



University of Reading

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Investigation of Variation and Genetic Basis of Traits Determining Forage Yield and Quality of Faba Bean

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DECLARATION

I sincerely declare that no unethical actions were taken for the creation of this thesis which is entirely the results of my own work and has not been shared with any institution (or publication).

06.09.2022

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LIST OF ACRONYMS

ADF: Acid Detergent Fiber

ADL: Acid Detergent Lignin

CP: Crude Protein

DM: Dry Matter

DTF: Days to Flowering

FB: Faba Bean

NDF: Neutral Detergent Fiber

OM: Organic Matter

PC: Protein Content

PH: Plant Height

ST: Stem Thickness

SUMMARY

The world population is increasing fast, and the food requirement by 2050 will be increased by 70 %. Protein production will also take up a big proportion of this increase. Animal production provides one-third of the required protein amount in especially developing countries. However, global warming and climate change have a mutually harmful relationship with animal production. Forage crop production is shown as one of the most challenging aspects of agriculture production. In this dissertation, faba bean (*Vicia faba* L.), which is sustainable and has a high potential for forage, was evaluated as a whole crop forage crop. As its seed is generally incorporated in concentrated animal feed, its forage capability is left less studied in recent years. In this study, therefore, fresh biomass and DM yield, PH, ST, DTF, and PC were examined to evaluate the forage quality of FB. The mean values of these traits were 4300 kg/da, 913 kg/da, 78.01 cm, 7.83 mm, 60.87 days, and 16.44%, respectively, in all plots. Among all traits, PH, ST, DTF, and PC have shown statistically significant variation across the genotypes. Genome-wide association scans were conducted with PH, ST, and DTF phenotypic data to find associations between those traits and SNP markers. No marker significantly associated with any characteristic was discovered possibly because of the low statistical power of a small population with a low level of replication. However, SNPs associated with DTF made a smooth peak near the threshold of 0.05 confidence interval. This is a hopeful result for future studies.

KEYWORDS: FABA BEAN, FODDER FORAGE, BIOMASS, GWAS, SNPs

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CHAPTER 1. INTRODUCTION

In 2022, The US Census Bureau estimated that the world population is more than 7.9 billion. While that was 3.68 billion in 1970 (The World Bank, 2019), more than two times more people require food in 2022 compared to fifty years ago. By 2050, Foyer *et al.* (2016) predict that food production should rise by 70 per cent under population growth (Figure 1.1). In addition, Henchion *et al.* (2017) call attention to the increased protein needs in the future.

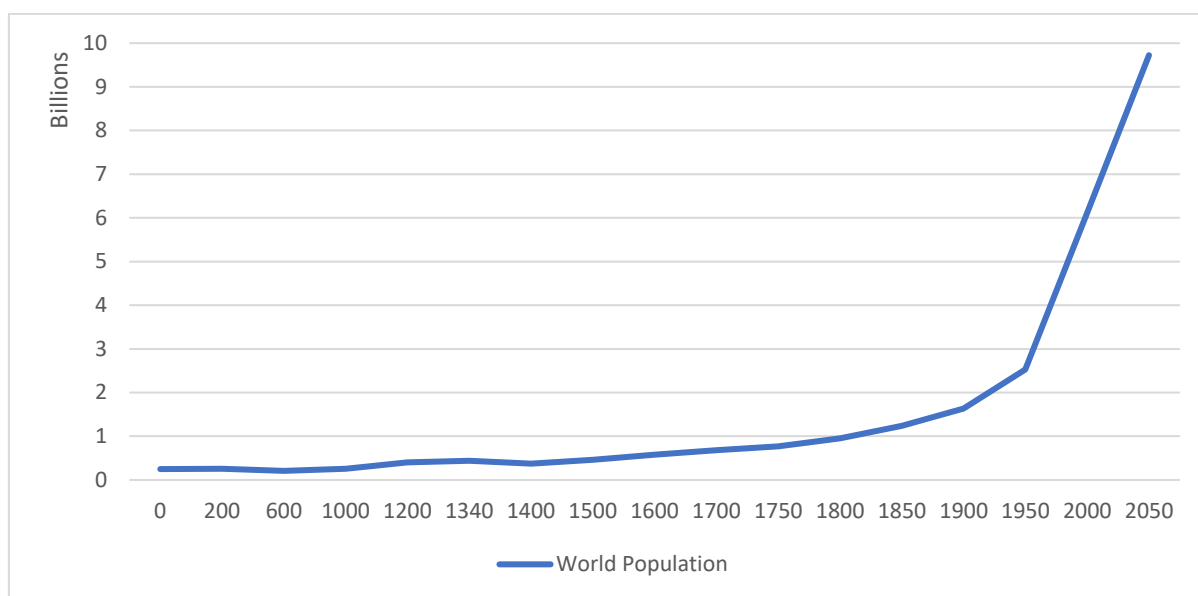


Figure 1. 1. The Change of World Population in the Past and Present, and the Prospect of Future Population (Bacci, 2017)

The dilemma is that while protein needs will increase, climate change and global warming, the biggest problems of the 21st century, cause pressure associated with temperature increase and precipitation variation on livestock production, which is an important protein source (Thornton, 2010; Wreford & Topp, 2020). Rotter and Van de Geijn (1996) have reported that climate change negatively affects the followings; i. grains as feed, ii. pastures, forage crop production, and quality, iii. health, growth, and reproduction, iv. diseases. Furthermore, more land usage for food production because of the 70% more food requirement by 2050, the idea of direct consumption of cereals as

human food instead of feed, and water scarcity will limit livestock production (Rojas-Downing *et al.*, 2017). Consequently, in addition to animals' physiological adaptation problems in more warm future climates, fodder production might be difficult because of the land competition for food production.

On the opposite side, livestock production impacts climate change and global warming directly or indirectly. It is reported that 14.5 per cent of total greenhouse gases (GHGs) and the usage of just above one-fourth of the earth's total land area are contributed to livestock (Sakadevan & Nguyen, 2017). As a direct impact (Figures 1.2 and 1.3), the lion's share of global livestock-induced GHGs comes from enteric fermentation and feed production (FAO, 2010; Gerber *et al.*, 2013). Indirect impacts of livestock in climate change are related to land use changes. The expansion of livestock production is more associated with deforestation. Published studies have found a positive correlation between livestock production and deforestation in Brazil (Seidle *et al.*, 2001; Barona *et al.*, 2010; Skidmore *et al.*, 2021). The root of that is the creation of new feed sources such as pastures and grasslands. Even though the rate of deforestation has gradually gone down since 2000, especially South America and Africa are still affected dramatically by deforestation (FAO, 2020A).

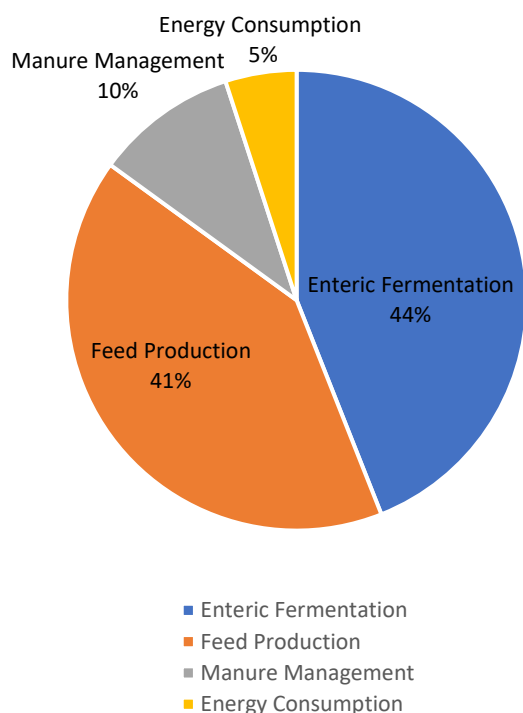


Figure 1. 2. The Parameters Causing GHGs in Livestock Production in 2010 (FAO, 2010)

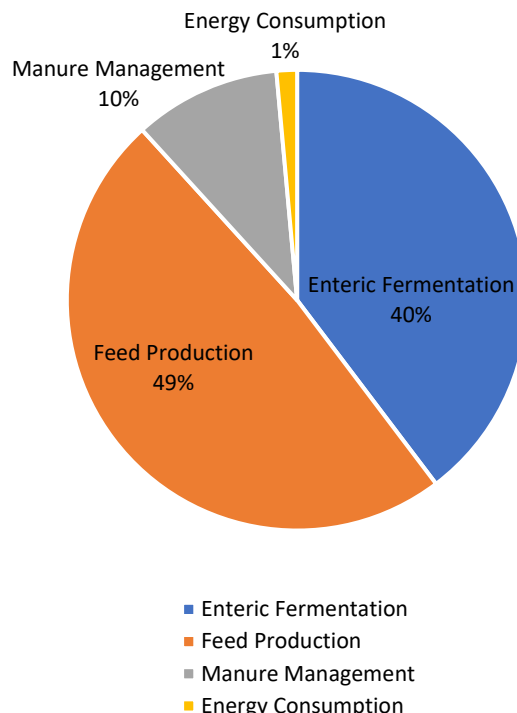


Figure 1. 3. The Parameters Causing GHGs in Livestock Production in 2013 (Gerber et al., 2013)

Although reducing livestock and substituting animal-based proteins with plant-based proteins are seen as new approaches to the struggle with climate change (Human Society International, 2022; Carrington, 2018; Levitt, 2021), livestock is a vital sector for many developing countries. It accounts for one-third of total protein sources in their diets (Havlík *et al.*, 2014). In addition to the expected protein demand increase next 30 years, forgoing livestock production might result in an extra burden on crop production in developing countries as people try to substitute protein from the plants. Although improvements in agronomy and plant breeding have made it possible to produce enough food in the past and today (Ameen & Raza, 2017), Berners-Lee *et al.* (2018) notify that future food security cannot be provided just by relying on production increases provided with efficiency increase because of the limits of potential improvements. Therefore, an extra burden on crop production might cause extra deforestation to open new farmlands (Davis *et al.*, 2016; Nuwer, 2016). Instead of reducing from livestock farming, solution-oriented approaches to the problems,

such as enteric fermentation and feed production, causing GHGs, would be better for protein production.

Pitesky *et al.* (2009) states that the livestock production footprint which is overwhelmingly GHGs depends on the type of livestock production system. In today's world, it can be separated into two groups which are intensive and extensive livestock production systems in terms of how animals are reared. It is reported that intensive livestock systems are more efficient and emit fewer GHGs than extensive production systems. (McAllister *et al.*, 2020; Lovarelli *et al.*, 2020). The reason behind that is the high efficiency of feed sources in intensive livestock systems. In contrast, animals reared in extensive systems require more feed to gain the same weight compared to the intensive system (Pitesky *et al.*, 2009). In addition, as extensive systems are dependent on the feed sources coming from the environment, it is predicted that a transition from extensive to intensive in developing countries with climate change and global warming is prospective (Rust, 2019). Therefore, improving the quality and efficiency of feed sources that can contribute to intensive livestock production systems might help mitigate livestock's adverse effects on climate change and enhance the efficiency of animals.

In this perspective, faba bean (*Vicia faba* L.) will be treated as a forage crop in this experiment. Warsame (2021) introduces faba bean (FB) as a promising crop because of its high protein content (PC) in seed and N fixation capability. Its fabulous characteristics can contribute to livestock production in the following three ways; i. it might be substituted for protein sources in animal diets, ii. its production can contribute to soil fertility, iii. being an annual crop makes FB favourable to add to crop rotation.

1. Faba bean

FB is one of the most cultivated legumes in the world. China and ancient Egypt used FB in human diets 5000 and 3000 years ago, respectively (Askar, 1986). Its production amount has barely

increased for half a century (Figure 1.4). It is used as both food and feed (Crépon *et al.*, 2010). In terms of nutritional content, such as high PC (Table 1.1), carbohydrates, and fibre contents, FB is reported as a staple food in many developing countries (Askar, 1986; Jamalain, 1999; Crépon *et al.*, 2010; Gnanasambandam *et al.*, 2012; Labba *et al.*, 2021) due to its affordability (Alghamdi, 2009).

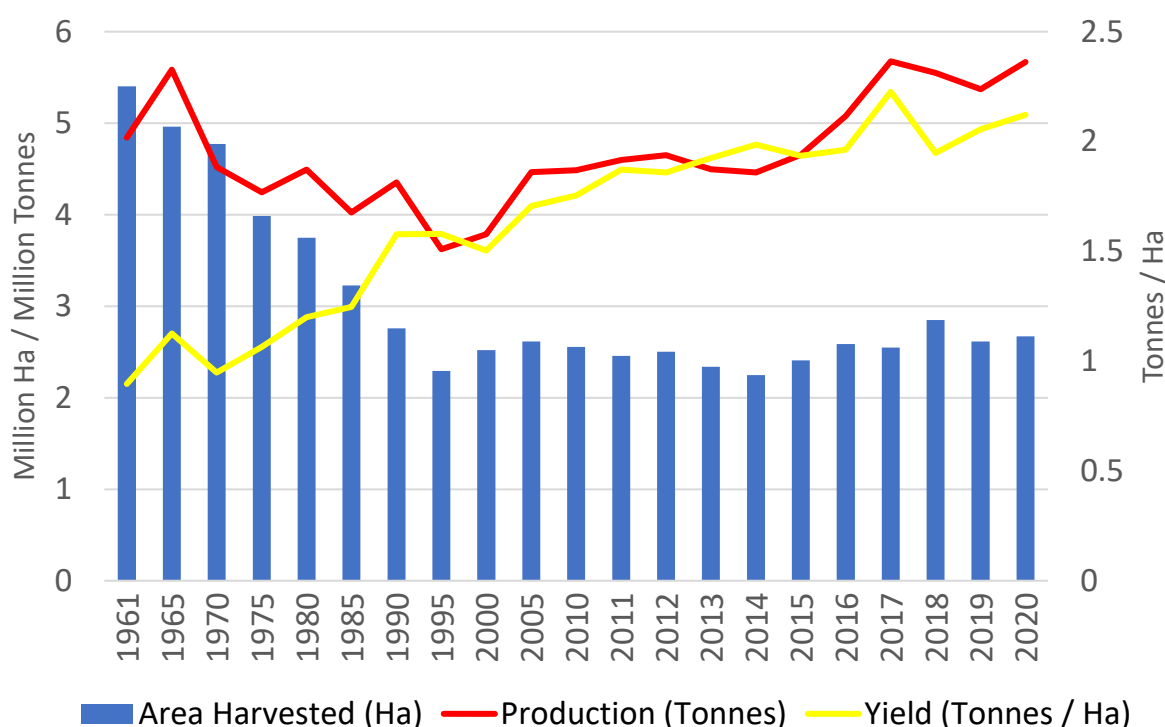


Figure 1. 4. Faba bean Production History (Source: Derived from FAO, 2020B)

Plus, Volpelli *et al.* (2012) have remarked that not only the rumen degradability of FB protein (overwhelmingly containing globulins and albumins) is higher than soybean, but also the dual advantages of using FB in animal feeding in terms of protein and energy content as FB is a starch-rich legume in contrast to soybean. However, some anti-nutritional factors, favism related to vicine and convicine, high tannin content, trypsin inhibitors, lectins, and saponins, limit more widespread use of faba grains (Degago, 2000; Labba *et al.*, 2021; Dhull *et al.*, 2021). Lastly, low methionine content, not an anti-nutritional factor, is also reported as a deficiency in its nutritional value. Even though it is introduced with high protein quality because of great lysine content (Warsame, 2021),

the low protein quality is also reported due to limited sulphur-containing amino acids, cysteine and methionine (Rahate *et al.*, 2021).

Table 1. 1. Protein Content of Faba Bean Seeds (Warsame, 2021)

Number of Genotypes	Protein Content (%)	References
33	22-38	(Griffiths & Lawes, 1978)
600	19-34	(Lafiandra <i>et al.</i> , 1981)
125	22-36	(Sjödin, 1982)
125	29-38	(Frauen <i>et al.</i> , 1984)
30	23-39	(Griffiths, 1984)
12	26-30	(Makkar <i>et al.</i> , 1997)
74	25-37	(Duc <i>et al.</i> , 1999)
-	22-29	(Degago, 2000)

2. Literature Review

2.1. Faba Bean Seeds as a Concentrate Feed

FB is an essential crop as a protein-rich feed source. Besides its high PC, FB is an excellent alternative to soybean because of its GMO-free cultivars (de Visser *et al.*, 2014). Furthermore, several published studies describe the high nitrogen fixation capability of FB compared to other pulses (Augustin and Cole, 2022; Ntatsi *et al.*, 2018). In this aspect, FB is a good option for sustainable animal diets by reducing environmental effects and providing biodiversity in plant rotation (Lamminen *et al.*, 2019). However, the anti-nutritional compounds limit their usage compared to other legumes. The utilisation of FB as a concentrate feed is going to be reviewed separately in the monogastric and ruminants.

2.1.1. Usage in Monogastric (Poultry)

After 1960, studies informed that the tannin content of crops caused an inhibitory effect on protein digestibility in chickens fed with tanning-containing crops (Chang & Fuller, 1966; Kendall, 1966). In 1976, Bond's comparative study found out that tannin-containing FB varieties (coloured flowered) have nearly 40 per cent lower digestibility of testa than tannin-free ones (white flowered), while the digestibility of cotyledon does not change between white and different coloured flowered FB (Bond, 1976). Even though the tannins content of FB had been known and some tannin-free varieties had been discovered during the 1950s (Dickinson *et al.*, 1957), this study was one of the first studies showing the condensed tannins content of FB in its testa. Furthermore, Ortiz *et al.* (1992) have confirmed that monogastric animals are more sensitive to tannin-related problems.

The existing literature on tannin says that feed intake drops might be recorded in animals fed with tannin-containing feed and fodder because of astringent taste (Jansman *et al.*, 1989). However, Jansman *et al.* (1993) have published their findings showing that the inclusion of 300 g/kg FB into the ration does not affect feed intake in chicken. The experiment carried out by Ortiz *et al.* (1992) has shown that when the tannins extracted from FB's testa are added to the chicken feeding ration, the digestibility significantly goes down (from 88.8% to 80.8%). Due to the restrictions and worries on the animal-based and GMO crop-based protein sources in the livestock diets in Europe, Moschini *et al.* (2005) have explored the use of pea, FB, and lupin seeds as an alternative to soybean and the other protein sources in broiler diets and found that only FB (low-tannin content, 0.47 mg/g) had a similar average daily gain compared to the control group. In further years, many studies on poultry have shown similar results that the high tannin content has a negative impact on energy and particularly on protein digestibility compared to tannin-free or low tannin content cultivars (Vilariño *et al.*, 2009; Crépon *et al.*, 2010). Despite the problem of tannins, especially in monogastric animals, farmers in the European continent had cultivated mainly high-tannin content FB cultivars by 2010 (Crépon *et al.*, 2010). Crépon *et al.* (2010) have believed that this hinders the use of more FB in

European countries and have advised 250g/kg concentrate FB in broiler diets, provided that they are tannin-free.

In contrast to earlier findings, a more recent article has shown that the partial inclusion of high tannin content FB in the starter (8-16%) and grower (15-22%) period diets did not show a statistical difference in body weight, body weight gain, feed intake and feed conversion rate compared to control and any other dietary treatment (Tomaszewska *et al.*, 2018). More interestingly, it has been reported that broilers with a diet including Fabelle, a tanning-containing and low vicine-convicine content FB cultivar, had the highest daily intake and feed efficiency (Cho *et al.*, 2019). The authors propagate that vicine-convicine content is a more critical component when FB is considered as an alternative feedstuff in poultry diets.

Moreover, the vicine-convicine content of FB has been reported as another obstacle causing small egg sizes in poultry feed (Olaboro *et al.*, 1981; Vilariño *et al.*, 2009; Crépon *et al.*, 2010). However, contradictory findings are present in the literature. Compared with these results, Magoda and Gous (2011) have found that when FB is dehulled (a method to remove tannin-rich testa from the beans), laying hens do not experience any yield loss. But, it was not stated whether the FB cultivars used in this experiment are high or low vicine-convicine content. Helpser *et al.* (1993) declared that even though tannin content is removed from the seed via genetic improvement, the vicine-convicine content might still negatively affect egg yield. By contrast, Perella *et al.* (2009) have declared that organic broilers fed with rations including, as a protein source, FB plus the declined amount soybean or just soybean performed the same final body weight on the 120th day despite high vicine-convicine content of FB.

O'Neil *et al.* (2012) have demonstrated pea and FB as two feedstuffs in poultry production to sustain reliable and sustainable production in Europe due to soybean prices and EU policy related to GMOs.

Nonetheless, they believe that available nutritional value variability between FB cultivars causes its cultivation instability.

2.1.2. Usage in Ruminants

In contrast to monogastric animals, ruminants are relatively better against the anti-nutritional content of FB. Therefore, the shortage in the 1971 soybean market in the world (USDA, 2009) whetted scholars' appetite for alternative substitutions instead of soybean. In 1974, FB grains were tested as a concentrate feed source as a substitute for soybean and rapeseed meal in lactating cows' rations. It has been concluded that the inclusion of FB with 17.5% and 35% in the total ration without other concentrate protein feed sources can sustain production yield in cows (Ingalls & McKirdy, 1974). A similar experiment was applied for 8-20 weeks of young calves in 1980. The results have shown that the inclusion of 24% or 8.5% FB + 0.8% urea to the ration did not cause any change in feed intake, weight gain, and feed efficiency compared to different rate soybean meals (Ingalls, 1980). The same article informed that when the inclusion of 35 % FB into lactating cows' ration, milk yield is not affected, but cows' weight decreases despite an increased in dry matter (DM) intake.

Turning to other ruminant species, Surra *et al.* (1992) tested FB together with bitter vetch (*Vicia ervilia*) instead of soybean cake in lamb ration. According to the results, while bitter vetch in the ration caused a loss in growth rate and feed conversion efficiency, the inclusion of FB into the ration did not affect the digestibility of DM, OM, and CP and the growth performance of lambs. An experiment comparing low and high anti-nutritional content in 20% substituted FB carried out on cows has shown that anti-nutritional content does not affect cows' health, feed intake, and milk production (Melicharová, *et al.* 2009). In line with previous studies, Leitgeb and Lettner (1992, as cited by Crépon *et al.*, 2010), Brunschwing and Lamy (2002, as cited by Crépon *et al.*, 2010), Lapierre *et al.* (2003, as cited by Crépon *et al.*, 2010) Di Grigoli *et al.* (2005, as cited by Crépon *et al.*, 2010), Delmotte and Rampanelli (2006, as cited by Crépon *et al.*, 2010) have expressed nearly similar results and emphasised that its use in cows and lamb ration does not cause any reduction in

feed intake, weight gain, milk production and composition, and lamb carcass composition. Volpelli *et al.* (2012) have also shared similar results for daily DM intake, milk yield and composition with the introduction of FB to cow diets instead of soybean meal.

Comellini *et al.* (2009) tested the mixture of 10% flaked FB and 7.5% dehulled soybean meal to only 12% dehulled soybean meal in lactating cow diet following the call of Parmigiano-Reggiano Consortium (Italy) not to use GMO feeds. No adverse effect on concentrate intake, milk yield, and quality has been observed.

By contrast, recent articles report that due to the limited presence of sulphur-containing proteins, rather than anti-nutritional content, FB might result in a loss in milk yield when it is added to the diet. (Puhakka *et al.*, 2016; Halmemies-Beauchet-Filleau *et al.*, 2018). However, these two studies differ from previous studies with the substituted protein source, rapeseed meal, not soybean meal. Such that the experiment carried out by Cherif *et al.* (2018) has shown one more time that when soybean is substituted with FB, the intake of DM, OM, and CP do not change, and milk production and composition do not alter between control and FB rations. Notably, for the first time, the impact of FB inclusion in the diets on the methane (CH₄) emissions from cows has been checked in the same study. However, no difference in methane emissions has been found out between control and FB diets.

2.2. Whole-Crop Forage (Fodder)

McKnight and MacLeod (1977) were one of the first to examine the whole-crop forage quality of FB. They have demonstrated that when FB is harvested in the period in which the first pod is turning black, the PC and the DM of FB silage are higher compared to grass-legume silage and have advised FB as protein-rich forage. In addition, lactating cows fed with FB silage in the trial had higher DM intakes and produced milk including more fat (4.09% - 3.77%) than the cows fed with grass-legume silage. After two years, another trial confirmed the increase in the milk fat and daily DM intake

when lactating cows are fed with wilted FB silages (Ingalls *et al.*, 1979). In the same experiment, it has also been reported that the total DM increase in cows is possible with the use of FB silage despite the decrease of the grain rate (from 56% to 43%) in the ration. Ingalls *et al.* (1979) have also informed that whole plant FB silage provides the highest daily weight gains ($P<0.01$) in beef cattle compared to maize, grass-legume, barley and barley-clover silages. Like lactating cows, steer calves consumed more daily DM consumption in the trial when they were fed with FB silage.

Faulker and Steen (1984) have obtained DM yields ranging from 7.85 ton/hectare to 9.90 ton/hectare and PC between 14.5% and 15.5% from different FB varieties. Despite these promising characteristics, the feeding value of FB silage was not found to be better than grass silages. It has been advised as a break crop (wheat-fallow) or nurse crop. A comparison study between FB and pea as spring forage crops has shown that the former has greater DM yield per hectare and DM concentration than the latter (Faulkner, 1985). However, the mixture sowing of FB with barley and oat tended to reduce DM yield, while peas increased the total yield in the mixture sowing with the same crops. Furthermore, the erect habit of FB has been propagated as a positive trait for harvesting in comparison with the peas. Another experiment carried out by Berkenkamp and Meeres (1985) has informed that the PC of whole-plant FB is on average 17.8 % (between 17.6% – 20%), and the average DM yield per hectare is 10310 kg in Lacombe (Canada) and 4381 kg in Bluffton (Canada). Even though FB seed has more PC than the green parts of the whole plant (Table 1.1), these values were more significant than in pea (16.6% PC).

The inclusion of whole-plant FB silage to legume-maize silage instead of alfalfa silage by 30% in the diet of steers was tested by Wagner *et al.* (1990). There has been no difference in feed intake, weight gain, and feed conversion between steers fed with FB-maize and alfalfa-maize. Rather than the whole-plant forage capability of FB, the authors have called attention to the agronomic and economic conditions to determine whether using it as a whole-plant forage crop is acceptable. A relatively more recent study carried out by Lamminen *et al.* (2015) confirmed the result of no

significant variation in feed intakes and milk yield between cows fed with grass silage and fed with the 50 % FB-included legume-grass silage. Mustafa and Sequin (2003) have checked FB, soybean, and pea for degradability. While FB had the highest crude protein (488g/kg), the pea was the best silage for in situ degradability in the experiment.

In contrast to the previous mixture sown, barley-FB or oat-FB, new studies have discovered a positive correlation in mixture sowing with FB. The experiment comparing the spring and autumn sown wheat-FB mixture and separately sown of both crops have figured out that average DM yields of the spring sown mixtures were greater than wheat sown in spring (Ghanbari-Bonjar and Lee, 2003). In addition, autumn sowing positively affected the DM yield in both mixtures (14 t/ha) and sole FB (12t/ha) cultivation.

In favour of Faulker and Steen (1984), Strydhorst *et al.* (2008) have shown a reduction in forage DM yield of barley intercropped with FB compared to sole barley. On the other hand, protein yield is better in the FB-barley mixture than in the sole barley, lupin-barley and pea-barley mixtures.

In terms of the optimum forage characteristic of stems in the FB, a maximum of two stems or two lateral branches per plant and plant stature between 1 and 1.2m have been specified by Mihailović *et al.* (2011). Otherwise, the increase in the stem or branch amount might cause high lignin content, which is not favourable for digestibility. Likewise, the stage of full flowering and first pods has been advised for forage harvesting. In contrast, Fraser *et al.* (2001) have announced that FB produced more fresh-biomass than peas when harvested at fourteenth week from the sowing in the mid-podding stage. In the same study, higher plant height (PH) was reported for FB than pea, between 115 cm and 146cm from the 10th week to the 14th week.

As well as whole-plant forage usage, the straw of FB is a necessary roughage in predominantly developing countries. Therefore, experiments to determine and evaluate the straw quality of FB have been carried out as it is one of the most cultivated plants in Ethiopia. Even though farmers

allow the growth of weeds in traditional FB cultivation to increase forage production in the country, Tadesse (2016) has reported that FB intercropped with oat had a higher straw yield compared to conventional and the weeded FB production practice. Alongside the management practices, the variation between FB cultivars used in the experiment (CS-20DK, Tumsa, Walki, Gebelchu, and Dosha) was also significant ($P < 0.01$) in straw DM, according to the results. With a similar perspective, Alktib *et al.* (2016) have checked four cultivars and one local variety to find differences in grain yield, straw yield, and the nutritional value of different fractions of straw. While DM, organic matter (OM), ash, and in vitro OM digestibility accounted for a strong variation between cultivars, the variations of different cultivars in CP, NDF, ADF, and ADL were not statistically significant. In addition, the botanical fractions of straw and their proportions in total highly determined the straw quality just as substantial as cultivars (Alkhtib *et al.*, 2016).

Recently, Guevara (2020) has tested the roughage capability of FB. Between 7.68 and 12.74 t/ha, DM yields obtained from different cutting stages were reported, and the highest yield of FB has been shown to be better than alfalfa and timothy DM yields. Moreover, CP content of whole-crop FB was found between 20-22 %, and again these values were reported as greater than alfalfa and timothy values, 18% and 5%, respectively. Lastly, the study showed that the tannin content of plants can affect the attractiveness of roughage and the voluntary feed intake of ruminants. Likewise, when the FB plants have been just mown and are fresh, the attractiveness of roughage for cows was greater compared to FB silage (Guevara Oquendo *et al.*, 2021).

Lastly, Palmio *et al.* (2022) have reported that the mixture of 75% whole-crop FB silage and 25% of grass silage increased DM intake and milk yield. In addition, no statistically significant difference between diets, which are FB-grass silage and grass silage plus rapeseed meal, was present. Most interestingly, the authors have disclosed the possibility of increased digestibility with late harvest in whole-crop FB forage.

2.3. Plant Breeding History in Faba Bean

FB cultivation has been steady for two decades (Figure 1.4). Plant breeding is one of the most important tools we have to ensure that FB production is competitive and profitable. The main objectives of FB breeding are yield enhancement, building resistant or tolerant lines against biotic and abiotic factors, and removing anti-nutritional factors (Khazaei *et al.*, 2021a; Khazaei *et al.*, 2021b). Although the former two aims are vital to increase production and meet the risen demand for food demand in the world, eliminating anti-nutritional factors is of priority in particular feed FB. As Degago (2000), Labba *et al.* (2021), and Dhull *et al.* (2021) have pointed out that the compounds found in its seed mitigate the interest of farmers in FB. To such an extent, Adhikari *et al.* (2021) determine the FB as ‘the orphan of orphans’.

Even so, remarkable progress in FB breeding has been achieved for four decades (Maalouf *et al.*, 2018). In fact, it has pretty rich genetic diversity (Maalouf *et al.*, 2018), and conventional plant breeding relying on phenotypic selection is the most significant stakeholder in this progress (Adhikari *et al.*, 2021). For example, the *zt1* and *zt2* genes discovered by Picard (1976, as cited in Duc *et al.*, 1999) and the *vc-* gene discovered by Dut *et al.* (1989) were the first few initiatives clarifying responsible genes related to the anti-nutritional content of FB. Despite many cultivars bred via conventional methods (Khazaei *et al.*, 2021a), because of ineffective selection criteria and low heritability in especially quantitative traits occurring interaction of genotype and environment, either these methods have limited ability to find out variation between germplasms or require long-term observation to catch the variation (Oladosu *et al.*, 2019).

Precision breeding has recently become a new approach (Salgotra & Stewart, 2020) as molecular breeding is more accessible thanks to the availability of relatively cheaper markers and the abundance of genome sequence maps of most cultivated crops (O'Sullivan & Angra, 2016). Markers have been classified into three main groups, i.) Hybridisation-based, ii.) PCR-based, iii.) DNA chip and sequencing-based markers, by Khlestkina and Salina (2006). RFLP for the first one; CAPS,

RAPD, AFLP, SSR, and SCAR for the second one; and SNPs (Single Nucleotide Polymorphisms) for the third one are different methods examples. However, SNPs markers are the most used and have gained acceptance by breeders. Salgotra and Stewart (2020) explain this situation by showing the declining use of other molecular markers due to their lower reproducibility compared to SNPs and the ability to select SNPs in genes controlling traits of interest.

SNPs are held responsible for the differences of the nucleotides in the DNA sequence of individuals within the same species (Mammadov *et al.*, 2012). New approaches using SNP markers have assisted the selection of FB, which is resistant to biotic and abiotic stress factors and tannin-free or less vicine-convicine included lines (Lavanaia *et al.*, 2015; Gutierrez and Torres, 2019; Khazaei *et al.*, 2019; Gutierrez and Torres, 2021; Tacke *et al.*, 2022). Lastly, Warsame (2021) have suggested to use marker-assisted selection to change legumin(methionine-rich)/vicilin(methionine-poor) ratio which can enhance methionine content. Even though this is unprecedented in FB, the progress on the SNP marker technology and linkage maps between traits and those markers might make it possible. Thanks to these improvements, after a decline in the cultivation area until 2000, FB has relatively stayed stable in terms of cultivation area (Figure 1.4).

2.3.1. Characteristics to Breed Faba Bean for Fodder Production

FB includes high content protein in its seeds. Therefore, most breeding projects have focused on high-yield grain production. Even though Vasiljević *et al.* (2009) and Mihailović *et al.* (2011) have expressed the ability of forage production of FB to 45 t/ha green forage and 13 t/ha forage DM, and 50 t/ha green forage and 13 t/ha forage DM respectively, a minimal breeding effort has been encountered in the literature to enhance new cultivars being high biomass production and high forage quality. The limited amount studies regarding fodder usage of FB were about its mixture sowing with grasses (Faulkner, 1985; Ghanbari-Bonjar and Lee, 2003; Strydhorst *et al.*, 2008) and its straw yield and quality (Tessema, 2016; Wegi, 2016). Alkhtib *et al.* (2016) have advised breeding both food and feed capability high cultivars. Unfortunately, there is no progress on this topic.

Recently, fodder quality has been carefully examined, and more effort to breed new varieties has been advised by Guevara Oquendo (2020) and Guevara Oquendo *et al.* (2021).

Regarding fodder yield and quality, PH, leaf to stem rate, PC, and digestibility (ADF and NDF) are generally interested characteristics in forage crops (Vasiljević *et al.*, 2009; Capstaff and Miller, 2018; Pauler *et al.*, 2020). Therefore, in the current experiment, PH and PC of FB were examined. In addition, stem thickness and days to flowering (DTF) were other characteristics included in the examination to check their effect on the forage crop capability of FB.

2.3.1.1. Plant Height (PH)

PH has been studied many times in different FB cultivars and FB accessions (Fraser *et al.*, 2001; Tanttawi *et al.*, 2007; Alghamdi, 2007; Matthews *et al.*, 2008; Siddiqui *et al.*, 2015; Guevara Oquendo, 2020), but not many of them associated the PH with green biomass or DM yield except Fraser *et al.* (2001), Alghamdi, (2007), and Guevara Oquendo (2020). PH is determined as a quantitative trait, and phenotype for PH results from genotype x environment combination (Al Barri, 2012; Cao *et al.*, 2019). Thus, studies about PH produced quite different results. For example, Alghamdi (2007) got a mean of 93.9 and 122.5 cm PH in respectively consecutive two years. Hughes *et al.* (2020) have reported the results of 81 cm average PH and the range from 71 to 96 cm. Moreover, heterosis can affect the PH positively. A study carried out in FB found that heterosis increased average PH from 89.40 cm to 105.70 cm (Tanttawi *et al.*, 2007).

Many studies indicate a correlation between PH and biomass when other forage crops are checked. Robins *et al.* (2007), Hamd Alla *et al.* (2013), Cavero *et al.* (2017), He *et al.* (2020), and Liu *et al.* (2021) have confirmed this correlation in alfalfa. Zewdu *et al.* (2002), Jain *et al.* (2010), Serba *et al.* (2015), Shukla *et al.* (2017), Rady (2018), and Gilliot *et al.* (2021) have also proved the same correlation in Napier grass, sorghum, switchgrass, Sudan grass, and maize respectively.

2.3.1.2. Stem Thickness (ST)

Stem thickness (ST) in crops has nearly always been studied in relation to lodging as the ST provides strength to plant stems (Madić *et al.*, 2016). Cubero and Nadal (2005) have taken ST into account just for advantages against lodging. However, many studies have examined it and have disclosed positive or negative correlations between ST and herbage yield or ST and forage quality. On the one hand, Avci *et al.* (2013) have reported a correlation between ST and green herbage yield in alfalfa. As a forage crop, Hungarian vetch (*Vicia pannonica* L.) accounts for an association between ST and green and dry herbage in both monoculture and mixture sowing (Demirhan *et al.*, 2018). Also, a high correlation between ST and PH ($r = 0.921$) has been informed in the same study. Another study has presented a correlation between ST and PH in sweet corn (Zhang *et al.*, 2020). On the other hand, ST is shown as a negative parameter for forage quality in terms of digestibility and PC because the increase in ST means a rise in the leaf-to-stem ratio (Sayar & Han, 2014).

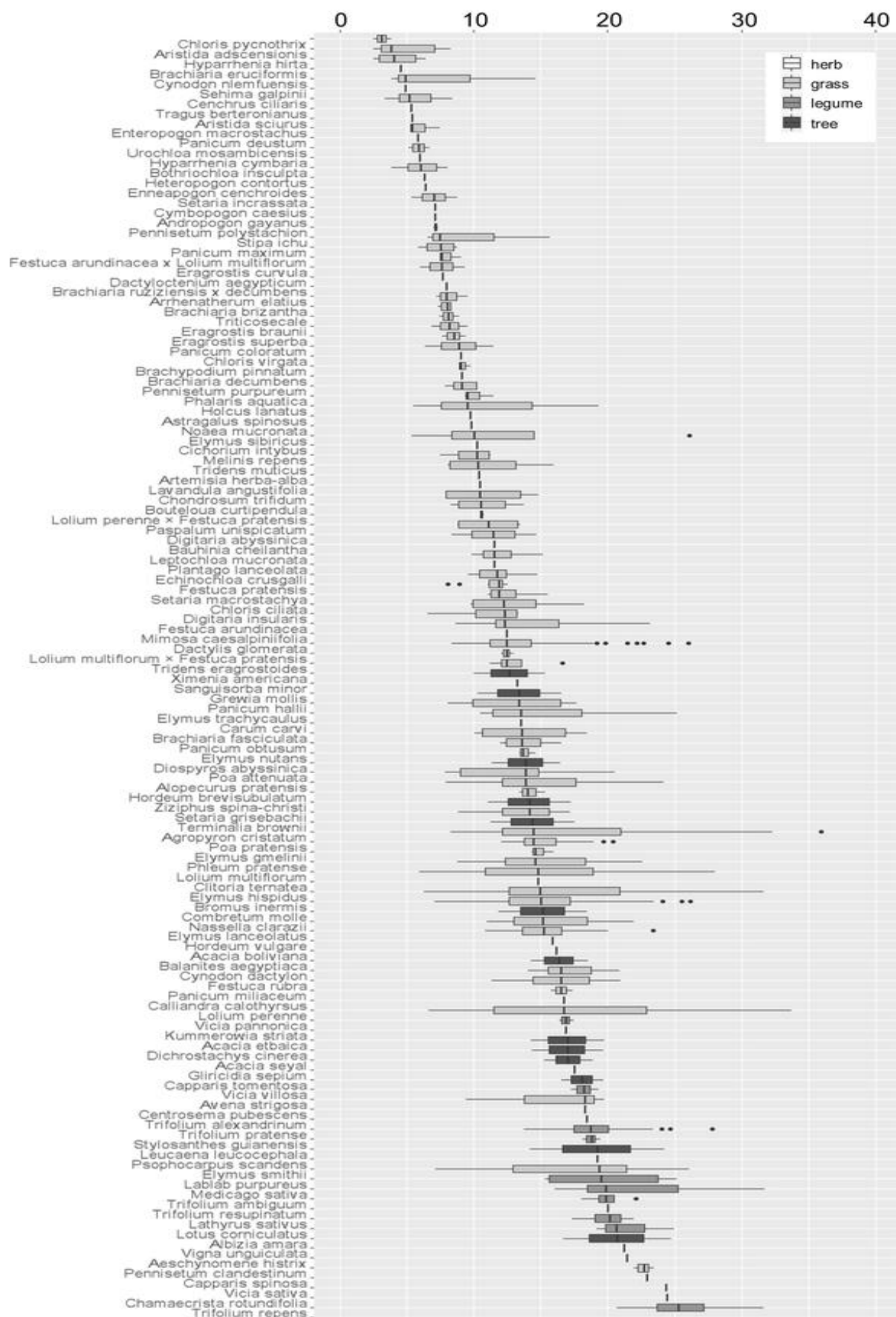
2.3.1.3. Protein Content (PC)

The importance of protein for animals has not been newly known (Schaefer, 1946). Diets should be balanced in terms of structural and non-structural components. Protein is one of these non-structural parameters and a critical parameter in forage DM (Ball *et al.*, 2001). Wu *et al.* (2014) have specified that proteins consist of amino acids, and these are traditionally divided into groups which essential and nonessential for animal feeding. Karimi *et al.* (2021) have informed that protein in forage is vital because of amino acids. Therefore, high PC is a desirable characteristic in forage crops. However, not only PC on DM is the primer selection criterion, but also which amino acids are in the forage DM have become another primer selection criterion.

The minimum protein requirement of cows for maintenance has been reported as an average of 300 gr digestible protein and 518 gr total crude protein per 500 kg cow, depending on the digestibility of proteins (Swanson, 1977). This means approximately 5% PC of daily forage DM in the ration. The PC of FB fodder has been reported by Faulker and Steen (1984) between 14.5 and 15.5%,

Berkeen and Meeres (1985) between 17.6 and 20%, and Guevara *et al.* (2020) between 20 and 20%. Furthermore, Alktib *et al.* (2016) have disclosed 5% PC in FB straws after harvest. Separately, Lee (2018) compared 129 forage crops for nutritional characteristics (Figure 1.5). The mean of PC for those crops is 15 %.





2.3.1.4. Days to flowering (DTF)

Flowering time is an important parameter when the yield is taken into account in both herbage and grain production, as it is directly related to how much any plant is able to make growth and development before the reproductive period (Materne & Siddique, 2009). However, whether or not early or late flowering is a favourable characteristic for plants depends on environmental conditions. For example, while early flowering has been reported as an escape mechanism from drought and better yields have been reported in the early flowered lentils in Australia (Thomson *et al.*, 1997) and Syria (Silim *et al.*, 1993), the biomass yield can increase on more DTF if the conditions are favourable (Figure 1.6). The day- to-flowering has been found highly heritable (97%) in FB (Toker, 2004). That means if the forage yield is associated with higher DTF in FB, the cultivar can be bred with higher DTF and might be a consistent characteristic for forage production from FB.

Up to now, even though the literature on the effect of DTF on grain yield is present (Tadele *et al.*, 2011), limited knowledge is present of its impact on biomass and DM yield. For example, Loss and Siddique (1997) tested the DTF by changing sowing dates and the plots sown early (ea. more days to the first flower) had more DM yield in FB. In other crops, a positive correlation between DTF and biomass have reported several times by Shanjani *et al.* (2013) in alfalfa, Shanjani *et al.* (2015) in wheatgrass, and Jebril (2018) in sorghum. In addition, DTF time positively correlated with in vitro OM digestibility and negatively associated with ADF, NDF, and ADL in lentil biomass.

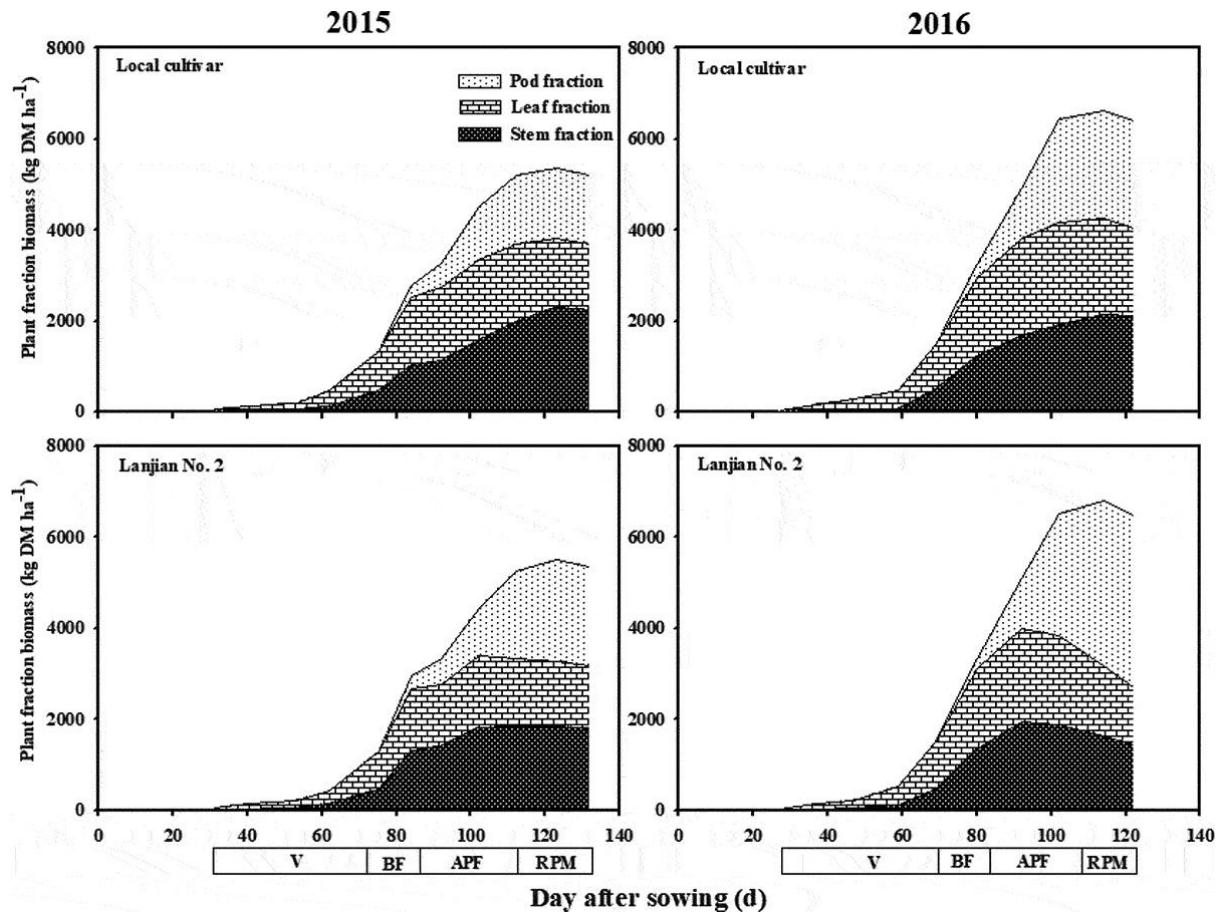


Figure 1. 6. The Relationship Biomass Yield and Day After Sowing in Common Vetch (*Vicia sativa* L.) (Huang et al., 2021) (V: Vegetative stage; BF: Before Flowering; APF: Advanced Pod Filling; RPM: Ripening to Pod Maturity)

3. Summary of Introduction

While evaluating FB as a feed crop, generally, it is the seeds which are thought. The studies approaching FB seed as feedstuff reported some anti-nutritional factors, especially tannin and vicine-convicine, according to the digestion system of target animals. Thanks to the breeding projects, new cultivars that are tannin free and less vicine-convicine including has been bred and released, and relatively current sources advise up to 250 g/kg concentrate FB substitution in chicken diets. In contrast, the studies focusing on cows, lambs, and steers have not reported any kind of restrictive impact of FB due to the anti-nutritional compounds. Only few articles show the low methionine content of FB and the variability between cultivars in terms of the nutritional value as the reason why it is preferred less compared to other protein concentrates in cow feeding.

Fortunately, the overwhelming majority of those problems, such as tannin and convicine content, have been fixed via plant breeding, or some studies are being carried out to find a way to resolve the deficiencies, such as cystine and methionine.

On the other hand, up to now, only one study has analysed the FB as roughage (Guevara Oquendo, 2020), although whole-plant forage capacity has been examined as silage and in mixtures with cereals and the highest crude protein value was reported from FB silages or FB included mixes. Guevara Oquendo (2020) has pointed out the necessity of new high fodder yield varieties for drought areas. Moreover, the need for FB cultivars for both food and feed reported by Alkhtib *et al.* (2016) has not been fulfilled yet. Though, if new lines and cultivars are improved for fodder usage of FB, they can be used as annual forage crops, which can enter the rotation plan quickly and provide N to next year's crops. Thus far, no progress has been made to improve hay yield and quality, and to find out which region of genes are responsible for high yield FB biomass production and its quality traits by taking advantage of GWAS and the associations between SNPs markers and characteristics.

We hypothesised that certain parts of the chromosome might be associated with PH, ST, biomass production, DM yield, the PC of FB. In addition, it was hypothesised that the linkages between SNPs and the genes of the interested-characteristic might be present. This experiment was planned to discover marker-assisted selection (MAS) possibilities for forage capability in FB breeding. The objective of this experiment was to phenotype some forage-specific characteristics, which are biomass production, DM yield, PH, ST, and PC in FB, and to perform GWAS analysis.

CHAPTER 2. MATERIAL AND METHODS

1. Plant Material and Field Experiment

The Reading Spring Bean Population (RSBP) derived from a PhD study carried out by Warsame (2021) on *Vicia faba* L. (Faba bean) was used in the experiment. 100 genotypes from 154 inbred lines were included in the experiment because of insufficient seed amount. Moreover, NN_Bayblon_K, NN_Espersso_AF, NN_Espresso_AG, LG Cartouche, Allison, and Hedin/2 cultivars were used as control (NN means non-nodulating which nodule does not grow in root). Furthermore, Fuego cultivar was used in the discard (check) row. Seed materials were stored at 4°C until the sowing. Once the seeds were taken out of storage, the damaged seeds by *Bruchus rufimanus* were separated. Depending on seed availability, different replicate numbers were applied for each genotype in the experiment.

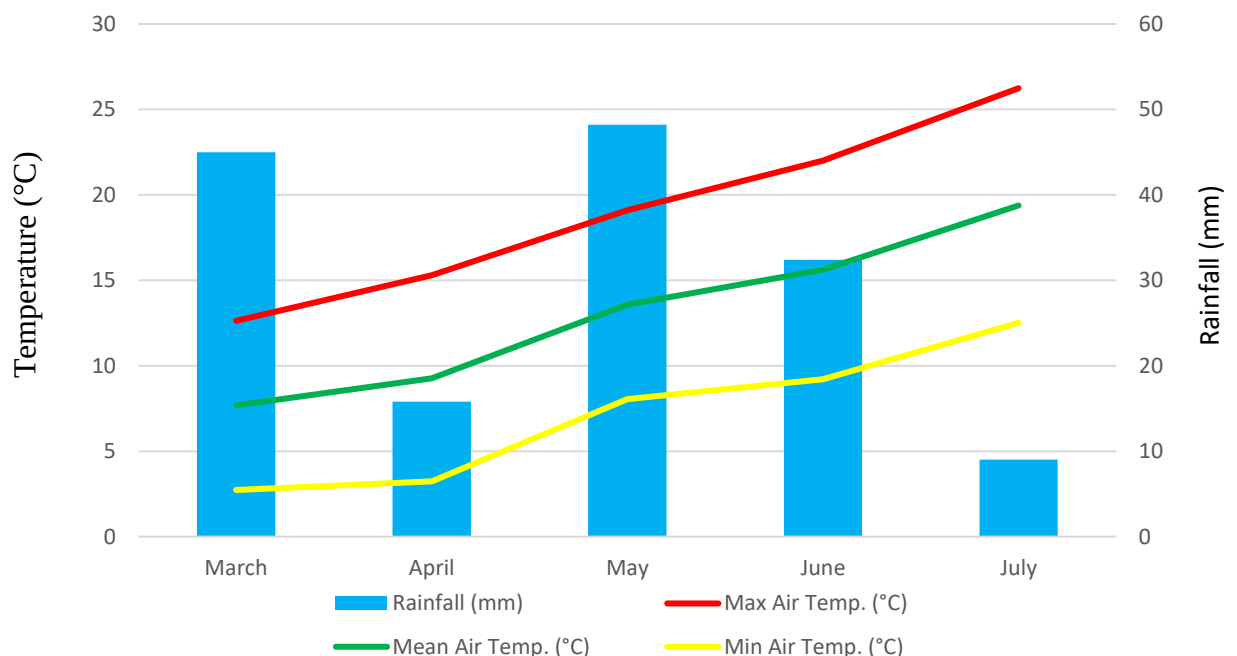


Figure 2. 1. The Temperature Data of Sonning Farm during the Experiment

The trial was carried out in University of Reading Sonning Farm's experiment field, UK (51°28'49.9"N, 0°54'02.9"W). The weather data record of the Sonning Farm is given in Figure 2.1. These data can be determined as anomaly with respect to the long-term the UK averages (Met Office, 2022). The soil characteristic was sandy loam.

2. Method Used in Sowing

An augmented experiment design method was used in the experiment. The experiment design was derived from R Studio 4.2.1 via agricolae package (Appex 1). 31, 36, and 33 different genotypes were included to experiment with 1, 2, and 4 replicates, respectively. Similarly, while Lg Cartouche, Allison, and Hedin/2 were replicated 4 times, the replicate amounts of NN_Bayblon_K, NN_Espersso_AF, and NN_Espresso_AG (NN means non-nodulating) were 2. 4 blocks were included to the experiment. Labelling was done upon envelopes via stickers created in accordance with the experiment plant. Each plot in the experiment plan was planned as 2 rows, each one accounting for 10 plants. Between plots, at the beginning of columns and the end of the columns, the discard (check) row, which is only 1 row, was applied since genotypes might have different competition capabilities (Warsame, 2021). Plants on these rows were not included in phenotyping and data analyses. The seeds were sown with 15 cm inter-row space and 10 cm space between plants on the row. The density in the experiment field was calculated as 66 plant / m². The depth of sowing was set as 8 cm. A planting board pursuant to these sowing norms was designed, produced, and used to make organised sowing. The sowing was completed in 3 days on the 30th, 31st of March, and the 1st of April 2022. After sowing, the experiment field was covered with fleece netting to protect the emerging seedlings from bird damage.

3. Phenotyped Traits and Processes

Data for day to the first flowering, stem height and thickness, fresh biomass weight, the separate weight of leaves, stem, and pods in fresh biomass, total DM weight, and the weights of the same plant parts in the DM were collected from the experiment. In order to accept as flowering started in any genotype, the threshold of at least 50 per cent of flowering plants was sought (IBPGR and ICARDA, 1985). PH was measured from the ground level to the top of the main shoot after the plant had bloomed. The mean of five PH was recorded in each plot. ST was determined by measuring the diameter of the middle point of the stem when the first pod emerged. The measurement was processed through the calliper, which mm precision measurement is able to do. It was repeated on 5 plants, and the mean of them was recorded. Due to the different replicate amounts and the lack of labour to harvest all plots, 33 genotypes and 3 cultivars were included in the destructive harvest. As each genotype has a different maturing time, the harvest was planned six times on 18 days according to the first flowering. The harvesting was performed during the stage of mid-podding. The plants were cut 4 cm above the soil. The first and last two plants in each row were not harvested due to the border effect. The fresh biomass weights were measured after the harvest. Later, harvested plants were separated into three parts which are stem, pod, and leaf. Fresh weights of these parts were measured. Then, the drying process was performed in the oven for 2 days at 80°C. After that, the DM weight of all parts was measured. In the next step, all samples were milled and prepared for nitrogen analysis. Samples weighed between 0.01 and 0.02 grams were analysed via Leco CHN628. The PC was calculated by multiplying the nitrogen content by 6.25 (Kjeldahl procedure).

4. Statistical Analysis of Data and GWAS Analysis

All statistical analyses were calculated in R Studio 4.2.1 version. Shapiro-Wilk test and Levene's Homogeneity test were used to check the normality and homogeneity of variables. Box plot, histogram plot, and quantile-quantile (Q-Q) plot were used to graphically exhibit the distribution of

variables. As the assumption of ANOVA was not presented in data of any characteristics, Kruskal-Wallis test, as a non-parametric test, was used to assess the inbred lines.

The correlation between variable pairs was analysed with Pearson's Correlation test. The results were evaluated according to the r-value ($r = 0$ means no correlation, r between 0 and 0.30 means weak, r between 0.30 and 0.60 means average, r between 0.60 and 0.90 means strong, r between 0.90 and 1 means very strong, and $r = 1$ indicates perfect correlation (Silveira *et al.*, 2021). The relation was graphically exhibited with a scatter smooth plot.

The phenotypic data were filtered via Best Linear Unbiased Predictor (BLUPs) before GWAS analysis in R studio via spATS package. GWAS analysis was executed in TASSEL 5.2.83 version. GLM approach was used, and the results were exhibited in the Manhattan plot and Q-Q plot. The threshold value of $-\log_{10}(\text{p-value})$ was calculated via the Bonferroni Correction method at the confidence interval of 0.05.

CHAPTER 3. RESULTS

1. Biomass Yield

1.1.Fresh Biomass (Herbage) Yield

52 plots (18 genotypes and 2 cultivars) were included in the analysis due to missing plants in the other plots. Drought damage occurred in the half part of the arm A in block 1 in June when the first pod was emerging. In the next period, severe defoliation was observed in the especially lower parts of the plant in the same block. The experiment's average biomass yield (BY) was 4300 kg/da. BY data were between 2249 and 6074 kg/da. The histogram graph was relatively uniformly spread (Figure 3.2).

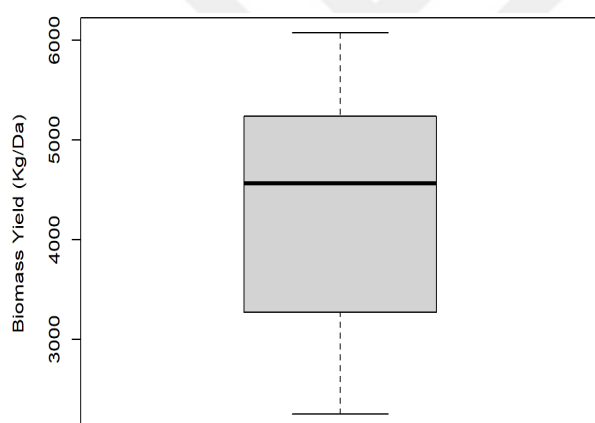


Figure 3. 1. The Boxplot of Biomass Yield

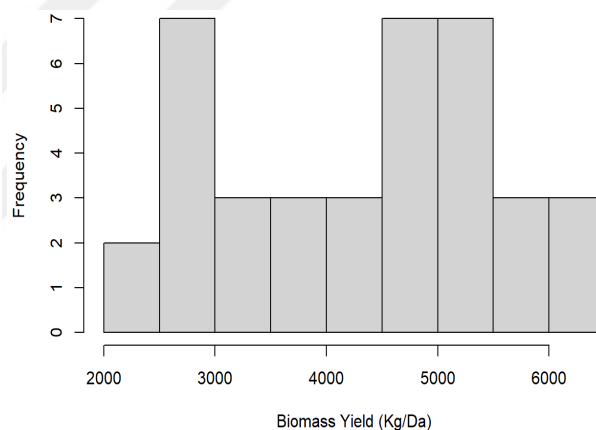


Figure 3. 2. The Histogram Graph of Biomass Yield

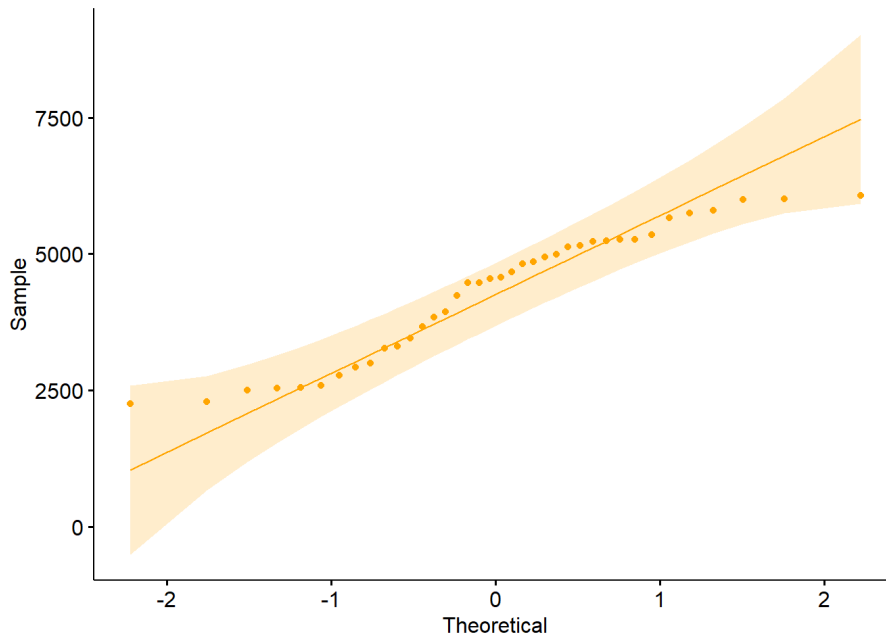


Figure 3. 3. Distribution of Biomass Yield (Q-Q Plot)

BY variables were not normally distributed according to the result of Shapiro-Wilk test ($p < 0.05$). Such that, Figure 3.3 does not conform to the normal distribution. Especially, the deviation was more intense in the lower left-hand and upper right-hand corners of the graph. Homogeneity was not found in the variables across the plots according to Levene's Test ($p < 0.01$). Finally, the result of the Kruskal-Wallis test presented that there is no statistically significant difference between genotypes in terms of BY. Therefore, the null hypothesis was accepted.

The number of plants harvested had a weak positive correlation ($r=0.21^*$) with biomass weight per plot. The plots where 8, 9, and 10 plants were harvested sometimes had higher biomass weight than the many plots where 11 or 12 plants were harvested (Figure 3.4). However, the highest biomass yield was generally achieved from the plots where 12 plants were harvested.

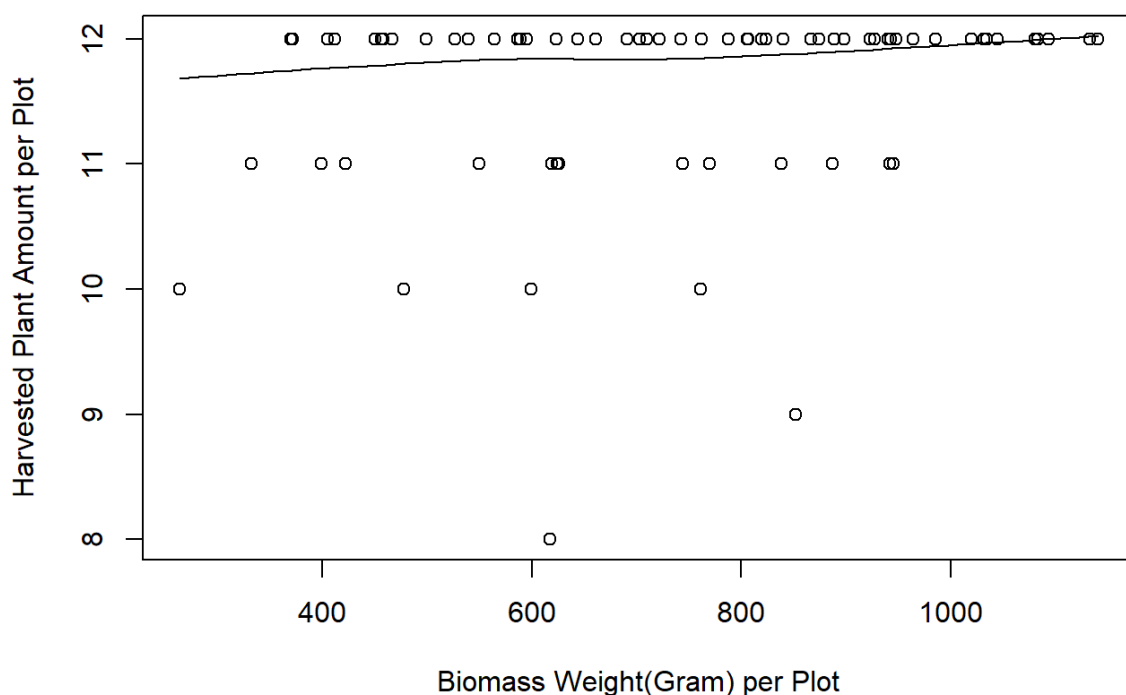


Figure 3. 4. Association between Harvested Plant Amount and Biomass Weight per Plot

1.1.1. Fresh Pod, Fresh Leaf, and Fresh Stem Weight

Different parts of the plant were also analysed. However, the variances of pod, leaf, and stem weights across genotypes were not found to be statistically significant. Fresh pod, fresh leaf, and fresh stem weights ranged from 173.1 to 554.3 grams, from 42.1 to 256.4 grams, and from 53.4 to 434.7 grams, respectively, in plots (Figure 3.5). Despite the different ratios in each genotype, pod, leaf, and stem accounted for an average of 42.42 %, 20.01%, and 36.44% of total biomass, respectively. The results of the correlation analysis between biomass weight and these plant parts and also between these plant parts themselves are shown in Table 3.1. Stem was the part which is the most correlated with biomass yield (Figure 3.6). Leaf and pod were the following parts with 0.87 and 0.85 correlation coefficient values, respectively. In addition, another comparably high correlation has been detected between stem and leaf.

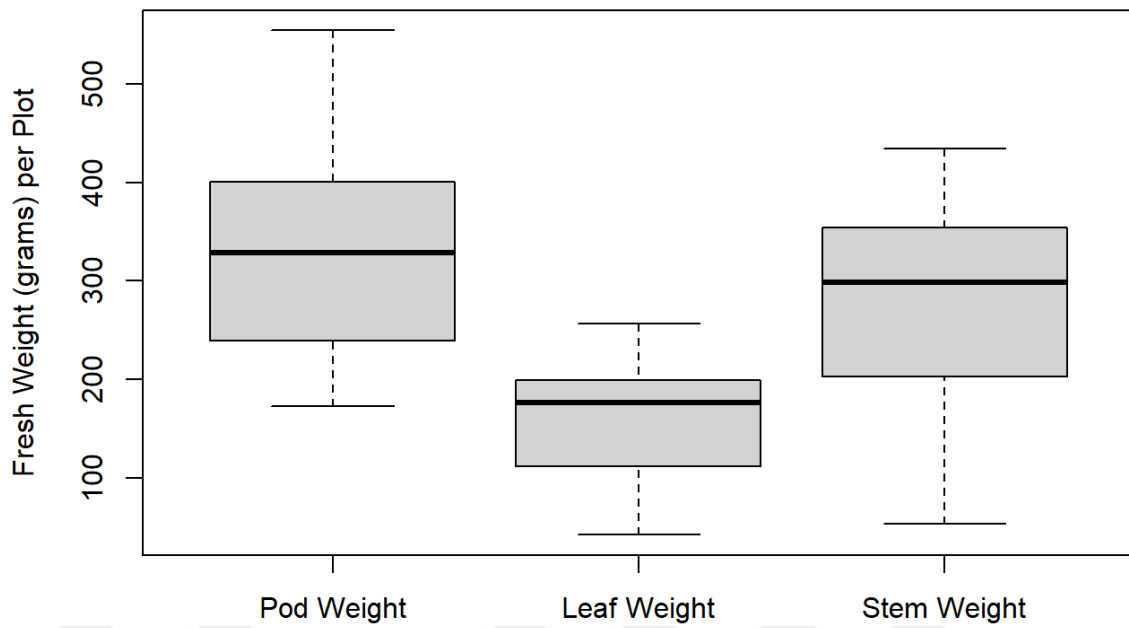


Figure 3. 5. Fresh Weight of Different Plant Parts

Table 3. 1. Correlation (r) Values between Fresh Biomass and Fresh Plant Parts

	Biomass	Pod	Leaf	Stem
	Weight	Weight	Weight	Weight
Biomass Weight	1			
Pod Weight	0.85**	1		
Leaf Weight	0.87**	0.60**	1	
Stem Weight	0.90**	0.61**	0.82**	1

*: P<0.05, **: P<0.01

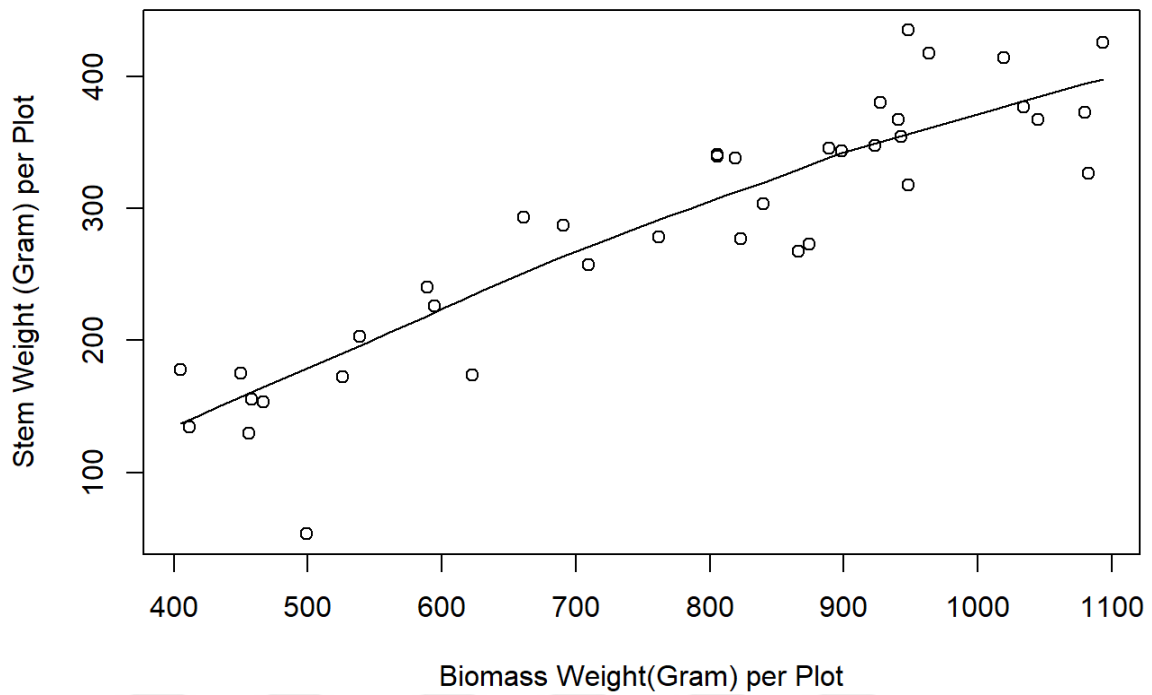


Figure 3. 6. The Association between Stem and Biomass Weight per Plot

1.2. Dried Matter Yield

Dried matter (DM) yield diversified between 622.2 and 1313.9 kg/da. The mean of DM was 913.8 kg/da. The weight decreased by between 3.43 and 5.68 times depending on genotype in conversion from fresh herbage to DM. While Shapiro-Wilk test found that there is a normal distribution ($p > 0.05$), Levene's test did not find homogeneity between variables as in herbage yield. Kruskal-Wallis test did not report statistically significant variation across genotypes ($p > 0.05$). Therefore, the null hypothesis was accepted.

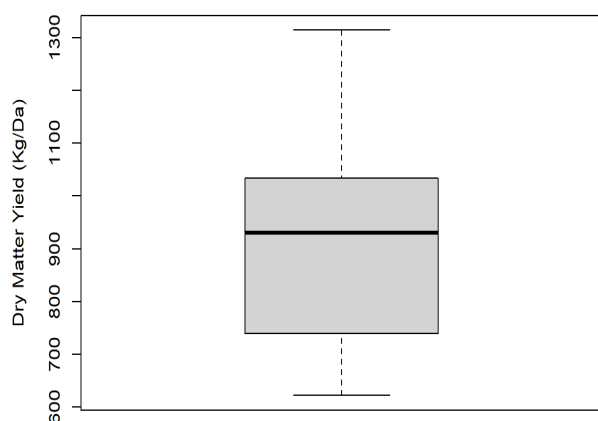


Figure 3. 7. The Boxplot of DM Yield

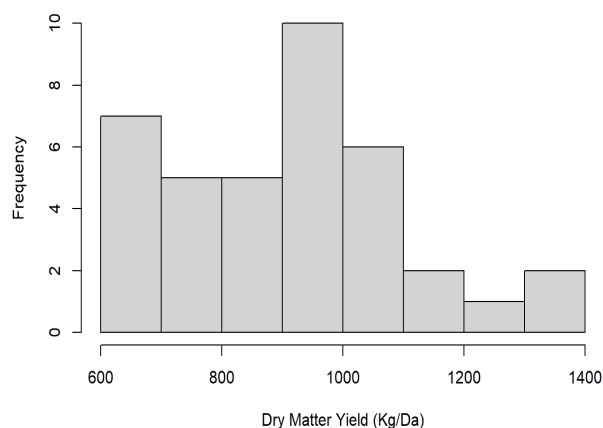


Figure 3. 8. The Histogram Graph of DM Yield

1.2.1. Dry Pod, Leaf, and Stem Weights

Dry plant parts were also analysed to check genetic variation between genotypes for the dry weight of those parts. However, a significant variation was not detected across genotypes for dry weights. Dry pod, dry leaf, and dry stem ranged from 34.80 to 128 grams, from 19 to 50.40 grams, and from 32 to 84.90 grams separately per plot (Figure 3.9).

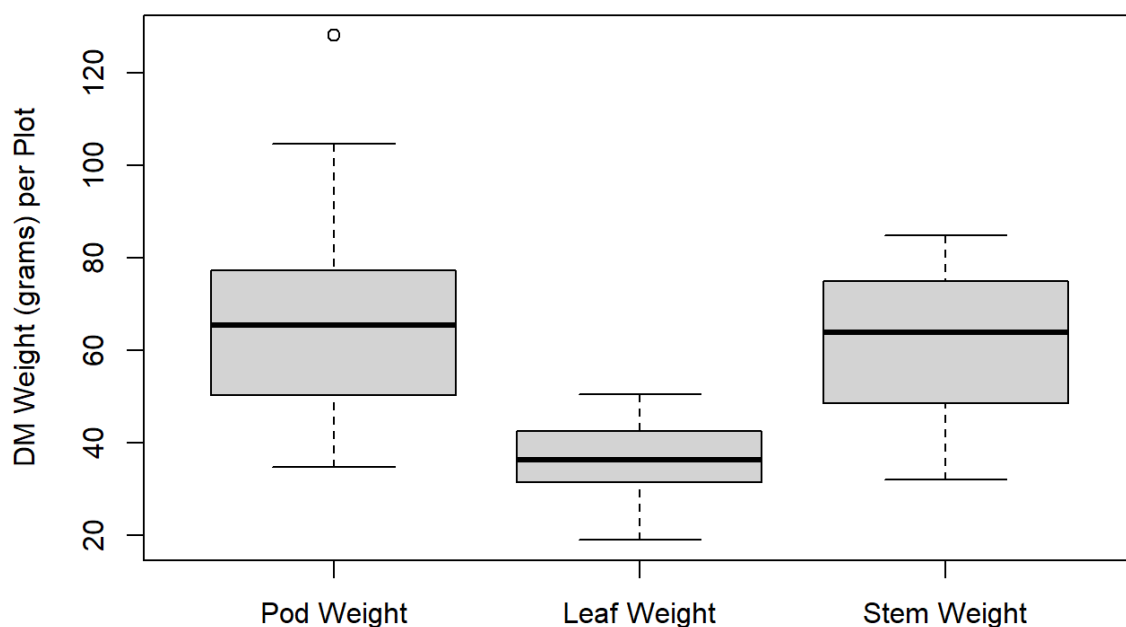


Figure 3. 9. Dry Weight of Different Plant Parts

Coefficients of correlation between dried stem, dried leaf, and dried stem weights with total DM yield were 0.83, 0.71, and 0.85, respectively. Even though the rates decreased slightly compared to fresh weights, the stem was still the part in which the increase of it positively affected the most total dried weight. However, dried leaves accounted for less DM yield than fresh herbage yield. The most striking result was that no statistically significant correlation between dried pod and leaf weight was present.

Table 3. 2. Correlation (r) Values between Total Dry Matter Weight and Dry Plant Parts

	Dry Matter Weight	Dry Pod Weight	Dry Leaf Weight	Dry Stem Weight
Dry Matter Weight	1			
Dry Pod Weight	0.78**	1		
Dry Leaf Weight	0.63**	0.13	1	
Dry Stem Weight	0.82**	0.33*	0.70**	1

*, P<0.05, **: P<0.01

2. Plant Height (PH)

Different PH were observed in the experiment field. The mean of PHs was recorded as 78,01 cm. The range was from 47,40 cm to 98.10 cm. No lodging was observed in any plots. Despite the vast range, half of the samples were between 72.60 and 83.80 cm (Figure 3.10). The histogram diagram of HT (Figure 3.11) is also slightly right skew.

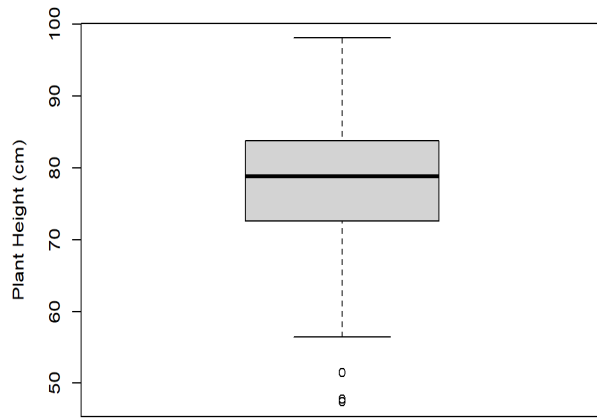


Figure 3. 10. Boxplot of Plant Height

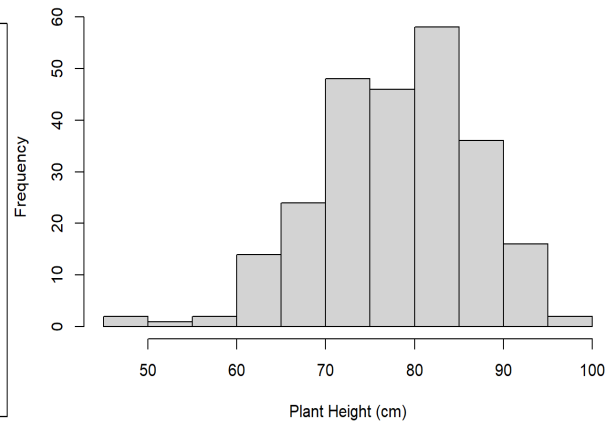


Figure 3. 11. Histogram Graph of Plant Height

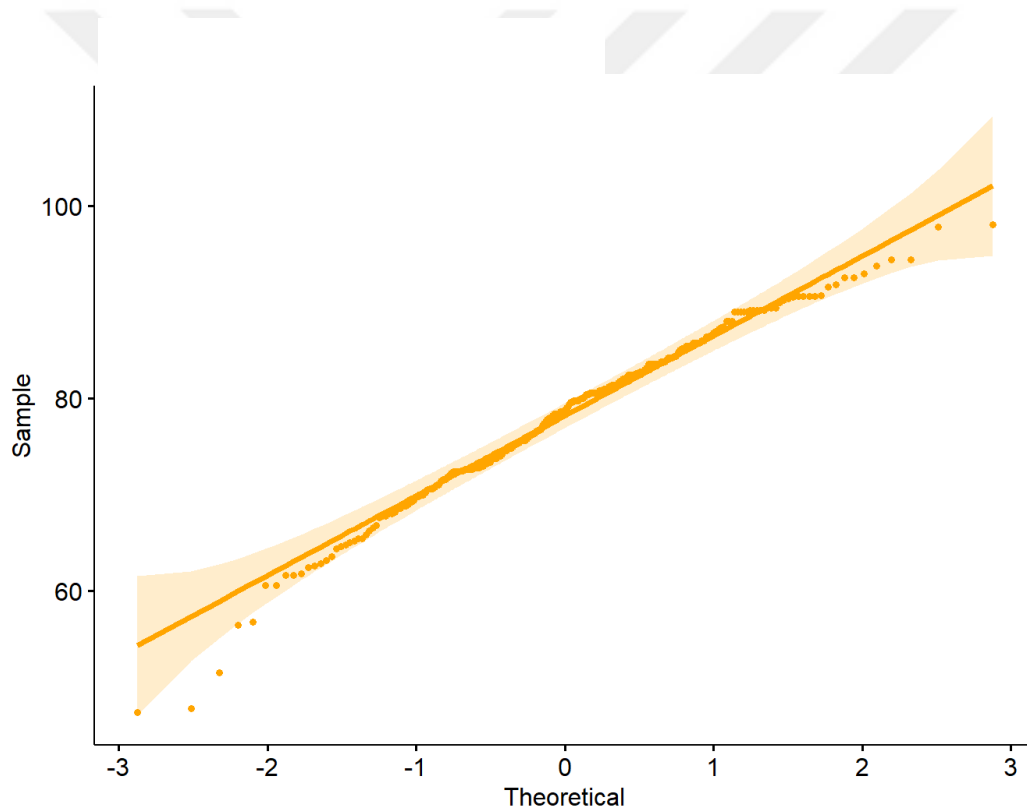


Figure 3. 12. The Distribution of Plant Height (Q-Q Plot)

Figure 3.12 shows the distribution of PH. In addition, Shapiro-Wilk normality test showed that variables were not normally distributed ($p < 0.01$), and Levene's test, in contrast, proved that variables ensured homogeneity. As the assumptions, which are normal distribution and homogeneity across variables, were not provided. Kruskal Wallis test, used in a line of these results, revealed that

there is statistically significant variation ($p<0.01$) across genotypes in terms of the PH. The genotypes of 434-1 and 367-1 were 91.15 cm and 90.6 cm. They accounted for the highest average PH, above 90 cm. 728-3, 291-3, 434-2, 582-3, 342-4, 835-3, 660-4, 362-2, 1124-4, 367-3, 1117-3, 367-4, 355-4, 367-1, and 60-3 were the genotypes from taller to shortest between 85-90 cm. In contrast, the other genotypes which are fewer than 70 cm were 1250-3, NN_Espresso_AG, 1000-2, 176-1, 1072-1, 506-4, 1250-1, 660-2, 670-3, Hedin/2, 668-3, 1346-2, 176-2, 1292-3, 1482-1, 1282-3, and 728-1 respectively from shorter to taller. The shortest three genotypes were outliers with 47.4, 49.62 and 56.8 cm, respectively. While LG Cartouche was greater than the mean, 78.24 cm, Allison and Hedin/2 were under the mean.

3. Stem Thickness (ST)

Various ST were observed in different plots. The mean of ST was 7.83 mm. The range was between 4.79 mm and 10.20 mm. However, the lowest two variables, 4.76 mm and 5.14, were outliers and distant from the minimum threshold of the box plot (Figure 3.13). Distribution nearly created a shape near to bell-shape in the histogram graph (Figure 3.14).

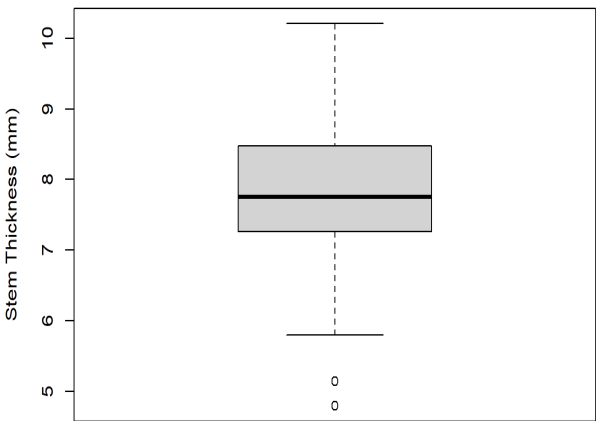


Figure 3. 13. Box Plot of Stem Thickness

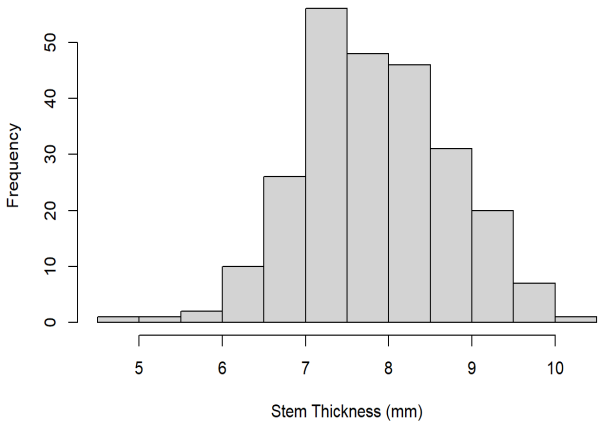


Figure 3. 14. The Histogram Graph of Stem Thickness

The distribution of ST was normal distributed (Figure 3.15). Shapiro-Wilk Test proved the normal distribution ($p > 0.05$). However, the variance was not distributed homogeneously over genotypes in terms of ST according to Levene's test ($p < 0.01$). The following analyse, Kuskal-Wallis test instead of ANOVA due to Levene's test result, showed an statistically significant variation of ST in genotypes ($p < 0.01$).

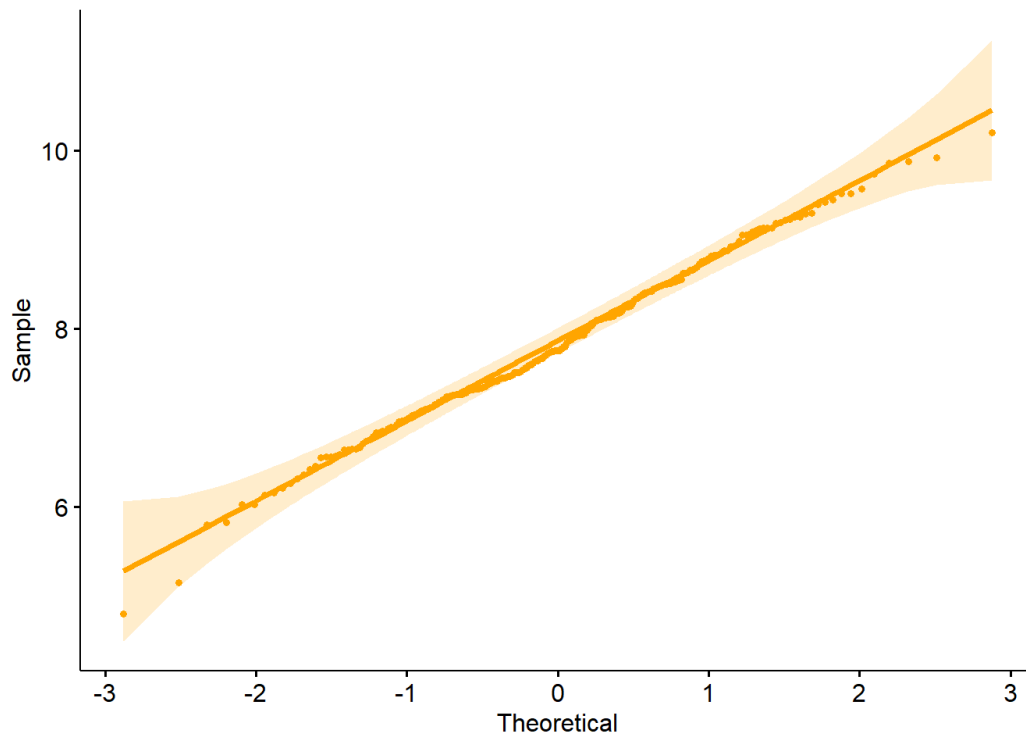


Figure 3. 15. The Distribution of Stem Thickness (Q-Q Plot)

Genotype 434-1 had by far the thickest stem which was 9.81 mm. It was followed by 1247-2 with 9.66 mm ST. The genotypes having thicker stem than 8.5 mm were 1216-3, 367-4, 1482-1, 367-2, 745-3, 1338-3, 434-2, 740-1_2, 1117-3, 582-3, 668-1, 1482-4, 668-4, 342-4, 82-4, 291-4, and 39-2 from high to low respectively. Against that, NN_Espresso_AG, 660-2, 1250-3, 331-4, 176-2, 1000-2, 626-4, 1282-4, 506-4, Hedin/2, 1072-1, 1135-1, 346-3, 745-4, 1250-1, and 60.4 were the genotypes having ST fewer than 7 mm. NN_Espresso_AG and 660-2 were the thinnest ones in terms of ST.

During the measurement, it was observed that the plots comprising more thick plants in terms of the stem also had longer plants. The result of the correlation test confirmed the observation with was 0.51 coefficient of correlation in the confidence interval of 0.01. In addition, ST showed a linear relationship with PH (Figure 3.16). Moreover, ST also positively contributed dry stem weight ($r = 0.49^{**}$).

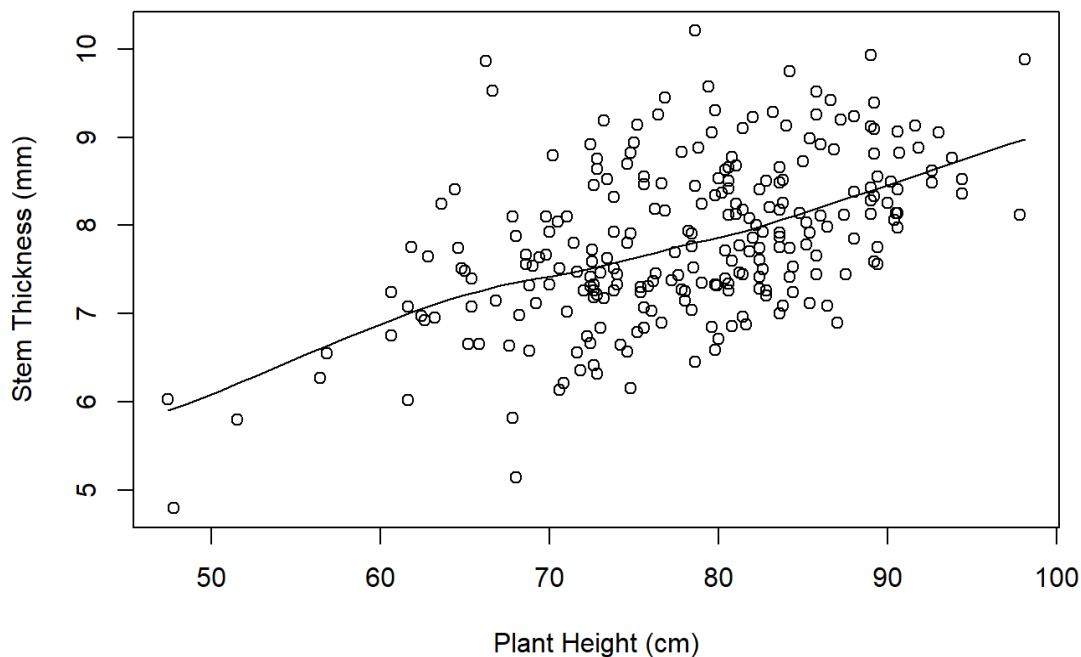


Figure 3. 16. The Relationship between Stem Thickness and Plan Height

4. The Association between Biomass Weight and Plant Height or Stem Thickness

A positive relation between biomass weight per plot and PH has been shown in Figure 3.17. This was accepted with Pearson's correlation test and the coefficient of correlation was calculated as $r = 0.60$ ($p < 0.01$). Although the same positive association was detected between biomass weight and ST (Figure 3.18), the coefficient of correlation, $r = 0.55$, was slightly fewer than the former ($p < 0.01$). The increase of DM weight on average was more prevalent when just ST increased from 7.5 to 8 mm.

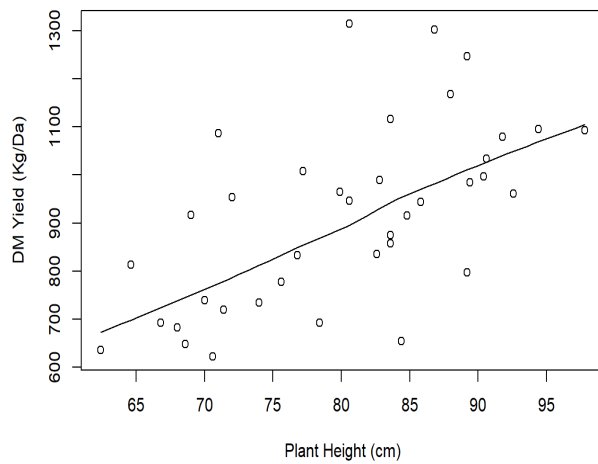


Figure 3. 17. The Positive Relation between Dry Matter Weight per Plot and Plant Height

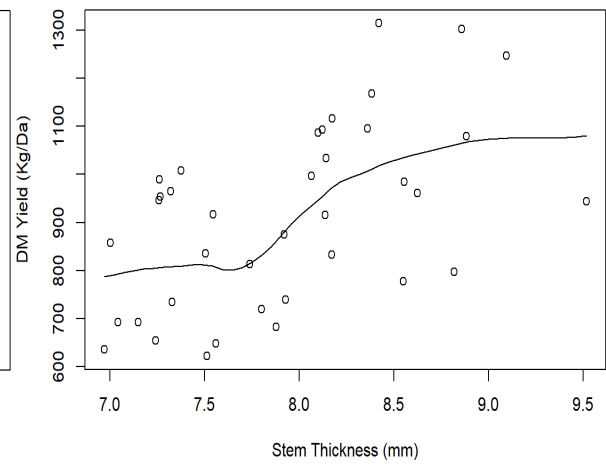


Figure 3. 18. The Positive Relation between Dry Matter Weight per Plot and Stem Thickness

5. Days to flowering (DTF)

Variables diversified in a wide range from 48 to 69 days in plots. The mean of DTF was calculated as 60.87 days. The distribution of DTF was non-symmetric in Figure 3.20. Non-normal distribution was approved with Shapiro-Wilk test ($p < 0.01$). In addition, homogeneity was not present among variables in Levene test ($p < 0.01$). The variation of DTF across genotypes was found significantly important in Kruskal-Wallis test rather than ANOVA due to non-parametric data. However, the impact of DTF on DM yield was not statistically significant ($p > 0.05$). Figure 3.21 shows the unrelated relationship between DTF and DM yield ($p > 0.05$).

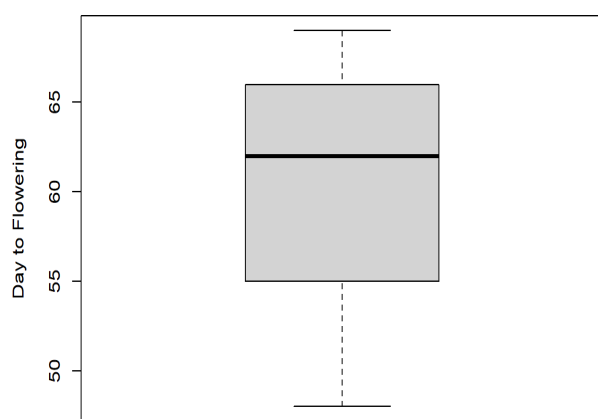


Figure 3. 19. The Boxplot of Days to Flowering

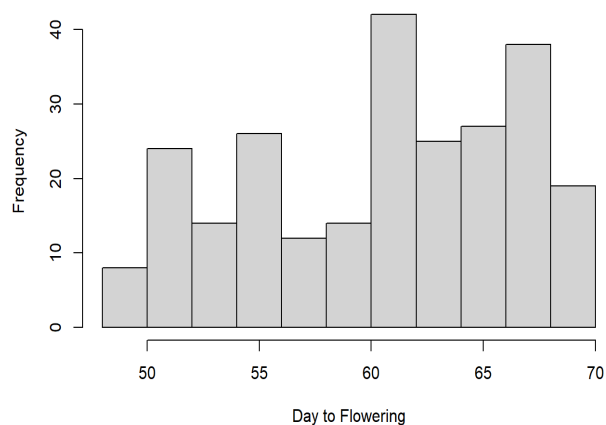


Figure 3. 20. The Histogram Graph of Days to Flowering

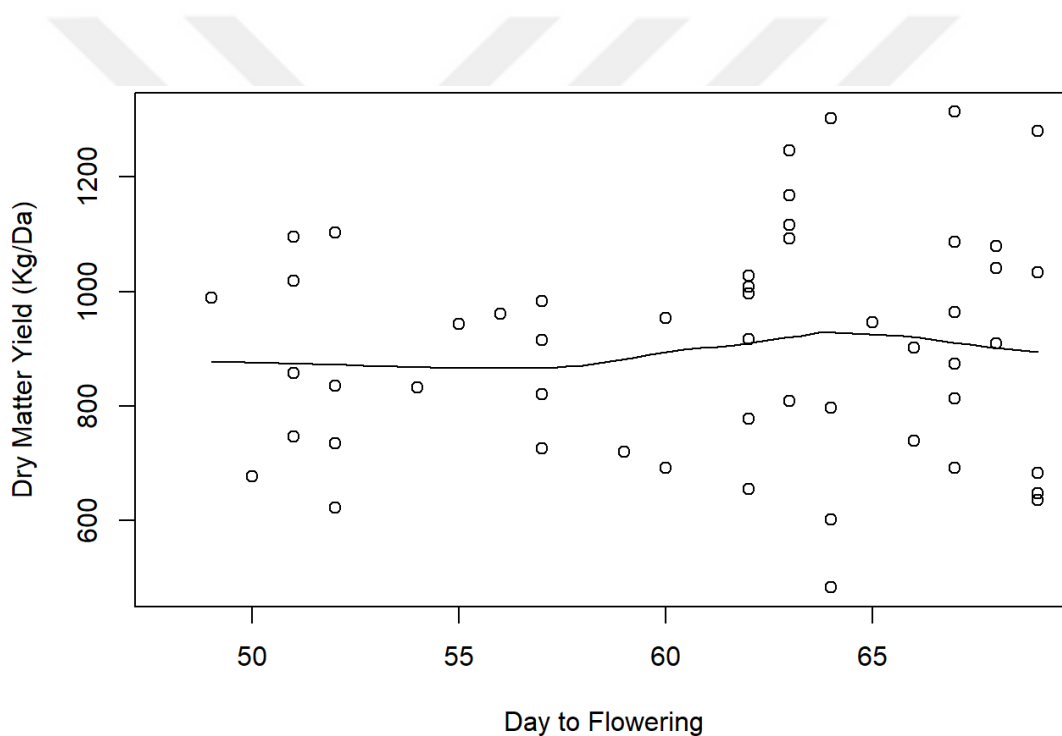


Figure 3. 21. Association between Day to Flower and DM Yield

6. The Protein Content of Dried Biomass

PC was tested just in the genotypes in which destructive harvest was applied. The average PC was 16.44 %. While the minimum PC was 13.97%, the maximum value was recorded as 18.90% among 72 plots. The histogram graph showed a relatively near bell-shaped model. The distribution of PC

has been demonstrated in Figure 24. In line with the figure, the normality test notified the normal distribution.

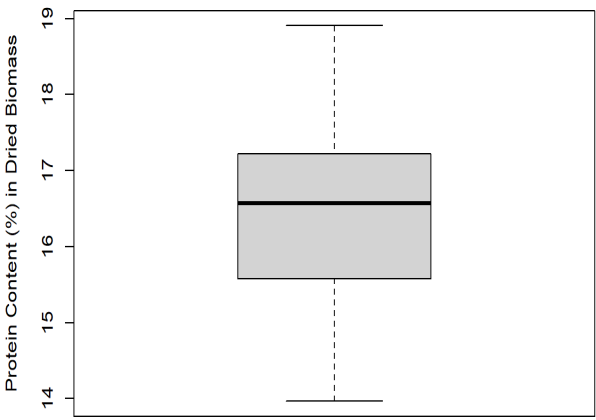


Figure 3. 22. The Boxplot of Protein Content

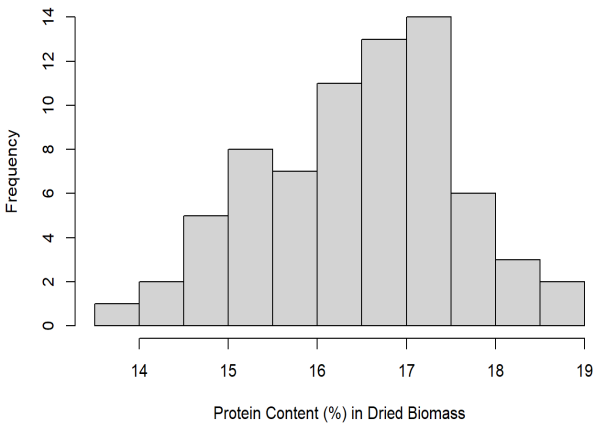


Figure 3. 23. The Histogram Graph of Protein Content

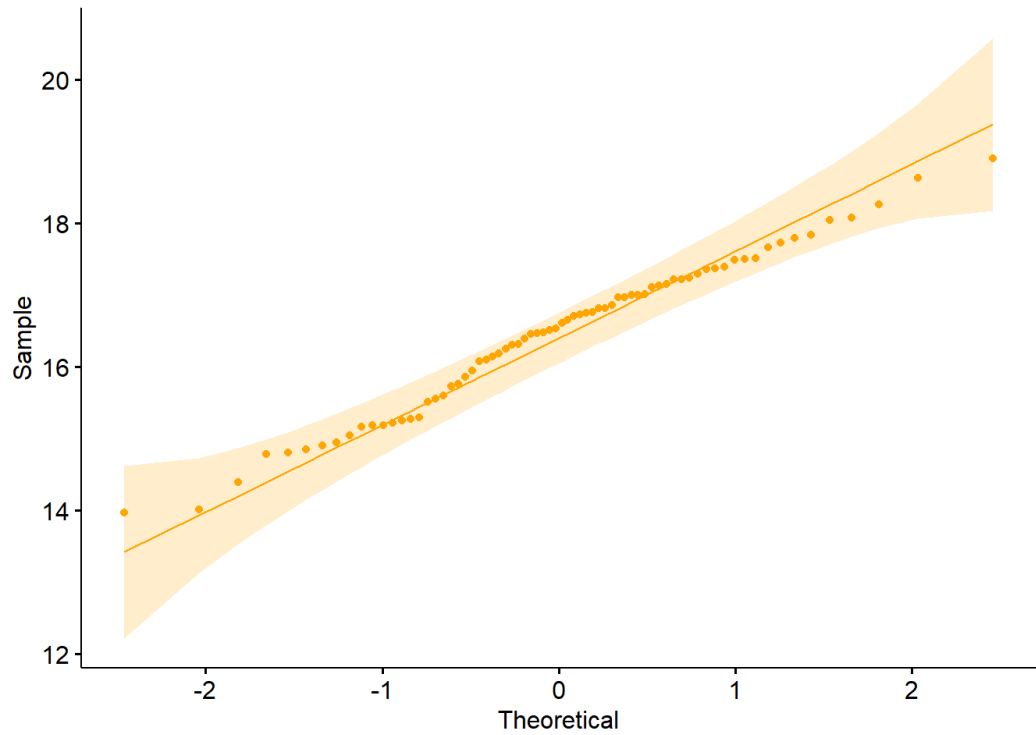


Figure 3. 24. Distribution of Protein Content (Q-Q Plot)

Homogeneity of PC variables across genotypes was not also provided ($p < 0.01$). The result obtained from Kruskal-Wallis test confirmed the statistically significant variation between genotypes in terms of PC ($p < 0.05$). Inbred lines had a quite wide range for PC between 14.37% and 18.21%. 291-4 and 1282-3 had more than 18% average PC. The genotypes between 17-18% PC were 1247-4, Allison, 355-4, 1000-1, 1127-3, 1117-3, 176-3, and 668-3 from low to high, respectively. 660-4 and 859-2, which had 14.37 and 14.99 % PC, respectively, were the protein poorest genotypes.

6.1. The Impacts of Pod, Leaf, and Steam on Total Protein Content

PC varied in each section (Figure 3.25). The pod part had the highest PC with a 24.91% average. The minimum and maximum rates were 21.24% and 28.22% in the pod. Leaves followed it. The mean of leaf PC was 18.5%. Compared to the pod section, a more comprehensive range, 12.99% - 24.52%, was present. The stem was the poorest and the least part contributing to the PC. While its average PC was just 6.09, the general pattern altered between 4.54% and 8.99%.

The effects of these plant parts on the overall PC were quite different. The increase of pod PC correlated nearly the same with total PC compared to dried pod weight. The coefficient of correlation of the former was 0.58 in the 0.01 confidence interval, while this rate was equal to 0.57 in the latter in the 0.05 confidence interval. Surprisingly, the rise of pod PC up to 25% boosted the total PC from 14% to 16%. However, this linear relationship slowed after the threshold of 25% was exceeded. Despite the change in pod PC from 25% to 28%, the total PC stayed nearly at the same level (Figure 3.26). Furthermore, dried pod weight only contributed to the total PC when it exceeded 80 grams per plot (Figure 3.27). The correlation between dried pod weight per plot and pod PC was also checked, but the variables did not provide statistical significance.

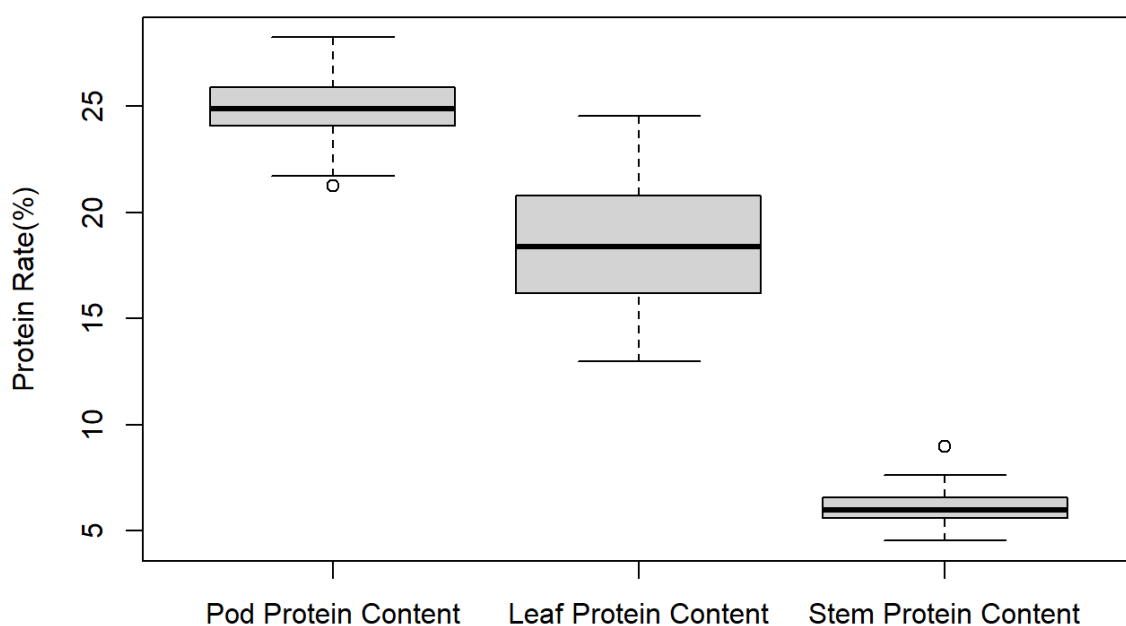


Figure 3. 25. The Protein Content of Pod, Leaf, and Stem

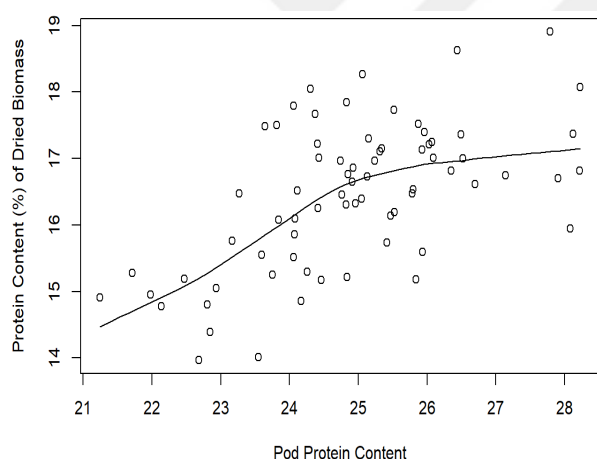


Figure 3. 26. The Relationship between Total Protein and Pod Protein Content

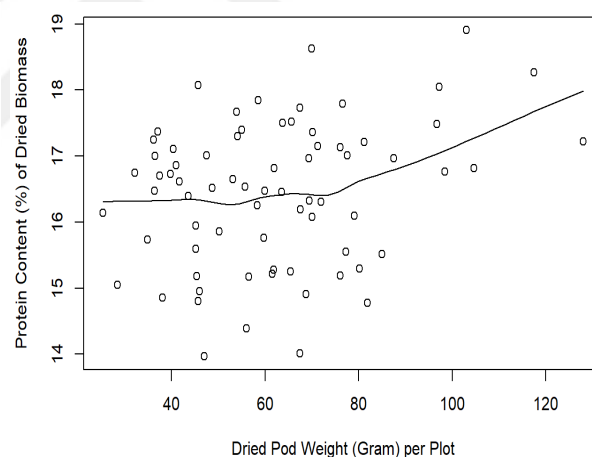


Figure 3. 27. The Relationship between Total Protein and Dried Pod Weight per Plot

The impact of leaf on total PC was comparatively limited. In spite of the statistically significant correlation ($p < 0.05$), the enhancement in leaf PC did not enormously improve total PC ($r = 0.27$) (Figure 3.28). Similarly, the inclusion of more leaf weight in the total DM did not change total PC (Figure 3.29), such that correlation was not detected ($p > 0.05$).

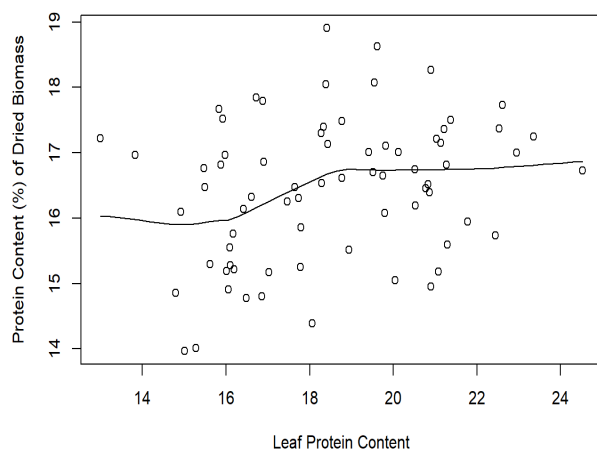


Figure 3. 28. The Relationship between Total Protein and Leaf Protein Content

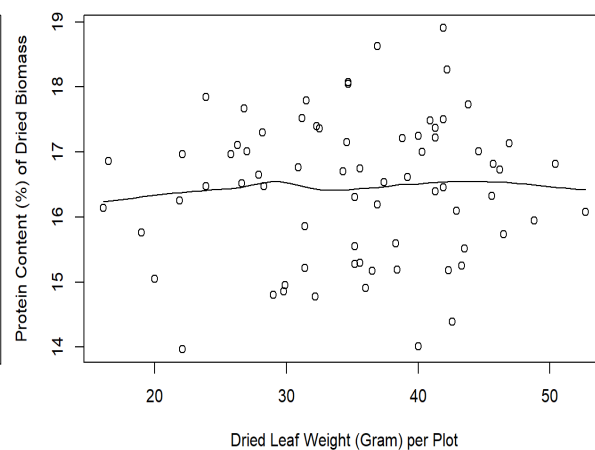


Figure 3. 29. The Relationship between Total Protein and Dried Leaf Weight per Plot

The stem was the only plant part causing a loss in overall PC when its weight went up within the total dried biomass. On average, the rise of stem weight per plot caused a gradual reduction in total PC, from 17% to below 16%. Figure 3.31 was confirmed with correlation, $r = -0.25$, in 0.05 confidence interval. In contrast, there was no correlation between total PC and stem PC ($p > 0.05$).

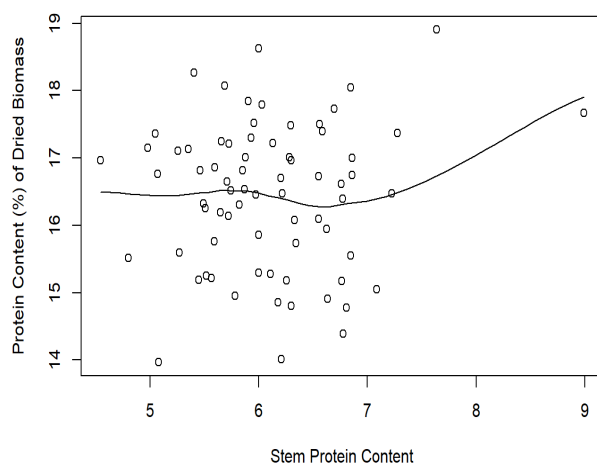


Figure 3. 31. The Relationship between Total Protein and Stem Protein Content

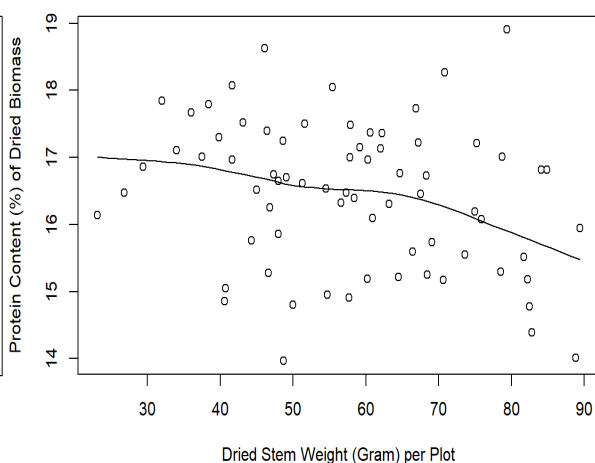


Figure 3. 30. The Relationship between Total Protein and Dried Stem Weight per Plot

7. GWAS Analysis for Plant Height, Stem Thickness, and Days to flowering

GWAS analyses gave different results for each characteristic. PH did not give any association in any chromosome or locus (Figure 3.32). Early separations in the p values of SNPs from the diagonal lines to expected values were observed.

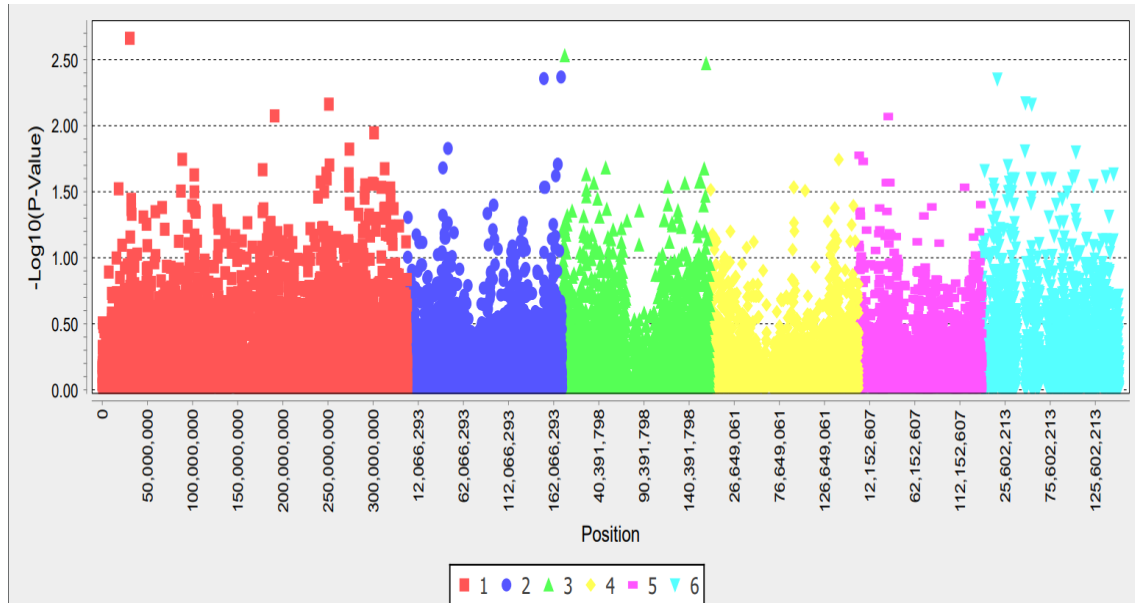


Figure 3. 32. Manhanttan Plot of GWAS Results For Plant Height

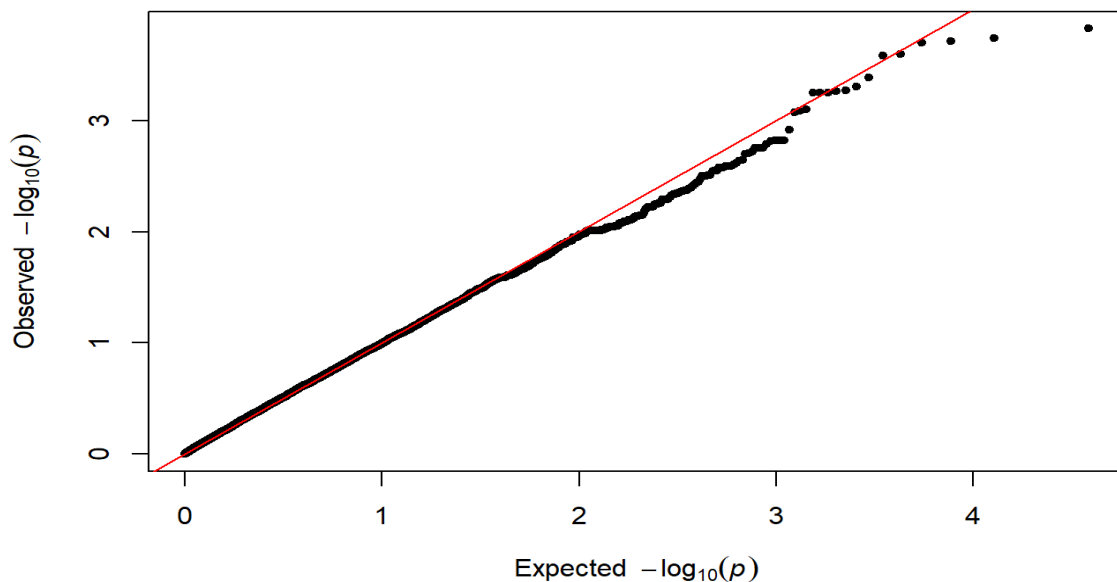


Figure 3. 33. Q-Q Observed and Expected P Values of Plant Height on GWAS

GWAS analysis of ST showed weak potential signals on all chromosomes (Figure 3.34). However, the signals was not strong enough to exceed the Bonferroni threshold of $-\log_{10}(p)$ at the confidence value of 0.05, which is 5.58 on the y-axis and is not shown as it is out of the y-axis scale. Q-Q plot demonstrated that SNPs' p values were distributed relatively more toward observed values (y-axis) in the ST (Figure 3.35).

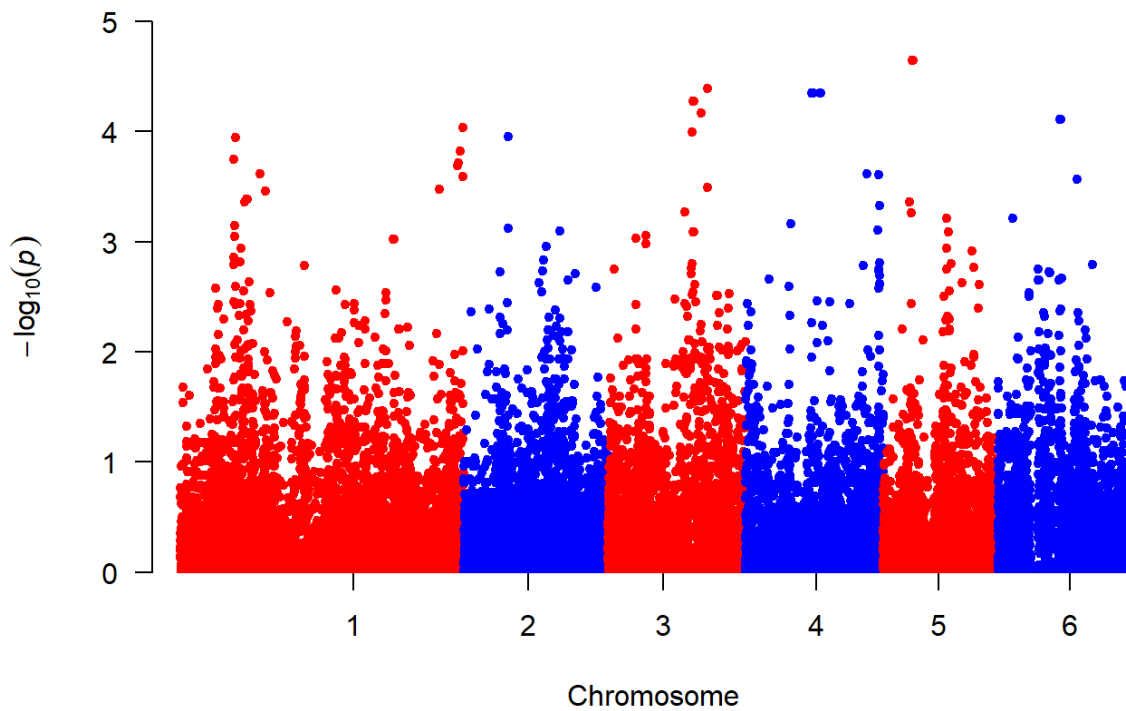


Figure 3. 34. Manhanttan Plot of GWAS Results For Stem Thickness

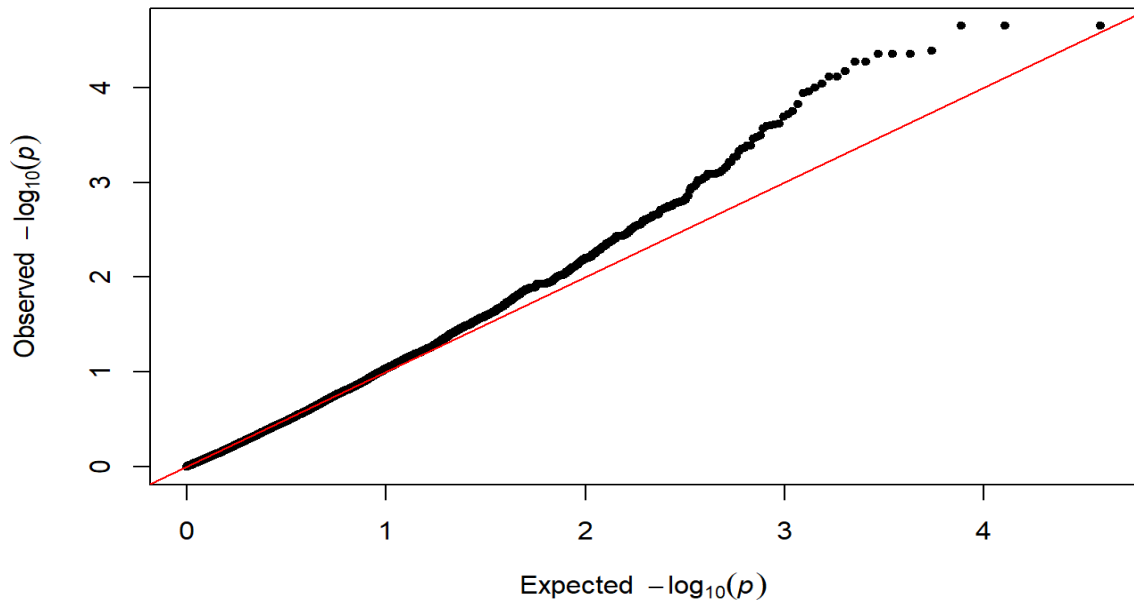


Figure 3. 35. Q-Q Observed and Expected P Values of Stem Thickness on GWAS

DTF became the only trait making a peak near the Bonferroni threshold of $-\log_{10}(p\text{-value})$ at the confidence value of 0.05, shown as a red line in chromosome 3 in GWAS analysis. P values of SNPs distributed toward the observed values for the days to flowering in the Q-Q plot (Figure 3.37).

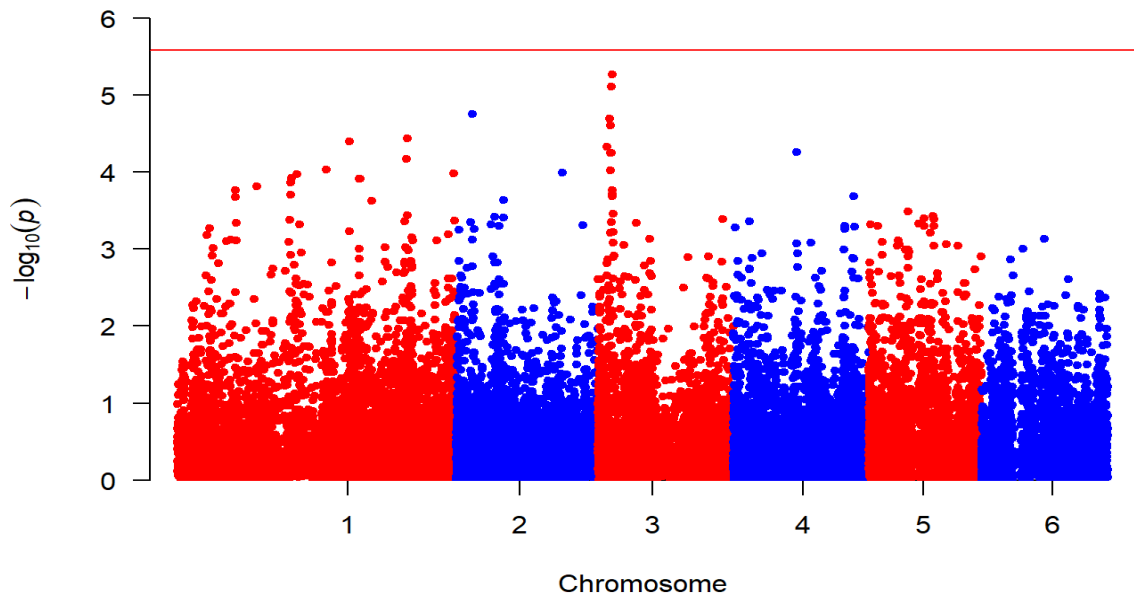


Figure 3. 36. Manhatttan Plot of GWAS Results For Days to flowering

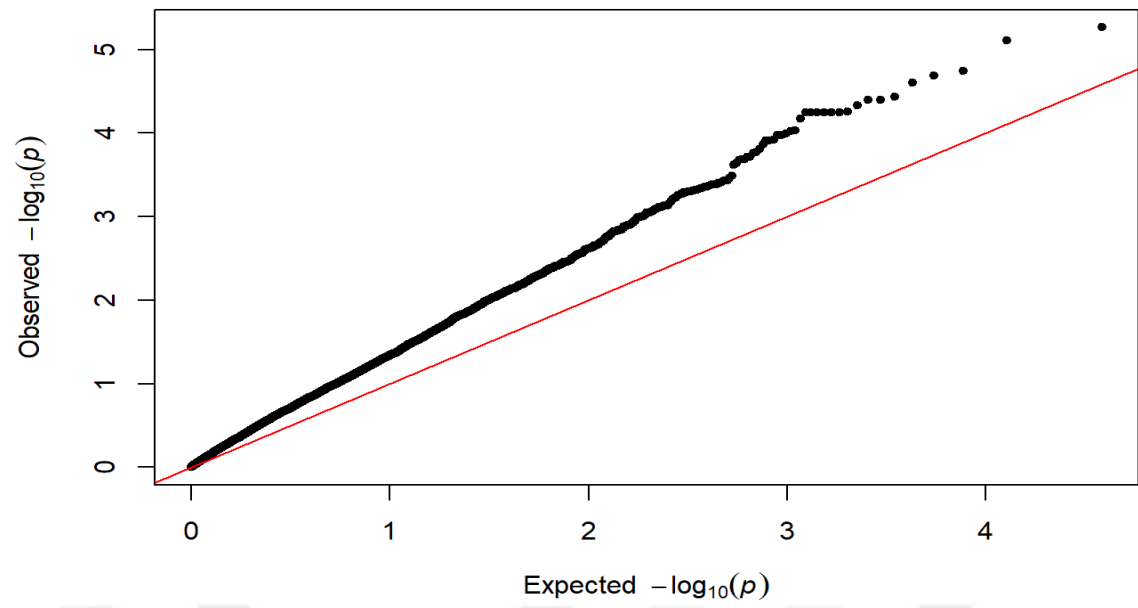


Figure 3. 37. Q-Q Observed and Expected P Values of Days to flowering on GWAS

CHAPTER 4. DISCUSSION

1. Fresh Herbage (Biomass) and Dry Matter Yield

In this experiment, the herbage yield and DM yield the Reading Spring Bean Population were determined. Statistically significant differences could not be found across genotypes. On average, 4300 kg/da biomass yield was obtained from the inbred FB lines. This value is below the average of 6770 kg/da obtained by Kusvuran *et al.* (2015) from a local variety. The rainfed condition and drought that occurred in June and July adversely affected plants' development. In addition, it has been reported that inbreeding depression causes a reduction in biomass up to 91 % in *A. vulneraria* (kidney vetch) (Rehling *et al.*, 2019). The same depression might have been another reason behind the lower biomass yield compared to the latter study.

Plant parts did make a different contribution to total BY. On average, the pod and stem were superior in total biomass in each genotype. In accordance with the present results, Huang *et al.* (2020) have reported an increased proportion of pod and stem in the total biomass and a slowdown, in contrast, of leaf in total biomass after flowering in common vetch (*Vicia sativa* L.) (Figure 1.6). According to the strong correlation between stem and fresh biomass, the ST could be a good potential proxy trait for total biomass. The correlation between these plant parts with biomass is more surprising when the proportion of pod is taken into account. Although all parts showed a strong correlation (Table 3.1), the leaf ($r = 0.87^{**}$) had a higher correlation with biomass than the pod ($r = 0.82^{**}$).

The current study also found a weak correlation between plant density and fresh biomass yield. Although the plots including fewer plants due to missing ones sometimes performed a higher yield than those not including missing plants, the higher fresh biomass yield was obtained from the plots there was no missing plant. This corroborated the knowledge that biological yield continues to increase together with high plant density until the point at which inter-plant competition obscures

the yield (López-Bellido *et al.*, 2005). Therefore, higher plant density can still be tried as the yield tends to increase with advancing plant density (Figure 3.4).

Forage crops are produced for herbage. As fresh herbage highly includes also moisture content, DM yield is a more critical parameter in forage production. In this experiment, an average 913.8 kg/da DM yield was obtained. Weight losses during the drying varied across genotypes, and the losses were higher than the result (3 times lower yield) demonstrated by Kusvuran *et al.* (2015). In terms of yield per da, this result was lower than the findings of a great deal of the previous works, which are 1031 kg (Berkenkamp & Meeres, 1986), 1200 kg/da (Ghanbari-Bonjar & Lee, 2003), and 2250 kg /da (Kusvuran *et al.*, 2015). At the same time, the current experiment was consistent with the DM yield literature for FB, which ranges from 750 kg/da to 990 kg/da (Faulkner & Steen, 1984) and from 768kg/da to 1274 kg/da (Guevara Oquendo, 2020).

Plant parts also lost weight during the drying, and the losses are proportional to the general loss rate, from fresh biomass to DM in each genotype. However, correlation results altered between total DM yield and plant parts compared to fresh biomass (Table 2.). The stem similarly had the strongest correlation ($r = 0.82^{**}$) with dried biomass. As opposed to fresh biomass, the pod was the second high part associated with total DM ($r = 0.78^{**}$). The leaf became the less correlated plant part ($r = 0.63^{**}$) with DM.

2. Plan Height (PH)

Genotypes have been found as statistically significant variance sources in this experiment. Previous studies have also demonstrated the variance for PH across genotypes (Daur *et al.*, 2010; Chaieb *et al.*, 2011; Etemadi *et al.*, 2018). These results mean that the genetic diversity of PH in FB has not been stable yet. In the current experiment, many inbred lines performed greater PH than control checks.

The result of the current experiment for PH agrees with the finding of Hughes *et al.* (2020), which are the results of 81 cm average PH and the range from 71 to 96 cm. The rainfed condition can explain the huge range between the lowest and highest value. On closer inspection of the current experiment, a relatively lower minimum value, 47.40 cm, was obtained compared to Hughes *et al.* (2020). The reason behind that might have been the drought occurring in June. Despite rainfed conditions in both experiments, the current experiment lagged far behind the results of Fraser *et al.* (2001), which were PH between 115-146 cm. In contrast to rainfed conditions, the range of PH is comparatively narrow in irrigated conditions. The wide range of this experiment was not matched with the relatively narrow PH range of irrigated experiments. While Alghamdi (2007) reported between 88.2-98.2 cm in the first year and 119-122.7 cm in the second year, Matthews *et al.* (2008) reported between 95-103cm. That means that irrigated conditions provide a more consistent PH yield in FB.

The high correlation ($r=0.60^{**}$) between PH and herbage DM yield is a good indicator for FB in terms of its forage crop capability. Similar associations have been reported in important forage crops such as alfalfa (Robins *et al.*, 2007; Hamd Alla *et al.*, 2013; Caverro *et al.*, 2017; He *et al.*, 2020; Liu *et al.*, 2021), maize (Gilliot *et al.*, 2021), and grasses (Majidi *et al.*, 2009). No lodging being present in this experiment is the other positive characteristic for FB because even though getting more green herbage is theoretically possible with high PH in the light of those studies, tall plants are more likely for the lodging and this causes adverse effects in plant development and difficulties in the harvest. ST is advised in the struggle with lodging (Cubero & Nadal, 2005). The absence of lodging in this experiment can be explained by the correlation ($r=0.45$) between PH and ST in the current study (Figure 3.16). This average correlation is in line with the high correlation between PH and ST in Hungarian vetch (Demirhan *et al.*, 2018).

3. Stem Thickness (ST)

Variation of ST and breeding new lines have been studied moderately less on FB. In the current experiment, genotypes have been found statistically significant for ST. This result is partially compatible with the results of Terzopoulos *et al.* (2008). They identified ST as a feature which uniformity is not yet an issue among 54 local Greek FB populations. In addition, the same study has shown local populations as gene sources to breed locally adapted new populations. However, the difference between this study and the current study is inbred lines and open-pollinated populations. While open-pollinated populations can get diverse genes from the gene pool within the population, this is not the case in the inbred lines. Therefore, the inbred genotypes with statistically thicker stems can be used to breed synthetic cultivars. In this approach, the thickest genotypes, greater than 9.5 mm, which were 434-1 and 1247-2 can be important lines.

A huge range, between 4.79 and 10.20 mm for ST, has been explored in the current experiment. It is wider than the result between 7 and 9 mm reported by El-Emam *et al.* (2014). The average ST of the current study, 7.8 mm, was higher than the mean of 7.12 mm notified by Yahia *et al.* (2012). When these results are compared with the ST of alfalfa, FB has a nearly two times thicker stem. Such that, comparatively lower alfalfa ST ranges between 1.86-4.76 mm and between 2.7-3.9 mm have been recorded by Basbag *et al.* (2009) and Avci *et al.* (2013), respectively.

In this experiment, ST was determined as a parameter for forage DM yield. This has been proved with correlation ($r = 0.55^{**}$) between them, shown in Figure 25. Even though it is not linear, and the yield increase is not distinct, like in PH, herbage yield increased with extension in the ST from 7 mm to 9.5 mm. While the same relationship has been reported in Hungarian vetch by Demirhan *et al.* (2018), it was not found in alfalfa (Avci *et al.*, 2013). On the other hand, ST is generally considered an essential characteristic once strong stems are wanted (Silveira *et al.*, 2021). Even though ST is a character associated with lodging resistance in many plants (Porqueddu 2001; Liu *et al.*, 2018; Silveira *et al.*, 2021), lignification and lignin content of stem is also determined as a

parameter which enhances the breaking strength of stem. While the increase is already known with the advancing stage of growth in lignin content, there is no article reporting the correlation between ST and the lignin content of stem in FB up to now. However, Mihailović *et al.* (2011) have already alerted that a high stem amount on total biomass might increase lignin content. Our finding revealed a positive correlation between fresh stem increase and ST. Therefore, even if structural components such as lignin might not have differed because of the growth in the ST, the boost in fresh stem weight with ST can negatively affect the forage quality.

4. Days to flowering (DTF)

In this experiment, DTF has been examined to find out its impact to the DM yield of herbage FB. In the UK, spring FB demonstrated 60.87 DTF. This result is near to DTF between 55-59 days reported in Ethiopia (Alkhtib *et al.*, 2016). In contrast to Los and Siddique (1997), DTF variation was not derived from different sowing times. Genotypes held a genetic variation for DTF ($p < 0.01$). Although Los and Siddique (1997) reported an increase in DM yield with more DTF, the result of the experiment demonstrated that DTF did not contribute to the DM yield of herbage FB. This result does not also agree with other studies informing statistically significant and strong correlation between these characteristics in alfalfa (Shanjani *et al.*, 2013), wheatgrass (Shanjani *et al.*, 2015), and sorghum (Jebril, 2018).

5. Protein Content of Herbage (Biomass) (PC)

As PC is one of the critical forage crop quality parameters, it is included in the experiment. The mean of PC OF DM of all plots was 16.44%. This rate is higher than the rate for FB between 14.5-15.5 % notified by Faulker and Steen (1984). However, it is lower than the rates for FB ranging from 17.6 to 20 % and from 20 to 22 % demonstrated by Beken and Meeres (1985) and Guevara (2020), respectively. Compared with other forage crops, FB is in a good position with grasses such as *Poa pratensis*, *Phleum pratense*, and *Lolium multiflorum* (Figure 1.5). According to the review

of Lee (2018), FB has relatively the same mean as *Vicia pannonica* for PC. However, there is a slightly lower PC compared to other legumes such as alfalfa, trifoliums, and hairy vetch.

The current experiment checked the fodder PC of FB for the first time for the variation between lines. The huge range from 14.37 to 18.21% PC in inbred lines can be helpful in breeding new cultivars having higher PC. While variation for PC within the different plant species can be based on different chemical compositions in plant cells, different plant parts (pod, leaf, and stem) importantly affected the total PC within the same species in this experiment. Pod and leaf included higher PC (Figure 3.25). However, their impact on total PC was not equal. The pod is a more important part of FB to increase total PC. As opposed to FB, leaf contributes more to the total PC in alfalfa. More interestingly, while the higher leaf PC caused only a minimal rise in total PC, more leaf weight did not cause any change in FB total PC. That means that an enhancement in the leaf-to-stem ratio of FB would not improve the total PC. Negatively, stem weight in total biomass reduced the total PC. Therefore, the positive contribution of the stem to fresh and DM yield (Table 3.1 and Table 3.2) is vitiated by its deficit in the quality of herbage.

6. GWAS Analysis Results

In this experiment, PH, ST, and the DTF traits of inbred lines were evaluated to find out the association with SNPs through GWAS analysis as these were the traits which had been measured on the entire panel and found to be significantly determined by genotype. No significant association has been found in GWAS analysis between any traits and markers. However, some markers for the DTF promised were almost at the significance threshold value (Figure 3.36). Despite some peaking of SNPs with the stem thickness, they were weaker compared to the DTF. Alqudah *et al.* (2020) specified that when performing genome-wide association scans, the size of the population should be at least 100 genotypes, and each genotype should be replicated. When the result is assessed from this scope, the population size in the present experiment was at the minimum level due to limited seed amounts. Moreover, because of the same reason, some genotypes could not be replicated.

Therefore, study may have been underpowered considering the complexity of the traits under study. If the population could be put through a round of seed multiplication, it would be possible to carry out a larger experiment with 154 lines all replicated, significantly increasing the prospects of detecting markers significantly associated with the DTF, ST, and other traits.



CHAPTER 5. CONCLUSION

The current experiment was planned to extend the limited knowledge of the whole plant forage capability of FB and fill the knowledge gap on the SNPs associated with forage-related characteristics. This study has shown that fresh forage (biomass) yield and DM yield did not statistically differ across genotypes. However, the investigation of the correlation between different plant parts has identified that the stem was the most critical part for both fresh biomass and DM weight. In contrast, the pod was the most vital part of the total PC. Leaf had a powerful impact on neither total yield nor PC. This experiment confirmed that PH, stem thickness, and DTF significantly differed across genotypes. One of the important findings to emerge from this study is that ST has been found as a strong parameter for the first time for herbage DM yield together with PH in FB. The results of this research support the approach that PH and ST can be evaluated to increase forage DM yield. The major finding of this experiment was that phenotyped data for DTF shows a signal not far from the threshold in GWAS analysis, and it is promising. The second major building was the signals from each chromosome for ST in GWAS analysis. This is the first experiment contributing to the literature by evaluating the ST and DTF for more forage DM in FB. The analysis of GWAS for the DTF undertaken here has extended our knowledge of the genetic linkage between the marker and a particular causative locus controlling the DTF on chromosome 3.

The major limitation of this study is the limited time given for the experiment. Therefore, the digestibility test could not be included in the investigation. This situation prevented the evaluation of stems in terms of digestibility. In addition, the present study was limited due to the insufficient seed amount of some genotypes. While the line amounts used in the experiment decreased to 100 from 154 lines, the replicate amounts also changed in some genotypes. The decrease in genotype amount in the experiment might have caused a weakness in the results of GWAS analysis. The lack

of labour limited the size of the destructive harvest. This restrained the GWAS analysis of PC of FB herbage due to the limited genotype amount.

Further studies need to be done to establish the same experiment with more genotypes amounts whether the GWAS analyses give linkage signals that are above the threshold value. Furthermore, studies which are bigger size should be carried out to establish the GWAS analysis result for PC and digestibility parameters. Finally, a future study investigating fresh and dry forage yield with more replicates would be very worthwhile to analyse the yield data via GWAS to determine whether a linkage between any marker and locus is present.



APPENDICES

APPENDIX 1. Data Input of Phenotypic Data

```
library(readxl)
FB110 <- read_excel("C:/Users/ahmet/OneDrive/FB110.xlsx")
View(FB110)
summary(FB110)
```

APPENDIX 2. Codes for Plant Height Analysis

Histograms and Boxplots

##1.Distrubition

```
boxplot(FB110$`Plant Height (cm)by 08.06.2022`, ylab="Plant Height (cm)")
hist(FB110$`Plant Height (cm)by 08.06.2022`, xlab = "Plant Height (cm)")

library("dplyr")
library("ggpubr")

ggqqplot(FB110$`Plant Height (cm)by 08.06.2022`, color = "orange", size = 1.25
)
```

##2.Normality

```
shapiro.test(FB110$`Plant Height (cm)by 08.06.2022`)
```

##3.Homogeneity

```
library(car)

k <- FB110$`Plant Height (cm)by 08.06.2022`
l <- FB110$`trt (Genotype)`
leveneTest(k ~ l, data = FB110)
```

Kruskal Wallis

```
kruskal.test(k, l, data=FB110)
```

APPENDIX 3. Codes for Stem Thickness Analysis

##1.Distrubition

```
boxplot(FB110$`Stem Thickness (mm)`, ylab = "Stem Thickness (mm)")
hist(FB110$`Stem Thickness (mm)`, xlab = "Stem Thickness (mm)")
ggqqplot(FB110$`Stem Thickness (mm)`, color = "orange", size = 1.25)
shapiro.test(FB110$`Stem Thickness (mm)`)
```

##2.Homogeneity

```
yy <- FB110$`Stem Thickness (mm)`
```

```
l <- FB110$`trt (Genotype)`
leveneTest(yy ~ l, data = FB110)

kruskal.test(yy ~ l, data = FB110)
```

APPENDIX 4. Codes for Days to Flowering Analysis

```
##1.Distrubution
boxplot(FB110$`Days to Flowering (DTF)`, ylab="Day to Flowering")

hist(FB110$`Days to Flowering (DTF)`, xlab = "Day to Flowering")

library("dplyr")
library("ggpubr")
ggqqplot(FB110$`Days to Flowering (DTF)`, color = "orange", size = 1.25)

##2.Normality
shapiro.test(FB110$`Days to Flowering (DTF)`)

##3.Homogeneity
library(car)
DTF <- FB110$`Days to Flowering (DTF)`
l <- FB110$`trt (Genotype)`
## 4.Kruskal Wallis
kruskal.test(DTF, l, data=FB110)

##5.Correlation
cor.test(DTF, FB110$`Dried Matter Yield (Area Based)`, data=FB110)
```

APPENDIX 5. Codes for Correlation Analysis between Biomass and Harvested Plant Amounts

```
scatter.smooth(x=FB110$`Fresh Green Weight (Gramme)`, y=FB110$`Harvested Plant
s Amount`, xlab = "Biomass Weight(Gram) per Plot", ylab = "Harvested Plant Amo
unt per Plot" )

cor.test(FB110$`Fresh Green Weight (Gramme)`, FB110$`Harvested Plants Amount`,
method = "pearson", alternative = "greater")
```

APPENDIX 6. Codes for the Analysis of Protein Content in Dried Biomass

```
##1. Distrubution
hist(FB110$`Total Dried Biomass Protein Content (%)`, xlab = "Protein Content
(%) in Dried Biomass")

boxplot(FB110$`Total Dried Biomass Protein Content (%)`, ylab="Protein Content
(%) in Dried Biomass")

ggqqplot(FB110$`Total Dried Biomass Protein Content (%)`, color= "orange")

label=c("Pod Protein Content", "Leaf Protein Content", "Stem Protein Content")
boxplot(FB110$`Pod Protein Content (%)`, FB110$`Leaf Protein Content (%)`, FB1
10$`Stem Protein Content (%)`, names = label, ylab="Protein Rate(%)" )
```

```
shapiro.test(FB110$`Total Dried Biomass Protein Content (%)`)
```

##2.Homogeneity

```
uu<-FB110$`Total Dried Biomass Protein Content (%)`  
leveneTest(uu ~ 1, data = FB110)
```

##Kruskal Wallis Test

```
kruskal.test(uu ~ 1, data = FB110)
```

APPENDIX 7. Codes for Protein Content Correlation Analysis with Pod, Stem, and Leaf Amount

```
scatter.smooth(x=FB110$`Dried Pod Weight(Gram)`, y=FB110$`Total Dried Biomass  
Protein Content (%)`, xlab="Dried Pod Weight (Gram) per Plot", ylab = "Protein  
Content (%) of Dried Biomass")
```

```
scatter.smooth(x=FB110$`Pod Protein Content (%)`, y=FB110$`Total Dried Biomass  
Protein Content (%)`, xlab="Pod Protein Content", ylab = "Protein Content (%)  
of Dried Biomass")
```

```
scatter.smooth(x=FB110$`Pod Protein Content (%)`, y=FB110$`Dried Pod Weight(Gr  
am)`, xlab="Pod Protein Content", ylab = "Dried Pod Weight per Plot")
```

```
scatter.smooth(x=FB110$`Dried Leaves Weight (Gram)`, y=FB110$`Total Dried Biom  
ass Protein Content (%)`, xlab = "Dried Leaf Weight (Gram) per Plot", ylab = "  
Protein Content (%) of Dried Biomass")
```

```
scatter.smooth(x=FB110$`Leaf Protein Content (%)`, y=FB110$`Total Dried Biomas  
s Protein Content (%)`, xlab = "Leaf Protein Content", ylab = "Protein Content  
(%) of Dried Biomass")
```

```
scatter.smooth(x=FB110$`Dried Stem Weight (Gram)`, y=FB110$`Total Dried Biomas  
s Protein Content (%)`, xlab = "Dried Stem Weight (Gram) per Plot", ylab = "Pr  
otein Content (%) of Dried Biomass")
```

```
scatter.smooth(x=FB110$`Stem Protein Content (%)`, y=FB110$`Total Dried Biomas  
s Protein Content (%)`, xlab = "Stem Protein Content", ylab = "Protein Content  
(%) of Dried Biomass")
```

```
cor.test(FB110$`Total Dried Biomass Protein Content (%)`, FB110$`Pod Protein C  
ontent (%)`, data=FB110)
```

```
cor.test(FB110$`Total Dried Biomass Protein Content (%)`, FB110$`Dried Pod Wei  
ght(Gram)`, data=FB110)
```

```
cor.test(FB110$`Total Dried Biomass Protein Content (%)`, FB110$`Leaf Protein  
Content (%)`, data=FB110)
```

```
cor.test(FB110$`Total Dried Biomass Protein Content (%)`, FB110$`Dried Leaves  
Weight (Gram)`, data=FB110)
```

```
cor.test(FB110$`Total Dried Biomass Protein Content (%)`, FB110$`Stem Protein  
Content (%)`, data=FB110)
```

```
cor.test(FB110$`Total Dried Biomass Protein Content (%)`, FB110$`Dried Stem We  
ight (Gram)`, data=FB110)
```

```
cor.test(FB110$`Pod Protein Content (%)`, FB110$`Dried Pod Weight(Gram)`, data
=FB110)
```

APPENDIX 8. Codes for Correlation Analysis between Plant Height and Stem Thickness

```
cor.test(FB110$`Plant Height (cm)by 08.06.2022`, FB110$`Stem Thickness (mm)`)
ph <- FB110$`Plant Height (cm)by 08.06.2022`
st <- FB110$`Stem Thickness (mm)`
scatter.smooth(x=ph, y=st, xlab = "Plant Height (cm)", ylab = "Stem Thickness
(mm)")
```

APPENDIX 9. Data Input of the Destructive Harvest

```
library(readxl)
biomass <- read_excel("C:/Users/ahmet/OneDrive/biomass.xlsx")

View(biomass)
summary(biomass)
```

APPENDIX 10. Codes for Fresh Biomass Analysis

```
##Distribution
ddc <- biomass$`Green Biomass Yield (Kg/Da) Area Base`
hist(ddc, main = NULL, xlab = "Biomass Yield (Kg/Da)")

boxplot(ddc, ylab = "Biomass Yield (Kg/Da)")
library("dplyr")

library("ggpubr")

library(car)

ggqqplot(ddc, color = "orange")

##Normality
shapiro.test(biomass$`Green Biomass Yield (Kg/Da) Area Base`)

##Homogeneity
p <- biomass$`trt (Genotype)`
leveneTest(ddc~p, data = biomass)

##Variation Check
kruskal.test(ddc~p, data = biomass)

##Histogram Pod, Leaf, and Stem Weight
hist(biomass$`Fresh Pod Weight (Gram)`)

hist(biomass$`Fresh Leaves Weight (Gram)`)

hist(biomass$`Fresh Stem Weight (Gram)`)
```

```

label=c("Pod Weight", "Leaf Weight", "Stem Weight")
boxplot(biomass$`Fresh Pod Weight (Gram)`, biomass$`Fresh Leaves Weight (Gram)`
, biomass$`Fresh Stem Weight (Gram)`, names = label, ylab="Fresh Weight (grams) per Plot")

kruskal.test(biomass$`Fresh Pod Weight (Gram)`~p, data = biomass)

kruskal.test(biomass$`Fresh Leaves Weight (Gram)`~p, data = biomass)

kruskal.test(biomass$`Fresh Stem Weight (Gram)`~p, data = biomass)

```

APPENDIX 11. Codes for Correlation Analysis in Fresh Plant Parts

```

pod<- biomass$`Fresh Pod Weight (Gram)`
leaf<- biomass$`Fresh Leaves Weight (Gram)`
stem1<- biomass$`Fresh Stem Weight (Gram)`
scatter.smooth(x=biomass$`Fresh Green Weight (Gramme)`, y=pod, xlab = "Biomass
Weight(Gram) per Plot", ylab = "Pod Weight (Gram) per Plot")

scatter.smooth(x=biomass$`Fresh Green Weight (Gramme)`, y=leaf, xlab = "Biomass
Weight(Gram) per Plot", ylab = "Leaf Weight (Gram) per Plot")

scatter.smooth(x=biomass$`Fresh Green Weight (Gramme)`, y=stem1, xlab = "Biomass
Weight(Gram) per Plot", ylab = "Stem Weight (Gram) per Plot")

linearModstem <- lm(biomass$`Fresh Green Weight (Gramme)`~ stem1, data = biomass)
summary(linearModstem)

cor.test(biomass$`Fresh Green Weight (Gramme)`, pod, method = "pearson", alternative = "greater")

cor.test(biomass$`Fresh Green Weight (Gramme)`, leaf, method = "pearson", alternative = "greater")

cor.test(biomass$`Fresh Green Weight (Gramme)`, stem1, method = "pearson", alternative = "greater")

cor.test(pod, leaf, method = "pearson", alternative = "greater")

cor.test(stem1, pod, method = "pearson", alternative = "greater")

cor.test(stem1, leaf, method = "pearson", alternative = "greater")

```

APPENDIX 12. Codes for Dry Biomass Analysis

```

ddk <- biomass$`Dried Matter Yield (Area Based)`
##Distribution
boxplot(ddk, ylab = "Dry Matter Yield (Kg/Da)")

hist(ddk , xlab = "Dry Matter Yield (Kg/Da)", main = NULL)

ggqqplot(ddk, color = "orange")

```

##Normality

```
shapiro.test(ddk)
```

##Homogeneity

```
leveneTest(ddk~p, data = biomass)
```

##Kruskal-Wallis Test

```
kruskal.test(ddk~p, data = biomass)
```

APPENDIX 13. Codes for Correlation Analysis in Dry Plant Parts

```
Dpod<- biomass$`Dried Pod Weight(Gram)`  
Dleaf<- biomass$`Dried Leaves Weight (Gram)`  
Dstem1<- biomass$`Dried Stem Weight (Gram)`  
label=c("Pod Weight", "Leaf Weight", "Stem Weight")  
boxplot(biomass$`Dried Pod Weight(Gram)`, biomass$`Dried Leaves Weight (Gram)`  
, biomass$`Dried Stem Weight (Gram)`, names = label, ylab="DM Weight (grams) per Plot")  
  
kruskal.test(Dpod~p, data = biomass)  
  
kruskal.test(Dleaf~p, data = biomass)  
  
kruskal.test(Dstem1~p, data = biomass)  
  
scatter.smooth(x=biomass$`Dried Matter Weight (Gram)`, y=Dpod, xlab = "Dry Matter Weight(Gram) per Plot", ylab = "Dry Pod Weight (Gram) per Plot")  
  
scatter.smooth(x=biomass$`Dried Matter Weight (Gram)`, y=Dleaf, xlab = "Dry Matter Weight(Gram) per Plot", ylab = "DryLeaf Weight (Gram) per Plot")  
  
scatter.smooth(x=biomass$`Dried Matter Weight (Gram)`, y=Dstem1, xlab = "Dry Matter Weight(Gram) per Plot", ylab = "Dry Stem Weight (Gram) per Plot")  
  
cor.test(biomass$`Dried Matter Weight (Gram)`, Dpod, method = "pearson", alternative = "greater")  
  
cor.test(biomass$`Dried Matter Weight (Gram)`, Dleaf, method = "pearson", alternative = "greater")  
  
cor.test(biomass$`Dried Matter Weight (Gram)`, Dstem1, method = "pearson", alternative = "greater")  
  
cor.test(Dpod, Dleaf, method = "pearson", alternative = "greater")  
  
cor.test(Dstem1, Dpod, method = "pearson", alternative = "greater")  
  
cor.test(Dstem1, Dleaf, method = "pearson", alternative = "greater")
```

APPENDIX 14. Codes for Correlation Between Biomass, Plant Height, and Stem Thickness

```
scatter.smooth(x=biomass$`Plant Height (cm)by 08.06.2022`, y=biomass$`Dried Matter Yield (Area Based)`, data=biomass, xlab="Plant Height (cm)", ylab="DM Yield (Kg/Da)")

scatter.smooth(x=biomass$`Stem Thickness (mm)`, y=biomass$`Dried Matter Yield (Area Based)`, data=biomass, xlab="Stem Thickness (mm)", ylab="DM Yield (Kg/Da)")

cor.test(biomass$`Plant Height (cm)by 08.06.2022`, biomass$`Dried Matter Yield (Area Based)`, method = "pearson", alternative = "greater")

cor.test(biomass$`Stem Thickness (mm)`, biomass$`Dried Matter Yield (Area Based)`, method = "pearson", alternative = "greater")
```

APPENDIX 15. Codes for Correlation Analysis between Stem Thickness and Stem Weight

```
##CORRELATION
cor.test(biomass$`Fresh Stem Weight (Gram)`, biomass$`Stem Thickness (mm)`, method = "pearson", alternative = "greater")

cor.test(biomass$`Leaf to Stem Rate (Fresh)`, biomass$`Stem Thickness (mm)`, method = "pearson", alternative = "greater")

scatter.smooth(x=biomass$`Stem Thickness (mm)`, y=biomass$`Fresh Stem Weight (Gram)`, data=biomass, xlab="Fresh Stem Weight", ylab="Stem Thickness (mm)")
```

APPENDIX 16. Codes for Bonferroni Correction Threshold

```
library(qqman)
library(dplyr)

# import TASSEL results
# note
TASSEL_GLM_Out <- read.table("GLMout.txt", header = T, sep = "\t")
#TASSEL_GLM_Out
STM<-subset(TASSEL_GLM_Out, TASSEL_GLM_Out$Trait == "Stemthick")
#STM
PH<-subset(TASSEL_GLM_Out, TASSEL_GLM_Out$Trait == "PH")
#PH
DTF<-subset(TASSEL_GLM_Out, TASSEL_GLM_Out$Trait == "DTF")
#DTF
# Number of traits
head(unique(TASSEL_GLM_Out$Trait))

# note: for each plot trait name must be specified

# first trait as example (i.e., EarHT)
#TASSEL_GLM_Out %>% filter(. $Trait == "PH")
```

```

#Plant Height
# Bonferroni correction threshold
nmrk <- nrow(PH)
(GWAS_Bonn_corr_threshold <- -log10(0.05 / nmrk))

## [1] 5.585303

#GWAS_Bonn_corr_threshold

# Manhattan plot
Mann_plot <- manhattan(
  PH,
  chr = "Chr",
  bp = "Pos",
  snp = "Marker",
  p = "p",
  col = c("red", "blue"),
  annotateTop = T,
  genomewideline = GWAS_Bonn_corr_threshold,
  suggestiveline = F
)

Mann_plot

## $xpd
## [1] FALSE

#Plant Height
# QQ plot
QQ_plot <- qq(PH$p)

#Stem Thickness

# Bonferroni correction threshold
nmrk <- nrow(STM)
(GWAS_Bonn_corr_threshold <- -log10(0.05 / nmrk))

## [1] 5.585303

# Manhattan plot
(Mann_plot <- manhattan(
  STM,
  chr = "Chr",
  bp = "Pos",
  snp = "Marker",
  p = "p",
  col = c("red", "blue"),
  annotateTop = T,
  genomewideline = GWAS_Bonn_corr_threshold,
  suggestiveline = F
)
)

## $xpd
## [1] FALSE

```

```

#GWAS_Bonn_corr_threshold

# QQ plot
QQ_plot <- qq(STM$p)

# Days to Flowering

# Bonferroni correction threshold
nmrk <- nrow(DTF)
(GWAS_Bonn_corr_threshold <- -log10(0.05 / nmrk))

## [1] 5.585303

# Manhattan plot
(Mann_plot <- manhattan(
  DTF,
  chr = "Chr",
  bp = "Pos",
  snp = "Marker",
  p = "p",
  col = c("red", "blue"),
  annotateTop = T,
  genomewideline = GWAS_Bonn_corr_threshold,
  suggestiveline = F
)
)

## $xpd
## [1] FALSE

#GWAS_Bonn_corr_threshold

# QQ plot
QQ_plot <- qq(DTF$p)

```

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