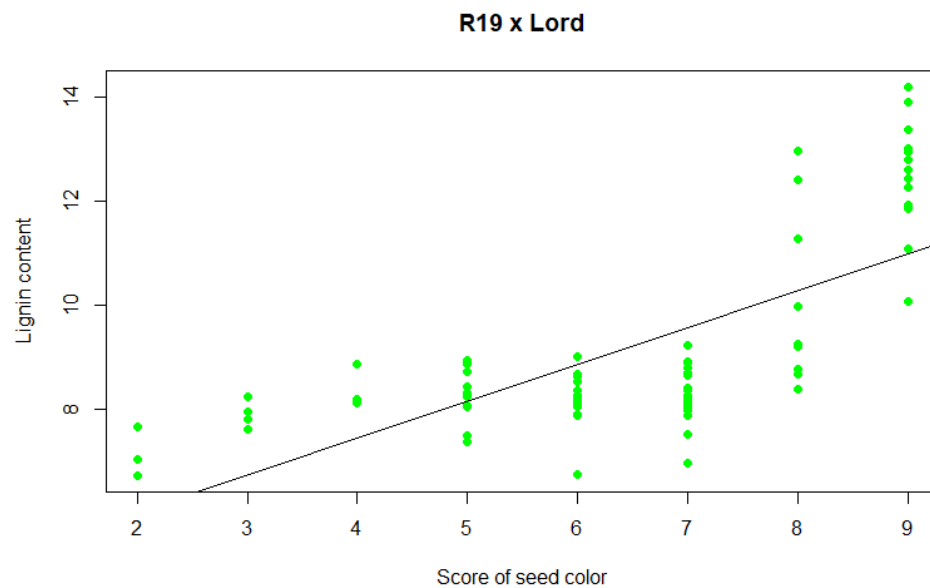


Figure 4.11. Relationship between seed color and lignin content in Population III.

#### 4.6 Population IV SGD14 x 4042xExpDH14

From the 170 F<sub>2</sub> plants sown, 147 were harvested and analyzed by NIRS for different quality traits. The main quality traits include protein content, oil content, NDF, ADF, ADL, hemicellulose, and cellulose content. In addition, total seed weight (TSW) and thousand kernel weight (TKW) were measured and seed color was scored. The results of the F<sub>2</sub>-population (F<sub>3</sub>-seeds) is shown in Table 4.7. Individual genotypes of the F<sub>2</sub>-population showed a much lower NDF, ADF and ADL contents than both parents. Also, there was a much larger variation in seed color compared to both parents which had quite similar seed color scorings. The parents had similar mean values for TKW, protein content, NDF and cellulose content, with great variation in TSW. Noteworthy, the extreme genotypes with low ADL content also showed a yellowish appearance (Fig. 4.12). Except for TKW, large differences in the F<sub>2</sub>-population were found in all traits.

Table 4.7. Statistics of TKW and TSW of Population IV

|            | F <sub>2</sub> (n=147) |       |       |      | SGDH14<br>(n=8) | 4042xExpDH14<br>(n=6) |
|------------|------------------------|-------|-------|------|-----------------|-----------------------|
| Traits     | Min                    | Max   | Mean  | SD   | Mean            | Mean                  |
| TSW        | 1.97                   | 12.42 | 6.63  | 2.15 | 9.12            | 3.69                  |
| TKW        | 2.54                   | 5.14  | 3.64  | 0.49 | 3.91            | 3.31                  |
| Seed color | 2.00                   | 9.00  | 7.33  | 1.60 | 7.13            | 7.83                  |
| Oil        | 43.28                  | 53.60 | 49.81 | 2.14 | 53.59           | 47.29                 |
| Protein    | 16.84                  | 21.37 | 18.61 | 1.02 | 17.91           | 18.24                 |
| NDF        | 20.91                  | 37.43 | 31.38 | 3.50 | 32.04           | 33.05                 |
| ADF        | 22.88                  | 33.80 | 28.60 | 2.61 | 26.88           | 31.02                 |
| ADL        | 8.21                   | 16.61 | 12.64 | 2.35 | 10.59           | 15.48                 |
| HemiC      | -5.34                  | 7.89  | 2.77  | 2.28 | 5.15            | 2.03                  |
| CellU      | 14.28                  | 18.56 | 15.96 | 0.80 | 16.29           | 15.54                 |

The frequency distribution of TKW, and cellulose content (Fig 4.12) resembled a normal distribution. Shapiro-Wilk normality test proved normal distribution ( $p < 0.05$ ). However, NDF, ADF and ADL, oil content, protein content and TSW were not normally distributed. Although both parents had similar seed color (Fig 4.12), yet their ADL content was very different (Fig 4.13). Interestingly, SGD14 had a lower ADL content but much higher hemicellulose and cellulose contents.

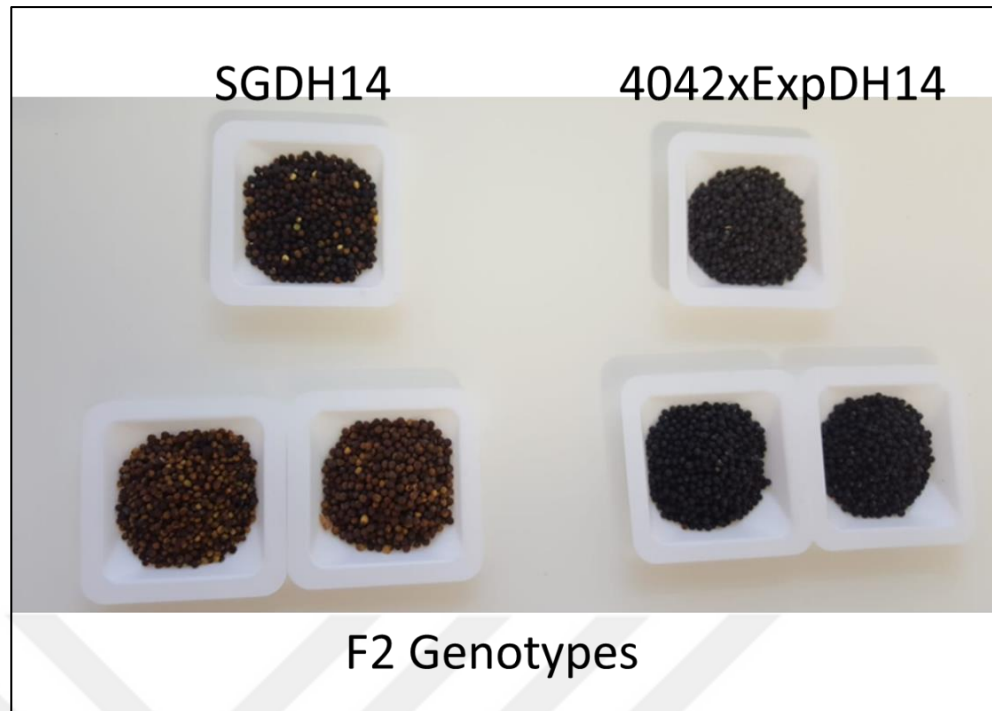


Figure 4.12. Seed color of parents and F2 from Population IV. only the two most yellow and the two most black seed samples are shown in the bottom. The most yellow seeds were scored 2 while the black seeds were scored 9.

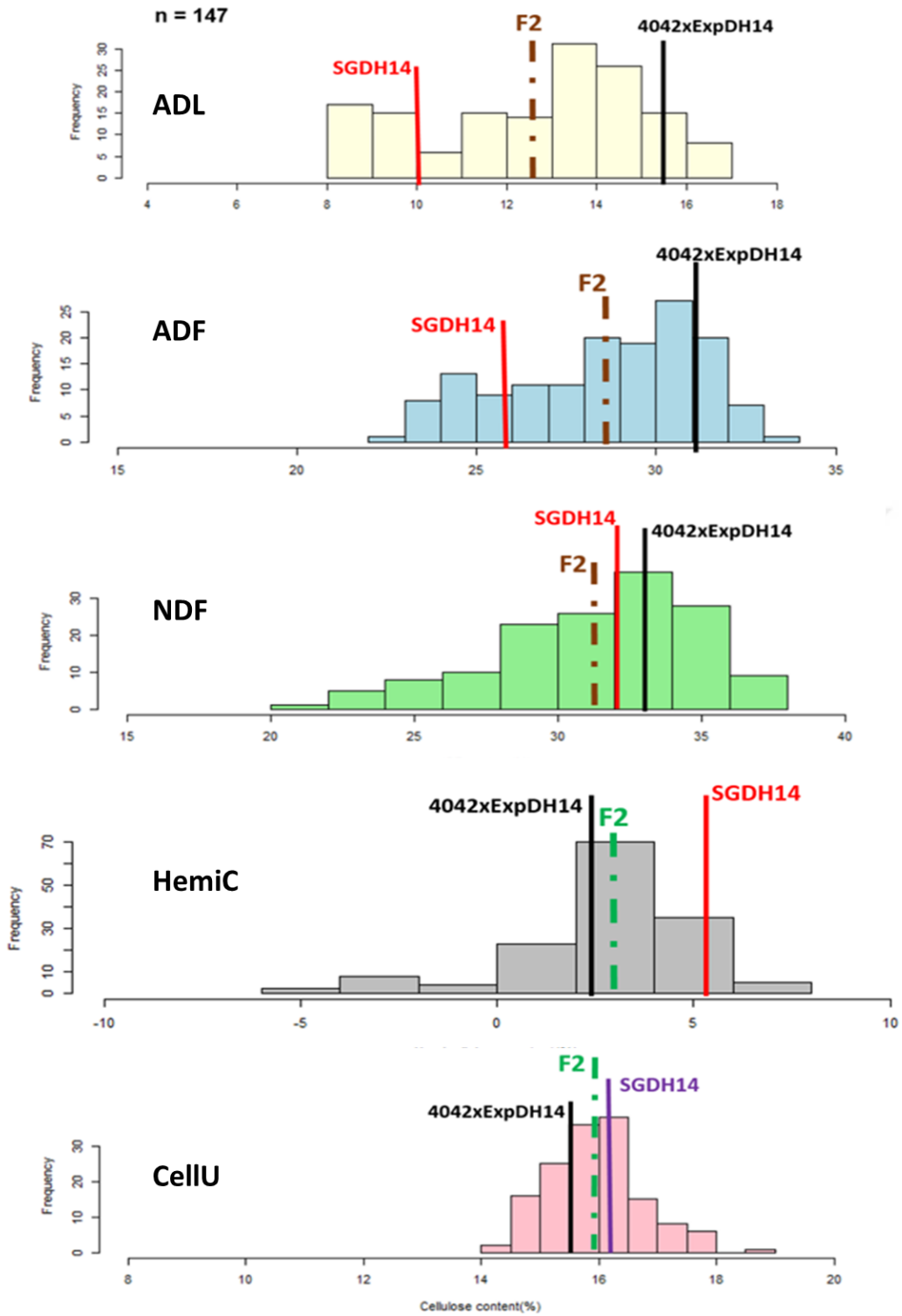


Figure 4.13. Frequency distribution of fiber fractions in Population IV.

Strong positive correlation with high significance was observed among NDF, ADF and ADL contents in the F2 population (Table 4.8). Excluding NDF, and ADF, ADL content showed a weak correlation with all traits with near zero value for hemicellulose and oil content. Surprisingly, ADF content had a nil correlation with hemicellulose content. As expected, protein content showed a strong negative correlation with oil content. In addition, all traits were significantly negative correlated with protein content.

Table 4.8. Spearman's Rank Correlation Coefficient of Population IV

|       | NDF             | ADF             | ADL             | HemiC           | CellU           | XP              |
|-------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| ADF   | <b>0.85***</b>  |                 |                 |                 |                 |                 |
| ADL   | <b>0.76***</b>  | <b>0.93***</b>  |                 |                 |                 |                 |
| HemiC | <b>0.49***</b>  | 0.01            | -0.09           |                 |                 |                 |
| CellU | <b>0.55 ***</b> | <b>0.49***</b>  | <b>0.19*</b>    | <b>0.30***</b>  |                 |                 |
| XP    | <b>-0.80***</b> | <b>-0.48***</b> | <b>-0.31***</b> | <b>-0.78***</b> | <b>-0.63***</b> |                 |
| XL    | <b>0.53***</b>  | <b>0.22**</b>   | 0.02            | <b>0.68***</b>  | <b>0.61***</b>  | <b>-0.77***</b> |

Fig 4.14 shows the positive correlation between seed color and ADL content

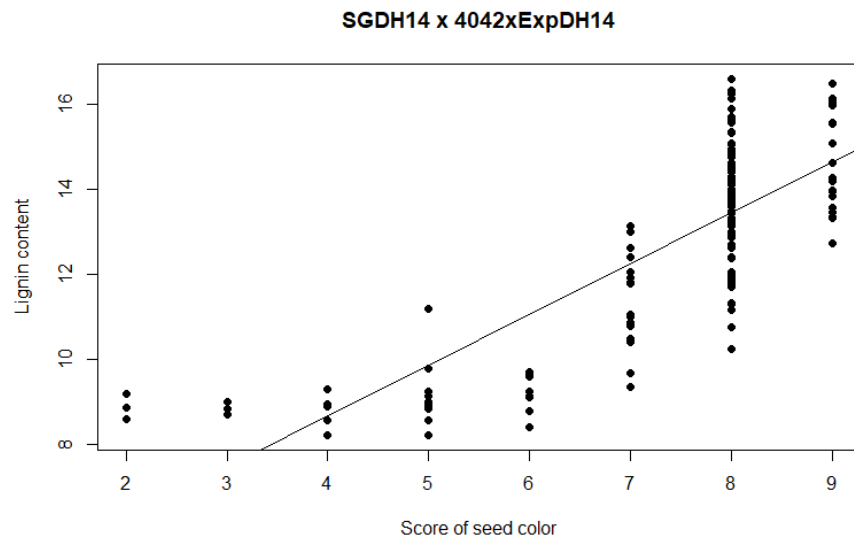


Figure 4.14. Relationship between seed color and lignin content in Population IV.

#### 4.7 KASP marker analysis of Population IV SGD14 x 4042xExpDH14

Population IV segregated for QTL for low ADL content on chromosome C05. As shown in section 4.6 SGD14 had a lower ADL content. In previous work performed by (Behnke et al., 2018), SNP markers have been identified in the vicinity of this QTL. Based on this SNP polymorphisms KASP markers on chromosome C05 were ordered and tested in the population. Contrary to expectations the second parent 4042xExpDH14 had a high ADL content, although it was presumed to have a low ADL content with a QTL on chromosome C03 (Widiarsih, 2017). Therefore, only KASP marker Bn-scaff\_17441\_3-p28950 on C05 was tested on the F2 Population. For a single locus KASP marker a 1: 2: 1 segregation could be expected. Results showed an 8: 31: 15 segregation of n=54 F2 plants in the KASP assay (Fig. 4.15). A Chi-Square test did not allow to reject the hypothesis of a 1: 2: 1 segregation. A comparison of the phenotypic data showed that homozygous parent SGD14 had a low ADL content and that homozygous parent 4042xExpDH14 had a high ADL content (Table 4.8). However, F2 plants which were homozygous for allele 1 (Fig. 4.14) were all low in ADL content (Table 4.8). Whereas F2 plants which were heterozygous or homozygous for allele 2 were found in both classes with high and low ADL content.

Furthermore, from Table 4.8, none of the F2 plants had an ADL content outside the range of the ADL content of both parents.

Table 4.9. The ADL contents and genotype of F2 plants from KASP

| ADL content/Genotype                    | Min   | Max   | Mean  | SGDH14 | 4042xExpDH14 |
|-----------------------------------------|-------|-------|-------|--------|--------------|
| Low ADL homozygous for allele 1 (n=8)   | 8.21  | 9.59  | 8.92  | 6.46   |              |
| Low ADL heterozygous (n=14)             | 8.21  | 9.31  | 8.90  |        |              |
| High ADL heterozygous (n=17)            | 14.81 | 16.50 | 15.46 |        |              |
| High ADL homozygous for allele 2 (n=10) | 15.08 | 16.61 | 15.87 |        | 16.95        |
| Low ADL homozygous for allele 2 (n=5)   | 8.83  | 9.18  | 8.96  |        |              |

Segregation ratio  
8:31:15

Parents & F2: Bn-scaff\_17441\_3-p28950

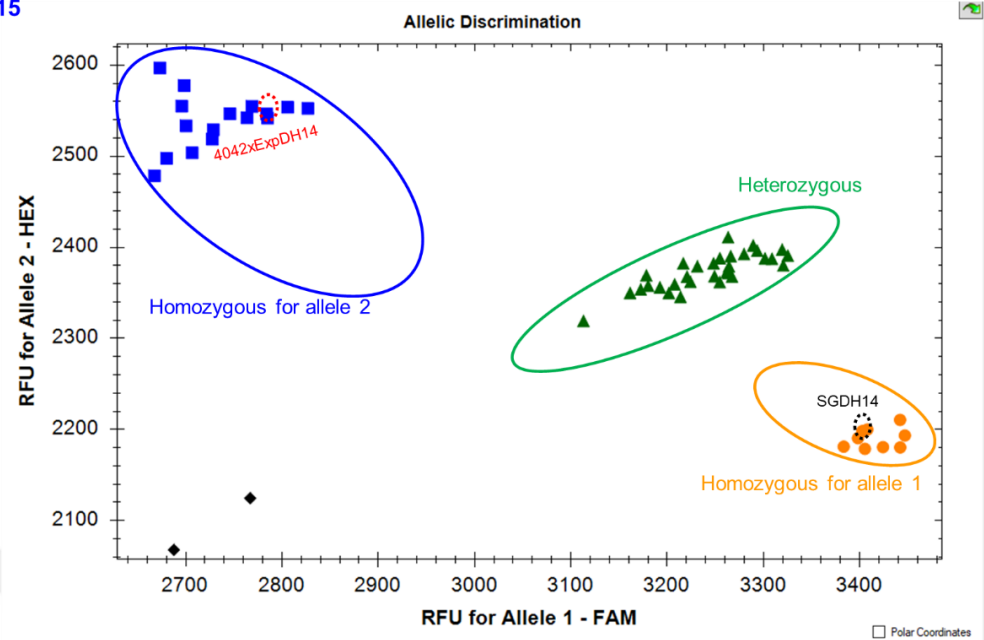


Figure 4.15. Allelic segregation of marker Bn-Scaff\_17441\_3-P28950 linked to C05.

## 5. DISCUSSION

### 5.1 Variation Among Traits

From this study, there was a large variation in F<sub>2</sub> for all measured traits in comparison with the parents. F<sub>2</sub> plants segregated for seed color, fiber related traits, TSW, TKW, oil and protein content. These segregations could also explain the number of genes controlling the traits, if there are one or two genes associated with a trait, there is less variation in the F<sub>2</sub>, however, a large variation means multiple genes are associated with a certain trait. Considering that the F<sub>2</sub> plants from all four populations were grown in greenhouse, these large variations could be because of environmental factors. Previous studies has shown that seed color which is associated with ADL content is greatly influenced by high temperature, stress among others (Burbulis and Kott, 2005; Deynze et al., 1993). It is therefore important to grow these individual F<sub>2</sub> genotypes showing wider variations for seed color and/or ADL content in field conditions in different environments to observe the genetic, environmental and interaction effects on traits.

In population I, ADL content showed a bimodal frequency distribution with Lord on the minor peak for low ADL and L239 at the major peak for high ADL, this distribution follows a 3:1 segregation ratio. Furthermore, in population I and III, Lord on average had a lower ADL content compared to either parent (L239 and R19), nevertheless, it showed a high hemicellulose and cellulose content. The similarities in the fiber related traits of these two populations could be as a result of been grown in the same greenhouse. In population II, Lord had a slightly higher ADL content than the other two populations as well as lower variation in seed color. More than 75% of the F<sub>2</sub> genotypes in population II were scored 8 or 9 for seed color.

Large segregation for seed color is found in all four populations, Spearman rank correlation also confirmed a positive correlation between seed color and ADL content, however, maternal traits such as seed color, is not a reliable indicator for breeding for quality oil (Wang et al., 2017).

Thousand kernel weight of all F3 seeds from the four populations showed a range of 2.38 – 5.75g, this is similar to the findings of Widiarsih (2017) who reported the TKW to be 2.6 – 7.9g. The lower TKW from this study could be because all populations were grown in greenhouse and not in field conditions.

The laboratory results of NDF, ADF and ADL content was positively correlated with NIRS results (Fig 4.1), however, this correlation is below  $r_s=0.7$ . Therefore, NIRS calibration should be improved to yield similar and accurate results. However, laboratory analysis needs to be performed with replicated samples to reduce the laboratory error. On one hand, the NIRS used for this current study was calibrated by Dimov et al. (2012) using a wide range of black seeded winter-type oilseed rape. As the current study consists of yellow seeded genotypes, inaccurate estimates of fiber related traits are unavoidable as the case of negative values of hemicellulose content in all populations. Thus, a new calibration method is needed which will comprise up-to-date samples containing both yellow and black seeded oilseed rape cultivars. On the other hand, not all seed samples were crushed with the coffee mill before samples were defatted, these uncrushed samples therefore gave an overestimate of the fiber related traits in the final measurement of the ANKOM methods. For breeding for low fiber content, the ranking of the genotypes is more important than absolute accurate results. The present NIRS calibration and future improved NIRS calibrations allows selection of extreme genotypes with low fiber content in segregating populations.

## 5.2 Spearman Rank Correlation

There was positive correlation between seed color and lignin content in all four populations, previous studies have also confirmed this relationship (Liu et al., 2013, 2012; Simbaya et al., 1995; Snowdon et al., 2010). This implies that seed color can be used as an indicator for lignin content, however, seed color is not a reliable assessment as it is greatly affected by environmental factors.

Seed color was positively correlated with NDF, ADL and ADL content with high significance ( $p$ -value < 0.001), this result is in agreement with earlier works

of Widiarsih (2017). Seed color was also shown to be negatively correlated with TKW in all four populations (data not shown). The relationship between TKW and seed color obtained from this study is contrary to the report of Tang et al. (1997) who showed that there was no relationship between seed color and TKW, but that TKW was influenced by genetic factors.

NDF, ADF, and ADL contents were positively correlated in all populations. This strong correlation is due to their individual composition. NDF consist mainly of hemicellulose, cellulose, and lignin, whereas ADF consist of cellulose and lignin while ADL consists of the indigestible lignin. Except for population II, there was no correlation between ADL and oil content in the different populations. Surprisingly, hemicellulose content was not correlated with ADF and ADL in all populations except population II. The ADF content was negatively correlated with protein content in all four populations and ranged between -0.41 to -0.73, this association is in agreement with the findings of Badani et al. (2006) who reported similar range (-0.55 and -0.56) when yellow seeded lines were crossed with black seeded cultivars of *B. napus*.

In the population IV, TKW was positively correlated with protein content and obviously negatively correlated with NDF, ADF and ADL content (-0.33, -0.34, -0.28 respectively) showing high significant differences (data not shown). However, using a DH population Widiarsih (2017) reported no association between TKW and other traits.

### 5.3 Molecular Data

The segregation ratio of the KASP marker in a 1: 2: 1 ratio was observed in using a marker related to C05 indicates that the QTL on C05 follows a normal Mendelian segregation. The results show that all F<sub>2</sub> plants that were homozygous for allele 1 were similarly low in ADL content as parent SGD14. Surprisingly, plants heterozygous for this marker and homozygous for allele 2 did not show consistent ADL contents. The plants could be grouped into the two classes with high and with low ADL content. This result indicates that recombination between the KASP marker and the QTL for low ADL content occurred in the F<sub>2</sub>

population. To elucidate this, more markers closely linked with the QTL (Islam et al., 2015; Widiarsih, 2017) need to be analyzed. It is also possible that the KASP assay did not yield in all cases correct results. Repeated analysis of the samples probably applying an improved assay protocol would be required to consolidate results (Islam et al., 2015; Widiarsih, 2017). The seed color score of genotypes with low ADL had a wide spectrum of between 2 – 7, whereas, the genotypes with high ADL content had a seed color score of either 8 or 9. All genotypes homozygous for allele 1 were having low ADL content and low color scores while a larger variation of color scores occurred in heterozygous genotypes and in those homozygous for allele 2.

PCR results showed amplification of the PAL gene in the parental genotypes (Lord, L239, R19 and 4042xExpDH15). Although no clear PCR products were found using primer 134/135, improving the optimization protocol could yield products in future experiments. Since clear amplification and expected product length was observed in primer 138/139 (Fig 4.1), the next stage of gel extraction and sequencing is encouraged. Due to limited time available for this study, PCR products from this reaction could not be sequenced and thereafter compared with sequences of *B. rapa*, *B. oleracea* or *B. napus* reference genome of Darmor *bzh*. It would be important in future studies to analyze the F2 plants of population I, II, and III in order to observe alignment of this candidate gene in *B. napus* cultivars. In addition, (Ni et al., 2008) showed that the expression of PAL gene was greatly suppressed in yellow seeded oilseed rape genotypes. Therefore, comparing PCR results with phenotypic data of seed color and other traits related to ADL content would be a feasible approach in understanding the inheritance of seed fiber traits in oilseed rape.

## 6. CONCLUSIONS

From this study, the laboratory results by ANKOM methods for the estimation of fiber related traits such as NDF, ADF and ADL were all very similar to the results from NIRS prediction. These two methods can both be applied in the determination of fiber traits when estimating and formulating feed composition in animal nutrition. Although the ANKOM method is very laborious and time consuming, yet it offers a more reliable value for NDF, ADF and ADL contents.

Generally, there was wide variation observed in the F2-populations from all four populations for different traits such as seed color, TKW, TSW, protein and oil content and most importantly fiber related traits such as NDF, ADF, ADL, hemicellulose and cellulose content. These variations provide a platform for selection of different traits for future breeding programs such as breeding for low fiber, seed size, protein or oil content

In addition, correlation of fiber related traits with other quality traits such as seed color, oil and protein content, TKW, TSW was observed across all four populations, most of these correlations were all positively correlated except for protein content which is negatively correlated with all traits in all four populations. This correlation also provides insight into the association of quality traits and how they are inherited. It is therefore important to note the effect of selection on one trait has on other traits.

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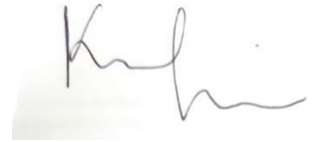
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18 / 09 / 2020

A handwritten signature in black ink, appearing to read 'Kefas', with a long horizontal flourish extending to the right.

Kefas Luka BAIYI



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