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Starch and protein accumulation during seed development of field grown faba beans (*Vicia faba* L. cv. Vertigo) in Norway

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1. ABSTRACT

The grain legume *Vicia faba* L. (faba bean) is a potential commercial protein crop in Norway. Little information is available concerning seed development and accumulation of storage compounds in *Vicia faba* seeds grown in Norwegian climate. The purpose of this thesis was to observe starch and protein accumulation, moisture content, fresh weight (FW) and dry weight (DW) changes during seed development in faba beans grown in southeast Norway. Pods of *Vicia faba* L. cv. Vertigo exposed to three treatments (untreated/fertilized with NPK/*Rhizobium* pre-inoculated) were harvested in the summer of 2019 in Ås, Norway at 7 stages of seed development with one-week incremented harvest dates (HD1-HD7). Treatment effects were not significant (p>0.05) on DW, FW, starch and protein contents, but significant for moisture content (p<0.05). HD was significant for all parameters measured (p<0.01). HD x Treatment interaction was not significant (p>0.05) for any parameter. Moisture content declined significantly from HD1 to HD7 (R_2=98.33%). The highest protein concentration was at HD1 (33.4%) and the lowest at HD5 (27.4%). Protein content per seed peaked at HD6 (178.5 mg) and was lowest at HD1 (29.4 mg). The highest starch concentration was at HD5 (40.5%) and the lowest at HD1 (12.4%). Starch content per seed peaked at HD7 (254.1 mg) and was lowest at HD1 (10.9 mg). The crop required a long growing season indicating late maturity in this climate. Seed desiccation was delayed due to heavy precipitation in the field.

Belgveksten *Vicia faba* L. (åkerbønne) er en proteinvekst med stort potensial i Norge. Det finnes lite informasjon om frøutvikling og oppsamling av lagringsstoffer i åkerbønner dyrket i Norge. Hensikten med denne oppgaven var å se på endringer under frøutvikling av åkerbønner dyrket i norsk klima i form av stivelse, proteiner, vanninnhold, friskvekt (FV) og tørrvekt (TV). *Vicia faba* L. cv. Vertigo såfrø (ubehandlet, NPK-gjødsling eller *Rhizobium* førinokulering) ble høstet sommeren 2019 i Ås, Norge på syv frøutviklingsstadier (HD1-HD7) med én ukes mellomrom. Frøbehandling ga ikke signifikant utslag (p>0,05) for TV, FV, innhold av stivelse og protein, men signifikant for vanninnhold (p <0,05). HD var signifikant for alle parametere (p<0,01). Interaksjon mellom HD x Behandling var ikke signifikant (p>0,05) for noen av parameterne. Vanninnholdet sank betydelig fra HD1 til HD7 (R_2 = 98,33%). Den høyeste proteinkonsentrasjonen var ved HD1 (33,4%) og lavest ved HD5 (27,4%). Proteininnhold per frø var høyest ved HD6 (178,5 mg) og lavest ved HD1 (29,4 mg). Den høyeste stivelseskonsentrasjonen var ved HD5 (40,5%) og lavest ved HD1 (12,4%). Stivelsesinnhold per frø var høyest ved HD7 (254,1 mg) og lavest ved HD1 (10,9 mg). Vertigo hadde lang modningstid, noe som innebærer at den er en sen sort i dette klimaet. Frøuttørking gikk langsomt på grunn av mye nedbør i feltet.

2. LITERATURE REVIEW

2.1 *Vicia faba* - a grain legume and protein crop

Plant-based proteins represent sustainably sourced protein alternatives, facilitating reduced dependence on animal-derived proteins (Lam et al., 2018; Multari et al., 2015). Over the last years there has been a shifting consumer trend towards increased consumption of plant-based proteins (Vasilean et al., 2018). In Europe, protein crops are predominantly grown in the Czech Republic, France, Germany and the United Kingdom. The grain legumes lupin, field pea and faba bean have lately gained increased popularity in Europe (Landry, 2014). Grain legumes belong to the Fabaceae family and are commonly used for their protein-rich seeds (Peltonen-Sainio et al., 2013; Rosa-Sibakov et al., 2018; Warsame et al., 2018; Coda et al., 2017). The Fabaceae family include the warm season grain legumes soybean (*Glycine max* L.), common beans (*Phaseolus vulgaris L.*), pigeon pea (*Cajanus cajan*), cowpea (*Vigna unguiculata*), peanut (*Arachis hypogaea*) and the cool season grain legumes field pea (*Pisum sativum*), faba bean (*Vicia faba* L.), chickpea (*Cicer arietnum*) and lentil (*Lens culinaris*) (Peltonen-Sainio et al., 2013; Multari et al., 2015; Parvin et al., 2019; Farooq et al., 2017; Witten et al., 2018; Meulen and Jansman 2010). Currently, grain legumes are less common in Norway compared to central Europe (Peltonen-Sainio et al., 2013). Apart from cereals, the major protein crops suitable for Northern Europe are rapeseed, faba beans and field peas (Peltonen-Sainio et al., 2013). Faba beans and field peas are good candidates to increase the seed-crop based protein production (Peltonen-Sainio et al., 2013). Faba bean has higher yield than pea in Finland, which also establishes potential for growth in Norway (Pulkkinen et al., 2018). *Vicia faba* can tolerate lower temperatures than other grain legumes (Maalouf et al., 2018). The Nordic countries have a great potential to expand faba bean cultivation as it would aid protein crop self-sufficiency instead of soybean import (Waalen et al., 2019; Peltonen-Sainio et al., 2013; Koivunen et al., 2016). Common names of *Vicia faba* include horse bean, broad bean, field bean, tic bean, faba bean and fava bean (Jensen et al., 2010; Warsame et al., 2018, Landry, 2014; Multari et al., 2015). Literature generally refer to *Vicia faba* as faba bean and will be used in this thesis (Sellami et al., 2019; Etemadi et al., 2019; Maalouf et al., 2018).

2.2 Socioeconomic benefits

The faba bean crop is among the oldest in the world and traces back to Neolithic times. The oldest faba bean seed was found in Syria and dates back to 10 000 BC (Jensen et al., 2010; Tanno and Willcox, 2006; Singh et al., 2013). Its exact origin is unknown, but it is believed to originate from South East Asia (Multari et al., 2015; Singh et al., 2013). Now, grain legumes are accountable for one third of the world’s plant protein supply, traditionally as dry grains in developing countries (Warsame et al., 2018). *Vicia faba* is an important grain legume crop because of its high nutrient content and yield (Multari et al., 2015; Maalouf et al., 2018). Faba beans are a staple food in many countries

(Alhajj Ali et al., 2018). In the world, *Vicia faba* is the fourth most widespread cool season legume crop after pea, chickpea and lentil (FAOSTAT, 2018). China is the leading faba bean grower worldwide, followed by Ethiopia, Australia and the United Kingdom (Warsame et al., 2018). Additionally, faba beans are widely cultivated in India, around the Mediterranean and the Middle East (Maalouf et al., 2018; Singh et al., 2013; Alhajj Ali et al., 2018). Faba bean is the third most important legume crop in India after soybean (*Glycine max)* and pea (*Pisum sativum)* (Singh et al., 2013).

In addition to food, faba bean has extensively been used as a protein source for animal feeds (Pulkkinen et al., 2015; Jensen et al, 2010; Perez-Maldonado et al., 1999; Peltonen-Sainio et al., 2013). In higher income areas of the world, meat is the primary source of protein. However, recent research suggests high intakes of red and processed meat to be unhealthy. For culinary purposes the seeds are most desirable, but the pods can additionally be used for animal feed (Multari et al., 2015). Faba been seeds are high in protein and a reliable animal protein substitute (Crépon et al., 2010; Multari et al., 2015). Nevertheless, faba bean as a protein source may be considered inferior to animal sources because the amino acid levels may be inadequate (Multari et al., 2015). Legumes are generally lower in sulfur containing amino acids (FAO/WHO/UNU, 2002). Possible products with faba beans for developed countries are protein rich snacks, flour in pasta or bread, protein isolate and oil. The seeds have gastronomic applications as hulls, grains, cooked mature or raw immature (Warsame et al., 2018; Vasilean et al., 2018; Multari et al., 2015). Previous studies have produced protein isolates of faba beans (Vioque et al., 2012). Dietary supplies with phytochemicals extracted from deseeded pods is also possible (Multari et al., 2015). Possible issues using plants as a protein source is limitations in functional properties and consumer flavor acceptance (Lam et al., 2018). Functional processing properties depend on physiochemical composition and structural protein types (Shevkani et al., 2019). Further, faba bean cultivation can contribute to sustainability and economic importance. Production of faba beans may target some of the UN Sustainable Development goals 2030 such as goal 2: zero hunger, goal 13: climate action and goal 15: Life on land (UN, 2019). Faba beans are a cost-effective crop, partially due to their characteristic biological nitrogen fixation. If used as a break crop, the residual nitrogen in the soil can be used by the following crop (Köpke and Nemecek, 2010; Landry, 2014).

2.3 Suitable climate and growing conditions

Vicia faba is a partially allogamous crop, both self-pollination and cross-pollination can occur (Meitzel et al., 2011; Maalouf et al., 2019). Faba bean pollinators are bees and other insects (Suso et al., 2016; Purves et al., 2018). The crop is indeterminant where flowers develop continuously over a period of time (Lake et al., 2019). The flowers of *Vicia faba* may be white or colored (Abrahamsen et al., 2018). Faba bean is a versatile, cold tolerant crop commonly grown in temperate and subtropical areas (Peltonen-Sainio et al., 2013; Warsame et al., 2018; Multari et al., 2015). Faba bean is a long-day plant and requires a cool season (Jensen et al., 2010). There are spring varieties and winter varieties of faba beans. Spring varieties are suitable for Nordic climate but depend largely on early summer precipitation (Jensen et al., 2010). In Norway the suitable geographic region is the southeast (Abrahamsen et al., 2019). *Vicia faba* is a late maturing crop in the Nordic countries and generally requires long growing season of more than four months. For this reason, early maturing cultivars are preferred for growth in Norway (Abrahamsen et al., 2018; Jensen et al., 2010). Optimal growth temperatures are 17 to 27 °C. Winter cultivars can tolerate -10°C and of -15°C (Jensen et al., 2010; Landry, 2014)

Faba beans are sensitive to drought (Farooq et al., 2017; Maalouf et al., 2018). Water availability is most important during the reproductive and seed filling phase (Farooq et al., 2017; Parvin et al., 2019). When well-irrigated the seed filling rate is higher which result in larger seeds (Ghassemi-Golezani and Hosseinzadeh-Mahootchy, 2009). Parvin and coworkers (2019) observed increased faba bean yield when exposed to elevated CO_2 concentrations (550 ppm) during growth in a Free-Air CO_2 Enrichment (FACE) facility. In their experiment, elevated CO_2 resulted in more efficient use of soil water content which may be significant for drought prone areas such as Mediterranean regions (Parvin et al., 2019).

2.3.1 Biological nitrogen fixation (BNF)

There is currently a terrestrial surplus of nitrogen (N). Old agricultural practices included manure and plant residues to obtain fertile soils, but ever since the green revolution and synthetic fertilizers became available the amount of terrestrial N has increased dramatically (Butler et al., 2016). Legumes have the agricultural advantage of biologically fixing atmospheric nitrogen, which contributes to improved soil fertility (Elsheikh and Elzidany, 1996; Parvin et al., 2019; Jensen et al., 2010; Köpke and Nemecek, 2010). Faba beans can fix the same amount of N as pea and soy combined (Warsame et al., 2018). In faba beans, up to 96% of total N fixed can derive from the atmosphere (Köpke and Nemecek, 2010). Faba beans can fix 180 kg N/ha by BNF in 4t/ha field with plants containing 4.5% nitrogen (Köpke and Nemecek, 2010). N-fixation up to 648 kg N/ha has been recorded in faba bean (Sprent et al., 1977). Atmospheric fixation capability depends largely on the presence of nitrogen-fixing *Rhizobia* in the soil, with which faba beans and other legumes form a symbiotic relationship (Jensen et al., 2010; Köpke and Nemecek, 2010; Agrios, 2005). The bacteria must trap atmospheric nitrogen and transform it to a functional organic form (Agrios, 2005). In contrast to other legumes, faba beans can fix atmospheric nitrogen even though there is a large amount of N in the soil, hence reducing utilization of preexisting nitrogen from fertilizers. However, startup N application may be beneficial in initial growth stages (Köpke and Nemecek, 2010).

Seeds can also be inoculated with *Rhizobium* bacteria before sowing to achieve more effective N-fixation (Jensen et al., 2010; Köpke and Nemecek, 2010). *Rhizobia* are gram negative, unflagellated, rod-shaped bacteria. In most arable soils, native strains of *Rhizobia* are commonly present (Köpke and Nemecek, 2010). Some strains are more effective than others on particular legumes. This is genetically determined by host specific nodulation genes (Agrios, 2005). Although not strictly required, faba bean seeds can be pre-inoculated with *Rhizobium leguminosarium viceae* before sowing (Etamadi et al., 2018; Belhadi et al., 2018; Köpke and Nemecek, 2010). The bacteria produce nodules on the root hairs to fix nitrogen (Singh et al., 2013). Nodulation occurs after bacterial penetration of root hairs on taproots and lateral roots. Nodulation is induced by bacterial *nod* genes in response to the release of flavonoids from the roots. Nod factors trigger faba bean host genes which activates root hair curling (Agrios, 2005). Other soil bacteria that can induce nodulation are *Brayrizobium, Frankia* and *Azorhrizobium* (Köpke and Nemecek, 2010; Agrios, 2005). Faba beans have a high phosphorus requirement because of ATP used during nodulation (Köpke and Nemecek, 2010).

2.3.2 Cropping systems

Faba beans can be used in different types of cropping systems (Jensen et al., 2010). Monoculture is extensively distributed around the world and impacts soil quality as it requires addition of fertilizers (Karkanis et al., 2018; Alhajj Ali et al., 2018). Crop rotation and intercropping are the main legume cropping systems (Karkanis et al., 2018). Crop rotation is when faba beans are grown as a break crop in the same field of another crop species, but in separate growing seasons. Intercropping is simultaneous growth of more than one crop in the same site during a growing season (Jensen et al., 2010; Köpke and Nemecek, 2010). Intercropping is the same as breaking monoculture and leads to better agroecosystems (Peltonen-Sainio et al., 2013). Like other legumes, *Vicia faba* can be used as a break crop with cereals and may limit the need of fertilizer application due to BNF (Karkanis et al., 2018; Alhajj Ali et al., 2018). In Norway and Finland cereals dominate arable land in which using faba bean as a break crop could be useful (Peltonen-Sainio et al., 2013; Waalen et al., 2019; Abrahamsen, 2018). Increased wheat yield was observed by Abrahamsen (2018) when a faba bean break crop was used. Furthermore, incorporating legume plant remains in the soil may increase the yield of following crops in the same field (Jensen et al., 2010). Cropping systems of cereals and faba beans could be a means to moderate soil quality decline. Crop rotation with faba bean can reduce soil erosion and nitrogen requirement. Faba beans are commonly grown in one out of three or four years in crop rotation regimes with wheat (Landry, 2014). Furthermore, using faba beans in cropping systems can prevent biotic stress factors (Köpke and Nemecek, 2010). Less pathogens have been recorded in the wheat crop the following year when using faba beans as a break crop (Waalen et al., 2019). Moreover, as observed by Patriquin and colleagues (1988), aphid (*Aphid fabae*) infestation was reduced in oat and barley when intercropped with faba beans.

2.4 Nutrient composition

A diet with more plant-based proteins has demonstrated lower phosphorus content in the urine excretion of human subjects with chronic kidney disease (Moorthi et al., 2014). Consumption of plant-based protein can be beneficial because of associated plant bioactive chemical profile to improve the gut metabolite flora. Faba beans contain nutritional compounds in many chemical families; carbohydrates (starch, sugars, dietary fibers), proteins and amino acids, polyphenols (flavonoids, tannins), alkaloids, terpenoids, jasmonates, organic acids, nucleosides/nucleotides (Abu-Reidah et al., 2014; Duc et al., 1999; El-Feky et al., 2018). Faba beans are low in sodium, free of cholesterol and low in fat (Adamu et al., 2015; Singh et al., 2014; Shevkani et al., 2019). The major seed nutritional constituents are starch, protein and fiber (Duc et al., 1999). Starch and proteins are the major storage compounds in faba bean seeds (Heim et al., 1993). Starch and protein content may vary with cultivar as contents were lower on cross pollinated embryos as demonstrated by Meiztler and colleagues (2011). Polyphenols in faba beans are primarily tannins, which can limit the availability of certain amino acids, making them generally undesirable. However, polyphenols have a good antioxidant capacity (Crépon et al., 2010 El-Feky et al., 2018). The *Vicia faba* plant may have therapeutic applications as it is a good source of levodopa (L-dopa), a precursor of dopamine which can be used to treat Parkinson’s disease. Levodopa is not evenly distributed in all tissues. Cooking may decrease levodopa content (El-Feky et al., 2018; Singh et al., 2013). Faba beans are a good source of mineral nutrients such as Cu, P, Mg, K, Ca, Zn, Fe and Mn (Singh et al., 2014; Karkanis et al., 2018; Etamadi et al., 2018).

2.4.1 Carbohydrates

Faba bean seeds are good sources of dietary fiber and starch (Ivarsson and Neil, 2018; Warsame et al., 2018; Multari et al., 2015). Starch concentrations are in the range 39-42% (dry weight) and is the dominant constituent of faba bean seeds followed by crude protein (Crépon et al., 2010; Duc et al., 1999; Ivarsson and Neil, 2018). Starch grains are located in chloroplast-derived plastids of the embryo (Viola et al., 1991). Zdunczyk and coworkers (2018) found a negative correlation between tannin content and starch content when investigating low-tannin cultivars against high-tannin cultivars. Faba bean cv. Vertigo has been previously analyzed to have a starch content of 39.4g/100g (Ivarsson and Neil, 2018).

2.4.2 Proteins

Faba beans accumulate a large proportion of proteins during seed development, up to 25-35% in dry seeds (Multari et al., 2015; Warsame et al., 2015; Karkanis et al., 2018; Parvin et al., 2019). In general, faba beans have a higher protein concentration than peas (Pulkkinen et al., 2018). The variation in protein concentration may be a result of cultivar, growing season and fertilizer (Meiztler et al., 2011; Multari et al., 2015; El Fiel et al., 2002). Protein analysis can be done by analyzing total nitrogen by Kjeldahl (Jung et al., 2003; Etamadi et al., 2018; Ivarsson and Neil, 2018) or Dumas method (aka nitrogen combustion method) (Jung et al., 2003; Rubio et al., 2014; Schwediauer et al., 2018). The Dumas method may be preferred because it omits the use of toxic compounds such as mercury and cadmium utilized in the Kjeldahl method (Müller, 2017). The protein content can be calculated from nitrogen % (approx. 16% of the protein molecule) by the universal factor 6.25 (Etamadi et al., 2018; Ivarsson and Neil, 2018). Previously analyses of Vertigo demonstrated a protein concentration of 30g/100g (Ivarsson and Neil, 2018).

2.4.3 Major proteins: globulin and albumin

In most food legumes, including faba beans, the main seed proteins are globulin and albumin (Lam et al., 2018; Shevkani et al., 2018). Albumin has the most nutritive amino acid composition. The major storage protein in faba beans is globulin which comprises 69-80% of the total protein content (Shevkani et al., 2018: El Fiel et al., 2002; Lam et al., 2018). Globulin is soluble in salt solution and consists of vicilin, convicilin and legumin (Lam et al., 2018). Albumin (5-80kDa) is water-soluble (Lam et al., 2018). Albumin may contain some antinutrients such as trypsin and amylase inhibitors (Shevkani et al., 2019). The most abundant faba bean proteins in order from high to low proportion are vicilin, legumin, albumin, prolamin and glutelins (Nikokyris and Kandylis, 1997). Legumin (300-400 kDa) and vicilin (150-180 kDa) are high-molecular weight proteins. Convicilin has a smaller molecular mass (70 kDa) and form trimers (Lam et al., 2018; Multari et al., 2015; Vioque et al., 2012). Globulin is synthesized in the endoplasmic reticulum (Warsame et al, 2018). During seed development vicilin is formed before legumin, but legumin is predominant in mature seeds. The ratio of [legumin]:[vicilin] will affect the protein functionality of products. Vicilin is held together by hydrophobic interactions and legumin is hexameric and held together by covalent disulfide bonds (Warsame et al., 2018; Lam et al., 2018).

2.4.4 Amino acid profile

Protein quality of faba bean is largely defined by the content of sulfur containing amino acids (Warsame et al., 2015). Faba beans are lysine-rich and the amino acid composition is considered to be good, however faba beans have low levels of tryptophan and the sulfur-containing amino acids cystine and methionine (Singh et al., 2013; Multari et al., 2015; Vioque et al., 2012). Hence, it might be beneficial to include cereals or other plant-based protein sources in the diet (Vioque et al., 2012; Lam et al., 2018). Vicilin (globulin) has low levels of methionine, cysteine and tryptophan, but higher levels of arginine, lysine, glutamic acid and aspartic acid (Lam et al., 2018). Albumins have higher proportions of cysteine and methionine than in globulins (Shevkani et al., 2018; Lam et al., 2018; Vioque et al., 2012). Prolamins have higher amounts of glutamic acid, leucine and proline (Multari et al., 2015).

2.5 Antinutrients

Antinutrients are compounds that result in nutritional constraints. Antinutrients inhibit digestion when binding to proteins and in turn limit their breakdown by protease (Warsame et al., 2018). The most limiting antinutrients in food legumes, widespread in faba bean and other *Vicia* sp. are tannins and the pyrimidine glycosides vicine and convicin (Pulkkinen et al., 2018; Warsame et al., 2018; Abu-Reidah et al., 2014). The issue with glycosides arises when they are hydrolyzed in the digestive tract producing aglycones. This causes oxidative stress in blood cells with G6PD (glucose-6-phosphate dehydrogenase) deficiency. The effect of this deficiency is favism (haemolytic anemia) (Rizzello et al., 2016). Additional antinutrients of faba beans are phytic acid, saponins, alpha-galacturosidase, protease inhibitors, lectins, alkaloids, trypsin inhibitors (Vioque et al 2012; Crépon et al., 2010; Warsame et al., 2018; Multari et al., 2015). Phytic acid can form stable compounds with proteins and limits proteolytic digestion (Rosa-Sibakov et al., 2018). Condensed tannins (proanthocyanidins) may affect digestibility (Warsame et al., 2018). There is conflicting evidence whether tannins, phytic acid, alkaloids and saponins are beneficial or not. Cultivars with lower levels of these phytochemicals may result in altered defense mechanisms against pathogens (Multari et al., 2015).

To reduce the risk of favism, breeding programs can produce cultivars with lower levels of vicine and convicine. Such efforts have been made to by targeting the *VC* locus (Vioque et al., 2012; Pulkkinen et al., 2018; Warsame et al., 2018). Processing techniques can also reduce tannins, vicine and convicine (Warsame et al., 2018). Germination may reduce antinutrients (Schwediauer et al., 2017). Processing, such as cooking, heating and radiation, can limit the amount of some antinutrients drastically. Protein isolates is a processing method that can improve the protein quality by antinutrient extraction and may have higher theoretical biological value (Vioque et al., 2010).

2.6 Seed development

Vicia faba is an annual grain legume and consists of green pods which typically has 3-6 seeds (Singh et al., 2013). Mature seed weight is usually between 0.3-0.8 g (Crépon et., 2010). Angiosperms have double fertilization. The fertilization of the egg leads to embryo development and the fertilization of the central cell leads to endosperm formation (Bushell et al., 2003). Compared with other legumes, faba beans have slow developing embryos reaching heart shape 12 days after anthesis (DAA) and early cotyledon stage 16 DAA as observed by Slater and colleagues (2013). Phytohormones (auxin, cytokinin and abscisic acid) may influence the rate of embryogenesis and mature seed size (Slater et al., 2013; Meitzel et al., 2011). The embryo is developed into a storage organ by a signaling network of sugars and hormones that regulate storage activity and seed maturation. Early cell proliferation may enhance storage capacity. Plastids are the major location for amino acid and starch biosynthesis (Meitzel et al., 2011).

2.6.1 Accumulation of storage compounds during seed development

Sucrose is the photoassimilate and the main substrate for the major storage products (Heim et al., 1993). As demonstrated by Heim and colleagues (1993), sucrose increased between 10-15 days after fertilization. In early seed development invertases are expressed in the seed coat parenchyma, the site of photoassimilate unloading (Weber et al., 1996). Sucrose is cleaved by invertases at the seed coat unloading area in early development (Heim et al., 1993; Weber et al., 1996). Starch is mainly synthesized from sucrose (Weber et al., 1995; Heim et al., 1993). In peas, sucrose supply to the embryo leads to formation of starch granules in amyloplasts (Smith and Denyer, 1992). Sucrose synthase assimilates sucrose from leaves and pods and directs it to the embryo (Weber et al., 1995; Heim et al., 1993). Fructose and glucose levels are high during the early stages of development (Heim et al., 1993). Sucrose synthase catalyzes the conversion of sucrose to hexoses (glucose and fructose) which provides substrate for starch synthesis (Angeles-Nunez and Tiessen, 2010). Starch accumulates as a result of ADP-glucose pyrophosphorylase (AGPase) and starch synthase activity (Weber et al., 1995). Starch synthase is derived from glucose-1-phosphate (Glc1P) and ADP-glucose. AGPase catalyzes starch synthase formation from Glc1P and ADP-glucose (Weber et al., 1995). Sucrose synthase activity in the seed coat is low in early seed development. In later stages of seed development higher activity of sucrose synthase in the seed coat leads to starch increase simultaneously with sucrose decrease, as demonstrated by Heim and colleagues (1993). During seed development, seed coats are early sucrose sinks and later, during the storage phase, the cotyledons are the dominating sinks (Heim et al., 1993; Weber et al., 2005). Storage activity and maturation starts when the [sucrose]:[hexose] ratio in the embryo is high. Sucrose feeding to the dividing cotyledon cells leads to appearance of storage proteins and starch grains (Weber et al., 1996; Weber et al., 2005). In grain legumes the endosperm is degraded, and the cotyledon parenchyma is the final storage organ (Patrick and Offler, 2001). Starch and protein accumulation lead to cell elongation in later developmental stages (Heim et al., 1993). Proteins synthesized during early seed development are ultimately degraded as the embryo grows, whereas proteins synthesized after the heart stage form storage protein bodies in the cotyledon (Warsame et al., 2018).

2.7 Project aims

In Norway there has been an increasing import of plant-based products with high protein content. However, there is a potential for certain protein crops in Norway which can contribute to economic benefits for Norwegian agriculture and food industry. Faba beans could be a good option. If used for food, a high protein content will undoubtedly be fundamental. However, faba beans may be late maturing in Norwegian climate due to low temperatures. There is currently limited information of faba bean growth and use for food in Norway. As there are no national breeding programs, cultivars must be obtained from abroad. Finnish early cultivars have been on the market for a while, but the availability of seeds is inconstant. Late cultivars have also been grown in Norway, such as Vertigo, which is usually high yielding. However slow maturation and low growing temperatures can delay the maturation process and lead to unfavorable harvesting conditions. To find the optimal agricultural practices there is need for better understanding of seed development and maturation. The purpose of this thesis was to investigate seed development and maturation of faba beans grown in Norway, with emphasis on protein and starch accumulation. The objectives of this thesis are as follows:

3. OBJECTIVES

1. Explain faba bean seed development in terms of moisture content, fresh weigh and dry weight
2. Describe protein accumulation during seed development of faba beans grown in Norway
3. Describe starch accumulation during seed development and maturation grown in Norway
4. Literature study of faba bean

4. MATERIALS AND METHODS

4.1 Plant material

For this thesis, seeds of *Vicia faba* L. cv. Vertigo were harvested from a field experiment conducted at the Norwegian University of Life Sciences, Vollebekk Research Farm (Ås, Norway) in the summer of 2019. The field experiment was part of the project FoodProFuture and aimed to investigate effects of *Rhizobium leguminosarium* seed inoculation prior sowing combined with different startup fertilizer regimes. Pre-inoculation with *Rhizobium* and startup fertilizer were used to facilitate early plant development for growth in lower soil temperatures, typical for Norwegian climate. *Vicia faba* L. cv. Vertigo seeds (origin Germany) were bought from Felleskjøpet (Norway). Vertigo is a medium sized seed, low-tannin cultivar with colored flowers (Ivarsson and Neil, 2018). In Canada Vertigo is considered a medium early cultivar with a typical height of 100 cm (Stamp Seeds, 2018). In Norway Vertigo is defined as a late maturing cultivar (Abrahamsen et al., 2018). Seeds were sowed on April 25, 2019 with sowing depth of 5-7 cm in 1.65 x 8.0 m (13.2 m_2) plots. Total field size was 1188 m_2 including border plots. Harvest was done between July 24 and September 4.

4.2 Experimental design

The field experimental was split plot with two factors with *Rhizobium* inoculation on the main plots and startup fertilizer regimes on the sub-plots. The factor combinations were randomized within the total six replicates for each inoculation factor (with or without *Rhizobium* pre-inoculation). Four of these were used for yield determinations after combiner harvest at maturity. For this experiment two replicates were used for harvesting of pods during the seed development and maturation period. For studies of seed development in this master thesis, three treatment combinations of *Rhizobium* seed pre-inoculation and start fertilizer was selected. Treatments were A1: no treatment; A2: 23.1 kg/daa 22-3-10 NPK fertilizer, uninoculated seeds and B1: pre-inoculated seeds, no fertilizer (Table 1). These treatments were selected because they were expected to have the most significant effects on seed development and the final chemical composition (pers. comm. Anne Kjersti Uhlen, November 26, 2019). No irrigation regime was used in the field. Harvest was done at 7 different harvest dates (hereafter referred to as HD) with one-week intervals between July 24 and September 4 in 2019 (Table 2) to investigate seed development changes over time. Pods were harvested from 3 different plots with 2 replications for each HD (Table 1). An untouched 0.5 x 0.5 m plot section was sampled from at each HD. The bottom pods of 20 randomly selected plants were sampled. The bottom pod was developed from the lowest pod-containing node. The lowermost of these was selected if more than one pod was developed at this node. A total number of 840 pods were harvested during the sampling period (20 pods x 7 HDs x 3 treatments x 2 replicates).

Table 1. Plots numbers, treatment code and treatment descriptions, Y=yes, - = none

Replicate	1			2		
Plot number	102	105	109	403	410	414
Treatment code	A1	A2	B1	B1	A1	A2
Fertilizer 23.1 kg/daa 22-3-10 NPK	-	Y	-	-	-	Y
Rhizobium pre-inoculation	-	-	Y	Y	-	-

Table 2. HD1 to HD7 and corresponding dates, 2019

HD1	HD2	HD3	HD4	HD5	HD6	HD7
24 July	31 July	7 Aug.	14 Aug.	21 Aug.	28 Aug.	4 Sept.

4.3 Moisture content, dry weight and fresh weight analysis

Immediately after harvest seeds were separated from a subsample of 12 pods, counted and used to determine fresh weight (FW). Thereafter, fresh seeds were dried at 60°C for 48 hours for calculations of dry weight (DW) and moisture content (%).

4.4 Freeze drying and milling

Fresh seeds of a subsample of the 8 remaining pods were stored at -80°C before freeze drying to complete dryness in a Gamma 1-16 LSCplus for 4 days (Martin Christ Freeze Dryers GmbH, Germany). Freeze dried material was thawed then milled to 0.5 mm particle size with a Retsch ZM 100 rotor mill (Retsch GmbH, Germany).

4.5 Quantification of protein, starch and water-soluble carbohydrates

Starch and protein were quantified in g/100g freeze dried material, and thus equivalent to g/100g on dry weight basis. Total protein (nitrogen % x 6.25) was determined by the Dumas method (Jung et al., 2003; Rubio et al., 2014). Total starch was analyzed after glucose removal by Total Starch Assay Procedure K-TSTA (Megazyme, Ireland) according to AOAC procedure 996.11 (Coda et al., 2016; Zdunczyk et al., 2018). Water-soluble carbohydrates were quantified for the A1 treatment samples by D-fructose/D-glucose Assay Procedure K-FRUGL (Megazyme, Ireland).

4.6 Temperature, heat sum, precipitation and flowering

Weather data as mean daily temperatures and precipitation was downloaded from NIBIO (https://lmt.nibio.no/). Heat sums were calculated from the sum of diurnal mean temperatures, using 0°C as base temperature. Beginning of first visible flowering was recorded plot wise. Days from first unfolded flower (DAF) and to the different HDs were also calculated. The average first flowering date of June 20 was used in the calculations.

4.7 Statistical analysis

A balanced analysis of variance (ANOVA) was performed using Minitab 19 (Minitab Ltd., United Kingdom). The fixed variables were HD and treatment, and random variables were replicates. A significance level of $p<0.05$ was used as criterion. All data were averaged across all plots for each HD.

5. RESULTS AND DISCUSSION

5.1 Flowering, temperature, heat sum and precipitation

The date for the first visible flower varied between plots from June 17 (plot 414) to June 20 (plots 109, 403, 410) and to June 24 (plots 102, 105). The mean value was June 21. June 20 was used for calculations of DAF and day degrees. End of flowering was recorded at July 15 for all plots. The average temperature was 14.5°C from sowing to last harvest (Table A2). The highest mean temperature was 24.1°C on July 27 and lowest mean temperature was 2.6°C on May 6 (Table A2). From sowing to last harvest, the total precipitation in the field was 362.4 mm. There was a lot of precipitation towards the end of harvest. At HD1, HD2 and HD3 the precipitation was miniscule. For the remainder of the sampling period the precipitation was substantial and more frequent (Fig. 1). The temperature was high during the week prior to HD2 (Fig. 1; Table A2), giving the highest heat sum (151°d) of all the subsequent HDs. Between HD2 and HD3 the temperature dropped and varied around 15ºC for the rest of the sampling period (Fig. 1). During the entire growing period the crop was subject to a heat sum of 1909°d from April 25 to September 3 (Table 3). Before the HD1 the total heat sum of the field was 1200°d. Relative humidity (RH) was high during the sampling period (Table A2).

Table 3. HDs and corresponding dates, days after flowering June 20 (DAF), seed moisture content and heat sums.

HD	HD1	HD2	HD3	HD4	HD5	HD6	HD7
Date, summer 2019	24 July	31 July	7 Aug.	14 Aug.	21 Aug.	28 Aug.	4 Sept.
DAF	33	40	47	54	61	68	75
Seed moisture content %	82.87	75.34	66.22	58.95	55.42	46.05	32.07
Heat sum after June 20	530	681	797	913	1014	1131	1239
Heat sum from sowing	1200	1351	1467	1582	1684	1800	1909

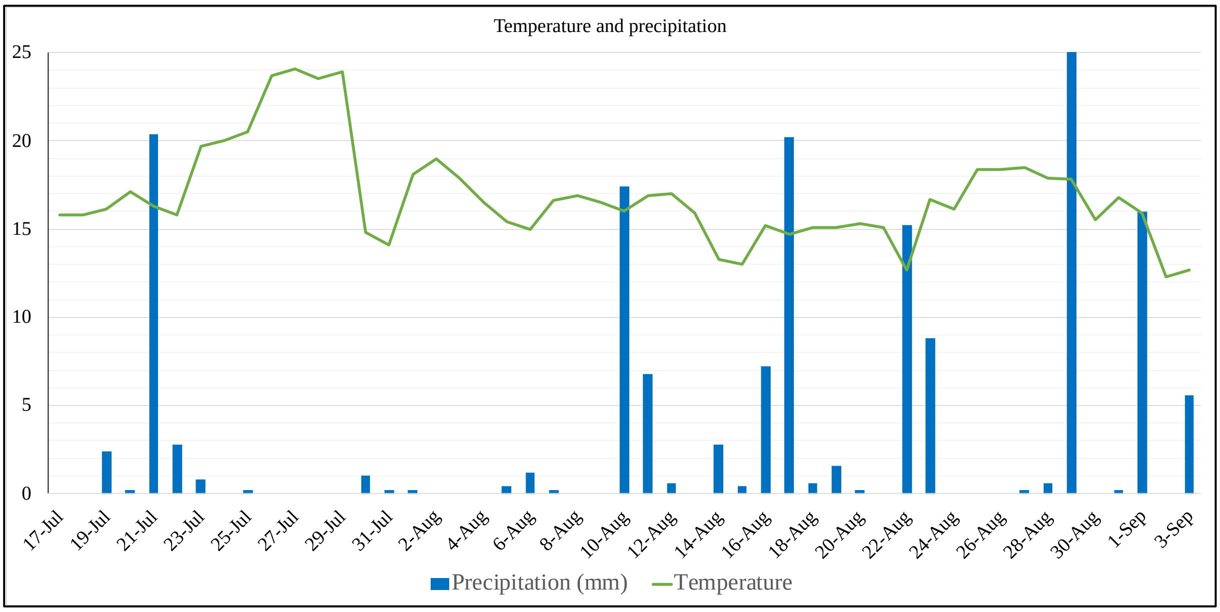

Figure 1. Precipitation (mm) and mean daily temperatures (°C) from July 17 (the week prior to HD1) to September 3 (the day before HD7) in Ås, Norway 2019.

5.2 Analysis of variance of seed moisture content, dry weight and fresh weight.

The *Rhizobium* inoculation/start fertilizer treatment had a significant effect on moisture content during seed development (Table 4), but not on DW and FW. Stage of seed development (HD) had a significant effect for moisture content, DW and FW. The interaction between 'HD' and 'treatment' was non-significant for moisture content, DW and FW.

Table 4. Analysis of variance (ANOVA) of moisture content, DW/seed and FW/seed.

	HD[1]	Treatment[2]	HD xTreatment[3]
Moisture content, %	0.000**	0.021*	0.356
DW/seed	0.000**	0.508	0.841
FW/seed	0.000**	0.721	0.635
df	6	2	12

*significant at p<0.05, **significant at p<0.01. [1]Stage of seed development according to the 7 different HDs of July 24 to September 4. [2]Fertilizer/no fertilizer and/or pre-inoculation with *Rhizobium*. [3]Interaction between HD and treatment.

5.3 Analysis of variance of starch and protein

Seed development stage had a strong, significant impact on protein and starch content, both when expressed as mg/seed and g/100 g (Table 5). 'Treatment' and interaction between 'HD' and 'treatment' did not have a significant effect in either starch or protein.

Table 5. Analysis of variance (ANOVA) for protein and starch content in DW.

Dry matter	Quantity	HD[1]	Treatment[2]	HD x Treatment[3]
Protein	mg/seed	0.000**	0.345	0.784
	g/100 g	0.000**	6.641	0.946
Starch	mg/seed	0.000**	0.293	0.293
	g/100 g	0.000**	0.153	0.469
df		6	2	12

*significant at p<0.05, **significant at p<0.01. [1]Stage of seed development according to the 7 different HDs of July 24 to September 4. [2]Fertilizer/no fertilizer and/or pre-inoculation with *Rhizobium*. [3]Interaction between HD and treatment.

5.4 The effect of fertilizer and *Rhizobium* treatments

There was no significant effect of treatment type i.e. application of fertilizer and *Rhizobium* inoculation for protein, starch, DW and FW (Table 4 and Table 5). These results suggested treatment type had little or no impact. Pre-inoculation with *Rhizobium* may be needed, but in this case, it is likely N-fixing bacteria already existed in the soil. In terms of protein and starch accumulation during seed development, pre-inoculation did not seem to have a significant effect. 'HD x treatment' was overall not significant and was not investigated further. 'Treatment' had a significant effect on moisture content during seed development (Table 4). Treatment A2 (no *Rhizobium* pre-inoculation, NPK 22-3-10) resulted in seeds with slightly lower moisture content after HD5 (Fig. 2). This suggest untreated seeds or seeds with *Rhizobium* pre-inoculation resulted in a slightly higher moisture content.

However, it cannot be concluded whether or not this was a result of random variation. Interestingly, Elsheikh and Elzidany (1996) discovered that inoculation with *Rhizobium* significantly increased moisture content, however, the stage of seed development was not stated in their analysis.

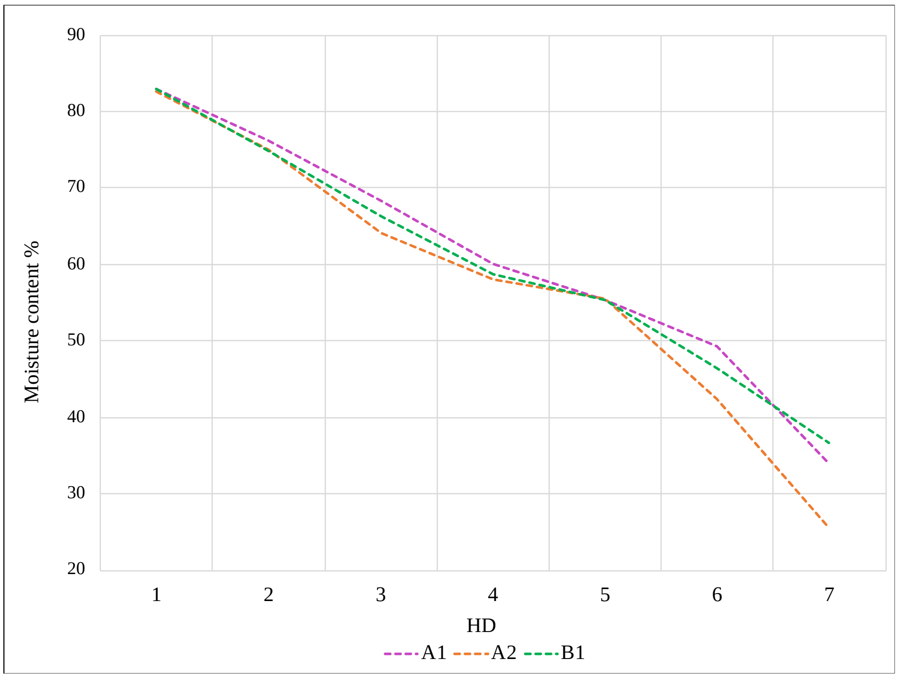

Figure 2. Moisture content (%) of treatments from HD1 to HD7. A1: no treatment; A2: 23.1 kg/daa 22-3-10 NPK fertilizer, uninoculated seeds and B1: pre-inoculated seeds, no fertilizer.

5.5 Moisture content, dry weight and fresh weight during seed development

FW increased until HD4, leveled off between HD4 and HD5, and started to decline somewhere between HD5 and HD6 (Fig. 3). FW continued to decline until HD7. Borisjuk and colleagues (1995) found similar results of FW in cv. Fribo with a rapid increase until HD6. Yet, their study did not present FW data past this time and FW decline. DW increased until HD6, but then seemed to stabilize at some point between HD6 and HD7. DW increased at the highest rate from HD1 to HD3. Moisture content decreased evenly from HD1 to HD7. Regression analysis of moisture content from HD1 to HD7 was significant (R_2=98.33%). A minor moisture content drop was observed at HD5 (Fig. 2). Moisture content would be expected to have a clearer breaking point as the accumulation of storage compounds stopped and desiccation started. FW started to decline sometime between HD5 and HD6 (Fig. 3), nonetheless, the lack of prominent seed desiccation, in terms of lowered moisture content, is considered to be a result of field weather conditions. There was heavy precipitation in the field from HD4 to HD7 (Fig. 1). There was high relative humidity (RH) in the later HDs due to the heavy rainfall in HD4 to HD7 (Table A2). High RH may also have contributed to delayed seed desiccation.

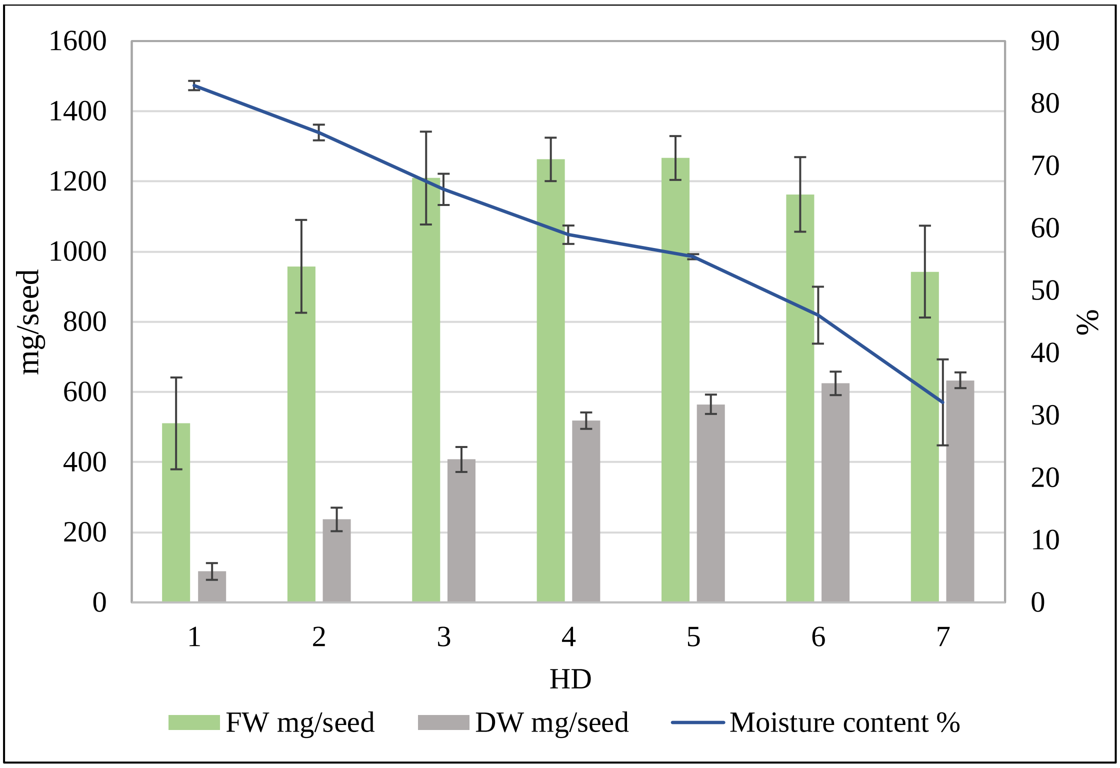

Figure 3. Moisture content (%), DW (dry weight) mg/seed and FW (fresh weight) mg/seed with standard deviation (mean ± *s*) bars from HD1 to HD7.

5.6 Changes in starch and protein concentrations during seed devevelopment

The highest starch concentration was at HD5 (40.5%) and the lowest at HD1 (12.4%). Protein concentration peaked at HD1 (33.4%) and was the lowest at HD5 (27.5%) (Fig. 6 and Table A1). The peak starch and protein concentrations observed in this thesis correspond to results by Ivarsson and Neil (2018) of cv. Vertigo. The concentration of protein was higher than that of starch until some point between HD2 and HD3. Thereafter, the accumulation (%) of starch was higher than for protein. Somewhere between HD1 and HD2 the protein concentration dropped and after HD3 the protein concentration stabilized. Starch concentration (g/100g) was at its lowest level in the beginning of seed development but increased rapidly from HD1 to HD4. After HD4, starch concentration leveled off at around 40% (Fig. 4). Both starch and protein concentration had stabilized after HD4 (Fig. 6). Starch accumulation compared to water-soluble carbohydrates were as expected. During the sampling period the concentration of water-soluble carbohydrates declined (Fig. A1).

5.7 Starch and protein accumulation in mg/seed during seed development

Starch mg/seed peaked at HD7 (254.1 mg) and was lowest at HD1 (10.9 mg) (Table A1). Protein per seed peaked at HD6 (178.5 mg) and was lowest in HD1 (29.4 mg). The amounts of protein and starch mg/seed are shown in Figure 7. Both starch and protein (mg/seed) accumulation increased rapidly from HD1 to HD6 (Fig. 7). Protein (mg/seed) was higher than starch (mg/seed) until somewhere between HD2 and HD3. Somewhere between HD2 and HD3 starch mg/seed began to accumulate at a slightly higher rate than protein mg/seed. Accumulation of starch and protein mg/seed leveled off after HD6 (Fig. 7).

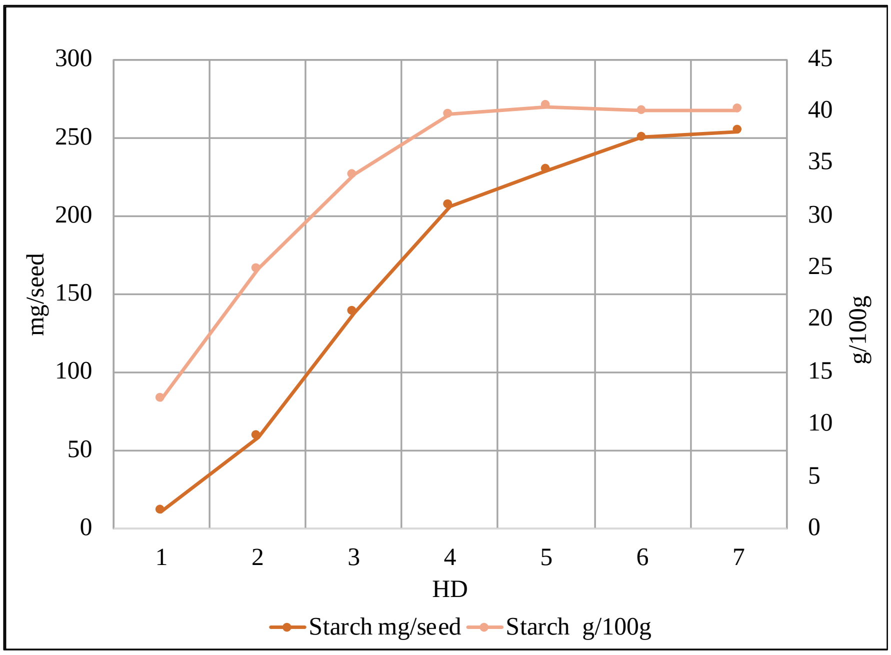

Figure 4. Starch content in g/100g and mg/seed at sampling times HD1 to HD7.

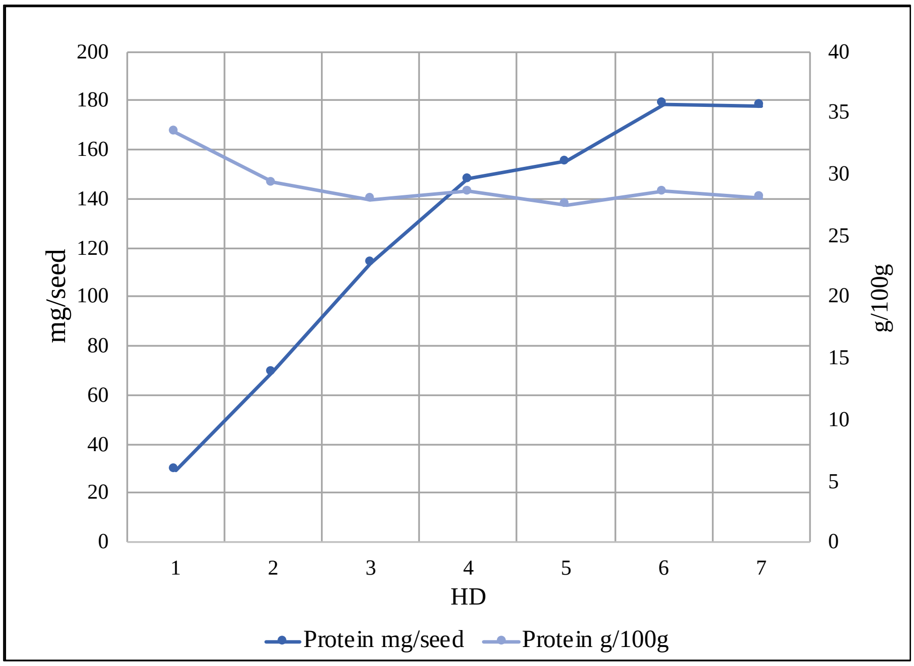

Figure 5. Protein content in g/100g and mg/seed at sampling times HD1 to HD7.

5.8 Data transformation and accumulation patterns

The total change in starch was higher than total change in protein content in both concentration and mg/seed (Fig.4 and 5, Table A1), but the overall trend was greater changes in mg protein/seed and mg starch/seed. Quantification in mg/seed shows prominent starch increase (x 23) from HD1 to HD7 whereas changes in starch concentration (g/100g) from its lowest (HD1) to highest (HD5) was just above 3-fold (Fig. 4). Similarly, the most drastic changes were also observed when protein content was quantified in mg/seed. Protein mg/seed had a 6-fold increase from its lowest (HD1) to its highest (HD6), whilst the change in concentration was only 1.2-fold (Fig. 5, Table A1). Protein concentration was more stable than starch concentration during development. Common for starch and protein content in terms of concentration and mg/seed was higher content of protein than of starch in HD1 and HD2 and a shift to a higher starch content from stage HD2 and HD3 (Fig. 6 and 7).

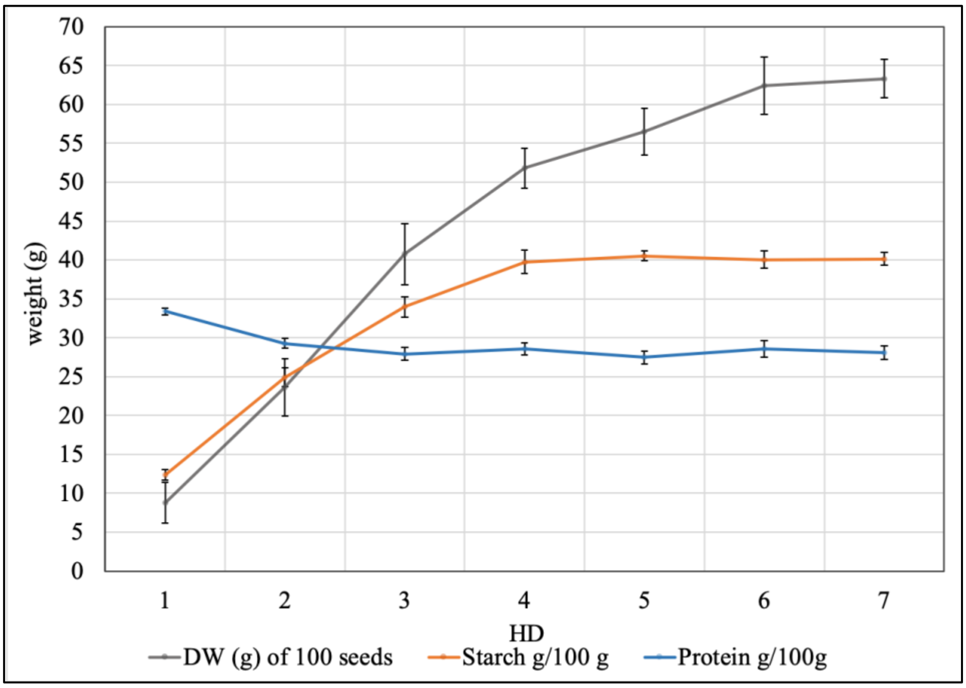

Figure 6. DW of 100 seeds (g), protein (g/100 g) and starch (g/100 g) with error bars (standard deviation, ± *s*).

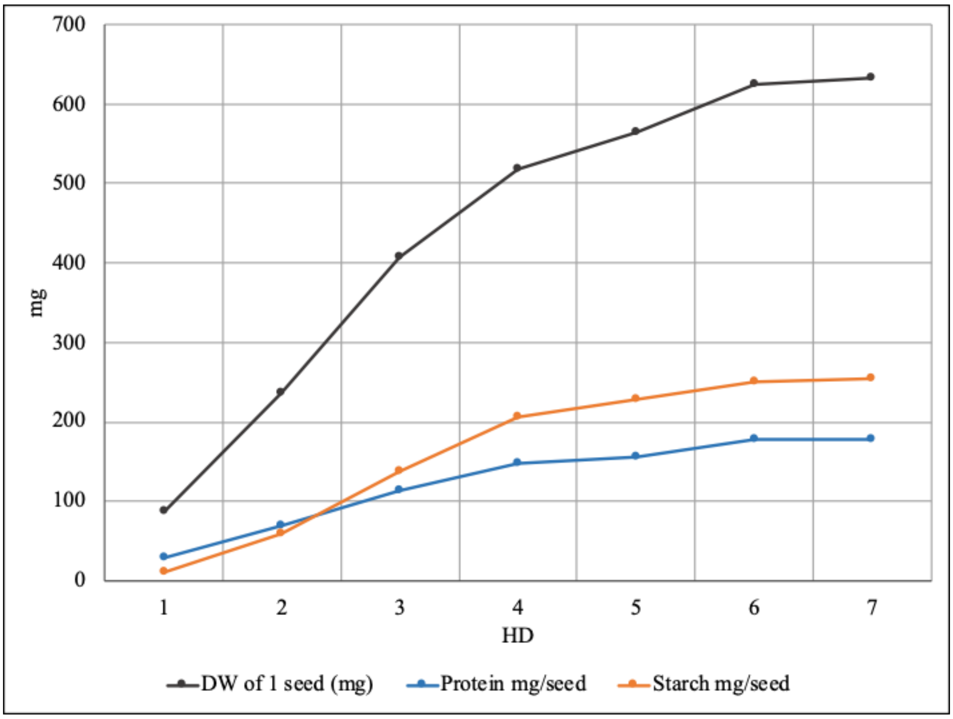

Figure 7. Dry weight (DW) per seed, protein and starch contents per seed at sampling times HD1 to HD7.

5.9 Seed filling trends

DW increased as the seeds were filled with starch and protein. DW, protein (mg/seed) and starch (mg/seed) measured suggest accumulation of storage compounds ceased after HD6 (Fig. 3 and 7). FW increased rapidly until HD4 and started to decline somewhere between HD5 and HD6. As FW decreased after HD5 (Fig. 3) it is suggested that dry matter accounts for an increasing part of the seed weight. Protein seemed to start to accumulate at a faster rate than starch until HD2, but starch accumulated at an overall higher rate from HD1 to HD7. Starch may be responsible for most of the seed weight increase in HD4 to HD7. Starch and protein combined does not account for the total DW (Fig.7). Thus, other compounds are suggested to make up for the remaining seed weight. Other components not investigated in this experiment but contributing to seed dry weight may be hexoses, minerals, dietary fiber, cellulose, phenolics, vicine, convicine, ash, phytic acid and fat (Mattila et al., 2018; Pulkkinen et al., 2015; Singh et al., 2014).

5.10 Faba bean growth in Norway

This investigation observed Vertigo to be a late maturing cultivar in Norway based on the long growing period, late developing flowers and slow desiccation. This corresponds with recent literature (Abrahamsen et al., 2018; Abrahamsen et al., 2019). For future faba bean growth in Norway it might be beneficial to use early maturing cultivars.

The results presented in this thesis are based on one field experiment, but several similar experiments are conducted in the FoodProFuture project to investigate seed maturation in faba beans under Norwegian conditions in different geographic locations. The limiting factor for faba bean cultivation in Norway is low temperature. The overall goal of this experiment was to achieve deeper insight of the maturation process of faba beans grown in cool temperatures and to identify measures of improvements. The results obtained from this thesis can be summarized as follows.

Pre-inoculation is commonly recommended in other geographic regions, but this has not been adapted in the general faba bean cultivation practice in Norway. Natural *Rhizobia* populations in the soil is considered to be sufficient, although this has not yet been investigated scientifically in Norway. Pre-inoculation with *Rhizobia* did not affect the seed weight, nor the accumulation of protein and starch in this experiment. This may support the hypothesis of the sufficiency of existing *Rhizobia* populations in the soil. Startup fertilizer was included to investigate whether a low dose of fertilizer would contribute to earlier plant development from planting and until the nodulation and N-fixation was established. Neither seed weight, protein nor starch accumulation were affected by startup fertilizer this experiment. The results indicate the adequacy of a basic agricultural practices without pre-inoculation and startup fertilizer.

The faba bean variety Vertigo is considered as a later variety when grown in Norwegian climate. Pods from the bottom node were harvested. Calculations of heat sums indicated that 1900 d^{o} was needed for cessation of dry matter accumulation in early September. For the same location in 2019, spring wheat reached yellow ripeness in mid-August, demonstrating the differences of the required maturation periods between these two crops. Moisture content decline was slow, particularly during the later HDs, but this could also be explained by the heavy precipitation in this period. Yet, the results indicate that Vertigo is late maturing and should only be grown in the most suitable regions in Norway with longer growth season.

The results provided novel and greater understanding of the progression of starch and protein accumulation. This information is relevant for harvesting immature faba beans for nourishment, in which the protein to starch ratio as well as other nutrients and antinutrients are important. These and other compounds will be analyzed further in FoodProFuture, and accordingly presented in future papers.

The aim of this investigation was to study the cotyledon development phase in terms of accumulation of the storage compounds starch and protein, which accumulate in the mid to later part of the seed development. The selected harvest period and intervals seemed successful. An additional follow-up of the HDs with microscopy to identify morphological changes can be recommended for future studies.

6. CONCLUSION

Maturation and accumulation of starch and protein from mid to late seed developmental period of Vertigo faba bean grown at relatively cool temperatures is described in this thesis. The information provided can be used to select the optimal harvest times in Norway for food usage. The results confirmed that Vertigo is a late maturing variety in Norway. The results showed no effect of pre-inoculation with *Rhizobia* and application of startup fertilizer on seed weight as well as protein and starch accumulation. This highlights the sustainable benefits of this crop as it can do well without energy-demanding synthetic fertilizers. Norwegian summers may have heavy precipitation which reduces watering requirements, although may delay seed desiccation. Follow-up studies will investigate other components that make up the DW, which could be investigated further. Future research should investigate the physiological and anatomical seed development to get a more complete picture of cotyledon accumulation. This could also include closer look at the seed coat composition and cotyledon composition during seed development.

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9. APPENDIX

Table A1. Range of starch and protein means from lowest to highest during the sampling period.

Dry matter	Unit	Range
Protein	mg/seed	29.439 - 178.472
	g/100 g	27.479 - 33.385
Starch	mg/seed	10.937 - 254.096
	g/100 g	12.382 - 40.512

Table A2. Weather data during the growing period from April 24 to September 3 at NIBIO weather station, Ås, Norway in 2019.

Date	Mean temp. °C	Max temp. °C	Min temp. °C	Precipitation (mm)	Mean RH %
25-Apr-19	13.8	18.0	9.9	0.8	53.3
26-Apr-19	14.3	21.1	8.3	0.4	63.4
27-Apr-19	14.2	20.1	10.1	7.2	74.3
28-Apr-19	14.1	22.0	9.4	6.0	66.7
29-Apr-19	15.1	24.1	3.7	0.0	59.1
30-Apr-19	16.4	21.8	10.4	0.0	57.5
01-May-19	12.8	18.3	5.7	0.0	61.5
02-May-19	7.4	12.2	3.4	1.6	47.2
03-May-19	4.0	9.7	-3.9	1.0	51.8
04-May-19	4.9	11.2	-0.7	0.6	49.3
05-May-19	4.7	12.1	-3.5	0.6	60.8
06-May-19	2.6	4.6	-0.5	1.0	81.8
07-May-19	4.4	10.7	-2.0	1.2	57.4
08-May-19	4.4	10.9	-5.0	0.0	58.5
09-May-19	6.2	7.6	2.8	12.4	87.9
10-May-19	8.4	9.9	7.2	11.4	94.5
11-May-19	8.5	13.1	5.5	0.8	80.5
12-May-19	7.9	12.9	0.8	0.0	47.7
13-May-19	7.7	15.1	-1.3	0.0	50.7
14-May-19	9.9	18.0	2.9	0.0	55.8

15-May-19	11.5	19.6	2.1	0.0	67.4
16-May-19	15.6	23.2	5.5	0.2	52.8
17-May-19	15.4	22.3	4.6	0.0	51.6
18-May-19	11.9	16.0	9.3	3.4	79.6
19-May-19	14.1	19.2	10.2	10.8	86.0
20-May-19	12.5	15.7	7.6	9.4	91.8
21-May-19	17.1	25.0	10.1	14.0	86.0
22-May-19	16.3	22.3	11.7	0.0	86.8
23-May-19	11.1	14.0	7.2	0.0	79.1
24-May-19	10.0	14.2	6.9	21.2	87.3
25-May-19	11.7	19.1	3.6	1.0	79.0
26-May-19	11.0	17.3	8.0	0.2	62.6
27-May-19	9.8	13.5	5.9	0.0	75.6
28-May-19	10.6	17.4	3.7	0.2	71.7
29-May-19	10.0	15.9	4.7	0.2	48.9
30-May-19	9.1	13.8	4.7	9.4	81.9
31-May-19	10.5	17.1	0.3	0.0	63.0
01-Jun-19	11.7	18.2	8.0	0.0	85.0
02-Jun-19	12.4	19.1	4.2	0.0	72.5
03-Jun-19	13.3	17.6	10.4	0.0	87.3
04-Jun-19	13.2	17.8	8.8	0.0	73.3
05-Jun-19	12.2	18.8	6.0	0.0	85.5
06-Jun-19	18.3	28.2	10.4	0.0	82.6
07-Jun-19	14.9	18.2	8.7	0.0	91.9
08-Jun-19	13.3	19.0	8.3	0.0	87.2
09-Jun-19	13.0	16.6	10.3	0.0	85.2
10-Jun-19	13.5	18.2	8.0	0.0	66.2
11-Jun-19	13.9	22.2	5.6	0.0	73.3
12-Jun-19	9.9	12.4	8.3	0.0	85.7
13-Jun-19	11.7	16.9	8.5	15.2	92.5
14-Jun-19	14.1	20.2	7.5	5.8	84.8
15-Jun-19	18.5	27.4	8.5	0.2	67.4
16-Jun-19	16.0	20.5	12.4	0.2	83.8
17-Jun-19	15.3	19.6	10.9	0.0	78.0
18-Jun-19	14.8	19.3	11.3	0.4	83.6

19-Jun-19	15.0	19.6	11.0	7.0	85.9
20-Jun-19	14.8	18.2	12.6	18.4	93.1
21-Jun-19	14.2	18.4	10.1	4.0	73.1
22-Jun-19	14.7	21.7	5.3	0.0	55.8
23-Jun-19	14.2	20.6	4.5	0.0	65.3
24-Jun-19	16.8	25.6	6.9	0.0	67.2
25-Jun-19	14.5	18.3	12.7	1.8	84.0
26-Jun-19	16.8	25.2	11.8	10.6	80.7
27-Jun-19	16.9	23.8	11.3	0.0	61.8
28-Jun-19	17.3	24.7	8.4	0.0	62.1
29-Jun-19	19.0	26.7	11.5	0.0	74.8
30-Jun-19	18.3	23.3	12.1	0.0	71.6
01-Jul-19	16.8	21.2	12.9	2.0	67.9
02-Jul-19	14.4	19.6	9.8	4.2	56.2
03-Jul-19	13.2	19.2	9.0	0.0	44.5
04-Jul-19	12.4	19.8	5.2	0.0	56.6
05-Jul-19	14.0	21.1	4.5	0.0	51.6
06-Jul-19	12.9	18.6	9.3	3.2	77.0
07-Jul-19	13.9	22.0	10.7	1.0	73.9
08-Jul-19	15.6	22.5	6.7	0.2	62.5
09-Jul-19	16.4	23.1	9.7	0.0	61.7
10-Jul-19	18.2	27.1	7.2	0.0	59.4
11-Jul-19	18.2	25.0	9.5	0.0	65.1
12-Jul-19	18.4	24.4	12.9	0.0	70.0
13-Jul-19	17.6	25.6	12.8	0.0	83.9
14-Jul-19	16.8	21.2	11.3	0.0	82.0
15-Jul-19	16.3	23.1	12.1	12.0	70.7
16-Jul-19	15.9	22.3	9.5	1.4	67.8
17-Jul-19	15.8	22.3	7.4	0.0	73.8
18-Jul-19	15.8	20.9	11.8	0.0	81.2
19-Jul-19	16.1	20.8	11.5	2.4	87.8
20-Jul-19	17.1	21.9	12.7	0.2	80.0
21-Jul-19	16.3	20.3	14.6	20.4	93.6
22-Jul-19	15.8	20.2	9.1	2.8	83.7
23-Jul-19	19.7	27.7	13.8	0.8	80.9

24-Jul-19	20.0	25.7	14.8	0.0	84.0
25-Jul-19	20.5	28.3	11.9	0.2	75.8
26-Jul-19	23.7	33.1	13.6	0.0	69.5
27-Jul-19	24.1	29.1	16.8	0.0	62.4
28-Jul-19	23.5	31.2	15.1	0.0	66.5
29-Jul-19	23.9	30.9	18.3	0.0	67.6
30-Jul-19	14.8	20.6	11.5	1.0	84.2
31-Jul-19	14.1	17.7	11.2	0.2	71.2
01-Aug-19	18.1	24.4	12.5	0.2	65.5
02-Aug-19	19.0	27.5	8.9	0.0	64.7
03-Aug-19	17.9	26.0	9.3	0.0	77.0
04-Aug-19	16.5	19.7	11.7	0.0	86.2
05-Aug-19	15.4	17.7	12.9	0.4	86.7
06-Aug-19	15.0	18.8	9.9	1.2	89.7
07-Aug-19	16.6	22.7	11.3	0.2	80.2
08-Aug-19	16.9	22.8	13.4	0.0	79.9
09-Aug-19	16.5	25.0	8.5	0.0	78.1
10-Aug-19	16.0	18.3	14.2	17.4	90.3
11-Aug-19	16.9	19.9	15.3	6.8	94.5
12-Aug-19	17.0	21.3	14.2	0.6	86.0
13-Aug-19	15.9	21.2	11.0	0.0	72.3
14-Aug-19	13.3	20.3	8.0	2.8	78.0
15-Aug-19	13.0	19.3	5.2	0.4	78.6
16-Aug-19	15.2	20.4	12.5	7.2	89.1
17-Aug-19	14.7	16.4	13.0	20.2	93.5
18-Aug-19	15.1	18.5	11.7	0.6	82.4
19-Aug-19	15.1	19.9	9.4	1.6	83.2
20-Aug-19	15.3	19.4	10.9	0.2	80.9
21-Aug-19	15.1	21.1	7.8	0.0	72.0
22-Aug-19	12.7	16.7	8.0	15.2	89.7
23-Aug-19	16.7	23.9	12.3	8.8	78.7
24-Aug-19	16.1	23.3	8.2	0.0	83.1
25-Aug-19	18.4	23.9	14.4	0.0	83.2
26-Aug-19	18.4	24.9	13.2	0.0	79.9
27-Aug-19	18.5	28.0	10.6	0.2	78.6

28-Aug-19	17.9	20.8	13.0	0.6	92.6
29-Aug-19	17.8	20.9	12.7	25.4	92.9
30-Aug-19	15.5	19.3	11.3	0.0	84.4
31-Aug-19	16.8	19.4	11.6	0.2	87.4
01-Sep-19	15.9	20.2	9.6	16.0	82.8
02-Sep-19	12.3	19.5	4.8	0.0	74.2
03-Sep-19	12.7	20.8	6.4	5.6	86.7
Sum of total growing period	1908.8	2664	1158.5	362.4	
Mean of total growing period	14.5	20.2	8.8	2.7	74.8
Sum 24 July to 3 Sept	708.8	938.8	480.9	133.2	
Sum 25 Apr to Jul 23	1200	1725.2	677.6	229.2	

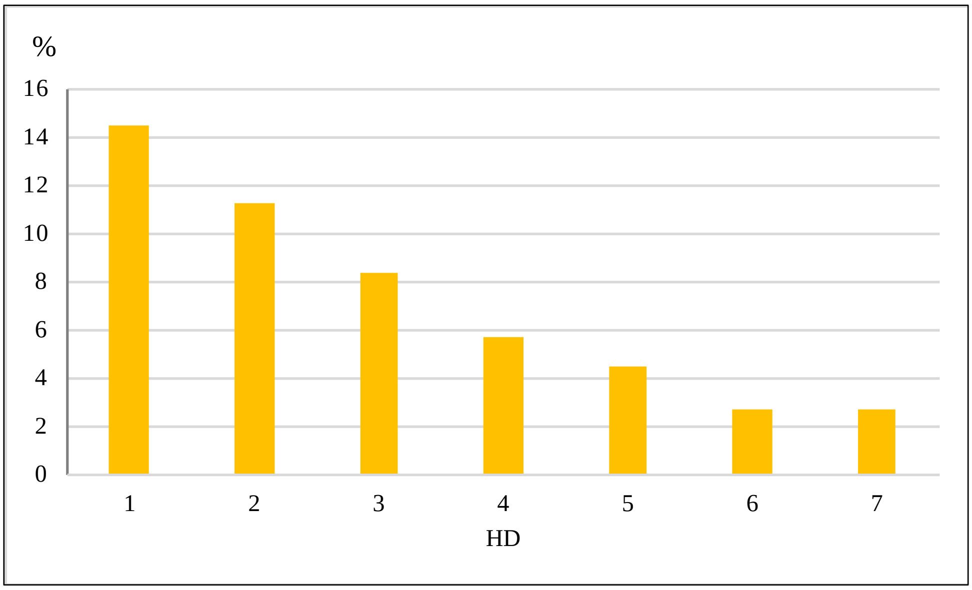

Figure A1. Water-soluble carbohydrates (%) in HD1-HD7 for treatment A1.

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