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NUTRITIVE VALUE OF FORAGE GRASSES: EVOLUTION DURING MORPHOGENESIS AND GENETIC BASES IN PERENNIAL RYEGRASS

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Acronyms

ADF: Acid detergent fibre

ADL: Acid detergent lignin

ADL.NDF: ADL proportion in NDF

ADM: Absolute dry matter

AFLP: Amplified fragment length polymorphism

ANRT: “Association nationale de la recherche et de la technologie”

National association for research and technology

BIC: Bayesian information criterion

BLUP: Best linear unbiased predictor

C: Cellulose

C.NDF: C proportion in NDF

CAD: Carbamoyl-Phosphate Synthetase 2, Aspartate Transcarbamylase, and Dihydroorotase

CIFRE: “Conventions industrielles de formation par la recherché”

Industrial agreement of training through research

COMT: Catechol-O-methyltransferase

CTPS: “Comité technique permanent de la sélection des plantes cultivées”

DUS: Distinctness, uniformity and stability

DM: Dry matter

DMD: Dry matter digestibility

DNA: Deoxyribonucleic acid

DSV: “Deutsche Saatveredelung AG”

ed: Effective day

GBS: Genotyping by sequencing

GDD: Growing degree day

GEVES: “Groupe d'étude et de contrôle des variétés et des semences”

GreenD: Mature green leaf duration

GWAS: Genome-wide association study

HC: Hemicellulose

HC.NDF: HC proportion in NDF

HD: Heading date

IBERS: Institute of biological, environmental & rural sciences

ILVO: “Instituut voor Landbouw-, Visserij- en Voedingsonderzoek”

Flanders research institute for agriculture, fisheries and food

INRAE: “Institut national de recherché pour l’agriculture, l’alimentation et l’environnement”

French national research institute for agriculture, food and environment

LED: Leaf elongation duration

LER: Leaf elongation rate

MAF: Minimal allele frequency

ML: Maximum likelihood

MLMM: Multi-locus mixed-modelling

NDF: Neutral detergent fibre

NDFD: Neutral detergent fibre digestibility

NIRS: Near infrared reflectance spectroscopy

NPK: Nitrogen, phosphorus and potassium

OM: Organic matter

OMD: Organic matter digestibility

PCA: Principal component analysis

QTL: Quantitative trait loci

SAMAE: “L'interprofession des semences et plants”

French Interprofessional organisation for seeds and plants

SenD: Leaf senescence duration

SNP: Single nucleotide polymorphism

TN : Total nitrogen

UFL: “Unité fourragère lait”

URP3F: “Unité de recherche pluridisciplinaire prairies et plantes fourragères”

VCU: Value for cultivation and use

WSC: Water soluble carbohydrates

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This thesis has been conducted in the frame of a CIFRE ("Conventions Industrielles de Formation par la REcherche") founded by the French ministry of "Enseignement supérieur, Recherche et Innovation" and supervised by ANRT ("Association Nationale de la Recherche et de la Technologie"). It has been the fruit of a collaboration between the breeding company: DSV France (Experimental center of Les Rosiers sur Loire) as part of DSV AG ("Deutsche Saatveredelung AG"), the INRAE ("Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement") unit of research: pluri-diciplinary, grasslands and forage species (URP3F Lusignan) and the ILVO (Instituut voor Landbouw-, Visserij- en Voedingsonderzoek") research unit: Plant Science (Ghent).

DSV AG was founded in 1923 in Germany for the production of seeds of fodder crops. It has a long experience in forage grass breeding with high digestibility and high yield being the main targets. Today, with a turnover of 203 million euros, DSV is located in several continents through subsidiaries. It is a European leader in multiple crops selection: rapeseed, winter wheat, six row winter barley, intercrops, forage grasses, forage legumes and turf grasses.

The idea of the present thesis came from a discussion after the defence of Frederik van Parijs thesis on "Cell wall digestibility of perennial ryegrass: an association mapping approach" (van Parijs, 2016), which answered some questions but, as always, also raised many other questions, in particular on the link between the plant morphogenesis through the seasons and the quality of the forage which could be harvested.

After an introduction including the importance of grasslands for milk, meat production and in agrosystems in general, a general presentation of perennial ryegrass, a presentation of the ways to measure forage quality and the different origins of its variability *i.e.* physiological, environmental and genetic ; this thesis consists of two chapters: i/ seasonal differences in structural and genetic control of digestibility in perennial ryegrass, and ii/ dynamics of the quality during the lifespan of a leaf and a stem of perennial ryegrass: a survey of genotypes contrasting for their precocity and their quality; eventually the thesis ends by a general conclusion and some perspectives in particular on the possibility to include quality in morphogenesis plant models and on the consequences for breeding.

1. Introduction

1.1. Importance of grasslands and perennial ryegrass

Grasslands are essential to ruminant feeding for ages. Depending on the soil and the climate, they could provide a good yield of a balanced ration (energy and protein) when exploited correctly (right physiological stage, fertilized and good species composition). Moreover, they are the cheapest source of feed when provided to ruminants directly through grazing pasture land (Huyghe et al., 2014). For these reasons, they are still largely used either grazed or mechanically harvested to produce conserved forage such as hay, wrapping forage or silage. Grasslands have other benefits than biomass production. They are providers of ecological services such as the preservation of water quality, biodiversity, landscape biodiversity and carbon sequestration.

Grasslands cover about 40 % of the agricultural area in Europe in 2013 (60 million ha) (van den Pol-van Dasselaar et al., 2019). Permanent grasslands (maintained more than five years) represent 85 % of these areas. The rest is composed of temporary grasslands, which are sown regularly with different bred forage species including legumes such as alfalfa or white and red clovers, and grasses such as Italian and perennial ryegrasses (*Lolium multiflorum L.* and *Lolium perenne L.*), timothy, fescues or cocksfoot. Temporary grasslands sown with elite varieties of Italian and/or hybrid ryegrass produce generally a better yield of a better quality than permanent grasslands and are mainly used in intensive systems in particular for milk production (Søegaard et al., 2007).

The area for seed production of forage plants in Europe represented 515 700 ha in 2020 which was at the first position just before soft wheat (GNIS, 2021). Forage grasses dominate the forage seed market and, in 2020, represented 262 963 ha with perennial ryegrass being the first one with 88 140 ha. In 2019, the exchange of forage grass seeds represented 325 Million € (SEMAE personal communication). Among grass forage species, perennial ryegrass is the main species used in temperate climate with about 70-90 000 tons of seeds used per year in Europe for forage and turf between 2007 and 2015 (Bruins, 2016). It is often sown in association with white clover and is well known for its high quality, its good persistency under temperate climate, its good response to nitrogen fertilizers and its very good behaviour under grazing (McDonagh et al., 2016; Sampoux et al., 2011).

1.2. General presentation of perennial ryegrass

Perennial ryegrass: *Lolium perenne L.*, belongs to the genus *Lolium*, the sub-tribe Loliinae, the tribe Poeae, the super tribe Poodae, the sub family Pooideae and the family Poaceae (http://lifemap-ncbi.univ-lyon1.fr/?tid=640630). Perennial ryegrass is phylogenetically close to major crops such as oat, wheat and barley and to the grass model species: *Brachipodium distachyon* (Figure 1).

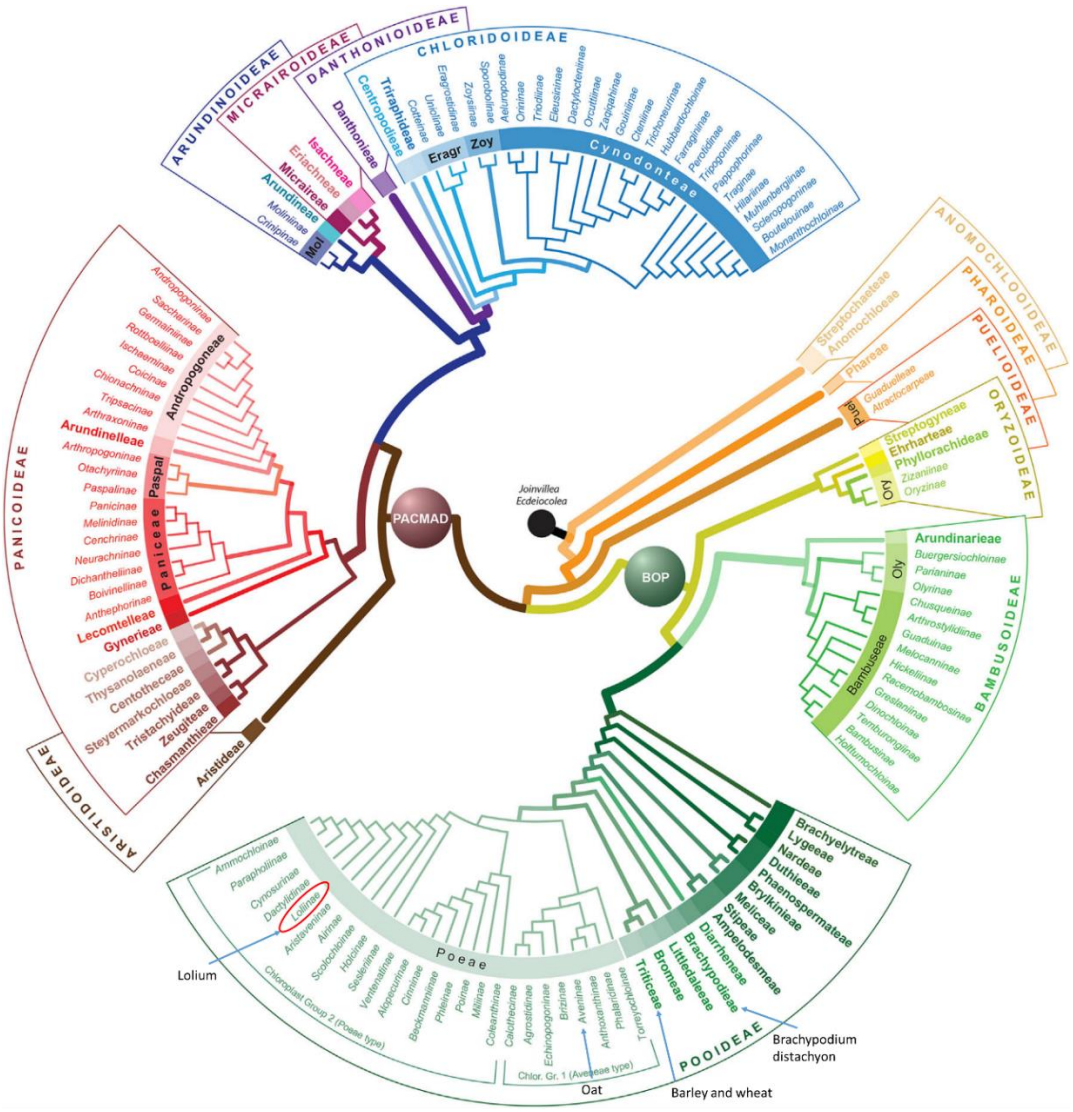

Figure 1: A phylogenetic ranking of the Poaceae evolving clockwise from Joinvillea and Ecdeiocolea. BOP: Bambusoideae, Oryzoideae, and Pooideae; PACMAD: Panicoideae, Aristidoideae, Chloridoideae, Micrairoideae, Arundinoideae, and Danthonioideae. Modified from Soreng *et al.* (2017).

Perennial ryegrass is a perennial herbaceous plant composed of roots, a crown with non elongated internodes during the vegetative stage (Figures 2 and 3) and tillers: main tiller and daughter tillers. The absence of internode elongation during the vegetative stage is a powerful way to protect apical meristems from defoliation by herbivores and to provide a good persistency under grazing. Each tiller consists of an apical meristem (apex), nodes with a leaf and a lateral meristem at the origin of a daughter tiller and internodes. A leaf is composed of a sheath and a lamina separated by a ligule (Figure 4). Each leaf grows within the sheath of its previous leaf. The sheaths and growing leaves within the sheaths during the vegetative stage are often called pseudo-stem by contrast with true stems corresponding to elongated internodes and nodes during the generative or reproductive stage. During this generative stage, the apical meristem produces lateral meristem which will produce spikelets instead of vegetative tillers and the internodes elongate. The increase of length of the successive internodes during the lifespan of a tiller from vegetative to generative stages is presented in Figure 5. The beginning of the generative stage is triggered by both a vernalisation and an elongation of day length (Rouet, 2021).

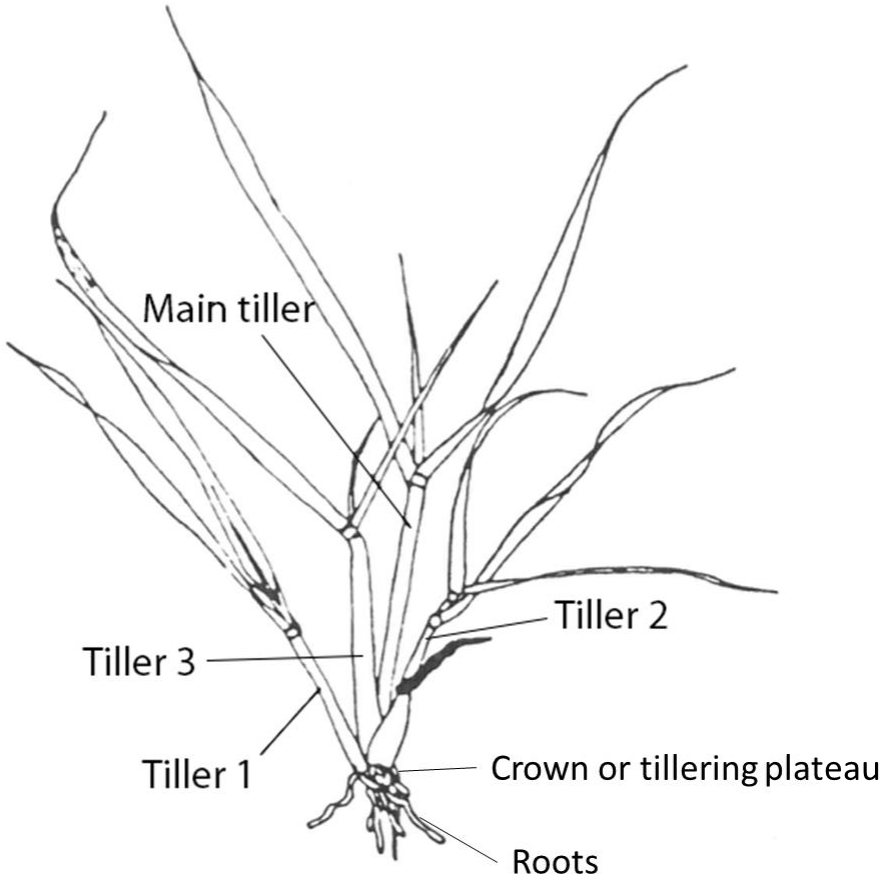

Figure 2: Drawing of a perennial ryegrass young vegetative plant (Gillet, 1980).

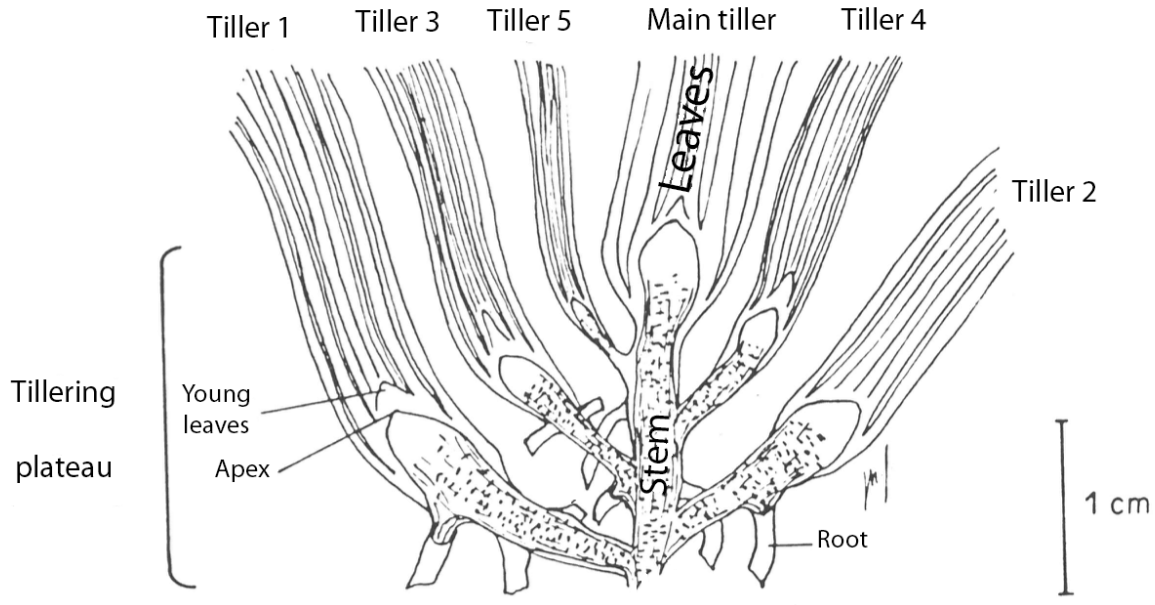

Figure 3: Focus on a tillering plateau (Gillet, 1980)

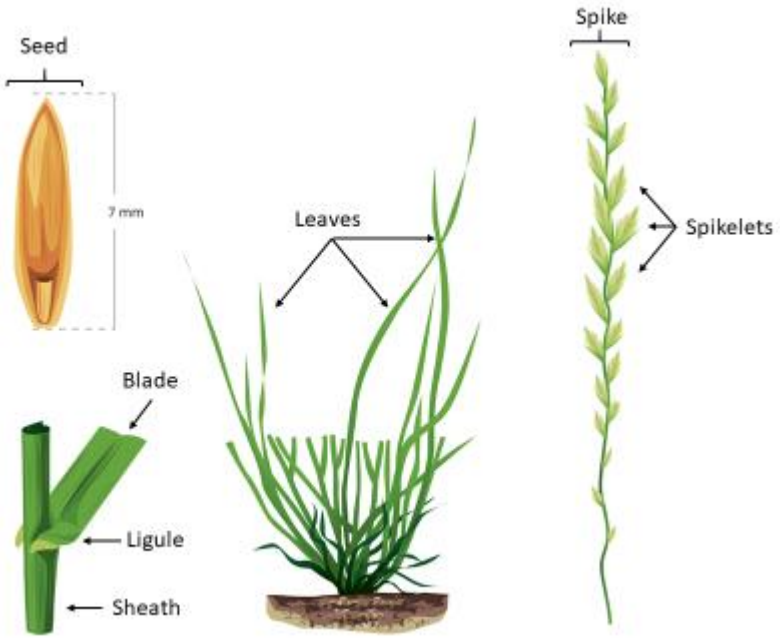

Figure 4: Presentation of perennial ryegrass modified from SEMAE (https://www.gnis-pedagogie.org/?s=ray-grass+anglais+gazon)

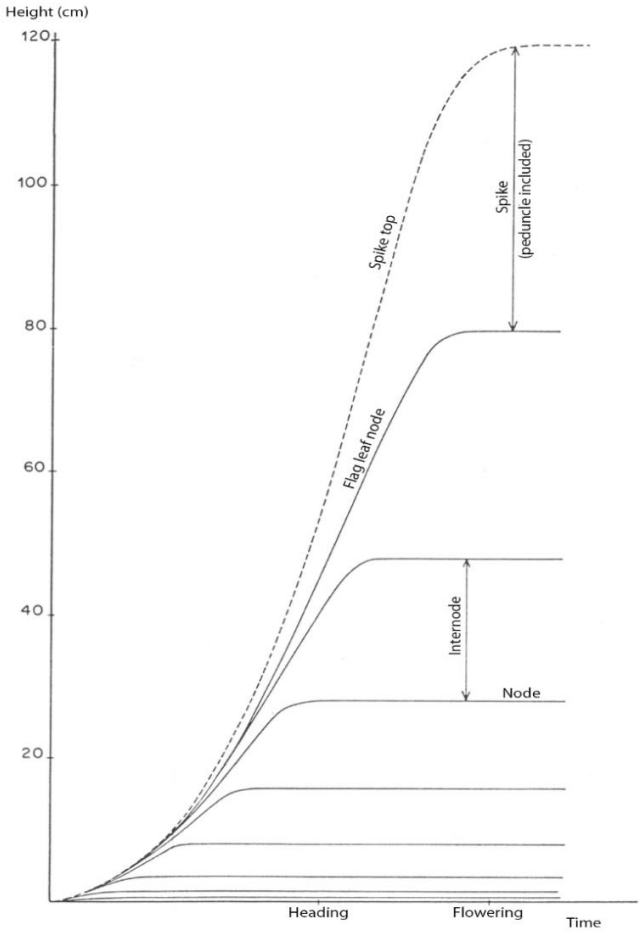

Figure 5: The height of nodes during the lifespan of a tiller (Gillet, 1980).

Naturally, perennial ryegrass is diploid with 2n = 2x = 14 chromosomes but tetraploid varieties have been created. Its haploid genome size corresponding to 1X is 2.68 pg = 2.62 Gb (Pellicer and Leitch, 2019). The genome of perennial ryegrass has been recently sequenced (Byrne et al., 2015) and assembled (Frei et al., 2021). A limited number of different molecular markers have been produced (microsatellite, AFLP, amplicon sequencing) (Forster et al., 2004; Roldan-Ruiz et al., 2019) but a major step was the production of several hundreds of thousands markers by a simple genotyping-by-sequencing (GBS) method based on restriction enzymes (Elshire et al., 2011). This method allows to genotype individual plants but also populations for which the allelic frequencies could be estimated with a single DNA pool per population (Byrne et al., 2013). This method allows to obtain markers all over the genome but not at selected positions. For this reason, new markers are developed based on previous knowledge (candidate genes, QTLs) to sequence selected loci by either high throughput amplicon sequencing or by sequencing capture (Roldan-Ruiz et al., 2019).

Perennial ryegrass is an obligate outcrossing species due to a self-incompatibility system and presents a strong inbreeding effect (Slatter et al., 2021; Thorogood et al., 2002). The absence of a simple method for inter-crossing selected plants at a large scale like in maize leads to the creation of synthetic varieties originating from the crossing of a few founders (between 4 to 20) followed by two to three generations of panmictic multiplication in order to produce enough seeds for the market need and to create a stable off spring from the synthetic progeny. These biological constraints with the practical and financial constraints lead to the definition of breeding schemes. A classical breeding scheme is proposed on Figure 6. Starting from a panel of diverse material (the construction of this panel is essential for the success of the breeding programme), plants are first selected only on their phenotype observed in spaced plant field trail (mass selection) during three years for highly heritable traits such as disease resistance, vigour, heading date, persistency, growth habit. The selected ones are crossed in polycrosses (inter-crossing of a set of plants) to obtain half-sib families (each mother plant is harvested separately) or alternatively in pair-crosses (inter-crossing of two plants) to obtain full-sib families. Then the families are selected based on their genetic value estimated in forage yield plots with replicates and possibly several locations during three years for low heritable traits such as yield and quality. Then randomly selected plants within the selected families are crossed in polycrosses to obtain synthetics which will be multiply for further phenotyping evaluations in forage plots. The best ones are selected to be evaluated by national institutions like GEVES (Groupe d'Etude et de contrôle des Variétés Et des Semences) in France for three years in order to be registered by CTPS (Comité Technique Permanent de la Sélection des plantes cultivées) if they pass the tests : DUS (distinctness, uniformity and stability) and VCU (value for cultivation and use). This breeding scheme could release a variety after about 15 years. This long process could be shortened be using genomic selection which is currently being implemented in some breeding companies (Barre et al., in press; Cericola et al., 2018; Fè et al., 2016, 2015; Hayes et al., 2013; Lin et al., 2016, 2014; Pembleton et al., 2018). Moreover when selecting on half-sib families, the genetic gain could be increased by using molecular markers for paternity tests in order to select siblings within a family having the best paternal parents instead of selecting randomly.

The main targets for selection are forage yield, quality, disease resistance, heading date (since for registration, varieties are classified within precocity groups) and persistency. The importance of forage quality in breeding programmes increased particularly when in 2013 it has been included in the variety testing for registration in France (including proteins, water soluble carbohydrates and lingo-cellulose (ADF) contents). One of the main difficulties to select for forage quality is that it depends on many factors including the stage of development when harvested which could hide genetic variation for quality behind variation for heading date.

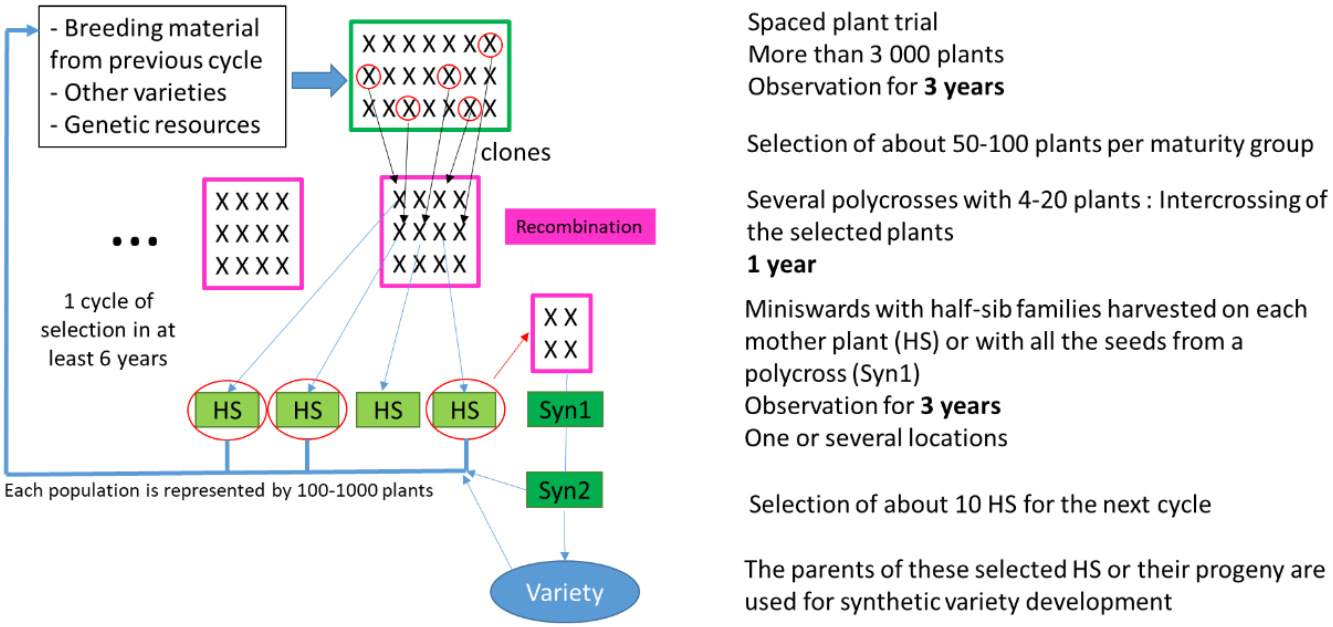

Figure 6: A classical example of breeding scheme for perennial ryegrass (Barre et al., in press).

1.3. Evaluation of forage quality

In order to build a ration to cover animal needs, it is necessary to evaluate the quality (equivalent to the digestibility in this thesis not considering the micro-nutriments) of the forage *i.e.* the energy and the protein content brought by the forage usable by the ruminant. For that purpose, many methods have been developed through years with the *in vivo* apparent digestibility being the reference method. In this part, we will go through all currently used protocols. Starting with the *in vivo* methods, we will continue with *in situ* (or *in sacco*) method, then we will see the large diversity of *in vitro* methods.

1.3.1. *In vivo* methods

The principle of *in vivo* methods consists in measuring the difference between the feed given to an animal and the produced feces: total collection. This method is performed since the beginning of forage digestibility measurement in the XIX century (Schneider and Flatt, 1975). It allows to measure "apparent digestibility" following one assumption: the proportion of feed intake not excreted is totally digested. We talk about "apparent digestibility" because the feces contain, in addition of undigested food, some materials produced by the animal body (Schneider and Flatt, 1975) and a part of carbohydrates, fermented into methane, is lost as eructation and is not absorbed by the animal (McDonald et al., 2011).

The main protocol can be divided in multiple steps:

- The feed is analysed for its chemical composition (% of dry matter, crude protein, ash, ether extract, crude fibre, nitrogen-free extract and gross energy);
- Animals are individually locked in a metabolism crate, a sort of cage used to facilitate feces collection, and fed with only the analysed feed. The feed intake is calculated as the weight of proposed feed minus the weight of refused feed;
- After a period of time necessary to rid the digestive system of the precedent feeds, feces are started to be collected and weighted separately of urines;
- Samples of feces follow the same chemical analysis path than feed;
- Differences between results from chemical analysis of feeds and feces allow to obtain the digested percentage of each component of the feed and the feed digestibility itself: (feed intake – feces) / feed intake x 100 in percent.

1.3.2. *In situ* method

Also called *in sacco* method, the *in situ* technique requires the use of fistulated ruminants. The method allows the estimation of small samples of 2 to 5 g, which are milled dry feed (to pass a 3 mm sieve) or minced wet feed (Ørskov, 2000). The samples are placed in nylon bags with a pore size around 40-60 µm, which are inserted inside the rumen for a maximum of 48 h. After that, bags are washed and weighted. The difference of sample weight gives an estimation of the dry matter digestibility. It is also possible to compare the amount of nitrogen or fibre between undigested samples and digested ones to determine their digestibility. This technique is particularly used for determining degradability kinetics of samples.

1.3.3. *In vitro* methods

Tilley and Terry (1963) proposed a two-stages *in vitro* technique that has become the reference. The principle consists in measuring the difference of weights of a sample before and after two incubations: a first incubation of the sample in rumen liquor from fistulated animals with synthetic saliva followed by a second incubation in an acid aqueous solution of pepsin. More precisely, the samples are dried, milled (to pass a 1 mm sieve), weighted and incubated with synthetic saliva (McDougall, 1948) and strained rumen liquor into 80-90 mL at 38°C for 48 h in a carbon dioxide atmosphere. The samples are agitated and the pH checked to be maintained between 6.7 and 6.9 to optimize conditions for microbial growth and development. After 48 h of incubation, mercuric chloride ($HgCl^2$) and sodium carbonate (Na^2CO^3) are added to inhibit further microbial activity and aid sedimentation. The supernatant is discarded following filtration or centrifugation. Acid pepsin is added to the residue and the mixture

incubated again for 48 h at 38°C. The sample is centrifuged again, the supernatant discarded and the residue washed. This undigested residue is dried. Digestibility is calculated by dividing the weight loss of the sample by its initial weight, after correction for the blank weight of the diluted rumen liquor (Omed et al., 2000). This method was adapted to use a filter bag system instead of digestion tubes allowing the analysis of several samples in a single jar (Wilman and Adesogan, 2000).

To avoid the necessity of fistulated cattle, a method was investigated by Balfe (1985) with the use of a liquor obtained from sheep feces. This method was modified by El Shaer et al. (1987) by the use of McCartney bottles fitted with wine fermentation locks. Next, Omed et al. (1989a, 1989b) found that the procedure could be carried out satisfactorily in sealed bottles, dispensing with the fermentation locks. The sample size was reduced to 180 mg and the faecal liquor volume scaled down correspondingly to 18 ml.

To avoid the use of animals, several enzymatic solubility protocols have been proposed on the basis of pepsin-cellulase technique (Aufrère, 1982; Donefer et al., 1963; Jarrige et al., 1969; Jones and Hayward, 1975; Kowalski et al., 2014). The large amount of protocols is due to each laboratory using its own buffer recipe, material (crucible, Erlenmeyer, filter bag for exemple) and incubation duration. Also, the provider of cellulase (mainly obtained from fungus *Trichoderma viride*) change following availability for each laboratory, giving the obligation to adapt the protocol to the enzymatic activity of cellulase and its optimum of pH and temperature. This technique is the most repeatable method with small amount of dry matter, as rumen liquor (or faecal liquor) is highly dependent on the animal (diet, sex, age). This technology contribute to facilitate researches performing digestibility evaluation. In France, the Aufrère protocol (1982) act as reference for pepsin-cellulase technique. Its adaptation for the use of Ankom filter bags was recently made by Méchin V. (not published).

In practice, even if the *in vivo* method stays the reference for all digestibility evaluations, *in vitro* methods are the most commonly used due to their rapidity, facility, low cost and reapetability. *In situ* methods are mainly used to observe digestibility kinetics instead of measuring general quality. But the future of digestibility measurement is in the use of spectrometry.

1.3.4. Spectrometry method

Near infrared reflectance spectroscopy (NIRS) is a technology that can predict various feed components without destruction (Deaville and Givens, 1998). NIRS is an analytical technique based on the absorption of infrared radiations by the chemical bonds in organic matter (Bastianelli, 2013). This absorption is linked to the chemical composition and can be measured with a spectrometer. In order to predict a component proportion, it is necessary to calibrate the NIRS with the development of mathematical models, serving as a translator between infrared spectrum absorbed and the sample composition. A model is specific for a component and more or less specific depending on the type of sample. To develop a robust model, it is necessary to integrate a large and varied dataset of samples. This model could then be updated with the addition of dataset (chemical composition or *in vitro* or *in vivo* digestibility) from new samples.

In conclusion, techniques to evaluate digestibility have been simplified with time but the *in vivo* technique stays the reference from which all other methods have to be calibrated. These techniques could be applied directly on forage or on fibres to evaluate their digestibility.

1.4. Origins of the quality variability in forage grasses

Since the 70s, thanks to *in vitro* methods and later to the use of NIRS to evaluate digestibility, many forage samples have been surveyed for their quality. Rapidly, it came out that many factors affect the forage quality including the plant maturity which is in close relationship with the type of organs (leaf, stem and inflorescence) and their morphology, the chemical composition of cells, the crop management (fertilisation in particular nitrogen, frequency of defoliation, use of fungicide, watering), the growth environment (temperature, drought, freezing…) and the genetic variability between and within species (reviewed in Jung et al., 1993). We propose here to give the main conclusions with an update.

1.4.1. Plant maturity, type of organs and their morphology

One of the major factors influencing forage quality is plant maturity (Nelson and Moser, 1994). This is mainly due to the evolution in the proportion of plant organs: leaves, sheaths and stems, which have different level of quality: leaves > sheaths > stems > dead leaves (Beecher et al., 2013; Chaves et al., 2006). For example, in big bluestem (*Andropogon gerardii*, Vitman) and switchgrass (*Panicum virgatum*, L.), at spike emergence, the dry matter digestibility (DMD), neutral detergent fibre content (NDF) and lignin contents of leaves were 60, 66 and 4.7 % DM whereas the ones of stems were 50, 75 and 7.2 % DM (Griffin and Jung, 1983). During the reproductive stage, the proportion of stems in percent of plant weight increases leading to a decrease of the quality of the harvested forage (Buxton and Redfearn, 1997). This difference of quality between leaf and stem evolves with the organ maturity *i.e.* its age (Wilman and Altimimi, 1982). The leaf quality evolves slightly during its lifespan with a cell wall mass almost constant but a decrease in cell wall digestibility (Groot et al., 1999). Young stems are generally of high quality (digestibility could be better than the leaves at the same stage) (Minson, 1990; Wilman and Altimimi, 1982) but their quality drops rapidly with internode elongation which is easily noticeable after spike emergence (Nelson and Moser, 1994). This could be attributed to anatomy differences, leaves being composed mainly of mesophyll cells with thin cell walls whereas stems containing many highly lignified xylem cells. These lignified cells are the major factor providing structural strength but it is also the greatest limitation to the breakdown of stems in the rumen (Akin, 1989). By comparison, stem parenchyma is easily digested.

Green leaf tissue is often the highest quality part of the forage (Hides et al., 1983). Nevertheless, the structure of leaves should be taken into account. An increase of the sheath/blade ratio decreases the quality since the quality of sheath is lower than the quality of blade with for example, after spike emergence, a blade DMD of about 80 % and 70 % in perennial ryegrass and cocksfoot, respectively, compare to 70 % and 65 % for sheath (Terry and Tilley, 1964). Moreover, leaves' quality decreases with their lengths; this effect is often confounded with the rank effect since leaf length increases with its rank (Agnusdei et al., 2011; Duru and Ducrocq, 2002; Gastal and Lemaire, 2015; Groot et al., 1999). This length or rank effect is due to an increase of the sheath/blade ratio and to an increase of structural tissue content (Insua et al., 2018, 2017). It has been shown that leaf length is more important than leaf age to explain the variation of leaf digestibility (Avila et al., 2010).

Precocity (date of spike emergence or heading date) has a major effect on the plant quality because of morphological differences. At a given date, when heading has started, the late heading genotypes present a better quality than the early heading genotypes because of a difference of leaf/stem ratio (Buxton and Casler, 1993). By contrast, when harvested at the same physiological stage: spike emergence, an effect of precocity is also observed but the late heading genotypes present a lower quality than the early heading genotypes (Christie and Mowat, 1968; van Parijs, 2016). This could come from the fact that late heading genotypes have more leaves of higher length and sheath than the early heading genotypes.

1.4.2. Cell-Wall structure

The differences of quality observed between organs and their maturity could be related to chemical composition differences. In particular, concerning digestibility, cell-wall structure and composition play an important role. During the lifespan of a leaf, indigestible cell walls accumulate following a negative exponential saturation curve (Groot and Neuteboom, 1997). Main structures that limit forage digestibility are the support tissues, which are highly lignified, like sclerenchyma and xylem (Akin, 1989). The structure of grass stems is more rigid than leaves, with the non-digestible epidermis, sclerenchyma ring, and vascular tissue occupying about 30 % of the tissues in the cross sections (Akin, 1989). An increase in plant maturity will increase the number of lignification sites, which will decrease plant digestibility (Akin, 1989).

During plant cell development, the primary cell wall is deposited initially and it contains cellulose, hemicellulose, and pectins. Lignin use ether bonding with ferulic acid and ester bonding with hemicellulose to create lignin-carbohydrate complexes. Lignin becomes part of the cell wall during formation and thickening of the secondary cell wall (Figures 7 to 9) (Theander and Aman, 1984). Lignin is often described as the key element that limits cell-wall digestibility, but its impact is conditioned by its structure and cross-linkage. In other hand, lignin composition (proportion of guaiacyl (G), p-hydroxyl phenol (H) and syringyl (S) lignin) is less likely to affect digestibility (Grabber, 2005; Jung and Allen, 1995; Zhang et al., 2011). The inhibiting effect on plant digestibility by lignin decrease at higher concentrations. Lignin concentration in cell walls influences negatively the digestibility of other cell wall components with a stronger effect on cellulose digestibility. Hemicellulose concentration in cell walls is negatively correlated to the cellulose concentration, but these two cell wall components don’t have a correlation with lignin concentration. (Jung and Vogel, 1986).

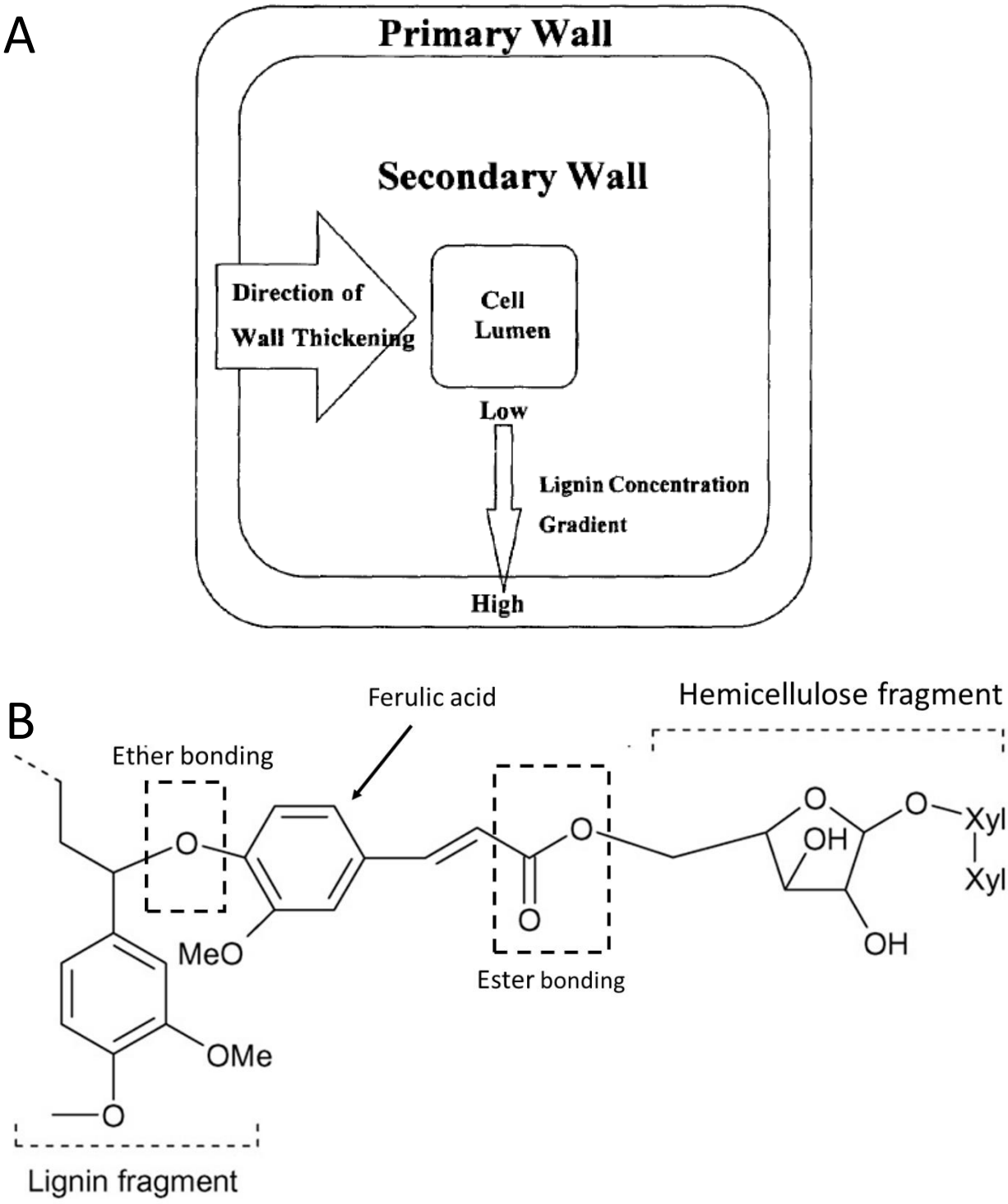

Figure 7: A: Schematic representation of a plant cell and wall development. Thickening of the wall proceeds from the primary cell-wall region toward the cell lumen. Lignin deposition begins in the primary wall and then progressively moves through the secondary wall. Lignin concentration is greatest in the primary wall region (from Jung and Allen, 1995). B: Lignin/phenolics–carbohydrate complex in wheat straw (modified from Buranov and Mazza, 2008)

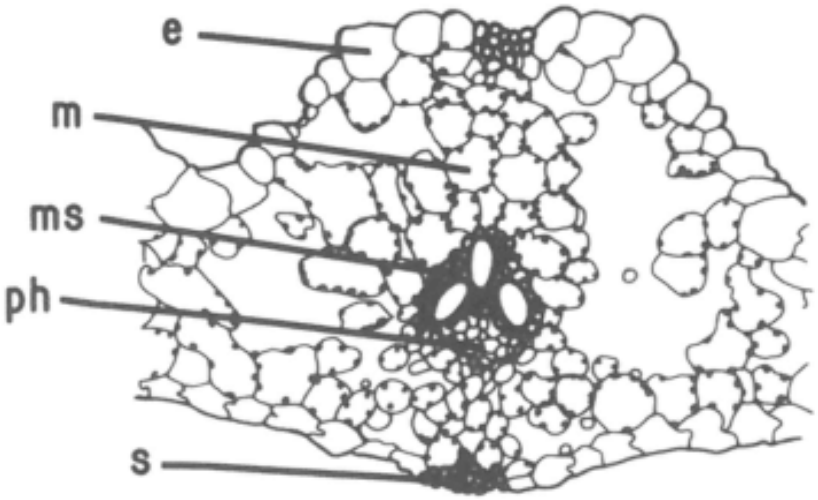

Figure 8: Cross section of a main vein for temperate grass (*Lolium multiflorum* L.). e = epidermis, m = mesophyll, ms = mestome sheath, ph = phloem and s = sclerenchyma (from Wilson, 1993).

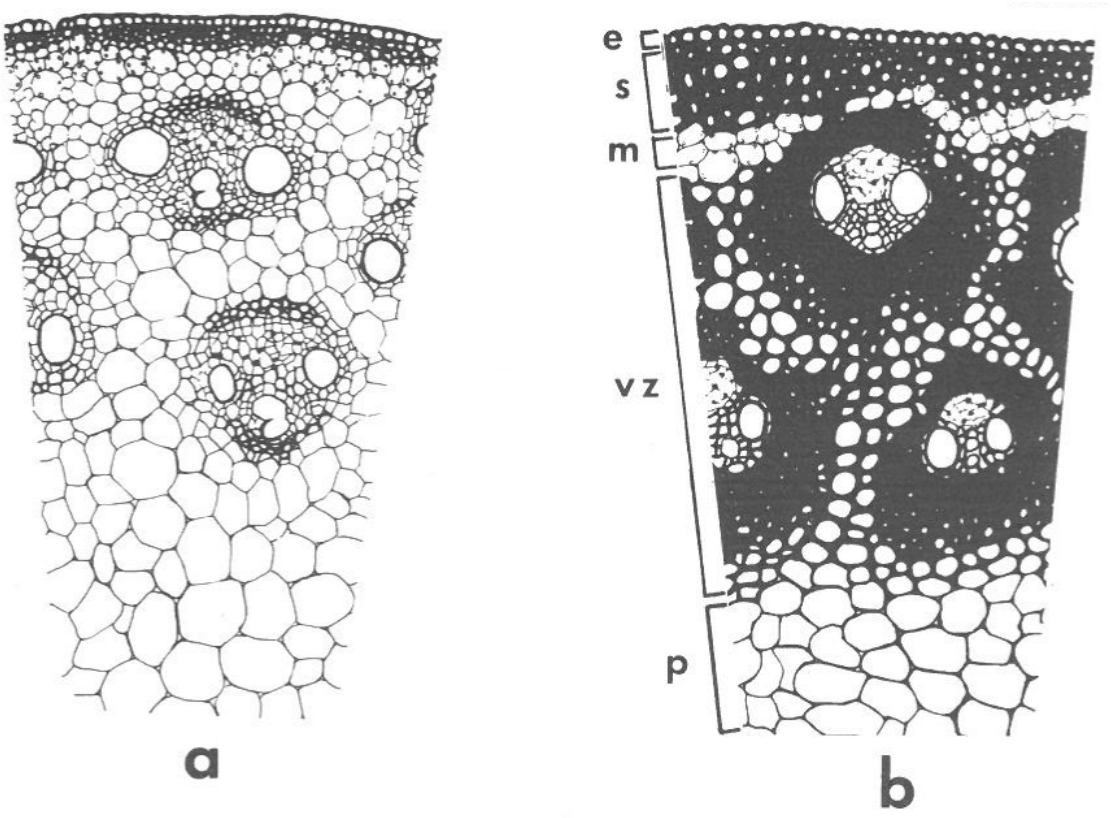

Figure 9: Cross sections of young and mature stems of a tropical grass (sorghum (a) head emergence and (b) grain maturity). Solid black walls indicate high lignification. e = epidermis, s = sclerenchyma, m = mesophyll, vz = lignified vascular zone and p = pith parenchyma (from Wilson, 1993).

1.4.3. Environmental factors

Plant environment including biotic and abiotic factors has a major impact on quality through their impact on plant morphogenesis and plant chemical composition (Buxton and Casler, 1993). Some major factors encountered in the field affecting plant quality are diseases, extreme temperatures, water availability, nitrogen fertilization and frequency of defoliation. Generally, a stress tends to decrease the quality of the plant, for example for rust susceptibility (Carr, 1975) and extreme temperatures (Akin et al., 1987) whereas an increase of fertilization tends to increase the quality (Duru et al., 2000) and drought tends to increase slightly the quality but with high side-effect on yield (Buxton and Fales, 1994). Concerning the frequency of defoliation, increased grazing pressure increases the digestibility of the following regrowth by decreasing the sheath length (Duru and Ducrocq, 2002). Moreover, an un-frequent defoliation regime tends to increase the herbage mass harvested with a decrease of digestibility due to an increase of cell wall lignification (Beecher et al., 2018; Jung and Allen, 1995; McEvoy et al., 2010) and with an increase of dead material which induces a decline of DMD (Abrahamson and Talbot, 1986; Lawrence et al., 2017).

In addition to these environmental factors, strong differences of quality have been observed between seasons, which involve various abiotic factors such as temperature and photoperiod but also the physiological stage of the plant. For example, in tall fescue, DMD of leaves was higher in cool season than in warm season (Insua et al., 2018). These DMD differences were related to changes in NDF and not to changes in cell wall digestibility.

1.4.4. Genetics

Despite strong environmental and physiological effects on the quality of the harvested forage, genetic variability has been observed between and within species (Buxton and Casler, 1993; Pontes et al., 2007). Effectively management practices have a major impact on forage quality with a good quality of forage harvested at early vegetative stage (young leaves), but it is not always possible to harvest at the right stage and it would be of great interest to dispose of varieties of high quality for a long period even at advanced stages of maturity. In addition to a better flexibility in timing of harvesting or grazing, these varieties could offer higher yield of digestible biomass.

Large genetic diversity for quality exists between grass forage species. For example, in spring, on 13 temperate species, Pontes et al. (2007) observed a range of DMD from 76.3 %DM for perennial ryegrass to 62.2 %DM for *Poa pratensis* for natural populations and a maximum of 80.4 %DM for the variety Clerpin of perennial ryegrass. Another example on five species shows a variation of organic matter digestibility (OMD) from 79 %OM for perennial ryegrass to 54 %OM for *Paspalum* on green blades (Chaves et al., 2006). Considering the main temperate grass species, on varieties registered in France, we observe the following ranking concerning the forage quality: perennial ryegrass (UFL 0.86-0.99 and water soluble sugar content 17.4-24.4 %DM) > cocksfoot (UFL 0.84-0.86 and water soluble sugar content 6.9-9.7 %DM) = tall fescue (UFL 0.82-0.86 and water soluble sugar content 10.5-12.7 %DM) (Herbe-book, 2021).

Genetic diversity also exists within species and could be used for breeding (Buxton and Casler, 1993; Capstaff and Miller, 2018; Casler and Vogel, 1999; Sampoux et al., 2011; Wilkins, 1997). A comparison of three perennial varieties for DMD over two years and several harvests revealed a range among varieties varying from 1 to 8.6 units with a conserved ranking

between varieties (Wilkins, 1997). A survey on 723 perennial ryegrass genotypes for DMD in spring on spaced plants harvested at spike emergence has shown a large genetic diversity (van Parijs, 2016). Interestingly, Reich and Casler (1985) observed a genetic variation for the response of plant to advancing maturity for fibre content (NDF) on smooth bromegrass revealing some families with a slow response to maturity which could have a great advantage for farmers. Casler and Vogel (1999) noted an increase of DMD of 0.7 – 2.5 % yr^{-1} on several grass forage species and interestingly without any effect on forage yield. This gain of quality was generally observed across a wide range of environment and management systems. In perennial species, selection is generally conducted on single spaced plants and typical heritability of DMD is about 0.2 – 0.3. In a study using perennial ryegrass varieties from 1971 to 2003, it has been observed a genetic gain of 0.49 % per decade for DMD, of 1 % per decade for the digestibility of NDF and - 0.77 % per decade for NDF (Sampoux et al., 2011). In addition a genetic gain of 3.18 % per decade for annual yield was measured.

1.5. Plant modelling: individual based model, the example of L-grass

Since the quality of a harvested forage depends greatly on the organ composition and their ages, it would be useful to have a model simulating the morphogenesis of each organ with its shape, biomass and quality in order to know at each moment the biomass and quality produced by the plant integrating all organs. The L-grass model allows the simulation of the shape (thus indirectly biomass) of each organ of a grass during its vegetative stage.

L-grass is an aerial growth simulation model for perennial ryegrass developed by (Verdenal, 2009) at INRAE Lusignan. This model is based on the Lindenmayer systems (L-systems) formalism (Lindenmayer, 1968), using the L-Studio LPFG simulation platform and the L+C modelling language (Karwowski and Prusinkiewicz, 2003). A L-system program contains a description of the initial system using an arrangement of plant components called modules (leaf, stem, etc.). Each module contains a list of declared parameters according to its type. When the program is running, it will read the initial system and modify the values of the parameters of each module and their arrangement according to rules defined in its code, which will give a new system. After, it will read and modify this new system until it has reached the number of repetitions requested, recording the state of the system at each loop. The program also contains instructions for the geometric interpretation of the system, which makes it possible to create its graphic representation.

In L-grass, the plant is simulated by the connection of four different module types. Three of them have the purpose to represent a virtual phytomer: an internode supporting a leaf, which is associated to an axillary bud (Figure 10); and the fourth module is the shout apical meristem. A list of parameters as age, position or length is attributed to each individual module depending of its type. When a leaf length reaches the value of the previous leaf sheath length, the production rules modify the shoot apical meristem to a new virtual phytomer with the shout apical meristem over it (Figure 11) and the axillary bud connected to the previous leaf is modified to a shout apical meristem over a new virtual phytomer.

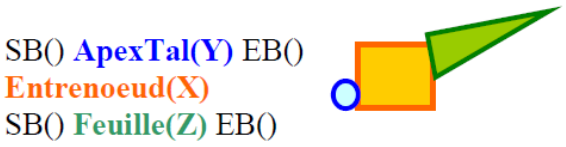

Figure 10: L-system code and its schematic representation of a virtual phytomer. “SB” means “Start Branching” and “EB” “End Branching” and are used as branching between modules. “ApexTal” is the axillary bud module, “Entrenœud” is the internode module and “Feuille” is the leaf module (from Verdenal, 2009).

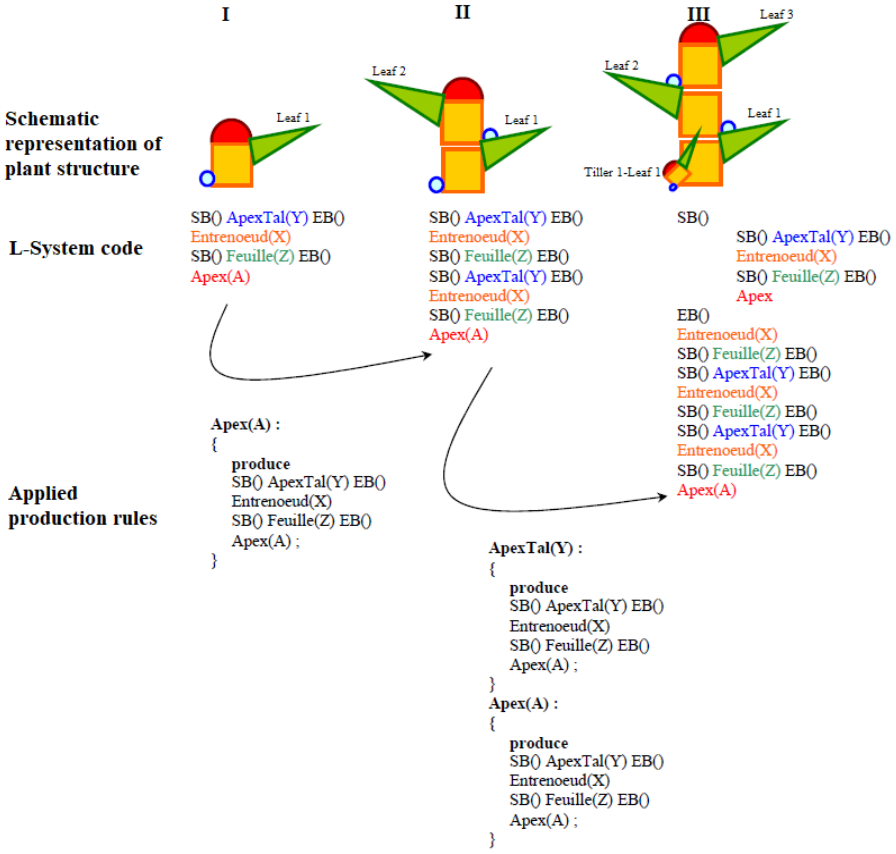

Figure 11: Schematic representation of ryegrass structure at three development stages (I, II, III) and the L-system code associated. Transition between stages is made by the rewriting of the L-system code following production rules (from Verdenal (2009)).

To control leaf growth, the model uses a sigmoid function inspired from function β integral (Yin et al., 2003): $Y = Ym\left(1 + \frac{te-t}{te-tm}\right)\left(\frac{t}{te}\right)^{\frac{t}{te-tm}}$ for $t < te$; $Y = Ym$ for $t \geq te$. With Y (mm) the leaf length at time t (°C.j), Ym (mm) the final leaf length, te (°C.j) the elongation duration and tm (°C.j) the time when growth speed reach its maximum (Figure 12).

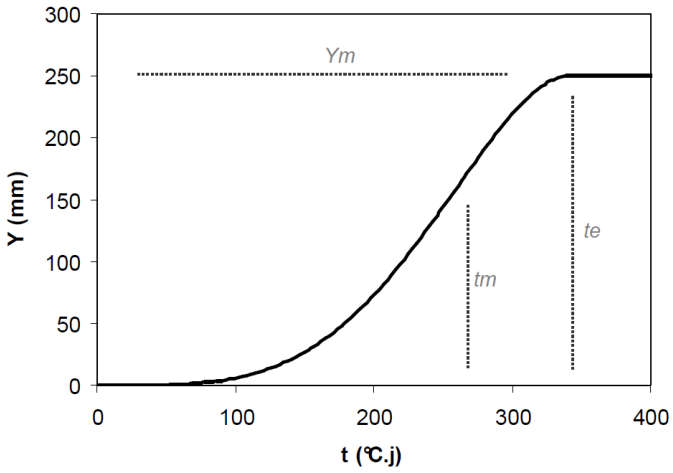

Figure 12: Graphical representation of β function used as growth function for leaves in L-grass model. The function is configured with *Ym* = 250mm, *te* = 340°C.j and *tm* = 260°C.j (from Verdenal, 2009).

In practice, the model uses two configurations per leaf for the β function: one for pre-emergence with a *Ym* set at 110mm and one post-emergence which used as *Ym* a value computed from a growth potential function depending of the time needed by the leaf to emerge (Figure 13).

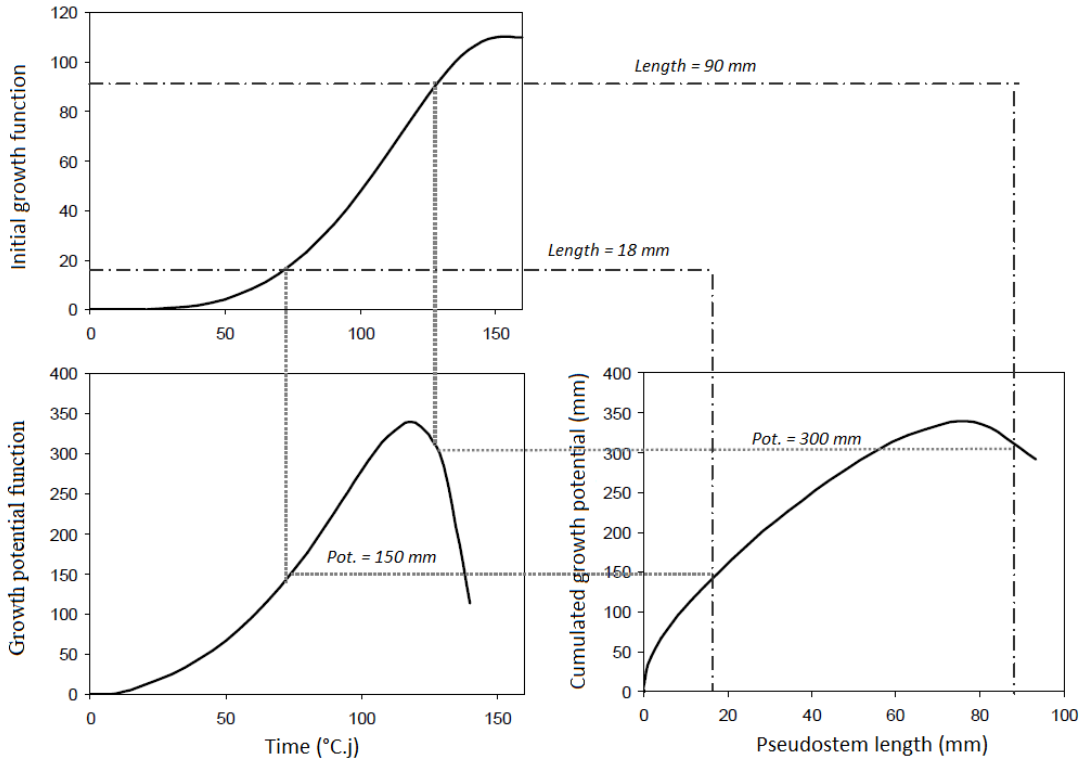

Figure 13: A) Initial growth function, B) Growth potential function and C) Cumulated growth potential thanks to B by a leaf growing following A during it hiding growth (from Verdenal (2009)).

Later, the model was improved with the addition of roots simulation by Migault (2015) and heading simulation by Rouet (2021).

1.6. Objectives of the thesis

Understand the biochemical origin of quality variation in a large panel of perennial ryegrass genotypes harvested in spring and in fall.

Identify the genetic bases for quality and its components in a large panel of perennial ryegrass genotypes in spring and in fall.

Understand the link between the aerial morphogenesis and the quality in spring and in fall by using four genotypes contrasting for their heading date and their quality.

Discuss the consequences of the findings for plant modelling and breeding.

2. Seasonal differences in structural and genetic control of digestibility in perennial ryegrass

This chapter has been published: Colas V., Barre P., van Parijs F., Wolters L., Quitté Y., Ruttink T., Roldán-Ruiz I., Escobar Gutiérrez A.J., Muylle H. (2022) Seasonal Differences in Structural and Genetic Control of Digestibility in Perennial Ryegrass. Frontiers in Plant Science 12 https://doi.org/10.3389/fpls.2021.801145. This chapter was also the subject of a presentation at EUCARPIA and an article in the Proceedings of the 34th Meeting of the EUCARPIA Fodder Crops and Amenity Grasses Section (Annexe 1).

Abstract

Perennial ryegrass (*Lolium perenne* L.) is an important forage crop in dairy farming, either for grazing or haying purposes. To further optimize the forage use, this study focused on understanding forage digestibility in the two most important cuts of perennial ryegrass, the spring cut at heading and the autumn cut. In a highly diverse collection of 592 Perennial ryegrass genotypes the organic matter digestibility (OMD) and underlying traits such as cell wall digestibility (NDFD) and cell wall components (cellulose, hemicellulose and lignin) were investigated during 2 years. A high genotype x season interaction was found for OMD and NDFD, indicating differences in genetic control of these forage quality traits in spring versus autumn. OMD could be explained by both the quantity of cell wall content (NDF) and the quality of the cell wall content (NDFD). The variability in NDFD in spring was mainly explained by differences in hemicellulose. A 1% increase of the hemicellulose content in the cell wall (HC.NDF) resulted in an increase of 0.81% of NDFD. In autumn, it was mainly explained by the lignin content in the cell wall (ADL.NDF). A 0.1% decrease of ADL.NDF resulted in an increase of 0.41% of NDFD. Within seasons, traits were highly heritable and showed a higher variation in autumn versus spring, indicating the potential to select for forage quality in the autumn cut. In a candidate gene association mapping approach, in which 503 genes involved in cell wall biogenesis, plant architecture and phytohormone biosynthesis and signalling, identified significant QTLs which could explain from 29% to 52% of the phenotypic variance in the forage quality traits OMD and NDFD, with small effects of each marker taken individually (ranging from 1 to 7%). No identical QTLs were identified between seasons, but within season, some QTLs were in common between digestibility traits and cell wall composition traits confirming the importance of hemicellulose concentration for spring digestibility and lignin concentration in NDF for autumn digestibility.

2.1. Introduction

Forage grasses are main sources of energy and protein for ruminants (Fick et al., 1994). The nutritive value of the forage has a significant impact on animal performance by influencing the intake rate, the volume of milk produced and its quality (Oba and Allen, 1999; Thomas et al., 1981). For example, Casler and Vogel (1999) concluded in a study comprising five forage grass species, that an increase of 1 % of dry matter digestibility (DMD, or the fraction of the dry matter (DM) that is digestible - closely linked to forage nutritive value as discussed below) leads to a 3.2% increase of daily gain of beef cattle. In addition, Oba and Allen (1999) observed in a study that a 1% increase of fibre digestibility was associated with a 0.25kg increase in 4% fat-corrected milk. Therefore, breeding for high nutritive value has become one of the main

objectives in forage grasses, in addition to forage yield and disease resistance (Sampoux et al., 2011; Wilkins and Humphreys, 2003). This is in particular the case for perennial ryegrass (*Lolium perenne* L.), which is one of the most used forage grass species in temperate regions thanks to its good tolerance to grazing and to its overall good nutritive quality (Semae, 2020; Wong, 2005).

The nutritive value of the forage is determined by the concentration of available energy (related to the concentration of digestible constituents) and the concentration of crude protein (Newman et al., 2006). The dry matter (DM) of grass forage consists for approximately 92.3% of an organic fraction (OM) and of a mineral fraction (7.7%), which is completely non-digestible (Figure 14). In the OM we can distinguish fibre constituents, which are partially digestible, and non-fibre constituents, which are almost completely digestible (Van Soest, 1967). Non-fibre constituents include water-soluble carbohydrates (WSC) and proteins. Fibres include hemicellulose, cellulose and lignin, in decreasing order of digestibility. Ideally, the available energy of forage should be measured in feeding experiments using animals, but this is time-consuming, costly and requires a large amount of forage (Khan et al., 2003), which are important limiting factors in breeding context. Therefore, *in vitro* methods have been established to quantify different constituents that influence the forage nutritive value. For example, a frequently used methodology to determine the properties of the fibre fraction is the 'Van Soest method' (Van Soest and Wine, 1967). This comprises determination of the neutral detergent fibre content (NDF), the acid detergent fibre content (ADF), the acid detergent lignin content (ADL) and the enzymatic solubility of the fibre fraction (Aufrère, 1982; De Boever et al., 1986; Terry et al., 1978; Van Soest and Wine, 1967). In the last decades, near infra-red spectrometry (NIRS) has been implemented to predict efficiently all parameters of forage nutritive value (Bastianelli, 2013; Corson et al., 1999; Jafari et al., 2003).

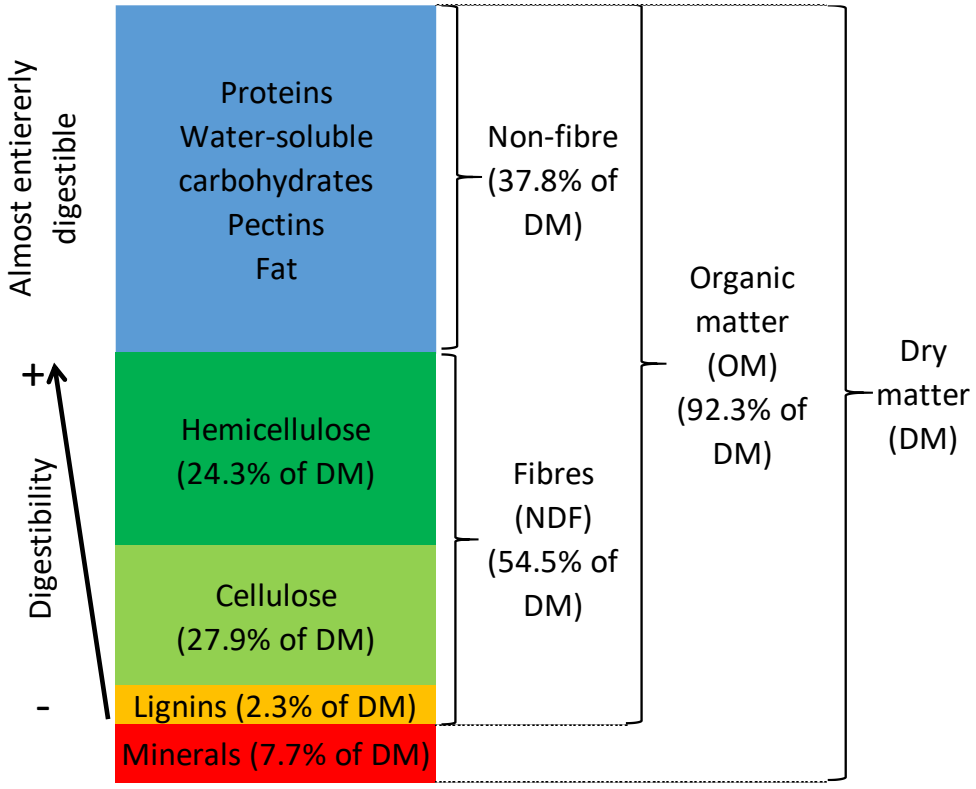

Figure 14: Average biochemical composition of forage grasses (data from Jančík et al. (2008)). Black arrow represents digestibility variation of fibre constituants.

An improvement of the forage nutritive value, can be achieved by increasing the proportion of non-fibre constituents and/or by increasing the digestibility of the fibre constituents (Buxton and Casler, 1993). Concerning the proportion of the different constituents, breeding efforts have resulted in an improvement of the forage nutritive value by decreasing the fibre content (Van Soest et al., 1991) or by increasing the WSC content (Humphreys, 1989). A higher amount of sugars in grass gives improvements to cattle as larger intakes, an increased milk production, less nitrogen excreted with urine and more protein in milk (Miller et al., 2001; Parsons et al., 2011). In addition, WSC in forage promote silage fermentation (McDonald and Henderson, 1964). However, a too high WSC content can lead to ruminal acidosis (González et al., 2012), and a too strong reduction of cell wall content can lead to a reduction of disease resistance for the plant (Smirnova and Kochetov, 2016). The protein content of grass forage is unequally partitioned between organs and is directly linked to nitrogen fertilisation and the environmental conditions (Durand et al., 2010; Lemaire et al., 1984). Therefore, protein content has not been subjected to a strong selection up to now (Sampoux et al., 2011).

The composition of the cell walls has also a strong impact on the forage nutritive value. As Keys et al. (1969) demonstrated, the proportion of cellulose to hemicellulose influences the cell wall digestibility (and as a consequence also the DMD) since hemicellulose is more digestible than cellulose in grasses. However, the major factor influencing the digestibility of the fibre fraction is its lignin content (Jung and Vogel, 1986; Woodman and Stewart, 1932). Lignin is not only difficult to digest, it also decreases cellulose and hemicellulose digestibility by decreasing their accessibility to enzymes through creation of ether bonds (Bonawitz and Chapple, 2010; Grabber, 2005; Grabber et al., 1997). Furthermore, the lignin structure has also a strong influence on the cell wall digestibility (Grabber, 2005). The relationship between cell wall composition and cell wall digestibility has been largely documented in maize (Barrière et al., 2003; Méchin et al., 2001), but has got less attention in perennial forage grass species (Vetharaniam et al., 2014).

Several studies have shown that in forage grasses DMD depends on several factors including species, genotype within species, plant organ/tissue, organ/tissue age, season, and environmental conditions (Akin et al., 1987; Beecher et al., 2018; Bruinenberg et al., 2002; Buxton and Casler, 1993; Carrère et al., 2010; Groot et al., 1999). A large variability exists between forage grass species with DMD ranging from less than 60% in tall fescue to more than 80% in perennial ryegrass (Buxton and Casler, 1993; Herbe-book, 2021; Pontes et al., 2007). Genetic variability within species is also available, and in perennial ryegrass low to moderate heritabilities have been reported depending on the studied germplasm (Arojju et al., 2020; Ullmann et al., 2017). This diversity is exploited in breeding (Wilkins, 1997), and a progress of 0.39 %DM per decade was observed for DMD during the period 1971-2004 in perennial ryegrass in Europe (Sampoux et al., 2011). This progress was realized with a 0.31 decrease of fibre content (% of DM) and a 0.55 increase of fibre digestibility (% of NDF). It is notable that this progress in forage quality was obtained simultaneously with a progress in dry matter yield and disease resistance. It is also important to consider that the DMD of different plant parts is different and that the age of the plant organ has a major impact on its DMD (Buxton and Casler, 1993). Stems are less digestible than leaves, and the reduction of the DMD with aging is more pronounced in stems than in leaves (Buxton and Casler, 1993; Terry and Tilley, 1964; Wilman and Altimimi, 1982). Therefore, DMD decreases strongly as the fraction of mature stems

increases. This could explain the strong negative correlation between precocity and spring DMD across perennial ryegrass cultivars (Sampoux et al., 2011). To avoid problems with quality while maintaining sufficient dry matter yield, grass forage should be harvested just before heading. Similarly, it can be anticipated that genetic progress to avoid aftermath heading could contribute to an increase of DMD for forage harvested in summer and autumn. Also seasonal differences have been reported in leaves, with a tendency for lower DMD under summer weather conditions (drought and high temperatures) (Pontes et al., 2007).

The genetic basis of DMD and more specifically the fibre content and its digestibility has been largely studied in species such as maize (Lopez-Malvar et al., 2021; Méchin et al., 2001; Riboulet et al., 2008), with some co-localization with candidate genes. In particular, genes involved in the biosynthesis of lignin have been associated to variation of DMD, and functional validation through transgenesis has been delivered for COMT (Catechol-O-methyltransferase) and CAD (Carbamoyl-Phosphate Synthetase 2, Aspartate Transcarbamylase, and Dihydroorotase) in maize and tall fescue (Chen et al., 2004, 2003; Hisano et al., 2009). In perennial ryegrass QTLs for DMD, fibre content and WSC have also been identified (Cogan et al., 2005). The 25 QTLs identified for WSC explained no more than 20% of the genetic variance suggesting a complex genetic determinism for this trait (Cogan et al., 2005; Gallagher et al., 2015; Shinozuka et al., 2012; Turner et al., 2006). To the best of our knowledge only few studies exist on the genetic control of fibre digestibility in perennial ryegrass.

The objective of this study was to obtain a better understanding of the variability of OMD in perennial ryegrass leaves harvested in spring and in autumn on a wide collection of 580 genotypes. The large number of genotypes analysed allowed us to obtain interesting variation for all studied traits. A particular emphasis was placed on the variability of fibre digestibility and its link with the composition of fibre for a better comprehension of their links with overall quality. We also investigated the genetic determinism of quality traits by a GWAS approach based on candidate genes. The results obtained could help to improve breeding programs by focusing on major indicators for quality in spring and/or autumn.

2.2. Material and Methods

2.2.1. Plant material

The plant material used in this study consisted of 580 genotypes from diverse origins chosen to represent a large genetic and phenotypic diversity (Veeckman et al., 2018). Among them, 42% were chosen among breeding material from ILVO, DSV and Barenbrug, 22% represented commercial cultivars and 36% originated from wild accessions (of which 76% of French origin).

2.2.2. Trial design

For each genotype, three clones were produced in trays by splitting tillers in September 2011 and 2012. The clones were moved to an unheated greenhouse (minimum temperature of 4°C) for vernalisation during the winter and then potted in 12L containers in early March 2012 and 2013. Each plant consisted of three tillers, which were cut to the same height (*ca*. 4 cm). The soil in the pots was a combination of white peat and garden peat (30% DM, 20% OM), fertilized with 0.3 Kg/m³ NPK 14-16-18. The pots were transferred to a container field in open-air in Melle, Belgium (latitude 50°59'39", longitude 3°47'5" and 24 m above sea level) and

arranged according to a randomized complete block design with three clonal replicates. The same set up was used for the two years. In 2012, the plants were fertilized with NPK (16-8-22) at the end of April, at the end of June, in the middle of July and in early August (at a rate of 80, 30, 70 and 100 Kg/ha respectively). In 2013, the plants were fertilized with NPK (16-8-22) at the begin of May, in the middle of June, in the middle of July and in the middle of August (at a rate of 100, 30, 70 and 70 Kg/ha respectively). The pots were watered using drip irrigation once to twice a day, depending on irradiance and air temperature.

2.2.3. Phenotyping

The plants were cut four times per year: once during the spring, twice during the summer and once during the autumn. Only plant materials collected at the spring and autumn cuts were analysed. The sampling in spring and autumn differed slightly.

Each year, the spring cut was harvested at the actual day of heading of each individual plant (*i.e.* when three ears were visible). This involved inspection of each individual plant every two or three days from the beginning of April to early July 2012 and from the beginning of May to the end of July 2013, to record the heading date of each individual plant. Plants were cut at 4 cm height in the time slot between 2 and 3 pm. The biomass was dried in a ventilated oven during 48 h at 70°C, hand separated in blade and "stem" (*i.e.* sheath and true stem together) fractions, and milled in a Fritsch cutting mill using a 0.5-mm sieve. In this study, we focused only on the blade samples which were harvested in both spring and autumn to allow comparison between seasons. Plants that were harvested with too many spikes (weight proportion of spikes larger than 5%) and plants with extreme heading dates were not considered for further analysis. We considered the extreme 0.6 % (*i.e.* 10 genotypes) of the distribution as "extreme heading dates", which corresponded to >17 days before and >28 days after the median heading date in both years.

The summer cuts were performed on 9 July 2012, 6 August 2012, 8 July 2013 and on 12 August 2013 to promote a good regrowth in September. On 7, 18 and 19 of September 2012 and on 17, 19 and 20 of September 2013, the autumn cuts were performed. Cuts were spread over three days because of the short time window to harvest (2 to 3pm). All plants were cut at 4 cm height in the time slot between 2 and 3 pm. The biomass was dried in a ventilated oven during 48 h at 70°C and milled in a Fritsch cutting mill using a 0.5-mm sieve. In this case blades and sheaths were not separated because of the absence of stems.

2.2.3.1. Wet chemical analysis and NIRS prediction

All samples from spring and autumn cuts were scanned by near-infrared spectroscopy (NIRS, FOSS XDS). Wet-chemical analyses were performed on four NIRS calibration sets (one for each season and year) for seven traits: Absolute Dry Matter (ADM) expressed in percentage of dry matter (DM), Organic Matter (OM) expressed as percentage of ADM, Organic Matter Digestibility (OMD), Neutral Detergent Fibre (NDF), Acid Detergent Lignin (ADL), Cellulose (C) and Hemicellulose (HC) expressed in percentage of OM. Because of lacking data, some genotypes (20) were removed and only 550 were kept for further phenotypic analysis.

ADM was determined gravimetrically by drying 2.5 g of sample at 103°C. This sub-sample was then ashed at 550°C, allowing calculation of OM as the proportion of ash-free ADM in ADM. OMD was determined following Goering & van Soest (1970). 0.5 g of sample was submerged in buffered ruminal fluid from three sheep and incubated for 48 h at 39°C. Then,

neutral detergent was added and the suspension was boiled for 30 minutes. After filtration in a P2 crucible (40-100 µm pore size), the residue was washed, dried at 103°C, weighed, ashed at 550°C and weighed again. OMD was calculated as $1 - \frac{residue - ashes}{0.5 \times OM}$.

The sequential van Soest method (Van Soest et al., 1991) was modified for use with the ANKOM Fibre Analyzer (ANKOM Technology Corporation, Fairpoint, NY) to measure NDF, ADF and ADL. To determine NDF, 0.5 g of sample was brought in a filter bag, and incubated in neutral detergent at 100°C for 1 hour in the ANKOM. The residue was then washed with water at 100°C and acetone, and dried at 103°C. NDF was calculated as the ash-free NDF residue (using the ash determined on the ADL fraction, see further), divided by the OM previously determined. ADF was determined by incubating the bags containing the NDF fraction in 5% H_2SO_4 and CTAB (20g/L) for 1 hour at 100°C in the ANKOM. The residue was then washed with water at 100°C and acetone, dried at 103°C, and weighed. ADF was calculated as the proportion of ash-free ADF residue divided by the OM previously determined. ADL was determined by submerging the bags containing the ADF fraction in a beaker with 72% H_2SO_4 for 3 hours. Ash content was determined by heating the ADL residue to 550°C in a muffle furnace. ADL was calculated as the proportion of ash-free ADL residue divided by the OM previously determined. Hemicellulose (HC) was calculated as NDF - ADF and cellulose (C) as ADF - ADL in %OM.

NIRS closed calibrations were built (one for each year and season) with wet-chemistry data obtained, including 72 + 68 blade samples in spring and 72 + 60 plant samples in autumn of the two years (2012 and 2013). In order to assess the extent to which a trait could be predicted for samples not included in the calibration model, a leave-one-out cross-validation was performed. The resulting NIRS predictabilities (Table 1) are variable among traits, years and seasons.

Table 1: NIRS statistics: number of samples used in the closed NIRS calibration models, R2 of the calibration model (r2 between fitted and observed values), R2 of the leave-one-out cross-validation (r2 between predicted and observed values, with prediction for each sample based on a model with all other samples). Samples in spring are composed of blades only and samples in autumn are composed of the blades and sheaths.

	Number of samples				**R^2 of calibration (%)**				**R^2 of cross-valid. (%)**			
	Spring		**Autumn**		**Spring**		**Autumn**		**Spring**		**Autumn**	
Variable	**2012**	**2013**	**2012**	**2013**	**2012**	**2013**	**2012**	**2013**	**2012**	**2013**	**2012**	**2013**
ADM (%DM)	72	69	72	59	98	81	96	97	97	76	94	96
OM (%ADM)	72	69	72	60	94	96	91	75	83	92	78	59
OMD (%OM)	72	68	72	60	89	93	88	92	82	89	81	84
NDF (%OM)	71	69	72	60	97	98	96	94	94	97	93	89
ADL (%OM)	72	67	72	59	77	87	89	70	63	70	78	65
C (%OM)	72	67	72	60	97	98	97	95	94	96	93	88
HC (%OM)	72	68	72	60	87	96	86	88	76	92	82	80

2.2.3.2. Derived biochemical data

Several traits were computed in order to study cell wall digestibility and its composition. Cell wall components (C, HC and ADL) were expressed in percentage of NDF: C.NDF, HC.NDF and ADL.NDF. Digestibility of NDF was computed using a formula derived from Struik (1983):

$$NDFD = 100 - 100 \times \frac{100-OMD}{NDF}$$ (Equation 1)

This formula is based on the assumption that all indigestible parts of the plant are contained in cell walls.

2.2.4. Statistical analysis of the phenotypic data

2.2.4.1. General models and heritability

The effect of genotype, year, season and block were analysed using a mixed model with R package *lme4* (Bates et al., 2015) as follows:

$$Y_{ijkl} = \mu + G_i + T_j + S_k + TS_{jk} + GT_{ij} + GS_{ik} + B_l(TS_{jk}) + e_{ijkl}$$ (Equation 2)

Where Y_{ijkl} = the phenotype of genotype i (1,…,550), in year j (2012, 2013), season k (spring, autumn) and block l (1, 2, 3),

μ = overall mean,

G_i = random effect of genotype i, $G_i \sim N(0,\sigma^2_g)$

T_j = fixed effect of year j,

S_k = fixed effect of season k,

TS_{jk} = interaction of year j and season k,

GT_{ij} = interaction of genotype i and year j, $GT_{ij} \sim N(0,\sigma^2_{gt})$

GS_{ik} = interaction of genotype i and season k, $GS_{ik} \sim N(0,\sigma^2_{gs})$

$B_l(TS_{jk})$ =fixed effect of block l nested within year j and season k,

and e_{ijkl} = residual, assumed to be normally distributed, $e_{ijkl} \sim N(0,\sigma^2_e)$.

The data set was further analysed by season using the following model for spring and for autumn separately:

$$Y_{ijl} = \mu + G_i + T_j + GT_{ij} + B_l(T_j) + e_{ijl}$$ (Equation 3)

Where Y_{ijl} = the phenotype of genotype i (1,…,550), in year j (2012,2013) and bloc l (1,2,3) ,

μ = overall mean,

G_i = random effect of genotype i, $G_i \sim N(0,\sigma^2_g)$,

T_j =fixed effect of year j,

GT_{ij} = interaction of genotype i and year j, $GT_{ij} \sim N(0,\sigma^2_{gt})$,

$B_l(T_j)$ = fixed effect of block l nested within year j,

and e_{ijl} = residual, assumed to be normally distributed, $e_{ijl} \sim N(0,\sigma^2_e)$.

For each season, the broad-sense heritability (H^2) for each trait was calculated as:

$$\frac{\sigma^2_G}{\sigma^2_G+\sigma^2_{GT}+\sigma^2_e}$$ (Equation 4).

For GWAS, Equation 3 was used to calculate the Best Linear Unbiased Predictor (BLUP) of the genotype effect ($\mu + G_i$) per season over two years. Another model per season and per year was also used:

$$Y_{il} = \mu + G_i + B_l + e_{il}$$ (Equation 5)

Where Y_{il} = the phenotype of genotype i (1,…,550) and bloc l (1,2,3),

μ = overall mean,

G_i = random effect of genotype i, $G_i \sim N(0,\sigma^2_g)$,

B_l = fixed effect of block l,

and e_{il} = residual, assumed to be normally distributed, $e_{il} \sim N(0,\sigma^2_e)$.

This model (Equation 5) was used to calculate the Best Linear Unbiased Predictor (BLUP) of genotype effect ($\mu + G_i$) per season and per year.

2.2.4.2. Drivers of OMD and NDFD

Linear models were built using the *lm* function in R (R Core Team, 2020). Two models were used: one with OMD as dependent variable and NDF and NDFD as independent variables, and one with NDFD as dependent variable and HC.NDF and ADL.NDF as independent variables. We did not use C.NDF in the model as C.NDF + HC.NDF + ADL.NDF = 1 and because C.NDF is highly negatively correlated to HC.NDF (-0.96 for all years and seasons). Models were built for each season and each year separately and were compared.

2.2.5. Genetic markers

The genetic markers reported in Veeckman et al. (2018) were used. These markers describe the sequence diversity in 503 candidate genes involved in plant development, plant architecture, phytohormone biosynthesis and response pathways and cell wall composition. This dataset consists of 252 406 SNPs and 5 074 indels identified in an association panel of 736 genotypes, including the 580 genotypes reported in this study (Veeckman et al., 2018). The reported markers are spread over 455 different scaffolds belonging to the 7 chromosomes of the species (Veeckman, not published).

Genotypic data were recoded numerically to denote allelic doses (e.g. 0, 1, 2 for AA, AT, TT (A/T SNP)). Imputation of missing genotype calls was performed independently for each group found by population structure analysis (see below). For each marker, a missing genotype call was replaced by the value of the most common allelic dose of its group. Minimal allele frequency (MAF) was set to 1% and markers with MAF below 1% were removed.

2.2.6. Correction for heading date in spring

Harvest date in spring was set at the heading date of each individual genotype, as this is also an agronomic important time point at which farmers tend to harvest perennial ryegrass. However, this individual harvest date had an impact on OMD, NDFD and NDF (Table 2). In particular, OMD and NDFD decreased at later heading dates (Figure 15). In order to detect QTL not related to heading date in GWAS, we corrected each trait for heading date (HD). After optimisation, HD-corrected BLUP values were calculated with a harvest date correction using two models:

per season and per year:

- $Y_{il} = \mu + G_i + B_l + HD_{il}^{-2} + HD_{il}^{-1} + HD_{il} + e_{il}$ (Equation 8)

per season over the two years:

- $Y_{ijl} = \mu + G_i + T_j + GT_{ij} + B_l(T_j) + HD_{ijl}^{-2} + HD_{ijl}^{-1} + HD_{ijl} + e_{ijl}$ (Equation 9),

Where HD_{il} = heading date of genotype i in block l per year

HD_{ijl} = heading date of genotype i in block l in year j

Table 2: Correlations coefficient (rho) between quality traits and heading date (HD.GDD0) in spring 2012 and 2013 using Spearman method and their significance (pval).

Trait	2012		2013	
	rho	pval	rho	pval
OMD (%OM)	**-0.73**	***	**-0.61**	***
NDF (%OM)	0.39	***	-0.05	ns
NDFD (%NDF)	**-0.80**	***	**-0.78**	***
HC (%OM)	0.19	***	-0.26	***
HC.NDF (%NDF)	**-0.49**	***	**-0.66**	***
C (%OM)	**0.49**	***	0.41	***
C.NDF (%NDF)	**0.51**	***	**0.68**	***
ADL (%OM)	0.06	*	-0.29	***
ADL.NDF (%NDF)	-0.32	***	-0.39	***

ns p > .05, * p < .05, ** p < .01, *** p < .001

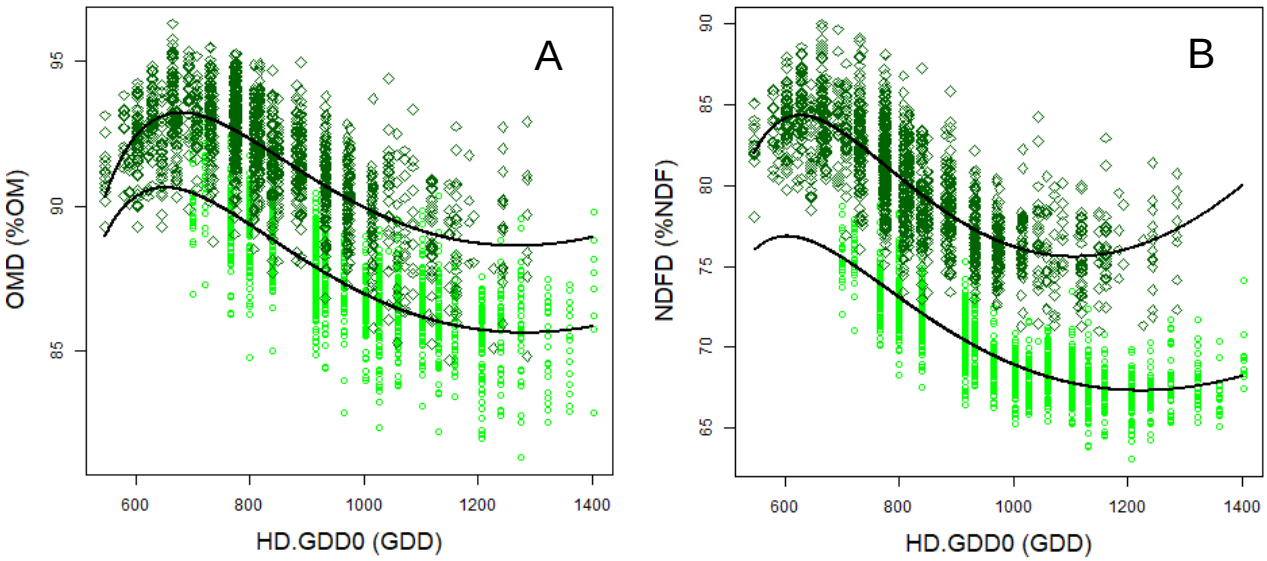

Figure 15: Organic matter digestibility (OMD) (A) and fibre digestibility (NDFD) (B) depending on heading date (HD.GDD0) in 2012 (light green circles) and in 2013 (dark green diamonds). Black curves represent Equation 8.

2.2.7. Population structure

To determine population structure, we used fastSTRUCTURE (Raj et al., 2014) with default parameters for one to ten groups over all 580 genotypes. Three runs were performed in order to obtain a good prediction for the choice of the number of populations to retain.

Four genetic groups were identified, based on 252 406 SNPs and 5 074 indels, using marginal likelihood (Figure 16). The first group, called “Temperate”, integrates all wild genotypes, except the ones from Spain and two from Italy, which belong to the second group named “Warm”. In addition, the “Temperate” group contains 16 genotypes from Barenbrug, 93 from DSV, 68 from ILVO, one from IBERS, nine from DLF and two from Mommersteeg. The group “Warm” contains 33 genotypes from Barenbrug and one from DSV. The third group contains all genotypes derived from Aberavon, Aberdert and Aberzest and is called “Aber”. It contains one genotype from Barenbrug, 27 from DSV, 41 from ILVO and 32 from IBERS. Most of the breeding genotypes and genotypes from commercial forage varieties are divided among these three groups. A fourth group, called “NZ”, contains 51 Barenbrug breeding genotypes from New Zealand.

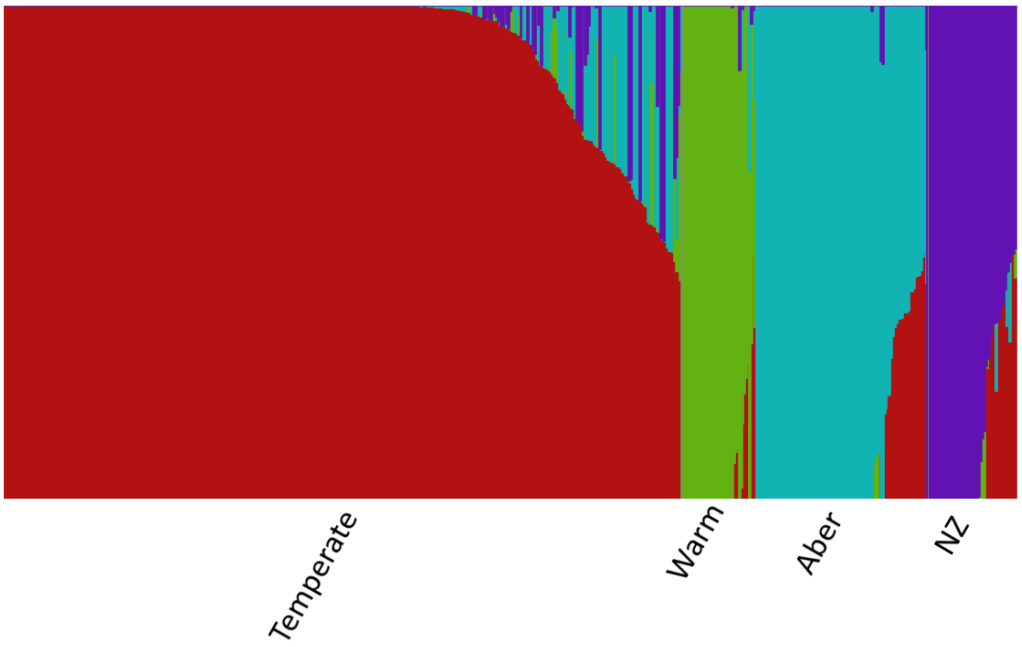

Figure 16: Graphical representation of FastStructure results. Each color represents an identified group

Principal component analysis (PCA) was performed using Plink software (Chang et al., 2015) with genotypic data. The principal component 1 (PC1) (Figure 17) indicated a divergence between Aber, Temperate and the two last groups (Warm and NZ). PC2 showed divergence between four wild genotypes (two from Italy and two from Spain). PC3 showed a divergence between the Temperate group and the others.

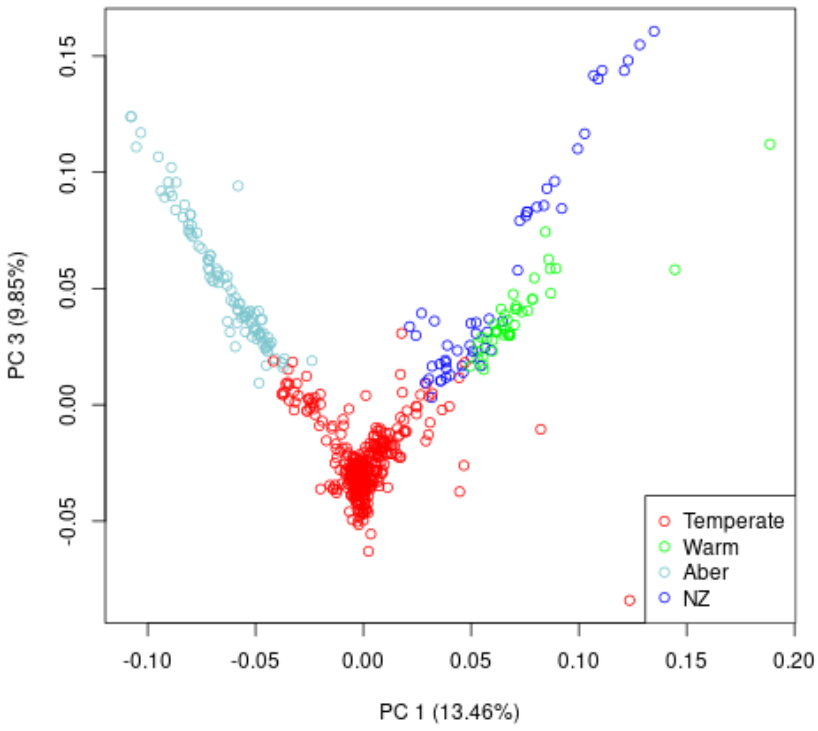

Figure 17: Principal component analysis (PCA) of 580 perennial ryegrass genotypes based on 252 406 SNPs and 5 074 indels. Groups from population structure are indicated by different colours

2.2.8. MLMM method

Multi-Locus Mixed-Modelling (MLMM) was used for GWAS analysis (Segura et al., 2012). We used an Identity-by-State (IBS) matrix as kinship, computed using the formula from Rincent *et al.* (2012):

$$IBS = \frac{GG' + G_2G_2'}{K}$$

where G is the matrix of genotypes (with individuals in rows and markers in columns) coded as 0, 0.5, and 1 for the homozygote, the heterozygote, and the alternative homozygote state, respectively; K is the total number of markers, and $G_2 = 1 - G$, where 1 is a matrix of ones.

MLMM was used for each trait with: a) the matrix of genotype calls coded as 0, 1 and 2; b) the BLUPs values; c) the population structure; and d) the kinship matrix. The MLMM R function (Segura et al., 2012) was used. The best MLMM model with the lowest BIC value was retained for each trait. In addition to the QTLs identified in the best model, other QTLs were identified by using a Bonferroni threshold computed with the number of genes (503) regarding that markers on a same gene had high linkage disequilibrium and the Bonferroni threshold is normally computed with the number of independent markers. The threshold for a global p-value of 5% was $0.05/503 = 9.94e10^{-5}$.

To evaluate the percentage of variance explained by the markers, first the most significant marker per gene was identified and then, the log-likelihood of the global model with all identified QTLs and the the log-likelihood of the model without the studied marker were computed with lmekin from the coxme R package using maximum likelihood (ML) (Therneau, 2020). Next, an estimation of the semipartial R^2 of each studied marker was computed by comparison of these models with the r.squaredLR function from the MuMIn R package (Bartoń, 2020).

For each trait, the effect of each QTL was estimated by the coefficient (slope) of the marker effect in the model.

To evaluate the effect of cell wall components QTLs on OMD and NDFD, two models were applied. For the spring cut, we evaluated the variance explained for OMD and NDFD by the QTLs identified for HC.NDF. In autumn, the same method was applied, but using QTLs identified for ADL.NDF.

2.3. Results

2.3.1. Impact of season on OMD and NDFD

The distribution of all traits per season and per year are summarised in Table 3. High variability among the 550 genotypes was observed for heading date, which was in this experiment the individual harvesting date per plant in spring.

Table 3: Descriptive data for organic matter digestibility (OMD), fibre content (NDF), fibre digestibility (NDFD), hemicellulose (HC), hemicellulose over NDF (HC.NDF), cellulose (C), cellulose over NDF (C.NDF), lignin (ADL), lignin over NDF (ADL.NDF), and heading date (HD) in growing degree days (GDD) from potting date by year and by season.

Trait	Spring 2012					Spring 2013				
	mean	min	max	var	sd	mean	min	max	var	sd
OMD (%OM)	87.42	81.34	92.9	3.65	1.91	91.67	84.69	96.3	3.66	1.91
NDF (%OM)	41.62	29.92	56.21	16.04	4.01	41.6	29.94	55.92	22.86	4.78
NDFD (%NDF)	69.86	63.1	78.84	7.77	2.79	80	71	89.94	13.41	3.66
HC (%OM)	23.27	18.06	30.76	3.81	1.95	23.98	16.76	37.79	11.17	3.34
HC.NDF (%NDF)	55.37	50.15	64.57	6.38	2.53	57.3	50.17	67.48	9.89	3.14
C (%OM)	17.35	11.19	25.54	5.39	2.32	16.23	10.99	24.75	4.67	2.16
C.NDF (%NDF)	42.08	32.48	47.45	6.64	2.58	39.27	27.34	46.85	12.66	3.56
ADL (%OM)	1.07	0.68	1.68	0.02	0.15	1.45	0.5	3.14	0.21	0.46
ADL.NDF (%NDF)	2.56	1.99	3.29	0.05	0.21	3.43	1.41	6.47	0.62	0.79
HD (GDD)	993	700	1402	26482	163	833	546	1286	23734	154
Trait	Autumn 2012					Autumn 2013				
	mean	min	max	var	sd	mean	min	max	var	sd
OMD (%OM)	78.12	66.46	87.04	12.06	3.47	84.49	67.98	93.53	12.57	3.55
NDF (%OM)	61.9	44.13	75.26	26.92	5.19	55.44	33.86	74.76	27.84	5.28
NDFD (%NDF)	64.83	53.07	73.5	10.28	3.21	72.25	53.04	83.27	18.38	4.29
HC (%OM)	33.63	25.38	41.2	9.02	3	29.95	18.99	39.16	9.55	3.09
HC.NDF (%NDF)	54.75	48.11	62.23	4.59	2.14	53.93	45.01	63.36	7.18	2.68
C (%OM)	25.69	18.37	33.89	5.79	2.41	23.74	15.85	32.65	6.6	2.57
C.NDF (%NDF)	41.52	32.24	49.18	6.8	2.61	42.65	32.41	51.78	8.41	2.9
ADL (%OM)	2.33	1.04	4.44	0.37	0.61	1.92	0.72	3.81	0.26	0.51
ADL.NDF (%NDF)	3.73	1.88	6.28	0.57	0.76	3.42	1.72	5.73	0.46	0.68

The heritability of OMD and NDFD, was very low or zero when both seasons where analysed together (Table 4, 0.13 and <0.01). Indeed a strong genotype x season interaction (G:S) was observed. In particular, for OMD the G:S interaction was more than seven times higher than the genotypic variance. This strong interaction led us to analyse the data per season. The heritability of OMD and NDFD increased when analysed separately per season (Table 4), suggesting a different genetic control for these traits per season and the potential to breed separately for higher spring and/or autumn digestibility (OMD and NDFD).

Table 4: Heritabilities and variance components calculated from the mixed model Equation 2 over seasons and Equation 3 by season for organic matter digestibility (OMD), fibre content (NDF), fibre digestibility (NDFD), hemicellulose (HC), hemicellulose over NDF (HC.NDF), cellulose (C), cellulose over NDF (C.NDF), lignins (ADL), lignins over NDF (ADL.NDF), and heading date (HD) in growing degree days (GDD) from potting date. Are indicated: genotype effect (G), genotype x year interaction (G:Y), genotype x season interaction (G:S).

Trait	**Spring and autumn** Variance G	G:Y	G:S	Residuals	H2
OMD (%OM)	0.42	1.15	3.05	3.6	0.05
NDF (%OM)	4.39	3.89	6.24	8.43	0.19
NDFD (%NDF)	<0.01	1.9	5.47	5.56	<0.01
HC (%OM)	1.13	1.57	2.54	3.34	0.13
HC.NDF (%NDF)	1.6	0.65	2.73	2.2	0.22
C (%OM)	1.6	0.57	1.63	1.64	0.29
C.NDF (%NDF)	2.06	0.86	3.4	2.59	0.23
ADL (%OM)	<0.01	0.04	0.09	0.09	<0.01
ADL.NDF (%NDF)	<0.01	0.07	0.18	0.19	<0.01

Trait	**Spring** Variance G	G:Y	Residuals	H2	**Autumn** Variance G	G:Y	Residuals	H2
OMD (%OM)	2.44	0.55	0.65	0.67	3.85	3.27	5.07	0.32
NDF (%OM)	7.99	6.99	4.93	0.4	11.36	5.53	8.92	0.44
NDFD (%NDF)	7.05	1.67	2.09	0.65	2.72	4.76	6.89	0.19
HC (%OM)	2.18	3.8	2.16	0.27	3.82	2.21	3.02	0.42
HC.NDF (%NDF)	5.51	1.51	1.6	0.64	2.86	0.84	2.19	0.49
C (%OM)	3.25	0.73	0.96	0.66	3.09	0.91	1.9	0.52
C.NDF (%NDF)	6.33	2.15	1.79	0.62	4.1	1	2.57	0.53
ADL (%OM)	0.02	0.06	0.04	0.17	0.14	0.07	0.1	0.45
ADL.NDF (%NDF)	0.06	0.14	0.14	0.18	0.23	0.12	0.17	0.44
HD (GDD)	21812	3319	1332	0.82				

The values of the digestibility traits OMD and NDFD were significantly different between the spring and autumn seasons. OMD was higher in spring than in autumn (8 % OM difference), but the range of OMD was broader in autumn than in spring (11.6 and 23.1 % OM, respectively). Similarly, NDFD was higher in spring than in autumn (6 % NDF difference), but the range of NDFD was broader in autumn. Nevertheless, weak positive and significant correlations between seasons for OMD and NDFD were observed (Figure 18, Table 5).

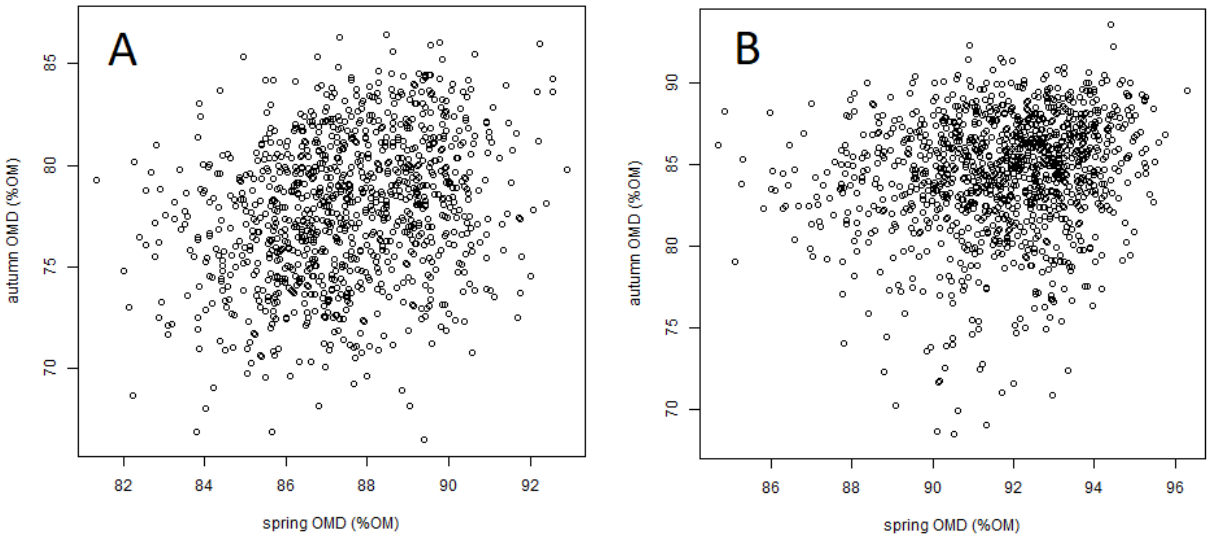

Figure 18: Organic matter digestibility (OMD) of blades in spring against OMD of leaves in autumn in 2012 (A) and 2013 (B)

Table 5: Correlations coefficient (rho) between seasons for each trait (organic matter digestibility (OMD), fibre content (NDF), fibre digestibility (NDFD), hemicellulose (HC), hemicellulose over NDF (HC.NDF), cellulose (C), cellulose over NDF (C.NDF), lignins (ADL) and lignins over NDF (ADL.NDF)), using Spearman method and their significance (pval).

Trait	2012		2013	
	rho	pval	rho	pval
OMD	0.27	***	0.17	***
NDF	0.38	***	0.31	***
NDFD	0.13	***	0.12	***
HC	0.29	***	0.20	***
HC.NDF	0.29	***	0.26	***
C	0.42	***	0.44	***
C.NDF	0.26	***	0.26	***
ADL	0.09	**	0.11	***
ADL.NDF	-0.03	ns	0.05	ns

^{ns} p > .05, * p < .05, ** p < .01, *** p < .001

2.3.2. Effect of cell wall content and cell wall digestibility on spring and autumn digestibility

We observed a significant and strongly positive correlation between OMD and NDFD for each season and year (0.79 in spring 2012, 0.87 in spring 2013, 0.92 in autumn 2012 and 0.92 in autumn 2013 ($p < 0.05$)) (Figure 19A). A significant and negative correlation was observed between OMD and NDF (-0.81 in spring 2012, -0.53 in spring 2013, -0.90 in autumn 2012 and -0.81 in autumn 2013 ($p < 0.05$)) (Figure 19B). OMD could be explained by both the cell wall content (NDF) and the quality of the cell wall (NDFD) (Table 6). NDFD showed a higher importance than NDF to explain OMD, except in spring 2012. Our data suggest that an increase of 1% OMD can be obtained by focusing on either a decrease of NDF or an increase of NDFD.

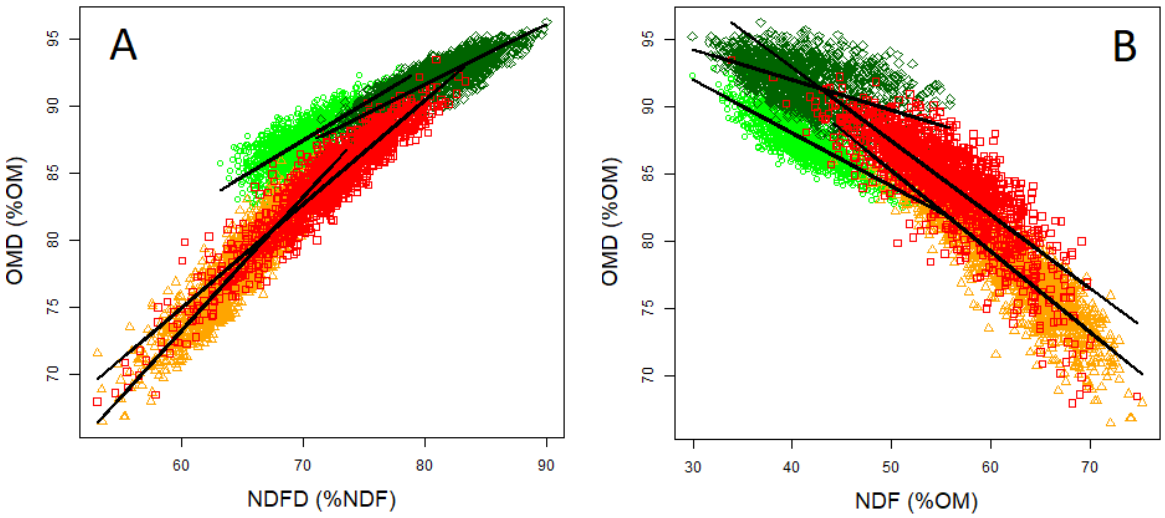

Figure 19: Relationship between OMD and A) NDFD and B) NDF. Light green circles indicate spring 2012 data, dark green diamonds indicate spring 2013 data, orange triangles indicate autumn 2012 data and red squares indicate autumn 2013 data.

Table 6: Influence of fibre content (NDF) and fibre digestibility (NDFD) on organic matter digestibility (OMD) in %OM for each season by year (equation 6): significance (pval) and semipartial R2 (sr2) for independent variables. The coefficient of determination of the model (R2) is given.

	spring 2012		spring 2013		autumn 2012		autumn 2013	
	pval	sr2	pval	sr2	pval	sr2	pval	sr2
NDF (%OM)	***	0.37	***	0.24	***	0.15	***	0.12
NDFD (%NDF)	***	0.31	***	0.67	***	0.19	***	0.33
R^2	>0.99		>0.99		>0.99		>0.99	

^{ns} p > .05, * p < .05, ** p < .01, *** p < .001

Negative correlations between NDFD and NDF were observed for each season, but the coefficients were stronger in autumn (-0.67 and -0.53 in 2012 and 2013, respectively) (Figure 20A) than in spring (-0.31 and -0.09 in 2012 and 2013, respectively) (Figure 20B). Despite this correlation, genotypes with similar NDF content were displayed in a wide range of NDFD values. Given the heritability of NDF and NDFD, breeding towards higher NDFD while maintaining the NDF content should be feasible in spring and holds promise for improving OMD in perennial ryegrass.

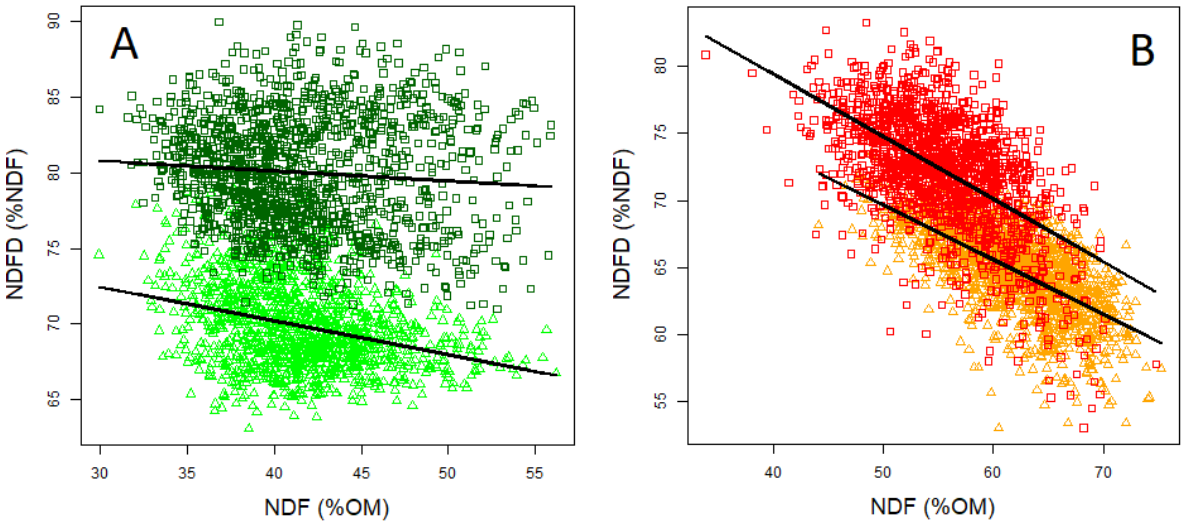

Figure 20: Relationship between NDF and NDFD in spring (A) and autumn (B). Light green circles indicate spring 2012 data, dark green diamonds indicate spring 2013, orange triangles indicate autumn 2012 and red squares indicate autumn 2013 data.

2.3.3. The effect of cell wall composition on NDFD

In order to understand better which aspects determine the variation in NDFD in spring and autumn, we analysed the cell wall components. In spring, a large part of NDFD variability could be explained by HC.NDF itself (or C.NDF) (Table 7). A 1% increase of HC.NDF resulted in an increase of 0.81% of NDFD. In autumn, NDFD was mainly explained by ADL.NDF. A 0.1% decrease of ADL.NDF resulted in an increase of 0.41% of NDFD. The studied population holds variation for HC.NDF in spring and ADL.NDF in autumn (Table 3). The heritabilities for these traits are high for HC.NDF (0.64 in spring), and moderate for ADL.NDF (0.44 in autumn).

Table 7: Influence of hemicellulose and lignin in fibres (HC.NDF and ADL.NDF) on NDFD in %NDF for each season by year (equation 7): significance (pval) and semipartial R2 (sr2) for independent variables. The coefficient of determination of the model (R2) is given.

	spring 2012		spring 2013		autumn 2012		autumn 2013	
	pval	sr2	pval	sr2	pval	sr2	pval	sr2
HC.NDF (%NDF)	***	0.39	***	0.46	ns	<0.01	***	0.04
ADL.NDF (%NDF)	***	<0.01	ns	<0.01	***	0.53	***	0.48
R^2	0.44		0.59		0.69		0.49	

^{ns} p > .05, * p < .05, ** p < .01, *** p < .001.

2.3.4. GWAS

In spring, in order to identify QTLs different from the ones for heading date, and due to the correlation between traits and heading date, GWAS was performed on data corrected and uncorrected for heading date (equations 8 and 9). QTLs found could explain between 29% and 52% of the phenotypic variance (Table 8).

Table 8: Number of QTLs and genes identified by MLMM analysis and phenotypic variance explained by all QTLs identified for each trait and season.

	Spring			Autumn		
	QTLs	Genes	sr2	QTLs	Genes	sr2
OMD (%OM)	67	28	47%	57	21	40%
NDF (%OM)	109	23	52%	83	20	43%
NDFD (%NDF)	36	16	29%	52	19	35%
HC.NDF (%NDF)	60	18	39%	67	17	41%
C.NDF (%NDF)	54	16	38%	59	16	38%
ADL.NDF (%NDF)	96	27	49%	58	23	46%

Comparing all identified genes, we noticed that two genes (NAC-13, ABI3-02) were in common between HC.NDF, NDFD and OMD in spring, and six genes (RGA-03, ABI1-01, ARF-03, OBE1-02, KAN-06, BIM2-01) were in common between ADL.NDF, NDFD and OMD in autumn. In spring, 8.3% of OMD variance was explained by the three QTLs identified for both OMD and HC.NDF (NAC-13, SPL3-02, ABI3-02). In autumn, 17.9% of OMD variance was explained by the seven QTLs identified for both OMD and ADL.NDF (RGA-03, ABI1-01, SPA-02, ARF-03, OBE1-02, MBD9-01, KAN-06, BIM2-01). In spring, 6.3% of NDFD variance was explained by the three QTLs identified for both NDFD and HC.NDF (LUG-02, NAC-13, ABI3-02). In autumn, 15.8% of NDFD variance was explained by the seven QTLs identified for both NDFD and ADL.NDF (RGA-03, ABI1-01, SPA-02, ARF-03, OBE1-02, KAN-06, BIM2-01).

2.4. Discussion

2.4.1. Seasonal differences in OMD and NDFD among genotypes

We observed strong interactions between genotype and season for both OMD and NDFD, which leads to inversions of ranking between genotypes when autumn data versus spring data are considered. This observation indicates that selection should be realised in both spring and autumn simultaneously to improve the overall, annual digestibility. Interestingly, some genotypes showed high values of OMD and NDFD in both seasons, and are of particular relevance for breeding purposes.

The OMD was higher in spring than in autumn. This cannot be explained by the fact that only blades were analysed in spring compared to blades and sheaths combined in autumn, since OMD of blades and "stems" (sheaths and flowering stems) was also higher in spring (van Parijs, 2016). This difference between seasons could be due to differences in environmental conditions (Buxton and Casler, 1993). Indeed, the autumn harvest is the result of the growth during summer (August and beginning of September), when temperatures were higher than in spring, which can lead to an increase of fibre content (Lee et al., 2017; Moyo and Nsahlai, 2021).

We observed higher heritability in spring than in autumn for OMD and NDFD. This can be due to the high correlation between OMD and NDFD with heading date, with heading date being a highly heritable trait ($h^2 = 0.82$) determined by a few genes with large effect (Wang and Forster, 2017).

The genetic variance of NDFD in spring was more than twice the value observed in autumn, while a higher genotype x year interaction was observed in autumn than in spring. This broad range of variation in spring NDFD suggests potential for selection.

2.4.2. NDFD and NDF determine OMD in both spring and autumn

The observed variation for OMD could be explained by both differences in the cell wall content (NDF) and of its digestibility (NDFD), as previously reported by Barrière et al. (2003) and Pembleton et al. (2013). Selection can be performed on both NDF and NDFD, but an appropriate selection approach should consider the fact that the correlation between NDFD and NDF was different in spring and in autumn. In spring, the correlation between NDFD and NDF was weak, showing near independency between these two traits and allowing simultaneous selection for both NDFD and NDF. In contrast, NDFD and NDF were strongly negatively correlated in autumn, what would hamper simultaneous selection. A potential explanation of this difference in the strength of the correlation between these traits in different seasons lies in the difference in NDF and its variability between seasons. Biomass harvested in spring displays lower and less variable NDF values compared to biomass harvested in autumn. NDFD is on average higher in spring compared to autumn. We can assume a different cell wall biogenesis in summer, when plants are resisting to high temperatures and start preparing for winter survival. The autumn NDF content is high compared to spring when a high leaf growth rate can be observed associated with lower NDF content (Wingler and Hennessy, 2016).

Indeed, variability of NDFD can be partially explained by fibre composition, with main contribution of different components in spring and autumn. In spring, an increase of NDFD was associated with an increase of hemicellulose (and a relative decrease of cellulose, given the negative correlation between both) but with almost no effect of lignin. In contrast, in autumn, an increase of NDFD was associated with a decrease of lignin concentration in the cell wall but with almost no effect of relative hemicellulose content. The link between cell wall lignin concentration and NDFD is normally observed in forage grasses (Jung and Vogel, 1986; Mowat et al., 1969), but not always (Lam et al., 2003). The absence of a link between cell wall lignin concentration and NDFD in spring is surprising, given that the same range for cell wall lignin concentration was observed in both spring and autumn. One explanation could be that the methodology used for cell wall characterisation is limiting because it is not precise enough. It is known that the ADL fraction determined with the Van Soest method detects the acid-insoluble lignin but does not allow to quantify the acid-soluble lignin (van Parijs et al., 2018). However, the effect of lignin on NDF digestibility depends not only on lignin concentration, but also on cross-linking and hydrophobicity (Grabber et al., 2004). The acid-soluble phenolics are important in monocot species for cross-linking lignin with hemicellulose, while they are not detected by the van Soest method (van Parijs et al., 2018). Furthermore, the hemicellulose content in this study was estimated by substracting the ADF from the NDF content. This represents the detectable acid soluble carbohydrates from the non-water-soluble biomass. Our hemicellulose content estimate may lack the hemicellulose fraction that is cross-linked with lignin. We can assume that in spring acid-soluble phenolics which are important in cross-linking lignin to hemicellulose play a role in the hemicellulose composition. Indeed, hemicellulose is a complex mixture of polysaccharides including xylans, β-glucans, xyloglucans and mannans. arabinoxylans are predominant in hemicellulose in monocots and the high degree of substitution on the xylan backbone with arabinose relates to a low digestibility (Li et al., 2021). We observed an increase of NDFD associated with an increase of hemicellulose concentration, consistent with previous reports by (Roussel et al., 2002). The effects observed in autumn, were probably due to a major effect of the acid-insoluble lignin on cell wall digestibility, and not on the hemicellulose fraction. This may indicate that in autumn, the acid-insoluble lignin is better to determining the cell wall digestibility compared to the acid-soluble phenolics.

We observed a strong effect of heading date on all traits determined in plant materials harvested in spring. OMD decreased with later heading date, which was associated with a decrease of NDFD, cell wall hemicellulose and lignin concentrations. In other words, leaves harvested on late flowering genotypes were less digestible than leaves harvested on early flowering genotypes. Since leaves were harvested at heading stage, the decrease in digestibility could be explained by the age of leaves on later heading plants. Indeed, it has previously been reported that digestibility decreases with increasing age of the organs (Buxton and Casler, 1993). It is therefore important to take into consideration the strong correlation between heading date and digestibility during breeding and selection. If this is not taken into account, breeding for highly digestible genotypes may indirectly lead to selection of early heading material, which may or may not be desirable in the global breeding program.

2.4.3. Genetic control of digestibility

The SNP dataset used in this study tagged genes from the pathways involved in plant cell wall biogenesis, plant architecture and development and phytohormone biosynthesis, signalling and response (Veeckman et al., 2018). GWAS identified multiple QTLs for each trait, spread across the genome, and explaining together from 29.2% to 51.7% of phenotypic variance. Each marker, taken individually, explaining a small part of phenotypic variance (from 1 to 7%). Markers identified by GWAS targeting to genes involved in different pathways. In general, the QTL effects were lower than the one observed in previous studies on *L. perenne*, as expected on mapping populations (Shinozuka et al., 2012), but also on diversity panel (Pembleton et al., 2013). A number of genes showing significant effects for the different quality traits considered in this study were also identified in other studies on digestibility and cell wall digestibility in forage grasses such perennial ryegrass and maize (Pembleton et al., 2013; Ralph et al., 2004). These include genes involved in lignin and cellulose biosynthesis (eg. HCT3 and CAD in autumn OMD and C3H, ALDH and CCoAOMT in autumn NDFD; CAD2, COMT-01, ALDH, CES-02, XylT4 and PAL-03 in spring OMD and CES-02 and HCT in spring NDFD), which have previously been described to be involved in digestibility traits (reviewed by Halpin, 2019). Associations were also found for transcription factors such as WRKY-02, MYB-10 and NAC-13 with spring related forage quality traits, and MYB-02 in autumn quality traits. These transcription factors have previously been described to be involved in cell wall biogenesis in grasses. For example, NAC transcription factors have been identified as master switches to grass secondary wall biogenesis by activating involved genes and transcription factors (Rao and Dixon, 2018). Furthermore, Myb transcription factors are considered as strong activators of cell wall biogenesis, though at a lower level than NAC (Nakano et al., 2015; Rao and Dixon, 2018). It has also been shown that members of the WRKY transcription factor family can repress lignification (Gallego-Giraldo et al., 2016; Rao and Dixon, 2018). A more in depth analysis of these gene families in cell wall biosynthesis, including transcription factor families, may shed light on the regulation of secondary cell wall biogenesis in *L. perenne.*

No identical QTLs were identified across seasons, except one QTL for ADL.NDF, C.NDF and OMD and four QTLs for NDF. Within each season, we identified some QTLs common between digestibility traits and cell wall composition traits. In spring, HC.NDF had three QTLs in common with OMD and/or NDFD, whereas no common QTLs were identified in autumn. In autumn, ADL.NDF had seven QTLs in common with OMD and/or NDFD whereas no common QTL was identified in spring. These GWAS results confirm the importance of hemicellulose concentration for the digestibility of the spring cut, and lignin

concentration in NDF for the digestibility of the autumn cut. QTLs only linked to NDFD or OMD could suggest there is other components, not considerated in this study, which have a link with NDFD or OMD.

In conclusion, we found that NDF and NDFD are major components of OMD in spring and autumn, but we observed a high interaction between genotypes and seasons. In spring, NDFD was mainly explained by HC.NDF and C.NDF and their related QTLs. In autumn, NDFD was mainly explained by ADL.NDF and their related QTLs. This new knowledge suggests that breeding for OMD should be performed by selecting both NDF and NDFD in spring and autumn with an emphasis on cell wall hemicellulose content in spring and cell wall lignin content in autumn. In future work, the diversity of candidate genes identified in this study could be surveyed more in detail and in other plant materials in order to identify interesting alleles useful for molecular assisted breeding.

2.5. Key points for chapter 2

- Strong genotype x season interaction for quality traits.
- Broad sense heritabilities were 0.67 and 0.32 for OMD in spring and in autumn, respectively.
- Almost no correlation between NDFD and NDF in spring.
- OMD variation was mainly explained by hemicellulose and cellulose content (HC.NDF and C.NDF) variation in spring.
- OMD variation was mainly explained by lignin content (ADL.NDF) variation in autumn.
- Markers linked to different quality traits can explain a large part of variance of OMD variation (47% in spring and 40% in autumn).

3. Dynamics of the quality during the lifespan of a leaf and a stem of perennial ryegrass: a survey of genotypes contrasting for their precocity and their quality

Abstract

Quality and yield are essential for determining the value of a forage variety. These two traits depend on genetic and environmental factors and are tightly linked to plant morphogenesis and date of harvest. Moreover the plant quality and its biomass results from the quality and biomass of its different organs: leaves (including sheath and blades) and stems at the reproductive stage. The objective of this study was to describe the leaves and the stems for their morphology and their quality (dry matter digestibility (DMD), fibre content (NDF) and its digestibility (NDFD)) during their lifespan on four genotypes contrasting for their heading date and quality. We observed that the main factor explaining the quality decrease of leaves with maturity was leaf senescence. Excluding senescence, green leaf quality decreased with an increase of leaf length and sheath proportion. Stem quality was lower than leaf quality and decreased strongly with maturity. The stem quality and its decrease rate with maturity differed between genotypes allowing a potential selection for genotypes with higher quality of mature stems. The ranking of genotypes for quality was different for leaves and stems. In spring, a compromise should be made between biomass production and its quality. This compromise differed between genotypes and harvest dates. In autumn, plants should be harvested immediately after the biomass reached a maximum in order to obtain the best quality.

3.1. Introduction

Quality and yield are essential for determining the value of a forage variety (Carr, 1975). Yield has been integrated as a main breeding target for years but quality has been considered more recently in particular with the access to new methodologies for high throughput phenotyping like NIRS. In 2013, quality has been added in the selection criteria for variety registration in France.

Nevertheless, quality depends on many factors including plant maturity, morphology, genetic and environmental factors (Buxton and Casler, 1993). Quality decreases with plant maturity because the proportion of structural tissues with low quality increases with plant growth and development. In particular, quality decreases with leaf length and sheath proportion and strongly with stem growth (Terry and Tilley, 1964; van Parijs, 2016). In other words, quality is strongly linked to plant morphogenesis, which depends on the genotype and the environment. Moreover, the produced biomass is also directly linked to plant morphogenesis. Depending on the harvest date, different ranking of genotypes for quality and biomass production could be obtained depending on their stage of development. A better description and understanding of the morphogenesis and its impact on the produced biomass and its quality could help defining ideotypes adapted for various cutting managements. Classically, it is advised to harvest forage grasses just before spike emergence in order to maximize the compromise between quality and biomass. However, the possibility to harvest after spike emergence without losing to much quality could be of great interest for farmers given them more flexibility for harvesting (Casler and Vogel, 1999).

The objective of this study was to describe the leaves and the stems for their morphology and their quality (dry matter digestibility (DMD), fibre content (NDF) and its digestibility (NDFD)) over their lifespan on four genotypes contrasting for their heading date and quality. The goal was to integrate these data in order to have cues on the evolution of the biomass and its quality at the plant level.

3.2. Material and methods

3.2.1. Plant material

Plant material consisted of four different perennial ryegrass genotypes obtained from DSV breeding material. Each genotype was cloned by splitting tillers to obtain 150 plants. These genotypes were chosen based on previous data obtained by DSV on spaced plants (personal communication Lukas Wolters) because they presented contrasting precocity and quality of the whole plant harvested in spring. For easier understanding, two-digit numbers (ID) will be used to refer to the four different genotypes (Table 9).

Table 9: List of the four genotypes used in this study

ID Genotype	Quality	Precocity
12	High	Early
13	Low	Intermediate
15	Low	Late
16	High	Late

3.2.2. Trial design

Clones were produced by splitting mother-plants, tillers were potted in 2L containers containing horticultural compost mixture (315 DO from Peltracom) with 10% of perlite and 3 g/L of Osmocote 12/24 month. After a period in a greenhouse (minimal temperature of 5°C) during winter, the plants were cut at 4 cm high and placed on ten cultivation tables outside at INRAE URP3F Lusignan, France (46.4052, 0.0811), the 28th of March 2019. The plants were cut again after spring measurements and the 28th and 29th of August before autumn measurements. Nitrogen fertilizer (40 U) was applied for each plant just after the cutting in August. On each table, the four genotypes were randomized on five latin squares of 4x3 pots and surrounded by a line of containers to avoid border effect. This way, 15 clones by genotype were placed by table. Tables were numbered and divided in two blocks (A and B) (Figure 21). Irrigation were made by sprinklers to avoid water stress. A set of 23 temperature captors were spread at soil level under the leaves giving a mean value every 15 minutes. The insecticide Confidor Vert (0.5 mL/L) was applied May 3, 2019 against aphids.

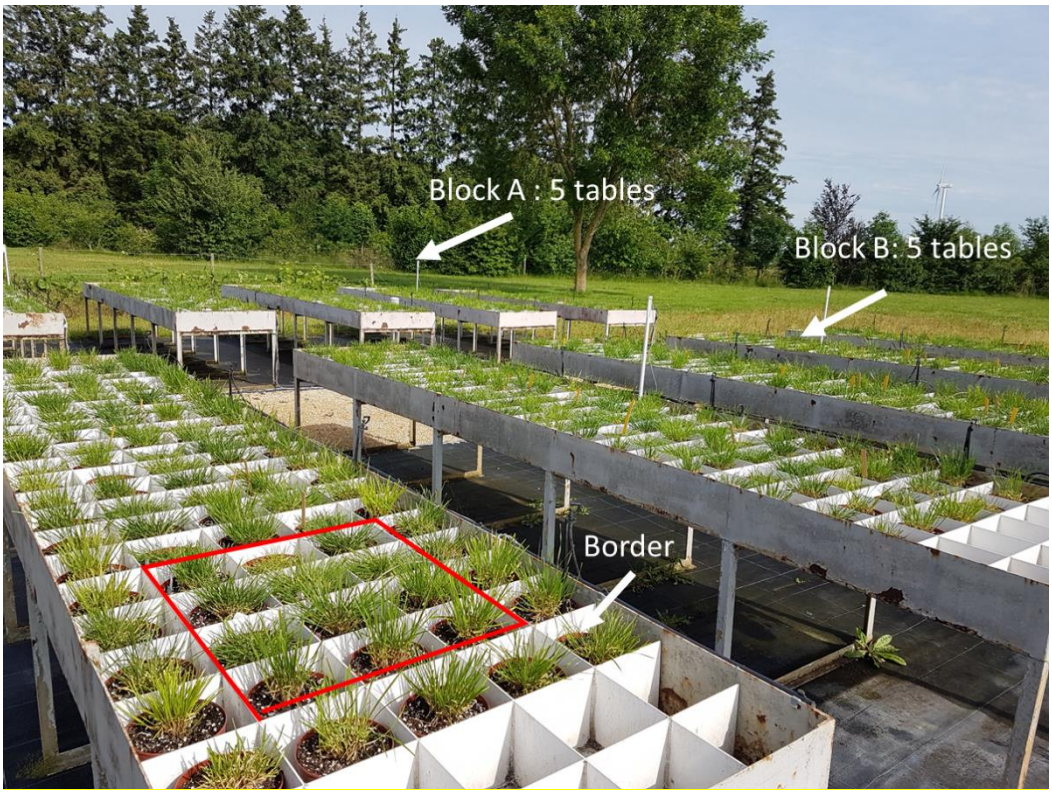

Figure 21: Picture of the experiment showing the two replicates (blocks A and B), the border line and one latin square of 12 plants (3 clones per genotype) in red.

3.2.3. Phenotyping

To follow tiller growth in spring and in autumn, 15 containers were selected (spread over the block) by genotype and by block to measure each leaf of a selected tiller every two or three days, starting with the first uncut leaf as the leaf n°1. Measures were made with a ruler with the 0 at soil level. When a leaf did not grow for four consecutive measures, its sheath was measured and the leaf was not followed anymore. Tiller growth was followed from the 1st of April to the 26th of July 2019 and from the 2nd of September to the 4th of December 2019. In addition, in spring, a notation of heading advancement was made from 0 to 3 with 0: vegetative tiller, 1: the base of the tiller began to harden, 2: emergence of the spike and 3: spike blooming.

To follow quality evolution of different organs for each growth period, one to two tillers by container were cut at 3 cm high every week starting when the third uncut leaf reached 10 cm high (in order to obtain enough tissues for chemical analysis) until the end of growing measurements. Tillers were divided following their genotype and block and were dissected to separate each leaf, composed of one blade and one sheath (Figure 22). For each date of harvest, we obtained one sample per genotype, per block and per leaf rank composed of leaves from about 70 clones in order to obtain enough plant material for chemical analyses. On reproductive tillers, stem and spike were separated from leaves. Stems were split by rank (node and internode corresponding to a leaf) when stem length was sufficient for chemical analyses (Figure 23). So on reproductive tillers, in addition to leaf samples, we obtained one stem sample per genotype, per block and per rank (considering peduncle and spike as a particular rank) also obtained from about 70 clones. All samples were measured for their average length and their average senescence (scoring of the proportion of brown leaf) and weighted after drying at 60°C during 72 h.

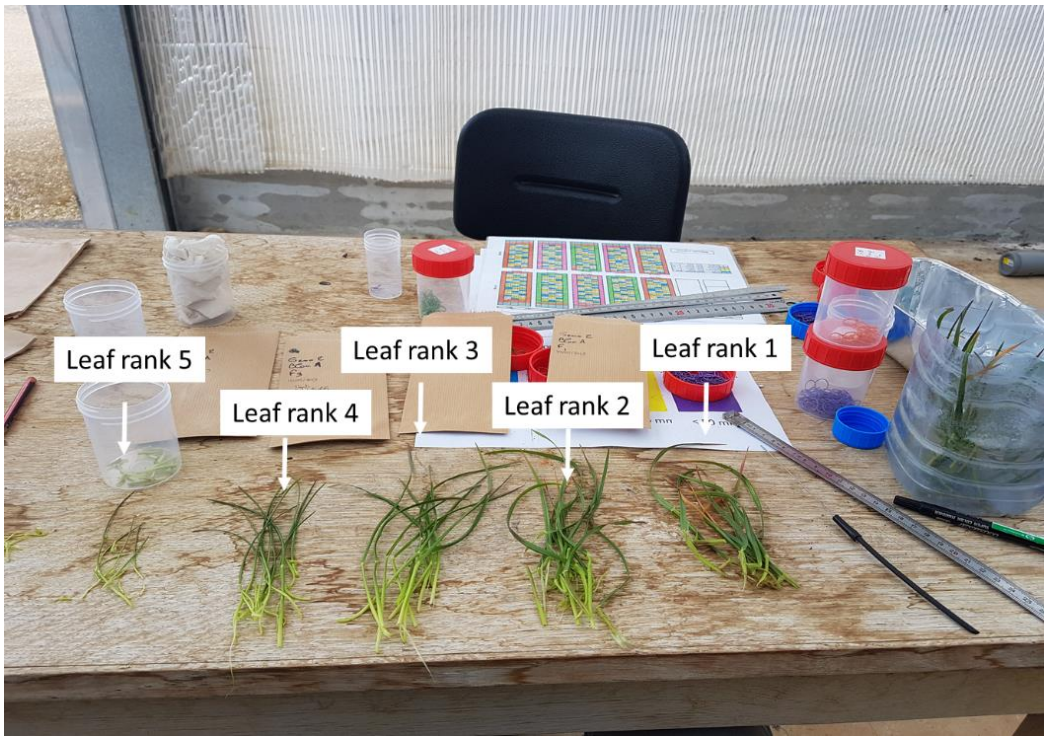

Figure 22: Example of the collect of leaf samples.

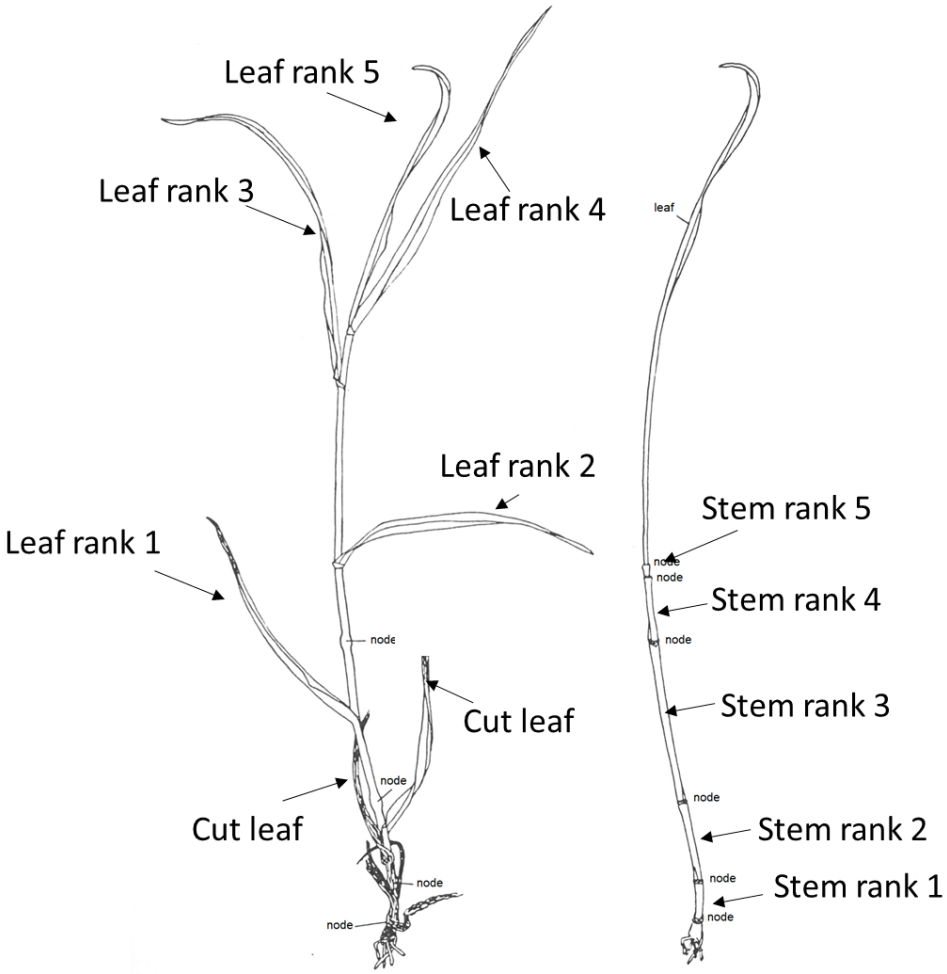

Figure 23: Description of the leaf and stem samples (drawing modified from Gillet (1980)).

3.2.4. Wet chemical analysis and NIRS prediction

All harvested samples of leaves and stems were dried at 60°C during 72 h, ground with a grid of 1 mm and scanned by near infrared reflectance spectroscopy (NIRS) with the MTA from BRUKER for Dry Matter Digestibility (DMD), Neutral Detergent Fibre (NDF) and Total Nitrogen (TN) using existing equations developed by INRAE URP3F with more than 6000 samples. Wet-chemical analyses were performed on a selection of samples (109 of leaves and 139 of stems from all genotypes, ranks, years and seasons) for Dry Matter Digestibility (DMD), Neutral Detergent Fibre (NDF), Neutral Detergent Fibre Digestibility (NDFD) and Total Nitrogen (TN).

NDF was measured with the sequential van Soest method (Van Soest et al., 1991) adapted for the ANKOM Fibre Analyzer (ANKOM Technology Corporation, Fairpoint, NY). To determine NDF, 0.15 g of sample was included into a filter bag, and incubated in neutral detergent at 100°C for 1 h in the ANKOM. The residue was then washed with water at 100°C and acetone, and dried at 60°C.

DMD and NDFD were estimated by the pepsin cellulase method developed by Aufrère (1982), with some modifications to allow the possibility to analyse small samples (40 mg) in 5 mL tubes (Méchin V., personal communication). In a tube, sample (dried harvest of NDF residue) was mixed with 2 mL of pepsin solution (420 mg of pepsin in 60mL of HCl 0.1 N) before 24 h incubation at 40°C in Daisy incubator (ANKOM). Next, tubes were opened and placed in an 80°C water bath for half an hour for amidon gelification. Tubes were mixed with 140 µL of NaOH 2N to neutralise acid before adding 2 mL of cellulase-amyloglucosidase solution (172 µL of glacial acetic at 99 %, 246 mg of anhydrous sodium acetate (CH3COONa), 120mg of cellulase and 180µL of amyloglucosidase in 60mL of distilled water) and to be incubated at 40°C for 24 h. Next day, tubes were centrifuged at 10°C for 20 minutes at 3500 rpm, supernatant were removed, then to rinse residue, 4 mL of distilled water were added by tube before centrifugation and supernatant removal were performed two times. To finish, tubes were dried at 70°C for 48 h before their weight were measured.

TN was measured with Dumas method with an elemental analyser FLASH from Thermo-Fischer.

NIRS equation used to estimate DMD, NDF and TN for leaf samples were elaborated by INRAE-URP3F with a large database of forage analysis obtained over years. This equation gave good prediction accuracy with leaf samples (R^2>80%) but less with stem ones. It was slightly modified with cross-validation with wet-chemistry data obtained from stems and spikes, giving a better estimation of these tissues.

All the analyses were performed in the chemistry laboratory of INRAE-URP3F.

3.2.5. Statistical analysis of phenotypic data

All the analyses were performed with R (R Core Team, 2020).

For each leaf observed, leaf length data in function of time (time was counted as effective days (ed) corresponding to one for one day at 23°C *i.e.* the optimum temperature for perennial ryegrass growth and below one for the other temperatures following a Gaussian function (Rouet, 2021)) were fitted with a beta function (Verdenal, 2009) allowing the evaluation of leaf elongation rate (LER) leaf initiation (leaf length equal 1 mm), of the time when leaf elongation rate is maximum, the time when leaf growth stops and the maximum leaf length. These parameters were averaged over the 15 clones per block. Then, the lifespan of a leaf was divided in leaf elongation duration (LED), mature green leaf (GreenD) and leaf senescence (SenD) (Figure 24).

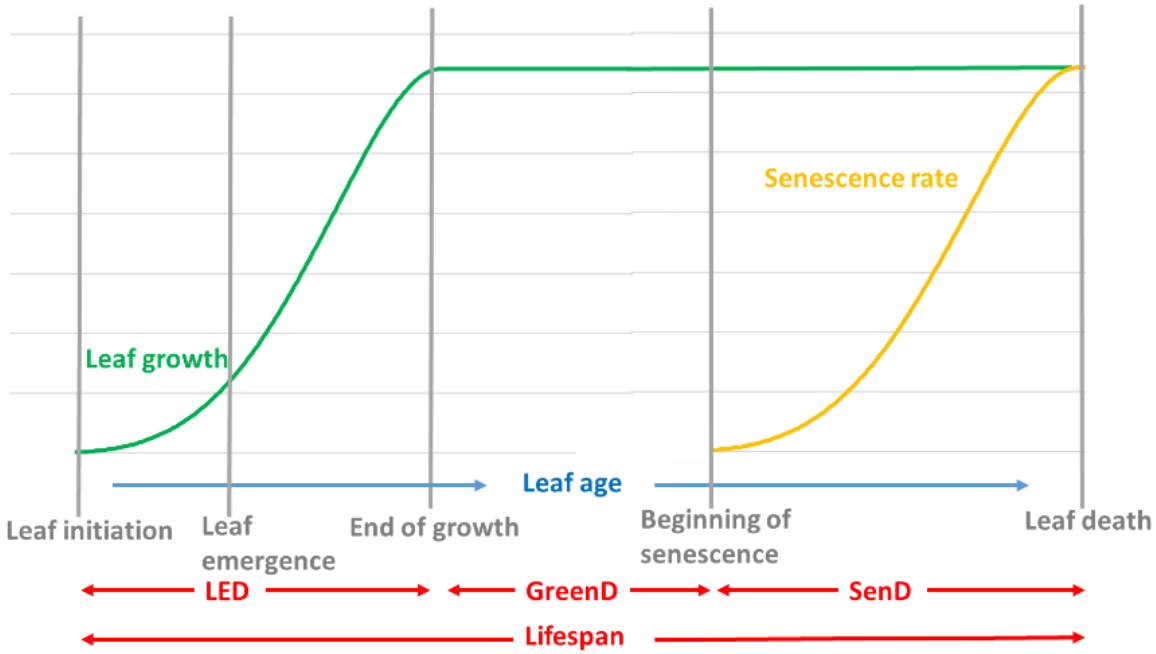

Figure 24: Leaf stages during its lifespan

In order to analyse an entire stem, data obtained for each rank were summed for dry weight and length, averaged taking into account the weight of each rank for NDF, DMD, TN and NDFD.

In order to analyse an entire tiller, data obtained for each leaf and stem rank were summed for dry weight and averaged taking into account the weight of each rank for NDF, DMD, TN and NDFD.

A variance analysis (R package stats: functions aov and anova (R Core Team, 2020)) was performed to test the effect of leaf ranks, genotypes, replicates and seasons on these variables and on the first two axes of the principal component analysis. A multiple comparison of means (Newmann and Keuls; package agricolae function SNK.test) was performed to compare genotypes in autumn for the number of ranks were identical among genotypes.

A principal component analysis (package FactoMineR) was performed on morphological parameters (maximum leaf length: Max_length, maximum leaf elongation rate: LER, leaf elongation duration: LED, duration of the period when the leaf is mature: GreenD, duration of the senescence: SenD, sheath length / leaf length ratio: sheath proportion, duration between two successive leaves: phyllochrone) and chemical parameters (maximum digestibility of dry matter max_DMD, minimum fibre content Min_NDF and maximum total protein content Max_TN) on all leaf ranks, genotypes, replicates and seasons. The missing data were imputed with R function imputePCA from package missMDA.

A linear model (lm and anova in R) with genotype (qualitative) effect, time (quantitative) effect and genotype : time (quantitative) interaction was used to test the difference of slopes and means between genotypes for a given trait over time.

3.3. Results

3.3.1. General description of the four genotypes

3.3.1.1. Description of leaves

For each genotype, for each leaf rank, we measured the kinetics of leaf growth (leaf length and leaf weight), of DMD, of fibre content (NDF), of fibre digestibility (NDFD), and of total proteins (TN) in spring (Figure 25) and in autumn (Figure 26). The same profile was observed for all leaves. Leaf length increased until a plateau and then after a period of time (GreenD) the green part of the leaf decreased due to senescence. DMD decreased slowly with time when the leaf was mature and then rapidly when the senescence started. This evolution was simultaneous with an increase of fibre content and a decrease of its digestibility. The total protein content decreased rapidly after the leaf reached its maturity. The dry leaf weight decreased with the senescence. Leaf length and sheath proportion increased generally with leaf rank and this is particularly marked in spring when the stem started to elongate (Figure 27 and 28). No significant correlation was observed between dry leaf weight and DMD (Figure 29).

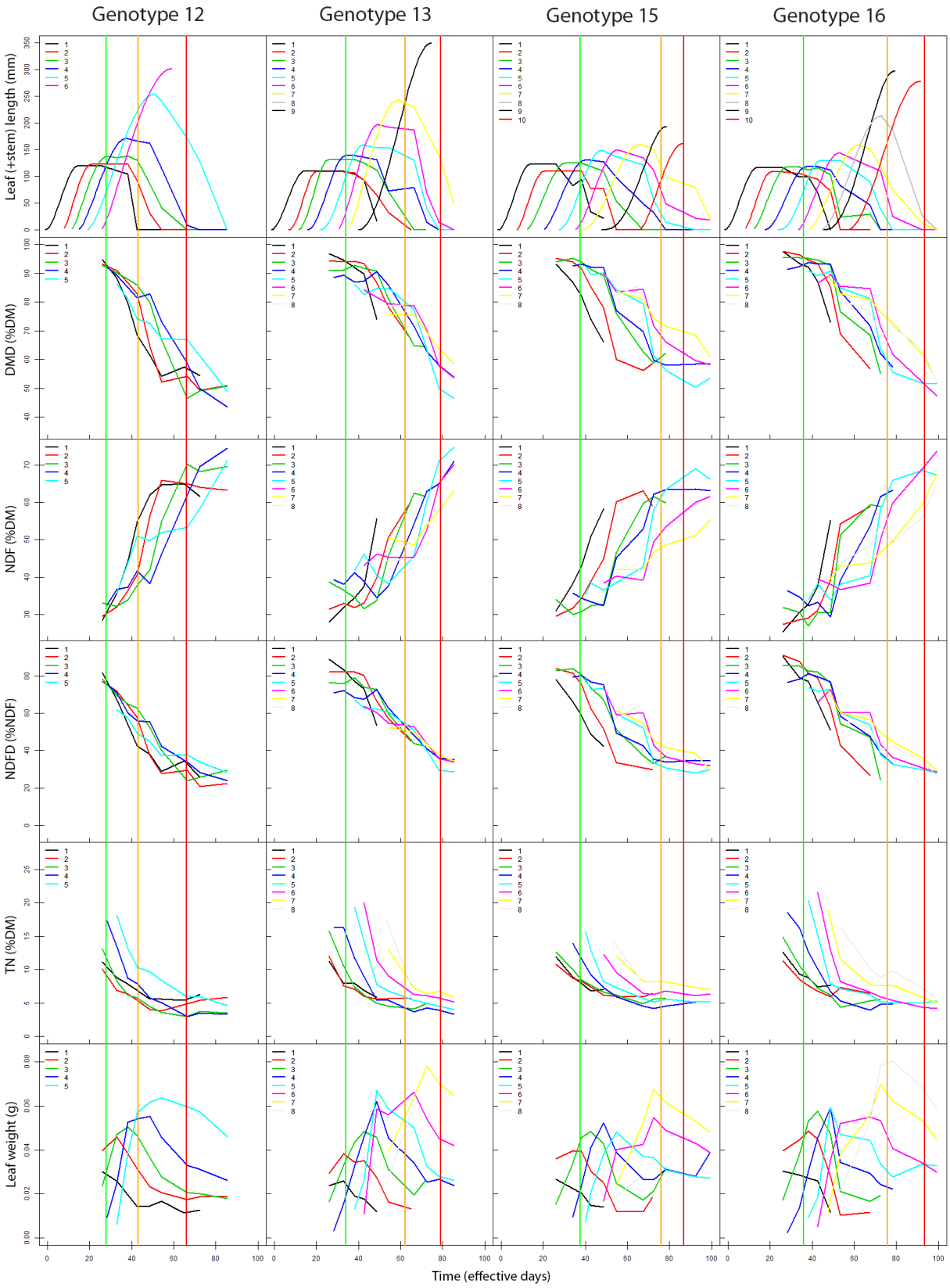

Figure 25: For each genotype (columns) and with a different colour for each leaf rank, various variables (lines) are shown over time in spring 2019. Variables are kinetics of the green length of leaf in mm (the length decrease comes from the senescence), DMD (%DM), NDF (%DM), NDFD (%NDF), TN (%DM) and weight (mg). Each leaf corresponds to the average over 30 clones. Vertical lines matches with heading stages: green is tiller stiffening, orange is spike emergence and red is blooming.

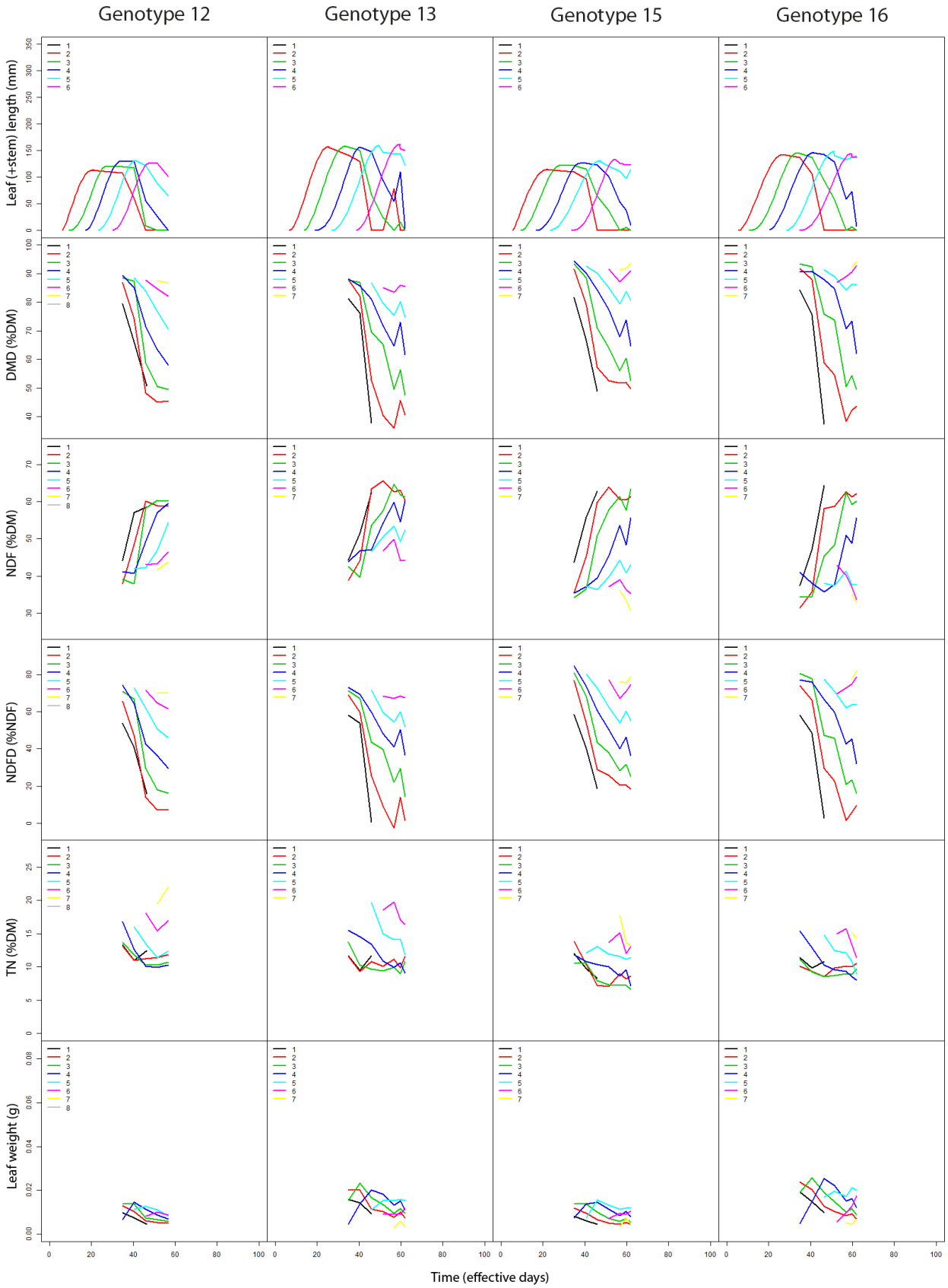

Figure 26: For each genotype (columns) and with a different colour for each leaf rank, various variables (lines) are shown over time in autumn 2019. Variables are kinetics of the green length of leaf in mm (the length decrease comes from the senescence), DMD (%DM), NDF (%DM), NDFD (%NDF), TN (%DM) and weight (mg). Each leaf corresponds to the average over 30 clones.

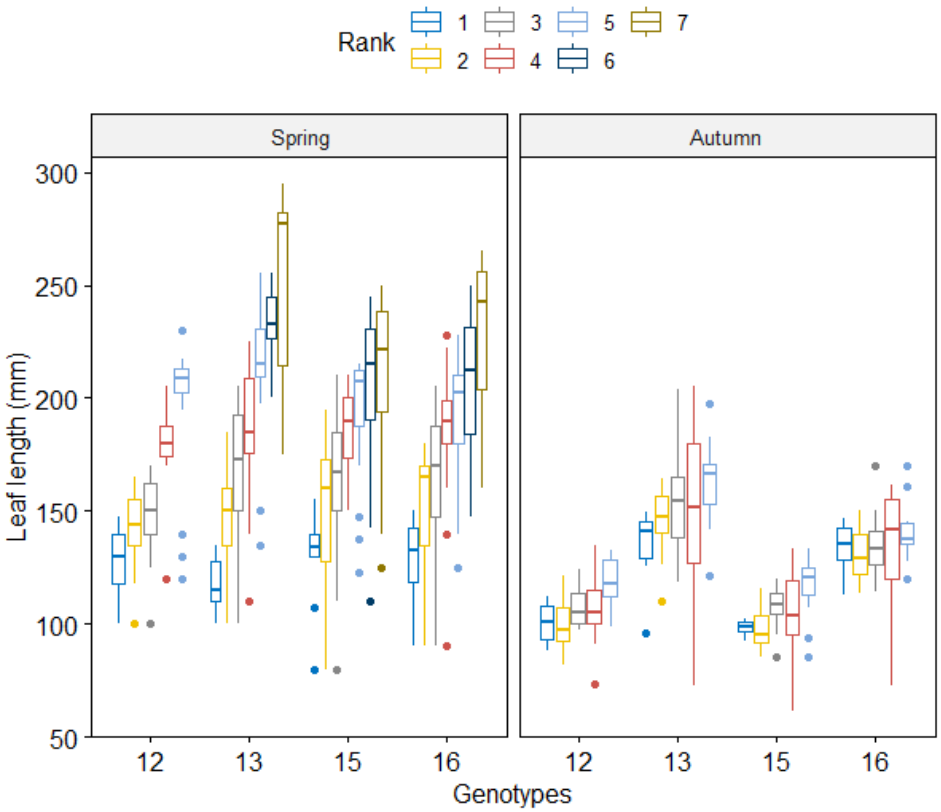

Figure 27: Leaf length of the harvested samples per rank, per genotype and per season.

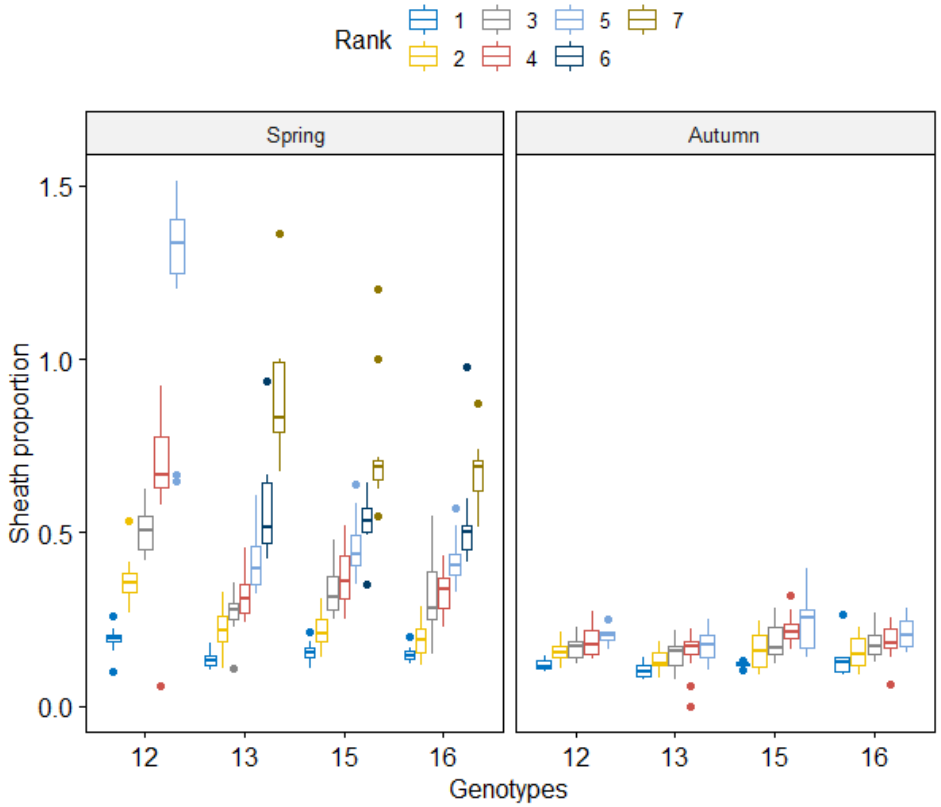

Figure 28: Sheath proportion (in length) of the harvested samples per rank, per genotype and per season.

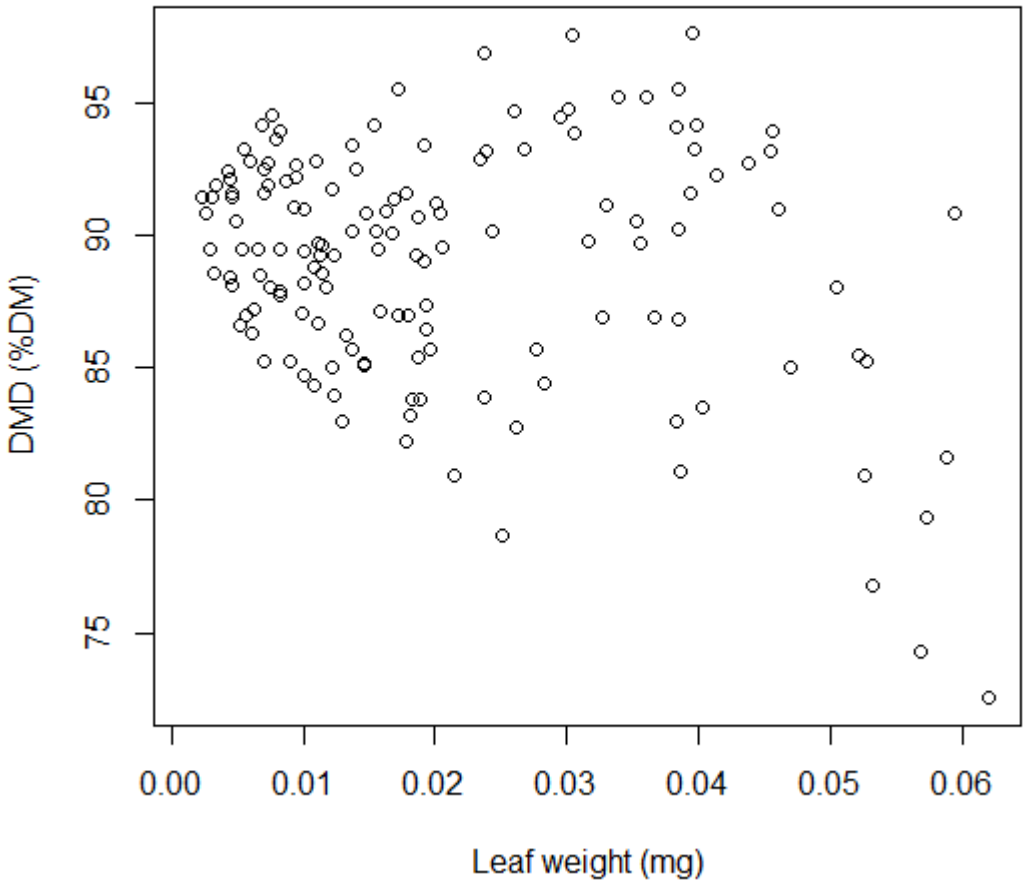

Figure 29: Relationship between dry matter digestibility and leaf weight on non senescent leaves.

The effects of genotype, rank, season and the rank:season interaction for all morphological and chemical parameters are presented in Table 10. We observed a large effect of leaf rank for all traits. Table 11 presents the averaged values over ranks per genotype for morphological and chemical parameters only in autumn because the number of leaves was different between genotypes. In autumn, genotype 13 showed longer leaves than genotypes 14 and 15, with genotype 16 being intermediate. Genotype 15 and 16 showed leaves of higher quality than genotype 12 and 13 which was linked with lower fibre contents.

Table 10: Effect of genotype, season, leaf rank, replicate and season x rank interaction on the different variables and the first two axis of the principal component analysis. ***: < 0.001, **: < 0.01, *: < 0.05

Effect	Genotype	Season	Rank	Block	Season:Rank
Trait	F value p value	F value p value	F value p value	F value p value	F value p value
Max_length	6.51 ***	24.19 ***	22.12 ***	0.34	3.78 **
Sheath_proportion	6.23 ***	11.51 **	41.78 ***	0.00	4.96 **
LER	3.75 *	1.20	6.31 ***	0.05	4.51 **
Phyllochrone	6.92 ***	14.47 ***	3.65 **	0.21	3.84 *
LED	2.18	2.17	21.21 ***	0.03	2.11
GreenD	1.14	11.34 **	10.72 ***	4.39 *	0.48
SenD	3.23 *	25.30 ***	4.74 ***	0.96	0.30
Max_DMD	20.02 ***	3.81	56.56 ***	2.32	10.97 ***
Min_NDF	0.00 ***	0.00 ***	0.00 ***	0.22	0.00 ***
Max_TN	8.69 ***	0.03	15.79 ***	10.19 **	1.89

Table 11: Description of the four genotypes for morphological and chemical parameters in autumn. Grp indicates Newman-Keuls mean differences between genotypes by season. ed: effective day with 23°C being the optimum.

Genotype Season	Max_length (mm)			Sheath_proportion (%)			LER (mm.ed)			Phyllochrone (ed)			LED (ed)			GreenD (ed)			SenD (ed)		
	mean	sd	Grp	mean	sd	Grp	mean	sd	Grp	mean	sd	Grp	mean	sd	Grp	mean	sd	Grp	mean	sd	Grp
12 Autumn	124.5	8.6	c	40.7	3.0	a	3.9	1.4	a	7.0	0.8	ab	17.2	2.1	b	8.3	4.8	a	13.1	4.0	a
13 Autumn	158.6	3.4	a	32.5	2.1	c	4.4	1.4	a	8.5	1.5	a	21.0	2.1	a	5.9	3.7	a	16.0	5.5	a
15 Autumn	125.5	9.1	c	39.6	1.4	a	3.5	1.6	a	8.6	1.7	a	20.4	3.2	a	9.8	4.0	a	16.7	5.6	a
16 Autumn	145.4	3.9	b	35.5	2.8	b	3.8	0.8	a	8.6	1.1	a	22.8	1.9	a	6.3	1.9	a	16.3	4.8	a

Genotype Season	Max_DMD (%DM)			Min_NDF (%DM)			Max_TN (%DM)		
	mean	sd	Grp	mean	sd	Grp	mean	sd	Grp
12 Autumn	87.9	2.0	b	39.9	2.3	b	15.4	2.2	ab
13 Autumn	87.4	1.3	b	42.5	3.5	a	16.1	3.4	a
15 Autumn	92.8	1.4	a	35.3	1.4	c	13.0	1.8	b
16 Autumn	92.2	1.2	a	34.1	2.3	c	13.6	2.3	ab

In order to characterise the multivariable diversity of leaves from different genotypes, different ranks, different replicates and different seasons, we performed a principal component analysis with morphological parameters (maximum leaf length: Max_length, maximum leaf elongation rate: LER, leaf elongation duration: LED, duration of the period when the leaf is mature: GreenD, duration of the senescence: SenD, sheath length / leaf length ratio: sheath proportion, duration between two successive leaves: phyllochrone) and chemical parameters (maximum digestibility of dry matter max_DMD, minimum fibre content Min_NDF and maximum total protein content Max_TN). The first two axes explained 57 and 12 % of the total variance; their correlations with the parameters are presented in Figure 30. The first axis discriminated leaves mainly based on their rank (Figure 31), with leaves from high ranks showing long leaves with a high proportion of sheath, a high leaf elongation duration, a high fibre content and a low maximum digestibility. The second axis discriminated leaves based on the season and the genotype. Leaves from spring showed a lower phyllochrone than leaves from autumn. The genotype 12 showed a lower LER and phyllochrone than other genotypes.

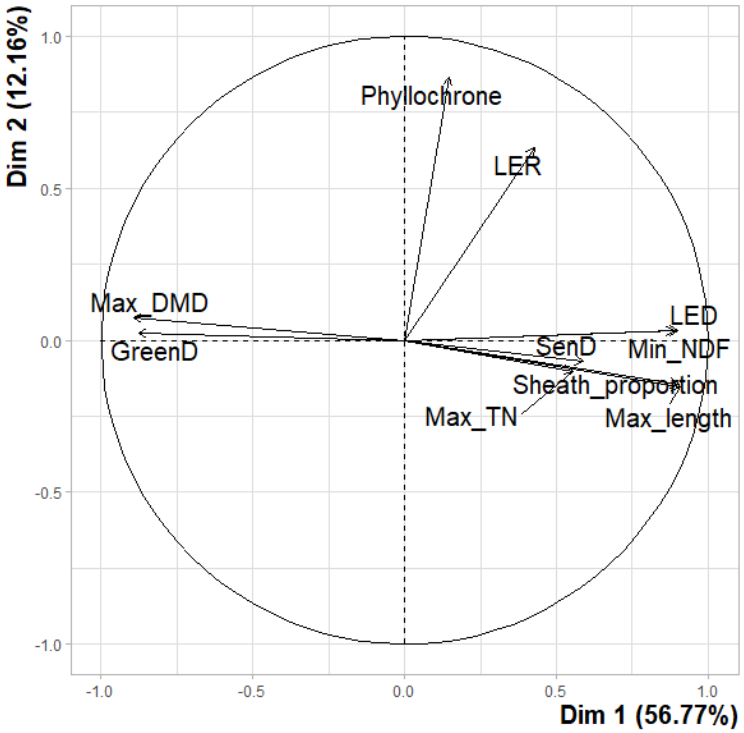

Figure 30: First axes of the principal component analysis of leaves with Max_length (mm), Sheath_proportion (%), Phyllochrone (ed), LER (mm/ed), LED (ed), GreenD (ed), SenD (ed), Max_DMD (%DM), Min_NDF %DM and Max_TN (%DM) as projected traits

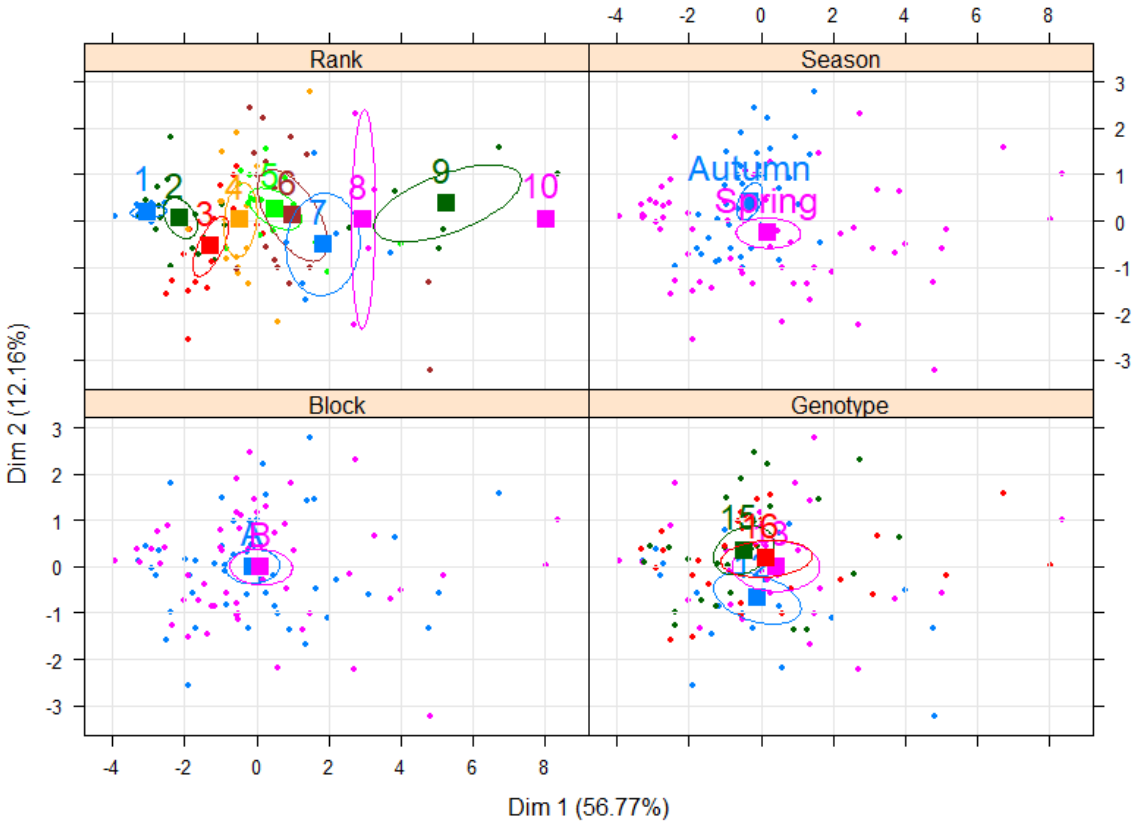

Figure 31: Representation of leaves on first axes of the PCA of leaves , highlighted for their appurtenance to different ranks, seasons, blocks and genotypes

3.3.1.2. *Description of stems*

First, the precocity for the four genotypes in spring 2019 is presented in Figure 32. It is in accordance with the previous data (Table 9). The number of uncut leaves on reproductive tillers varied between genotypes (Figure 33), with an average of 5.6 leaves for the genotype 12, 8.2 leaves for the genotype 13, 9.2 leaves for the genotype 15 and 9.5 leaves for the genotype 16.

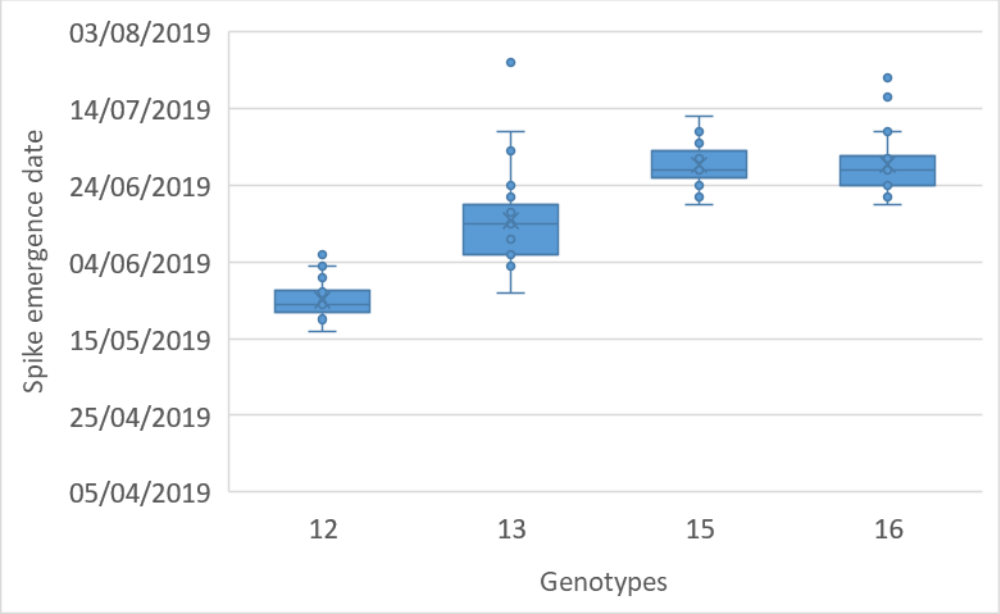

Figure 32: Variability for spike emergence in spring 2019

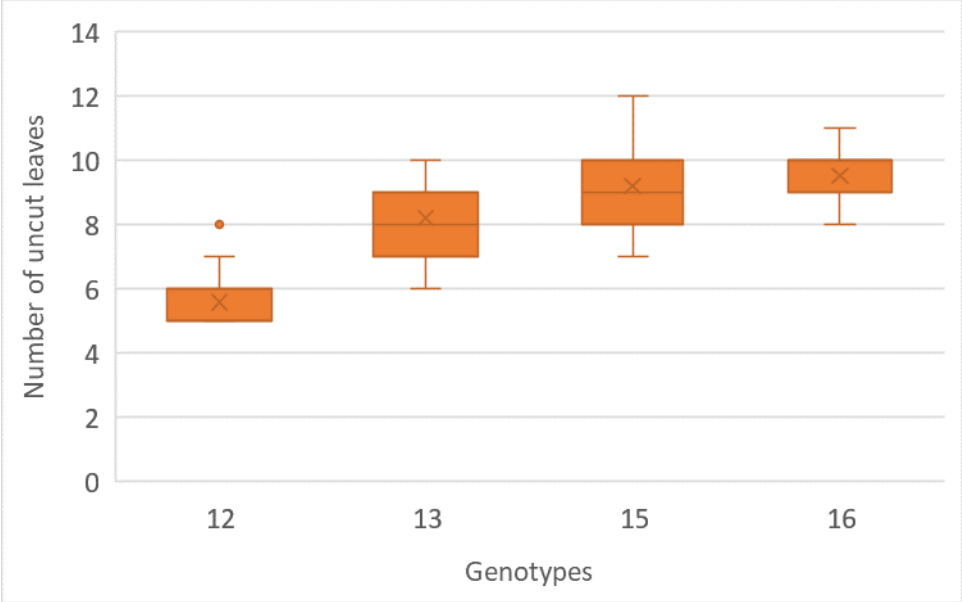

Figure 33: Variability of the uncut leaf number of reproductive tillers for four genotypes in spring 2019

Concerning the digestibility of stem, young stems before heading presented the highest DMD from 70 to 78 %DM of the stem, which was lower than green leaf digestibility of about 90 %DM (Figure 34). Then, DMD increased with the rank, showing lower DMD for basal internodes (except the last rank of genotype 16). The peduncle, carrying the spike, showed the

lowest digestibility of the stem. The spike itself presented a large variability of DMD certainly due to the degree of maturity with a strong decrease of DMD after spike emergence.

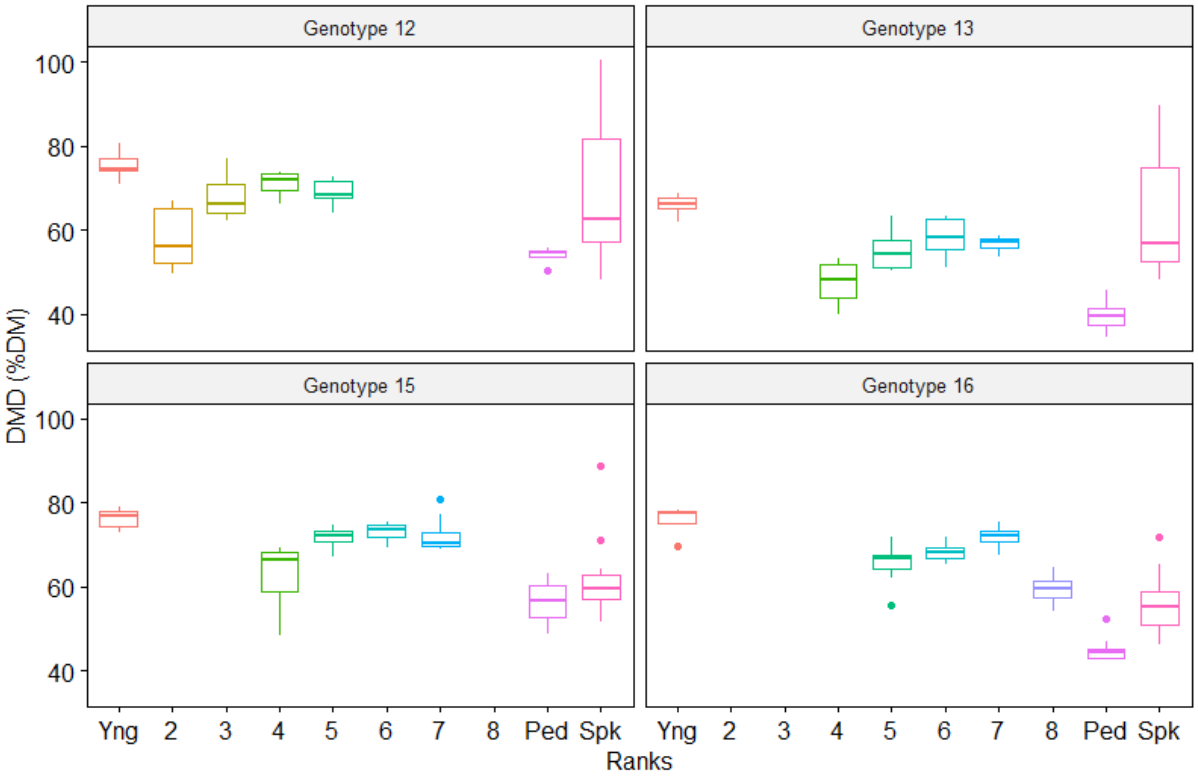

Figure 34: Variation of dry matter digestibility for each stem section by genotype in spring 2019. Yng: Young stems (not enough tissues to analyse by rank), Pad: spike peduncle and Spk: Spike

DMD decreased with maturity (Figure 35) at different rate depending on the genotype ($p<0.05$). Genotype 15 showed a lower decrease of DMD with time than the genotypes 12 and 13. Moreover, for a medium time, the ranking of genotypes for DMD was: 15 = 16 > 12 > 13. Dry weight increased with maturity (Figure 36) at the same rate for the four genotypes. For a medium time, the ranking of genotypes for dry weight was: 12 > 13 = 16 > 15.

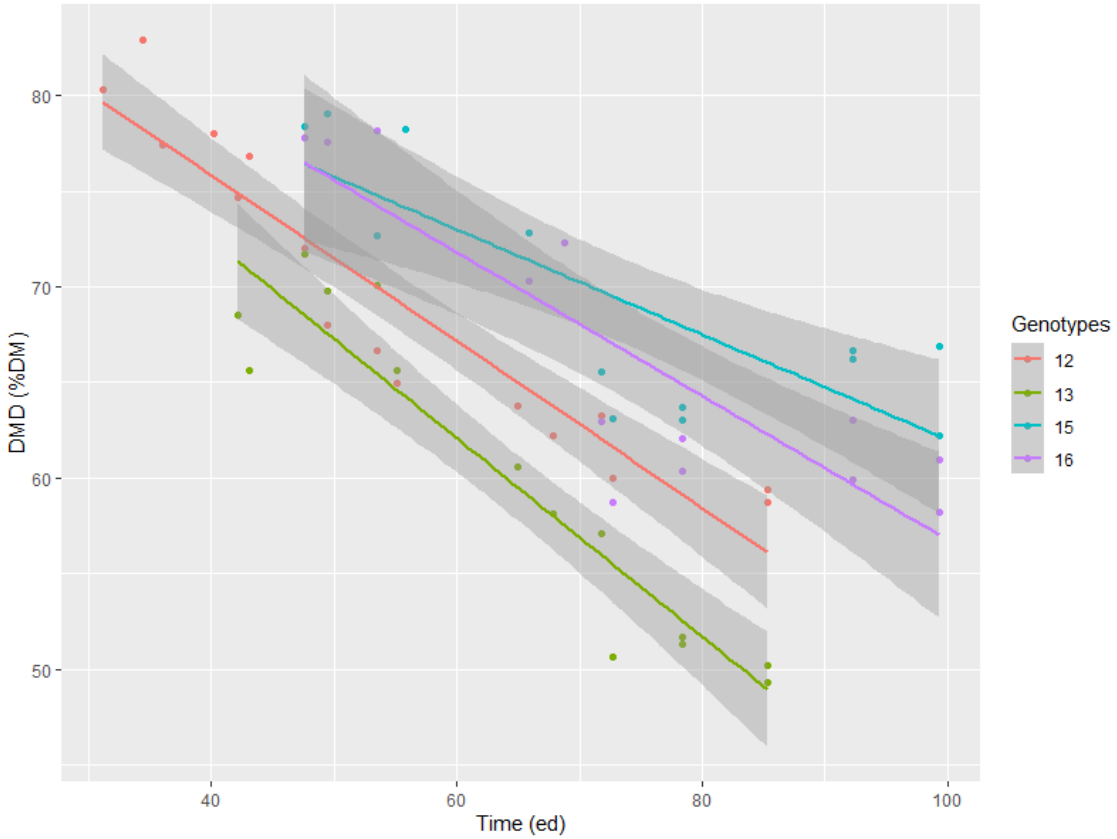

Figure 35: Variation of dry matter digestibility (DMD) of stem with time for four genotypes in spring 2019

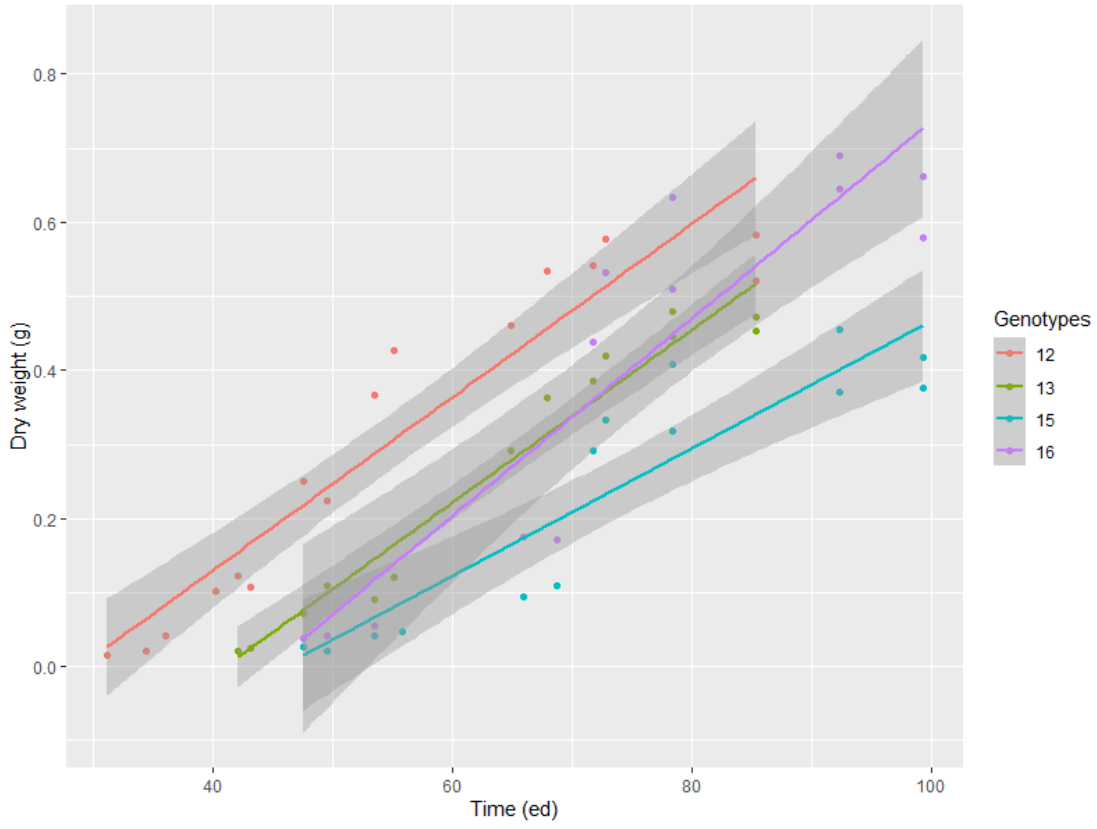

Figure 36: Variation of dry weight of stem with time for four genotypes in spring 2019

The first principal component of the PCA made on full stem data explained 72.6% of the observed variation and was highly positively correlated to NDF, time, length and weight and was highly negatively correlated to DMD and NDFD (Figure 37). The second principal component was positively correlated to TN and explained 13.0% of the observed variation. The projection of all stem samples on these principal components showed a small block effect for TN and a genotype effect (Figure 38). First, the genotype 16 had less nitrogen than the genotype 12 with genotypes 13 and 15 being intermediate. To finish, the genotypes 13 and 16 showed higher NDF, length and weight and lower DMD and NDFD than genotypes 12 and 15.

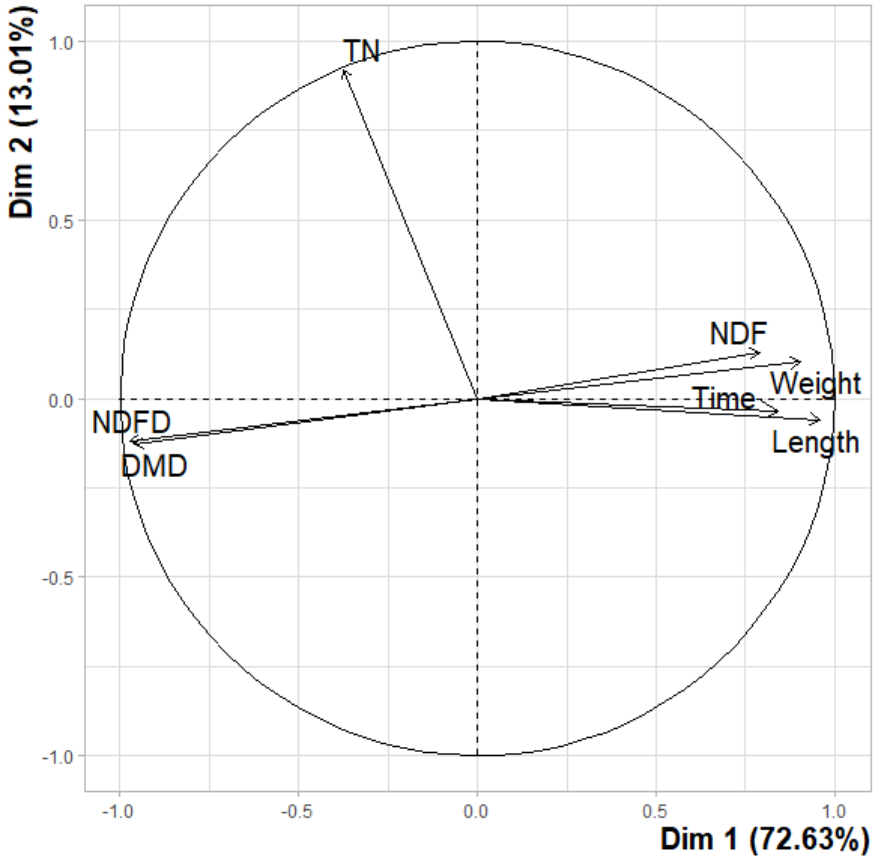

Figure 37: Representation of the two first Principal components of a principal component analysis (PCA) made on full stem data.

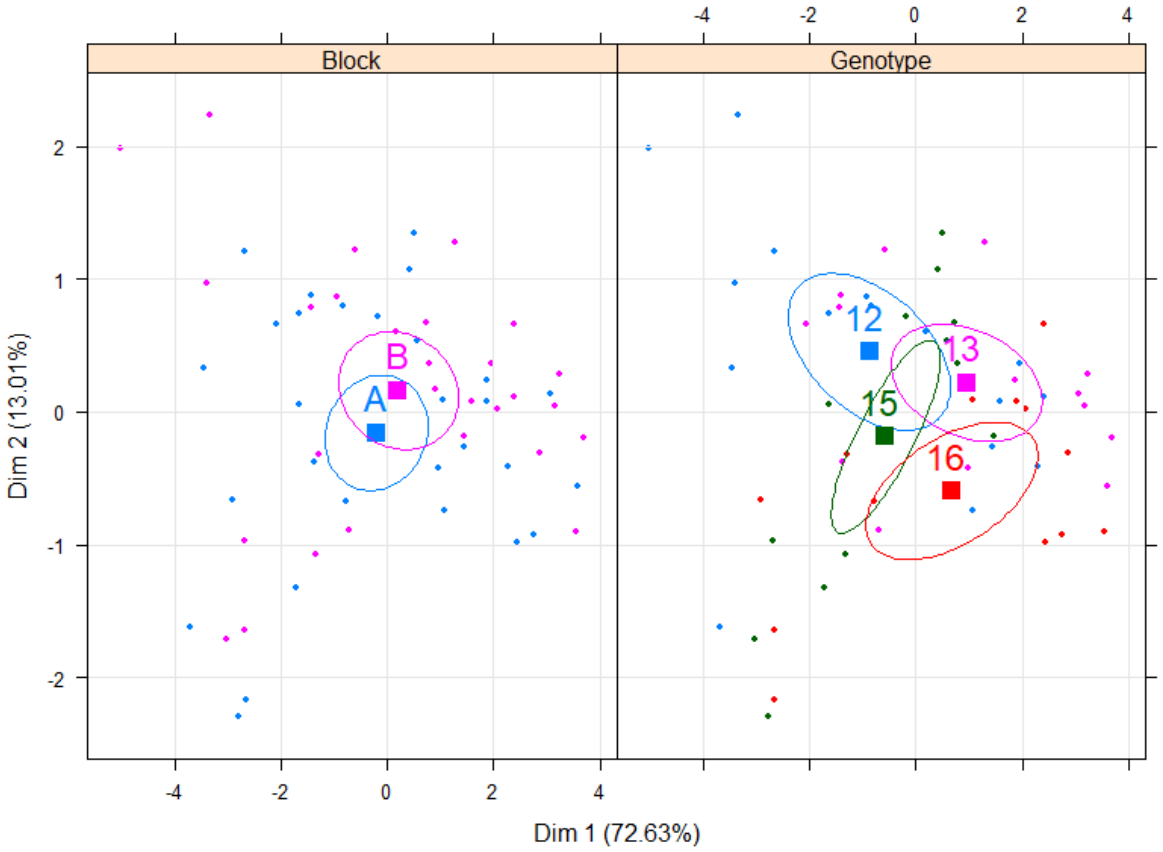

Figure 38: Block and Genotype projection on the two first Principal components of a principal component analysis (PCA) made on full stem data

3.3.2. Origin of DMD variability

3.3.2.1. In leaves: Senescence effect and leaf length

DMD was highest from the first harvest and stayed maximum (DMD of 90% of DM) until a dramatic decrease at the beginning of senescence followed by a plateau (DMD of 50% of DM) (Figure 25 and 26).

Senescence was highly negatively correlated to dry-matter digestibility (DMD) for each season (-0.88 in spring and -0.92 in autumn 2019 with p-value<0.05) (Figure 39) and explained a large part of DMD variability (about 80%). In spring, we observed no significant difference of DMD decreasing rate with senescence between genotypes. But, in autumn, we observed a higher DMD decreasing rate with senescence for the genotype 16 than genotypes 12 and 15. This decrease of DMD with senescence was highly link with both the increase of NDF (Figure 40) and the decrease of NDFD (Figure 41). NDF and NDFD showed a negative correlation in spring and autumn 2019 (Figure 42), except in autumn for NDF higher than 55 %DM and NDFD lower than 65 %NDF.

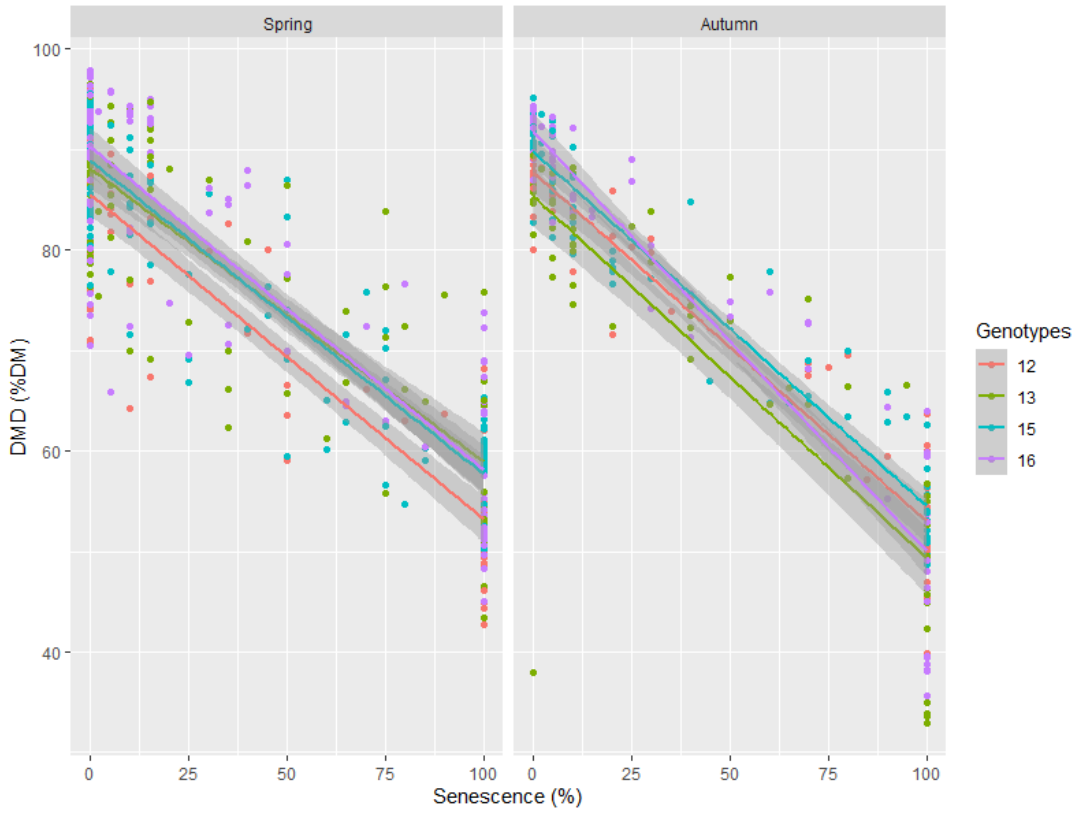

Figure 39: Variation of dry matter digestibility (DMD) of leaf samples as a function of senescence for each genotype in spring and autumn 2019

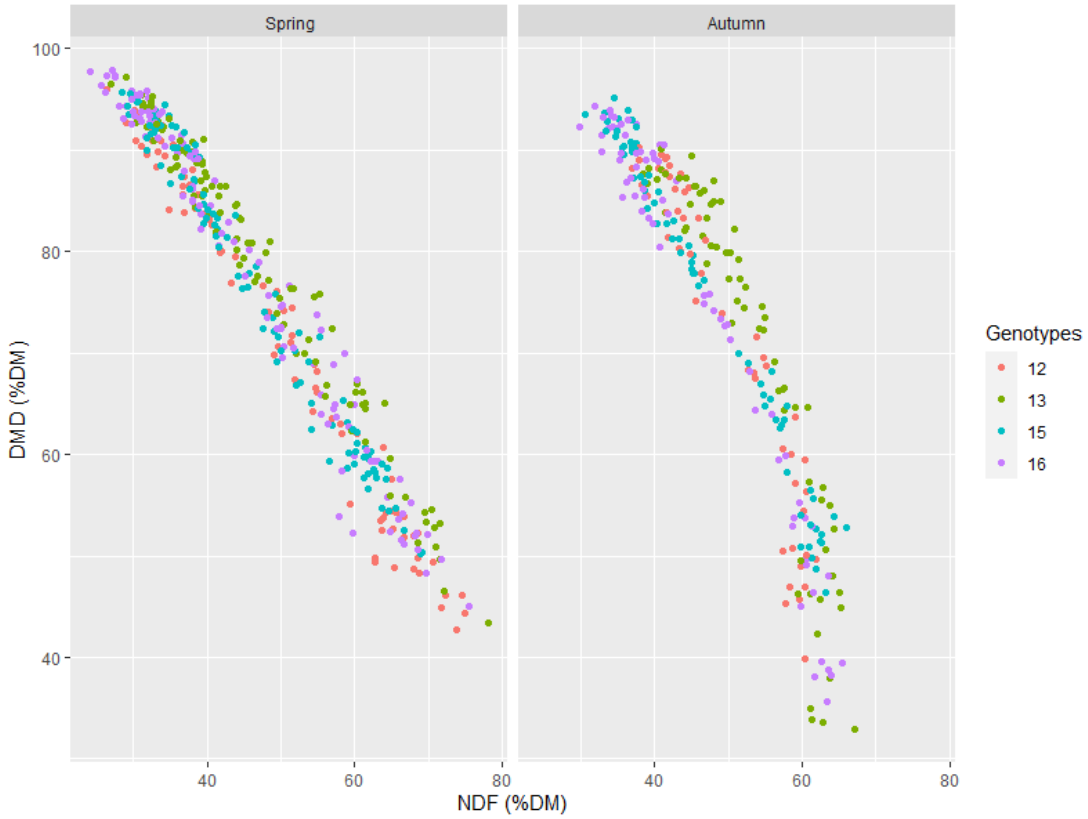

Figure 40: Variation of dry matter digestibility (DMD) of leaf samples as a function of fibre content (NDF) for each genotype in spring and autumn 2019

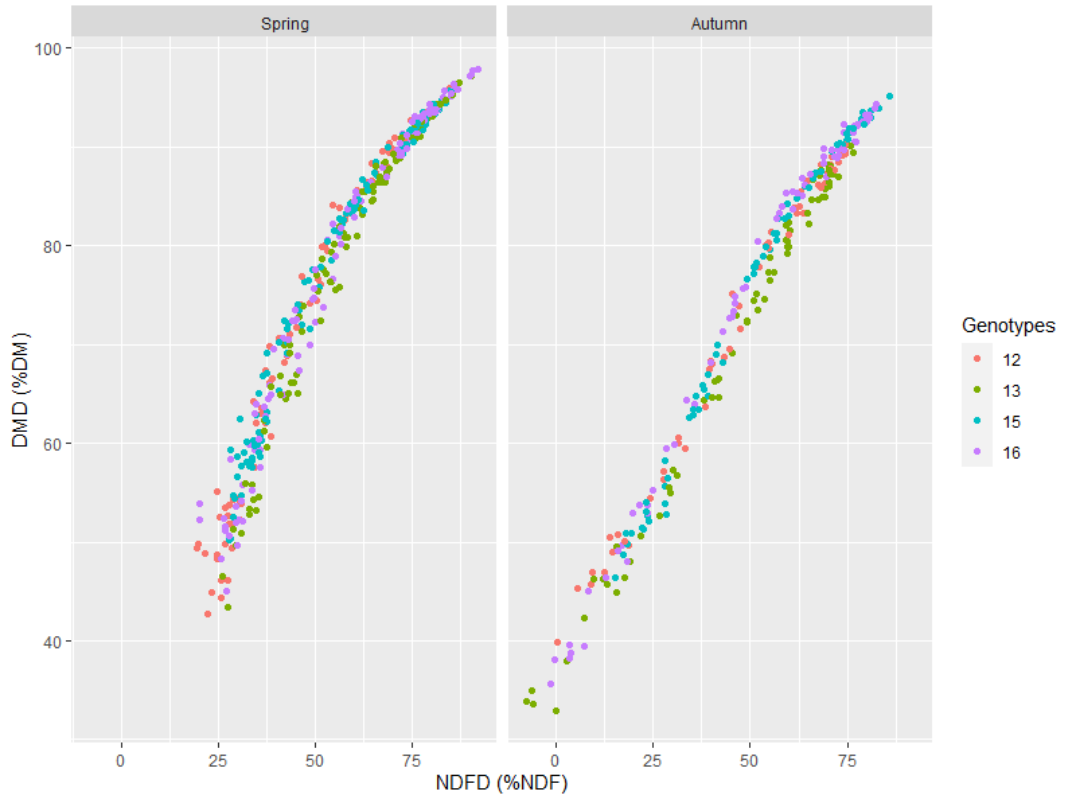

Figure 41: Variation of dry matter digestibility (DMD) of leaf samples as a function of fibre digestibility (NDFD) for each genotype in spring and autumn 2019

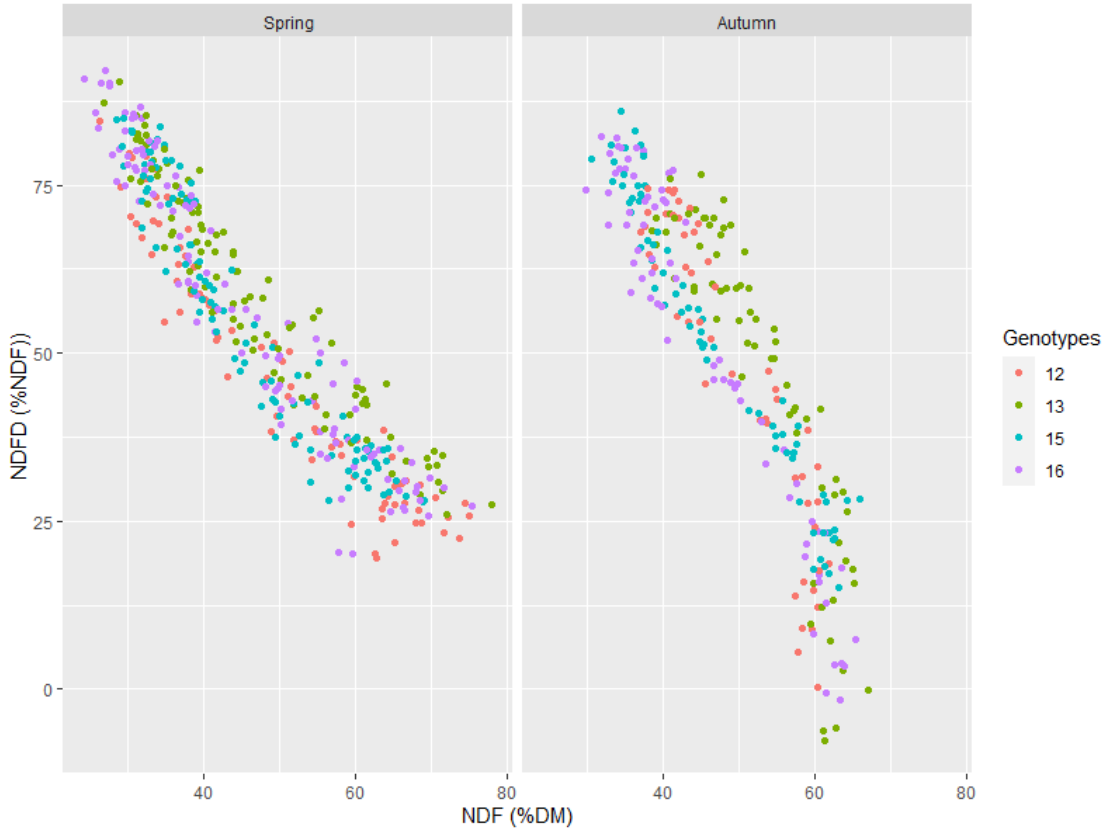

Figure 42: Variation of fibre content (NDF) of leaf samples as a function of fibre content (NDF) for each genotype in spring and autumn 2019

Focusing on non-senescent leaves, we investigated on the relationships between the highest value of DMD obtained for each leaf and others measured physiological parameters such as leaf mature length and sheath proportion. In spring 2019, we observed a negative correlation between DMD and leaf maximal length for all genotypes (Figure 43, -0.82 with p-value<0.05) and with sheath proportion (Figure 44). A negative correlation was spotted between DMD and sheath proportion (-0.81 with p-value<0.05) with a weaker slope for the genotype 12. We also observed, in spring 2019, a positive correlation between leaf maximal length and sheath proportion (0.68 with p-value>0.05) with a slightly weaker slope for the genotype 12. No correlation was observed in autumn 2019.

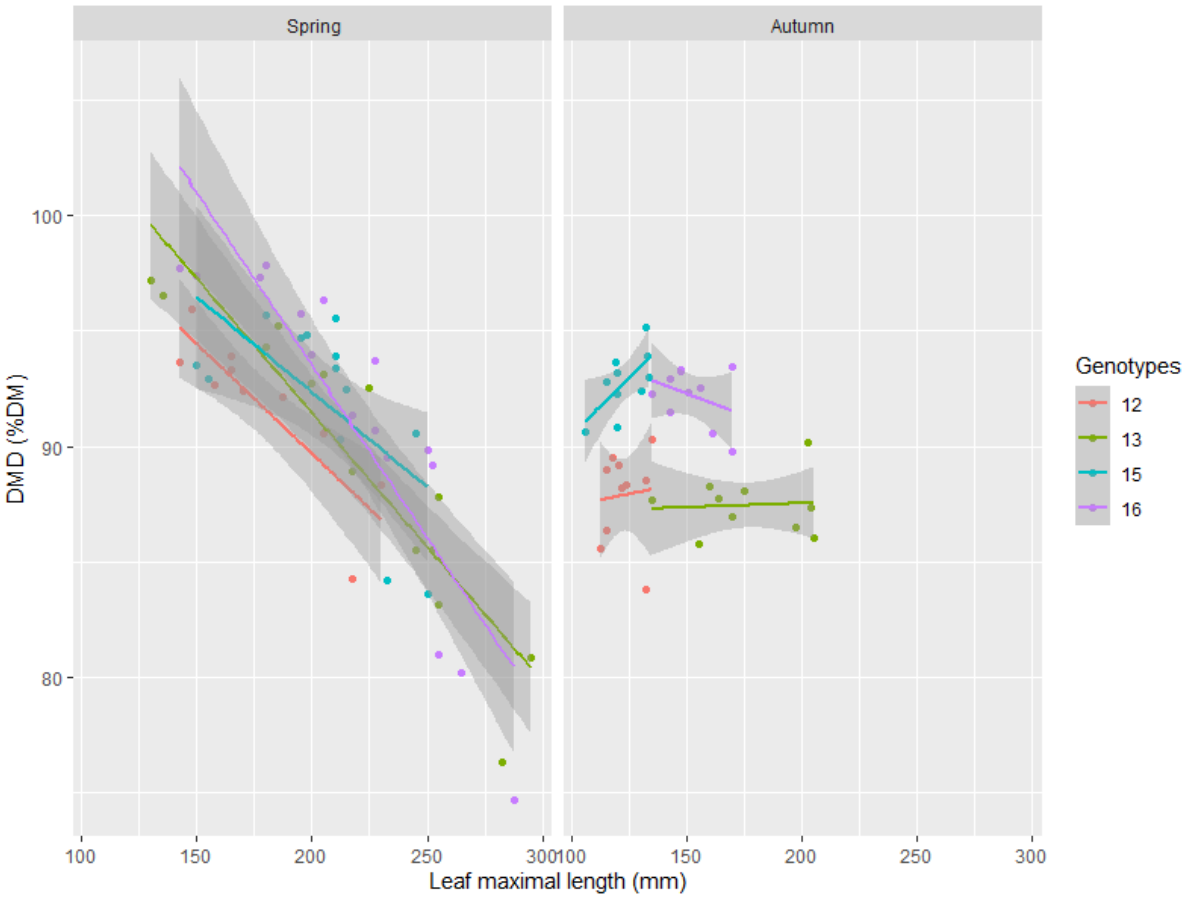

Figure 43: Variation of dry matter digestibility (DMD) of leaves as a function of leaf maximal length for each genotype in spring and autumn 2019

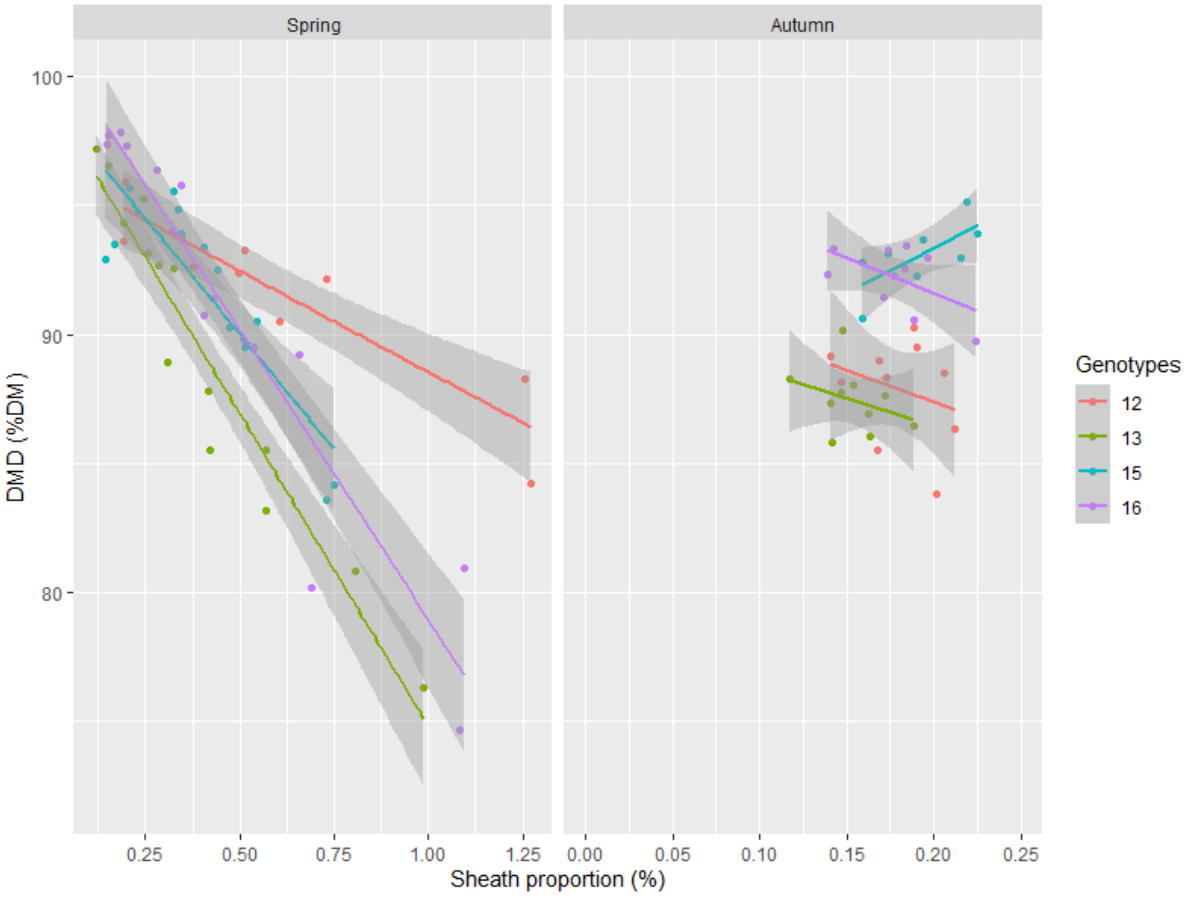

Figure 44: Variation of dry matter digestibility of leaves as a function of sheath proportion for each genotype in spring and autumn 2019

3.3.2.2. In stems: structural tissues and their digestibility

The rate of stem DMD decrease with time was significantly lower for genotype 15 than for genotypes 12 and 13 (Figure 35). At a medium time, stem DMD of genotype 13 was lower than genotypes 15 and 16 with genotype 12 being intermediate. The rate of stem NDF decrease with time was not significantly different between genotypes (Figure 46). Stem NDF of genotype 13 showed higher level of NDF than genotype 15 with genotypes 12 and 16 being intermediate. The rate of stem NDFD decrease with time was significantly lower for genotype 15 than for genotypes 12 and 13 (Figure 46). At a medium time, stem NDFD of genotypes 12 and 13 were lower than genotypes 15 and 16.

This decrease of DMD with time was highly link with both the increase of NDF (Figure 48) and the decrease of NDFD (Figure 48). NDF and NDFD showed a negative correlation (Figure 49).

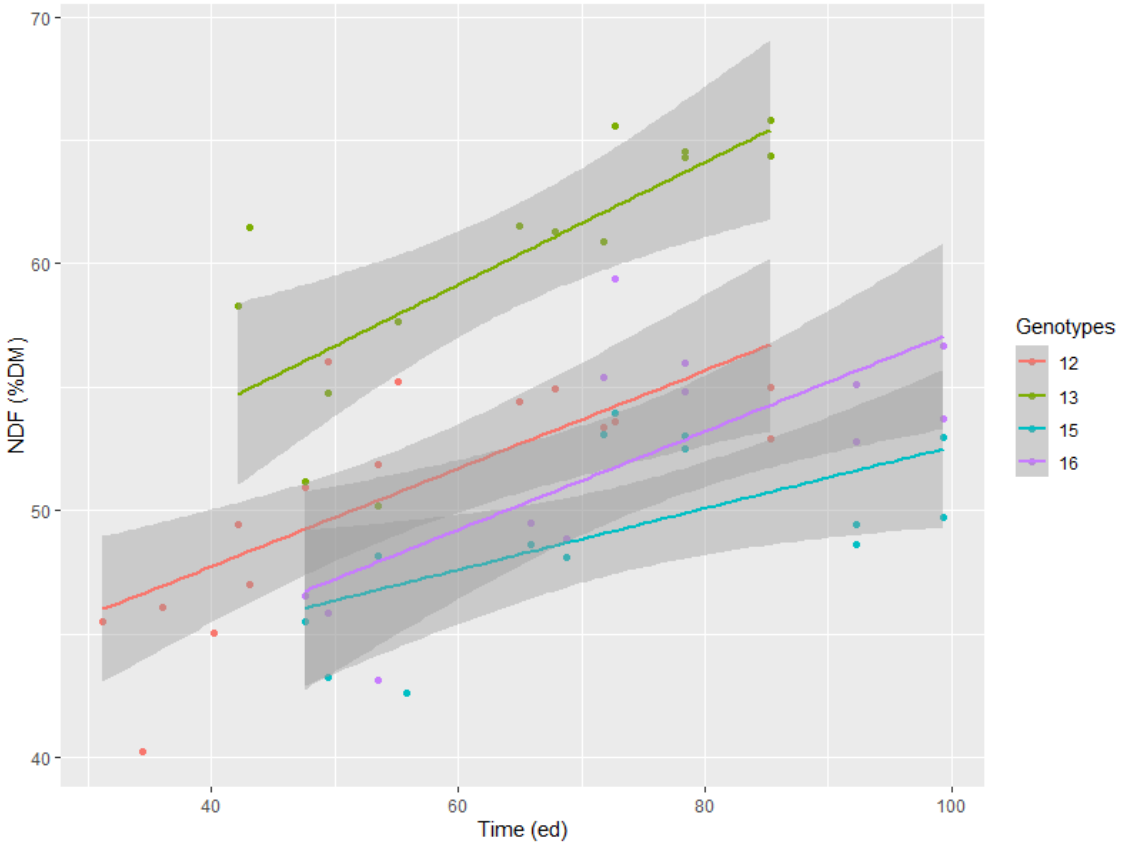

Figure 45: Fibre content (NDF) of stems as a function of time for each genotype

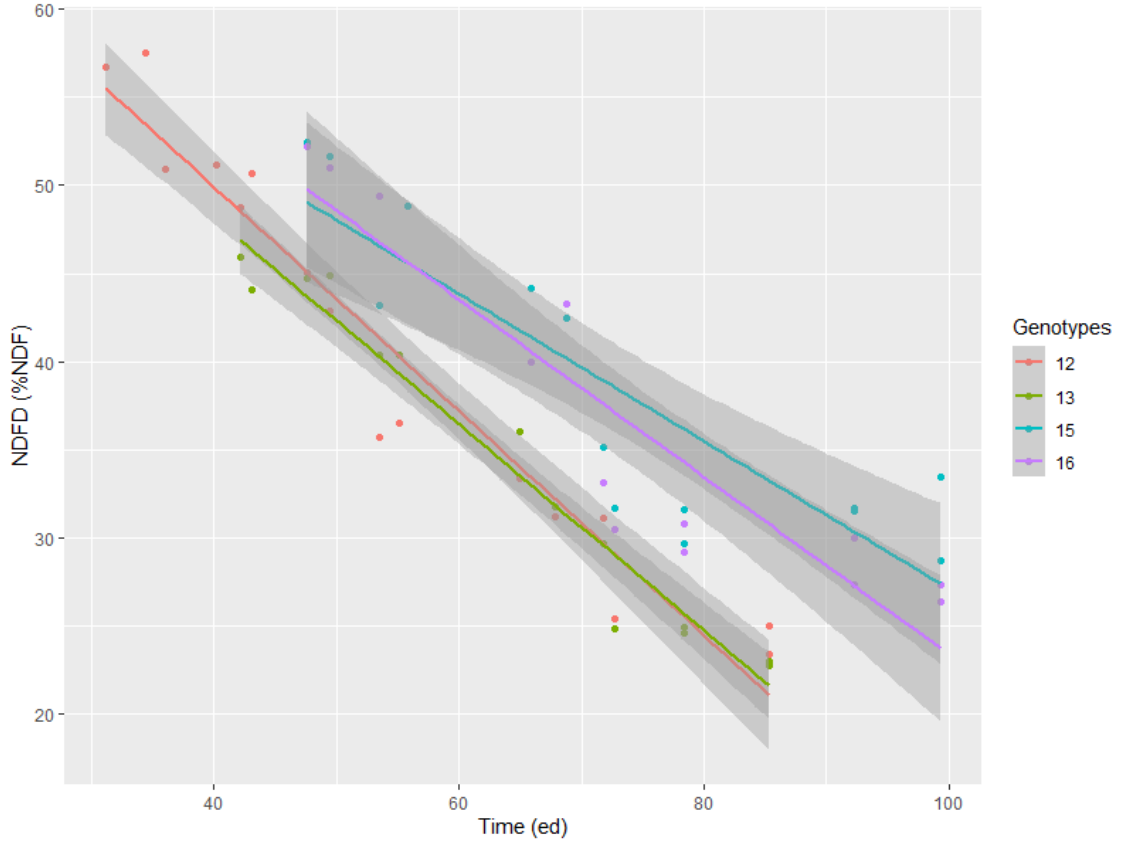

Figure 46: Fibre digestibility (NDFD) of stems as a function of time for each genotype

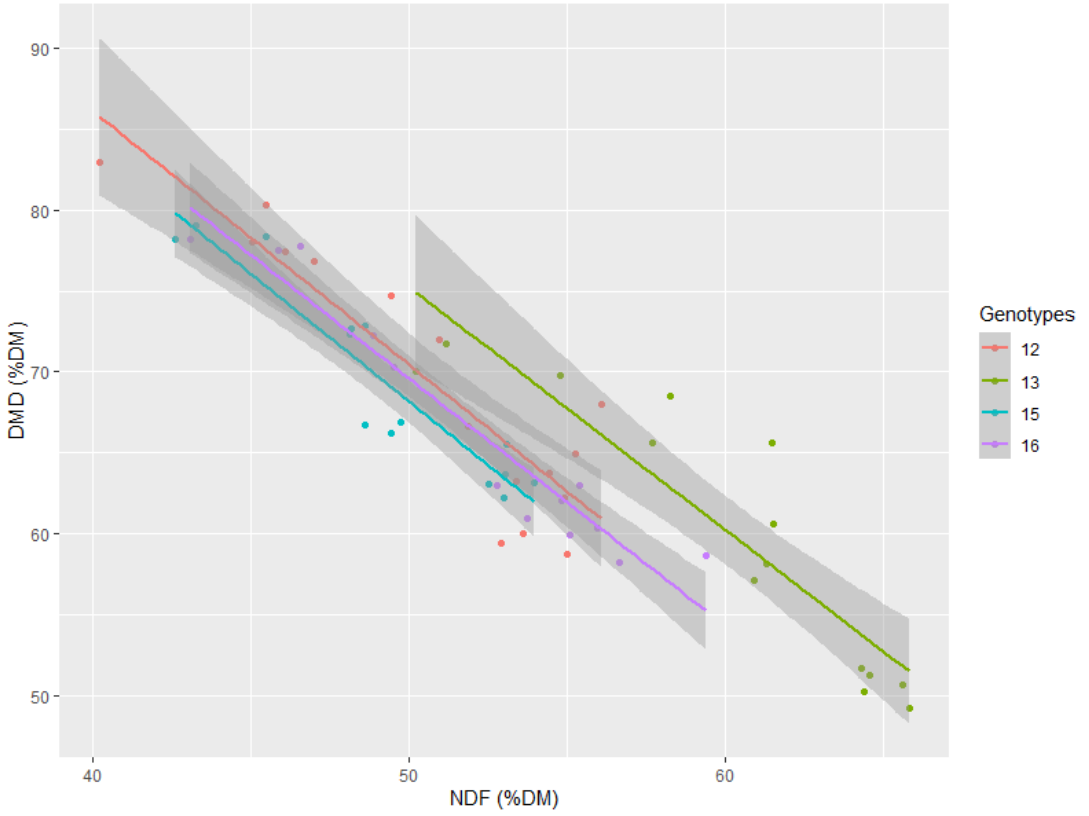

Figure 47: Dry matter digestibility (DMD) of stems as a function of fibre content (NDF) for each genotype

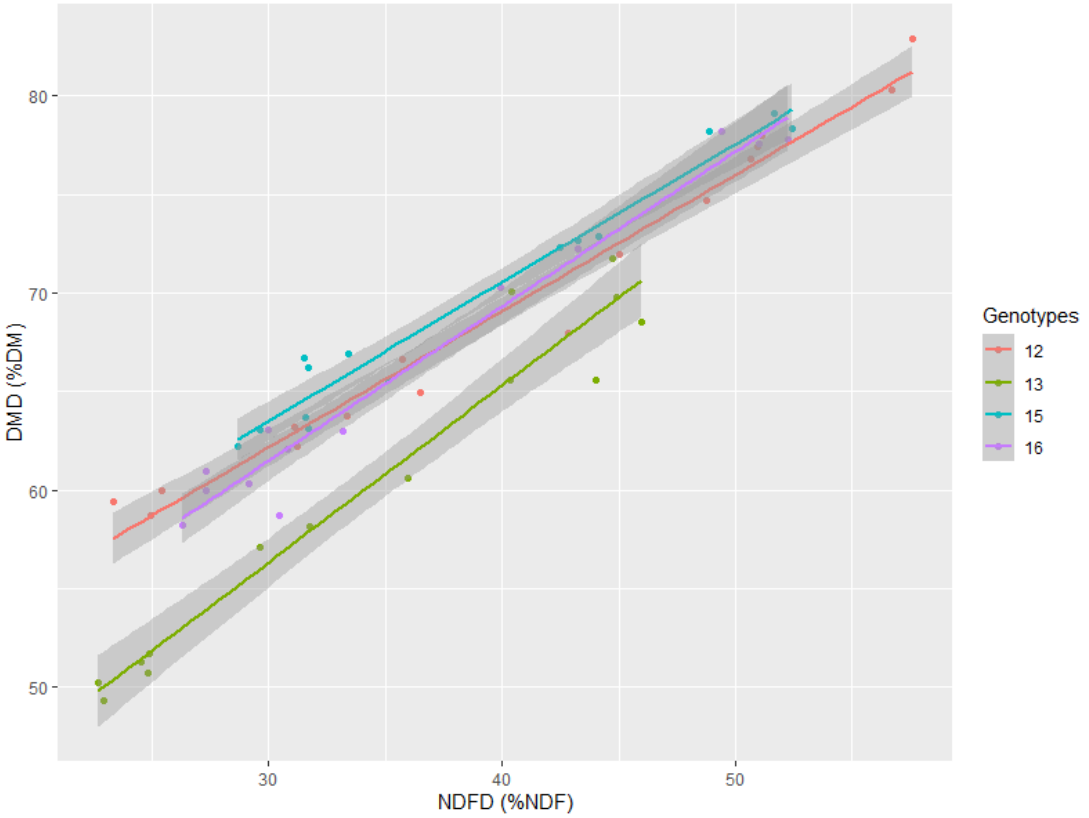

Figure 48: Dry matter digestibility (DMD) of stems as a function of fibre digestibility (NDFD) for each genotype

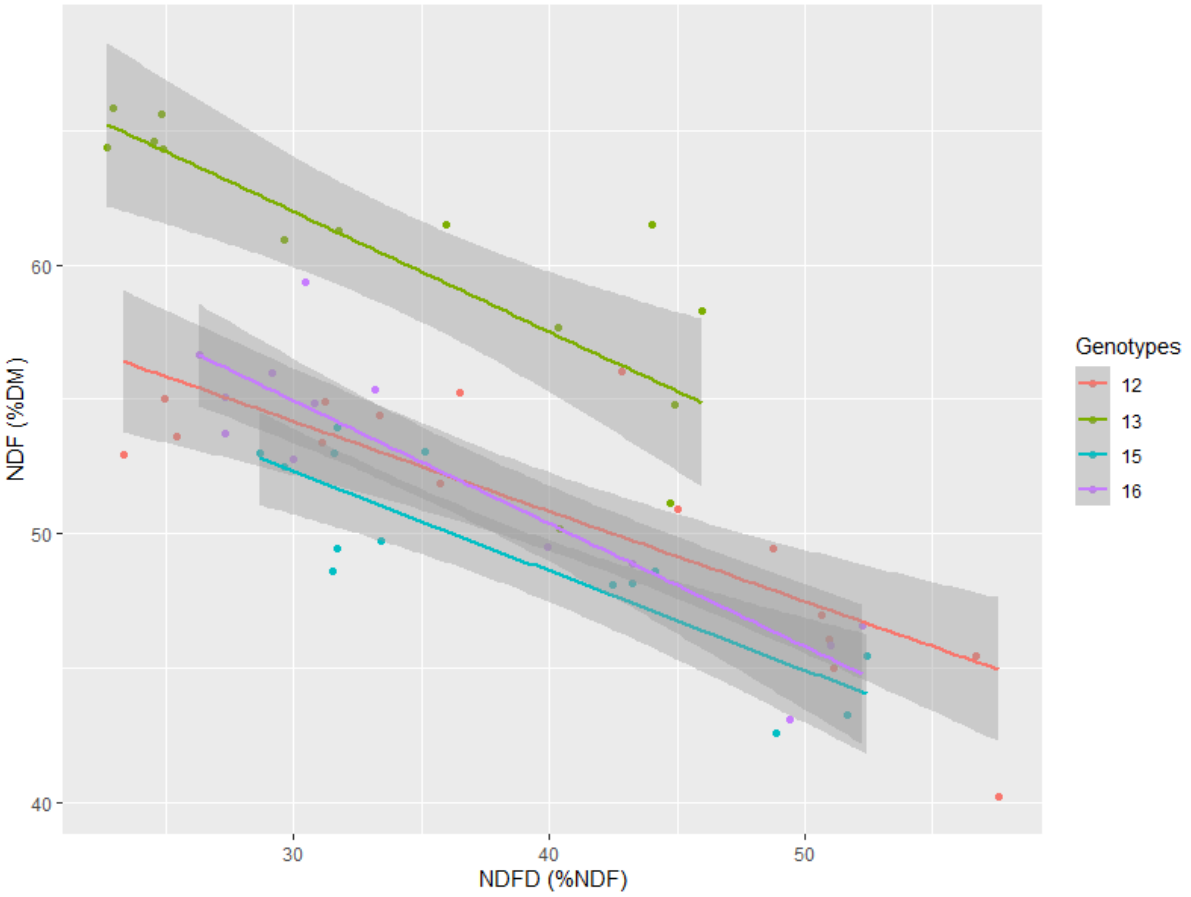

Figure 49: Fibre content (NDF) of stems as a function of fibre digestibility (NDFD) each genotype

3.3.3. Consequences of DMD variation at the tiller level

Using the data obtained on the different leaves and on the stems, we calculate the weight and the DMD of an average tiller over time.

In spring, the DMD of an average tiller of genotype 13 decreased more rapidly than genotype 15 with genotypes 12 and 16 being intermediate (Figure 50). The dry weight of an average tiller of genotype 15 increased less rapidly than the other genotypes (Figure 51). For all genotypes, DMD decreased with an increase of dry weight (Figure 52). Nevertheless DMD decreased less rapidly with increasing dry weight for genotype 16 than the others.

In autumn, the DMD of an average tiller of genotypes 12 and 13 decreased more rapidly than genotypes 15 and 16 (Figure 50). No variation of dry weight of an average tiller was observed over time (Figure 51). Moreover, no correlation was observed between DMD and dry weight (Figure 52).

Different scenarios of defoliation were made to evaluate the quantity and the quality of biomass produced by a tiller (Table 2). Scenario 1: a cut at the spike emergence date showed a higher DMD for the genotype 12 than the others but with a lower dry weight. Scenario 2: an early cut in spring showed a high DMD for all genotypes except the 12 which presented the highest biomass. Scenario 3: a late cut in spring showed a better DMD for the genotypes 15 and 16 than the genotypes 12 and 13. The genotype 12 presented a higher biomass. Scenario 4: In spring, for a low harvested biomass (125 mg/tiller), the genotype 12 was the first to reach the weight, followed by the genotypes 13 and 16, then by the genotype 15. The quality didn't show high differences between genotypes. Scenario 5: In spring, for a high harvested biomass

(625 mg/tiller), the order of the genotypes to reach the weight was the same as for a low harvested biomass, but we observed a difference of quality between genotypes with the genotype 16 showing a higher quality than genotypes 13 and 15, with the genotype 12 as intermediate. Scenario 6 and 7: In autumn, for an early (6) and a late (7) cutting date, the genotypes 15 and 16 had higher DMD than the genotypes 12 and 13.

For all genotypes, the harvested digestible biomass increased with the harvested biomass but more and more slowly (Figure 53). For a given harvested biomass, genotype 16 showed the highest harvested digestible biomass followed by genotype 12 , 15 and 13. This biomass was reached first by genotype 12 followed by genotypes 13 and 16 and later by genotype 15. In spring, genotype 12 produced higher digestible biomass than the other genotypes at the beginning of the regrowth but it was caught up by genotype 16 later (Figure 54). For example, almost at the same time, genotype 16 and 12 produced 0.4 g per tiller of digestible biomass but from 0.54 g of biomass for genotype 16 before heading and from 0.69 g of biomass for genotype 12 after heading.

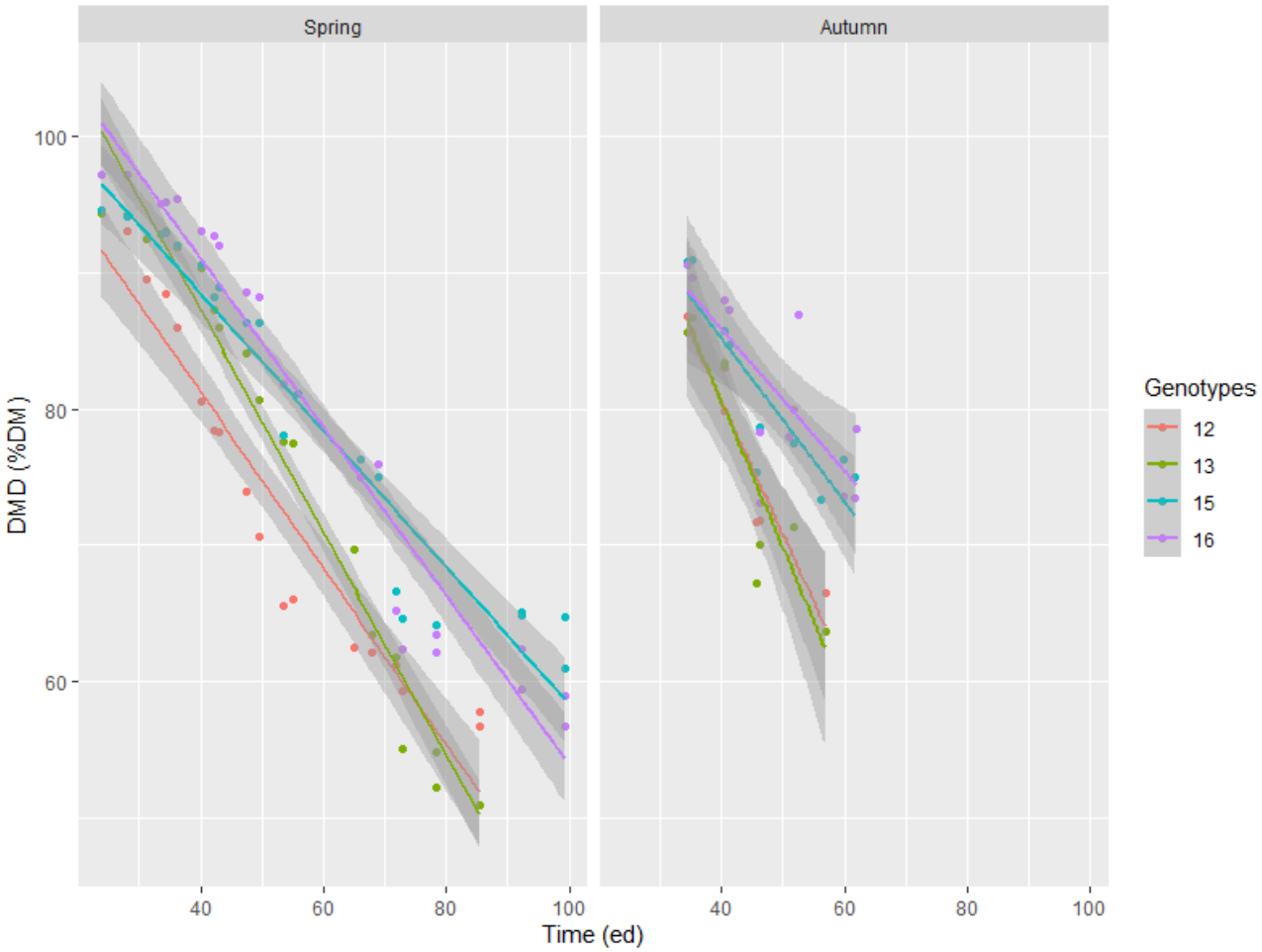

Figure 50: Variation of dry matter digestibility (DMD) of an average tiller as a function of time in effective days (ed) for each genotype in spring and autumn 2019

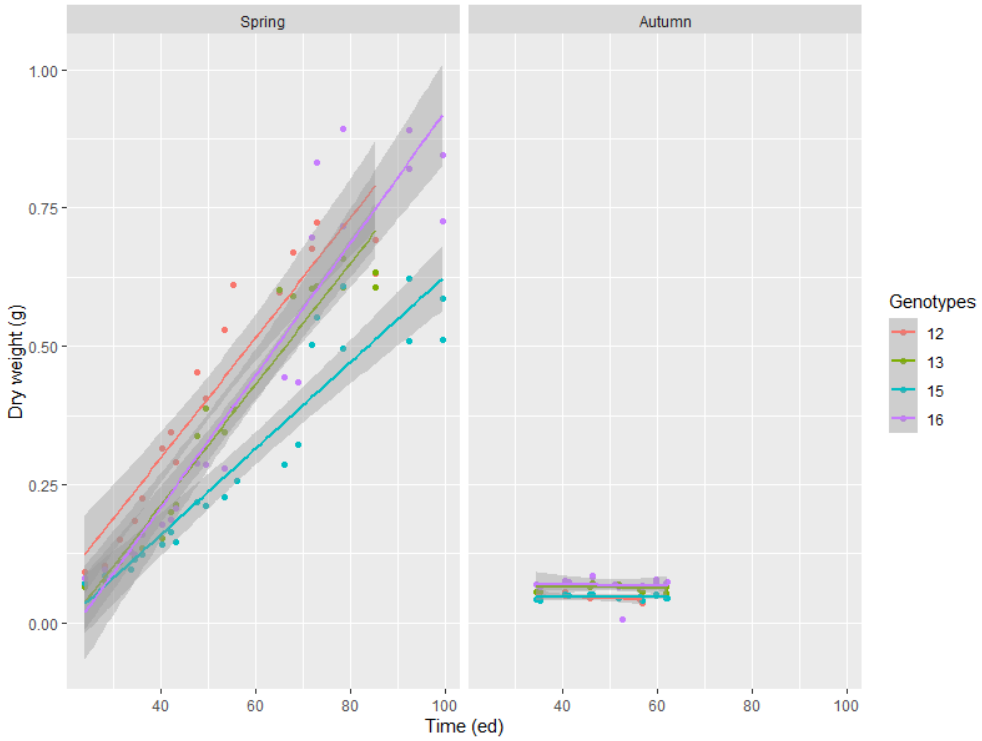

Figure 51: Variation of dry weight of an average tiller as a function of time in effective days (ed) for each genotype in spring and autumn 2019

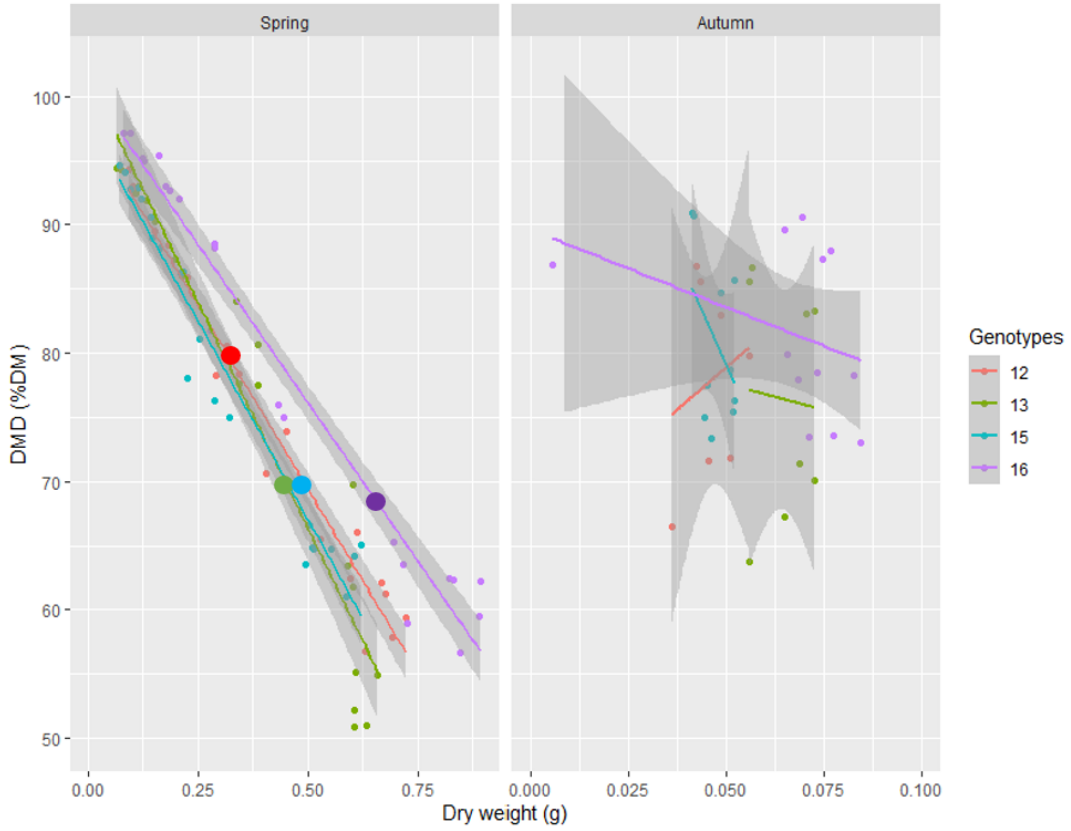

Figure 52: Variation of dry matter digestibility (DMD) of an average tiller as a function of dry weight for each genotype in spring and autumn 2019. Dots are placed at values obtained at spike emergence date with colours matching lines.

Table 12: Values of time, dry matter digestibility and dry weight of a averaged tiller cut following different scenarios for each genotype. In grey are the values set by the scenarios.

Genotypes		12				13			
Seasons	Defoliation scenarios	Time (ed)	DMD (%DM)	Dry weight (g)	Digestible dry matter (g)	Time (ed)	DMD (%DM)	Dry weight (g)	Digestible dry matter (g)
Spring	1/ Cut at spike emergence	43	79.3	0.33	0.26	62	69.3	0.45	0.31
Spring	2/ Cut at 30 ed	30	87.7	0.19	0.17	30	95.4	0.10	0.10
Spring	3/ Cut at 80 ed	80	55.4	0.73	0.41	80	54.6	0.65	0.36
Spring	4/ Cut to obtain 0.125 g	27.2	90.9	0.13	0.11	33.3	92.8	0.13	0.12
Spring	5/ Cut to obtain 0.625 g	67.9	62.3	0.63	0.39	76.2	57.6	0.63	0.36
Autumn	6/ Cut at 35 ed	35	85.4	0.05	0.04	35	85.8	0.06	0.06
Autumn	7/ Cut at 55 ed	55	65.9	0.04	0.03	55	64.5	0.06	0.04

Genotypes		15				16			
Seasons	Defoliation scenarios	Time (ed)	DMD (%DM)	Dry weight (g)	Digestible dry matter (g)	Time (ed)	DMD (%DM)	Dry weight (g)	Digestible dry matter (g)
Spring	1/ Cut at spike emergence	76	70.4	0.44	0.31	76	68.8	0.64	0.44
Spring	2/ Cut at 30 ed	30	93.5	0.08	0.08	30	97.2	0.09	0.09
Spring	3/ Cut at 80 ed	80	68.4	0.47	0.32	80	66.3	0.69	0.46
Spring	4/ Cut to obtain 0.125 g	38.1	90.2	0.13	0.11	35.7	94.7	0.13	0.12
Spring	5/ Cut to obtain 0.625 g	95.3	59.4	0.63	0.37	73.1	70.0	0.63	0.44
Autumn	6/ Cut at 35 ed	35	88.1	0.05	0.04	35	88.4	0.07	0.06
Autumn	7/ Cut at 55 ed	55	76.2	0.05	0.04	55	78.1	0.07	0.05

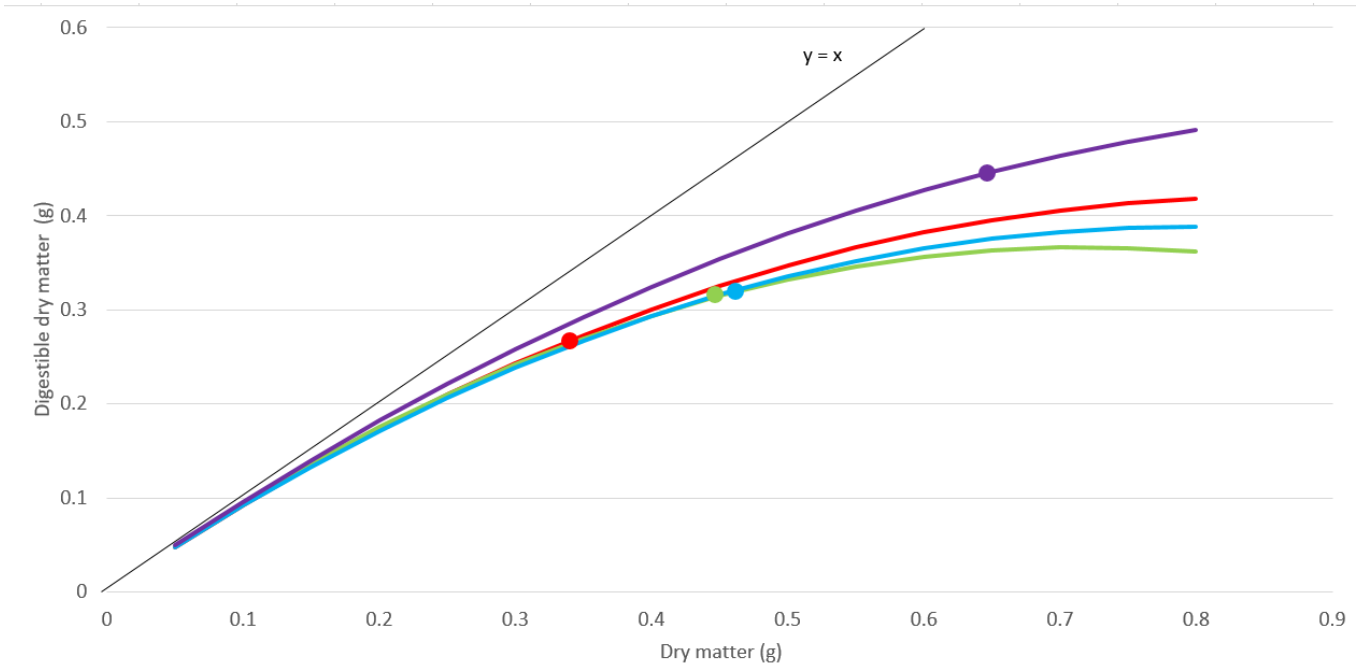

Figure 53: Predicted digestible dry matter as a function of dry matter for genotype 12 (red), 13 (green), 15 (blue) and 16 (purple) in spring. Dots are placed at values obtained at spike emergence date with colours matching lines.

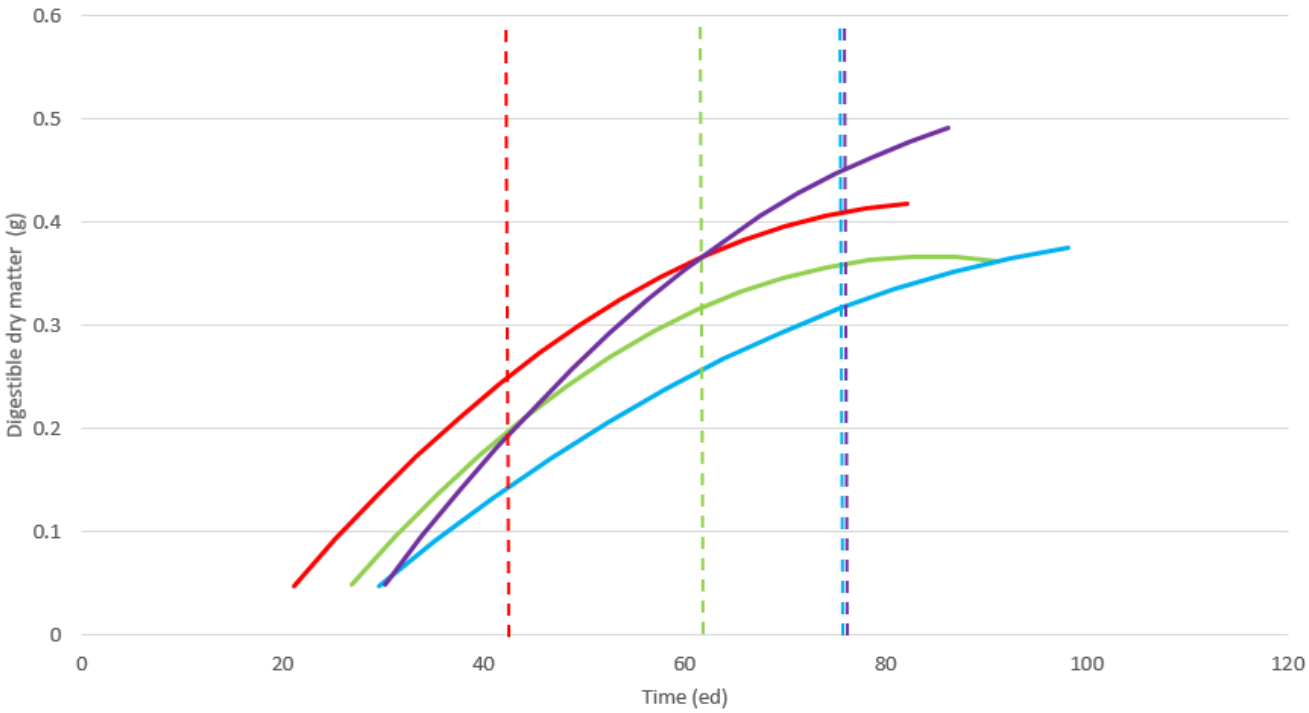

Figure 54: Predicted digestible dry matter (solid lines) as a function of time for genotype 12 (red), 13 (green), 15 (blue) and 16 (purple) in spring. Dotted lines indicates the date of spike emergence for each genotype.

3.4. Discussion

3.4.1. Leaves morphogenesis and quality

In both seasons, the main factor affecting DMD of leaves over their **complete lifespan** was the senescence proportion explaining about 80 % of the variation (Figure 39). We observed the same negative correlation between DMD and the senescence proportion for all genotypes. This effect could be due to a decrease of water soluble sugars and nitrogen content with senescence (Gregersen et al., 2008).

In spring, the major factor of variation for **green leaf** digestibility (DMD) was the rank of the leaf (Table 10). The higher ranks were associated with lower DMD. This effect was directly linked to the length of the leaves and to the sheath proportion (Figures 30, 31, 43 and 44). This is often observed in forage grasses (Agnusdei et al., 2011; Groot et al., 1999; Insua et al., 2018). The global variation of leaf length, considering the whole leaf series, was observed mainly within genotypes in spring with a huge effect of stem elongation and mainly among genotypes in autumn (Figure 27).

Concerning the biochemical composition of the leaves, the variation of DMD was explained by both the fibre content (NDF) and its digestibility (NDFD) (Figures 41 and 42). This has been already observed in forage grasses (Jančík et al., 2008).

3.4.2. Stems morphogenesis and quality

Stems digestibility was almost always lower (DMD 65-80 %DM) than green leaves digestibility (DMD 75-97 %DM) (Figures 35 and 39). This is often observed in the literature but in some cases very young stems could have a better quality than mature leaves (Terry and Tilley, 1964). We didn't have a chance to observe this phenomenon certainly because the stems were harvested too late in order to have enough dry matter for chemical analyses. Moreover, we observed that stem quality decreased rapidly with maturity due a decrease of quality of the basal internodes and a lower quality of the spike and the peduncle (Figures 34 and 35). The rapid decrease of stem quality with maturity is known in forage grasses, but literature didn't show a lower quality of spike and its peduncle (Terry and Tilley, 1964; Wilman and Altimimi, 1982). The evolution of stem quality has a direct impact on the date of forage harvest which is preconized just before or at the spike emergence (Duru et al., 2000). At this stage, plants are still leafy and the stem quality has not dramatically dropped yet.

Interestingly, we observed that the rate of quality decrease with maturity was not the same among genotypes (Figure 35). This offers the possibility to select for stem quality at an advancing maturity. This has already been proposed by (Reich and Casler, 1985) on smooth bromegrass. This selection could produce new varieties with a less negative impact of stems on the harvested forage quality providing a higher flexibility on grassland management for the farmer.

3.4.3. Tillers morphogenesis and quality

Since the level and the evolution of leaf and stem quality differed among genotypes, it was essential to reconstruct tillers in order to evaluate the quality of the entire plant. By doing so, we observed in spring that the ranking of the genotypes for DMD at an early harvest was: genotype 16 = 13 = 15 > 12 and at a late harvest was: genotype 15 = 16 > 12 = 13 (Figure 50). This ranking doesn't correspond to previous data (Table 9) where the ranking was: 16 = 12 > 13 = 15. Nevertheless, at spike emergence, we observed a different ranking: genotype 12 > 13 = 15 = 16. This result emphasises clearly the importance of earliness and cutting management on the measured quality of genotypes which is well known by breeders (Buxton and Casler, 1993). In our case, the evolution of stem quality with maturity (Figure 35) was essential to explain the downgrading of genotype 13 among the other genotypes for quality.

The quality is important but it goes with the produced biomass. If we look at the harvested digestible biomass (Figure 51), we observed that the ranking of genotypes changed with at an early cut: genotype 12 > 13 = 15 = 16 and at a late cut: genotype 16 >= 12 > 13 = 15. Moreover, for a given harvested biomass genotype 16 provided a higher digestible biomass than the other genotypes (Figure 53). This late heading genotype 16, thanks to a rapid growth with long leaves, provided a good level of biomass per tiller (potentially yield) with a good quality. The late heading genotype 15 showed a higher quality than the others but produced less biomass which downgraded it when considering the harvested digestible biomass. We found out that a good way to compare genotypes for both quality and biomass, independently of the harvest date, would be to select for the ratio between the percentage of non-digestible dry matter and the yield (Figure 52) with the hypothesis than DMD tends to 100% when biomass tends to 0g. The genotype with the lowest value for the formula (100 - DMD) / biomass will be the best in digestible biomass production.

These results showed that in spring the ranking of genotypes for quality was largely dependent on the harvest date and maturity stage. Moreover, it is important to take into account not only the quality but also the biomass which increases on reproductive tillers (Korte et al., 1984).

In autumn, we observed no harvested biomass evolution with time, like the vegetative tillers in the study of (Korte et al., 1984). We observed a decrease of quality faster for genotypes 12 and 13 than genotypes 15 and 16 (Figure 50). For all genotypes, the harvested biomass in autumn was largely lower than the one harvested in spring. The ranking of genotypes for DMD didn't change over time and was similar to the one in spring at a given date: genotype 15 = 16 > 12 = 13 but different from the one in spring at spike emergence: genotype 12 > 13 = 15 = 16. These results showed that in autumn, the ranking of genotypes for quality was stable with time and the grasses had a better quality (and digestible biomass) for a cut as soon as the plant reached its maximal harvestable biomass.

In this study, we focused on the development of tillers and organs within tillers. It would be necessary to integrate the morphogenesis of many tillers to have a better evaluation of the plant quality and biomass. Plant modelling could be of great interest for this purpose.

3.5. Key points for chapter 3

- Senescence of leaves decreased strongly their quality.
- Leaf length and sheath proportion decreased green leaf quality.
- Stem quality was lower than leaf quality and decreased strongly with maturity.
- The stem quality and its decrease rate with maturity differed between genotypes allowing a potential selection for genotypes with higher quality of mature stems.
- The ranking of genotypes for quality was different for leaves and stems.
- In spring, a compromise should be made between biomass production and its quality. This compromise differed between genotypes and harvest dates. A good way to compare genotypes for both quality and biomass, independently of the harvest date, would be to select for the ratio between the percentage of non-digestible dry matter and the yield
- In autumn, plants should be harvested immediately after the biomass reached a maximum in order to obtain the best quality.

4. General discussion

In this study, we surveyed different sources of variation of forage quality in perennial ryegrass: genetic origins, seasons of harvest, plant organs and their age. As in other studies on grass forage species (Barrière et al., 2003; Casler and Vogel, 1999; Grabber et al., 2004), we observed that a significant part of the quality variation was explained by genetic variation which is encouraging for breeding. Nevertheless, we observed an interaction between genotype and season (spring and fall) so that the ranking of genotypes for leaf quality differed between spring and fall. This has the consequence that for breeding quality over the year, it would be essential to select for both spring and fall seasons. This could be obtained by including quality of both seasons in the selection index with other traits such as yield, disease resistance or persistency. Knowing that the yield in fall is considerably lower than the one in spring (Figure 55), once could wonder on the economics efficiency of selecting for fall quality. Considering the lack of forage in fall, we think it could be worthy to have a high quality forage at this season. Moreover, the genetic variance in fall for quality was higher than the one in spring despite a larger environmental variance. A solution for breeding could be to give a larger weight in the index to spring forage quality than to the fall one. Another point concerning the difference between seasons was that the biochemical characteristics explaining the variation of leaf quality were different in both seasons. In spring, the variation of leaf quality was mainly explained by the hemi-cellulose and cellulose contents (highly negatively correlated together) whereas it was explained by lignin content in fall.

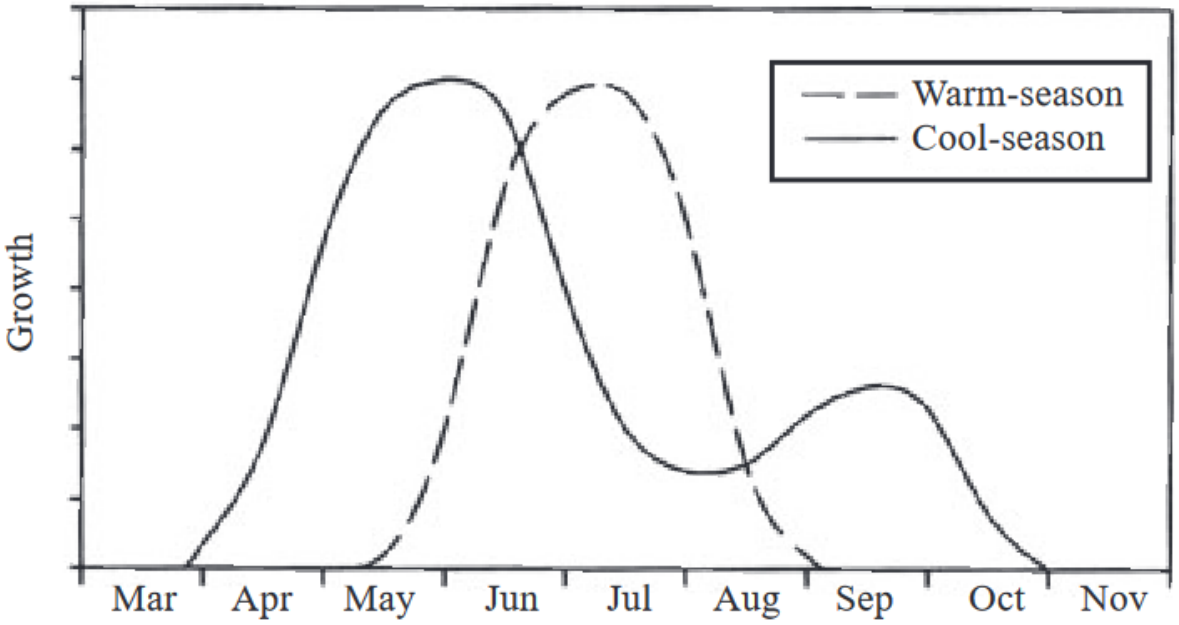

Figure 55: Seasonal growth distribution of cool (*e.g.* ryegrass, smooth bromegrass, orchardgrass, tall fescue) and warm season grasses (*e.g.* indiangrass, switchgrass, bermudagrass, bluestem) from Volesky et al. (2003)

In spring, there is a major difficulty to rank the genotypes for their quality potential because quality, biomass and heading date are strongly correlated (Beecher et al., 2018; Carrère et al., 2010). Indeed, the reproductive stage decreases the quality of the harvest biomass through two processes: the production of longer leaves with a higher sheath/leaf length ratio and the production of stems, which have a lower quality than leaves. Effectively, we observed that comparing genotypes harvested at spike emergence (different dates of harvest for each genotype), the late heading genotypes presented a lower quality than the early heading genotypes. Whereas comparing genotypes harvested just before spike emergence of the late heading genotypes (same date of harvest for all genotypes), the late heading genotypes presented a higher quality than the early genotypes which contained already stems of low quality. This phenomenon is well known and is one of the reason that genotypes are clustered in precocity groups to be compared for variety registration by CTPS. Nevertheless, genetic variation for quality, biomass production and heading date remains present within the precocity groups hampering an optimal ranking of genotypes for quality or yield. The survey of quality and biomass of each organ during plant maturity allowed us to follow the relationship between plant quality and biomass. From this, we found out that **a good way to compare genotypes for both quality and biomass, independently of the harvest date, would be to select for the ratio between the percentage of non-digestible dry matter and the yield**.

Also in spring, we observed that it should be possible to select for stem digestibility. Effectively, differences of stem quality and its decrease rate with aging were observed between genotypes. For example, genotype 13 showed leaves of high quality but stems of low quality whereas genotype 15 showed high leaf and stem quality. This result is particularly interesting since it could enhance the creation of varieties with better stem quality allowing more flexibility for the date of harvest. The farmer could harvest his grassland after spike emergence keeping a relatively high forage quality and a good yield with **the presence of "heavy" digestible stems**.

In autumn, it was easier to compare genotypes for their quality potential since there was no confounding effect such as heading date in spring. We observed that the plant biomass increased rapidly up to a physiological stage of about three mature uncut leaves and then stayed stable, the production of new leaves balancing the senescence of others. Since the main factor decreasing leaf quality was the senescence, **it would be better to harvest when reaching the third uncut mature leaf in order to optimize the yield and the quality of the harvested forage**. This is the grassland management advised in dynamic rotating grazing: Herby method (Cliquet et al., 2019).

In both seasons, we observed that digestibility was explained by both cell wall content and its digestibility. Selection could be applied directly on organic matter digestibility but it would be **more efficient to select against cell wall content and for cell wall digestibility** keeping in mind that for ruminants it is healthy to be fed with forage containing a certain amount of digestible fibres (for milk production, diet should containing a minimum of 27 % DM as fibres (Woodford et al., 1986)) rather than soluble components (sugars, proteins).

From this study, we can have a better understanding of quality kinetics at organ and tiller level. However, to apprehend the evolution of quality at plant level, which consists of many tillers of different ages, or at field level, it is necessary to use a plant growth model. This model will have to simulate each aerial organ (leaf with blade and sheath, stem) and their growth rules before adding their quality. The L-grass model presented in introduction matches these requirements, so we will explain how it is possible to improve this model by quality integration. It will allow simulating the quality of a simulated plant (entirely or only harvested part) during its growth.

First, it will be necessary to add two new parameters for leaf modules: dry matter digestibility (DMD) and senescence ratio. The senescence ratio of the leaf n will start at a value of 0 % and will start to decrease when the leaf n+3 appears. A value for DMD will be attributed for each leaf at its creation. This value will depend on four factors: the maximal leaf length, the leaf rank, a coefficient depending on genotype, and one other depending on season. A fifth factor is the senescence ratio in order to decrease the DMD value with the senescence progression. A leaf will be removed when its value for senescence ratio reaches 100 %.

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Annexe 1: Communication at the 34th meeting of the EUCARPIA Fodder Crops and Amenity Grasses Section

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Variability for organic matter digestibility at heading date in a collection of perennial ryegrass genotypes

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Abstract. Digestibility is essential to determine the value of forage perennial ryegrass cultivars. We observed a large variability for Organic Matter Digestibility (OMD) in a panel of 580 perennial ryegrass genotypes when harvested at heading date. This variability was explained by the proportion of blades and by both, the cell wall content (NDF content) and its digestibility (NDFD). These last two traits showed low correlation in this experiment. The broad sense heritabilities of OMD, NDF content and NDFD were 0.77, 0.76 and 0.73, respectively. Blades showed slightly higher OMD than 'stems' (sheaths and flowering stems) but some plants 'stems' showing higher OMD than their blades. These results found on the whole collection were also obtained within maturity groups and are encouraging for breeding for high OMD *via* a decrease of NDF content and an increase of NDFD in both blades and 'stems'.

Keywords: digestibility, cell wall content, *Lolium perenne* (L.).

1 Introduction

Digestibility of organic matter (OMD) is a major selection criterion in forage grass breeding [1]. Indeed, OMD influences fodder intake, milk production and meat production. For example, 1% increase of OMD generally leads to a 3.2% increase in average daily gains of beef cattle [2]. Organic matter consists of water-soluble components, which are almost completely digestible, and cell walls (NDF content) which are only partially digestible. Increase in OMD could be obtained by decreasing NDF content and/or by increasing the cell wall digestibility (NDFD).
The objective of this study was to survey the variability of OMD in a large set of perennial ryegrass genotypes. As OMD varies throughout the season according to the developmental stage of the ryegrass plant, we monitored closely the development of each plant and harvested plant materials for biochemical analysis when each particular plant had reached the 'heading stage'. The origin of this variability was explored: NDF content *versus* NDFD and blades *versus* 'stems' (sheaths and flowering stems).

2 Material and methods

A set of 580 perennial ryegrass genotypes (209 from natural populations) [3] was cultivated outdoors in pots (12 L) through vegetative multiplication at Melle in Belgium starting at the beginning of March 2012. The experiment was set-up as a randomized block design with three replicates. At its respective heading date, each plant was harvested and the plant material was split in two fractions: blades and 'stems' (sheaths and flowering stems). Each part was dried for 48 h at 70°C and grinded. NIRS was used to determine the organic matter content (OM), the organic matter digestibility (OMD) and the NDF cell-wall content [5]. Digestibility of NDF (NDFD) was calculated as 100 - 100 x (100 - OMD)/NDF [5, 6]. Correlation tests, linear models and heritabilities were computed using R software. Heritabilities estimation used a mixed linear model with genotypes as random effect and block as fixed effect.

3 Results and discussion

As expected, given the large genetic diversity present in perennial ryegrass, we observed a large phenotypic variability for quality traits (Table 1). The results are consistent with the findings of previous studies in which the high nutritive value of perennial ryegrass in comparison to other forage grasses was demonstrated, but showing also interesting variation range [1, 7, 8]. The variability of OMD was partly explained by heading date ($p<0.05$, $R^2=0.40$, not shown). In particular, a group of early plants (before May 26 or 900 GDD) showed a higher OMD (88% OM) than the rest of the genotypes (86% OM).

Table 1. Variability of traits. Std: standard deviation. GDD: growing degree days with 0°C as base temperature and starting March 1.

Traits	min	max	mean	std
Heading date (GDD)	700	1402	982	159
Blade proportion (% w/w)	23	93	47	9
OMD (% OM)	79	92	87	2
NDF content (% DM)	35	58	45	3
NDFD (% NDF)	61	80	70	2

The variability of OMD was partly linearly explained by the proportion of blades ($p<0.05$, $R^2=0.32$) because blades showed a slightly higher OMD than 'stems' (87% OMD *vs* 86% OMD) and OMD increased with blade proportion in both blades ($p<0.05$, $R^2=0.19$) and 'stems' ($p<0.05$, $R^2=0.20$). The higher digestibility of blades compared to 'stems' is generally observed in grasses [7]. In our case, this higher OMD of blades compared to 'stems' was due to a lower NDF content (42% DM *vs* 48% DM) but not to a higher NDFD (70% NDF *vs* 71% NDF) in blades compared to 'stems'. Nevertheless, we observed that for some plants, OMD were higher for 'stems' than blades, keeping in mind that the 'stems' were composed of sheaths and flowering stems which were not mature. A large variability of OMD remained within blades and 'stems', which could be explained by both NDF content and NDFD (Fig. 1). These findings for the whole collection were also obtained within maturity groups (not shown). It is worthy to note that there was low negative correlation following Kendall method between NDF content and NDFD in both blades and 'stems' ($p<0.05$, $tau=-0.21$ and -0.17, not shown). Finally, a large part of the variability of OMD, NDF and NDFD could be explained by genetic variability with broad sense heritabilities of 0.77, 0.76 and 0.73, respectively.

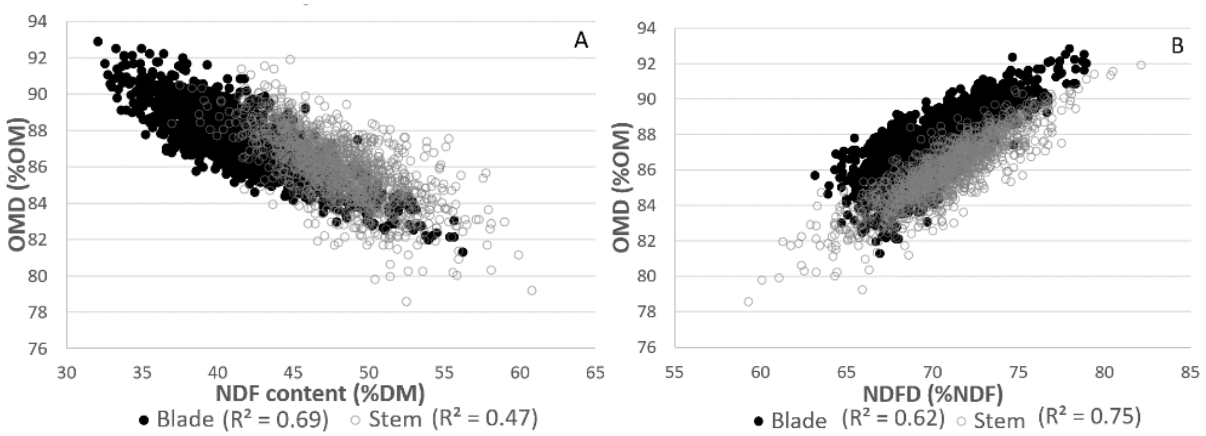

Fig. 1. Relationships between the digestibility of organic matter (OMD) and (A) the NDF content and (B) the NDFD in blades (black) and in 'stems' (grey).

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Annexe 2: Résumé français détaillé

Valeur nutritive des graminées fourragères : évolution au cours du développement et bases génétiques chez le ray-grass anglais

1. Introduction

1.1. Importance des prairies

Les prairies ont toujours été essentielles à l'alimentation des ruminants en leur fournissant une ration équilibrée en énergie et protéines pour un faible coût (Huyghe et al., 2014). Elles peuvent être pâturées ou fauchées pour ensuite être conservées sous forme de foin, par enrubannage ou par ensilage. En plus de leur intérêt en production de biomasse, les prairies apportent aussi divers services écologiques tels que la séquestration du carbone, la préservation de la qualité de l'eau, de la biodiversité et de la diversité des paysages.

Les prairies recouvrent 40% des surfaces agricoles en Europe en 2013, soit 60 millions d'hectares (van den Pol-van Dasselaar et al., 2019). Les prairies permanentes (= maintenues pendant plus de cinq années) représentent 85% des prairies, le reste étant composé de prairies temporaires qui sont semées régulièrement avec diverses espèces fourragères (graminées et/ou légumineuses).

1.2. Présentation générale du ray-grass anglais

Le ray-grass anglais, ou *Lolium perenne L.* appartient au genre *Lolium*, à la sous-tribu *Loliinae*, à la tribu *Poeae*, à la super-tribu *Poodae*, à la sous-famille *Pooideae* et à la famille *Poaceae* (http://lifemap-ncbi.univ-lyon1.fr/?tid=640630). Il s'agit d'une plante herbacée pérenne composée de racines, d'un plateau de tallage avec des entre-nœuds non allongés durant la phase végétative et de talles (une talle principale et plusieurs talles filles) (Figures 1 et 2). L'absence d'élongation des entre-nœuds lors de la phase végétative est un moyen de protéger les méristèmes apicaux des herbivores. Chaque talle est composée d'un méristème apical, de plusieurs nœuds, où se trouvent une feuille et un méristème secondaire, et d'entre-nœuds. Les talles filles sont initiées par les méristèmes secondaires. Chaque feuille est composée d'une gaine et d'un limbe séparés par une ligule (Figure 3). Chaque feuille croît à l'intérieur de la gaine de la feuille qui la précède. L'ensemble des gaines et des feuilles en croissance à l'intérieur sont souvent appelées pseudo-tiges en contraste de la vraie tige qui correspond à la somme des entre-nœuds allongés et des nœuds lors de la phase reproductive. Durant la phase reproductive, les entre-nœuds s'allongent (Figure 4) et le méristème apical produit des méristèmes secondaires qui produiront des épillets plutôt que de nouvelles talles. L'initiation de la phase reproductive est provoquée par une vernalisation suivie d'un rallongement de la durée du jour (Rouet, 2021).

A l'état naturel, le ray-grass anglais est une plante diploïde avec 2X = 14 chromosomes, mais des variétés tétraploïdes ont été créées. Son génome a été récemment séquencé mais n'a pas encore été complètement assemblé en pseudo-molécules (Byrne et al., 2015).

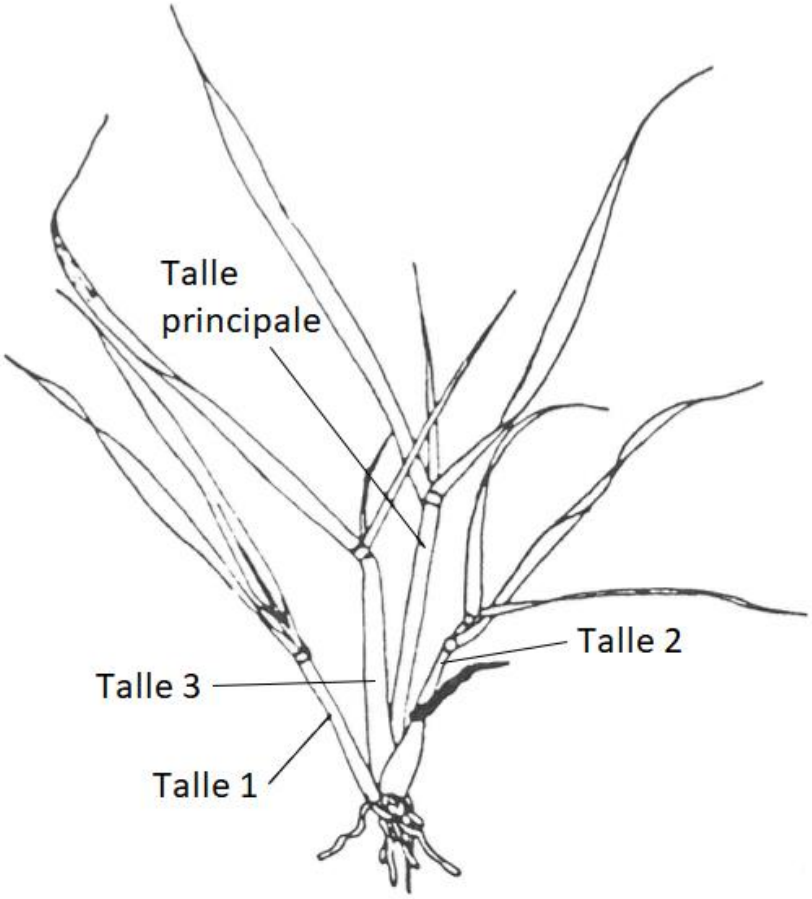

Figure 1 : Dessin d'un jeune plant végétatif de ray-grass anglais (Gillet, 1980).

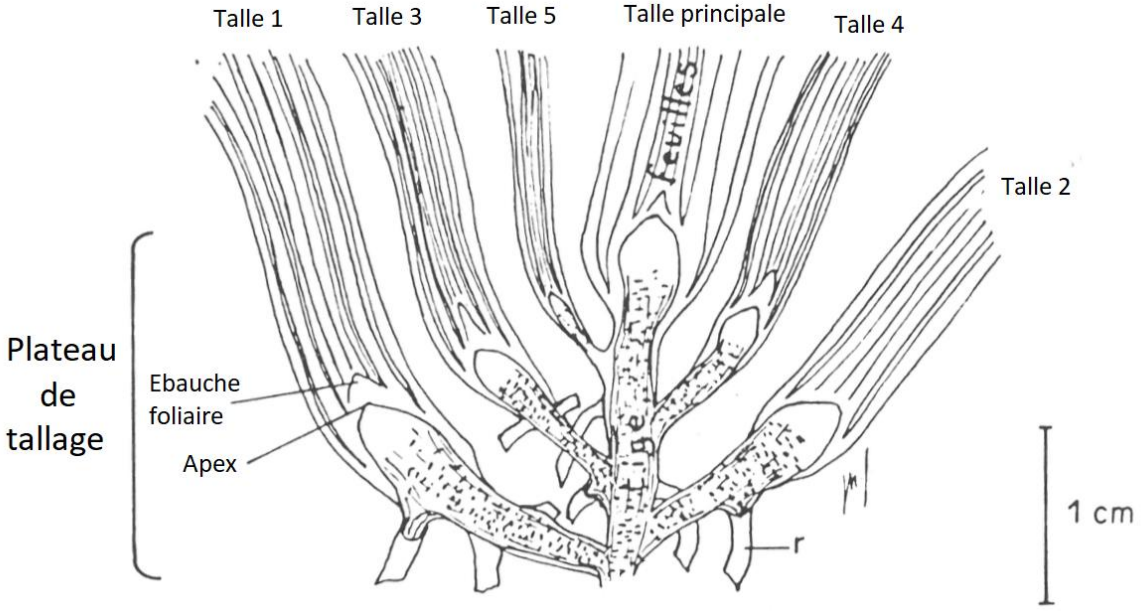

Figure 2 : Structure interne d'une touffe végétative (Gillet, 1980)

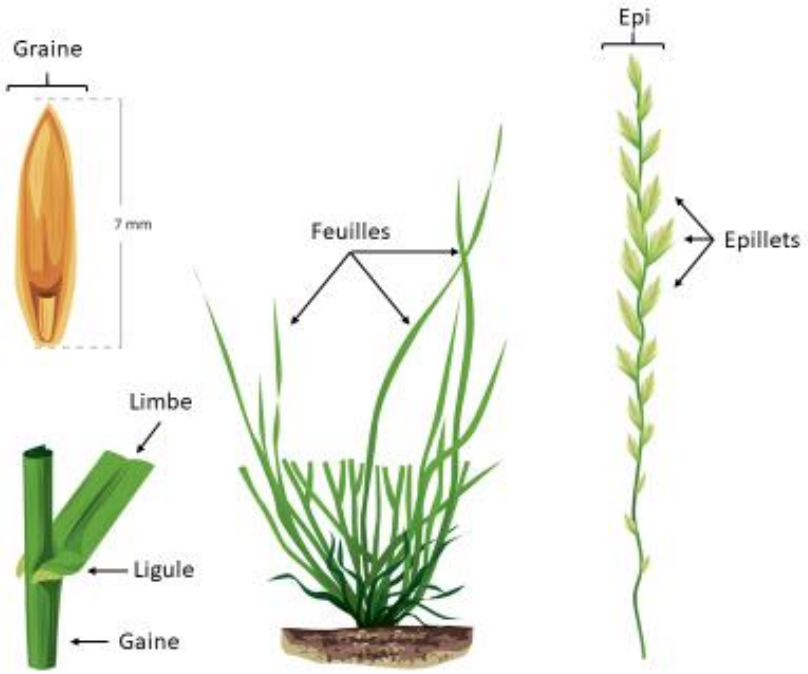

Figure 3 : Présentation du ray-grass anglais (modifié depuis SEMAE : https://www.gnis-pedagogie.org/?s=ray-grass+anglais+gazon)

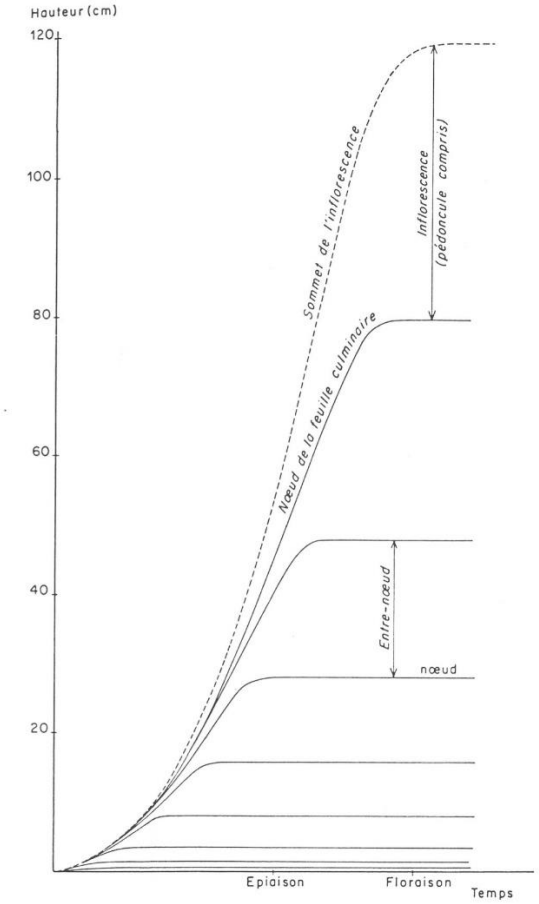

Figure 4 : Croissance en hauteur d'une tige de graminée (Gillet, 1980). Hauteur des différents nœuds en traits pleins et hauteur totale de la plante en tirets.

1.3. Evaluation de la qualité des fourrages

Afin de définir au mieux la ration nécessaire aux besoins des ruminants, il est nécessaire d'évaluer la qualité (ici équivalente à la digestibilité comme cette thèse ne tient pas compte des micro-nutriments) du fourrage *i.e.* l'énergie et les protéines apportées par le fourrage et assimilés par le ruminant. Pour ce but, plusieurs méthodes ont été développées au cours du temps en commençant par les méthodes *in vivo*, suivies par la méthode *in situ* (ou *in sacco*) avant d'être complétées par une grand gamme de méthodes *in vitro*. Enfin, la spectrométrie en proche infra-rouge (SPIR) a été utilisée.

La méthode *in vivo* est considérée comme la méthode de référence concernant la mesure de la digestibilité d'un fourrage et est utilisée depuis les commencements des mesures de digestibilité des fourrages au XIXème siècle (Schneider and Flatt, 1975). Elle consiste à mesurer la différence entre la biomasse ingérée par un animal et celle excrétée *via* les fèces. Cette méthode donne une mesure de la « digestibilité apparente » car les fèces contiennent, en plus de la nourriture non digérée, différents résidus venant de l'animal (Schneider and Flatt, 1975). De plus, une partie des éléments carbonés ingérés fermentent et sont ensuite eructés par l'animal sous forme de méthane (McDonald et al., 2011). Cette méthode présente plusieurs inconvénients, elle nécessite d'isoler plusieurs animaux dans des cages afin de contrôler le fourrage ingéré ainsi que les fèces (il est néanmoins possible d'utiliser des « couches » pour récupérer les fèces) sur une période de plusieurs semaines. Le fourrage dont on étudie la qualité doit être disponible de façon assez abondante pour nourrir plusieurs animaux sur la durée de l'expérimentation. De plus, il y a un fort effet dû à l'individu (chaque animal a sa propre flore intestinale). Enfin, c'est une méthode coûteuse en temps et en moyens humains. C'est pourquoi on lui préfère des méthodes plus rapides et moins coûteuses.

La méthode *in situ*, aussi appelée *in saco*, nécessite des animaux fistulés. Elle peut être réalisée sur des échantillons préalablement broyés de 2 à 5 g (Ørskov, 2000). Les échantillons sont placés dans des sachets nylons, eux-mêmes placés dans le rumen d'un animal pour une durée maximale de 48 h. La mesure de la digestibilité du fourrage se fait par le calcul de la différence de poids du sachet nylon entre avant et après son séjour dans le rumen. Comme pour la méthode *in* vivo, les résultats varient en fonction de l'individu. Cette technique est principalement utilisée pour étudier les cinétiques de digestibilité des fourrages.

Les méthodes *in vitro* sont les plus communément utilisées car elles nécessitent de faibles échantillons (entre 0,150 mg et 1 g) qui peuvent être analysés en grande quantité (plusieurs dizaines d'échantillons par manipulation), ce qui baisse le coup de l'analyse. Ces méthodes peuvent être divisées en deux principaux groupes selon dans quels liquides l'échantillon à analyser est incubé, les différentes méthodes au sein de chaque groupe divergent sur le contenant utilisé (*e.g.* creuset, sachet, tube) et/ou sur les concentrations des différents produits utilisés et leur provenance et/ou la durée d'incubation. Le premier groupe repose sur la technique en deux étapes de Tilley and Terry (1963) qui consiste à réaliser une première incubation dans de la salive synthétique et une seconde dans du jus de rumen prélevé sur un animal fistulé. Ceci nécessite d'avoir des animaux fistulés proches du laboratoire, car le jus de rumen doit être frais. Comme les méthodes précédemment présentées, un effet dû à l'individu d'où vient le jus de rumen est présent. Les méthodes du second groupe permettent de mesurer la solubilité enzymatique, qui est une approximation de la digestibilité. Les plus populaires reposent sur la technique pepsine-cellulase (Aufrère, 1982) qui comprend deux incubations : la première dans une solution de pepsine et la seconde dans une solution de cellulase. Ces dernières méthodes permettent de s'affranchir de l'animal et d'obtenir des valeurs plus répétables.

La spectrométrie en proche infra-rouge est une technologie permettant la prédiction non-destructive de nombreux composants des fourrages (Deaville and Givens, 1998). La SPIR est une technique analytique reposant sur l'absorption des radiations infra-rouges par les liens chimiques dans la matière organique (Bastianelli, 2013). Cette absorption est liée à la composition chimique et peut être mesurée par un spectromètre. Afin de prédire une variable, il est nécessaire de calibrer la SPIR avec le développement d'un modèle mathématique spécifique à cette variable et plus ou moins spécifique au type d'échantillon analysé. Afin que le modèle soit robuste, il est nécessaire d'intégrer une grande quantité de mesures effectuée en laboratoire sur des échantillons diversifiés.

1.4. Origines de la variabilité de la qualité des graminées fourragères

Depuis les années 70, de nombreux fourrages ont été évalués pour leur qualité, ce qui a permis d'identifier plusieurs facteurs affectant la digestibilité d'un fourrage. Un des facteurs majeurs est la maturité des plantes (Nelson and Moser, 1994), qui va agir sur la qualité *via* la variation en proportion des différents organes qui ont des qualités moyennes différentes (limbes > gaines > tiges > feuilles mortes) (Beecher et al., 2013; Chaves et al., 2006). Les variations de digestibilité sont aussi dues à la structure des parois cellulaires et leur composition (Akin, 1989; Groot and Neuteboom, 1997), notamment leur proportion en lignines. L'environnement et les pratiques culturales vont jouer un rôle sur la morphogenèse et la composition chimique des plantes, et onc sur leur qualité (Buxton and Casler, 1993). On peut mentionner comme effet environnementaux ayant un impact sur la digestibilité : les maladies, les températures extrêmes, la disponibilité en eau, la saison, la fertilisation azotée et la fréquence de coupe. Enfin, la génétique a aussi un impact sur la qualité des plantes (Buxton and Casler, 1993; Pontes et al., 2007). Sur les graminées fourragères, on peut observer une grande variabilité génétique dans de nombreuses espèces (Pontes et al., 2007).

1.5. Modélisation des plantes : exemple de L-grass

La qualité d'un fourrage récolté dépendant fortement de la composition des différents organes et de leur âge, l'utilisation d'un modèle simulant la morphogenèse de chaque organe avec sa forme, sa biomasse et sa qualité permettrait de connaître à chaque instant la biomasse et la qualité produite par la plante intégrant tous les organes. Le modèle L-grass est un modèle de simulation de croissance aérienne du ray-grass anglais développé par Verdenal (2009) à l'INRAE de Lusignan. Ce modèle permet de simuler l'état (donc indirectement la biomasse) de chaque organe d'une graminée au cours de sa phase végétative. Il est basé sur le formalisme des systèmes Lindenmayer (L-systems) (Lindenmayer, 1968), utilisant la plateforme de simulation L-Studio LPFG et le langage de modélisation L+C (Karwowski and Prusinkiewicz, 2003).

1.6. Objectifs de la thèse

Comprendre l'origine biochimique de la variation de la qualité dans un large panel de génotypes de ray-grass anglais récoltés au printemps et à l'automne.

Identifier les bases génétiques de la qualité et de ses composantes dans un large panel de génotypes de ray-grass anglais au printemps et à l'automne.

Comprendre le lien entre la morphogenèse aérienne et la qualité au printemps et à l'automne en utilisant quatre génotypes contrastés pour leur date d'épiaison et leur qualité.

Discuter des conséquences de ces résultats pour la modélisation et la sélection des plantes.

2. Différences saisonnières sur le contrôle structurel et génétique de la digestibilité du ray-grass anglais

Ce chapitre a fait l'objet d'un article : Colas V., Barre P., van Parijs F., Wolters L., Quitté Y., Ruttink T., Roldán-Ruiz I., Escobar Gutiérrez A.J., Muylle H. (2022) Seasonal Differences in Structural and Genetic Control of Digestibility in Perennial Ryegrass. Frontiers in Plant Science 12 https://doi.org/10.3389/fpls.2021.801145

2.1. Matériel et méthode

Les plantes utilisées dans cette étude consistent en 580 génotypes d'origine diverses et représentant une vaste diversité génétique et phénotypique (Veeckman et al., 2018). Parmi ces génotypes, 42% proviennent du matériel de sélection d'ILVO, DSV et Barenbrug, 22% sont issus de cultivars commercialisés et 36% sont issus d'accessions sauvages (parmi lesquelles 76% sont d'origine française).

Pour chaque génotype, trois clones ont été produits par éclatement de talles en septembre 2011 et 2012. Ces clones ont été placés en serre froide (température minimale de 4°C) pour leur vernalisation avant d'être rempotés en pots de 12 L en début mars 2012 et 2013. Chaque plante est constituée de trois talles coupées à la même hauteur (*ca.* 4 cm). Les pots ont été placés au sol en extérieur à Melle en Belgique (latitude 50°59'39", longitude 3°47'5" et 24 m au-dessus du niveau de la mer) sur trois blocs randomisés. Un apport d'engrais et d'eau par goutte-à-goutte afin que les plantes ne soient pas en conditions limitantes.

Les plantes sont récoltées quatre fois par an : une coupe au printemps, deux à l'été et une à l'automne. Seules les coupes de printemps et d'automne ont été étudiées. La coupe de printemps a été réalisée à la date d'épiaison pour chaque plante (moment où trois épis sont visibles). Les coupes sont faites à 4 cm de hauteur entre 14 et 15 h. Après la séparation entre limbes et « tiges » (tiges + gaines) (seulement pour la coupe de printemps), la biomasse récoltée pour chaque plante est séchée en étuve pendant 48 h à 70°C avant d'être broyée à 0.5 mm dans un broyeur à couteaux Fritsch. Dans cette étude, nous nous sommes concentrés sur l'études des limbes récoltés au printemps et à l'automne afin de pouvoir comparer les saisons. Les plantes contenant trop d'épis (plus de 5% de leur biomasse) et celles ayant épié à des dates extrêmes n'ont pas été incluses dans l'étude.

Tous les échantillons récoltés ont été analysés par spectrométrie en proche infra-rouge (SPIR, FOSS XDS). Des analyses chimiques ont été réalisées sur une sélection d'échantillons pour sept caractères : la matière sèche absolue (ADM) exprimée en pourcentage de matière fraîche, la matière organique (OM) exprimée en pourcentage de matière sèche (DM), la digestibilité de la matière organique (OMD), les fibres insolubles dans les détergents neutres (NDF), l'« acid detergent lignin » (ADL), la cellulose (C) et l'hémicellulose (HC) exprimées en pourcentage de OM. Dû à un manque de données, 20 génotypes ont été supprimés de l'étude des caractères phénotypiques, établissant le nombre de génotypes étudiés à 550. L'ADM a été déterminée par gravitation en séchant 2.5 g d'un échantillon à 103°C, puis en le brûlant à 550°C, permettant la détermination de l'OM en tant que proportion sans cendres de l'ADM. L'OMD a été déterminé selon Goering & van Soest (1970). La méthode Van Soest (Van Soest et al., 1991) a été adaptée pour l'utilisation de l'ANKOM Fibre Analyzer (ANKOM Technology Corporation, Fairpoint, NY) afin de mesurer le NDF, l'ADF et l'ADL (permettant ensuite de déterminer les quantités d'hémicellulose et de cellulose). Afin de mieux étudier la composition des fibres, ses composant (HC, C et ADL) ont été exprimés en pourcentage du NDF (ce qui donne (HC.NDF, C.NDF et ADL.NDF). La digestibilité du NDF (NDFD) a été calculée selon

une formule adaptée de Struik (1983) basée sur l'hypothèse que tous les composés indigestes de la plante sont contenus dans les parois cellulaires :

$$NDFD = 100 - 100 \times \frac{100 - OMD}{NDF}$$

Pour chaque caractère étudié, les effets du génotype, de l'année et du bloc ont été analysés par l'utilisation d'un modèle mixte sous R (R Core Team, 2020) avec le package *lme4* (Bates et al., 2015). Un second modèle mixte a été ensuite utilisé en séparant les données des deux saisons. Pour permettre l'étude d'association pangénomique (GWAS), le modèle mixte saisonnier a été utilisé pour calculer le meilleur prédicteur linéaire sans biais (BLUP) de l'effet génotypique par saison sur les deux années combinées. Afin d'identifier l'importance de chaque caractère dans la variation de l'OMD et la NDFD, plusieurs modèles linéaires ont été lancés sous R (R Core Team, 2020). Dans ces modèles, nous n'avons pas pris en compte le caractère C.NDF dû à sa forte corrélation avec HC.NDF (-0.96 sur les deux années et saisons).

Les marqueurs génétiques utilisés sont issus de Veeckman et al. (2018). Ces marqueurs décrivent la diversité des séquences de 503 gènes candidats impliqués dans le développement, l'architecture, les phytohormones et la composition des parois cellulaires de la plante. Ils sont au nombre de 252 406 « Single Nucleotide Polymorphism » (SNP) et 5 074 indels (insertion délétion) et ont été identifiés sur un panel de 736 génotypes, incluant les 580 de cette étude. L'imputation des allèles manquants a été réalisée par leur remplacement par l'allèle le plus commun dans le groupe obtenu par l'analyse de structure (voir ci-dessous) du génotype concerné. La fréquence minimale allélique (MAF) a été fixée à 1%.

La date de coupe au printemps a été fixée à la date d'épiaison de chaque plante, qui est aussi une date clé d'un point de vue agronomique à laquelle les producteurs essaient de réaliser leur récolte. Néanmoins, cette date de récolte a un impact sur l'OMD, la NDFD qui vont atteindre des valeurs plus faibles à une date plus avancée. Afin de ne pas détecter les QTLs impliqués dans l'établissement de la précocité lors de la GWAS, il a été décidé de corriger chaque caractère pour la date d'épiaison (HD).

Afin de déterminer la structure de la population étudiée, nous avons utilisé fastSTRUCTURE (Raj et al., 2014), ce qui nous a conduit à l'obtention de quatre groupes : le premier appelé « Temperate » regroupe l'ensemble des génotypes sauvages, à l'exception des espagnols et de deux italiens qui appartiennent au second groupe « Warm ». Le troisième groupe, contenant tous les génotypes dérivés des variétés Aberavon, Aberdert et Aberzest, est appelé « Aber ». Ces trois groupes regroupent aussi une grande partie des plantes commercialisées et faisant partie des pépinières de sélection des obtenteurs. Le dernier groupe, appelé « NZ » contient 51 génotypes issus des pépinières de sélection néo-zélandaises de Barenbrug.

La méthode MLMM pour « Multi-Locus Mixed-Modelling » a été utilisée pour la GWAS (Segura et al., 2012). Nous avons calculé une matrice d'apparentement selon la formule de Rincent *et al.* (2012). La méthode MLMM a été utilisée sur chaque caractère en utilisant les données génétiques, les valeurs BLUPs, la structure de la population et la matrice d'apparentement. Le modèle MLMM avec la plus faible valeur BIC (Bayesian information criterion). Les QTLs dont la p-value est inférieure à un seuil de Bonferroni calculé avec le nombre de gènes plutôt que le nombre de marqueurs indépendants car on retrouve un fort déséquilibre de liaison entre les marqueurs liés à un même gène.

Afin d'évaluer le pourcentage de variance expliquée par les marqueurs, une estimation du R^2 semi-partiel de l'ensemble des QTLs identifiés comme significatifs puis de chaque QTL indépendamment est réalisée à l'aide des fonctions *lmekin* et *r*.squaredLR des paquets R *coxme* et *MuMIn* respectivement (Bartoń, 2020; Therneau, 2020).

2.2. Résultats

L'héritabilité de l'OMD et du NDFD sont très basses lorsque les deux saisons sont analysées ensemble (Tableau 1, 0,13 et <0,01). En effet, on observe une forte interaction génotype *x* saison (G:S). Ceci nous a conduit à préférer l'analyse des données par saison. L'héritabilité de l'OMD et du NDFD augmentent lorsque l'analyse se fait séparément entre les deux saisons (Tableau 1), ce qui suggère un contrôle génétique différent pour ces caractères selon la saison.

Tableau 1 : Héritabilités et variances des différents effets calculés depuis un modèle mixte sur les deux saisons et par saison pour la digestibilité de la matière organique (OMD), le taux de fibres (NDF, la digestibilité des fibres (NDFD), le taux d'hémicellulose (HC), la proportion d'hémicellulose dans les parois (HC.NDF), le taux de cellulose (C), la proportion de cellulose dans les parois (C.NDF)), le taux de lignine (ADL), la proportion de lignine dans les parois (ADL.NDF) et la data d'épiaison (HD) en degré-jours (GDD) depuis la date de mise en pot. Sont indiqués : l'effet génotype (G), l'interaction génotype x année (G:Y) et l'interaction génotype x saison (G:S).

Trait	**Spring and autumn**				
	Variance				H2
	G	G:Y	G:S	Residuals	
OMD (%OM)	0.42	1.15	3.05	3.6	0.05
NDF (%OM)	4.39	3.89	6.24	8.43	0.19
NDFD (%NDF)	<0.01	1.9	5.47	5.56	<0.01
HC (%OM)	1.13	1.57	2.54	3.34	0.13
HC.NDF (%NDF)	1.6	0.65	2.73	2.2	0.22
C (%OM)	1.6	0.57	1.63	1.64	0.29
C.NDF (%NDF)	2.06	0.86	3.4	2.59	0.23
ADL (%OM)	<0.01	0.04	0.09	0.09	<0.01
ADL.NDF (%NDF)	<0.01	0.07	0.18	0.19	<0.01

Trait	**Spring**				**Autumn**			
	Variance			H2	Variance			H2
	G	G:Y	Residuals		G	G:Y	Residuals	
OMD (%OM)	2.44	0.55	0.65	0.67	3.85	3.27	5.07	0.32
NDF (%OM)	7.99	6.99	4.93	0.4	11.36	5.53	8.92	0.44
NDFD (%NDF)	7.05	1.67	2.09	0.65	2.72	4.76	6.89	0.19
HC (%OM)	2.18	3.8	2.16	0.27	3.82	2.21	3.02	0.42
HC.NDF (%NDF)	5.51	1.51	1.6	0.64	2.86	0.84	2.19	0.49
C (%OM)	3.25	0.73	0.96	0.66	3.09	0.91	1.9	0.52
C.NDF (%NDF)	6.33	2.15	1.79	0.62	4.1	1	2.57	0.53
ADL (%OM)	0.02	0.06	0.04	0.17	0.14	0.07	0.1	0.45
ADL.NDF (%NDF)	0.06	0.14	0.14	0.18	0.23	0.12	0.17	0.44
HD (GDD)	21812	3319	1332	0.82				

Les valeurs des caractères de digestibilité OMD et NDFD sont significativement différentes entre les saisons. Ces caractères sont plus élevés au printemps qu'à l'automne (une différence de 8% d'OM et de 6% de NDF), mais avec une plus grande diversité à l'automne. Néanmoins, on a pu observer une faible corrélation positive entre les deux saisons pour l'OMD et le NDFD (Figure 5).

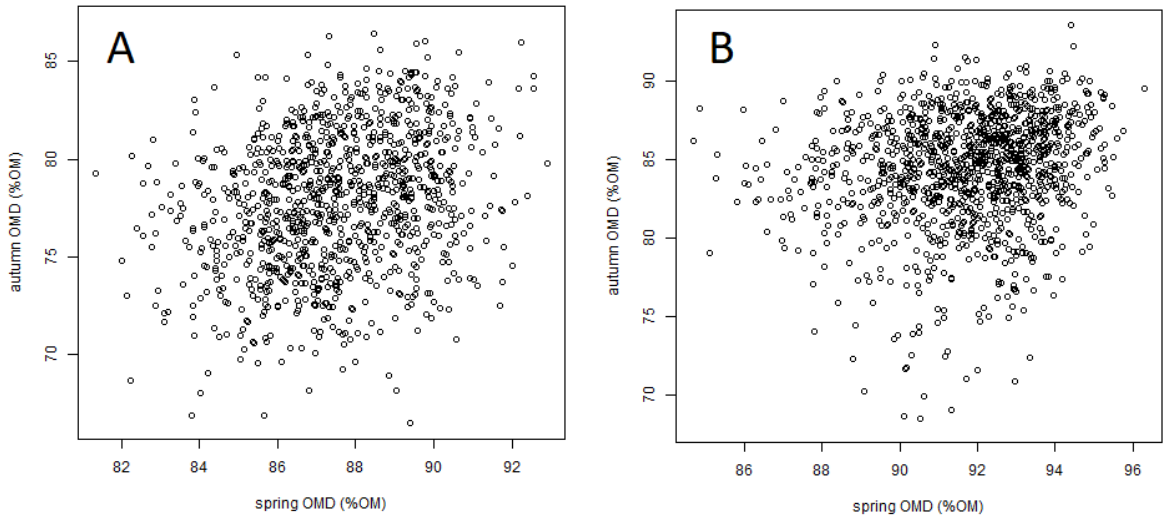

Figure 5 : Digestibilité de la matière organique (OMD) des limbes au printemps en fonction de l'OMD des feuilles à l'automne en 2012 (A) et 2013 (B)

Nous avons observé une forte corrélation positive entre l'OMD et le NDFD pour chaque saison et année (0,79 au printemps 2012, 0,87 au printemps 2013, 0,92 à l'automne 2012 et 0,92 à l'automne 2013) (Figure 6A). Une corrélation négative a été observée entre l'OMD et le NDF (-0,81 au printemps 2012, -0,53 au printemps 2013, -0,90 à l'automne 2012 et -0,81 à l'automne 2013) (Figure 6B).

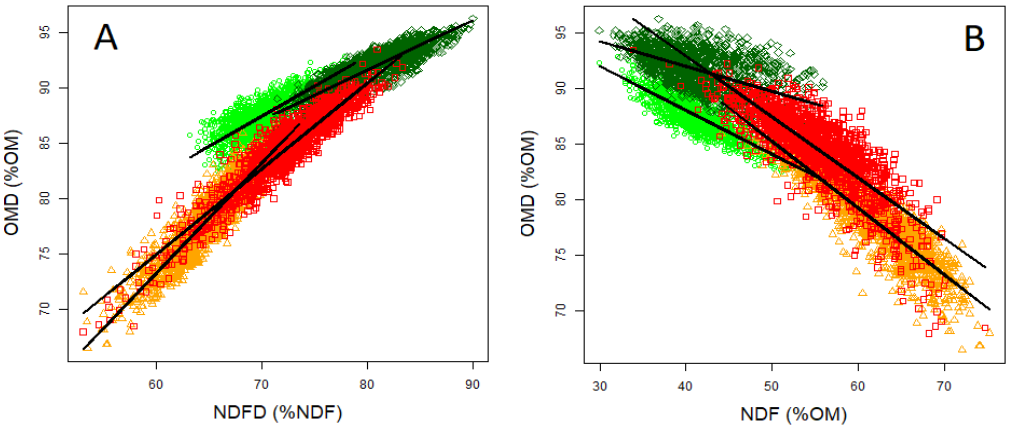

Figure 6 : Relation entre l'OMD et A) le NDFD et B) le NDF. Les cercles vert clair représentent les données du printemps 2012, les losanges vert foncé représentent les données du printemps 2013, les triangles oranges représentent les données de l'automne 2012 et les carrés rouges représentent les données de l'automne 2013.

En analysant l'influence de la composition des fibres sur le NDFD, nous avons observé qu'une grande partie de la variabilité du NDFD pouvait être expliquée par la variabilité de HC.NDF (ou C.NDF) au printemps et par la variabilité de ADL.NDF à l'automne.

Les QTLs identifiés lors de l'analyse pangénomique permettent d'expliquer de 29 à 52% de la variance phénotypique des différents caractères étudiés (Tableau 2). En comparant les différents gènes identifiés, deux gènes (NAC-13 et ABI3-02) sont apparus en commun entre HC.NDF, NDFD et OMD au printemps et six gènes (RGA-03, ABI1-01, ARF-03, OBE1-02, KAN-06, BIM2-01) entre ADL.NDF, NDFD et OMD à l'automne.

Tableau 2: Nombre de QTLs et de genes identifies par l'analyse MLMM et variance phénotypique expliquée par tous les QTLs identifies pour chaque caractère et saison.

	Spring			Autumn		
	QTLs	Genes	sr2	QTLs	Genes	sr2
OMD (%OM)	67	28	47%	57	21	40%
NDF (%OM)	109	23	52%	83	20	43%
NDFD (%NDF)	36	16	29%	52	19	35%
HC.NDF (%NDF)	60	18	39%	67	17	41%
C.NDF (%NDF)	54	16	38%	59	16	38%
ADL.NDF (%NDF)	96	27	49%	58	23	46%

2.3. Discussion

Nous avons observé une forte interaction génotype x saison pour l'OMD et le NDFD, conduisant à des changements de rangs entres les valeurs de printemps et celles d'automne. Ces observations indiquent que la sélection devrait être réalisée sur les valeurs obtenues pour chaque saison afin d'améliorer la qualité du fourrage sur l'année complète. Les valeurs pour l'OMD sont plus élevées au printemps, que l'on considère les limbes seuls ou qu'ils soient combinés aux « tiges ». Cette différence entre les saisons peut être expliquée par un plus fort taux de sucres au printemps et/ou des différences environnementales. En effet, la récolte d'automne est le résultat d'une croissance durant l'été (août et début de septembre) où les températures sont plus élevées qu'au printemps, ce qui peut conduire à une augmentation du taux de fibres (Lee et al., 2017; Moyo and Nsahlai, 2021).

La variation de l'OMD peut être expliquée par les variations en fibres (NDF) et leur digestibilité (NDFD) comme précédemment démontré par Barrière et al. (2003) et Pembleton et al. (2013). La sélection peut se faire sur ces deux variables, mais il serait préférable d'aussi tenir compte que la corrélation entre le NDF et le NDFD est différente selon la saison. Au printemps, cette corrélation est faible, montrant presque une indépendance des deux variables ce qui permet de sélectionner sur le NDFD et le NDFD. Tandis qu'en automne, la corrélation est fortement négative, ce qui rend moins utile de sélectionner sur ces deux variables et d'en préférer une à l'autre.

La variation du NDFD peut être partiellement expliquée par la composition des parois cellulaires. Au printemps, l'augmentation du NDFD est associée à l'augmentation de la concentration en hémicellulose (et la baisse de la concentration en cellulose) avec un très faible effet de la concentration en lignine. Au contraire, à l'automne, l'augmentation du NDFD est liée à la baisse de la concentration en lignine et la concentration en hémicellulose (et cellulose) a peu d'effet. Habituellement, la concentration en lignine est liée à le NDFD chez les graminées fourragères (Jung and Vogel, 1986; Mowat et al., 1969), mais pas toujours (Lam et al., 2003). Cette absence de lien entre le NDFD et la concentration en lignine à l'automne est surprenante, étant donné que la gamme de valeurs pour la concentration en lignine est la même au printemps et à l'automne. Une explication pourrait être que la méthode utilisée pour évaluer la lignine ne soit pas assez précise. Il est en effet connu que l'ADL déterminé par la methode Van Soest détecte la lignine insoluble dans l'acide mais ne peut quantifier la lignine soluble dans l'acide (van Parijs et al., 2018). De plus, l'effet de la lignine sur le NDFD ne dépend pas uniquement de sa concentration, mais aussi de sa réticulation et de son hydrophobicité (Grabber et al., 2004).

Un fort effet de la date d'épiaison a été observé sur les différentes variables étudiées. Les plantes tardives sont moins digestes que les plantes précoces. Comme les plantes ont été récoltées à la date d'épiaison, cette baisse de digestibilité pour les plantes tardives peut être expliqué par le fait que les feuilles récoltées sont plus âgées que celles récoltées sur les plantes précoces. Il est en effet connu que les organes perdent en digestibilité avec l'âge (Buxton and Casler, 1993). Il est donc important de tenir compte de la date d'épiaison pour évaluer différentes variétés qui sont récoltées au même stade phrénologique.

L'analyse pangénomique (GWAS) a permis d'identifier plusieurs QTLs pour chaque caractère, expliquant de 9,2 à 51,7% de leur variance phénotypique. Pris individuellement, chaque marqueur explique une petite part de la variance phénotypique (de 1 à 7%). Les QTLs identifiés sont liés à des gènes impliqués dans différentes voies de synthèse. Plusieurs des gènes identifiés comme ayant un effet significatif dans cette étude ont déjà été identifiés dans de précédentes études sur la digestibilité et le NDFD de graminées fourragères comme le ray-grass anglais et le maïs (Pembleton et al., 2013; Ralph et al., 2004). Ceci inclus les gènes impliqués dans la biosynthèse de la lignine et de la cellulose (*e.g*. HCT3 et CAD pour l'OMD en automne ; C3H, ALDH et CCoAOMT pour le NDFD en automne ; CAD2, COMT-01, ALDH, CES-02, XylT4 et PAL-03 pour l'OMD au printemps ; CES-02 et HCT pour le NDFD au printemps), qui ont déjà été décrits comme étant liés à des caractères de digestibilité (Halpin, 2019).

Aucun QTL n'a été identifié pour deux saisons à la fois à l'exception d'un QTL pour ADL.NDF, C.NDF et OMD et quatre QTLs pour le NDF. Pour chaque saison, nous avons identifié des QTLs en commun entre les caractères de digestibilité et les caractères de composition des parois cellulaires. Au printemps, HC.NDF a trois QTLs en commun avec OMD et/ou NDFD, tandis qu'aucun QTL commun n'a été identifié à l'automne. A l'automne, ADL.NDF a sept QTLs en commun avec OMD et/ou NDFD, tandis qu'aucun QTL commun n'a été identifié au printemps. Ces résultats de la GWAS confirment l'importance de la concentration en hémicellulose sur la digestibilité de la coupe du printemps et de la concentration en lignine de la coupe d'automne. Les QTLs qui sont seulement liés au NDFD ou à l'OMD suggèrent que d'autres caractères, qui n'ont pas été considérés dans cette étude, pourraient avoir un effet sur le NDFD ou l'OMD.

3. Dynamique de la qualité durant la vie d'une feuille et d'une tige de ray-grass anglais : une étude de génotypes contrastés pour leur précocité et leur qualité

3.1. Matériel et méthode

Dans ce chapitre, nous avons utilisé 600 plantes issues de quatre génotypes différents provenant du matériel de sélection de DSV, soit 150 clones pour chaque génotype. Ces génotypes ont été choisis à partir de données précédemment obtenues par DSV sur des plantes espacées (communication personnelle de Lukas Wolters). Ces quatre génotypes sont contrastés pour leur précocité et leur qualité. Pour une meilleure compréhension, un nombre à deux chiffres (ID) a été attribué à chacun d'entre eux (Tableau 3).

Tableau 3 : Liste des quatre génotypes utilisés dans cette étude

ID	Génoype	Qualité	Précocité
12	PC-100-102-01	Haute	Précoce
13	Aberavon 5	Basse	Intermédiaire
15	AA-VZH-08	Basse	Tardif
16	AA-VZH-10	Haute	Tardif

Les clones ont été produits par division de talle des pieds-mères qui sont ensuite plantés dans des pots de 2L en serre froide (température minimale de 5°C) durant l'hiver. The 28 mars 2019, les plantes ont été coupées à 4 cm de hauteur et ont été placées sur dix tablards extérieurs à INRAE URP3F Lusignan, France (46.4052, 0.0811). Les plantes furent coupées une seconde fois après les mesures de printemps puis une autre fois les 28 et 29 août 2019, avant les mesures d'automne. Sur chaque table, les quatre génotypes furent randomisés en cinq carrés latins de 4x3 pots (soit 15 clones de chaque génotype par tablard) et furent entourés de plantes de bordure. Les tables ont été numérotées de 1 à 10 et séparées en deux blocs (A et B). L'irrigation s'est faite par aspersion *via* douze sprinklers. Vingt-trois capteurs de température ont été dispersés sur l'ensemble des tablards au niveau de la base des plantes, sous les feuilles.

Afin de suivre la croissance des talles au cours du temps à chaque saison, 15 pots ont été sélectionnés sur chaque bloc pour effectuer des mesures de chaque feuille d'une talle tous les deux à trois jours en commençant par la feuille n°1, correspondant à la première feuille non-coupée. Suite à quatre mesures consécutives identiques, la gaine de la feuille est mesurée et cette feuille ne sera plus suivie. La mesure de la croissance des talles a été réalisée du 1er avril au 26 juillet 2019 et du 2nd septembre au 4 décembre 2019. De plus, une notation sur l'avancement de l'épiaison a été réalisée au printemps avec des notes allant de 0 à 3 avec 0 : talle végétative ; 1 : durcissement de la base de la talle ; 2 : émergence de l'épi ; 3 : floraison.

Pour mesurer l'évolution de la qualité des différents organes à chaque saison, une à deux talles par pot ont été coupées à 3 cm de hauteur chaque semaine à partir du moment où la première feuille non-coupée mesure 10 cm (afin d'obtenir assez de matière pour les analyses) jusqu'à la fin des mesures de croissance. Les talles furent séparées selon le génotype et le bloc de leur plante d'origine et ont été disséquées afin d'isoler chaque feuille, composée d'un limbe et d'une gaine). Sur les talles reproductives, les tiges ont été séparées des feuilles et divisées en fonction de leur rang (nœud et entrenœud associés à une feuille ; le pédoncule de l'épi et l'épi sont considérés comme des rangs spéciaux) lorsque leur longueur est suffisante (Figure 7).

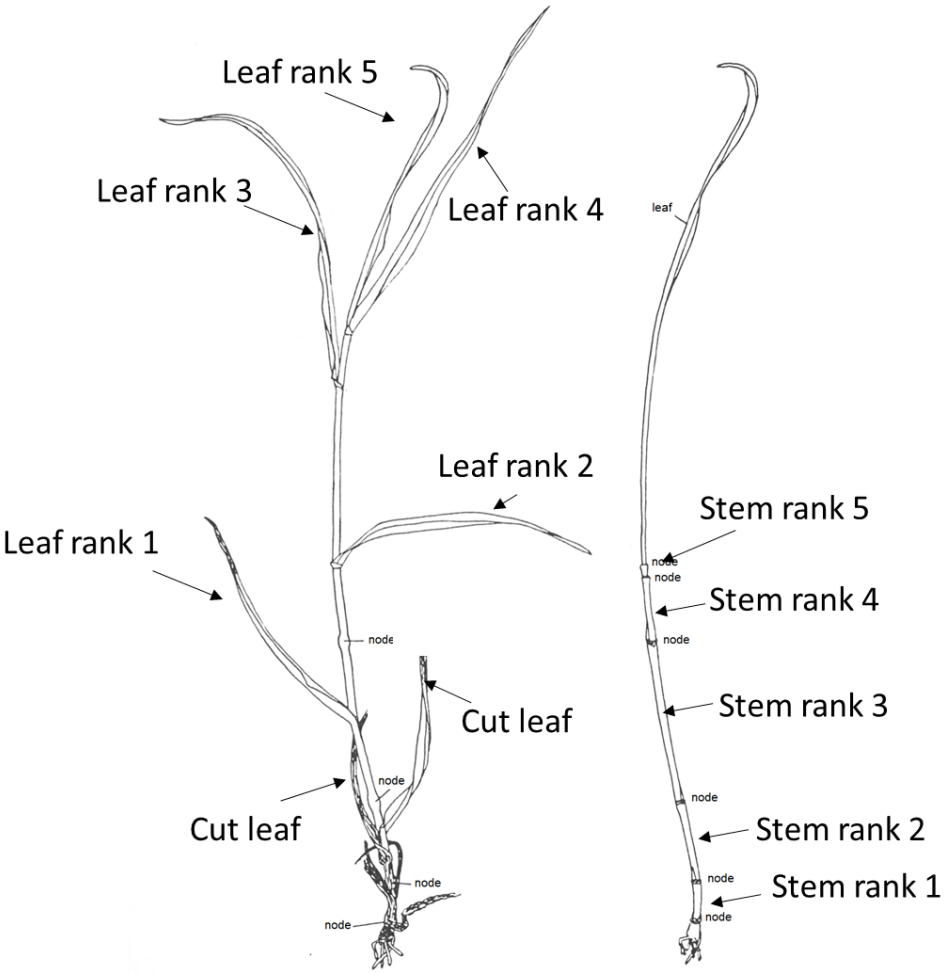

Figure 7: Description des rangs d'échantillonnage des feuilles et tiges (schéma de Gillet (1980) modifié).

Chaque échantillon obtenu est mesuré pour sa longueur moyenne et sa senescence moyenne avant d'être séchés en étuve à 60°C pendant 72 h et pesés.

Tous les échantillons ont été broyés à 1 mm avant d'être scannés par SPIR avec le MTA de BRUKER pour estimer le DMD, le NDF et l'azote grâce aux équations développées par INRAE URP3F à l'aide d'une grande base de données enrichie sur plusieurs années. Des analyses chimiques ont été réalisées sur une sélection d'échantillons pour la digestibilité de la matière sèche (DMD), le taux de fibres (NDF), la digestibilité des fibres (NDFD) et l'azote total (TN) et ont permis d'affiner les équations du SPIR, notamment pour une meilleure prédiction des tiges. Les mesures de NDF ont été réalisées en suivant la méthode Van Soest (Van Soest et al., 1991) adaptée pour l'utilisation de sachets ANKOM. La DMD et le NDFD ont été mesurés en suivant la méthode pepsine-cellulase développée par Aufrère (1982) adaptée pour l'utilisation de tubes de 5 mL. L'azote est mesuré en suivant la méthode Dumas. L'ensemble des analyses ont été réalisées au laboratoire de chimie de INRAE URP3F, Lusignan.

Pour chaque feuille observée, les longueurs en fonction du temps (le temps est mesuré en jours effectifs (ed) où un jour effectif correspond à un jour avec une température moyenne de 23°C et à moins d'un jour pour des températures moyennes différentes suivant une fonction Gaussienne (Rouet, 2021)) ont été régressées avec une fonction beta (Verdenal, 2009), permettant l'estimation de la vitesse d'élongation (LER), du moment où la feuille est initiée (ici, moment où la feuille atteint 1 mm), du moment où la vitesse d'élongation atteint son maximum, le moment où la feuille atteint sa taille maximale et la taille maximale de la feuille. Ces paramètres sont ensuite moyennés sur les 15 clones du même génotype de chaque bloc. Puis la durée de vie de la feuille est divisée entre la durée de croissance (LED), la durée où la feuille est mature et verte (GreenD) et la durée de senescence (SenD) (Figure 8).

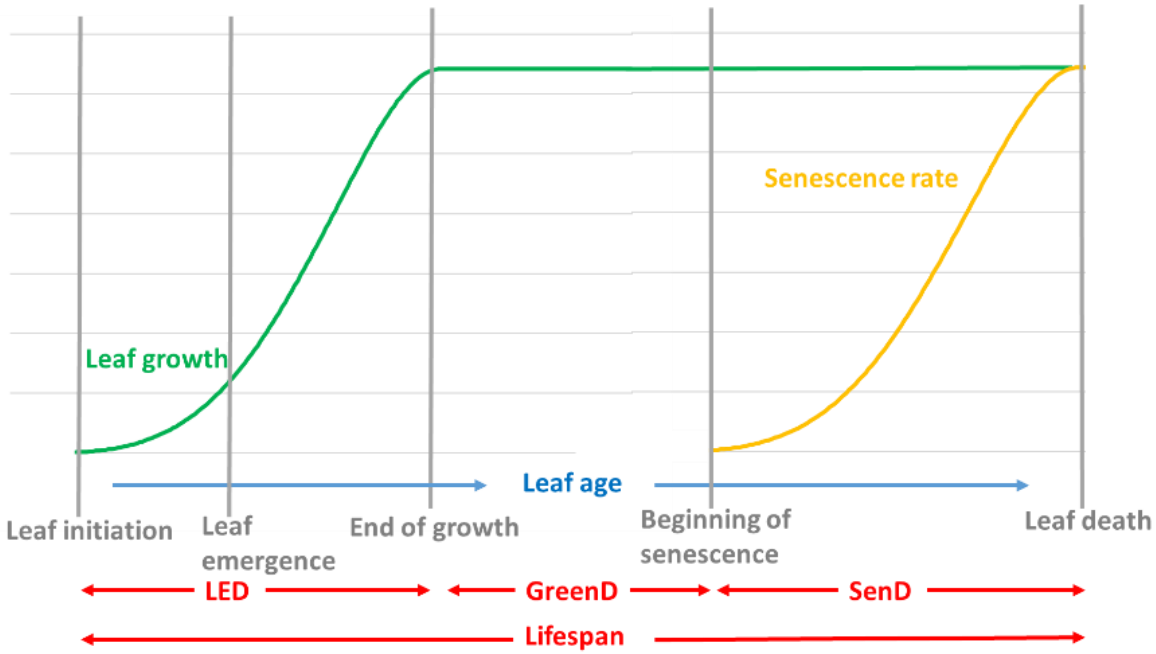

Figure 8 : Différentes étapes de la vie d'une feuille

Afin d'analyser une tige entière ou une talle entière, les données obtenues pour leurs composants (partie de tige, feuilles de rangs différents) sont additionnées pour la longueur (tige seulement) et la matière sèche et moyennés en tenant compte de leur poids pour le NDF, DMD, TN et NDFD.

Une analyse en composantes principales a été réalisée sur les paramètres morphologiques (longueur maximale (Max_length), vitesse de croissance maximale (LER), durée d'élongation (LED), durée où la feuille est mature et verte (GreenD), durée de senescence (SenD), ratio gaine/limbe (sheath_proportion) et phyllochrone) et chimiques (digestibilité maximale de la matière sèche (max_DMD), taux de fibres minimum (Min_NDF) et le taux maximum de protéines (Max_TN) pour chaque rang de feuille, génotype et saison. Une analyse de variance a été effectuée afin de tester l'effet du rang, du génotype, du bloc et de la saison sur ces variables. Un modèle linéaire avec l'effet génotypes (qualitatif), l'effet du temps (quantitatif) et l'interaction génotype *x* temps (quantitatif) a été utilisé pour comparer les différences de pentes et de moyennes entre les génotypes pour un caractère donné en fonction du temps.

3.2. Résultats

La mesure de la croissance des feuilles et de leur composition a dégagé un profil identique pour chacune d'elles. La longueur d'une feuille augmente jusqu'à atteindre un plateau et suite à une période donnée (GreenD), la partie verte des feuilles diminue à cause de la sénescence. La DMD diminue lentement avec le temps avant d'accélérer lors de la sénescence. La DMD diminue de façon simultanée à l'augmentation du NDF et la diminution du NDFD. La longueur des feuilles et la proportion de gaine augmente généralement avec leur rang et de façon plus marquée au printemps au début de l'élongation de la tige. Aucune corrélation n'a été observée entre le poids sec des feuilles et leur digestibilité.

Les effets du génotype, du rang, de la saison et de l'interaction rang *x* saison pour l'ensemble des caractères étudiés sont présentés dans le Tableau 4. On observe un très fort effet du rang sur l'ensemble des caractères. On retrouve cette effet lors d'une analyse en composantes principales (ACP) qui va associer les feuilles de hauts rangs avec une longueur plus élevée, une plus grande proportion de gaine, une plus longue durée de croissance, un taux de fibre plus élevé ainsi qu'une digestibilité plus faible.

Tableau 4 : Effet du génotype, de la saison, du rang et de l'interaction saison *x* rang sur les différentes variables. ***: < 0.001, **: < 0.01, *: < 0.05

Effect	Genotype		Season		Rank		Block		Season:Rank	
Trait	F value	p value	F value	p value	F value	p value	F value	p value	F value	p value
Max_length	6.51	***	24.19	***	22.12	***	0.34		3.78	**
Sheath_proportion	6.23	***	11.51	**	41.78	***	0.00		4.96	**
LER	3.75	*	1.20		6.31	***	0.05		4.51	**
Phyllochrone	6.92	***	14.47	***	3.65	**	0.21		3.84	*
LED	2.18		2.17		21.21	***	0.03		2.11	
GreenD	1.14		11.34	**	10.72	***	4.39	*	0.48	
SenD	3.23	*	25.30	***	4.74	***	0.96		0.30	
Max_DMD	20.02	***	3.81		56.56	***	2.32		10.97	***
Min_NDF	0.00	***	0.00	***	0.00	***	0.22		0.00	***
Max_TN	8.69	***	0.03		15.79	***	10.19	**	1.89	

Nous avons observé un lien entre la précocité d'un génotype et le nombre de feuilles produites avant l'apparition de l'épi, les génotypes les plus tardifs ayant un nombre plus élevé de feuilles.

Concernant la digestibilité des tiges, on constate que les jeunes tiges sont les plus digestes (70 à 78% DM) mais restent moins digestes que les feuilles vertes (about 90% DM). Puis on observe que les rangs les plus bas (la base de la tige) sont moins digestes que les rangs élevés. La digestibilité de l'ensemble de la tige diminue avec le temps, mais à différentes vitesses selon les génotypes.

Le taux de sénescence est fortement corrélé négativement à la digestibilité des feuilles pour chaque saison (-0,88 au printemps et -0,92 à l'automne 2019 avec une p-value<0,05) (Figure 9). En se concentrant sur les feuilles non-sénescentes, nous avons observé une corrélation négative entre la DMD et la longueur maximale des feuilles au printemps mais pas en automne (Figure 10).

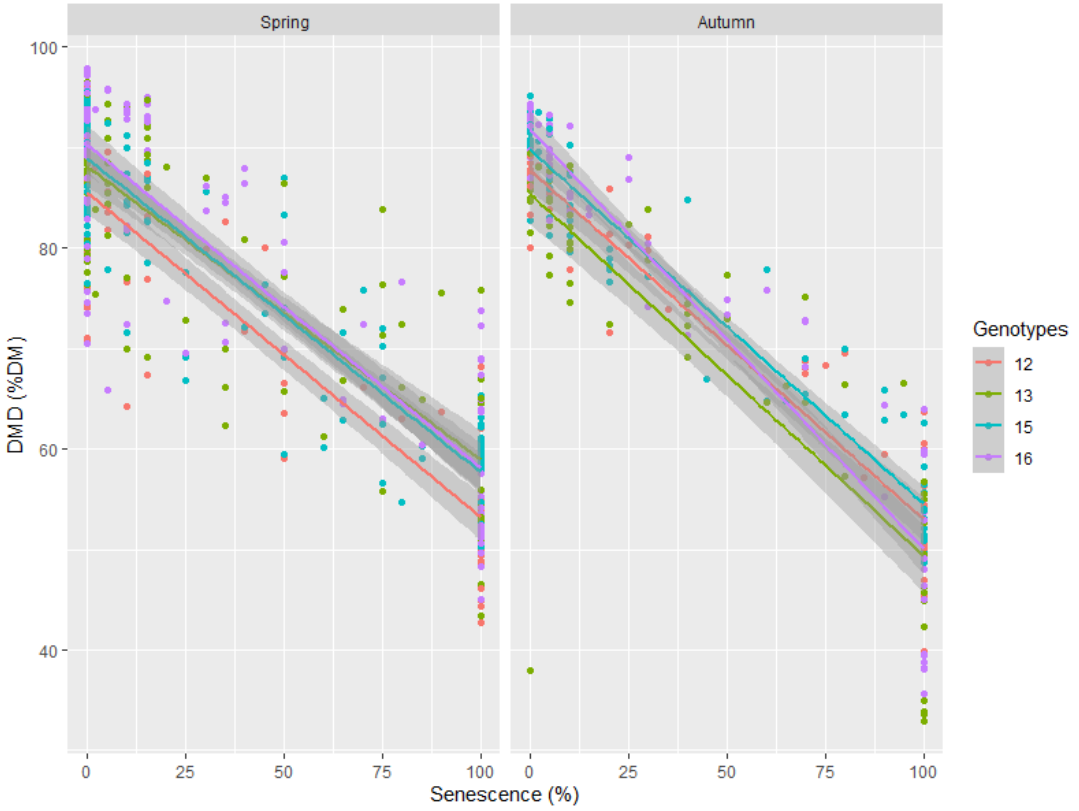

Figure 9 : Variation de la digestibilité de la matière sèche (DMD) des échantillons de feuilles en fonction de la sénescence pour chaque génotype au printemps et à l'automne 2019

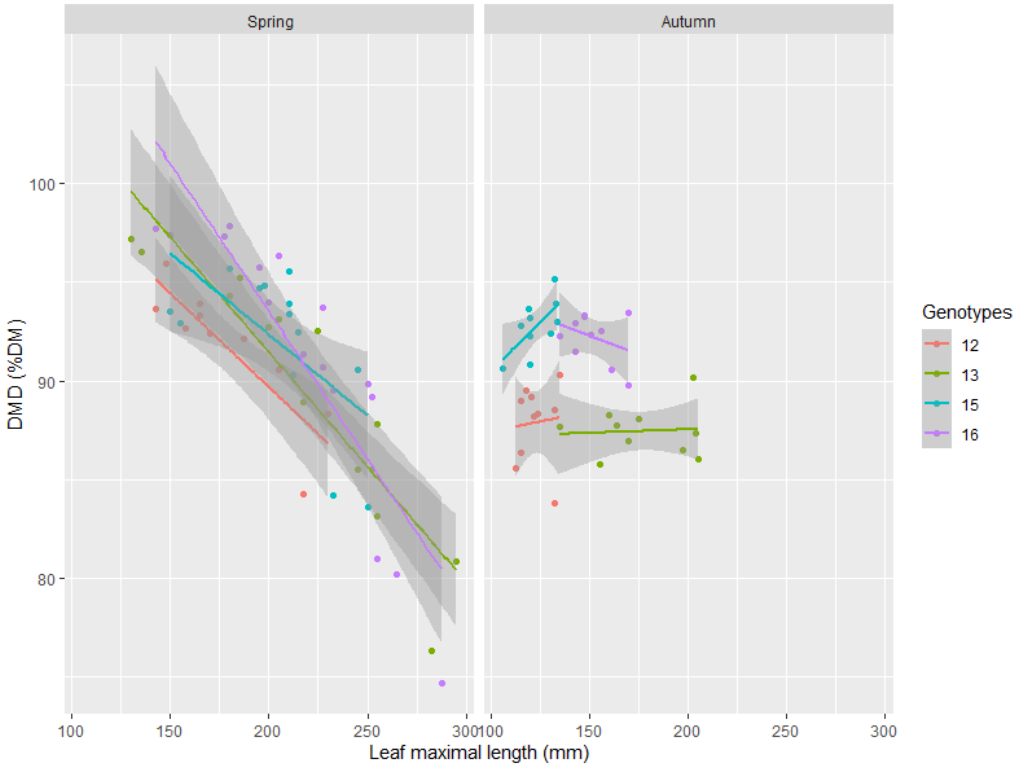

Figure 10 : Variation de la digestibilité de la matière sèche (DMD) des échantillons de feuilles en fonction de la longueur maximale des feuilles pour chaque génotype au printemps et à l'automne 2019

En combinant les données de l'ensemble des feuilles et tiges prélevées à une même date pour chaque bloc et génotype, il est possible d'obtenir les différentes caractéristiques d'une talle en fonction du temps.

Au printemps, la DMD d'une talle moyenne du génotype 13 décroit plus rapidement que le génotype 15 avec les génotypes 12 et 16 étant intermédiaires (Figure 11). Quant à la biomasse, elle augmente avec le temps, mais de façon moins rapide pour le génotype 15. A l'automne, ce sont les génotypes 12 et 13 qui ont leur digestibilité qui décroit le plus rapidement. Mais la biomasse des talles ne varie pas dans le temps.

On observe une corrélation négative entre la qualité d'une talle et son poids sec, mais seulement au printemps (Figure 12)

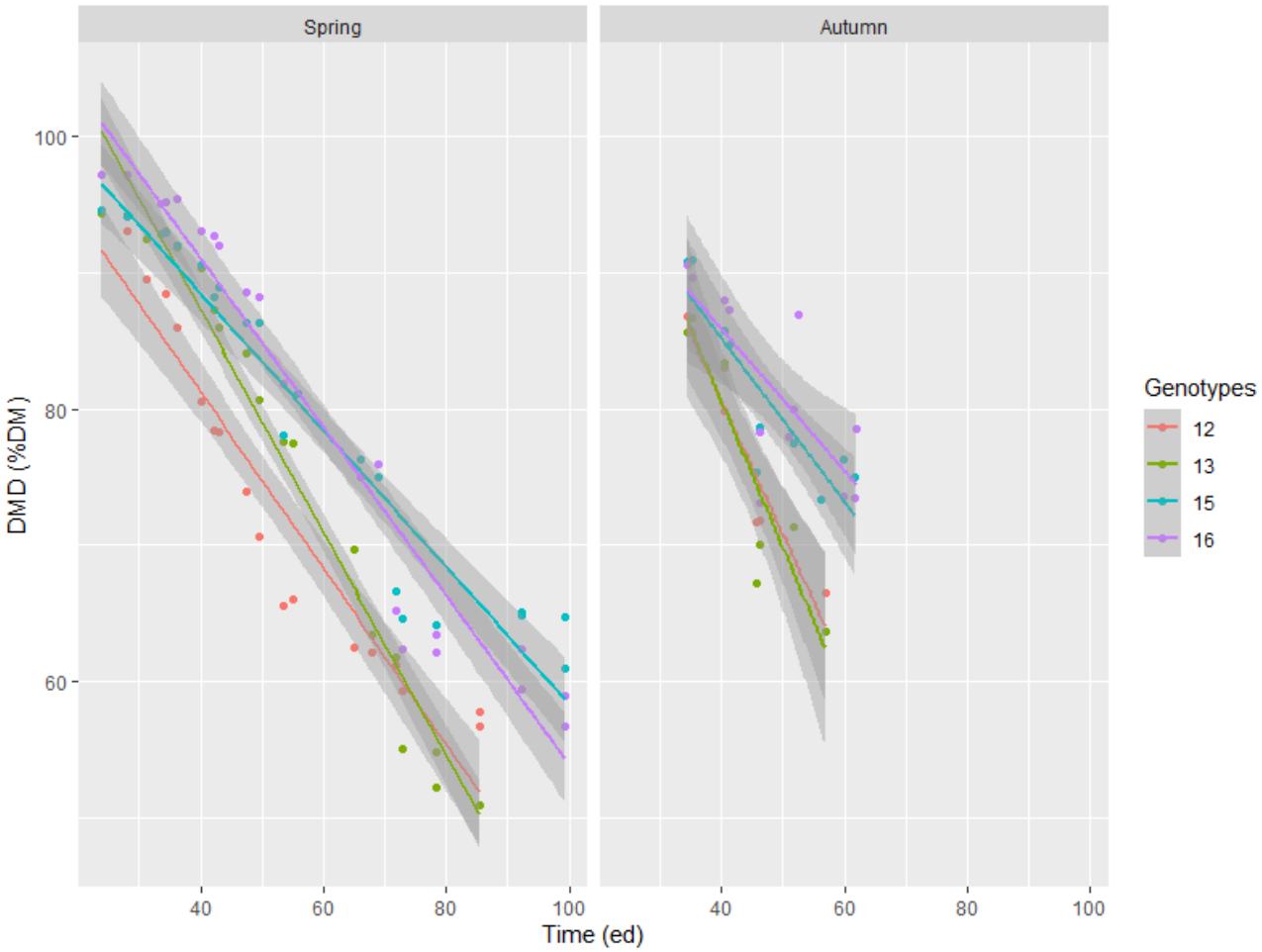

Figure 11 : Variation de la digestibilité de la matière sèche (DMD) d'une talle moyenne en fonction du temps en jours effectifs (ed) pour chaque génotype au printemps et à l'automne 2019

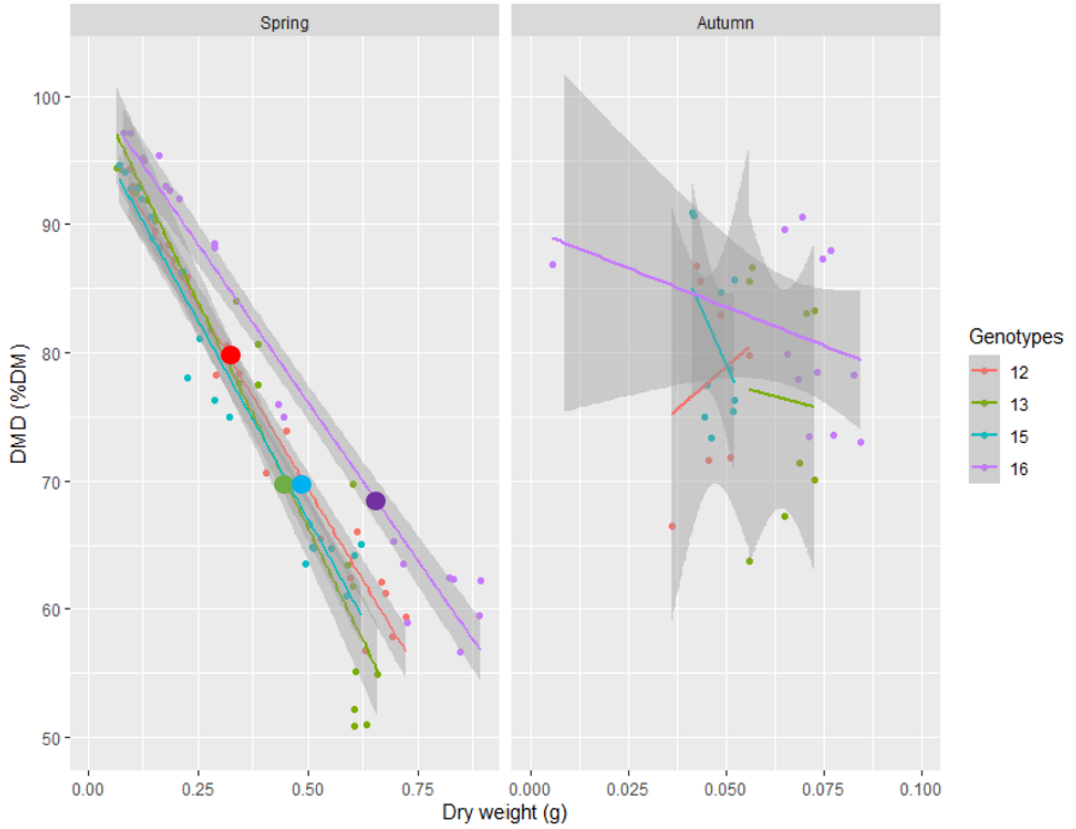

Figure 12 : Variation de la digestibilité de la matière sèche (DMD) d'une talle moyenne en fonction du poids sec pour chaque génotype au printemps et à l'automne 2019. Les points sont placés aux valeurs obtenues à la date d'émergence.

3.3. Discussion

Sur les deux saisons, le principal facteur affectant la DMD des feuilles sur l'ensemble de leur durée de vie serait la proportion de sénescence, expliquant environ 80% de la variation. Cet effet pourrait être dû à la baisse de sucres solubles et d'azote pendant la sénescence (Gregersen et al., 2008).

Au printemps, le facteur majeur de variation de la DMD des feuilles vertes (= non-sénescentes) serait le rang de la feuille. Les rangs les plus élevés ont une digestibilité plus faible. Cet effet serait directement lié à la longueur des feuilles et à la proportion de gaine et a déjà été observé chez les graminées fourragères (Agnusdei et al., 2011; Groot et al., 1999; Insua et al., 2018).

Concernant les facteurs biochimiques, la variation de DMD des feuilles serait expliquée par le taux de fibres (NDF) et la digestibilité des fibres (NDFD), ce qui a déj été observé (Jančík et al., 2008).

La digestibilité des tiges était presque toujours plus faible (65-80% DM) que les feuilles vertes (75-97% DM), ce qui est souvent observé dans la littérature, mais dans certains cas, les très jeunes tiges peuvent montrer une meilleure qualité que les feuilles matures (Terry and Tilley, 1964). De plus, nous avons observé que la qualité de la tige décroissait rapidement avec le temps, dû à une baisse rapide de la qualité des entrenœuds de faible rangs et à la faible qualité de l'épi et de son pédoncule. L'évolution de la qualité de la tige a un impact sur la date de coupe, qui est recommandée de faire avant l'émergence de l'épi (Duru et al., 2000). A ce stage, les plantes sont toujours feuillues et la qualité de la tige n'a pas encore violemment chutée. La chûte de digestibilité des tiges n'a pas été identique entre les génotypes observés, ce qui offre la possibilité de sélectionner pour la qualité des tiges à des stades avancés (après épiaison). Cette sélection pourrait produire de nouvelles variétés dont la date de récolte serait plus flexible.

Puisque l'évolution de la qualité des feuilles et des tiges diffèrent selon les génotypes, il était nécessaire de reconstruire les talles prélevées afin de pouvoir estimer la qualité de la plante entière. Ceci nous a permis de constater que le classement des génotypes, au printemps, en fonction de leur digestibilité variait en fonction de la date ou stade de coupe. Bien que la qualité soit importante, il est aussi nécessaire de s'intéresser à la biomasse produite. Nous avons donc constaté qu'une bonne méthode pour comparer différents génotypes pour leur qualité et leur biomasse, indépendamment de leur date de récolte, serait d'utiliser le ratio entre le pourcentage de matière sèche non-digestible et le rendement avec l'hypothèse que la DMD tende vers 100% lorsque la biomasse tend vers 0 g. Le génotype ayant la plus faible valeur pour la formule (100 – DMD) / Biomasse serait le meilleur en production de biomasse digestible.

A l'automne, nous n'avons pas observé de variation de biomasse avec le temps, comme pour les talles végétatives de l'étude de Korte et al. (1984). Le classement des génotypes suivant leur qualité ne varie pas suivant la date de coupe. Mais il a été observé que la qualité des talles baissait avec le temps.

4. Discussion générale

Dans cette étude, nous avons examiné les différentes sources de variation de la qualité du ray-grass anglais : la génétique, la date de récolte, le type d'organe et l'âge. Comme dans d'autres études sur des espèces de graminées fourragères (Barrière et al., 2003 ; Casler et Vogel, 1999 ; Grabber et al., 2004), nous avons observé qu'une part importante de la variation de la qualité était expliquée par la variation génétique, ce qui permet de faire des travaux de sélection de variétés. Néanmoins, nous avons observé une interaction entre le génotype et la saison (printemps et automne) de sorte que le classement des génotypes pour la qualité des feuilles diffère entre le printemps et l'automne. Ceci a pour conséquence que pour une sélection de qualité sur l'année, il serait nécessaire de sélectionner sur chacune des saisons. Cela pourrait être obtenu en incluant la qualité des deux saisons dans l'indice de sélection avec d'autres caractères tels que le rendement, la résistance aux maladies ou la persistance. Sachant que le rendement en automne est considérablement plus faible qu'au printemps, on peut s'interroger sur l'efficacité économique de la sélection sur la qualité automnale. Compte tenu du manque de fourrage en automne, nous pensons qu'il pourrait être intéressant d'avoir un fourrage de haute qualité à cette saison. De plus, la variance génétique observée de la qualité en automne était plus élevée que celle du printemps malgré une variance environnementale plus importante. Une solution pour l'élevage pourrait être de donner un coefficient plus important dans l'évaluation de la qualité du fourrage au printemps qu'à l'automne. Un autre point concernant la différence entre les saisons est que les caractéristiques biochimiques expliquant la variation de la qualité des feuilles étaient différentes dans les deux saisons. Au printemps, la variation de la qualité des feuilles était principalement expliquée par les teneurs en hémicellulose et en cellulose (fortement corrélées négativement) alors qu'elle était expliquée par la teneur en lignine en automne.

Au printemps, il existe une difficulté majeure pour classer les génotypes selon leur potentiel car la qualité, la biomasse et la date d'épiaison sont fortement corrélées (Beecher et al., 2018 ; Carrère et al., 2010). En effet, le stade reproductif diminue la qualité de la biomasse récoltée à travers deux processus : la production de feuilles plus longues avec un ratio longueur de gaine/feuille plus élevé et la production de tiges, qui sont de moins bonne qualité que les feuilles. Nous avons observé qu'en comparant les génotypes récoltés à la date d'épiaison (dates de récolte différentes pour chaque génotype), les génotypes à épiaison tardive présentaient une qualité inférieure à celle des génotypes à épiaison précoce. En revanche, en comparant les génotypes récoltés juste avant l'émergence des épis des génotypes à épiaison tardive (même date de récolte pour tous les génotypes), les génotypes à épiaison tardive présentaient une qualité supérieure à celle des génotypes précoces qui contenaient déjà des tiges de faible qualité. Ce phénomène bien connu constitue l'une des raisons pour lesquelles les génotypes sont regroupés en groupes de précocité afin d'être comparés pour l'enregistrement des variétés par le CTPS. Néanmoins, les variations génétiques de la qualité, de la production de biomasse et de la date d'épiaison restent présentes au sein des groupes de précocité, ce qui empêche un classement optimal des génotypes pour la qualité ou le rendement. L'étude de la qualité et de la biomasse de chaque organe pendant la maturité de la plante nous a permis de suivre la relation entre la qualité de la plante et la biomasse. Nous en avons déduit qu'**une bonne façon de comparer les génotypes pour la qualité et la biomasse, indépendamment de la date de récolte, serait de sélectionner le rapport entre le pourcentage de matière sèche non digestible et le rendement**.

Au printemps également, nous avons observé qu'il devrait être possible de sélectionner la digestibilité des tiges. En effet, des différences de qualité de la tige et de sa vitesse de décroissance avec le vieillissement ont été observées entre les génotypes. Par exemple, le génotype 13 a montré des feuilles de haute qualité mais des tiges de faible qualité alors que le génotype 15 a montré une haute qualité des feuilles et des tiges. Ce résultat est particulièrement intéressant car il pourrait favoriser la création de variétés avec une meilleure qualité de tige permettant plus de flexibilité pour la date de récolte. L'agriculteur pourrait récolter sa prairie après la levée des épis en conservant une qualité de fourrage relativement élevée et un bon rendement avec la **présence de tiges digestibles « lourdes »**.

En automne, il était plus facile de comparer les génotypes pour leur potentiel de qualité puisque les plantes restaient en stade végétatif. Nous avons observé que la biomasse végétale augmentait rapidement jusqu'à devenir stable à un stade physiologique d'environ trois feuilles matures non coupées. La production de nouvelles feuilles équilibrant la sénescence des anciennes. Puisque le principal facteur diminuant la qualité des feuilles est la sénescence, **il serait préférable de récolter lorsque l'on atteint la troisième feuille mature non coupée afin d'optimiser le rendement et la qualité du fourrage récolté**. C'est la gestion des prairies conseillée dans le cadre du pâturage tournant dynamique : méthode Herby (Cliquet et al., 2019).

Au cours des deux saisons, nous avons observé que la digestibilité était expliquée à la fois par la teneur en paroi cellulaire et sa digestibilité. La sélection pourrait être appliquée directement sur la digestibilité de la matière organique mais **il serait plus efficace de contre-sélectionner la teneur en parois cellulaires et de sélectionner la digestibilité des parois cellulaires** en gardant à l'esprit que les ruminants ont besoin d'un fourrage contenant une certaine quantité de fibres digestibles (pour la production de lait, le régime doit contenir un minimum de 27 % de MS sous forme de fibres (Woodford et al., 1986)) plutôt que des composants solubles (sucres, protéines).

Cette étude nous permet de mieux comprendre la cinétique de la qualité au niveau des organes et des tiges. Cependant, pour appréhender l'évolution de la qualité au niveau de la plante, qui est constituée de nombreuses talles d'âges différents, ou au niveau du champ, il est nécessaire d'utiliser un modèle de croissance végétale. Ce modèle devra simuler chaque organe aérien (feuille avec limbe et gaine, tige) et leurs règles de croissance avant d'ajouter leur qualité. Le modèle L-grass présenté en introduction répond à ces exigences, nous allons donc expliquer comment il est possible d'améliorer ce modèle par l'intégration de la qualité. Il permettra de simuler la qualité d'une plante simulée (entièrement ou seulement la partie récoltée) pendant sa croissance.

Tout d'abord, il sera nécessaire d'ajouter deux nouveaux paramètres pour les modules foliaires : la digestibilité de la matière sèche (DMD) et le ratio de sénescence. Le ratio de sénescence de la feuille n commencera à une valeur de 0 % et commencera à diminuer lorsque la feuille n+3 apparaîtra. Une valeur de DMD sera attribuée pour chaque feuille à sa création. Cette valeur dépendra de quatre facteurs : la longueur maximale de la feuille, le rang de la feuille, un coefficient dépendant du génotype, et un autre dépendant de la saison. Un cinquième facteur est le ratio de sénescence afin de diminuer la valeur du DMD avec la progression de la sénescence. Une feuille sera supprimée lorsque sa valeur pour le ratio de sénescence atteindra 100 %.

Résumé

Le ray-grass anglais (*Lolium perenne* L.) est une espèce fourragère importante pour l'alimentation des ruminants. Les sélectionneurs ont pour objectif d'améliorer la qualité du fourrage, son rendement, sa résistance à la sécheresse et aux maladies. La qualité et le rendement dépendent de facteurs génétiques et environnementaux et sont étroitement liés à la morphogenèse de la plante et à la date de récolte ce qui rend difficile le classement des génotypes selon leur qualité. La partie de la plante récoltée est composées de feuilles et de tiges au stade reproducteur. L'objectif de la thèse était de mieux comprendre les sources de variation de la qualité du fourrage au cours du développement de la plante.

Premièrement, nous avons étudié la diversité génétique et saisonnière pour des caractères liés à la qualité. Nous avons utilisé une collection de 592 génotypes de ray-grass anglais phénotypés pour la digestibilité de la matière organique (OMD), la digestibilité des parois cellulaires (NDFD) et les composants de cette paroi cellulaire (cellulose, hémicellulose et lignine) sur 2 années au printemps à la date d'épiaison et en automne. Une forte interaction génotype x saison a été observée pour l'OMD et la NDFD, indiquant des différences dans le contrôle génétique de ces traits entre saisons. La variation d'OMD est expliquée à la fois par la teneur en paroi cellulaire (NDF) et par sa qualité. La variation de la NDFD au printemps s'explique principalement par la teneur en hémicellulose. Une augmentation de 1% de la teneur en hémicellulose dans la paroi cellulaire (HC.NDF) a entraîné une augmentation de 0,81% de la NDFD. En automne, la variation de la qualité s'explique principalement par la teneur en lignine dans la paroi cellulaire (ADL.NDF). Une diminution de 0,1 % de l'ADL.NDF a entraîné une augmentation de 0,41 % de la NDFD. Pris par saison, les différentes variables ont montré une forte héritabilité et certaines ont présenté une variation génétique plus élevée en automne qu'au printemps. Dans une étude d'association basée sur 503 gènes candidats, nous avons identifié des QTLs expliquant de 29% à 52% de la variance phénotypique des variables OMD et NDFD. Chaque marqueur expliquait de 1 à 7% de la variance. Aucun QTL identique n'a été identifié entre les saisons, mais au sein d'une même saison, certains QTLs étaient communs entre les traits de digestibilité et les traits de composition de la paroi cellulaire, confirmant l'importance de la concentration en hémicellulose pour la digestibilité au printemps et la concentration en lignine dans le NDF pour la digestibilité en automne.

Dans un second temps, nous avons décrit la cinétique de croissance et de qualité de quatre génotypes contrastés pour la date d'épiaison et la qualité pendant deux saisons différentes (printemps et automne). Nous avons observé que le principal facteur expliquant la diminution de la qualité des feuilles avec la maturité était la sénescence des feuilles. Ce facteur exclut, la qualité des feuilles vertes diminue avec l'augmentation de la longueur des feuilles et de la proportion de gaines. La qualité des tiges était inférieure à celle des feuilles et diminuait fortement avec la maturité. La qualité de la tige et la vitesse à laquelle elle décroissait avec la maturité différait entre les génotypes, ce qui permet une sélection potentielle pour les génotypes ayant une meilleure qualité des tiges matures. Au printemps, le choix des génotypes à sélectionner diffère selon les objectifs de production de biomasse et de qualité. En automne, les plantes devraient être récoltées immédiatement après que la biomasse ait atteint un maximum afin d'obtenir la meilleure qualité.

Finalement, nous suggérons d'introduire la qualité de la tige dans l'évaluation du fourrage au cours du processus de sélection et d'effectuer des mesures de la qualité au printemps et à l'automne.

Abstract

Lolium perenne L. (perennial ryegrass) is an important forage crop in dairy farming, either for grazing or haying purposes. Breeders goals are to improve the forage quality, yield, drought resistance and diseases resistance. Quality and yield depend on genetic and environmental factors and are tightly linked to plant morphogenesis and to the date of harvest hampering the ranking of genotypes for their quality. The harvested plant part is composed of leaves and stems at reproductive stage. The objective of the thesis was to understand better the origins of quality variation during the plant growth.

First, we have investigated genetic and seasonal influences on quality traits. We used a highly diverse collection of 592 perennial ryegrass genotypes phenotyped for organic matter digestibility (OMD) and underlying traits such as cell wall digestibility (NDFD) and cell wall components (cellulose, hemicellulose and lignin) during 2 years for two different cuts : in spring at heading date and in autumn. A high genotype x season interaction was found for OMD and NDFD, indicating differences in genetic control of these forage quality traits in spring *versus* autumn. OMD was explained by both the quantity of cell wall content (NDF) and the quality of the cell wall content (NDFD). The variability in NDFD in spring was mainly explained by differences in hemicellulose. A 1% increase of the hemicellulose content in the cell wall (HC.NDF) resulted in an increase of 0.81% of NDFD. In autumn, the variation of quality was mainly explained by the lignin content in the cell wall (ADL.NDF). A 0.1% decrease of ADL.NDF resulted in an increase of 0.41% of NDFD. Within seasons, OMD was highly heritable and showed a higher genetic variation in autumn *versus* spring, indicating the potential to select for forage quality in the autumn cut. In a candidate gene association mapping approach, based on 503 genes involved in cell wall biogenesis, plant architecture and phytohormones, we identified significant QTLs explaining from 29% to 52% of the phenotypic variance in the forage quality traits OMD and NDFD. Taken individually each QTL had a small effect ranging from 1 to 7%. No identical QTLs were identified between seasons, but within season, some QTLs were in common between digestibility traits and cell wall composition traits confirming the importance of hemicellulose concentration for spring digestibility and lignin concentration in NDF for autumn digestibility.

Secondly, we described kinetics of growth and quality in four genotypes contrasting for heading and quality, during two different seasons (spring and autumn). We observed that the main factor explaining the quality decrease of leaves with maturity was leaf senescence. Excluding senescence, green leaf quality decreased with an increase of leaf length and sheath proportion. Stem quality was lower than leaf quality and decreased strongly with maturity. The stem quality and its decrease rate with maturity differed between genotypes allowing a potential selection for genotypes with higher quality of mature stems. In spring, a compromise should be made between biomass production and its quality. This compromise differed between genotypes and harvest dates. A good way to compare genotypes for both quality and biomass, independently of the harvest date, would be to select for the ratio between the percentage of non-digestible dry matter and the yield. In autumn, plants should be harvested immediately after the biomass reached a maximum in order to obtain the best quality.

To conclude, we suggest including stem quality to forage evaluation during breeding process and to take into account quality at both spring and autumn seasons.

Key-words: Forage grasses, Perennial ryegrass, Digestibility, Quality, Biomass, Genetic variation, Fibres, Season effect, Aerial morphogenesis, GWAS