The effects of temperature during grain filling on gluten quality of bread wheat (*Triticum aestivum* L.)

Effekter av temperatur under kornfylling på glutenkvalitet i brødhvete (*Triticum aestivum* L.)

Philosophiae Doctor (PhD) Thesis

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Abstract

Wheat production in Norway occurs at the highest latitudes among wheat production areas worldwide, and with lower growth temperature. The proportion of Norwegian wheat in total wheat consumed for food has increased from almost zero to over 70 % in good seasons over the last 45 years. However, the industries involved are still recurrently facing serious problems with a large and unpredictable variation in protein quality, primarily caused by weather conditions during the growing season. Earlier studies showed the association between cool and humid seasons and weaker gluten. Hence, this thesis aims to understand the effects of low temperature on gluten quality in order to produce wheat with good and stable baking quality. Experiments were carried out in climate chambers, growth tunnels and fields. The composition and the size distribution of gluten proteins and the viscoelastic properties of gluten were analyzed.

The climate chamber experiments were carried out with four Norwegian cultivars and one UK cultivar at temperatures between 13-23 °C during grain development. The changes in the composition of gluten proteins due to temperature were similar among all five cultivars. The proportions of ω-gliadins and D-type LMW-GS increased and the proportions of α- and γ-gliadins, and B-type LMW-GS decreased with temperature, while temperature had little influence on the proportion of HMW-GS. On the other hand, the temperature effects on the size distribution of glutenin polymers were cultivar dependent. For the four Norwegian cultivars, low temperature had little effect on the assembly of large glutenin polymers, while it increased with temperature in the UK cultivar. Changes in gluten strength due to temperature were associated with changes in the assembly of glutenin polymers rather than changes in the composition of gluten proteins. For the four Norwegian cultivars, strong gluten was obtained at the lowest temperature of 13 °C. The growth tunnel experiments made it possible to study the effects of temperatures from 21.6-25.6 °C during grain filling with plants grown in conditions more similar to the field. Under these conditions gluten strength generally increased with higher temperatures. The field experiments were carried out both in Norway and the USA with nine Norwegian cultivars and three hard red spring wheat cultivars from USA . The average mean daily temperatures during grain filling ranged from 14.3-16.9 °C and 21.5-24.2 °C, respectively. Wheat grown in the USA had significantly higher protein content and generally stronger gluten than wheat grown in Norway. Variations in gluten strength were, however, observed between locations within both mega-environments.

Wheat grown in some locations in Norway had gluten strength that was similar to or even stronger than the gluten strength measured in wheat grown in locations in the USA.

Field trials with winter wheat were carried out in several locations in the period 2005-2013 in Norway. The results showed a large variation in gluten quality and extremely weak gluten was observed in several locations in 2007 and 2011. Therefore, two cultivars from four locations with contrasting quality within the 2011 season were selected, and the gluten proteins from these samples were characterized. Samples with extremely weak gluten had a considerably lower proportion of large glutenin polymers. Characterization of gluten proteins in these samples also indicated protease activity. The results imply that protease seem to be the cause for extremely weak gluten in these samples. The origin of the proteases is still unconfirmed, however, proteases derived from *Fusarium* spp. seem to be a major candidate.

The conclusion from the present thesis is that wheat with strong gluten quality suitable for breadmaking is achievable in areas with low temperatures. Since low temperature in itself seems to have little effect on gluten quality, other environmental factors associated with cool and humid weather conditions are suggested to cause a variation in gluten quality. Biotic stress such as *Fusarium* infection can be one such factor. This is because low temperatures during wheat growing season usually coincide with precipitation in Norway, and such weather conditions are favorable for *Fusarium* infection.

Sammendrag

Hveteproduksjon i Norge skjer på høyere breddegrader enn andre hveteproduksjonsområder i verden, og under lavere temperaturer. Andelen norsk mathvete av totalt forbruk har økt fra nesten null til over 70 prosent i gode sesonger i løpet av de siste 45 år. Likevel har verdikjeden for matkorn utfordringer med til dels store og uforutsette variasjoner i proteinkvaliteten i norskprodusert mathvete. Tidligere studier har påvist svakere gluten i sesonger med kjøligere og fuktigere vær. Formålet med denne avhandlingen er å undersøke hvordan lave temperaturer i kornfyllingsperioden påvirker glutenkvaliteten. Det er utført forsøk med vårhvete i klimakammer, i veksttunnel og i felt der temperaturen gjennom kornfyllingen har vært en forsøksfaktor. Hveteprøvene er analysert for sammensetning og størrelsesfordeling på glutenproteiner og glutens viskoelastiske egenskaper.

Forsøkene i klimakammer ble utført med fire norske og en britisk hvetesort, og med temperaturregimer som varierte mellom 13 og 23 grader gjennom kornfyllingen. Resultatene viste at økende temperatur ga de samme endringer i proteinsammensetning for alle fem sorter. Andelen av ω-gliadins og D-type LMW-GS økte, mens andelen av α- og γ-gliadins og B-type LMW-GS ble redusert ved økende temperaturer. Temperaturen hadde derimot liten virkning på andelen av HMW-GS. Sortene responderte forskjellig på temperatur med hensyn til oppbygningen av store polymere proteiner. For de fire norske sortene ga lav temperatur kun små effekter på oppbygningen av store polymere proteiner, mens for den britiske sorten økte andelen av disse proteinene med økende temperaturer. Endringene i glutens styrke fulgte endringene i andelen av store polymerer. Dette tyder på at temperatureffektene kan knyttes til oppbygningen av glutenin til store polymerer, heller enn til endringene i sammensetning av ulike proteintyper. Resultatene fra klimaveksthusforsøkene viste at for de fire norske sortene ble det dannet glutenproteiner som ga en sterk og god glutenkvalitet ved den laveste temperaturen (13 grader). I veksttunnelen varierte temperaturer fra 21.6–25.6 grader gjennom kornfyllingsperioden. Her ble plantene dyrket under forhold som var tilnærmet lik forholdene i felt. Resultatene fra tunnelforsøkene viste at økende temperaturer ga sterkere gluten. Feltforsøkene ble utført med 9 norske sorter og 3 sorter fra USA på lokaliteter både i Norge og i USA. Gjennomsnittlig døgnmiddeltemperatur under kornfyllingen varierte fra 14.3 til 16.9 grader i Norge og fra 21.5 til 24.2 grader i USA. Hvete dyrket i USA hadde betydelig høyere proteininnhold og i gjennomsnitt sterkere gluten enn hvete dyrket i Norge.

Det var imidlertid variasjoner i glutenstyrke mellom ulike dyrkningsfelter både i Norge og i USA. Prøvene fra noen av dyrkningsfeltene i Norge hadde gluten som var på samme nivå eller enda sterkere enn gluten fra prøvene dyrket i USA.

I perioden 2005-2013 ble det utført feltforsøk med høsthvetesorter på flere lokaliteter i Norge. Resultatene viste stor variasjoner i glutenkvalitet, og i flere felt fra sesongene 2007 og 2011 ble det avdekket ekstremt svak glutenkvalitet. For å undersøke dette nærmere ble to sorter fra fire felt som viste store variasjoner i glutenkvaliteten i 2011-sesongen valgt ut, og glutenproteinene i disse prøvene ble karakterisert. Prøvene med ekstremt svak gluten hadde betydelig lavere andel av store polymerer. Resultatene fra proteinkarakteriseringen tydet på at prøvene inneholdt proteaser som degraderer glutenproteiner. Resultatene antydet at proteaser kan være årsaken til ekstremt svak gluten i disse prøvene. Opprinnelsen til proteaser er fortsatt ubekreftet, men resultater indikerer at proteaser kan være dannet av *Fusairum* spp.

Avhandlingen konkluderer med at det er mulig å dyrke hvete med sterk glutenkvalitet som er velegnet for brødbaking i områder med lavere temperatur. Resultatene fra disse undersøkelsene viste at lav temperaturer i seg selv synes å ha liten effekt på glutenkvaliteten. Andre miljøfaktorer, som forekommer i samspill med lave temperaturer og økt nedbør kan være årsak til variasjon i glutenkvalitet. Resultatene tyder på at angrep av *Fusarium* som har økt forekomst i regnfulle og da også kjøligere vekstsesonger kan være en slik faktor, og dette bør undersøkes videre.

要約

高緯度地帯に位置するノルウェーでの小麦栽培は、他の多くの小麦生産地域に比べ栽培時期の気温が低いのが特徴である。ノルウェーにおける食用小麦の自給率は、過去 45 年間でゼロから栽培条件が良好な年には 70 %までに増加した。しかしながら、栽培時期の気象条件による子実タンパク質（グルテン）の品質変動がおおきく、関連業界は未だに国産小麦の製パン性が安定しないという深刻な問題に直面している。これまでの研究で、栽培時期の低温および高湿度な気象条件がグルテンの非弾性化（弱化）に関与していることが報告されている。そこで本研究は、小麦登熟期の低温がグルテンの品質に与える影響について解明することを目的とし、グロースチャンバー、ビニールトンネル、及び圃場において小麦の栽培を行い、収穫した種子のグルテンタンパク質の組成と分子量分布、またグルテン粘弾性特性を調べた。

グロースチャンバー試験では、ノルウェーの 4 品種およびイギリスの 1 品種を栽培し、チェンバー内温度を 13～23℃に設定することで、登熟期温度が子実タンパク質に与える影響を検討した。温度によるグルテンタンパク質の組成の変化は 5 品種共に類似していた。チャンバー内温度が高いほど、ω-グリアジンおよび D-タイプ低分子量グルテニンサブユニットの割合は増加し、α-および γ-グリアジン、B-タイプ低分子量グルテニンサブユニットの割合は減少していたが、高分子量グルテニンサブユニットの変化は小さかった。その一方で、登熟期の気温がポリマータンパク質の分子量に与える影響は品種により異なっていた。ノルウェーの 4 品種においては、登熟期の低温はポリマータンパク質の重合度に影響を与えなかったが、イギリスの品種においては、ポリマータンパク質の重合度は温度上昇と共に高くなった。これらの実験結果から、温度によるグルテン弾性の変化は、タンパク質の組成変化によるものではなく、ポリマータンパク質の重合度の変化に起因すると示唆された。また、登熟期 13℃で栽培された北欧の 4 品種から得られたグルテンは高い弾性を有していた。ビニールトンネル試験では、圃場と類似した環境条件下で小麦を栽培し、かつ、ビニールトンネル内の温度勾配を利用して、登熟期温度が子実タンパク質に与える影響を検討した。ビニールトンネル内の登熟期平均温度は 21.6～25.6℃で、グルテン弾性は温度の上昇と共に高くなる傾向にあった。圃場試験はノルウェーの 9 品種とアメリカの 3 品種を用いて、ノルウェーおよびアメリカの両国で行われた。登熟期の平均気温はノルウェーで 14.3～16.9℃、アメリカで 21.5～24.2℃であった。アメリカで栽培された小麦は、ノルウェーで栽培された小麦に比べ子実タンパク質含量が高く、一般的に高弾性のグルテンを有していた。しかしながら、グルテン弾性は各国内で大きな栽培地間差がみられ、

ノルウェーの数箇所の栽培地で栽培された小麦のグルテンは、アメリカで栽培された小麦のグルテンに匹敵する、あるいはそれよりも高い弾性を有していた。

ノルウェーの一般的な小麦栽培地域で 2005～2013 年にかけて冬小麦の圃場試験を行い、グルテン粘弾性特性を調べた結果、栽培年及び栽培地間でグルテン弾性に大きな差異が認められた。特に 2007 年と 2011 年度に栽培された小麦は、他の栽培年度に比べ極めて弱いグルテンを有していた。そこで、2011 年度の圃場試験のサンプルから異なるグルテン弾性を示した 2 品種および 4 栽培地を選択し、子実タンパク質の解析を行った。きわめて弱いグルテンを有するサンプルは重合度の高いポリマータンパク質の割合が著しく減少していた。また、それらのサンプルでタンパク質加水分解酵素の活性が示唆され、グルテンの著しい品質低下はタンパク質加水分解酵素に起因するものではないかと考えられた。タンパク質加水分解酵素の由来は未確定であるが、赤かび病菌に由来する可能性が高いと考えられる。

本研究は、低温地帯でも製パン性に適した高弾性のグルテンを有する小麦を生産することが可能であることを示した。登熟期の低温自体がグルテンの品質に直接与える負の影響は小さいことから、低温及び高湿度の気象条件に関連する他の要因が、タンパク質の品質に影響を与えているのではないかと考えられる。本研究結果から、生物的ストレスである赤かび病の感染が、その要因のひとつではないかと示唆された。ノルウェーにおける小麦栽培時期の低温は、通常降雨を伴うことから、赤かび病の感染に好ましい気象条件であると考えられる。

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List of papers

I. Influence of temperature during grain filling on the composition and the polymerization of gluten proteins. (Journal of Cereal Science, Submitted December 2014)

II. Influence of temperature during grain filling on gluten viscoelastic properties and gluten protein composition. (Journal of the Science of Food and Agriculture, Submitted September 2014 and published online February 2015)

III. Temperature variations during grain filling obtained in growth tunnel experiments and its influence on protein content, polymer build-up and gluten viscoelastic properties in wheat. (Journal of Cereal Science 60 2014:406-413)

IV. Variation in gluten quality parameters of spring wheat varieties of different origin grown in contrasting environments. (Journal of Cereal Science 62 2015:110-116)

V. Investigating environmental factors that cause extreme gluten quality deficiency in winter wheat. (Manuscript)

Abbreviations

daa= days after anthesis

d°aa= day degrees after anthesis

DTT= Dithiothreitol

Ext = Extensibility

Exp= Experiment

FHB= *Fusarium* head blight

HMW-GS= High molecular weight glutenin subunits

LMW-GS= Low molecular weight glutenin subunits

MW= Molecular weight

N= Nitrogen

PCA= Principal component analysis

PCs= Principal components

%PC= Protein content (%)

%UPP= Proportion of SDS-unextractable polymeric proteins in total polymeric proteins

R_{max}= Maximum resistance to extension

RMS= Radial microtubule system

RT= Room temperature

S= Sulfur

SE-FPLC= Size exclusion fast performance liquid chromatography

SDS= Sodium dodecyl sulfate

SDS-PAGE= Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TGW= Thousand grain weight

TBS= Tris-buffered saline

1. General introduction

1.1. Wheat

Wheat is one of the most important food crops, and is produced under a wide range of edaphic and climatic conditions worldwide. Total wheat production is estimated to be 717 million tonnes for the 2013/2014 season, with about 70 % being consumed as a food worldwide (FAO, 2014). The production of hexaploid bread wheat (*T. aestivum* L.) dominates among the cultivated wheat species. Bread wheat is an allohexaploid (AABBDD), derived by two allopolyploidization processes (hybridization between different spp. followed by chromosome doubling). The first formation of allotetraploid emmer wheat (*T. turgidum*, AABB) was the result of hybridization between *T. urartu* (AA) and *Aegilops speltoides* (BB) ~0.5 million years ago, and the second between the *T. turgidum* (AABB) and *Ae. tauschii* (DD), which until recently was considered to have happened 10,000 years ago (reviewed by Feldman and Levy (2005). A recent study by Marcussen et al. (2014) has provided new insights in the evolution events of bread wheat and the phylogenetic history, giving rise to new theory about the hybridization events and the genome divergence times. The chromosomes of the allohexaploid bread wheat comprise of three sets of seven chromosomes (numbered 1-7) originating from the three different donor spp. (A, B and D). Each chromosome derived from different species (e.g., 1A, 1B and 1D) is homoeologous.

Wheat is usually consumed as a processed food such as bread, pasta, noodles or different types of baked products because of the unique properties of its storage proteins. When flour and water are mixed into dough, gluten proteins form a viscoelastic network. There is a variation in viscoelastic properties between different wheat types because various products require different viscoelastic properties. For breadmaking, the viscoelastic network is essential for retaining carbon dioxide released by yeast during dough leavening/fermentation and proving, and thus giving a porous structure to bread. As they are the important parameters for both processing and the quality of end products, several rheological methods are developed. Farinograph and Mixograph measure the dynamic properties of dough during mixing, and Extensograph measure the properties of dough after resting. The parameters from these measurement associate with the quality of end products (e.g., loaf volume or loaf shape of bread) (Aamodt et al., 2004, Faergestad et al., 1999, Khatkar et al., 2002, Roels et al., 1993, Tronsmo et al., 2003a, 2003b, Uthayakumaran et al., 1999).

1.2. Endosperm development

The wheat grain is botanically classified as a caryopsis. This is a single seeded fruit consisting of the maternal fruit and seed coats, termed the pericarp and testa, respectively, that enclose the seeds tissues: the triploid endosperm, and the diploid embryo which has a single cotyledon called the scutellum. The endosperm is the main storage tissue accounting for more than 80 % of the grain mass and the starchy endosperm is economically the most important part of the grain that is milled to white flour. The formation of endosperm is initiated by a double fertilization event within the central cell of the embryo sac, where two polar nuclei fuse with the pollen nucleus. Following fertilization, mitotic division of the fertilized triploid nucleus occurs rapidly without cytokinesis (cytoplasmic divisions and cell wall formation). The nuclei are distributed around the surface of a single cell, surrounding a central vacuole (formation of a syncytium/coenocyte). The first step of cellularization is the formation of a radial microtubule system (RMS) emerging from the surface of endosperm nuclei. An alveolar cell wall (a tube-like structure) is formed around each nucleus where the RMSs from the nuclei located next to each other meet. Finally, a periclinal cell wall is formed at the next nuclear mitosis (Brown et al. 1994; Brown et al. 1996). Cell divisions are repeated towards the inner part of the endosperm until the central vacuole of the endosperm is completely filled by cells (reviewed by Olsen (2001), (2004)). Complete cellularization takes three to six days after pollination in cereals (Sabelli and Larkins, 2009). The differentiated endosperm contains four cell types; starchy endosperm cells, transfer cells, aleurone cells and embryo surrounding region cells. Starchy endosperm cells tend to be large and irregularly shaped, storing starch granules and storage proteins that provide nutrients to the germinating seed. Wan et al. (2008) observed approximately 33,000 genes, including genes associated with starch and storage protein deposition, are expressed in wheat cv. Hereward, during seed development between the period of 6 and 42 days after anthesis (daa). Recently, Pfeifer et al. (2014) studied gene expression profile by using a RNA sequencing approach and found in total 46,487 genes to be expressed during endosperm development in wheat cv. Chinese Spring. Toward grain maturation and desiccation, starchy endosperm cells undergo cell death, while aleurone cells remain alive in mature grain (reviewed by Young and Gallie (2000)).

1.3. Gluten proteins

1.3.1. Protein contents

Protein content (%PC) of wheat flour is one of the most important characteristics for wheat quality as a positive correlation exists between loaf volume and %PC (Finney and Barmore, 1948, Uthayakumaran et al., 1999). Therefore, a minimum value for %PC is required at the grain delivery (e.g., 11.5 % is the current minimum requirement for wheat for breadmaking in Norway). Storage proteins are accumulated during grain development and their amount can vary greatly from about eight % to 20 % largely depending on the environment such as soil condition, temperature during the growing season and amount of fertilizer application, particularly nitrogen (N). Despite efforts, improvement of %PC by traditional breeding has been difficult because environmental factors strongly influence %PC compared to genotype. Although some cultivars have higher %PC at the same yield level according to their genetic based deviations (Bogard et al., 2010, Mosleth et al., 2014, Oury et al., 2003), there is a general negative correlation between %PC and yield (Lawlor, 2002). Not only the amount of protein but also protein quality is an important factor determining the properties of dough as well as loaf volume and the shape of hearth bread (Aamodt et al., 2004, 2005, Uthayakumaran et al., 1999).

1.3.2. Classification and composition of gluten proteins

Proteins accumulated in cereals were traditionally classified into four groups according to their solubility; water-soluble albumins, salt-soluble globulins, alcohol-soluble prolamins and alcohol-insoluble glutelins (Osborne, 1907). Gluten proteins consist of prolamins and glutelins called gliadins and glutenins, respectively in wheat. Analyses of 19 European wheat varieties showed that about 80% of storage proteins were gluten proteins (Seilmeier et al., 1991). Gliadins and glutenins accounted for about 60-70 % and 30-40 % of total gluten proteins, respectively, depending on genotypes as well as fertilizer applications (Wieser and Seilmeier, 1998, Wieser et al., 1998, 2004).

Gliadins and glutenins differ in their structure and they are associated with different functional properties. Gliadins exist as monomeric proteins and contribute to dough extensibility and viscosity. Gliadins are further classified into three groups; α-type, γ-type, and ω-type, according

to their electrophoretic mobility at acidic pH, with molecular weights (MWs) being around 28,000-55,000 (Wieser, 2007). Two major loci, *Gli-1* and *Gli-2*, control gliadins with ω-gliadins and γ-gliadins being encoded by clusters of tightly linked genes located at *Gli-1* loci of the homoeologous chromosomes 1 and α-gliadins being controlled by *Gli-2* loci at homoeologous chromosomes 6 (Payne, 1987). Analysis of the proportions of each gliadin subgroups within the total gliadin fraction showed that α-gliadins accounted for the highest proportion ranging from 43.9-59.9 %, γ-gliadins were the second highest ranging from 30.5-45.6 %, and ω-gliadins were least ranging from 7.1-20.0 % (Wieser et al., 1994). α-gliadins and γ-gliadins have six and eight cysteine residues, respectively, in their C-terminal domains forming intra-chain disulphide bonds that stabilize their protein structure (Shewry and Tatham, 1997). Wieser (1996) further divided ω-gliadins into ω1,2-gliadins and ω5-gliadins according to their amino acid sequences, amino acid compositions and MWs. ω5-gliadins, encoded by the B-genome (*Gli-B1*), have higher MWs (≈50,000) than ω1,2-gliadins (≈40,000) which are encoded by the A and D genomes (*Gli-A1 and Gli-D1*, respectively) . ω-gliadins differ from α-gliadin and γ-gliadins in amino acid composition, lacking cysteine residues and thus do not form disulphide bonds. Hence, the monomeric gliadins interact by non-covalent forces such as hydrogen bonds and hydrophobic interactions, to the gluten network.

Glutenins exist as polymers and contribute to dough elasticity. Their MWs are ranging from about 500,000 to more than 10 million (Wahlund et al., 1996). Glutenins are insoluble in aqueous alcohols because of cross-linking in their structure, however, they can be solubilized by reducing disulphide bonds, resulting in two subgroups, high molecular weight-glutenin subunits (HMW-GS) and low molecular weight-glutenin subunits (LMW-GS).

The HMW-GS account for about 10 % of total gluten proteins (Wieser and Kieffer, 2001) with MWs ranging from 67,000-88,000 (Wieser, 2007). They are further divided into two types according to their MWs and their amino acid and gene sequences, x-type and y-type with MWs being 83,000-88,000 and 67,000-74,000, respectively. HMW-GS are encoded by genes at the long arm of the homoeologous chromosomes 1 (1A, 1B and 1D) and are called *Glu-A1*, *Glu-B1* and *Glu-D1*, respectively (Bietz et al., 1975, Orth and Bushuk, 1974). Genes encoding for x-type and y-type subunits are tightly linked at each locus. Hence, bread wheat in theory could contain six HMW-GS, however, because of silencing of specific genes the number of HMW-GS varies from three to five (Payne et al., 1987). The amino acid sequences of the HMW-GS reveal that they

consist of three distinct domains: a large central repetitive domain flanked by the two non-repetitive N- and C-terminal domains. The repetitive domains are composed by three major repetitive motifs, tri- (being only present in x-type), hexa- and nona-peptides. Since the x-type subunits have a higher number of tri- and hexa-peptide repeat units in their repetitive domain (Shewry et al., 1992), their MWs are larger than the y-type subunits. These repetitive motif form repetitive β-turns in the central domain, which, in turn, form a β-spiral structure contributing the elasticity (Shewry et al., 1992, Tatham et al., 1984, 1985). The x-type and y-type subunits have four and six conserved cysteine residues, respectively, which form both intra- and inter-chain disulphide bonds (Shewry and Tatham, 1997).

Although the proportion of HMW-GS in total gluten proteins is relatively small, their contribution to the breadmaking performance is large (Lawrence et al., 1988). Payne et al. (1987) calculated the quality score according to the relationship between HMW-GS from each locus and quality measured as a SDS-sedimentation test. HMW-GS 1Dx5+1Dy10 performed the best in the quality tests and received the highest score in their scoring system. The better quality observed in genotypes with 1Dx5+1Dy10 was later shown to be associated with an extra cysteine residue found in 1Dx5. This extra cysteine residue forms inter-chain disulphide bonds and thus increases the MW of glutenin polymers compared to genotypes with HMW-GS 1Dx2+1Dy12 (Gupta and MacRitchie, 1994, Larroque et al., 1999, Shewry et al., 1992).

LMW-GS represent the major group of glutenin subunits, with their proportion in total gluten proteins being about 20 % (Wieser and Kieffer, 2001) and their MWs 32,000-35,000 (Wieser, 2007). They are encoded by genes at the short arm of chromosome 1 called *Glu-A3*, *Glu-B3* and *Glu-D3* (Jackson et al., 1983). The LMW subunits are further divided into three subgroups; B-type, C-type and D-type according to their mobility on SDS-PAGE (Jackson et al., 1983, Payne and Corfield, 1979). The B-type subunits are the major group of LMW-GS having eight cysteine residues, two of which are assumed to form inter-disulphide bonds and therefore they can act as polymer chain extenders (Kasarda, 1990). The C-type LMW-GS are similar in their amino acid sequences to the monomeric α-gliadins and γ-gliadins (Tao and Kasarda, 1989), but they have an odd number of cysteine residues. D-type LMW-GS are forms of ω-gliadins which possess a single cysteine residue (Masci et al., 1993, 1999). Hence, these unpaired cysteine residues form an inter-molecular disulphide bonds and interact with glutenin polymers, but act as terminators (Lew et al., 1992, Masci et al., 1993, 1999). B-type LMW-GS are mainly encoded by the *Glu-3* loci, while C-

type and D-type LMW-GS are suggested to be encoded by the *Glu-3* loci as well as by genes tightly linked to and also included within the *Gli-1* and *Gli-2* loci (Gupta and Shepherd, 1993, Masci et al., 2002, Pogna et al., 1990, 1995, Tao and Kasarda, 1989). The allelic variation in LMW-GS and gliadins also influence the breadmaking quality. Gupta et al. (1994) studied a set of 74 recombinant inbred lines homozygous for glutenin subunit loci (*Glu-1* and *Glu-3*), and ranked these loci as *Glu-D1>Glu-B1>Glu-B3>Glu-A3>Glu-D3=Glu-A1* according to their dough strength (R_{max}). They observed also a rank of allelic combination within each locus being: 1Dx5+1Dy10 > 1Dx2+1Dy12 in *Glu-D1*, 1Bx17+1By18 > 1Bx20 in *Glu-B1*, c > e in *Glu-A3* and b > c *Glu-B3* (Gupta et al., 1994). Flaete and Uhlen (2003) studied the progeny from three different crosses of wheat genotypes possessing similar HMW-GS compositions, and reported the relationships between gliadin(*Gli-1*)/LMW-GS(*Glu-3*) allelic combination and protein quality measured by SDS-sedimentation and 10 g Mixograph analysis.

Shewry et al. (1986) classified gluten proteins into three groups, the sulphur(S)-rich, the S-poor and the high molecular weight (HMW) prolamins according to their amino acid sequences. α-gliadins, γ-gliadins and LMW-GS have relatively higher contents of S-containing amino acids (cysteine and methionine), hence they were classified as the S-rich prolamins. On the other hand, ω-gliadins are classified as the S-poor prolamins having few or no cysteine and methionine but higher proportions of glutamine, proline and phenylalanine in their amino acid compositions. HMW prolamins comprise HMW-GS and contain intermediate amount of S.

1.3.3. Accumulation of gluten proteins during grain development

Gluten proteins are accumulated in the endosperm during grain filling. Direct staining of gluten proteins on fixed grain sections made it possible to detect the protein bodies as early as eight daa (Tosi et al., 2009, 2011). Shewry et al. (2009) reported the time course of transcripts encoding gluten proteins as well as synthesis and accumulation of gluten proteins in hexaploid wheat cv. Hereward grown at 18/15 °C. The transcripts encoding LMW-GS and gliadins rapidly accumulated between eight daa and 14 daa, thereafter the levels appeared to be stable. The synthesis and accumulation of LMW-GS and gliadin accumulated most rapidly between 12 daa and 35 daa and it plateaued after 42 daa. The patterns of synthesis and accumulation were similar within these protein groups with one exception, ω5-gliadins accumulated more rapidly at the

beginning of grain development compared to ω1,2-gliadins. The profile of HMW-GS synthesis differed from the other gluten protein groups. The accumulation of transcripts encoding HMW-GS started two days later than the other groups of gluten proteins, while it continued until later in the grain development. Similarly, the accumulation of HMW-GS was slower at the beginning and the middle of grain development, while it continued for longer period of time. This accumulation pattern of gluten proteins is in agreement with a previous study by Carceller and Aussenac (2001).

Tosi et al. (2011) reported the quantitative and qualitative distributions of gluten proteins in wheat grain. They observed clear gradients in protein concentration across the endosperm, the highest concentration being in the sub-aleurone cells and gradually decreasing towards the central starchy endosperm cells. The spatial distribution of gluten proteins also differed depending on the protein groups. HMW-GS and γ-gliadins were abundant in the central part of the endosperm, while the proportion of LMW-GS, α-gliadins and ω-gliadins were higher in the outer layer of endosperm including sub-aleurone cells.

Glutenin subunits are initially assembled into glutenin polymers as they are synthesized, while the MW of glutenin polymers increases rapidly during the desiccation/maturation phase at the end of seed development (Bechtel et al., 1982, Carceller and Aussenac, 1999, Gupta et al., 1996, Shewry et al., 2009). Those glutenin polymers with high MW are classified as glutenin macropolymers (Don et al., 2003) or SDS-unextractable polymeric proteins (UPP) (Gupta et al., 1993) and are associated with the viscoelastic properties of dough.

1.3.4. Protein compositions and the viscoelastic properties of dough

The rheological properties of dough or end-product quality parameters are influenced by the composition of gluten proteins as well as the size distribution of glutenin polymers. Since glutenins and gliadins contribute to dough functionality in different ways, their ratio can influence the baking quality. Uthayakumaran et al. (1999) modified the ratio of glutenin to gliadin at a constant %PC and showed that increases in glutenin to gliadin ratio resulted in increases in dough strength as well as loaf volume, with decreases in dough extensibility.

A highly positive correlation was reported between the proportion of UPP in total polymeric proteins (%UPP) and dough strength (R_{max}) (Gupta et al., 1993, MacRitchie and Gupta, 1993). The

proportion of UPP in total polymeric proteins was highly reduced in the line lacking all HMW-GS (*Glu-1* loci), while the absence of the all LMW-GS (*Glu-3* loci) showed small changes in %UPP. Hence, an increase in the HMW-GS:LMW-GS ratio results in higher MW in glutenin polymers (Gupta et al., 1995, Gupta and MacRitchie, 1994). Gupta and MacRitchie (1994) observed differences in the size distribution of the polymeric proteins between the *Glu-3* alleles, which they suggested was associated with the ratio of B-type LMW-GS to C-type LMW-GS. As previously mentioned, B-type LMW-GS act as polymer extenders, but C-type and D-type LMW-GS act as polymer-chain terminators. Changes in the ratio of B-type to C-type and D-type LMW-GS alter the MW distribution of polymeric proteins (Kasarda, 1990), and thus influence the dough strength.

1.4. Abiotic (environmental) effects on gluten quality

Both genotype, environment and their interaction are known to affect wheat quality, such as grain weight, quantity and quality of starch and gluten proteins (reviewed by Williams et al. (2008)). Although the relative contributions of genotype, environment and their interaction varied between studies, some studies clearly showed that environmental effects exceeded cultivar effects for protein quality parameters (Moldestad et al., 2011, Panozzo and Eagles, 2000, Peterson et al., 1992). Environment influences the %PC, the composition of gluten proteins and the size distribution of gluten proteins, hence affecting the functional properties of dough and the quality of end-products (Faergestad et al., 2004, Graybosch et al., 1995, Johansson et al., 2000, 2002, Panozzo and Eagles, 2000, Zhu and Khan, 2001). Temperature, the availability of plant nutrients (particularly N and S) and water availability (drought) are the major environmental factors that have been studied to determine their effects on bread-making quality of wheat (reviewed by DuPont and Altenbach (2003) and Altenbach (2012)).

1.4.1. Effects of temperature during grain filling

The temperature during grain development has been recognized as a major factor influencing grain quality (Blumenthal et al., 1993, Johansson and Svensson, 1998, Moldestad et al., 2011). It had large effects on the duration of grain filling, and thus influenced the grain weight (Sofield et al., 1977a, Wheeler et al., 1996). Increased temperature shortened the periods of both starch and

protein accumulation (DuPont et al., 2006a, 2006b, Hurkman et al., 2003). Higher temperature resulted in lower starch content, while temperature had little effect on protein accumulation, and thus the %PC increased with temperature (Altenbach et al., 2003, DuPont et al., 2006a, 2006b, Gooding et al., 2003).

Large parts of the wheat cultivation area in the world experience higher than optimal temperature for wheat cultivation during the growing season. This can lead to high temperature shock or stress influencing gluten quality. Previous studies reported that temperatures up to 30-35 °C during grain development increased dough strength, while temperature above 30-35 °C had a weakening effect on dough strength (Blumenthal et al., 1991, Borghi et al., 1995, Randall and Moss, 1990). DuPont et al. (2006b) reported that the expression of gluten protein genes was not affected by temperature as the timing and pattern of gluten protein transcripts in developing grain were similar between high and moderate temperatures when they were compared in terms of thermal time. Hence, the synthesis and the accumulation of gluten proteins are presumed to be genetically regulated. However, temperature during grain development was reported to cause changes in the composition of gluten proteins (Daniel and Triboi, 2000, Don et al., 2005b, DuPont et al., 2006a, Hurkman et al., 2013). A recent study by Hurkman et al. (2013) showed that HMW-GS, α-gliadins and ω-gliadins increased, while LMW-GS decreased and γ-gliadins showed little change in response to high temperature (37/24 °C compared to 24/17 °C), and this caused an increase in the ratio of gliadin to glutenin. Moreover, the weakening of dough properties as a result of high temperatures during grain filling was associated with a decrease in the proportion of large glutenin polymers (Ciaffi et al., 1996, Don et al., 2005a, Blumenthal et al., 1995).

By contrast, temperatures during wheat production in Northern Europe can be lower than the optimal temperature for wheat cultivation. Weak gluten strength has been reported from field grown wheat that experienced cool and humid conditions during grain filling in Scandinavia (Faergestad et al., 2004, Johansson and Svensson, 1998, Johansson et al., 2002, Moldestad et al., 2011). Moldestad et al. (2011) reported a weakening effect of low temperature (< 18 °C) during grain filling on gluten strength. Uhlen et al. (1998) reported that the proportion of total polymeric proteins decreased and the dough mixing properties measured by a Mixograph were negatively affected by low temperatures. On the other hand, Johansson et al. (2005) reported inconsistent effects of low temperature on the size distribution of glutenin polymers.

1.4.2. Effects of nitrogen and sulphur

Increased N application increases %PC and alters the composition of gluten proteins. Changes in the compositions of gluten protein groups associated with increased N application were inconsistent across reports. These changes can be summarized as HMW-GS and γ-gliadins showing little change, LMW-GS decreased, and α-gliadins and ω-gliadins increased with higher N application (Daniel and Triboi, 2000, DuPont et al., 2006a, Pechanek et al., 1997, Wan et al., 2013, Wieser and Seilmeier, 1998). The majority agreed that higher N application increased the proportion of gliadins, and thus increased the ratio of gliadin to glutenin (Chope et al., 2014, Daniel and Triboi, 2000, Godfrey et al., 2010, Jia et al., 1996, Kindred et al., 2008), which then led to increased dough extensibility. Chope et al. (2014) reported that the proportion of polymeric proteins decreased in mature grain with increased N application, hence decreased dough strength. By contrast, Pechanek et al. (1997) did not find the association between the increased N application and the gliadin:glutenin ratio, while they observed an increase in the ratio of LMW-GS to HMW-GS with high N application, which showed a negative correlation with SDS sedimentation and bread volume.

S deficiency also affects gluten quality by altering the composition of gluten proteins. When S is limited, the composition of gluten proteins changes as the proportions of S-poor prolamins (ω-gliadins) and relatively S-poor HMW-GS increase, while S-rich prolamins (α-gliadins, γ-gliadins and LMW-GS) decrease (Moss et al., 1981, Wan et al., 2014, Wieser et al., 2004, Wrigley et al., 1980, 1984). MacRitchie and Gupta (1993) reported that decreasing flour S content was associated with an increase in the HMW-GS:LMW-GS ratio which caused an increase in %UPP as well as gluten strength. Moss et al. (1981), (1983) reported similar observations as dough extensibility was correlated positively, while dough resistance was correlated negatively with increasing S content in the flour. Zhao et al. (1999b) found a positive correlation between the S content and loaf volume and showed that grain S status was as better indicator of loaf volume than grain N status, although the relationship between S content and loaf volume depends on the breadmaking methods (Zhao et al., 1999a).

1.4.3. Effects of water availability

Water availability or precipitation is another factor influencing the wheat quality. Drought resulted in grain yield loss (Ciais et al., 2005, Wardlaw, 2002). Drought during grain filling shortened the duration of grain filling and reduced the final grain weight. These effects of drought were more pronounced when drought was combined with high temperature (Altenbach et al., 2003, Gooding et al., 2003). Drought mainly changed the duration of starch accumulation, thus the amount of starch in a single grain decreased and the proportion of protein increased (Altenbach et al., 2003). Yang et al. (2011) reported that the composition of gluten proteins was altered as α-gliadins, γ-gliadins and one type of LMW-GS decreased and one type of LMW-GS increased by water deficiency. Drought during grain filling reduced the SDS-sedimentation volume (Gooding et al., 2003), which indicates that drought stress has weakening effects on the dough and gluten.

Excess precipitation leads to waterlogging and reduces cereal growth and yield (Musgrave, 1994), and high precipitation during grain maturation/desiccation causes pre-harvest sprouting. Pre-harvest sprouting (PHS) increases both α-amylase and protease in germinated grain and decreases the quality of bread (Lorenz et al., 1983). α-amylase causes starch degradation, resulting in sticky dough, and bread with higher loaf volume but with poor texture compared to the control sample (Lorenz et al., 1983). Kulp et al. (1983) observed dough weakening in PHS samples in which the effects of α-amylase were eliminated, hence suggested that PHS influences gluten proteins. Cysteine proteases are the typical endogenous proteases induced during cereal germination (Bottari et al., 1996, Poulle and Jones, 1988, Watanabe et al., 1991) and degrade gluten proteins, especially gliadins (Bottari et al., 1996, Kiyosaki et al., 2007, Oita et al., 2009, Prabucka and Bielawski, 2004).

1.5. Biotic effects of *Fusarium* on gluten quality

Fusarium head blight (FHB) is a disease caused by *Fusarium* spp. and *Microdochium* spp. which causes problems in most cereal-growing areas of the world. *Fusarium* infection gives shriveled and discolored grains, sometimes with a chalky white or pink appearance or sterile florets, and may cause severe yield loss (reviewed by Parry et al. (1995)). The most serious concern of the *Fusarium* infection is the ability of the fungi to produce and accumulate mycotoxins in grain, which are highly toxic to human and animals. Moreover, *F. graminearum* greatly affects the

quality of wheat grain by digesting starch granules, storage proteins and cell walls (Bechtel et al., 1985). Proteases produced by *Fusarium* spp. have been found to drastically reduce the breadmaking quality of wheat (Dexter et al., 1996, Nightingale et al., 1999, Papouskova et al., 2011, Wang et al., 2005). Proteases derived from *Fusarium spp.* affect storage protein accumulated in the endosperm. Bechtel et al. (1985) and Nightingale et al. (1999) observed absence of the cell protein matrix in the most severely infected areas of the endosperm. Glutenins were the most strongly reduced by fungal proteases (Eggert et al., 2010, Wang et al., 2005), with the HMW-GS being the most severely affected fraction among the glutenins (Eggert et al., 2010). Moreover, fungal proteases are presumed to degrade gluten proteins during dough preparation. Eggert et al. (2011) observed digestion of gluten proteins when they were incubated with proteases derived from *F. graminearum*. Consequently, undesirable dough properties as well as poor bread quality such as decreased loaf volume and deformed loaf shape were observed from samples that were highly contaminated with *Fusarium* (Nightingale et al., 1999, Wang et al., 2005).

FHB has been an increasing problem in Norwegian cereal production. The number of cereal seeds infected with *Fusarium* has increased more than twofold over the last decade compared to the three previous decades (Bernhoft et al., 2013). They found that high precipitation during flowering, which is the most susceptible period for infection, as well as during grain maturation and desiccation promoted fungal infection and growth. *F. avenaceum*, *F. graminearum*, *F. culmorum* and *M. nivale* are the most commonly observed species causing problems for the Norwegian cereal production in recent years (Bernhoft et al., 2013).

1.6. Experimental approaches

The effects of environmental factors on wheat quality, including baking quality, have been studied by growing wheat in different experimental systems. The choice of the experimental system depends on the purpose of the study and on the environmental factor(s) to be investigated. Temperature, nutrients (particularly N and S) and water availability discussed above are the major factors studied among environmental factors.

Field experiments allow us to study the effects of environmental factors on the wheat quality in plants grown under conditions similar to commercial production. Collecting data for environmental factors affecting wheat quality makes it possible to observe associations between

individual factors and quality parameters. However, because these environmental factors interact in complex manner, it can be difficult to draw conclusions on whether the effects are caused by a single factor or a combination of several factors. The effects of nutrient availability are amenable to study with field experiments since nutrition can be manipulated by applying different amount of fertilizer (Chope et al., 2014, Jia et al., 1996, Pechanek et al., 1997, Zhao et al., 1999b), although other factors such as soil type and precipitation can cause the variation in N availability. Some environmental factors (e.g., weather conditions) show seasonal variation and may occur randomly. Therefore, it is both difficult and time/cost-consuming (as many experiments in different environments are needed) to study the relationships between such environmental factors and wheat quality in the field experiments.

Experiments in growth tunnels can be carried out to create variation in some environmental factor(s) and at the same time obtain growth conditions closer to the field conditions as plants experience daily variation in temperature and light intensity. This system has been used to study the effects of environmental factors that can be manipulated within the growth tunnel such as temperature, precipitation (drought) and CO_2 concentration in atmospheric air (Borghi et al., 1995, Daniel and Triboi, 2000, Triboi et al., 2003, Wheeler et al., 1996). As an example, higher temperature than outside temperature or temperature gradients inside the growth tunnel can be obtained with this experimental approach.

Experiments in climate chambers have been used to study the effects of a single or combinations of a few environmental factor(s) in controlled environments (Hurkman et al., 2013, Don et al., 2005b, Blumenthal et al., 1995) . Hence, it is possible to draw conclusions on how the environmental factor(s) of interest influence(s) the wheat quality. Plant growth conditions in the climate chambers are artificial and differ from those in the field as firstly, plants were grown in pots, which influences the their morphology (e.g., root development) and N availability, and secondly, weather conditions such as light intensity and temperature differ from the field.

1.7. Background to the present study

The wheat production in Norway occurs at the highest latitudes among wheat production areas worldwide. Typical weather conditions during grain filling are characterized by a mean temperature between 14.5-18.5 °C and relatively high and/or frequent precipitation are common,

particularly during grain maturation and harvesting. Historically, Norway has been dependent on imported wheat. High proportions of strong wheat were traditionally imported from North America during the decade before and after the Second World War. However, wheat production in Norway was encouraged to secure a reliable supply of grain to the domestic market. Plant breeders started with the evaluation of Norwegian landraces at the beginning of the 20th century. Intensive breeding led to the introduction of resistances to lodging, pre-harvest sprouting (PHS) and diseases as well as traits for good baking quality in Norwegian cultivars. Expansion of the wheat production area from the mid-1970s to the mid-2000s also contributed to a large increase in wheat production. The whole value chain has been actively involved in the improvement of both yield and quality in Norwegian wheat by funding research programs, contributing to improved cultivation techniques, and by developing grading and sorting systems. For gluten quality, cultivars are divided into five classes according to their gluten quality potential, as determined on wheat samples grown under Norwegian climate conditions for several years.

Figure 1.7.1 shows data on Norwegian wheat production, consumption and imports from 1990 to recent years. There is a large variation in wheat production area and wheat production itself over the years (Figure 1.7.1, A). The purchase of domestic wheat varies due to production quantity, and the proportion of domestic wheat for food varies due to quality in addition to production quantity (Figure 1.7.1, B). One of the main reasons for the quality variation is PHS caused by frequent rain during maturation and harvesting in autumn. Consequently, the proportion of domestic wheat in total quantity consumed for food fluctuated between 20-70 % in the period between 1990 and 2013, and high and stable proportions were achieved for the seasons ’04/’05 to ’08/’09. Thereafter a dramatic decrease is observed from ’08/’09 to ’12/’13 (Figure 1.7.1, C), caused by a decrease in production area, but also by frequent precipitation leading to PHS in these years. An extremely difficult harvest occurred in 2011, as seen by the very low proportion of Norwegian wheat in total wheat consumption for food seen in the ’11/’12 season. In that season, the proportion of Norwegian wheat consumed for food was as low as during the mid-1970s. Figure 1.7.1, C also shows that total wheat consumption for food in Norway has been decreasing in recent years, mainly due to an increase in imports of processed wheat products.

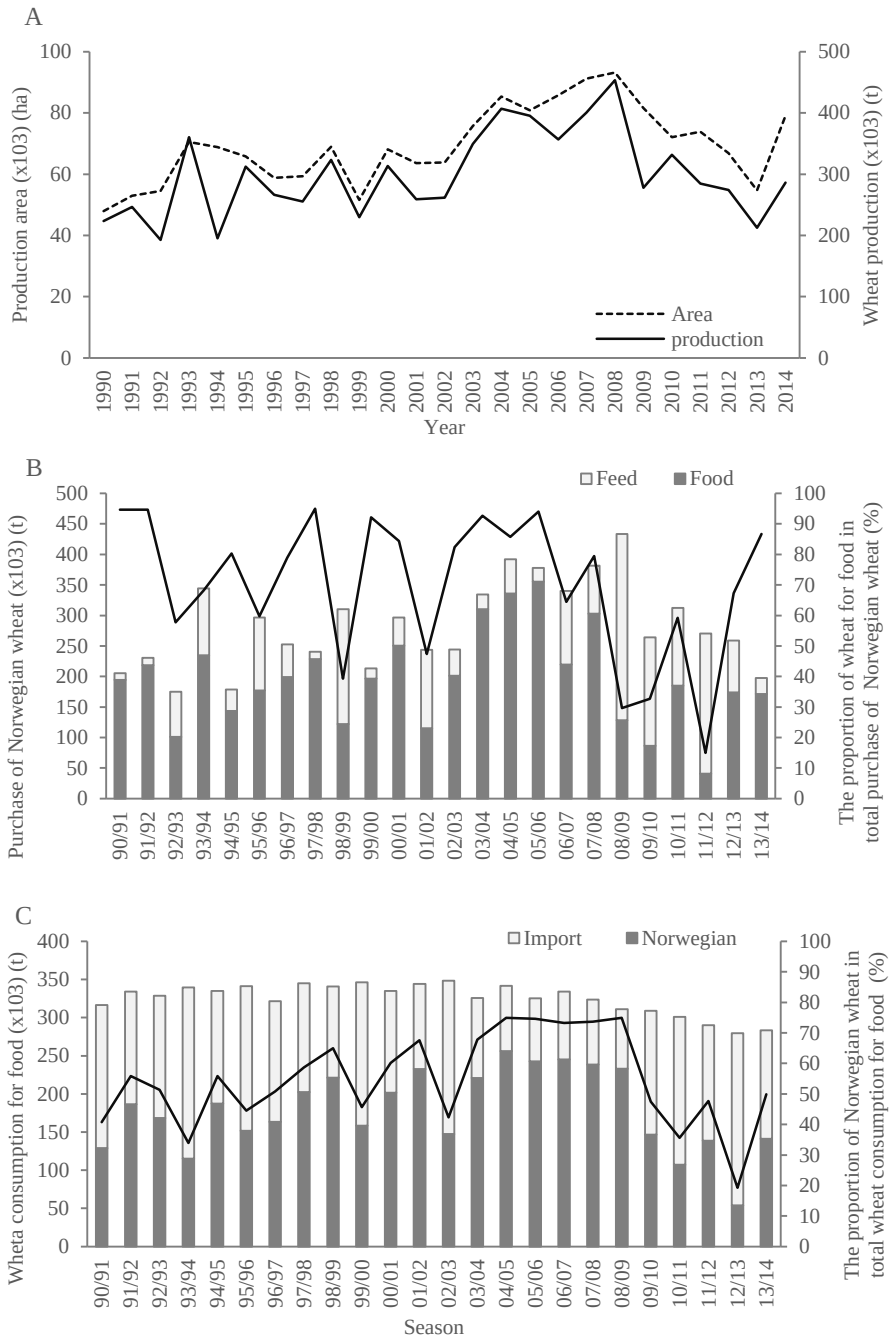

Figure 1.7.1 Wheat production area (ha) and produnctin (t) in Norway (A), purchase of Norwegian wheat (t) (food and animal feed) and the proportion of wheat for food in total purchase of Norwegian wheat (%) (B), and wheat consumption for food (t) (import and Norwegian wheat) and the proportion of Norwegian wheat in total wheat consumption for food in Norway (C). Data from 1990 to the last available year. Data are from Statistics Norway (http://www.ssb.no) and Norske Felleskjøp.

The protein quality of Norwegian wheat important for breadmaking is also affected by environmental factors, and variation in weather conditions are suggested to be the main cause. The purchase of cultivars for breadmaking in Norway is based on their division into five classes (class 1-5), rather than the direct measurement of protein quality at purchase. Therefore, variation in protein quality caused by the environment is usually not discovered at grain delivery. Moreover, domestically produced wheat is protected by the current agriculture policy and it must be used for food when the grain meets the requirements for food quality. With these two reasons, the milling and baking industry need to deal with varying protein quality in Norwegian wheat. Especially when the proportion of Norwegian wheat holding food quality is high, the protein quality becomes more important for industries, as limited amount of imported wheat can be used to adjust the protein quality at the milling.

To improve the protein quality of Norwegian wheat, the large and unpredictable variations in gluten quality caused by environmental factors need to be reduced. To investigate which environmental factors are causing the variation in gluten quality, field trials have been conducted with several spring and winter wheat cultivars in different locations in southeast Norway since 2005. The analyses of spring wheat cultivars obtained from field trials conducted between 2005 and 2008 showed a large variation in gluten quality, which was associated with the temperature during grain filling (Moldestad et al., 2011). Weaker gluten was observed when the temperature during grain filling dropped below 17-18 °C, and this was most pronounced when plants experienced low temperature (<18 °C) from the early to the middle stage of grain filling (Moldestad et al., 2011). However, it has not yet been confirmed that temperature is the direct cause of variation in gluten quality. Moreover, other environmental factors, possibly associated with cool and humid weather, and thereby being further possible causes for the variation in gluten quality should be investigated more in depth.

2. Aim of the thesis

The main aim of this study was to investigate the effects of low temperature during grain filling on wheat gluten quality. To determine the effects of low to moderate temperature on gluten quality, the experiments were carried out in climate chambers with controlled temperatures (Papers I and II). The objectives of these studies are to investigate whether temperature during grain filling influences the synthesis and composition of gluten proteins and the assembly of large glutenin polymers. Growth tunnel experiments were conducted to study the effects of temperature during grain filling on the composition and the viscoelastic properties of gluten in wheat grown in the environmental conditions (e.g., light, soil and humidity) similar to the field. (Paper III). In addition, field experiments were carried out to investigate the relationship between the effects of temperature and the viscoelastic properties of gluten in wheat grown under two mega-environments with contrasting weather conditions (Paper IV). Other environmental factors that interact with cool and humid weather may also affect gluten quality. Such environmental factors that cause poor gluten quality were also investigated by analyzing samples of winter wheat with extremely weak gluten obtained from field trials (Paper V).

3. Material and methods

The description of three experimental approaches with a range of temperature achieved for each study as well as materials and methods used in each study are described briefly in this section. Detailed information on material and methods is available in each paper.

3.1. Cultivars

Table 3.1.1 Summary of cultivars used in the study, breeder and country or origin, sorting class in Norway and allelic composition of HMW-GS.

Cultivar	Breeder, Country	Class[e]	Wheat type[f]	HMW-GS			Paper				
				1A	1B	1D	I	II	III	IV	V
Bastian	Graminor, NO[b]	1	S	2*	7+9	5+10				x	
Berserk	Graminor, NO	2	S	1	14+15	5+10		x		x	
Bajass	Graminor, NO	-	S	2*	14+15	5+10				x	
Demonstrant	Graminor, NO	3	S	2*	7+8	5+10				x	
Vinjett	Lantmännen SW Seed, SW[c]	-	S	2*	7+9	5+10				x	
Zebra	Lantmännen SW Seed, SW	3	S	2*	7+9	5+10		x	x	x	
Avle	Lantmännen SW Seed, SW	2	S	2*	7+9	5+10		x		x	
Bjarne	Graminor, NO	2	S	2*	6+8	5+10	x	x	x	x	
Quarna	Delley Semences et Plantes SA, CH	2	S	1	7+8	5+10				x	
Olivin[a]	RAGT Seeds Ltd., UK	4	W	null	7+9	5+10					x
Finans	Lantmännen SW Seed, SW	5	W	2*	7+9	2+12					x
Cadenza	Cambridge Plant Breeders Ltd, UK	-	S	null	14+15	5+10	x				
Sabin	MN[d], USA	-	S	1	7+8	5+10				x	
Tom	MN[d], USA	-	S	2*	7+9	5+10				x	
RB07	MN[d], USA	-	S	2*	7+9	5+10				x	

[a]: Bred by Monsanto, [b]: NO; Norway, [c]: SW; Sweden, [d]: MN; Minnesota Agricultural Experimental Station, [e]: Class 1-4 (1 is the strongest); strong protein quality and class 5; week protein quality, -: Cultivars were not sorted in Norwegian wheat sorting system, [f]: S; Spring wheat and W; Winter wheat

The cultivars used in this thesis are listed in Table 3.1.1. Temperature effects on gluten quality were studied with cultivars possessing 1Dx5+1Dy10 in all experiments (Papers I-IV) to reduce the effects of allelic variation at the *Glu-1D* locus. Nine cultivars bred or grown in Norway with strong gluten quality (class 1-3) and three strong hard red spring wheat cultivars from Minnesota, USA were used in the field experiments (Paper IV). The growth tunnel experiments included Bjarne and Zebra which are at present the most widely grown cultivars in Norway (Paper III). Two cultivars bred in different production areas within Northwestern Europe were used in Paper I. These were Bjarne bred in Norway, and Cadenza which is a breadmaking cultivar bred and grown in the UK. Moreover, the climate chamber experiment was carried out with four Norwegian cultivars selected from the field experiments (Paper II). They represent Norwegian wheat cultivars as they covered the variation in gluten quality in spring wheat grown under the field conditions.

Olivin and Finans are winter wheat cultivars selected to determine the causes of the extremely weak gluten that was observed in specific locations from field trials (Paper V). Finans showed extremely poor gluten quality, while Olivin showed better quality among the winter wheat cultivars grown in the field experiment in 2011.

3.2. Temperature response experiments

Table 3.2.2 Overview of experiment types and the ranges of average daily temperature that were obtained during grain filling. The papers covering the results are listed.

Paper	Experiment type	Average mean daily temperature during grain filling (°C)
I	Climate chamber	12.3-22.3 (3)[a]
II	Climate chamber	12.3-22.3 (6)[a]
III	Growth tunnel	18.1-19.6[b] 20.8-23.1[c]
IV	Field	14.3-16.9[d] 21.5-24.2[e]
V	Field	13.4-18.5[f]

[a]; three (3) or six(6) temperature regimes were used, [b]; experiment carried out in 2010, [c]; experiment carried out in 2011, [d]; experiment carried out in Norway between 2009 and 2011, [e]; experiment carried out in USA in 2011 and [f]; experiment carried out in Norway between 2005 and 2013

The effects of temperature were investigated using three different experimental approaches; in climate chambers, in a growth tunnel and field trials. An overview of the experiments and the ranges of average mean daily temperatures during grain filling is shown in Table 3.2.2.

3.2.1. Climate chamber experiments (Papers I and II)

The plants were grown in the greenhouse until flowering and then moved to temperature controlled climate chambers. Three experiments, Exp1, Exp2 and Exp3, were carried out with two complete biological replicates (in separate climate chambers). All experiments were conducted during the same time of year in order to have as similar growing conditions as possible. All experiments included three constant day/night temperatures of 13/10 °C, 18/15 °C and 23/20 °C, respectively, throughout the grain filling period (Figure 3.2.1). In addition, three temperature regimes with changes in temperature during grain development were included in Exp1 and Exp3. In these experiments plants were moved from lower to higher temperatures at 207 day degrees after anthesis (d°aa). The temperature changes were (from the lower to the higher temperatures): 13/10-18/15 °C, 13/10-23/20 °C and 18/15-23/20 °C (Figure 3.2.1). The temperature regimes are denoted 13-13, 18-18, 23-23, 13-18, 13-23 and 18-23 (Figure 3.2.1) in the Results and Discussion below. The early phase (flowering to 207 d°aa) mainly corresponded to the differentiation phase whereas the later period correspondent to the accumulation of storage compounds and the maturation phase. The choice of temperatures during grain filling in the climate chambers was based on temperatures observed during the growing season in Norway and the relationship with

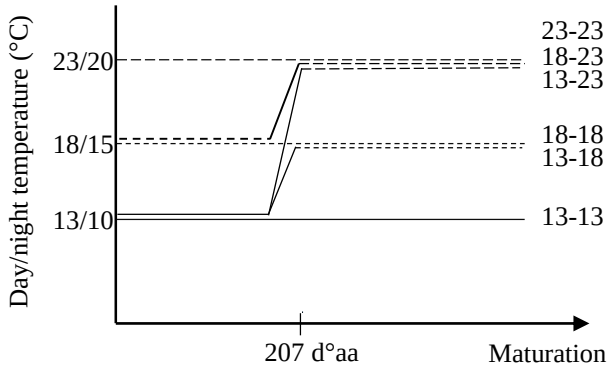

Figure 3.2.1 Schematic temperature regimes during grain filling in the climate chamber experiments

gluten quality based on field experiments. 18 °C is equivalent to the average mean day temperature observed during seasons that resulted in good gluten quality, and is defined as the middle temperature. Low and high temperatures were defined as ± 5 °C from the middle temperature. Moreover, 13 °C can be observed for short periods during grain filling in Norway, and 23 °C is a common mean day temperature during grain filling in large parts of the wheat production areas worldwide. Fertilizer was first applied at the fifth-leaf stage (Zadoks (Z) 15, (Zadoks et al., 1974)) and then weekly until just before the anthesis (Z59) in Exp1 and Exp2 and until late milk stage during grain filling (Z77, 340 d°aa) in Exp3. Exp1 and Exp3 were carried out with the Norwegian cultivar Bjarne, and the UK cultivar Cadenza. In addition to these two cultivars, Avle, Berserk and Zebra were included in Exp2. All cultivars possess HMW subunits 5+10, indicating that they have a potential to produce strong gluten. The effects of lower to moderate temperatures on the composition of gluten proteins and gluten quality were studied with two cultivars (Bjarne and Cadenza) in Paper I, covering results of all three experiments. In Paper II, the effects of the three constant temperatures during grain development were investigated with four Norwegian cultivars.

3.2.2. Experiments in the growth tunnel (Paper III)

Although climate chamber experiments are suitable to study the effects of a few selected environmental factors, such as temperature in this study, the growth conditions differ between climate chambers and field trials. Therefore, filed experiments covered with polypropylene tunnels were conducted in 2010 and 2011 (T2010 and T2011, respectively) (Paper III). This experimental approach created a temperature gradient through the longitudinal direction of the tunnels and enabled to study the effects of temperature during grain filling on gluten quality in plants grown under conditions similar to field. The plants were grown in natural soils, and were subjected to natural variation in solar radiation, humidity and temperatures. Two Norwegian spring wheat cultivars (Bjarne and Zebra) were sown and grown in the field with common agronomic practices (application of fertilizers, herbicide, fungicide, and insecticide). A propylene tunnel was erected at heading, just before anthesis, and irrigation thereafter, was performed by using a sprinkler system. By covering the field with a polypropylene tunnel, a temperature gradient was obtained in the longitudinal direction of the tunnel. Differences in mean daily (00:00-24:00) and mean day (07:00-20:00) temperatures were approximately 1.5 and 2-2.5 °C, respectively, from the opening

to the inner part of tunnel in both years. The average mean daily temperature during grain filling was higher in 2011 compared to 2010 (Table 3.2.2). The ranges of mean day temperature during grain filling were 21.6-23.4 and 23.5-25.4 °C in 2010 and 2011, respectively (Paper III).

3.2.3. Field trials (Paper IV)

Field experiments were conducted at four locations in the southeastern part of Norway between 2009 and 2011 as well as at three locations in Minnesota, USA in 2011. The effects of temperature on gluten quality were studied by comparing the viscoelastic properties of gluten between the locations within the two mega-environments as well as between the mega-environments (Norway vs. USA) (Paper IV). Nine spring wheat cultivars either bred in Norway or recommended for the Norwegian climate, and three hard red spring wheat cultivars from Minnesota, USA were included in the field experiments. Average mean daily temperatures during grain filling from the four fields in Norway in 2009-2011 ranged from 14.3 to 16.9 °C, while those from the three fields in the USA in 2011 ranged from 21.5 to 24.2 °C during grain filling.

3.2.4. Environmental factors causing extreme gluten quality (Paper V)

Annual field experiments have been carried out in Norway at several locations in the period 2005-2013 to determine the gluten quality of winter wheat cultivars. Environmental factors caused large variation in the viscoelastic properties of gluten between and within growth seasons. The viscoelastic properties of gluten were different between locations and seasons, and extremely weak gluten was observed in plants grown in some locations in 2007 and 2011. The protein quality in these samples were considerably decreased, and presented challenge for the milling and baking industries. Therefore, two winter wheat cultivars from four fields, which showed contrasting gluten quality in the 2011 season, were selected. The composition of gluten proteins in those samples was characterized further to investigate these phenomena. Possible environmental factors causing the extremely weak gluten are addressed in Paper V.

3.3. Analytical methods

3.3.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Gluten proteins were separated by SDS-PAGE, and quantified to study the effects of temperature and genotype on deposition of gluten proteins (Papers I and II). Gluten proteins were extracted in three fractions according to Tosi et al. (2011), 70% ethanol soluble gliadins, 50% propan-1-ol and dithiothreitol (DTT) soluble glutenins, and remaining proteins are extracted by extraction buffer (62.5 mM Tris-HCl, 2 % (w/v) SDS, 10 % (v/v) glycerol, 0.005 % (w/v) Bromphenol Blue and 1.5 % (w/v) DTT buffer (pH 6.8)) (residue fraction) are separated on the 4-12% polyacrylamide gel. SDS-PAGE is widely used to separate proteins according to their molecular size. Gluten proteins are denatured by SDS, which disrupts the non-covalent bonds, so that the protein structure becomes linear. Moreover, DTT was used to reduce the covalent bonds (disulphide bonds) within and between the gluten proteins.

HMW-GS as well as ω-gliadins are separated from other protein groups by SDS-PAGE because they differ in mass. However, it is still necessary to confirm the identities of the bands corresponding to the ω-gliadins. The bands corresponding to LMW-GS, α-gliadins and γ-gliadins are not readily separated by SDS-PAGE as they overlay in their masses. Moreover, not all HMW-GS and LMW-GS could be extracted by 50% propan-1-ol extraction buffer containing DTT with similar bands being observed in the residue fraction. Therefore, western blot analysis was carried out according to Tosi et al. (2011) to identify the bands corresponding to the different groups of gluten proteins, including to confirm the identities of glutenin subunits in the residue fractions (see 4.1 Western blot analysis of gluten proteins separated by SDS-PAGE).

3.3.2. Size exclusion fast performance liquid chromatography (SE-FPLC)

The size distribution of gluten proteins was analyzed by SE-FPLC. Extracted gluten proteins were injected into a Superose ®12HR 10/30 column connected to ÄKTA SE-FPLC (GE Healthcare life science, UK), and eluted according to their molecular size. The gluten proteins were extracted sequentially to obtain extractable and unextractable proteins using SDS solution. The SDS-unextractable proteins were obtained by sonication using a Sonics VC130 (Sonics and Materials, Newton, CT 06470 - 1614, USA). Sonication shears large glutenin polymers and makes them

extractable in the SDS solution. However, the polymers solubilized by sonication still represent the large polymers (Singh et al., 1990) and their retention times were similar to those of the largest SDS-extractable polymers. The two step extraction procedure allowed the proportion of SDS-unextractable polymeric proteins in total polymeric proteins (%UPP) to be calculated: this has a high and positive correlation with dough strength (Gupta et al., 1993) (Papers I-III and V). Chromatograms of the SDS-unextractable and SDS-extractable protein fractions show one main peak (F1*) and four main peaks (F1 to F4), respectively (Figure 4.2.1, A and B), where F1* and F1-F2 were considered as containing polymeric proteins, and F3-F4 were considered as containing monomeric proteins. To determine the components present in the peaks obtained by SE-FPLC, each peak was fractionated and western blot analyses were carried out on reduced and unreduced samples (see 4.2 Western blot analysis of SE-FPLC fractions).

3.3.3. The SMS/Kieffer Dough and Gluten Extensibility Rig

Gluten quality was measured by the SMS/Kieffer Dough and Gluten Extensibility Rig (Kieffer et al., 1998) (Papers II-V). The Kieffer-rig stretches a dough or gluten in uniaxial direction measuring the maximum resistance to extension (R_{max}) and extensibility (Ext). R_{max} is the maximum force needed to rupture the dough or gluten, and Ext is the distance until the point when the dough or gluten ruptures. Analysis of gluten with the Kieffer-rig makes it possible to measure gluten quality regardless of %PC. Large differences in %PC were observed from the climate chamber experiments because temperature had great effects on %PC. Flour material was limited, especially from the climate chamber and tunnel experiments. Therefore, the Kieffer-rig test on gluten was the best choice for the present study. Gluten was prepared from 10 g wholemeal flour in a Glutomatic 2100 (Perten Instruments AB, Sweden) and stretched after 45 min rest with the Kieffer-rig connected to the TA.XT plus Texture Analyzer.

3.3.4. Incubation of gluten proteins

The presence and extent of protease activity were indirectly measured by incubating the gluten proteins with the residue extract in an *in vitro* system according to Morel et al. (2000). They reported that polymeric proteins decreased after incubation (over 19 hours) when proteins were

extracted from proteolytically-active samples at ambient temperature, while they did not observe protein degradation when samples were extracted at 60 °C (Morel et al., 2000). The gluten proteins and remaining proteins (residue fraction) were sequentially extracted from wholemeal flour by 50% propan-1-ol + DTT and extraction/loading buffer (62.5 mM Tris-HCl, 2 % (w/v) SDS, 10 % (v/v) glycerol, 0.005 % (w/v) Bromphenol Blue with 1.5 % (w/v) DTT buffer (pH 6.8)), respectively. The gluten protein fraction was freeze dried and re-suspended in the residue fraction and incubated for 4 and 16 h. As a control, one of the sample mixtures was heated at 60 °C for 15 min to inactivate the enzyme activities prior to 16 h incubation. The proteins were then separated by SDS-PAGE to determine the degree of protein degradation. The comparison was based on the effects on the HMW-GS fraction since the degraded proteins overlapped with gliadins and LMW-GS fractions.

3.4. Statistics

Principal component analysis (PCA) was carried out to give an overview of the relationships between various parameters such as %PC, protein quality parameters (protein compositions, size distribution of gluten proteins and viscoelastic properties of gluten), cultivars, seasons or environmental factors such as temperature (Papers I-IV). In this method, the original variables are projected down to a new set of variables, called principal components (PCs). These are linear combinations of the original variables describing the most important variation in the data set. The PCs are expressed in decreasing order and represent independent characteristics. PCA gives a score plot of samples and a loading plot of variables, in which the PCs are used as new axes. They give an overview of how the properties are related to each other and how the properties predominantly distinguish the samples (Martens and Martens, 2001).

Data from each experiment were analyzed by analysis of variance (ANOVA), pairwise comparison was carried out by Tukey's test and simple linear regressions were performed (Papers I-III). Finlay-Wilkinson regressions (Finlay and Wilkinnson, 1963) for the R_{max} were calculated for the varieties against the environment (location*year) mean (Paper IV).

4. Studies of gluten proteins with western blot analysis

4.1. Western blot analysis of gluten proteins separated by SDS-PAGE

4.1.1. Material and methods

Western blot analysis of gluten proteins was carried out to identify each band on the gel. Gliadin, glutenin and remaining proteins (residue fraction) were sequentially extracted with 70% ethanol, 50% propan-1-ol + 4.5% DTT and extraction/loading buffer (62.5 mM Tris-HCl, 2 % (w/v) SDS, 10 % (v/v) glycerol, 0.005 % (w/v) Bromphenol Blue and 1.5 % (w/v) DTT (pH 6.8)), respectively from five cultivars (Avle, Berserk, Bjarne, Cadenza and Zebra) (see detail description in Paper I). They were separated on the gels and blotted onto nitrocellulose paper with an iBlot® Transfer system (Invitrogen, Life technology, USA) according to the manufacturer's instructions. The membrane was stained with Ponceau S solution (Sigma P7170, UK) and protein bands were marked on the membrane so that the bands recognized by the different antibodies could be identified. Membranes were then destained with Tris-buffered saline (TBS) (20 mM Tris, 500 mM NaCl, pH adjusted to 7 with HCl) and blocked with 5 % (w/v) skimmed dried milk in TBS for 1.5 h with gentle shaking at room temperature (RT). Following blocking, membranes were incubated in primary antibody solution [1 % (w/v) bovine serum albumin in 0.05 % (v/v) Tween in TBS] for 2 h with gentle shaking at RT. The primary antibodies used and their dilutions were as follows:

1) R2-HMG rabbit polyclonal, raised against an HMW subunit peptide (GYYPTSPQQPGC) and specifically recognizes HMW-GS (Denery-Papini et al., 1996), 1:8000.
2) F98-11 mouse monoclonal, raised against the N-terminal sequence (SHIPGLERPSGC) of LMW-GS (Institut National de la Recherche Agronomique, France INRA, Nantes, France), 1:3000.
3) IFRN 0610 mouse monoclonal antibody that recognizes an epitope (QQSF) common to many gliadins and LMW-GS, but not to HMW-GS (Institute of Food Research (IFRN), UK) (Brett et al., 1999), 1:8000.
4) Anti-NT1-ω rabbit polyclonal antibody, raised against the N-terminal sequence (ARELNPSNKELGC) recognises ω2-gliadins (Denery-Papini et al., 1994, 2000), 1:8000
5) Anti-NT2-ω rabbit polyclonal antibody, raised against the N-terminal sequence (SRLLSPRGKELGC) of ω5-gliadins (Denery-Papini et al., 2000, Tosi et al., 2011), 1:8000.

6) S3B512 mouse monoclonal antibody, raised against a peptide from the γ-gliadin repetitive domain (PEQPFPQGC) and specific for γ-gliadins (INRA, Nantes, France), 1:3000.

The membranes were washed three times in 0.05 % (v/v) Tween in TBS and incubated in a secondary antibody solution (either anti-mouse or anti-rabbit alkaline phosphatase conjugated at a 1:6250 dilution) for 1.5-2 h with gentle shaking at RT. The membranes were rinsed with 0.05 % (v/v) Tween in TBS for 5 min with gentle shaking, and twice in TBS. Protein bands recognized by the antibodies were visualized with a ready-made NBT/BCIP developing solution (Sigma-Aldrich, USA) in the dark for 5-10 min. To stop further reactions, the membranes were rinsed three times with distilled water.

4.1.2. Results and discussion

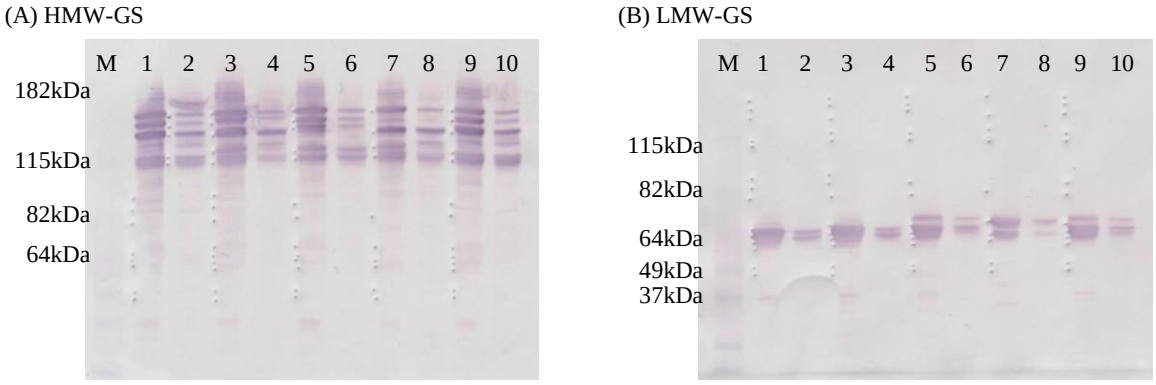

Figure 4.1.1 Western blot analysis of glutenin and the residue fractions with the antibodies for HMW-GS (A) and for LMW-GS (B). M:Marker, 1,2: Avle, 3,4: Berserk, 5,6: Bjarne, 7,8: Cadenza and 9,10: Zebra. 1,3,5,7 and 9: glutenin fraction and 2,4,6,8 and 10: residue fraction.

Analysis of the glutenin and the residue fractions with antibodies for HMW-GS and LMW-GS identified both HMW-GS and LMW-GS not only in the glutenin fractions, but also in the residue factions (Figure 4.1.1). The same observation was made even after the number of extraction steps with 50% propan-1-ol + DTT was increased (data not shown). The results indicated that some polymers could not be extracted by the reducing agent DTT alone, but needed to be solubilized by SDS. Hence, HMW-GS and LMW-GS were quantified in both the glutenin fractions and the residue fractions. Antibodies for gliadins (ω5-gliadins, ω2-gliadins and γ-

gliadins) recognized bands present in both the glutenin and in the gliadin fractions (Figure 4.1.2, A-C), but they did not recognize any bands in the residue fractions (Data not shown). The bands recognized by antibodies for gliadins in the glutenin fraction could result partially from contamination with gliadins in the glutenin fraction, however, they are more likely to be C-type and D-type LMW-GS that interacted with glutenin polymers by inter-chain disulphide bonds. The antibody for ω5-gliadins recognized more bands in Norwegian cultivars, especially Avle and Berserk which had three and four strong bands, respectively, compared to Cadenza (a U.K. cultivar) which had one single strong band (Figure 4.1.2, A). A previous study of total gluten proteins (extracted with 50% propan-1-ol followed by 50% propan-1-ol + 4.5% DTT) analyzed

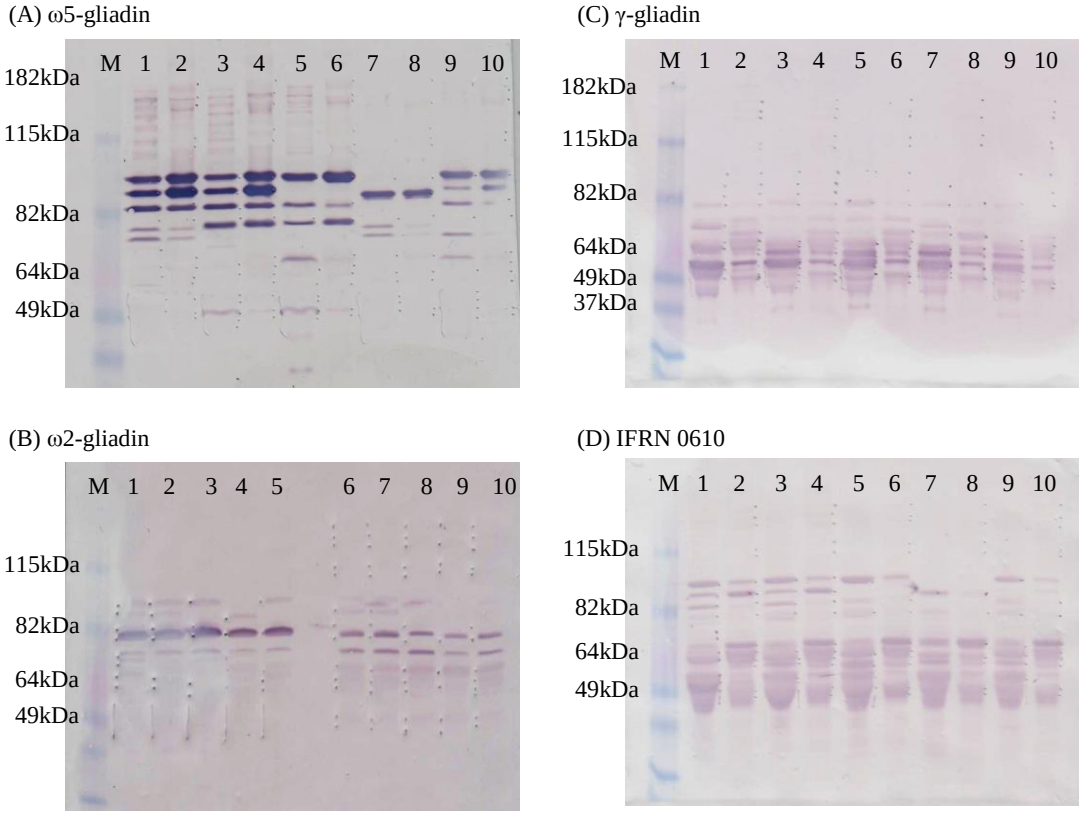

Figure 4.1.2 Western blot analysis of gliadin and glutenin fractions with the antibodies for ω5-gliadin (A), ω2-gliadin (B), γ-gliadin (C) and IFRN 0610 for all gluten proteins except HMW-GS (D).
For (A), (C) and (D); M:Marker, 1,2: Avle, 3,4: Berserk, 5,6: Bjarne, 7,8: Cadenza and 9,10: Zebra. 1,3,5,7 and 9: gliadin fraction and 2,4,6,8 and 10: glutenin. For (B); M:Marker, 1,6: Avle, 2,7: Berserk, 3,8: Bjarne, 4,9: Cadenza and 5,10: Zebra. 1-5: gliadin fraction and 6-10: glutenin fraction.

with antibodies for α-gliadins and γ-gliadins showed that they recognized bands in the same area, thus, α-gliadins and γ-gliadins were quantified together.

Comparing the results of western blotting and the gels stained with Coomassie Brilliant Blue allowed the identification of the bands (Figure 4.1.3) and the gluten proteins to be quantified more precisely. HMW-GS and LMW-GS were quantified both from the glutenin (marked with red in Figure 4.1.3, lane 1, 3, 5, 7 and 9) and the residue fractions. The bands recognized by the antibodies for ω5-gliadins and ω2-gliadins (marked with yellow and green, respectively in Figure 4.1.3) in the gliadin (lane 2, 4, 6, 8, and 10) and glutenin (1, 3, 5, 7 and 9) fractions were quantified as ω-gliadins and D-type (ω-type) LMW-GS, respectively. The rest of the bands below about 70kDa in the gliadin (lane 2, 4, 6, 8, and 10) and glutenin (1, 3, 5, 7 and 9) fractions were quantified as α- and γ-gliadins (combined) and C-type (α- and γ -type) LMW-GS, respectively.

Quantification of gluten proteins separated by SDS-PAGE gives an overview of the quantities or the proportion of each protein group, and western blot analysis improved the identification of each band. The method clearly separates HMW-GS and ω-gliadins from other gluten proteins, and it is relatively easy and inexpensive, thus it is useful especially for studying HMW-GS and ω-gliadins. However, SDS-PAGE does not clearly separate LMW-GS, α-gliadins and γ-gliadins from each other.

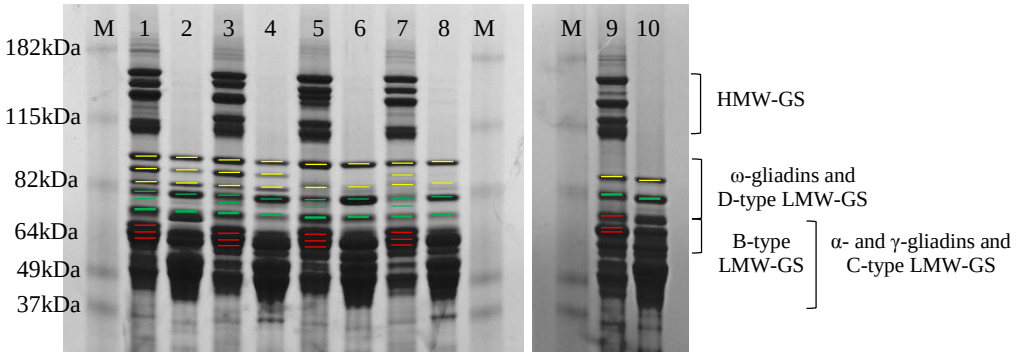

Figure 4.1.3 Gliadin and glutenin fractions separated by SDS-PAGE and stained with Coomassie Brilliant Blue. M: Marker, 1,2: Avle, 3,4: Berserk, 5,6: Bjarne, 7,8: Zebra and 9,10: Cadenza.1,3,5,7 and 9: glutenin fraction and 2,4,6,8 and 10: gliadin fraction. The bands marked with yellow were recognized by the antibody for ω5-gliadins, those with green were recognized by the antibody for ω2-gliadins and those with red were recognized by the antibody for LMW-GS in glutenin fractions.

4.2. Western blot analysis of SE-FPLC fractions

4.2.1. Material and methods

Analysis of each peak obtained by SE-FPLC were carried out by the fractionation, SDS-PAGE and western blotting. SDS-extractable and SDS-unextractable protein fractions were extracted from Bjarne (see detail description in Paper I). Both fractions were then separated on a Superose ®12HR 10/30 column connected to ÄKTA SE-FPLC (GE Healthcare life science, UK) and each peak was collected with a fraction collector. The collected fractions were then freeze-dried and re-dissolved with loading buffer [62.5 mM Tris-HCl, 2 % (w/v) SDS, 10 % (v/v) glycerol, 0.005 % (w/v) Bromphenol Blue with/without 1.5 % (w/v) DTT buffer (pH 6.8)]. The fractions were separated on NuPAGE® NOVEX 4-12% Bis-tris Mini Gels (Invitrogen AS, USA). The gels were either stained with Coomassie Brilliant Blue to visualize the protein bands, or analyzed further by western blotting (described above in 4.1) to identify each protein band in each peak.

4.2.2. Results and discussion

SE chromatography separates protein polymers and monomers based on their molecular size as described in section 3.3.2. Chromatograms of SDS-unextractable and SDS-extractable fractions from Bjarne which were separated by SE-FPLC are shown in Figure 4.2.1, A and B, respectively. The SDS-unextractable fraction gives one main peak, denoted F1*, which consists of large polymeric proteins. The SDS-extractable fractions were separated into four main peaks, denoted F1-F4. F1 and F2 were considered to contain polymeric proteins, while F3 and F4 were considered to contain monomeric proteins. The fractionated peaks were reduced by DTT, separated by SDS-PAGE and visualized by western blotting with antibodies for HMW-GS, LMW-GS, γ-gliadins, ω5-gliadins, ω2-gliadin and IFRN 0610 which recognized gliadins and LMW-GS (Figure 4.2.1, C). Most of the HMW-GS and the LMW-GS were present in F1*and F1, and only minor amounts were present in F2. Only traces of ω-gliadins were present in F1*, and some observed in F1. The antibody for ω5-gliadins revealed high concentrations in F2 and F3, while the antibody for ω2-gliadins showed high concentrations in F3 and F4. The antibody for γ-gliadins detected the highest concentration in F3, followed by F4 and minor amounts in F1*, F1 and F2. Comparisons between the results with IFRN0610 and other gliadins indicated that α-gliadins were most abundant in F4. Separation of unreduced F1* and F1 showed no clear band on the gels, indicating that they

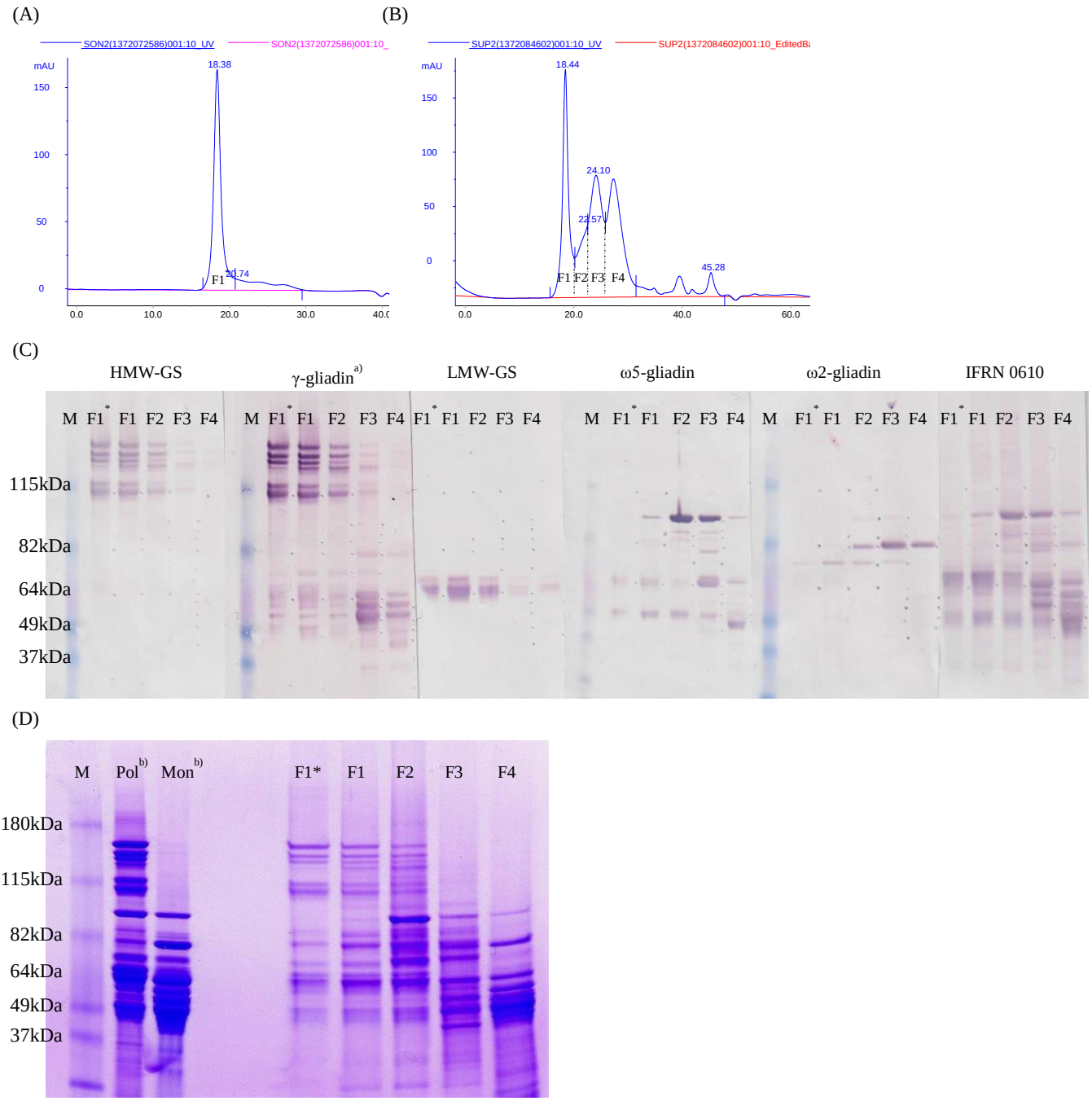

Figure 4.2.1 Chromatograms of SDS-unextractable fraction (A) and SDS-extractable fraction (B) extracted from Bjarne and separated by SE-FPLC. Western blot analysis (C) and SDS-PAGE (D) of each peak from the SDS-unextractable (F1*) and the SDS-extractable (F1-F4) fractions. Samples were reduced by DTT. M:Marker

a) The membrane was used for western blot with the antibody for HMW-GS previously. The bands recognized by γ-gliadin antibody were below 82 kDa. b) SDS-PAGE included polymeric and monomeric proteins extracted from flour by 50% propan-1-ol+DTT and 70% ethanol, respectively.

comprised large polymers that could not migrate into the gels, and the antibodies for HMW-GS and LMW-GS did not detect any bands (Figure 4.2.2, B). These results show that F1* and F1 contained polymeric proteins. The bands recognized by the antibodies for ω-gliadin in F1*and F1 were assumed to be D-type LMW-GS, however, IFRN 0610 also recognized some ω-gliadin bands in unreduced F1* and F1 fractions (Figure 4.2.2, B). This suggests that some ω-gliadins exist as monomeric proteins which bind tightly to large polymeric proteins by non-covalent bonds. IFRN 0610 did not recognize any other gliadins and LMW-GS in the unreduced F1* and F1 fractions. Hence, the bands recognized by the antibody for γ-gliadin and IFRN 0610 in the reduced F1* and F1 fractions were assumed to be C-type LMW-GS which interacted with polymers by inter-chain disulphide bonds. There appeared to be no differences between F1*and F1 fractions in their contents of HMW-GS, while F1 contained more LMW-GS (include C- and D-type). Hence, the ratio of HMW-GS to LMW-GS appeared to be lower in F1 resulting in lower molecular weight polymers compared to those in F1*. This may explain why they were more readily extractable in the SDS solution. F3 and F4 contained monomeric proteins, which in F3 was represented mainly by ω-gliadins and γ-gliadins and in F4 by α-gliadins. F2 appeared to contain a mixture of polymeric proteins and ω5-gliadins. Further analysis of F2 peak would be required to quantify the monomeric and polymeric components present in this fraction.

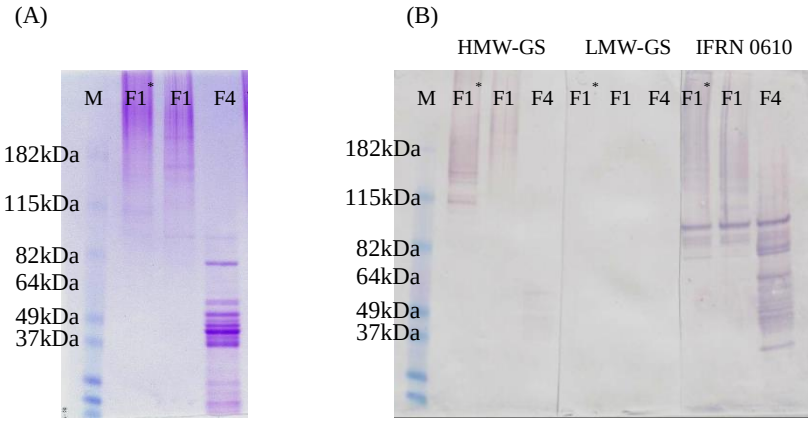

Figure 4.2.2 SDS-PAGE (A) and western blot analysis (B) of unreduced F1*, F1 and F4. Antibodies for HMW-GS, LMW-GS and IFRN 0610 which recognizes LMW-GS and gliadins were used for western blot analysis.

5. Main results and discussion

5.1. Temperature effects

Detailed results from each experiment are described in each Paper. In this section, the main results from different experimental approaches were assessed with focus on temperature effects, and the results were compared between the different experimental approaches to draw conclusions. Since Bjarne was included in all experiments (Papers I-IV), the results for Bjarne are extracted from each experimental approach and compared.

5.1.1. Grain weight and protein content

The results from the climate chamber experiments showed that temperature during grain filling had a large effect on the duration of grain filling, thousand grain weight (TGW) and %PC (Papers I and II, Figure 5.1.1, A and D). The increase in TGW was more gradual at the beginning of grain filling when plants were grown at lower temperatures compared to higher temperatures, while, lower temperatures prolonged the duration of grain filling (Paper I) which caused an increase in TGW (Papers I and II). However, low temperature had little effect on the amount of protein accumulated per grain when plants did not receive post-anthesis fertilization, and %PC in grain grown at low temperature consequently decreased (Papers I and II). The observed results support previous reports (Altenbach et al., 2003, DuPont et al., 2006b, Gooding et al., 2003, Sofield et al., 1977a, 1977b, Uhlen et al., 1998, Wheeler et al., 1996). When post-anthesis fertilization was applied in climate chamber experiments (Exp3 in paper I), the protein content per grain greatly increased in grain grown at low temperatures. Plants grown at low temperatures could benefit the available N for a longer period. Plants grown at higher temperatures, on the other hand, had a shorter period of grain filling, and post-anthesis fertilization did hardly affect the %PC in mature grain. Thus, differences in %PC between Exp1 without post-anthesis fertilization and Exp3 with post-anthesis fertilization were larger at lower temperatures compared to higher temperatures. Our results are in agreement with Altenbach et al. (2003) who reported that post-anthesis fertilization increased %PC at lower temperature (24/17 °C), but not at higher temperature (37/28 °C).

The temperature gradient inside the growth tunnel also influenced the period of grain filling. Grain from plants located close to the open end of the growth tunnel, had higher moisture content at harvest compared to those from plants located in the inner parts of the growth tunnel (Paper III). TGW decreased and %PC increased with temperature within seasons (Paper III). There was also year to year variation in %PC. The %PC decreased from 2010 to 2011 even though the temperature during grain filling was higher in 2011 than in 2010 (Paper III, Figure 5.1.1, E). This was most likely caused by higher yield in 2011 compared to 2010 (Paper III).

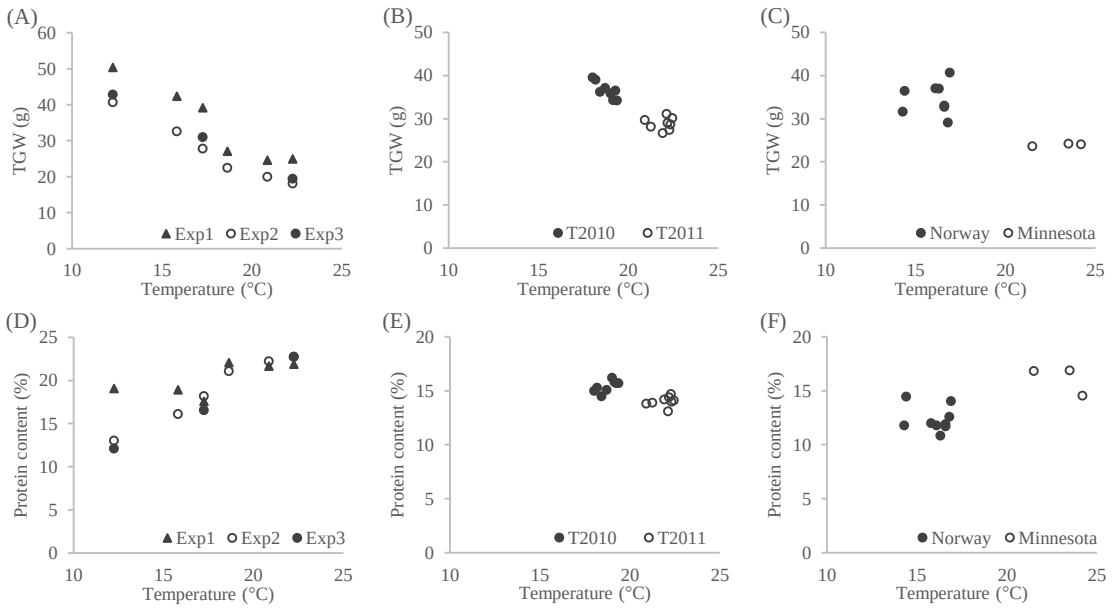

Figure 5.1.1 Thousand grain weight (TGW) (g) on fresh weight basis (A, B and C) and protein content (%) (D, E and F) of Bjarne from climate chamber (from Papers I and II) (A and D), growth tunnel (from Paper III) (B and E) and field (Paper IV) (C and F). Exp1 and Exp2 from Paper I and Exp3 from Paper II.

The relationship between temperature and TGW as well as %PC observed from the field experiments were consistent with the results obtained from the climate chamber experiments when TGW and %PC were compared between the two-mega environments. Lower temperatures in Norway resulted in higher grain weight and lower %PC compared to grain grown in the USA (Paper IV, Figure 5.1.1, C and F). However, when they were compared between the locations within each environment, inconsistent relationships with temperature were observed (Paper IV, Figure 5.1.1, C and F).

The results from the field and the growth tunnel experiments clearly showed that not only temperature but also other environmental factors influence the grain weight and %PC.

5.1.2. The composition of gluten proteins

The climate chamber experiments carried out without post-anthesis fertilization (Exp1 and 2 in Papers I and paper II) showed that the proportions of ω-gliadins and D-type LMW-GS increased, and the proportions of α- and γ-gliadins and B-type LMW-GS decreased with temperature increase. The proportion of HMW-GS slightly increased with temperature, but significant temperature effects were observed only from Exp1 ($P<0.05$) (Papers I and II). Although temperature had little influence on the protein content per grain, Paper II showed that the N:S ratio increased with temperature even though available S in soil for plants was assumed to be sufficient. The higher N:S ratio in grain grown at higher temperature (C, 37/28 °C compared to 24/17 °C) was also reported by DuPont et al. (2006a). DuPont et al. (2006a) applied post-anthesis S but it did not influence the composition of gluten proteins, thus the authors suggested that high temperature is one of the environmental conditions that increase the proportion of S-poor proteins in flour similar as S-deficiency or abundant N.

No relationship was found between temperature and the ratio of monomeric to polymeric proteins in the climate chamber experiments or in the growth tunnel experiments (Papers I-III). Moreover, insignificant temperature effects were found on the ratio of HMW-GS to LMW-GS and the ratio of B-type LMW-GS to C- and D-type LMW-GS (Papers I and II). Hence, the changes in the proportion of gluten proteins due to temperature seemed to occur within the protein groups, for example the increase in ω-gliadins was compensated for by a decrease in α- and γ- gliadins and thus did not change the gliadin:glutenin ratio. Moreover, temperature had little influence on the proportion of HMW-GS, the most important contributor to gluten strength. The temperature effects on the proportion of gluten proteins were not observed when plants received post-anthesis fertilization (Paper I).

5.1.3. Size distribution of glutenin polymers

The temperature effects on the size distribution of glutenin polymers were dependent on cultivar. %UPP clearly increased with temperature for Cadenza (UK cultivar) (Paper I). In other words, Cadenza samples grown at low temperatures (13-13 °C and/or 13-18 °C) during grain filling had considerably lower %UPP compared to those grown at 23-23 °C (Paper I). In two Norwegian cultivars, Avle and Bjarne, %UPP showed a slight increase from the samples grown at 13-13 °C to those grown at 18-18 °C, while it decreased in samples grown at 23-23 °C (Papers I and II) with Avle showing larger differences. Temperature had little effect on %UPP in Berserk and Zebra (Paper II). No significant differences were observed in %UPP in the four Norwegian cultivars grown between 13-13 °C and 18-18 °C (Paper II), indicating that low temperatures did not have negative effects on the assembly of glutenin polymers. Malik et al. (2011), (2013) observed a positive correlation between %UPP and temperature in comparable temperature ranges (17/14 °C compared to 22/19 °C). The results from the growth tunnel experiments showed a positive linear relationship between temperature during grain filling and %UPP in Bjarne and Zebra (Paper III). Even though the temperature gradient obtained inside the growth tunnel was smaller compared to that used in the climate chamber experiments, positive and significant temperature effects were found on %UPP in both years, except for Bjarne grown in 2010 (Paper III). These results indicate that temperature during grain filling influenced the assembly of glutenin polymers, however, its effects seem to be cultivar dependent (Papers I-III).

5.1.4. Viscoelastic properties of gluten

The viscoelastic properties of gluten were measured in samples from Exp2 (Papers I and II). Significant differences were not observed between grain developed at 13-13 °C and 18-18 °C in the four Norwegian cultivars (Paper II). Notably, the highest R_{max} value was observed from Berserk, Bjarne and Zebra grown at 13-13 °C (Paper II). Hence, detrimental effects of low temperature (< 18 °C) on gluten quality was not observed, at least in cultivars bred and/or grown under Norwegian climate conditions. The R_{max} value in Avle grown at 23-23 °C decreased considerably (Paper II). A positive correlation was obtained between %UPP and R_{max} (r=0.73, P<0.001) (Paper II), hence the temperature effects on the viscoelastic properties of gluten were associated with changes in the

assembly of glutenin polymers rather than with changes in the proportion of gluten proteins due to temperature (Papers I and II).

An inconsistent relationship between temperature and the viscoelastic properties of gluten was observed from the growth tunnel experiments (Paper III). High and significant positive linear relationships were found between temperature and R_{max} for both Bjarne and Zebra in one season, but not in the other season, even though positive and significant relationships were found between temperature and %UPP (Paper III). Considerably lower values for %UPP as well as for R_{max} were obtained from Zebra grown at the lower temperatures (Paper III), although the results of R_{max} value differed from what is generally obtained for this cultivar (unpublished data). Since the average mean day temperature during grain filling was 22 °C (Paper III) during this growing season, it is unlikely that temperature was the factor determining the low %UPP and R_{max}. Therefore, other environmental factors must be involved.

The relationship between temperature and the viscoelastic properties of gluten was more complex in the field experiments (Paper IV and Figure 5.1.2, C). A large variation in R_{max} was observed between environments (i.e. locations, seasons and two mega-enviroments) (Paper IV). Generally, stronger gluten was observed from grain grown in the USA compared to grain grown in Norway, as the average R_{max} was higher in samples grown in the USA (Paper IV, Figure 5.1.2, C). However, when average R_{max} was compared between field locations and/or seasons within and between the mega-environment(s), inconsistent relationships between temperature and R_{max} were observed (Paper IV). Vollebekk in 2011 and Rød in 2009 and 2010 had higher average values for R_{max} that were comparable to the R_{max} values obtained from three locations in the USA, although the temperature differences between two mega-environments were large (Paper IV).

5.1.5. Overall discussion of the temperature effects on gluten quality

The results from three different experimental approaches show the complexity of temperature effects on gluten quality. The low temperature effects on gluten quality in the Norwegian wheat cultivars studied in the three experimental systems show two conflicting results. Papers I, II and one part of Paper IV showed that lower temperatures had little effect on gluten quality. Constant low temperature (13-13 °C) did not have detrimental effects on gluten quality (%UPP and R_{max}) in the four Norwegian cultivars (Papers I and II, Figure 5.1.2, A). Moreover, comparable R_{max}

values were also observed from grain grown in Norway and the USA, even though temperature differences between the two mega-environments were large (Paper IV, Figure 5.1.2, C). On the other hand, Paper III and other part of Paper IV showed that low temperature was associated with negative effects on gluten quality. Experiments in the growth tunnel showed that both %UPP and R_{max} increased with temperature (Paper III, Figure 5.1.2, B), and the R_{max} values were generally lower in grains grown in Norway compared to those grown in the USA (Paper IV, Figure 5.1.2, C).

The climate chamber experiments clearly revealed that strong gluten could be obtained even if the temperature during grain filling was as low as 13 °C (Papers I and II, Figure 5.1.2, A). However, weaker gluten was observed both in the growth tunnel and field experiments when temperature during grain filling was in the lower ranges. Therefore, other environmental factors related to lower temperatures were presumed to cause poor gluten quality.

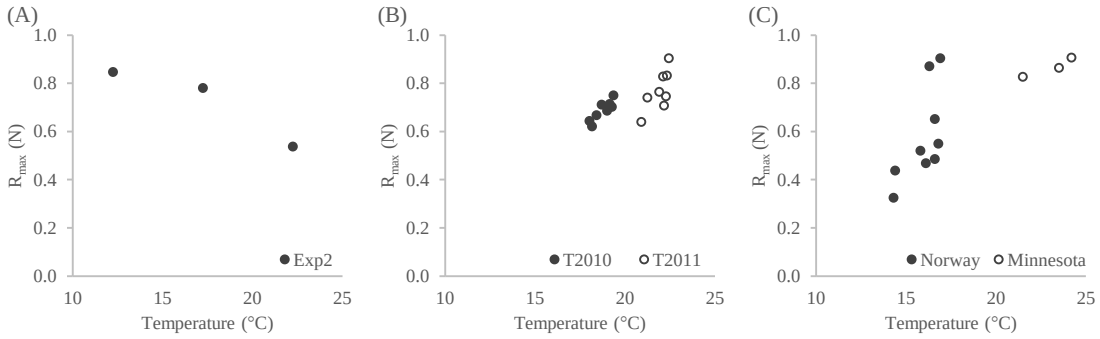

Figure 5.1.2 R_{max} (N) of Bjarne grown in the climate chamber (A) (Paper II), in the growth tunnel (B) (Paper III) and in field (C) (Paper IV) experiments.

5.1.6. Cultivar difference

Lower %UPP was observed in the UK cultivar Cadenza at lower temperatures, thus low temperature seems to have a weakening effect on gluten quality in this cultivar (Paper I). However, the weakening effect of low temperature on gluten quality was not observed in the four Norwegian cultivars (Papers I and II). Among the four Norwegian cultivars, Berserk and Zebra showed no changes in %UPP due to temperature, while some changes were observed for Avle and Bjarne (Paper II). An earlier study showed that Berserk had the stronger gluten among Norwegian

cultivars, and the present study revealed that it also had a more stable gluten strength across the environments (Papers IV) as well as across different temperatures (Paper II). Hence, Berserk seemed to be a good candidate to use in breeding programs, although more research is needed to confirm this observation.

5.2. An environmental factor causing extreme gluten quality deficiency (Paper V)

Field experiments with winter wheat carried out between 2005 and 2013 showed a large variation in the viscoelastic properties of gluten (Paper V). In general, higher R_{max} values were obtained when the weather during grain filling was warm and dry, while lower R_{max} values were obtained when the weather was cool and wet (Paper V), which supports the previous report by Moldestad et al. (2011). However, a considerably lower average R_{max} values (< 0.3 N) were observed in several locations in the 2007 and 2011seasons compared to other seasons. The weather conditions in these seasons were relatively similar in temperature to the other seasons (2008-2010 and 2012), but considerably higher precipitation was recorded especially in 2007 (Paper V). Moreover, samples with strong gluten were observed from other fields within the seasons (Paper V). Therefore, two cultivars, Finans and Olivin, from four fields, three fields with considerably low average R_{max} and one field with relatively high R_{max}, in the 2011 season were further analyzed to determine the composition and the characteristic of gluten proteins (Paper V).

Gluten prepared from Finans from three fields showed extremely low R_{max} values and these samples also had considerably low %UPP in flour (Paper V). Incubating gluten proteins with the residue fraction in the *in vitro* system resulted in degradation of gluten proteins in the samples possessing extremely low R_{max} (Paper V). The results implied protease activity. Re-polymerization of large glutenin polymers takes place during dough rest (Aamodt et al., 2005), however, %UPP in gluten prepared from these samples did not increase after 45 min rest. Hence, extremely low R_{max} in these samples were caused by low MW of the glutenin polymers in the flour and by protease activities in hydrated flour. The origin of these proteases remained unclear, but exogenous proteases derived from *Fusarium* seemed to be plausible candidates for observed protein degradation. This is because the samples with extremely low R_{max} values, low %UPP and severe protein degradation after 4 h incubation also had high proportions of grain to be infected by *Fusarium* (Paper V). Our observation agrees with previous reports showing that exogenous

proteases derived from *Fusarium* have negative effects on gluten protein as well as a weakening effect on dough (Capouchova et al., 2012, Dexter et al., 1996, Nightingale et al., 1999, Wang et al., 2005). Olivin seemed to be more resistant to the factors that adversely affect gluten quality compared to Finans (Paper V).

Temperature and moisture are important weather factors for *Fusarium* infection and development (reviewed by Parry et al. (1995)). Bernhoft et al. (2013) reported that high precipitation at flowering and during maturation in recent seasons resulted in increases in *Fusarium* infection in Norway. Low temperatures during grain filling are usually associated with precipitation in Norwegian summers. It can therefore be concluded that the poor gluten quality observed in the seasons with low temperature was at least to some extent caused by *Fusarium* infection.

6. Conclusions and further perspectives

Temperature affect the proportion of gluten proteins in a similar manner for all cultivars investigated in the climate chamber experiments. However, temperature effects on the size distribution of glutenin polymers were cultivar-dependent. The high and positive correlation between %UPP and R_{max} indicated that changes in the viscoelastic properties due to temperature were associated with the assembly of glutenin polymers rather than changes in the proportion of gluten proteins. Papers I and II showed that the detrimental effect of low temperatures was not observed in the four Norwegian cultivars. The results from three experimental approaches (Papers I-IV) revealed that low temperature in itself had little or no direct negative effect on gluten quality. However, other environmental factors associated with low temperature are assumed to cause weak gluten. Hence, low temperatures are suggested to have "indirect effects" on gluten quality. Paper V suggested that *Fusarium* infection was one such environmental factor. Further studies are necessary to confirm the relationships between the weather conditions, *Fusarium* infection and their effects on gluten quality in Norwegian wheat cultivars, including both spring and winter wheat. Moreover, it would be of great interest to understand the mechanisms that determine how Olivin has a higher tolerance to unfavorable conditions compared to Finans.

Climate chamber experiments showed a detrimental effect of low temperature only in Cadenza (UK cultivar), while it was not observed in the four Norwegian cultivars. Between the four

Norwegian cultivars, Beserk was shown to be the most promising genetic source in future breeding programs as a source of both strong and stable gluten quality. Further research is needed to understand why (genetic background) and how (mechanisms) the gluten quality of Berserk is better and more stable to environmental factors that result in weaker gluten compared to other cultivars.

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Paper I

Influence of temperature during grain filling on the composition and the polymerization of gluten proteins

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Abstract

One Norwegian and one UK spring wheat cultivar, Bjarne and Cadenza, respectively, were grown in climate chambers during grain filling to investigate the effects of lower to moderate temperatures on the gluten quality. Two experiments were carried out with fertilization until anthesis, while post-anthesis fertilization was applied in a third experiment.

The proportion of different gluten proteins was affected by temperature in a similar manner for both cultivars when grown without post-anthesis fertilization. However, whereas low temperature strongly decreased %UPP for Cadenza, Bjarne had high %UPP at all temperature regimes. The results indicated that the assembly of glutenin polymers in Bjarne was less sensitive to variation in temperature than in Cadenza. Thus, our results suggested that the temperature influenced the proportion of different gluten proteins in both cultivars, while its effects on the assembly of the glutenin polymers were cultivar dependent.

The duration of grain filling was longer at the lower temperatures, and this was associated with increased grain weight. Temperature had little effect on the amount of protein accumulated per grain, thus the proportion of proteins was strongly decreased at lower temperatures. This was to some extent, but not fully counteracted by post-anthesis fertilization.

Keywords:

Wheat, Gluten quality, Low temperature, Post-anthesis fertilization, Grain weight

Introduction

Wheat is one of the most important crops worldwide, being adapted to a wide range of edaphic and climatic conditions including soil properties, day length, temperature and precipitation. However, the quality of wheat for making bread and other end products can be severely influenced by its growth conditions. Environmental effects on baking quality may have diverse causes and are still not fully understood. The temperature during grain filling has been considered as one of the most important factors affecting gluten quality (Blumenthal et al., 1993). Since large areas of wheat cultivations experience high temperatures during the growing season, the effects of high temperature on wheat quality are well documented. High temperatures (>30-35 °C) during grain filling are reported to have a weakening effect on the dough strength (Blumenthal et al., 1991; Randall and Moss, 1990) which is associated with changes in composition of gluten proteins (Daniel and Triboi, 2000; DuPont et al., 2006a; Hurkman et al., 2013) and a decrease in the glutenin to gliadin ratio. High temperatures have also been reported to decrease the proportion of large glutenin polymers (Blumenthal et al., 1995; Don et al., 2005).

Although wheat cultivation is well established in areas with lower temperatures, such as Scandinavia, the effects of low temperature on gluten quality are still poorly understood. Greater knowledge is important in order to produce wheat with good and stable baking quality under such environments. This may be even more important for the future as wheat cultivation areas might need to be expanded to cooler regions as a consequence of climate change and the projected increase in wheat demand.

Baking quality is largely dependent on the gluten proteins which are classified into two groups: the monomeric gliadins that contribute to dough extensibility and viscosity, and the polymeric glutenins that contribute to dough elasticity. The monomeric gliadins are further classified into three subgroups, α-gliadin, γ-gliadin and ω-gliadins while the glutenin polymers consist of two groups of subunits, high molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS) linked by inter-chain disulphide bonds. The LMW-GS are further divided into B-, C- and D-types. The B-type LMW-GS are the major group of LMW-GS and have two cysteine residues that form inter-molecular disulphide bonds and therefore act as extender of the glutenin polymer chains. By contrast, the C- and D-type LMW-GS have sequences related to the monomeric gliadins, but have an additional unpaired cysteine residues allowing their incorporation into glutenin polymers. Hence they are considered to act as terminators of glutenin polymer (reviewed by D'Ovidio and Masci (2004)).

Gluten proteins are deposited from the early grain filling phase. Shewry et al. (2009) detected their deposition at 10 days after anthesis (daa) by SDS-PAGE, and their accumulation continued throughout grain filling. They are initially assembled into polymers as they are synthesized but the proportions of large polymers increase during the desiccation/maturation phase at the end of seed development (Shewry

et al., 2009). The viscoelastic properties of gluten are correlated with the proportion of larger glutenin polymers, which have also been classified as SDS-unextractable polymeric proteins (UPP) (Gupta et al., 1993), as well as the ratio of gliadin to glutenin (Uthayakumaran et al., 2000). The molecular weight of glutenin polymers is related to the ratio of B-type LMW-GS to C-type and D-type LMW-GS as well as the ratio of HMW-GS to LMW-GS (MacRitchie and Gupta, 1993). Hence, allelic variations in those gluten proteins play an important role in dough strength, as Payne (1987) and Shewry et al. (1992) reported the relationships between allelic variation in HMW-GS composition and dough strength.

It has been reported that cool and humid weather during grain filling in Scandinavia results in weak gluten strength (Johansson and Svensson, 1998; Moldestad et al., 2011). A climate chamber experiment with temperatures ranging from 9 to 21 ºC during grain filling also showed negative effects of low temperature on dough properties (Uhlen et al., 1998). Field experiments carried out in Norway showed strong correlations between temperatures during grain filling and gluten strength, with temperatures below 17-18 °C resulting in weaker gluten (Moldestad et al., 2011). This observation was most pronounced when plants experienced low temperatures from early to middle stage of grain filling. Similarly, Panozzo and Eagles (2000) reported that high temperature (>30 °C) during the first 14 daa significantly affected the composition of gluten proteins and dough functionality in a negative way. Hence, both studies suggest that the beginning of grain filling is sensitive to temperatures (both low and high) with effects on the final gluten quality at maturity. We have therefore explored the effects of low to moderate temperatures on the proportions of gluten proteins and the assembly of glutenin polymers, using temperatures ranging from 13 to 23 °C and combinations of low temperatures during the early period of grain filling.

Material and Methods

Plant materials

The Norwegian spring wheat (*Triticum aestivum L.*) cultivar Bjarne and the British cultivar Cadenza were grown in temperature controlled climate chambers at the Norwegian University of Life Sciences, Ås, Norway. Three experiments (named Exp1, Exp2 and Exp3) were carried out with two complete biological replicates (separate growth chambers) for each experiment. Plants were raised in a glasshouse until anthesis under natural light with supplemented high-pressure sodium lights to maintain a day length of 16 h. Seeds were sown in early March for all experiments in order to make the growing condition until flowering as equal as possible. The temperature was set to at minimum day/night temperature of 15/10 °C. Four plants were grown in a 3 L pot (L.O.G. AS, Oslo, Norway) filled with 1.15 kg of commercial plant soil (pH 5.5-6.5) containing 86% Sphagnum peat, 10% fine sand and 4% clay (L.O.G. AS, Oslo, Norway containing 900mg/L nitrogen (N), 190 mg/L potassium, 35 mg/L phosphorus and mixture of micronutrient (L.O.G. AS, Oslo, Norway). The pots were rotated weekly to minimize positional effects in the greenhouse. Two types of nutrient solution (dissolved Superba^{TM} Rød

and Ks Calcinit™) were mixed and applied as N: 0.148, P: 0.037, K: 0.185 g/ pot/ time. Fertilizer was first applied at Zadoks (Z) 15 (Zadoks et al., 1974) and continued weekly with the last application at Z59 (just befor anthesis) in Exp1 and Exp2 and at Z77 (340 day degrees after anthesis (d°aa)) in Exp3. Just before anthesis the plants were moved to the temperature controlled climate chambers where they grew further at a long day regime (day/night of 18/6 h respectively) until maturity. Three constant day/night regimes of 13/10 °C (denoted 13-13), 18/15 °C (denoted 18-18) and 23/20 °C (denoted 23-23) during the whole grain filling period were tested in all experiments. Additionally, three regimes combining two temperatures were used in Exp1 and Exp3: 13/10 °C in the first phase combined with 18/15 or 23/20 °C (denoted 13-18 and 13-23, respectively) until maturation, and 18/15 °C in the first phase combined with 23/20 °C (denoted 18-23). Plants were moved from lower to higher temperature at 207 d°aa to achieve the temperature shift. The first phase was mainly covering the differentiation phase and the later phase was covering most of the accumulation of storage compounds and maturation period. Relative humidity (RH) in the climate chambers were registered hourly and average RH were about 75-80% in Exp1, and 80-90 % in Exp2 and Exp3.

Average light intensity in the chamber was 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the position of ears. The date of anthesis of main stems and equally developed tillers were tagged and 10 tagged ears were harvested at 204, 272, 340 and 544 d° in Exp1 and at 102, 136, 204, 272, 340 and 544 d° in Exp3. The remaining tagged ears were harvested shortly after maturation in both experiments, pooled, dried and threshed for further analyses. Ears from main stems and equally developed tillers were harvested shortly after maturation in Exp2, and prepared as for Exp1 and Exp3.

Immature grain

For the samples harvested during grain development, two grains located at the base of each spikelet were collected from well-developed spikelets (about seven to ten spikelets) from the middle of a spike resulting in 14-20 grains per ear, and their fresh weight (mg) was measured. The moisture content was measured from grains collected from one of the ten ears and grain dry weight (GDW) (mg) was calculated based on the water content. Average GDW (g) was calculated from the results of ten ears for each temperature regime for each cultivar.

Mature grain and flour analysis

Grain weights of mature grains were measured based on a representative sample of approximately 300 grains, and is presented as GDW (mg). Mature grains were milled to wholemeal in a Laboratory Mill 3100 (Perten Instruments AB, Huddinge, Sweden) with a 0.8 mm sieve. Total N content of flour was measured by the Dumas method according to Bremner and Mulvaney (1982) and expressed as percent dry matter. Protein content in flour (%PC) was then calculated by multiplying the N content by

5.7. Protein content per grain (PCG) was calculated on a dry weight basis and expressed as mg protein/grain.

SDS-PAGE

Gluten proteins were extracted sequentially in three fractions according to Tosi et al. (2011) with modifications. Monomeric proteins were extracted by adding 0.5 ml of 70% ethanol to 20 mg wholemeal flour and stirred for 30 min at room temperature. The mixture was centrifuged for 15 min at 3700 g. The extraction procedure was repeated twice and the supernatants were pooled in an Eppendorf tube and dried by Speedy Vac (gliadin fraction). Polymeric proteins were then extracted by adding 0.5 ml of 50 % propan-1-ol and 4.5 % DTT to the remaining flour pellet and heating for 15 min at 60 °C. Homogenization was then carried out twice for 20 s at 5500 rpm speed with 10 s pause between the homogenization steps using a Precellys ®24 (Bertin Technologies), and centrifuged for 15 min at 3700 g. The extraction procedure was repeated twice and the supernatants were pooled into an Eppendorf tube and dried by Speedy Vac (glutenin fraction). The remaining flour pellet was dried by Speedy Vac, and non-gluten proteins were extracted with extraction/loading buffer: 62.5 mM Tris-HCl, 2 % SDS, 10 % glycerol, 0.005 % Bromphenol Blue and 1.5 % DTT buffer (pH 6.8) (residue fraction). Dried gliadin and glutenin fractions were re-suspended in extraction/loading buffer (see above). Samples were denatured at 90 °C for one min, and each fraction was separated on NuPAGE®NOVEX 4-12% Bis-tris Mini Gels (Invitrogen AS). Electrophoresis was carried out with 40 V for 20 min followed by 140 V for 105 min. The gels were stained with 40 % (v/v) methanol, 10 % (w/v) trichloroacetic acid (TCA) and 0.1 % (w/v) Coomassie Brilliant Blue R-250 for 3 hours, and destained with 10% (w/v) TCA for 18 hours.

Gels were scanned with an Epson perfection 4990 PHOTO scanner and quantified by densitometry with ImageQuant TL software (GE Healthcare life science, UK). The volume of each protein band was determined after background subtraction using the rubber band method. All gels contained a duplicate reference sample. To optimize the amount of sample for loading on the gel, a dilution series of each protein preparation was analyzed, and concentrations were selected within the linear response region for all protein bands. Each protein band in the sample was quantified relative to the density of a specific band in the reference sample from the same gel. This made it possible to compare the samples between the gels. Western blot analyses were carried out to identify bands using a series of antibodies for gluten proteins according to Tosi et al. (2011). The analyses showed that HMW-GS and LMW-GS were present in both the glutenin and the residue fractions, thus they were quantified from both fractions and the values combined. Total gluten proteins were determined as the sum of all quantified bands from the three fractions. Each group of gluten proteins was then divided by the value of total gluten proteins and expressed as proportion (%). The ratios of gliadins to glutenins (Gli:Glu), HMW-GS to LMW-GS

(HMW:LMW) and B-type LMW-GS to C- and D-type LMW-GS (B-type:C- and D-type LMW) were also calculated.

Size exclusion Fast performance liquid Chromotagrophy (SE-FPLC)

Wholemeal flour samples were extracted sequentially to obtain SDS-extractable and SDS-unextractable fractions according to Morel et al. (2000) with modifications described by Tronsmo et al. (2002). 30 mL of 1 % SDS, 0.1 M sodium phosphate extraction buffer (pH 6.9) was added to 240 mg wholemeal flour and continuously stirred for 80 min at 60 °C. The mixture was centrifuged at 37,000 g for 30 min at 20 °C (Beckman L-80 Ultracentrifuge, Rotor 50.2Ti) and the supernatant was recovered (SDS-extractable protein fraction). 30 mL extraction buffer was added to the remaining flour pellet and sonicated by Sonics VC130 (Sonics and Materials, Newton, CT 06470 - 1614, USA) for 3 min (with 70% automatic amplitude compensation). The SDS-unextractable protein fraction was obtained after centrifugation (same condition as above). Each fraction was filtered (Millex-HV 0.45 µm, Millipore, Irland) and 100 µl of each sample was separated on Superose ®12HR 10/30 column at ÄKTA SE-FPLC (GE Healthcare life science, UK) with 0.1% SDS, 0.08 M NaCl, 0.05 M Sodium phosphate elution buffer (pH 6.9) at flow rate 0.4 ml/min. All samples were extracted and run twice (technical replicates) and average from the technical replicates were used in the statistical analysis.

The SDS-unextractable fraction gives one main peak, denoted F1*, and consists of large polymers. The SDS-extractable fraction gives four main peaks, with F1-F2 consisting of polymeric proteins and F3-F4 consisting of monomeric proteins. The proportion of SDS-unextractable polymeric proteins in total polymeric proteins (%UPP) was calculated as [F1*/(F1*+F1)×100] and the ratio of monomer to polymer (Mon:Pol) as [(F3+F4)/(F1*+F1+F2)].

Statistical analyses

Data from each year was analyzed by analysis of variance (ANOVA), and pairwise comparison was carried out by Tukey's test using R commander modified by Norwegian University of Life Sciences (Ås, Norway).

Principal component analysis (PCA), was performed to obtain an overview over the multicorrelated data using the software The Unscrambler® X, v 10.2 (CAMO Software AS, Oslo, Norway) on data set from each year.

Results and Discussion

Temperature effects without post-anthesis fertilization (Exp1 and Exp2)

Two cultivars bred in different production areas within Northwestern Europe were used in this study to investigate the effects of lower to moderate temperature during grain filling on gluten quality.

Bjarne is a Norwegian cultivar and one of the most widely grown wheat cultivars in the country. Cadenza is a bread-milling cultivar grown in UK under higher temperature ranges than in Norway. Both cultivars possess HMW-GS 5+10 indicating strong gluten quality (Payne, 1987; Shewry et al., 1992). Bjarne is an early-maturity type and its maturity was 5-10 days earlier than Cadenza at the highest temperature in this study.

PCA provided an overview of the relationship between cultivars, temperature during grain filling and gluten quality parameters. The first two principal components (pc) accounted for 58 % and 18 % in Exp1 and 49 % and 25 % in Exp2 (Figure 1). The samples clustered according to cultivar along the first pc as Bjarne clustered on one side of the score plot, while Cadenza clustered on the opposite side for both experiments (Figure 1). The results indicated that differences in genetic background were the most important factors influencing gluten quality parameters such as %UPP and the composition of gluten proteins. Samples were distributed according to temperature within each cultivar along the first pc indicating that temperature had effects on %PC as well as on parameters related to gluten quality such as the composition of gluten proteins and %UPP (Figure 1, C and D).

The period from anthesis to maturation was influenced by temperatures with the duration of grain filling being twice as long at 13 °C as at 23 °C. Grain dry weight increased more slowly at first three harvest of immature grain during grain filling at lower temperatures than at higher temperatures when compared in chronological time (daa) (Figure2, C and G), while it was similar when compared in thermal time (d°aa) (Figure 2, D and H). The longer period of grain filling resulted in higher GDW at lower temperatures (Figure 2 and 3). On the other hand, grain filling ceased earlier and the increase in GDW was more gradual from the middle of grain filling, especially for Bjarne, which resulted in lower GDW at higher temperatures (Figure 2 and 3). Hence, GDW decreased as temperature increased in both cultivars and experiments (Figure 2, A). These observations agreed with previous reports by Sofield et al. (1977a) and Weegels et al. (1996).

Temperature influenced the PCG as it was decreased in grain grown at 18-23 and 23-23 compared to 13-13 °C ($P<0.05$) in Exp1 (Table 1, Figure 3). This was clearly seen in Bjarne but not in Cadenza (Figure 3, C). The same trend was observed in Exp2 as PCG was decreased at 23-23 °C, although significant differences were not observed (Table 1). Sofield et al. (1977b) reported similar results as N per grain was less affected by temperatures ranging lower to moderate (15/10 compared to 21/16 °C). The %PC increased with increasing temperature (Figure 3, B). Hence, temperature had small effects on storage protein accumulation per grain, but greater effects on GDW. The lower %PC observed at lower temperatures was presumably caused by dilution of storage proteins by other storage components such as starch, as Altenbach et al. (2003) reported that starch accumulation was increased by prolonged grain filling at lower temperatures.

Significant temperature effects were found on the proportions of gluten proteins, as the proportion of ω-gliadins and D-type LMW-GS increased, while the proportion of α- and γ-gliadins and B-type LMW-GS decreased with increasing temperature for both cultivars in both Exp1 and Exp2 (Table 2). Changes in the proportion of HMW-GS were inconsistent between Exp1 and Exp2 with slight increase with increasing temperature in Exp2 ($P<0.05$) (Table 2). The Gli:Glu ratio and the HMW:LMW ratio did not change with temperature, while the B-type:C-, and D-type LMW ratio decreased as temperature increased in Exp1 (Table 2). Changes in the composition of gluten proteins were reported previously at higher temperature ranges, but were inconsistent between reports (Daniel and Triboi, 2000; DuPont et al., 2006b; Hurkman et al., 2013). Recently, Hurkman et al. (2013) presented a detailed study of high temperature and fertilizer effects on the composition of gluten proteins analyzed by two-dimensional electrophoresis combined with mass spectrometry. They observed that ω-gliadins, HMW-GS and α-gliadin increased, LMW-GS decreased and γ-gliadins showed little change with increasing temperature (37/28 compared to 24/17 °C). The ω-gliadins showed the greatest changes, while HMW-GS and α-gliadin showed smaller changes of the proteins that were increased at high temperature (Hurkman et al., 2013). The changes in ω-gliadins, HMW-GS and LMW-GS observed in this study were similar to the results of Hurkman et al. (2013), while our observed temperature effects on the proportion of α-gliadins and γ-gliadins were inconsistent to their study.

Highly significant temperature effects were observed on %UPP. Changes in %UPP with temperatures were depended on cultivar, as significant temperature and cultivar interactions were found by ANOVA. The %UPP in Cadenza increased with temperature in both experiments (Figure 4, B) and was significantly lower (by 29 %) in grain grown at 13-13 compared to 23-23 °C in both experiments (Figure 4). The results indicated that lower temperature resulted in weaker gluten for this cultivar, as there is a strong positive correlations between %UPP and dough strength (maximum resistance to extension) (Gupta et al., 1993). The %UPP in Bjarne was 13 % lower at 13-13 °C in Exp1 compared with the proportion at 13-23 °C (Figure 4, A). However, the values for %UPP in Bjarne were comparable to %UPP reported for strong wheat (Gupta et al., 1993; Moldestad et al., 2011) at all temperatures indicating that the temperature had little effects on gluten quality in Bjarne between the temperature regimes tested in this study. Low temperatures, even as low as 13 °C, did not result in negative effects on gluten quality as the %UPP value in grain grown at 13 °C was relatively high. Bjarne is a wheat cultivar bred in Norway and consequently can be considered to be better adapted to lower temperatures compared to the U.K cultivar, Cadenza. Also another study performed in a growth tunnel, where temperature gradients were obtained during grain filling, revealed that the %UPP and resistance to extension of gluten in Bjarne was relatively stable to temperature (Moldestad et al., 2014). The differences in the proportions of gluten proteins at different temperatures were similar between the two cultivars, while temperature effects on %UPP were greater in Cadenza compared to Bjarne. Hence,

these results indicated that assembly of glutenin polymers in Bjarne was less sensitive to variation in temperature.

Changes in temperature between the early and later phases of grain development were applied to determine how low temperatures during the early phase influenced the quality of gluten proteins. Previous work showed that the early phase of grain filling was temperature sensitive with low (<18 °C) temperatures having negative effects on gluten quality in mature grain (Moldestad et al., 2011; Panozzo and Eagles, 2000). However, the low temperature during the early phase of grain filling did not seem to have negative effects on %UPP in this study. In field studies, such as that reported by Moldestad et al 2011, a number of other factors will vary in addition to the temperature, typically higher rainfall and humidity in periods with lower temperatures are typical in this region. This contrasts to experiments in the climate chambers where such factors can be controlled separately.

No temperature effects were found on the Mon:Pol ratio, which was consistent to the SDS-PAGE results showing no temperature effects on the Gli:Glu ratio. The results indicate that temperatures affected the proportions of some gluten proteins, however the changes occurred within the gliadins or within the glutenins, with the ratio of Mon:Pol or Gli:Glu not being affected.

Temperature effects under post-anthesis fertilization (Exp3)

Low temperatures prolonged the duration of grain filling resulting in an increase in grain weight. However, accumulation of storage proteins (PCG) did not increase with prolonged grain filling, indicating limitation of N in the soil at low temperature in Exp1 and Exp2. Post-anthesis fertilization was therefore applied to investigate whether it would increase the accumulation of storage protein and thus increase %PC. Moreover, the effects of temperature on the gluten quality were studied when plants were supplied with N during grain filling in Exp3.

PCG clearly increased at lower temperatures with post-anthesis fertilization. Because low temperature prolonged the duration of grain filling, grain grown at low temperatures had access to higher N due to post-anthesis fertilization for longer period. Post-anthesis fertilization directly increased the protein content (%). By contrast, changes in PCG due to post-anthesis fertilization at higher temperatures were considerably smaller than at lower temperatures. The period of grain filling was shorter at higher temperatures, and it is therefore assumed that the grain could not fully exploit the higher available N at high temperatures. Similar observations were reported by Altenbach et al. (2003) and DuPont et al. (2006b) as PCG was increased two-fold with post-anthesis NPK at 24/17 °C, but did not differ at 37/28 °C with or without NPK. They concluded that the effects of fertilizer and temperature were not additive under a high temperature regime (Altenbach et al., 2003). Although PCG greatly increased with post-anthesis fertilization at low temperature, %PC was significantly lower in grain grown at lower

temperatures (13-13, 13-18 and 18-18 °C) compared to that grown at higher temperatures (13-23, 18-23 and 23-23 °C).

Significant temperature effects were not found for any of the parameters related to gluten quality when post-anthesis fertilization was applied (Exp3), including %UPP and the proportions and the ratios of gluten proteins (Supplemental Table a). The results indicate that the post-anthesis fertilization modulated the effects of temperature.

The effects of cultivar on the proportion and the ratio of gluten proteins as well as size distribution of glutenin polymers were large regardless of post-anthesis fertilization (Figure 1 and Supplemental Table a). Hence, this study revealed that cultivar (genotype) had greater effects on the quality of gluten proteins than the temperature.

Conclusions

Temperature effects on gluten quality were studied without and with post-anthesis fertilization. Temperature influenced the proportions of gluten proteins in a similar way for both cultivars. The proportions of ω-gliadins and D-type LMW-GS increased, while the proportion of α- and γ-gliadins and B-type LMW-GS decreased with increasing temperature without post-anthesis fertilization. The temperature effects on %UPP were cultivar dependent. Cadenza showed a clear increase in %UPP with increasing temperature, and the low %UPP at 13-13 °C suggested the weak gluten quality under these conditions. On the other hand, Bjarne had higher %UPP at all temperature regimes indicating that the assembly of glutenin polymers in Bjarne was less sensitive to temperature compared to Cadenza. No negative effects of the low temperature during the early phase of grain filling were found on %UPP, and no temperature effects on parameters related to gluten quality were observed when post-anthesis fertilization was applied. Moreover, the cultivar effects on the gluten quality parameters were larger than the temperature effects.

Temperature and/or post-anthesis fertilization influenced the GDW, %PC and PCG. Lower temperature increased grain weight, while it did not influence PCG, thus %PC decreased without post-anthesis fertilization. Post-anthesis fertilization increased PCG and thus %PC, especially in grain grown at lower temperatures, but not at higher temperatures.

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Table 1. Significance level calculated with ANOVA of grain dry weight (GDW), protein content in flour (%PC), protein content per grain (PCG), the proportion of SDS-unextractable polymeric proteins in total polymeric proteins (%UPP) and the ratio of monomer to polymer (Mon:Pol) of Bjarne and Cadenza grown at six temperature regimes in Exp1 and Exp3 and three constant temperature regimes in Exp2 with two biological replicates. ANOVA was carried out for data set from each experiment (2 cultivar, 3 or 6 temperatures and 2 biological replicates for each experiment). Temp.: temperature, Cult.: cultivar and TxC: temperature and cultivar interaction

Exp		GDW	%PC	PCG	%UPP	Mon:Pol
1	Temp.	***	***	**	***	n.s.
	Cult.	***	***	***	***	*
	TxC	n.s.	*	***	***	n.s.
2	Temp.	***	***	n.s.	***	n.s.
	Cult.	***	***	***	***	n.s.
	TxC	n.s.	n.s.	**	***	n.s.
3	Temp.	***	***	***	n.s.	n.s.
	Cult.	***	**	**	***	***
	TxC	***	**	n.s.	n.s.	n.s.

***, **, * indicate significance at 0.1, 1 and 5 %, respectively, and n.s. indicates not significant.

Table 2. The proportions of ω-gliadins, α- and γ-gliadins, HMW-GS, D-type LMW-GS, B-type LMW-GS and C-type LMW-GS in the total gluten proteins, the ratios of gliadin to glutenin (Gli:Glu),HMW-GS to LMW-GS (HMW:LMW) and B-type LMW-GS to C-, and D-type LMW-GS (B-type:C-, D-type LMW). Protein was extracted from mature grain which was exposed to six and three temperature regimes during grain filling in Exp1 and Exp2, respectively, separated by SDS-PAGE and quantified. ANOVA was carried out for data set from each experiment and significance levels were presented under the data from each experiment. Temp.: temperature, Cult.: cultivar and TxC: temperature and cultivar interaction.

Exp.	Cult.	Temp. (°C)	ω-gliadin (%)	α- and γ-gliadin (%)	HMW-GS (%)	D-type LMW-GS (%)	B-type LMW-GS (%)	C-type LMW-GS (%)	Gli:Glu	HMW: LMW	B-type: C-, D-type LMW
1	Bjarne	13-13	9.12	30.09	21.74	7.05	17.92	14.09	0.64	0.57	0.85
		13-18	9.88	29.83	21.71	7.66	16.21	14.71	0.66	0.56	0.72
		18-18	11.88	29.74	22.58	8.07	15.41	12.32	0.71	0.63	0.76
		13-23	11.88	30.31	22.65	8.18	14.26	12.72	0.73	0.65	0.68
		18-23	12.18	29.61	24.43	8.14	13.38	12.26	0.72	0.74	0.65
		23-23	11.47	27.83	22.83	9.04	15.37	13.46	0.65	0.61	0.68
	Cadenza	13-13	5.40	35.56	19.49	5.46	23.05	11.04	0.69	0.49	1.41
		13-18	5.20	35.44	19.57	5.51	21.90	12.38	0.69	0.49	1.22
		18-18	5.94	34.07	20.20	6.09	21.12	12.59	0.67	0.51	1.13
		13-23	6.37	33.30	22.25	6.13	19.36	12.59	0.66	0.59	1.03
		18-23	7.12	32.94	23.13	7.11	16.67	13.03	0.67	0.63	0.83
		23-23	7.18	30.27	21.55	7.74	20.10	13.15	0.60	0.53	0.96
	Temp.		**	*	n.s.	***	*	n.s.	n.s.	n.s.	**
	Cult.		***	***	*	***	***	n.s.	n.s.	*	***
	TxC		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2	Bjarne	13-13	11.53	34.60	21.86	5.92	15.15	10.94	0.86	0.69	0.90
		18-18	12.90	32.45	22.08	7.11	14.77	10.69	0.83	0.68	0.83
		23-23	14.12	31.74	23.22	6.82	13.92	10.17	0.85	0.75	0.82
	Cadenza	13-13	8.73	40.62	20.99	4.25	14.96	10.44	0.98	0.71	1.03
		18-18	9.00	37.38	20.51	5.51	15.74	11.85	0.87	0.63	0.93
		23-23	10.98	33.19	23.04	6.31	14.54	11.94	0.79	0.70	0.80
	Temp.		**	*	*	**	*	n.s.	n.s.	n.s.	n.s.
	Cult.		***	**	n.s.	**	*	n.s.	n.s.	n.s.	n.s.
	TxC		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

***, **, * indicate significance at 0.1, 1 and 5 %, respectively, and n.s. indicates not significant.

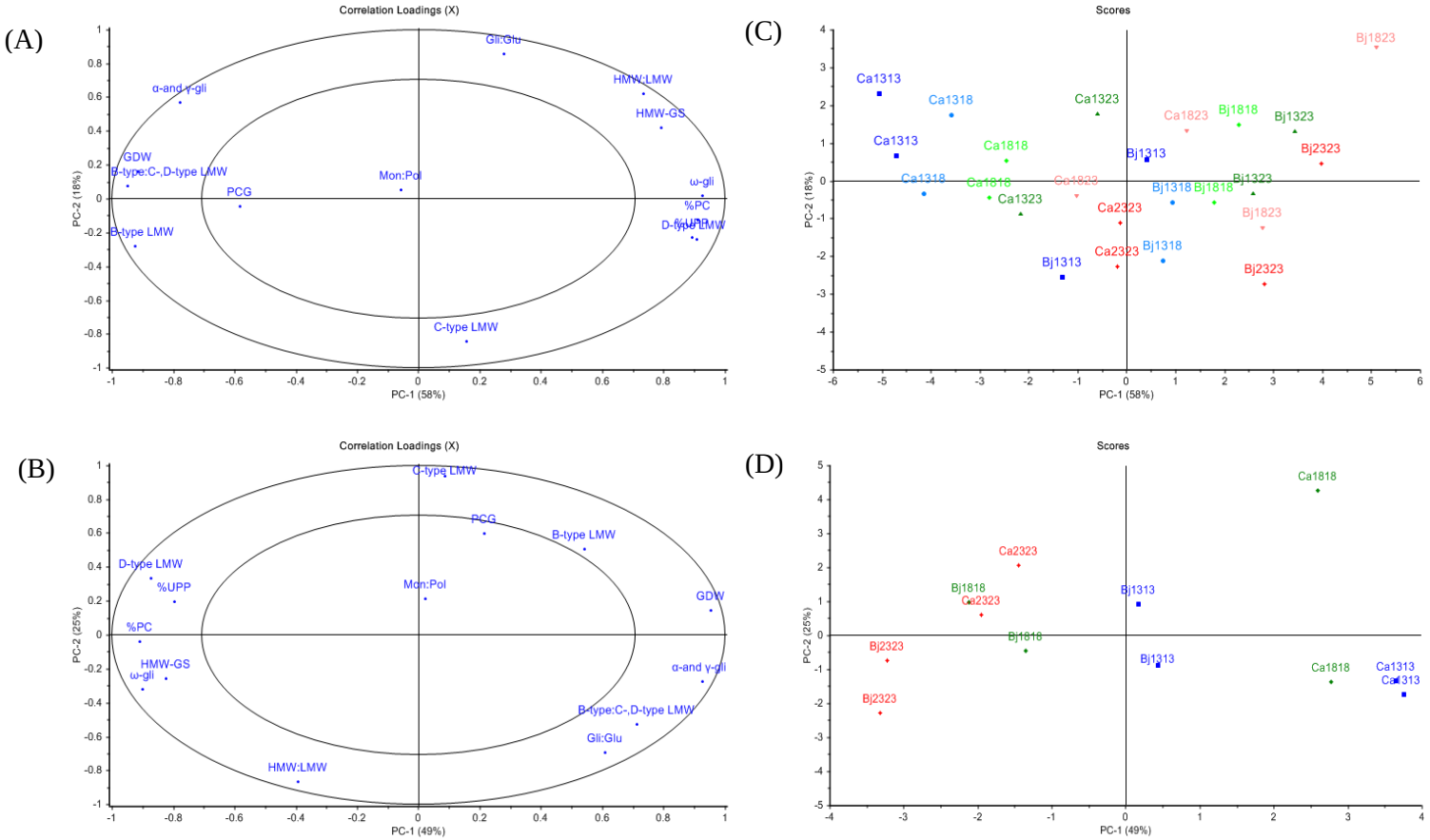

Figure 1. PCA loading plots (A) and (C) showing the distribution of x- and y-variables along the first and second principal components (pcs) and score plots (B) and (D) showing the distribution of the samples along the first and second pcs. (A) and (B) show the results of data set from Exp1, (C) and (D) show the results of data set from Exp2. The samples from (two) lowest temperature(s) were colored in blue, those from the middle temperature(s) in green and from the (two) highest temperature(s) in red in score plots. Abbreviations: Grain dry weight (GDW), protein content (%PC), protein content per grain (PCG), the proportions of ω-gliadin (ω-gli), α- and γ-gliadin (α- and γ-gli), HMW-GS (HMW-GS), D-type LMW-GS (D-type LMW), B-type LMW-GS (B-type LMW) and C-type LMW-GS (C-type LMW) in total gluten proteins, the ratios of HMW-GS to LMW-GS (HMW:LMW), Gliadin to Glutenin (Gli:Glu) and B-type LMW-GS to C- and D-type LMW-GS (B-type:C-, D-type LMW), Bjarne (Bj) and Cadenza (Ca). Numbers behind the name of cultivars indicat the temperatures during grain filling.

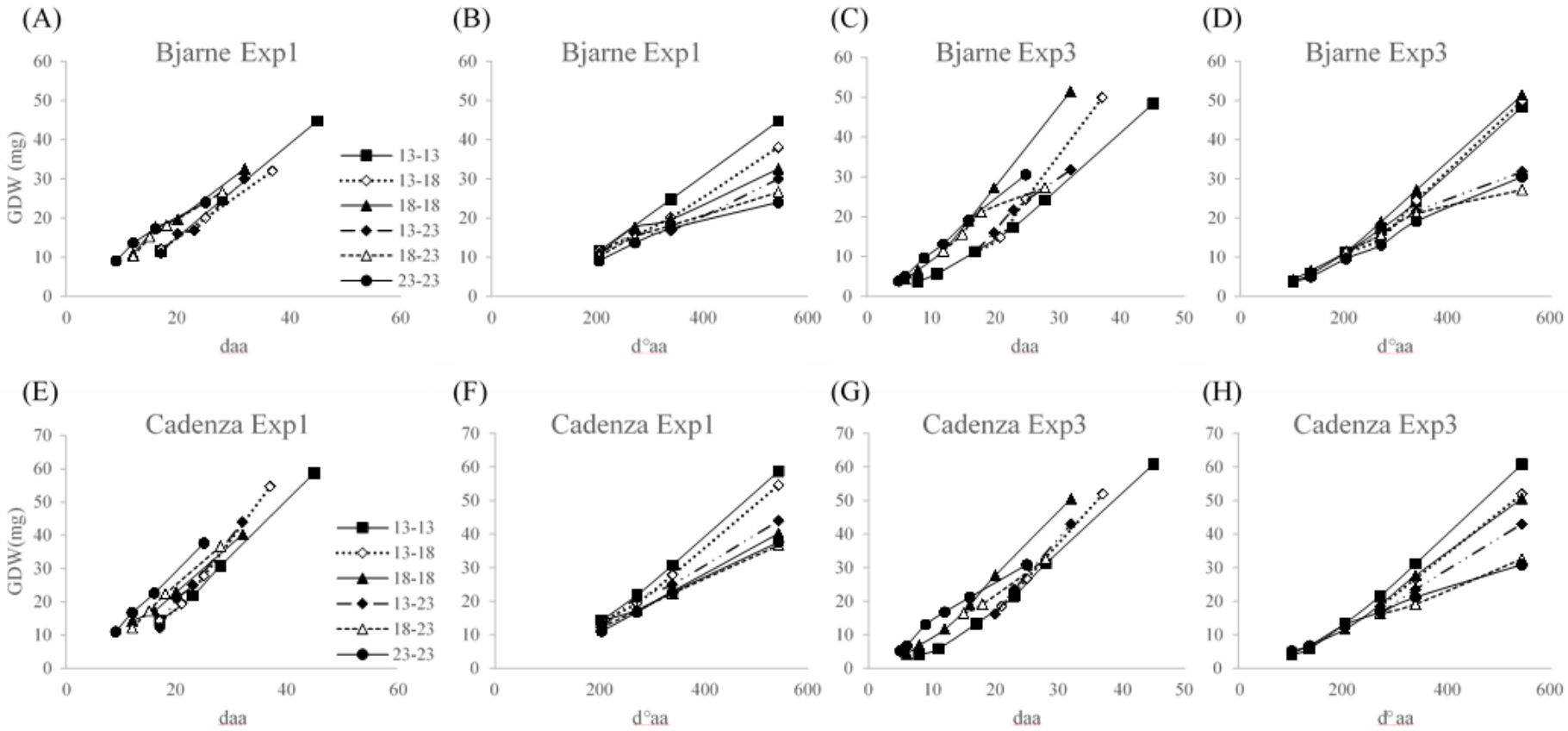

Figure 2. Grain dry weight (GDW) (mg) during grain filling in chronological time (day after anthesis, daa) (A, C, E and G) and in thermal time (d°aa) (B, D, F and H) for Bjarne (A-D) and Cadenza (E-H) from Exp1 (A,B,E and F) and Exp3 (C, D, G and H).

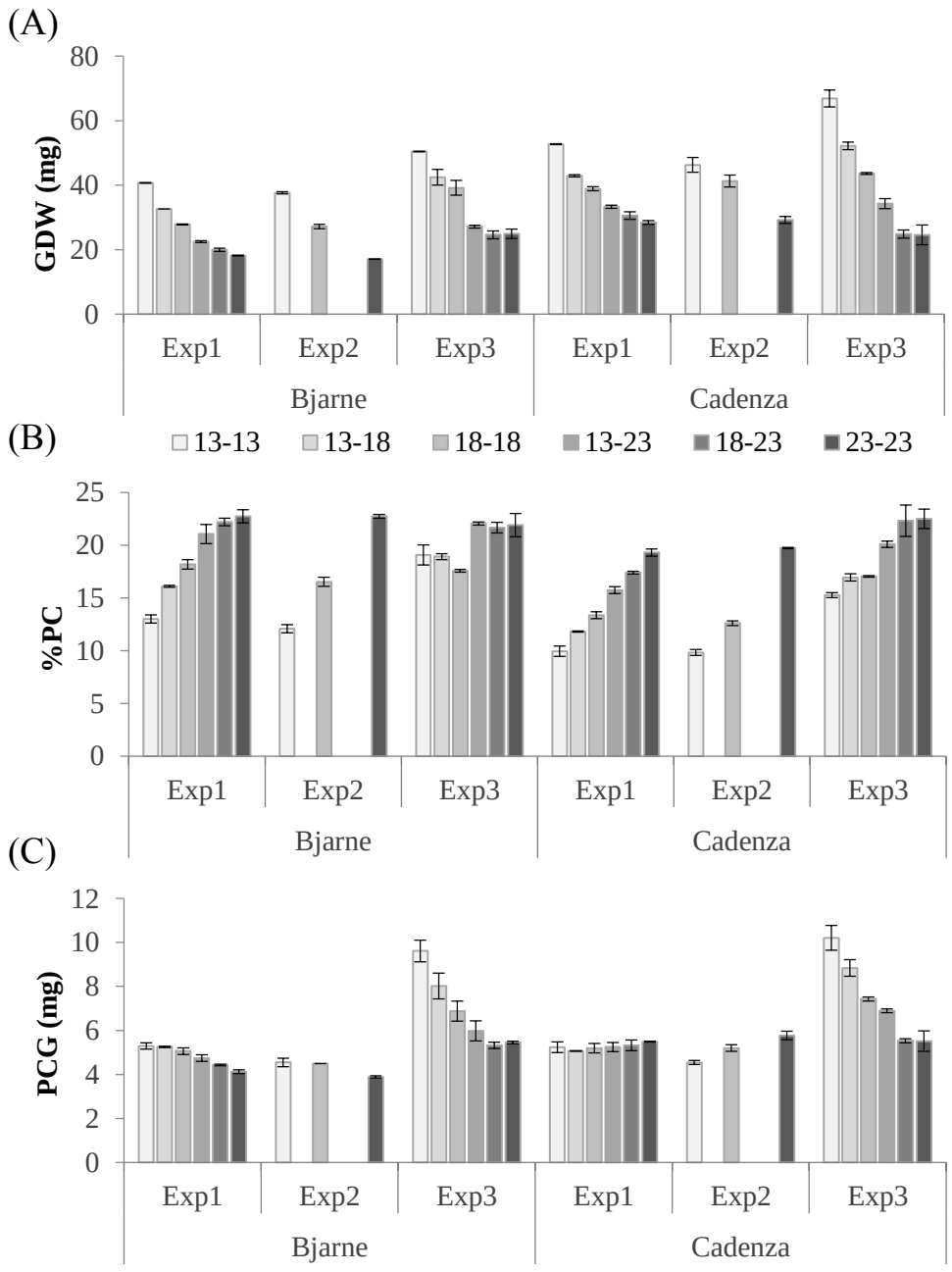

Figure 3. Grain dry weight (GDW) (mg) (A), protein content (%PC) in flour (B) and protein content per grain (PCG) (mg) (C) in Bjarne and Cadenza in Exp1, Exp2 and Exp3. The plants were grown at six different day/night temperature regimes during grain filling in Exp1 and 3, and three constant temperature regimes in Exp2. Grain was harvested at the maturity.

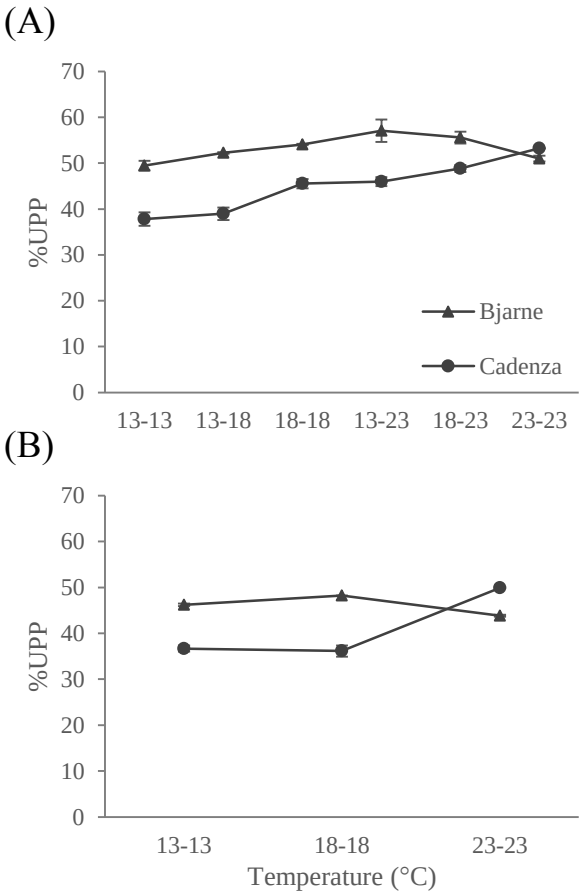

Figure 4. The proportion of SDS-unextractable polymeric proteins in total polymeric proteins (%UPP) in Bjarne and Cadenza from Exp1 (A) and Exp2 (B). Plants were grown in six temperature regimes in Exp1, while three constant temperature regimes in Exp2. Bars show standard deviation.

Supplemental Table a. The proportions of ω-gliadins, α- and γ-gliadins, HMW-GS, D-type LMW-GS, B-type LMW-GS and C-type LMW-GS in the total gluten proteins, the ratios of gliadin to glutenin (Gli:Glu), HMW-GS to LMW-GS (HMW:LMW) B-type LMW-GS to C-, and D-type LMW-GS (B-type:C-, D-type LMW) that were analyzed by SDS-PAGE and the proportion of SDS-unextractable polymeric proteins in total polymeric proteins (%UPP) and the ratio of monomer to polymer (Mon:Pol) analyzed by SE-FPLC. Protein was extracted from mature grain, which was exposed to six temperature regimes during grain filling in Exp3, separated by SDS-PAGE or SE-FPLC and quantified. ANOVA was carried out and significance levels were presented under the data. Temp.: Temperature, Cult.: Cultivar and TxC: temperature and cultivar interaction

Exp.	Cult.	Temp. (°C)	ω-gliadin (%)	α- and γ-gliadin (%)	HMW-GS (%)	D-type LMW-GS (%)	B-type LMW-GS (%)	C-type LMW-GS (%)	Gli:Glu	HMW: LMW	B-type: C-, D-type LMW	%UPP	Mon:Pol
3	Bjarne	13-13	15.03	31.51	22.93	9.45	11.03	10.05	0.87	0.75	0.57	53.88	1.20
		13-18	13.79	29.22	25.08	9.92	11.89	10.11	0.75	0.79	0.59	56.62	1.20
		18-18	13.57	30.68	23.62	9.84	11.94	10.36	0.79	0.74	0.59	57.05	1.20
		13-23	14.89	31.54	23.73	9.26	11.23	9.35	0.87	0.80	0.60	57.81	1.20
		18-23	13.17	30.11	24.13	9.32	12.54	10.73	0.76	0.74	0.63	57.65	1.18
		23-23	13.06	30.30	23.94	9.33	12.44	10.92	0.77	0.73	0.61	54.91	1.23
	Cadenza	13-13	8.21	34.85	19.91	6.01	19.34	11.68	0.76	0.54	1.09	44.52	1.30
		13-18	8.99	33.75	19.99	6.93	18.56	11.78	0.75	0.54	0.99	47.97	1.31
		18-18	8.69	33.47	20.16	6.68	19.06	11.93	0.73	0.54	1.03	45.34	1.29
		13-23	8.82	32.22	21.14	7.25	18.09	12.48	0.70	0.56	0.92	51.68	1.29
		18-23	8.57	32.41	20.52	6.92	19.20	12.39	0.69	0.53	0.99	46.15	1.27
		23-23	9.16	32.71	20.75	7.41	17.63	12.34	0.72	0.56	0.89	49.89	1.26
		Temp.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
		Cult.	***	***	***	***	***	***	***	***	**	***	***
		TxC	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

***, **, * indicate significance at 0.1, 1 and 5 %, respectively, and n.s. indicates not significant.

Paper II

Research Article

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Influence of temperature during grain filling on gluten viscoelastic properties and gluten protein composition

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Abstract

BACKGROUND: The aim of this study was to investigate the effects of low to moderate temperatures on gluten functionality and gluten protein composition. Four spring wheat cultivars were grown in climate chambers with three temperature regimes (day/night temperatures of 13/10, 18/15 and 23/20 °C) during grain filling.

RESULTS: The temperature strongly influenced grain weight and protein content. Gluten quality measured by maximum resistance to extension (R_{max}) was highest in three cultivars grown at 13 °C. R_{max} was positively correlated with the proportion of sodium dodecyl sulfate-unextractable polymeric proteins (%UPP). The proportions of ω-gliadins and D-type low-molecular-weight glutenin subunits (LMW-GS) increased and the proportions of α- and γ-gliadins and B-type LMW-GS decreased with higher temperature, while the proportion of high-molecular-weight glutenin subunits (HMW-GS) was constant between temperatures. The cultivar Berserk had strong and constant R_{max} between the different temperatures.

CONCLUSION: Constant low temperature, even as low as 13 °C, had no negative effects on gluten quality. The observed variation in R_{max} related to temperature could be explained more by %UPP than by changes in the proportions of HMW-GS or other gluten proteins. The four cultivars responded differently to temperature, as gluten from Berserk was stronger and more stable over a wide range of temperatures.

Supporting information may be found in the online version of this article.

Keywords: wheat; gluten quality; low temperature, viscoelastic properties; glutenin; gliadin

INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important staple crops worldwide together with rice and maize. Wheat is mostly consumed as processed foods such as pasta, noodles, bread and different types of baked products. The ability to make this range of products is determined by the grain storage proteins, which form a viscoelastic network, called gluten, when flour is hydrated and mixed into dough. Thus the quantity as well as the quality of gluten proteins is important for the quality of final products.[1,2]

Gluten proteins are classified into two groups, glutenins and gliadins.[3] Glutenins are large polymers that contribute to dough elasticity, while gliadins are monomers that contribute to dough extensibility and viscosity. The monomeric gliadins are further classified into three groups, α-gliadins, γ-gliadins and ω-gliadins. The glutenin polymers comprise two groups of subunits linked by inter-chain disulfide bonds. These are called low-molecular-weight glutenin subunits (LMW-GS) and high-molecular-weight glutenin subunits (HMW-GS), with the LMW-GS being further divided into B-, C- and D-types. The B-type LMW-GS are the major group of LMW-GS and have two cysteine residues forming intermolecular disulfide bonds. The C-type (α- and γ-type) and D-type (ω-type) LMW-GS are similar to the monomeric gliadins in their amino acid sequence but have an additional unpaired cysteine residue, allowing their incorporation into glutenin polymers. Hence they are considered to act as terminators of glutenin polymer chains (reviewed by D'Ovidio and Masci[4]).

The viscoelastic properties of gluten are related to the ratio of glutenin to gliadin[5] and to the proportion of larger polymeric proteins, which have also been classified as glutenin macropolymer (GMP)[6] or sodium dodecyl sulfate (SDS)-unextractable polymeric proteins (UPP).[7] There is a clear increase in these large polymers during the desiccation/maturation phase at the end of

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seed development,[8–10] and the proportion of UPP in total polymeric proteins (%UPP) is highly correlated with dough strength.[7] The proportion of large glutenin polymers is also related, at least in part, to the amount and properties of the HMW subunits of glutenin, with well-established relationships between allelic variation in the HMW subunit composition and dough strength.[11,12] Genetic variation in the gluten protein composition is therefore important in determining the end use quality for different products, including bread.

Not only genotype but also environmental conditions such as temperature, nutrient availability (particularly of sulfur and nitrogen) and water availability are reported to influence the properties of gluten proteins.[13–15] The temperature during grain development has been recognized as a major factor influencing grain quality.[16] Temperature strongly influences the duration of grain filling and the grain growth rate.[17,18] Lower temperature reduce the rate of grain filling while prolonging the duration of grain filling and thus increasing the grain weight compared with higher temperatures.[17] Accumulation of nitrogen in the grain is little influenced by low to moderate temperatures,[14,19] while higher temperature (>30 °C) or extended heat shock (35–40 °C) reduce nitrogen per grain.[20,21] DuPont *et al.*[22] showed that accumulation of dry weight and starch was reduced by higher temperatures (37/28 °C compared with 24/17 °C), while accumulation of protein was not affected by temperature. Consequently, the protein content of flour is higher in grain grown at higher temperatures than in grain grown at lower temperatures.[14,23,24]

Large areas of wheat cultivation in the world experience high temperatures during the growing season, and the effects of high temperature (shock or stress) on gluten quality have been well documented both in field experiments as well as in studies at higher temperature ranges under controlled environment conditions.[16,20,25,26] Temperatures above 30 °C during grain development have a weakening effect on the dough strength[27,28] resulting from changes in composition of the gluten proteins,[21,29,30] which affect the glutenin-to-gliadin ratio and the proportion of large glutenin polymers.[31,32]

By contrast, temperature during wheat production in Northern Europe can be lower than the suboptimal temperature for wheat cultivation, and weak gluten strength was observed when plants were exposed to cool and humid conditions in Scandinavia.[33–36] Field trials of spring wheat varieties in Norway have shown wide variation in gluten resistance both between and within seasons, with weaker gluten being observed in samples that had experienced temperatures below 18 °C during grain filling.[36] Based on their observations, the authors suggested that temperatures below 18 °C during grain filling can result in weaker gluten. Previously, Uhlen *et al.*[24] performed experiments in climate chambers to investigate the effects of temperatures ranging from 9 to 21 °C on gluten quality. The results showed that the proportion of total polymeric proteins in total gluten proteins was slightly lower in the grains grown at lower temperatures and that dough mixing properties measured by a Mixograph were negatively affected by low temperatures. However, Johansson *et al.*[37] reported inconsistent temperature effects on %UPP in comparable temperature ranges. Hence the effects of lower temperatures on wheat gluten structure and processing quality are still unclear.

We have therefore carried out climate chamber experiments with four Norwegian wheat cultivars with lower to moderate temperature ranges in order to increase our knowledge of the effects of temperature as well as temperature × cultivar interactions on grain development, the composition of gluten proteins and the viscoelastic properties of gluten.

MATERIALS AND METHODS

Wheat samples

An experiment with four Norwegian spring wheat (*T. aestivum* L.) cultivars, Avle (HMW-GS: 2*, 7 + 9, 5 + 10), Berserk (2*, 14 + 15, 5 + 10), Bjarne (2*, 6 + 8, 5 + 10) and Zebra (2*, 7 + 9, 5 + 10), three growth temperatures and two biological replicates was conducted in 2012 at the Center for Plant Research in Controlled Climate, Norwegian University of Life Sciences, Ås, Norway. Eight seeds were sown in a 3 L pot (L.O.G. AS, Oslo, Norway) filled with 1.15 kg of commercial plant soil (pH 5.5–6.5) consisting of 860 g kg^{-1} sphagnum peat, 100 g kg^{-1} fine sand and 40 g kg^{-1} clay and containing 900 mg L^{-1} nitrogen (N), 190 mg L^{-1} potassium (K), 35 mg L^{-1} phosphorus (P) and a mixture of micronutrients (L.O.G. AS). Plants were grown in a glasshouse at ambient temperature but with a controlled minimum day/night temperature of 15/10 °C until anthesis under natural light with supplemented high-pressure sodium lights to maintain a day length of 16 h. Pots were rotated weekly to minimize positional effects in the glasshouse. After emergence, when the seedlings had two to three leaves, they were thinned to four plants per pot. Diluted solutions of two types of fertilizer (Superba™ Rød and Ks Calcinit™) were mixed and applied as N 0.148/P 0.037/K 0.185 g per pot per time. Fertilization was first applied at Zadoks (Z) 15[38] and then carried out weekly, with the last application at Z59 (total of five applications). Just before anthesis, plants were moved to temperature-controlled climate chambers. Eight pots of each cultivar were grown further at day/night (18/6 h respectively) temperatures of 13/10 °C as lower, 18/15 °C as medium and 23/20 °C as higher temperature until maturation. Relative humidity (RH) in the chambers was measured hourly; the average RH was 91% at 13/10 °C, 86% at 18/15 °C and 77% at 23/20 °C. The average light intensity in the chambers was 300 µmol m^{-2} s^{-1} at the position of the ears. The ears from the main stems or from equally developed tillers were harvested shortly after maturation, dried and threshed. The experiment was carried out with two growth chambers of each temperature regime as biological replicates. Two to three ears were harvested from each plant from eight pots (32 plants) within each chamber and were pooled before threshing.

Grain and flour analysis

Grain weight was measured from a representative sample of approximately 300 grains and expressed as grain dry weight (GDW) in mg per grain. Harvested grains were milled as wholemeal in a Laboratory Mill 3100 (Perten Instruments AB, Huddinge, Sweden) with a 0.8 mm sieve. Total N content (g kg^{-1}) of wholemeal flour was measured by the Dumas method according to Bremner and Mulvaney.[39] Protein content of flour was then calculated by multiplying the N content by 5.7 and expressed as g kg^{-1}. Protein content per grain (PCG) was calculated on a dry weight basis and expressed as mg protein per grain. Sulfur (S) content of wholemeal flour was determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (5300 DV, Perkin Elmer, Waltham, MA, USA). Samples were decomposed in an Ultraclave with 100 mL L^{-1} HNO_3 ultrapure at 260 °C and 5 MPa before analysis by ICP-OES. Certified reference material Wheat Flour 1567a (National Institute of Standards and Technology, Gaithersburg, MD, USA) was analyzed with the series of samples, and the S content expressed as g kg^{-1}. The ratio of N to S (N:S) was calculated.

Kieffer Dough and Gluten Extensibility Rig

Viscoelastic properties of gluten were determined with the SMS/Kieffer Dough and Gluten Extensibility Rig.[40] Gluten was prepared from 10 g of wholemeal flour in a Glutomatic 2100 (Perten Instruments AB, Hägersten, Sweden). A 20 g L^{-1} NaCl solution was used for mixing and washing out starch, bran particles and salt-soluble components. Dough was mixed for 1 min prior to 10 min of washing out. Two types of filter were used for gluten preparation: an 840 μm filter was used for mixing and 2 min of washing out starch; thereafter the filter was changed to 88 μm to wash out the bran particles. The gluten was centrifuged in a special centrifuge mold in a swing-out rotor (Rotor TS-5.1-500) at 3000 × *g* for 10 min at 20 °C (TJ-25 Centrifuge, Beckman, Los Angeles, CA, USA), placed in the standard mold and incubated for 45 min at 30 °C before further analysis. Three pieces of gluten from each gluten preparation were stretched with the Kieffer rig extensograph. The maximum resistance to extension (R_{max}) and extensibility (Ext) were recorded using a TA.XT Plus texture analyzer (Stable Micro Systems, Godalming, UK) with test parameters of 3.3 mm s^{-1} test speed, 5 g trigger force and 1 g break sensitivity. All samples were analyzed twice (technical replicates) and the average results were used in the statistical analysis.

Size exclusion fast performance liquid chromotagrophy (SE-FPLC)

Wholemeal flour samples were extracted sequentially to obtain two extracts (SDS-extractable and SDS-unextractable) according to Morel *et al.*[41] with modifications described by Tronsmo *et al.*[42] A 240 mg sample of wholemeal flour was extracted with 30 mL of 10 g L^{-1} SDS and 0.1 mol L^{-1} sodium phosphate extraction buffer (pH 6.9) for 80 min at 60 °C with continuous stirring. An SDS-extractable protein fraction was obtained by centrifuging the sample at 37 000 × *g* for 30 min at 20 °C (L-80 Ultracentrifuge, Rotor 50.2Ti, Beckman). An SDS-unextractable protein fraction was subsequently extracted by suspending the pellet in 30 mL of extraction buffer as above with sonication. The sonication was done with a Sonics VC130 (Sonics and Materials, Newton, CT, USA) for 3 min (with 70% automatic amplitude compensation). Centrifugation of samples was carried out as described above after sonication. Each fraction was filtered (Millex-HV 0.45 μm, Millipore, Cork, Ireland) and separated on a Superose® 12HR 10/30 column connected to ÄKTA SE-FPLC system (GE Healthcare Life Sciences, Little Chalfont, UK) with 1 g L^{-1} SDS, 0.08 mol L^{-1} NaCl and 0.05 mol L^{-1} sodium phosphate elution buffer (pH 6.9). All samples were extracted and run twice (technical replicates) and the average results were used in the statistical analysis.

The SDS-unextractable fraction gave one main peak, denoted $F1^*$, which consisted of large polymeric proteins. The chromatogram of the SDS-extractable fraction was divided into four main peaks, denoted F1 – F4. The proportions of each peak (%$F1^*$ and %F1 – %F4) were calculated as percentages of the total areas of the two chromatograms (SDS-extractable and SDS-unextractable fractions). The proportion of SDS-unextractable polymeric proteins in total polymeric proteins (%UPP) was calculated as $[F1^*/(F1^* + F1)] \times 100$ and the ratio of monomeric to polymeric proteins (Mon:Pol) was calculated as $(F3 + F4)/(F1^* + F1 + F2)$.

Protein extraction and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Three different fractions of gluten proteins were extracted sequentially according to Tosi *et al.*[43] with modifications. First, monomeric gliadins were extracted from 20 mg of wholemeal flour using 0.5 mL of 700 mL L^{-1} ethanol. The mixture was stirred for 30 min at room temperature and centrifuged for 15 min at 3700 × *g*. The extraction procedure was repeated twice and the supernatant was pooled into an Eppendorf tube and freeze-dried (gliadin fraction). Then the polymeric glutenins were extracted from the remaining flour pellet with 0.5 mL of 500 mL L^{-1} propan-1-ol and 45 g L^{-1} dithiothreitol (DTT). The sample was heated for 15 min at 60 °C and extraction was carried out in a Precellys® 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Homogenization was carried out twice for 20 s at 5500 rpm with 10 s pause between homogenization steps, followed by centrifugation for 15 min at 3700 × *g*. The extraction procedure was repeated twice and the supernatant was pooled into a new Eppendorf tube and freeze-dried (glutenin fraction). The remaining flour pellet was dried using a Speedy Vac, and the remaining non-gluten proteins (residue fraction) were extracted with extraction/loading buffer: 62.5 mmol L^{-1} Tris – HCl, 20 g L^{-1} SDS, 100 mL L^{-1} glycerol, 0.05 g L^{-1} Bromophenol Blue and 15 g L^{-1} DTT buffer (pH 6.8) with Precellys® 24. Freeze-dried gliadin and glutenin fractions were re-suspended in extraction/loading buffer (see above). Samples were denatured at 90 °C for 1 min and each fraction was separated on NuPAGE® NOVEX 4 – 12% Bis-Tris Mini Gels (Invitrogen AS, Waltham, MA, USA). Electrophoresis was started at 40 V for 20 min, then the voltage was increased to 140 V for a further 105 min. The gels were stained for 3 h with 400 mL L^{-1} methanol, 100 g L^{-1} trichloroacetic acid (TCA) and 1 g L^{-1} Coomassie Brilliant Blue R-250 and destained for 18 h in 100 g L^{-1} TCA.

The gels were scanned with an Epson Perfection 4990 PHOTO scanner, and the electrophoretic patterns of the gel images were quantified by densitometry with ImageQuant TL software (GE Healthcare Life Sciences). To optimize the amount of sample for loading on the gel, a dilution series of each protein preparation was analyzed and concentrations were selected within the linear response region for all protein bands. All gels contained a duplicate reference sample. The volume of each protein band was determined after background subtraction using the rubber band method. Each protein band in the sample was quantified as the density of each band relative to the density of one specific band in the reference sample from the same gel. This makes it possible to compare the samples between the gels. Western blot analyses were carried out to identify bands on the gels according to the method of Tosi *et al.*[43]. The analyses showed that HMW-GS and LMW-GS were present in both the glutenin and the residue fractions, thus they were quantified from both fractions and the data combined. The bands of α-gliadins and γ-gliadins overlapped on the gel in the gliadin fraction, therefore they were quantified together as α- and γ-gliadins. Total gluten proteins were calculated as the sum of all quantified bands from the three fractions. Each group of gluten proteins was then divided by the value of total gluten proteins and expressed as a proportion. The ratios of HMW-GS to LMW-GS (HMW-GS:LMW-GS), gliadin to glutenin (Gli:Glu) and B-type LMW-GS to C-type and D-type LMW-GS (B-type:C- and D-type LMW-GS) were calculated. All samples were extracted in duplicate (technical replicates) and run on separate gels. The averages from the technical replicates were used in the statistical analysis.

Statistical analyses

Data were subjected to analysis of variance (ANOVA), and pairwise comparison was carried out by Tukey's test using R Commander modified at the Norwegian University of Life Sciences (Ås, Norway).

Principal component analysis (PCA), which is a multivariate approach designed for multicorrelated data, was performed using the software The Unscrambler® X, v. 10.2 (CAMO Software AS, Oslo, Norway) on the whole data set.

RESULTS

Four Norwegian spring wheat cultivars, all with the HMW-GS 5 + 10 associated with strong gluten, were grown in climate chambers and then exposed to three daytime temperatures (13, 18 and 23 °C) from anthesis to maturity to study the temperature effects on protein quality and viscoelastic properties of gluten.

PCA was performed on the results of the grain, flour and gluten analyses to provide an overview of the relationships between the temperature during grain development and wheat quality parameters. The first two principal components (PCs) accounted for 41 and 17% of the total variability respectively (Fig. 1). Protein content, GDW, S content, N:S ratio and protein quality parameters such as R_{max}, Ext and the proportion of ω-gliadins varied along PC-1, while PCG varied along PC-2 (Fig. 1(a)). The proportions of D-type LMW-GS and B-type LMW-GS, Mon:Pol ratio and %UPP varied along both PC-1 and PC-2. The proportions of gluten protein monomers and subunits (α- and γ-gliadins, C-type LMW-GS, HMW-GS) and the ratios HMW-GS:LMW-GS, B-type:C- and D-type LMW-GS and Gli:Glu clustered at the center of the loading plot. R_{max} of gluten was clustered with %UPP, GDW and the proportion of B-type LMW-GS located on the right side of the loading plot. Ext of gluten was located on the opposite side, clustered with Mon:Pol ratio, the proportion of ω-gliadins, protein content, S content and N:S ratio. R_{max} and %UPP were located on the opposite sides of Ext and Mon:Pol ratio respectively. The samples clustered according to temperature as well as to cultivar (Fig. 1(b)). Grains grown at 13 °C clustered on the right side, at 18 °C in the middle and at 23 °C on the left side of the score plot. Berserk was located in the lower part, Zebra in the upper right part, Bjarne in the upper part and Avle mainly on the left side of the score plot area.

Grain dry weight and protein content

Lower temperature prolonged the duration of grain development, with the duration of grain filling at 13/10 °C being twice as long as that at 23/20 °C. The temperature during grain development strongly affected GDW, protein content and S content of flour (Table 1). The highest GDW was observed at 13 °C and it decreased linearly as the temperature increased. Protein content was lowest for grain grown at 13 °C and increased with temperature. Protein content per grain was constant between temperatures for Avle, Berserk and Zebra, while it was lower at 23 °C for Bjarne. Sulfur content was increased as temperature increased. The N:S ratio was higher at 23 °C than at 13 °C ($P < 0.05$), the highest value being obtained for Berserk grown at 23 °C. Strong negative correlations were found between GDW and both protein content and S content ($r = 0.95$, $P < 0.001$ and $r = 0.95$, $P < 0.001$ respectively), and a strong positive correlation was found between protein content and S content ($r = 0.99$, $P < 0.001$).

Viscoelastic properties of gluten

The viscoelastic properties of gluten were measured using the Kieffer Dough and Gluten Extensibility Rig. The R_{max} values varied between 0.26 and 1.00 N, and significant effects of temperature, cultivar and their interaction were found for R_{max} (Table 2). Although significant differences were not observed between R_{max} of grain grown at 13 and 18 °C, the R_{max} value was highest in Berserk, Bjarne and Zebra grown at 13 °C (Table 2). R_{max} decreased when grain was grown at 23 °C ($P < 0.01$), with the effect being most pronounced for Avle and to a lesser extent for Bjarne. Both temperature and cultivar had significant effects on Ext (Table 2). No differences were found between 13 and 18 °C, while Ext was increased at 23 °C ($P < 0.05$). Avle had the lowest R_{max} and the highest Ext among the four cultivars.

Size distribution of gluten proteins

The size distribution of gluten proteins (gliadin monomers and glutenin polymers) of flour was determined by SE-FPLC. %UPP and Mon:Pol ratio were calculated from the SDS-unextractable fraction (F1*) and the SDS-extractable fractions (F1 – F4) (Table 2). Both temperature and cultivar as well as their interaction (T × C) significantly influenced %UPP. No difference was observed in %UPP between grain grown at 13 and 18 °C, while %UPP was decreased in grain grown at 23 °C ($P < 0.01$). Significant T × C interactions showed that the relationship between temperature and %UPP was not consistent among the four cultivars (Table 2). Temperature had little effect on %UPP in Berserk and Zebra. On the other hand, %UPP was clearly decreased, by about 10%, from 18 to 23 °C in Avle. Bjarne showed the same tendency as Avle, and %UPP was decreased moderately from 18 to 23 °C. %F1*, which consists of large polymers which are only extracted using sonication, showed a similar trend to %UPP, while %F1 was not affected by temperature. Cultivar and temperature had significant effects on Mon:Pol ratio (Table 2), which was increased at 23 °C ($P < 0.01$). %F3, consisting of SDS-extractable monomeric proteins, was significantly increased as temperature increased (Table 2). Berserk had the highest %UPP and the lowest Mon:Pol ratio among cultivars.

Composition of gluten protein

A correlation was found between protein content of flour and total gluten proteins quantified from SDS-PAGE analyses ($r = 0.93$, $P < 0.001$). Gliadins and glutenin subunits were quantified and the proportions of gluten protein groups were calculated. Western blot analyses with specific antibodies for gluten proteins allowed us to identify C-type LMW-GS and D-type LMW-GS in glutenin fractions (Supplemental Figures a and b in 'Supporting information'). The results are shown in Table 3 along with mean squares obtained by ANOVA. Significant temperature effects were found for the proportions of D-type LMW-GS, B-type LMW-GS, ω-gliadins and α- and γ-gliadins. The proportions of D-type LMW-GS and ω-gliadins increased with increasing temperature without significant changes in the proportion of D-type LMW-GS from 18 to 23 °C. The proportions of B-type LMW-GS and α- and γ-gliadins were lower at 23 °C ($P < 0.05$). Temperature did not have any effect on the proportions of HMW-GS and C-type LMW-GS or the ratios HMW-GS:LMW-GS, Gli:Glu and B-type:C- and D-type LMW-GS. Significant effects of cultivar were found for the proportions of all gluten protein groups, except C-type LMW-GS, and the ratios HMW-GS:LMW-GS and B-type:C- and D-type LMW-GS (Table 3).

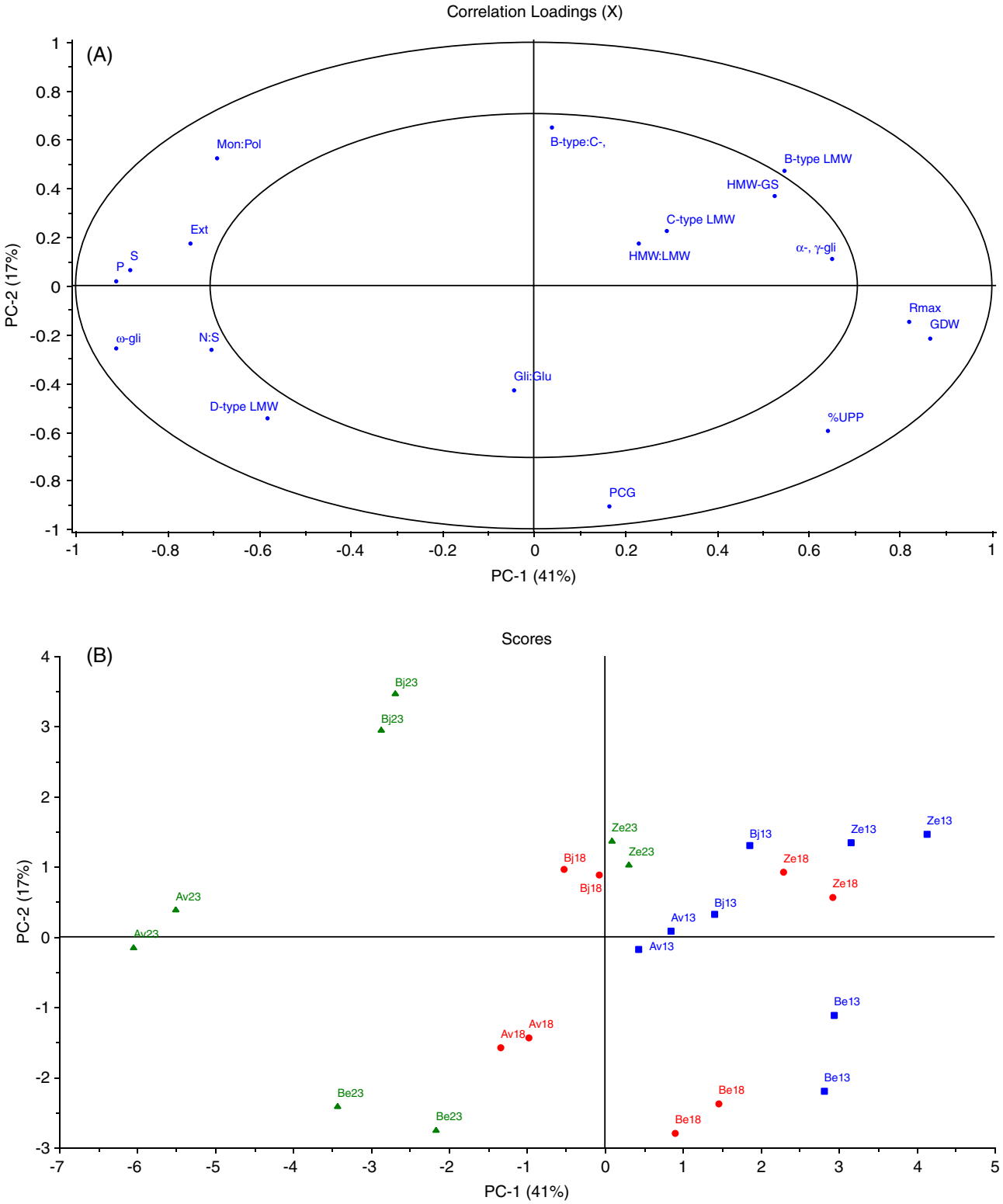

Figure 1. Loadings (A) and scores (B) for the two first principal components (PCs) in principal components analysis performed on the quality parameters measured on grain, flour and gluten. Abbreviations: grain dry weight (GDW), protein content (P), protein content per grain (PCG), sulfur content (S), N:S ratio (N:S), maximum resistance to extension (R_{max}) and extensibility (Ext) of gluten, proportions of ω-gliadins (ω-gli), α- and γ-gliadins (α-, γ-gli), HMW-GS (HMW-GS), D-type LMW-GS (D-type LMW), B-type LMW-GS (B-type LMW) and C-type LMW-GS (C-type LMW) in total gluten proteins, ratios of HMW-GS to LMW-GS (HMW:LMW), gliadins to glutenins (Gli:Glu) and B-type LMW-GS to C- and D-type LMW-GS (B-type:C-, D-type LMW) and cultivars Avle (Av), Berserk (Be), Bjarne (Bj) and Zebra (Ze). The numbers (13, 18 and 23) after cultivar names indicate the temperature during grain filling.

Table 1. Grain dry weight (GDW), protein content, protein content per grain (PCG), sulfur content and N:S ratio of four Norwegian spring wheat cultivars. The plants were grown at three different day/night temperatures (13/10, 18/15 and 23/20 °C) during grain development, and grain was harvested at maturity. Mean squares from ANOVA of each parameter are shown at the bottom of the table

		GDW (mg)		Protein content (g kg^{-1})		PCG (mg)		Sulfur content (mg g^{-1})		N:S ratio	
Cult.	Temp. (°C)	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD
Avle	13	40.10	1.46	1.216	0.003	4.87	0.17	1.35	0.07	15.82	0.87
	18	31.39	1.61	1.564	0.064	4.91	0.05	1.60	0.00	17.15	0.70
	23	20.31	1.97	2.418	0.122	4.90	0.23	2.45	0.21	17.34	0.63
Berserk	13	41.88	0.09	1.239	0.013	5.19	0.07	1.30	0.00	16.72	0.17
	18	34.92	2.13	1.589	0.129	5.53	0.11	1.70	0.14	16.40	0.03
	23	21.87	1.62	2.335	0.114	5.10	0.13	2.35	0.07	17.45	1.37
Bjarne	13	37.61	0.50	1.208	0.056	4.54	0.27	1.35	0.07	15.70	0.09
	18	27.19	0.98	1.652	0.060	4.49	0.00	1.70	0.00	17.05	0.62
	23	17.08	0.14	2.274	0.025	3.88	0.08	2.35	0.07	16.98	0.32
Zebra	13	42.37	0.84	1.108	0.041	4.69	0.08	1.25	0.07	15.56	0.30
	18	36.19	2.25	1.312	0.024	4.74	0.21	1.50	0.00	15.34	0.28
	23	24.70	1.43	1.820	0.021	4.50	0.21	1.95	0.07	16.38	0.40
Source	DF	MS		MS		MS		MS		MS	
Temp.	2	768.14	***	215.78	***	0.221	**	1.929	***	2.371	*
Cult.	3	57.69	***	14.33	***	0.997	***	0.078	**	1.502	*
T × C	6	2.21	NS	2.60	**	0.062	NS	0.022	NS	0.453	NS

Ave., average of two biological replicates; SD, standard deviation; Temp., temperature; Cult., cultivar; T × C, temperature × cultivar interaction; DF, degrees of freedom; MS, mean square calculated by ANOVA. Asterisks indicate significance at ***0.1, **1 or *5%; NS indicates no significance at 5%.

Table 2. Maximum resistance to extension (R_{max}) and extensibility (Ext) of gluten measured by Kieffer Dough and Gluten Extensibility Rig and size distribution of gluten proteins in flour determined by SE-FPLC. Four Norwegian spring wheat cultivars were grown at three constant day/night temperatures (13/10, 18/15 and 23/20 °C) during grain development, and grain was harvested at maturity. %UPP was calculated as $[F1^*/(F1^* + F1)] \times 100$ and the Mon:Pol ratio was calculated as $(F3 + F4)/(F1^* + F1 + F2)$. Mean squares from ANOVA of each parameter are shown at the bottom of the table

		R_{max} (N)		Ext (mm)		%UPP		Mon:Pol		F1* (%)		F1 (%)		F2 (%)		F3 (%)		F4 (%)	
Cult.	Temp. (°C)	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD
Avle	13	0.59	0.17	123.67	2.85	48.14	0.40	1.07	0.04	17.75	0.35	19.12	0.69	7.30	0.23	22.33	0.25	24.70	0.52
	18	0.75	0.12	115.21	8.71	49.21	0.45	1.09	0.01	17.93	0.29	18.50	0.03	7.80	0.06	23.54	0.01	24.53	0.35
	23	0.26	0.04	134.75	12.36	41.00	0.89	1.21	0.02	13.60	0.75	19.58	0.35	8.47	0.13	24.62	0.66	25.60	0.37
Berserk	13	1.00	0.10	94.66	7.19	53.01	1.14	1.05	0.02	19.54	0.75	17.31	0.12	7.34	0.31	18.42	0.35	28.03	0.25
	18	0.77	0.04	94.63	3.05	52.86	0.22	1.07	0.02	19.73	0.18	17.61	0.33	6.96	0.15	18.91	0.09	28.36	0.18
	23	0.76	0.02	116.27	14.03	51.14	0.49	1.13	0.01	18.24	0.41	17.42	0.05	7.40	0.28	19.01	0.31	29.53	0.28
Bjarne	13	0.85	0.07	112.37	3.63	46.21	0.30	1.12	0.02	16.31	0.34	18.99	0.16	7.73	0.10	21.43	0.05	26.82	0.26
	18	0.78	0.02	106.61	0.37	48.23	0.00	1.14	0.01	16.76	0.06	17.99	0.06	8.36	0.17	22.95	0.17	26.13	0.23
	23	0.54	0.07	117.70	5.17	43.83	0.19	1.18	0.02	14.84	0.26	19.01	0.20	8.49	0.13	24.11	0.06	25.71	0.50
Zebra	13	0.97	0.11	103.52	5.03	49.47	2.67	1.11	0.02	17.41	0.99	17.77	0.87	7.57	0.18	20.44	0.32	26.84	1.14
	18	0.84	0.07	95.35	5.08	49.32	0.12	1.10	0.02	17.89	0.29	18.38	0.38	7.30	0.27	21.11	0.16	26.76	0.38
	23	0.79	0.05	106.05	9.70	49.60	0.15	1.15	0.02	17.30	0.08	17.59	0.19	7.54	0.01	21.35	0.01	27.54	0.45
Source	DF	MS		MS		MS		MS		MS		MS		MS		MS		MS	
Temp.	3	0.151	***	509.2	**	27.69	***	0.014	***	10.05	***	0.164	NS	0.517	***	5.34	***	0.91	*
Cult.	2	0.138	***	705.5	***	54.30	***	0.004	**	12.19	***	3.175	***	1.081	***	26.85	***	14.40	***
T × C	6	0.026	*	39.5	NS	8.02	***	0.001	NS	1.83	**	0.501	*	0.216	**	0.53	**	0.65	NS

Ave., average of two biological replicates; SD, standard deviation; Temp., temperature; Cult., cultivar; T × C, temperature × cultivar interaction; DF, degrees of freedom; MS, mean square calculated by ANOVA. Asterisks indicate significance at ***0.1, **1 or *5%; NS indicates no significance at 5%.

DISCUSSION

Wheat-producing areas in Scandinavia and parts of Northwestern Europe are exposed to lower temperatures compared with most other wheat production areas in the world. For example, mean daily temperatures of 15–16 °C are normally achieved during grain filling in the southeast of Norway, and periods with mean daily temperatures during grain filling as low as 13 °C are frequently observed. To increase our knowledge of how such temperatures affect gluten protein quality, the present study was carried out in climate chambers with day/night temperature regimes of 13/10, 18/15 and 23/20 °C during grain filling.

Table 3. Proportions of ω-gliadins, α- and γ-gliadins, HMW-GS, D-type LMW-GS, B-type LMW-GS and C-type LMW-GS in total gluten proteins and ratios of HMW-GS to LMW-GS (HMW:LMW), gliadins to glutenins (Gli:Glu) and B-type LMW-GS to C-type and D-type LMW-GS (B-type:C-, D-type LMW) of four Norwegian spring wheat cultivars grown at 13, 18 and 23 °C during grain filling. Mean squares from ANOVA of each parameter are shown at the bottom of the table

		Gliadin fraction				Glutenin fraction													
		ω-Gliadins		α-, γ-Gliadins		HMW-GS		LMW-GS						HMW:LMW		Gli:Glu		B-type:C-, D-type LMW	
								D-type		B-type		C-type							
Cult.	Temp. (°C)	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD	Ave.	SD
Avle	13	12.6	1.3	31.9	1.9	22.1	0.7	8.4	1.3	13.9	1.2	11.1	1.4	0.67	0.10	0.74	0.01	0.72	0.03
	18	15.1	0.8	29.8	1.6	22.0	0.8	9.6	0.4	13.3	1.2	10.3	1.6	0.67	0.09	0.88	0.00	0.74	0.00
	23	16.8	0.3	29.1	0.8	21.7	0.1	9.9	0.2	12.6	0.6	10.0	0.0	0.67	0.00	0.80	0.06	0.69	0.03
Berserk	13	10.7	0.5	32.9	1.5	23.2	1.0	7.8	0.1	14.2	0.4	11.1	0.5	0.70	0.01	0.83	0.01	0.67	0.01
	18	11.9	1.0	32.8	0.5	23.1	0.5	8.7	0.4	13.4	0.5	10.2	0.1	0.72	0.01	0.85	0.03	0.65	0.03
	23	16.7	0.3	34.5	1.4	21.3	0.2	7.8	0.1	11.6	1.0	8.1	0.3	0.78	0.02	0.86	0.00	0.61	0.00
Bjarne	13	11.7	0.9	33.9	0.7	22.3	0.2	6.2	0.1	15.2	0.9	10.8	0.9	0.70	0.05	0.84	0.01	0.66	0.00
	18	13.0	0.4	32.1	1.6	22.3	0.4	7.3	0.5	14.7	0.9	10.7	0.3	0.69	0.02	0.80	0.02	0.73	0.05
	23	14.1	0.5	31.7	0.0	23.2	0.6	6.8	0.6	13.9	0.1	10.2	0.5	0.75	0.05	0.78	0.00	0.76	0.03
Zebra	13	9.4	1.0	37.5	1.6	23.6	1.7	5.5	0.2	14.4	0.8	9.5	1.4	0.80	0.03	0.78	0.04	0.73	0.01
	18	10.4	0.3	35.6	0.5	23.0	0.9	6.3	0.1	14.7	1.4	10.1	0.2	0.75	0.05	0.82	0.01	0.71	0.01
	23	11.1	0.3	33.5	0.1	23.9	0.3	7.1	0.8	13.5	0.0	10.9	0.6	0.76	0.01	0.87	0.11	0.74	0.06
Source	DF	MS		MS		MS		MS		MS		MS		MS		MS		MS	
Temp.	2	26.04	***	7.69	*	0.17	NS	2.47	**	4.91	*	1.55	NS	0.0029	NS	0.0035	NS	0.0003	NS
Cult.	3	21.07	***	28.80	***	2.48	*	10.90	***	3.20	*	0.69	NS	0.0104	*	0.0018	NS	0.0094	***
T × C	6	2.52	**	3.04	NS	1.08	NS	0.40	NS	0.35	NS	1.71	NS	0.0015	NS	0.0039	NS	0.0032	*

Ave., average of two biological replicates; SD, standard deviation; Temp., temperature; Cult., cultivar; T × C, temperature × cultivar interaction; DF, degrees of freedom; MS, mean square calculated by ANOVA. Asterisks indicate significance at ***0.1, **1 or *5%; NS indicates no significance at 5%.

Our results supported previous reports that lower temperatures prolonged the duration of grain filling and increased GDW.[17,18] PCG was not affected by temperature, while the lower to moderate temperatures had little effect on storage protein accumulation, which supports the result of Sofield *et al.*[19] Protein content was consequently decreased at low temperature. As both protein and S contents were negatively correlated with GDW, the decrease in grain weight due to higher temperatures was also a major cause of the high S content of flour at higher temperatures. As most of the S in grain is present in proteins, a strong correlation was observed between protein content and S content. The high N:S ratio at 23 °C indicated that S was limited compared with N in grain grown at higher temperatures.

The rheological properties of gluten were determined using the Kieffer rig extensograph in order to eliminate effects of the variation in protein and starch contents of the flour. A positive correlation was found between R_{max} and %UPP ($r = 0.75$, $P < 0.001$), and PCA also showed that there were close associations between R_{max} and %UPP and between Ext and Mon:Pol ratio. Our results indicated that the viscoelastic properties of gluten could to some extent be explained by %UPP and Mon:Pol ratio, supporting previous reports.[5,7,44] Changes in %UPP could be explained better by effects of temperature on the SDS-unextractable polymeric proteins (F1* fraction) rather than on the SDS-extractable polymeric proteins (F1 fraction), and changes in Mon:Pol ratio could be explained by effects on the SDS-unextractable polymeric proteins and the SDS-extractable monomeric proteins, especially %F3. Thus temperature seemed to influence the assembly of large glutenin polymers which takes place during the maturation/desiccation phase, as well as the Mon:Pol ratio.

All samples had strong gluten, even those grown at day/night temperatures of 13/10 °C, indicating that constant low temperature did not negatively affect the assembly of large glutenin polymers and the viscoelastic properties of gluten. These results contrast with previous data from field trials which showed weaker gluten in cooler seasons,[33,34,36] especially when temperatures during grain development fell below 18 °C.[36] Thus the results from the present study indicate that low temperature *per se* cannot explain the poor gluten quality. Other environmental factors associated with lower temperatures in the field are probably causing weak gluten. Precipitation could be one such factor, as low temperatures are usually associated with precipitation in Northern Europe. Frequent precipitation may have negative effects on the polymerization of gluten proteins, as discussed by Johansson *et al.*[45] Although temperature itself may not have direct effects on gluten quality, it probably has indirect effects, as large variations in R_{max} were explained by temperature variation.[36] The constant day/night temperatures used in the climate chamber experiments differ from those in the field, where temperatures fluctuate widely both within a day and during the grain-filling period and where rapid temperature changes or drops may occur. Corbellini *et al.*[20] tested the effects of very high temperature (35–40 °C) for short to long periods at different stages of grain filling and found effects of temperature shock on gluten quality. To our knowledge, it has not yet been investigated whether rapid decreases in temperature during grain filling can cause stress and affect gluten quality.

The proportions of ω-gliadins and HMW-GS in total gluten proteins determined by gel scanning in this study were higher than those determined by reverse phase high-performance liquid chromatography (RP-HPLC) by Wieser *et al.*[46,47] A possible reason is limited availability of S compared with N.[13,48–51] Analysis of N and S in flour gave N:S ratios ranging from 15.5:1 to 17.5:1, which were relatively high compared with the ratio reported from grains grown in fields with balanced fertilization of N and S.[52] Therefore the proportion of S-poor proteins in the grains grown in climate chambers in the present study may be higher than that in grain grown in the field with balanced nutrient supply. Another reason could be the different methodology. RP-HPLC separates proteins through their hydrophobicity, while SDS-PAGE separates proteins by their molecular size. Nevertheless, all samples were analyzed using the same methods and showed similar differences related to temperature treatments and genotypes.

Temperature effects on the composition of gluten proteins have been studied intensively,[21,22,29,30] showing that the proportion of ω-gliadins generally increases with increasing temperature. However, the effects of temperature on the proportions of other gluten proteins were small and inconsistent between studies.[15] DuPont *et al.*[22] observed only small changes in the relative amount of different gluten proteins due to temperature, while transcriptomic studies of gliadin and glutenin subunit gene expression showed no effect of temperature in their study. The authors therefore suggested that synthesis of the gluten proteins is tightly controlled by genetic rather than by environmental factors. In the present study, PCA showed associations between the N:S ratio and the proportions of ω-gliadins and D-type LMW-GS, with a significant correlation being found between the N:S ratio and the proportion of ω-gliadins plus D-type LMW-GS ($r = 0.69$, $P < 0.001$). These results imply that higher N:S ratio at higher temperatures may have resulted in the increase in S-poor proteins. This observation is similar to the results of DuPont *et al.*,[29] who reported that both the N:S ratio and the proportion of S-poor proteins were higher at (day/night) 37/28 °C compared with 24/17 °C.

R_{max} and %UPP were reduced at 23 °C for Avle and Bjarne. Earlier studies showed that high temperatures had a weakening effect on gluten properties which was associated with a decrease in molecular weight of large glutenin polymers accompanied by an increase in Gli:Glu ratio or a decrease in HMW-GS:LMW-GS ratio.[31,32] A clear association between %UPP and the ratios Gli:Glu, HMW-GS:LMW-GS and B-type:C- and D-type LMW-GS was not observed in this study, but PCA showed associations between ω-gliadins and gluten extensibility or the Mon:Pol ratio. The result imply that increases in the proportions of ω-gliadins at high temperatures had a weakening effect on dough properties. Our results support those of DuPont *et al.*,[29] who reported that high temperatures increased the proportion of S-poor proteins and decreased mixing tolerance. The F3 fraction is enriched in ω-gliadins, hence effects of temperature on the proportion of F3 may have contributed to the changes in Mon:Pol ratio.

Among the four cultivars, Berserk had the highest %UPP with high R_{max} values that were little influenced by temperature. The results agree with earlier studies in which Berserk had the highest gluten resistance among Norwegian spring wheat cultivars. By contrast, Avle had the lowest R_{max} and the highest Ext, which were strongly influenced by temperature compared with the other three cultivars, indicating that this cultivar was more sensitive to a wider range of temperatures than the other cultivars.

CONCLUSIONS

The effects of low to moderate temperatures during grain filling on gluten quality were investigated with four Norwegian cultivars in controlled climate chambers. The results showed that constant low temperature had no direct negative effects on gluten quality, as strong gluten was obtained from grain grown at 13 °C. Our results suggest that the effects of temperature on the viscoelastic properties of gluten were associated with changes in the assembly of large glutenin polymers rather than changes in the composition of gluten proteins. Significant temperature × cultivar interaction effects were found for %UPP and R_{max}. Berserk had the strongest gluten and its quality was more stable to temperatures, while Avle had weaker gluten which was more sensitive to temperatures investigated in this study. Temperature during grain filling had strong effects on the duration of grain filling and grain weight as well as on the protein and S contents of flour.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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Paper III

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Temperature variations during grain filling obtained in growth tunnel experiments and its influence on protein content, polymer build-up and gluten viscoelastic properties in wheat

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ABSTRACT

The aim of this study was to investigate effects of temperature during grain filling on gluten quality characteristics at a lower to moderate temperature range. Experiments with two wheat varieties grown in field covered by polypropylene tunnels during grain filling were performed in two seasons. Mean day temperature differences achieved within the tunnel were approximately 2–2.5 °C from the open to the closed end. There were significant effects of temperature on grain maturity time, thousand grain weight and protein content. The resistance to stretching of the gluten doughs increased with the increasing day temperatures. This was reflected in the proportion of unextractable polymeric proteins (UPP). The results suggest that increases in temperature within this temperature range affect the polymerization of polymeric proteins, giving higher molecular weights, and hence increased Rmax and stronger gluten. The two varieties differed in their response to temperature. In addition, there were seasonal variations in gluten functionality that may be associated with fluctuations in day temperatures between the seasons.

1. Introduction

The viscoelastic property of gluten is crucial for the baking quality of wheat flour. The well-known variation between wheat varieties is utilized to obtain flours of different gluten strengths adapted for different baked products. Not only the variety itself can affect the viscoelasticity of gluten, but also environmental factors linked to the growth conditions during plant development. Several studies have shown large variation between wheat samples of the same variety when grown in different growth environments (Graybosch et al., 1995; Johansson et al., 2002; Moldestad et al., 2011; Uhlen et al., 2004). Such variation is challenging for the industry as it causes an inconsistency in gluten strength that may impede the sorting of the wheat into uniform quality classes. Hence, environmentally induced variation in gluten quality presents a challenge for the milling industry making it difficult to maintain a stable quality, as well as for the bakeries that need to adjust baking processes to compensate for the variations.

The viscoelastic properties of gluten are primarily related to the ratio of monomeric to polymeric proteins (Uthayakumaran et al., 2000) and to the proportion of glutenin aggregates above a certain molecular weight (Southan and MacRitchie, 1999). Particularly, the proportion of the large and unextractable polymeric proteins (UPP) is related to gluten strength of flours. This fraction increases rapidly during the desiccation phase in the developing grain, through post-translational polymerisation of the glutenin subunits (Carceller and Aussenac, 1999). Allelic variation, particularly in the genes encoding the HMW glutenin subunits, is known to affect the degree of polymerisation of the glutenins and thus relate to differences in gluten quality between varieties (Payne, 1987; Shewry et al., 1992). Whereas the varietal differences in gluten viscoelastic properties are well studied and have been linked to specific alleles, the differences caused by environmental factors are less understood. It is suggested that environmental factors may influence both the synthesis of gliadins and glutenin subunits during grain development as well as their polymerisation and formation of the large and insoluble glutenin aggregates (Carceller and Aussenac, 2001; Johansson et al., 2005).

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List of abbreviations

%P	protein concentration in % d.m.
Ext	extensibility as measured by SMS/Kieffer Dough and Gluten Extensibility Rig
FN	Falling Number
Mono/Poly	ratio of monomeric to polymeric proteins
PCA	Principal component analysis
PC	Principal component
Rmax	Resistance to stretching as measured by the SMS/Kieffer Dough and Gluten Extensibility Rig
SE-FPLC	Size Exclusion Fast Performance Liquid Chromatography
SDS	Sodium Dodecyl Sulphate sedimentation volume
TGW	Thousand Grain Weight
TW	Test weight
UPP	Unextractable Polymeric Protein

Temperature during grain filling is among the environmental factors most studied for its impact on the gluten viscoelastic properties. It is well documented that periods of heat stress (>32–35 °C), particularly during the later part of grain filling, reduces the dough strength (Blumenthal et al., 1991). This was found to be due to increased monomer to polymer ratio and/or to increased ratio of HMW to LMW glutenin subunits, leading to lower UPP (Ciaffi et al., 1996). Based on experiments with moderate temperatures in both field and controlled climate chambers, Randall and Moss, 1990 concluded that dough strength generally increases with a temperature up to 30 °C. Other investigations have found results in line with this. For instance, field experiments in Scandinavia have shown that the warmer growth seasons generally give better wheat quality and stronger gluten compared to the cooler seasons (Johansson and Svensson, 1998; Moldestad et al., 2011; Uhlen et al., 2004). However, a positive relationship between growth temperature and gluten quality has not always been supported. Georget et al. (2008) found only limited variation in gluten protein composition from experiments in field and in polythene tunnels mimicking a hot/dry and a wet/cool climate. Several studies have used experiments in controlled climate chambers and analysed the composition and polymerisation state of the gluten proteins. Uhlen et al. (1998) analysed wheat samples from climate chamber experiments with temperature range from 13 to 21 °C, and found increased proportions of polymeric proteins with increasing temperatures, but the differences were moderate. Johansson et al. (2005) did experiments in controlled climate chambers at temperatures of 17/14 °C and 22/19 °C during grain filling, and found no consistent changes in the size distribution of polymeric proteins due to the temperature differences. More recently, Malik and co-workers (Malik et al., 2013, 2011) found increased UPP at the higher temperature regime when analysing grain samples from growth chambers of 22/19 °C and 17/14 °C. In a recent review, Johansson et al. (2013) state that the influence of the temperature on gluten protein structure is complex as it may involve a number of interacting biochemical mechanisms. Varieties may respond differently, but few studies have investigated temperature*genotype interactions when it comes to gluten polymer structure and viscoelastic properties.

The existing literature shows inconsistent results regarding the impact of temperature on gluten quality. Furthermore, few studies have investigated impacts of temperature on gluten polymer structure under field conditions. The approaches taken in the former investigations are primarily experiments in climate chambers with different temperature regimes, or field experiments laid out at different locations and/or in different growing seasons, which are expected to result in different temperature ranges. Both types of experiments may be challenging, the former because the defined conditions in climate chambers may be very different from field conditions, and the latter because a range of abiotic and biotic factors, as well as their interactions, may interfere with the results.

An alternative approach to investigate temperature effects is to use experiments in long growth tunnels, where a temperature gradient is achieved in the longitudinal direction of the tunnel. In such tunnels, plants can be grown in plots in natural soils and be subjected to similar but natural variations in solar radiation and humidity, as well as the seasonal and daily (day/night) variations in temperature. This investigation reports on the effects of temperature variations on gluten quality characteristics that were achieved by using experiments in polypropylene covered tunnels in two different growth seasons. The aims were to investigate the relationships between temperature during grain filling and gluten viscoelastic properties at lower to moderate temperatures, and to explore differences in gluten protein composition and molecular weight distribution of the polymeric proteins.

2. Materials and methods

2.1. Field experiments with polypropylene tunnels during grain filling

Field experiments were performed at Bioforsk Øst, Apelsvoll (60° 42″ N, 10° 51″ E) in Norway in 2010 and 2011. The experimental site is on an imperfectly drained brown earth (Gleyed melanic brunisoil, Canada Soil Survey) with predominantly loam and silty sand textures. Apelsvoll has a mean annual precipitation of 600 mm, a mean annual temperature of 3.6 °C and a mean growing season (May–September) temperature of 12 °C. Two spring wheat varieties, cv. Bjarne (Graminor, Norway) and cv. Zebra (Lantmannen SWseed, Sweden), were used. The field trial covered an area of 6*40 m^2 with a plot size of 1.5*5 m^2. The plots were sown in 4 rows in the longitudinal direction, each containing 8 plots. Sub-blocks of 4 plots having the same variety were randomized in the longitudinal direction of the tunnel, giving a total of 16 plots per variety and two replicates. Thus, each replicate comprised both varieties grown at each of the eight positions in the longitudinal direction of the tunnel. Sowing dates were May 7th (2010) and April 29th (2011). 90 kg nitrogen (N) per ha in a compound NPK fertiliser (Fullgjødsel® 19-4-12, Yara Norge AS) was band-placed in the seedbed at sowing as a basal dressing. In addition, a top dressing of 40 kg N per ha was surface-applied as calcium ammonium nitrate with sulphur (OPTI-NS 27-0-0™, Yara Norge AS) at stem elongation (Zadoks 31). Annual weeds were controlled by spraying once per season, with Ariane S (*fluorxypyr* + *clopyralid* + *MCPA*). In 2010 it was sprayed with fungicides Proline (*prothioconazole*) + Amistar Duo Twin (*azoxystrobin* + *propiconazole*) at the start of flowering. In 2011 the fungicide Stereo (*cyprodinil* + *propiconazole*) was applied together with the insecticide Fastac (*alpha-cypermethrin*) at stem elongation. In addition, it was sprayed at anthesis with fungicide Proline (*prothioconazole*) + insecticide Fastac (*alpha-cypermethrin*). The polypropylene tunnel was established at heading, just prior to anthesis. The tunnel used was a Viking Cathedral tunnel with the size 8 m * 40 m and maximum height of approximately 4 m. The tunnel was closed at the western end, also by polypropylene cloth. Temperature loggers (Temprecord Multitrip™, Multi use temperature recorder) were placed in every second plot (Fig. 1), and positioned at the height of the ears. The experimental area was irrigated at July 13th 2010 with 40 mm, and July 15th 2011 with 25 mm. Irrigation was performed by using a sprinkler system with

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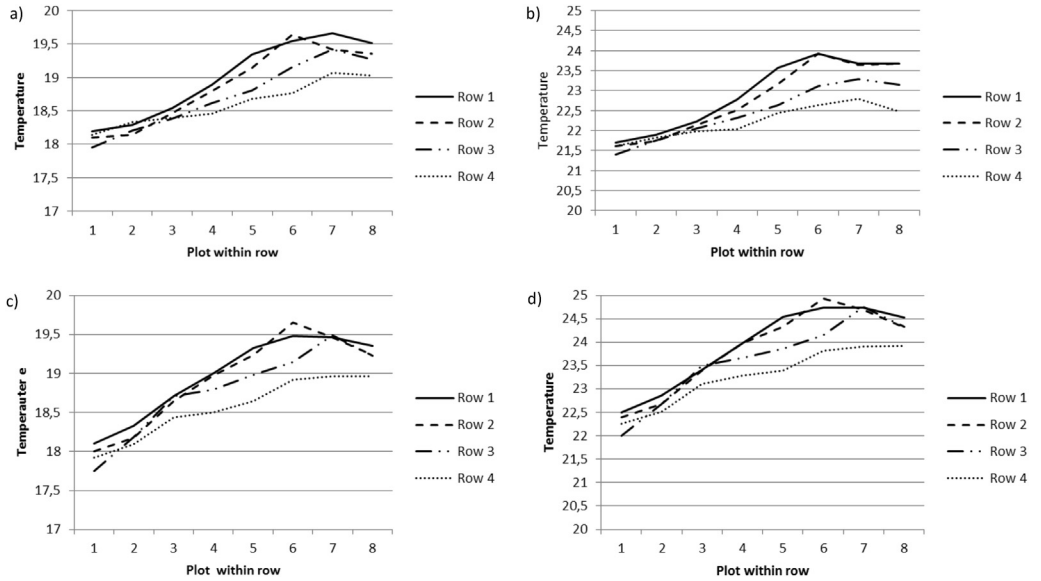

Fig. 1. Temperature gradients obtained wthin the tunnel for each of the 4 rows. a) and c) show mean daily temperature for the period from anthesis to yellow ripening in 2010 (7. July–27. August) and 2011 (5.July–2. August), b) and d) show the mean day temerature (calculated from 07:00-2000 h) for the same periods in 2010 and 2011. The plot within row on the x-axis show the longitudinal direction from the opening (plot 1) to the closure (plot 8).

nozzles mounted with 1 m intervals in the centre of the roof alongside the tunnel.

Dates for heading, anthesis and yellow ripeness were recorded. Each plot was harvested with a plot combine. On each plot, grain moisture content was measured using standard procedures and grain yields were corrected to 15% grain moisture.

2.2. Physical grain analyses and milling

1000 grain weight (TGW) and test weight (TW) were determined in samples from each plot. Whole meal flour was milled on a Laboratory Mill 3100 (Perten Instruments AB, Huddinge, Sweden) using a 0.8 mm screen. Samples of 50 g were milled from each plot.

2.3. Flour analysis

Falling Number (FN) was determined for all flour samples using Falling Number 1800 (Perten Instruments AB, Huddinge, Sweden). Sodium dodecyl sulphate sedimentation volume (SDS) was determined according to the AACC method 56–70 (AACC 2000). Protein content (%P) of wholemeal flours was determined by near infrared reflectance (NIR) using Perten Inframatic 9200 (Perten Instruments AB, Huddinge, Sweden).

2.4. Gluten micro-extension test

Gluten micro-extension test was performed as described by Moldestad et al. (2011) using the SMS/Kieffer Dough and Gluten Extensibility Rig for the TA.XT *plus* Texture Analyser (Kieffer et al., 1998) (Stable Micro Systems, Godalming, UK). Gluten was prepared from whole meal flour in a Glutomatic 2100 (Perten Instruments AB, Huddinge, Sweden) by using a 2% NaCl solution to remove salt soluble components. The dough was mixed for 1 min before 10 min of washing. To remove bran particles, two different filters were used in the process: An 88 μm sieve was changed after 2 min and replaced by an 840 μm sieve. To remove excess water, the gluten dough was centrifuged in a custom-made centrifuge mould at 4100 rpm for 10 min at 20 °C. Subsequently, it was pressed in the standard Teflon mould and rested for 45 min at 30 °C before the analysis with the Kieffer-rig. The parameters resistance to extension (Rmax), extensibility (Ext) and total area under the curve (Area) were recorded from the extensograms according to Kieffer et al. (1998).

2.5. Size exclusion fast performance liquid chromatography (SE-FPLC)

Flour samples of 240 mg were extracted sequentially to obtain two extracts (SDS-extractable and SDS-unextractable) according to the method of Morel et al. (2000) with modifications described by Tronsmo et al. (2002). The sonication was done by Sonics VC130 ultrasonic processor (Sonics and Materials, Newton, CT 06470 – 1614, USA) for 3 min (with 70% automatic amplitude compensation). Samples were extracted in duplicate. Flour extracts were separated on a Superose® 12HR 10/30 column at SE-FPLC (ÄKTAFPLC, GE Healthcare Biosciences, Uppsala). The proportion of unextractable polymeric protein (%UPP) was calculated as the percentage of unextractable polymeric protein of the total polymeric protein. SE-FPLC was performed on all samples from all plots in 2010 and on samples from plots with temperature logger in 2011.

2.6. Temperature data

Temperature data (recorded every hour) were downloaded from the temperature loggers positioned inside the tunnel. Mean daily temperatures as well as day temperatures (from 07:00–20:00 h) were calculated. For the plots without temperature loggers, values were estimated by using all neighbouring plots (i.e. for plot 11, the average of plot 3, 10, 12 and 19 was calculated). The temperature gradients across the longitudinal direction of the tunnel for the four rows, averaged for the dates from anthesis to yellow ripeness were calculated.

2.7. Statistical analysis

PCA (program packet Unscrambler v 10.1, CAMO Software AS, Oslo, Norway) was run in order to resolve and to visualize the main variation in the data. By PCA, the main variation in the data are visualised in the correlation loading plot for the interpretation of

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the variation among the variables for the two first and most important PCs, and in the score plot for the interpretation of the relationships between samples for the corresponding PCs.

ANOVA was performed for both growth seasons individually, and combined for the two growth seasons. The input in the model was both cultivar which defines two classes, and the temperature which was used in the model as a quantitative variable. The *p*-values were adjusted for false discovery rate (FDR) using the rotation test (Langsrud, 2005). Simple linear regressions were performed by Minitab (Minitab Inc., USA) using temperature data as input and quality characteristics as responses.

3. Results

The temperature gradients achieved in the longitudinal direction of the tunnel are shown in Fig. 1. Differences of approximately 1.5 °C in mean daily temperature from the opening to the inner part of the tunnel were achieved in both seasons (Fig. 1 a and c). The gradients showed a linear increase from the opening to approximately the 6th plot, and thereafter no further increase in temperature was found. Differences in temperature were found between the rows in the tunnel, where row 1 and 2, positioned to the south had similar and steeper temperature gradients compared to the other rows. Row 3 showed lower temperature compared to rows 1 and 2, and this was even more pronounced for row 4, positioned along the northern side of the tunnel. Larger temperature differences were obtained during day time, particularly on sunny days. Thus, steeper temperature gradients were found when calculating day averages from 07:00 to 20:00 h (Fig. 1, b and d), and temperature increases of approximately 2–2.5 °C from the open to the closed end were found in both seasons. For mean day temperatures, slightly higher temperatures were achieved in 2011 compared to 2010. The actual day temperatures during the grain filling period for the two seasons are shown in Fig. 2, and are represented as averages for all plots in the tunnel. For both seasons, the day temperatures fluctuated around 20 °C from anthesis and were slightly decreased in the period before yellow ripeness. A 10-day period approximately mid-way in the grain filling period had higher day time temperatures in 2011 compared to 2010 (Fig. 2), caused by a period of more sunny days.

The increase in temperature inside the tunnel affected plant development and maturation time (Table 1). The moisture contents at harvest reflected closely the temperature gradients in the tunnel, showing decreasing moisture contents from the opening to approximately the 6th plot within the tunnel, from where the moisture content was similar. The temperature gradient was also reflected in %P, as the %P increased with temperature inside in the tunnel.

Fig. 3 shows the PCA correlation loading and score plot for the two first and most important Principal Components (PCs). The two first PC accounted for 48% and 28%, respectively, of the total variability in the observed parameters. The score plot (Fig. 3a) shows two distinct clusters of samples corresponding to the year of growth. All samples from the growth year 2011 are located towards the lower right of the score plot, and the samples from 2010 are located in the upper left direction. Within each cluster, the variety Bjarne is located towards the upper right and Zebra towards the lower left direction. Each field plot in the tunnel is represented by the corresponding average day temperature in the score plot. Within each variety, there is a variation corresponding to the average day temperature; the higher temperature the more upwards to the right in the cluster within each cluster representing growth year. These two orthogonal patterns of variation between the samples are marked by arrows in the corresponding correlation loading plot, (Fig. 3b); solid arrow for the first direction, and dotted for the second). The first direction, marked with the solid arrow, which increases towards the lower right of the plot (high in PC1 and low in PC2) dividing the material into the two growth seasons, is characterised by higher specific SDS, higher yield and higher Rmax. This direction is also characterised by lower Ext and lower %P. The other direction marked with the dotted arrow in the loading plot, upper right (high in PC1 and high in PC2) vs. lower left are characterised by higher Rmax and higher %P, but not higher specific SDS value. This direction both reflects the variety differences but also the temperature variation within each variety with higher values for Bjarne compared with Zebra, and higher values for higher temperatures within each growth year and each cultivar. The day and daily temperatures were included in the analysis as down-weighted variables, allowing their relation to the other parameters to be visualised in the correlation loading plot without influencing the analysis. Both temperature variables were located along the first PC. Thus, the two directions in the plot described by the two arrays in the correlation loading plot represent two independent sources of variation which both increased with increasing temperature.

The analysed quality traits for the two varieties and in the two seasons (as averaged for all plots within the tunnel) are shown in Table 2. Bjarne was higher in protein content, SDS and Rmax, and lower in Ext and TGW compared to Zebra. These differences correspond to earlier results from spring wheat variety testing in Norway. The UPP values were also higher for Bjarne, reflecting the higher gluten strength as seen in Rmax. Zebra had a lower Falling Number than Bjarne, most prevalent in 2010. Differences in quality between the two seasons were also present. The protein contents were higher in 2010, whereas the gluten quality parameters SDS, Rmax and UPP were higher and the Ext were lower in 2011.

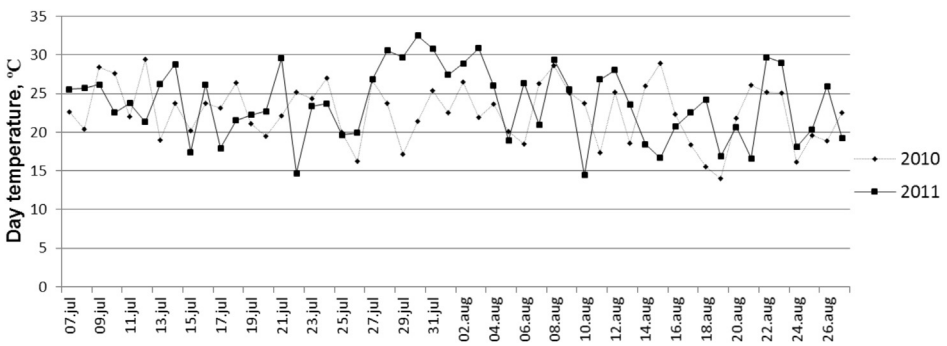

Fig. 2. Day temperatures (07:00–20:00 h) from heading to yellow ripening in 2010 and 2011, averaged for all temperature loggers in the tunnel.

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Table 1
Means of yield and quality traits for the different positions (1–8) in the longitudinal direction in the tunnel, averaged over varieties and replicates.

Season	Position	Day Temp.,° C	Moisture at harvest, %	Yield,kg/ha	TGW,g	Protein, %	TW, kg/hl	FN,s	SDS,ml	Rmax,N	Ext, mm	UPP,%	Mono/poly
2010	1	21.58	27.93	374	41.13	14.66	82.17	329	84.3	0.562	139.7	43.52	1.13
	2	21.80	28.24	392	40.81	14.93	82.07	311	84.5	0.486	155.7	44.27	1.11
	3	22.10	24.20	379	39.85	14.29	82.71	317	87.3	0.601	142.0	44.50	1.11
	4	22.41	20.53	341	39.25	14.74	82.74	346	85.3	0.542	121.7	44.63	1.10
	5	22.95	18.97	283	35.91	15.47	81.56	391	88.0	0.674	136.5	45.60	1.14
	6	23.40	18.20	273	34.66	15.40	81.37	399	89.5	0.705	137.0	47.07	1.17
	7	23.36	17.63	287	34.81	15.18	82.11	400	88.5	0.739	126.4	47.48	1.14
	8	23.25	18.26	302	38.08	15.13	83.33	358	89.5	0.671	125.1	45.81	1.13
2011	1	23.45	21.19	449	34.53	12.88	82.40	387	90.3	0.634	117.6	49.14	1.04
	2	23.79	18.70	406	32.28	13.38	81.04	429	91.8	0.743	122.5	48.24	1.07
	3	24.48	17.59	418	31.87	13.49	80.67	401	92.3	0.668	106.8	51.12	1.08
	4	24.72	16.99	373	29.56	13.88	79.57	429	93.3	0.704	98.1	47.75	1.12
	5	24.97	16.35	390	29.06	13.80	80.38	444	93.3	0.784	107.0	53.43	1.06
	6	25.34	16.42	460	31.15	13.72	82.38	436	94.0	0.823	99.8	52.43	1.06
	7	25.39	16.59	454	32.02	13.88	82.68	415	94.8	0.779	94.8	54.04	1.07
	8	25.14	16.51	485	32.50	14.39	83.02	449	95.0	0.789	112.4	53.90	1.08

The relationships between mean day temperature and the gluten quality variables (Rmax, Ext and UPP) are shown in Fig. 4 where varieties and growth seasons are visualized by different symbols. Rmax varied differently with day temperature in the two seasons and for the two varieties (Fig. 4a). Significant effects of day temperature on Rmax were found in 2010 ($p < 0.001$) but not in 2011. The combined ANOVA from both seasons gave a highly significant effect of the day temperature on Rmax ($p < 0.001$). More details on the relation between temperature and gluten quality were revealed by simple linear regression. In 2010, high and significant positive linear relations were found between mean day temperature and Rmax for both varieties. The highest correlation coefficient was observed for Zebra ($R^2 = 0.70$, $y = -3.623 + 0.180x$), which also had a steeper slope of the regression line compared to Bjarne ($R^2 = 0.54$, $y = -0.373 + 0.0458x$). The samples of Bjarne varied moderately in Rmax, having values from 0.6 to 0.8 N, whereas the samples of Zebra had a large variation from 0.25 to 0.82 N. In 2011, no significant regressions were found between Rmax and day temperature. The day temperatures were higher in 2011 compared to 2010, but the Rmax values did not increase further from the highest values in 2010. The Ext values decreased with increasing temperature, and this was found for both varieties and both seasons, as seen in Fig. 4b. The combined ANOVA from both seasons gave a highly significant effect of the day temperature on Ext ($p < 0.001$). UPP was significantly affected by the day temperature in both seasons ($p = 0.036$ and $p = 0.023$ in 2010 and 211, respectively), whereas no significant temperature effects was found for the ratio of monomeric to polymeric proteins. In 2010, a positive and significant relation was found between day temperature and UPP for Zebra ($R^2 = 0.62$), whereas no significant relation was found for Bjarne, having very similar UPP values for all plots within the tunnel. In 2011, positive and significant relations were found between day temperature and UPP for both varieties.

4. Discussion

The aim of this study was to investigate effects of temperature during grain filling on gluten quality characteristics at a lower to moderate temperature range, as typically experienced in Scandinavia and regions of North Western Europe. The choice of using experiments in growth tunnels was done to be able to grow the plants as similar as possible to field conditions and to create

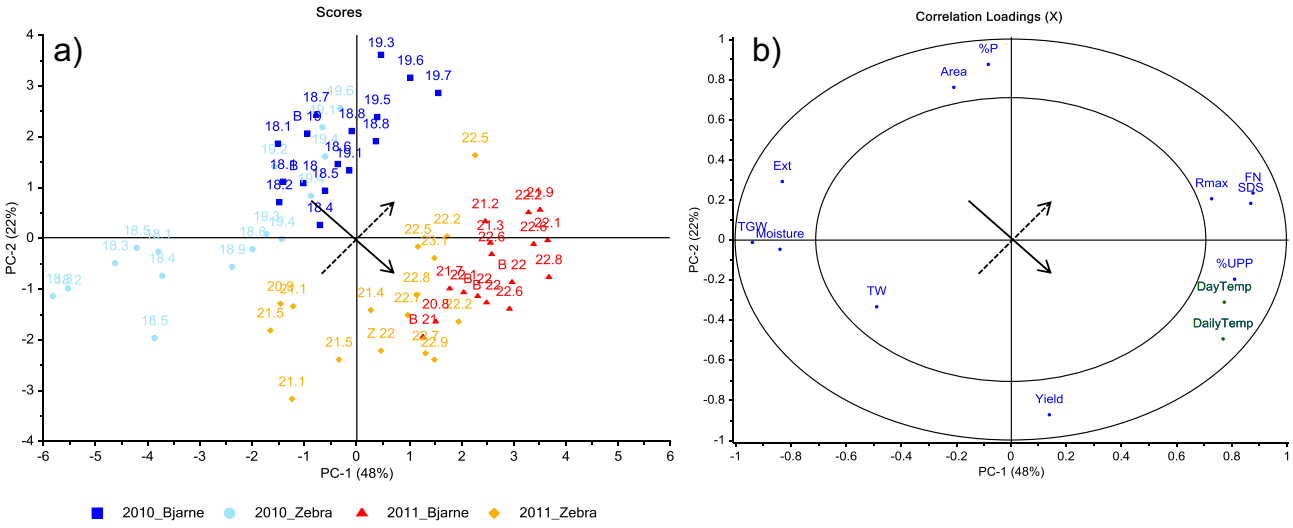

Fig. 3. PCA of the quality parameters. (a) Score plot of the samples where the two varieties and seasons are shown in different colours, and the sample point are labelled with their temperature. (b) Loading plot of the variables (the quality parameters and the yield), where daily and day temperatures, used as downweighted variables in the PCA analysis, are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Table 2
Means of yield and quality traits of the two varieties grown in the two growth seasons, averaged over the plots in the longitudinal direction of the tunnel. Significant differences as analysed by Tukey test is shown by the letters.

Season	Variety	Moisture at harvest, %	Yield, kg/ha	TGW,g	Protein, %	TW, kg/hl	FN,s	SDS,ml	Rmax,N	Ext,mm	Mono/poly	UPP,%
2010	Bjarne	20.7 b	307 b	36.6 b	15.4 a	82.0 a	423 b	93 ab	0.69 a	130 ab	1.12 ab	46.4 b
	Zebra	22.8 a	350 b	39.5 a	14.5 b	82.5 a	289 c	81 c	0.57 b	142 a	1.14 a	44.4 b
2011	Bjarne	17.1 d	422 a	29.2 c	14.0 b	80.6 b	462 a	96 a	0.77 a	97 c	1.06 c	51.8 a
	Zebra	18.0 c	437a	34.0 b	13.3 c	82.4 a	385 b	90 b	0.71 a	118 b	1.08 ab	50.8 a

variation in temperature during grain filling as it will appear in the longitudinal direction of the tunnel. Hence, the temperature effect could be investigated under natural conditions with similar soil and general weather conditions. The plants were subjected to natural soil conditions, and common fertilisation and management practices were used. Night/day fluctuations as well as fluctuations due to time of year in temperature and solar radiation were reflecting the natural fluctuations for the specific seasons. The polypropylene cloth caused a higher mean temperature within the tunnel compared to the temperature outside of the tunnel. The polypropylene cloth may also affect the light conditions within the tunnel compared to the outside.

In both years, a mean daily temperature gradient of 1.5 °C was achieved inside the tunnel in the period from anthesis to yellow ripening, allowing mean daily temperature differences from 18 °C to 19.5 °C to be investigated. Steeper gradients were achieved during day time and particularly on sunny days. When calculating mean day temperatures from 07:00 to 20:00 h during grain development, being characteristic for the long days during the Norwegian summer, temperature gradients of 2–2.5 °C were achieved. These temperature differences are smaller than those commonly used in most greenhouse studies, but they gave pronounced effects on maturation time (recorded as moisture content at harvest), TGWs and the protein contents of the grains. A mean daily temperature difference of 1.5 °C during grain filling covers well the natural temperature variations that are normally experienced in different seasons within the wheat producing area in Norway.

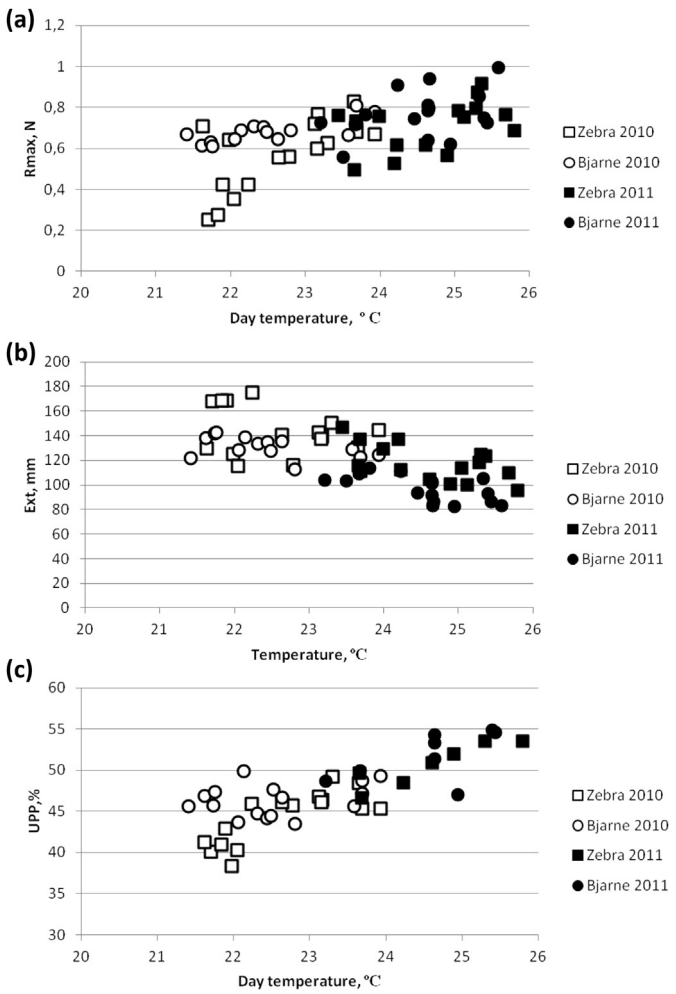

Fig. 4. Relationships between mean day temperature during grain filling and Rmax (a), Ext (b) and UPP (c). The different seasons and varieties are visualized with different symbols.

The observed effects of the temperature on TGW were expected because of the earlier ripening and thus the shorter period of grain filling. Similar relationships are well described in earlier experiments (Altenbach et al., 2003; Uhlen et al., 1998), and this reflects the fact that a shorter grain filling due to higher temperature cannot be compensated for by higher accumulation rates that are expected due to higher temperature. Increases in protein contents with increasing temperature are also found from other experiments in temperature controlled chambers (Altenbach et al., 2003; Uhlen et al., 1998). Significant temperature effects on the gluten viscoelastic properties were found. A strong relationship was found between day temperatures and Rmax in 2010 for both varieties, showing increasing Rmax values with increasing day temperatures. This was reflected in the UPP analyses, showing increased UPP values with increasing temperatures in both years, indicating a higher degree of polymerisation of the polymeric proteins at higher temperatures. The Ext values decreased with increasing temperatures, giving more extensible gluten when the temperature was lower. Thus, the results from this study support the earlier findings in that increases in temperature during grain filling can lead to increased gluten strength (Johansson and Svensson, 1998; Moldestad et al., 2011; Randall and Moss, 1990; Uhlen et al., 2004), most likely caused by an increased proportion of unextractable polymeric proteins as also found by previous studies (Malik et al., 2013, 2011; Uhlen et al., 1998). The stronger gluten in 2011 may be an effect of a higher temperature during a responsive stage during grain development, giving rise to a higher degree of polymerisation of the polymeric glutenins. This hypothesis may be supported by results of Moldestad et al. (2011) who found stronger temperature responses at lower temperatures and to different periods during grain filling. Other unknown growth conditions may also have influenced the Rmax differently in the two seasons. Both temperature, nutrient availability, and the grain filling duration have been suggested as important for gluten polymer structure that determines bread-making quality (Johansson et al., 2013).

Seasonal differences in the quality parameters were found, and stronger gluten was generally obtained in 2011 compared to 2010. The protein concentrations are slightly higher in 2010, and these are combined with lower yields and higher TGWs. This indicates slightly increased N availability during grain filling in 2010 compared to 2011, possibly caused by different weather conditions

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during the pre-anthesis period. Other studies have shown negative relationships between the N availability during grain filling and gluten strengths, possibly related to a higher gliadin/glutenin ratio (Godfrey et al., 2010; Wieser and Seilmeier, 1998). Thus, the stronger gluten in 2011 may be caused by differences in N availability during grain filling. The ratio of monomeric to polymeric proteins in the present study is slightly higher in 2010 compared to 2011, which support this theory. However, it is also documented that there were slightly higher mean day temperatures in 2011, mainly due to a period mid way in the grain filling period with higher temperatures. However, changes in other environmental factors could also have been present between the two seasons, both in the pre- and post-anthesis.

Another phenomenon that might cause the difference in Rmax response between 2010 and 2011 is linked to gluten protein degradation. Some samples of Zebra in 2010 had low Rmax values that were abnormal compared to the general gluten quality of this variety. These samples were from plots located close to the opening of the tunnel, and hence subjected to lower temperatures. In 2010, symptoms of *Fusarium* infestation were recognized in the tunnel, and also in field trials just outside the tunnel. Negative associations have been found between *Fusarium* spp. infection and baking quality of wheat (Capouchova et al., 2012; Wang et al., 2005). Therefore, statistical analysis was performed also without these abnormal samples. The results still showed a strong and significant correlation between Rmax and mean day temperature for Zebra in 2010.

The two varieties showed different temperature responses for Rmax in 2010. Zebra obtained lower Rmax values at the lower temperatures, but had a steeper slope of the regression equation. Both varieties are classified as having strong gluten, and they both carry the HMW-GS GluD1-d allele (5 + 10). In earlier quality investigations, Zebra has shown a slightly weaker gluten compared to Bjarne (unpublished results), similar to the results in this study. The results from 2010 indicated that Bjarne had a larger stability in Rmax upon the temperature variations. These differences between the varieties should be investigated further, as varieties having a better stability in gluten quality can be an important step to reduce quality variations in wheat.

The results showed that increases in temperatures during grain filling (mean day temperatures in the range from 21 to 25 °C) gave stronger gluten, having a higher resistance to stretching with less extensibility. This increase in temperature was also reflected in increased UPP values, indicating that polymerisation to higher molecular weights giving a higher proportion of the larger polymeric proteins is part of the explanation. The biological mechanisms cannot be unravelled from this study. However, the results may suggest that the increased temperature either caused an improved polymerisation of the polymeric proteins at the later stages of grain filling and maturation, or it caused changes in the gluten protein composition through their synthesis and accumulation that caused improved polymerisation at the later stage of grain filling.

5. Conclusions

Temperature gradients of 2–2.5 °C in day temperature during grain filling were achieved in the longitudinal direction of the tunnels, giving a temperature range (day temperature) of 21–25 °C. The increases in temperature affected maturation time, TGWs and the protein contents of the grains. Positive correlations were found between day temperature and Rmax, and between day temperature and UPP. The temperature responses differed between varieties and seasons. The results suggest that increases in temperature within this temperature range affect the polymerization of polymeric proteins to higher molecular weights, and hence give increased Rmax and stronger gluten.

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Paper IV

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Variation in gluten quality parameters of spring wheat varieties of different origin grown in contrasting environments

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ABSTRACT

The aim of this study was to investigate variation in protein content and gluten viscoelastic properties in wheat genotypes grown in two mega-environments of contrasting climates: the southeast of Norway and Minnesota, USA. Twelve spring wheat varieties, nine from Norway and three HRS from Minnesota, were grown in field experiments at different locations in Norway and Minnesota during 2009–2011. The results showed higher protein content but lower TW and TKW when plants were grown in Minnesota, while the gluten quality measured as Rmax showed large variation between locations in both mega-environments. On average, Rmax of the samples grown in Minnesota was higher than those grown in Norway, but some locations in Norway had similar Rmax values to locations in Minnesota. The data showed inconsistent relationship between the temperature during grain filling and Rmax. Our results suggest that the weakening effect of low temperatures on gluten reported in this study are caused by other environmental factors that relate to low temperatures. The variety Berserk showed higher stability in Rmax as it obtained higher values in the environments in Norway that gave very weak gluten for other varieties.

1. Introduction

Environmental factors that affect grain development in wheat may also have implications for the functionality of the gluten proteins that eventually will affect the end-use quality. Studies have documented that environmental variations in gluten quality can be large, and this represents a great challenge for the milling and baking industry. Comprehensive knowledge exists on the variability of gluten proteins, their inheritance and influence on gluten functional properties. In contrast, the impacts of environmental factors and their interactions with genotype affecting gluten quality are still only scarcely understood.

Gluten quality is determined by the viscoelastic properties of the dough, which are mainly related to the ratio of monomeric to polymeric proteins (Uthayakumaran et al., 2000) and to the proportion of glutenin aggregates above a certain molecular weight (Southan and MacRitchie, 1999). The fraction of large and unextractable glutenin aggregates, known as SDS-unextractable polymeric proteins (UPP), are found to correlate strongly with dough elasticity (Gupta et al., 1993). Large variation in gluten viscoelastic properties is found between varieties. In particular, the genes encoding the HMW glutenin subunits are known to affect the degree of polymerisation of the glutenins, causing differences in baking quality between varieties (see Shewry et al., 1992 for review).

Variation in protein content and gluten quality caused by the environment (E), the genotypes (G) and the G*E interaction have

Abbrevations: ANOVA, Analysis of Variance; Ext, Extensibility measured by the Kieffer Extensibility Rig; FN, Falling Number; GMP, Glutenin MacroPolymers; HMW-GS, High Molecular Weight Glutenin Subunits; HRS, Hard Red Spring; LSD, Least Significant Difference; NIR, Near InfraRed; PC, Principal Component; PCA, Principal Component Analysis; Rmax, Resistance to extension measured by the Kieffer Extensibility Rig; SDS, Sodium Dodecyl Sulphate Sedimentation Volume; TKW, Thousand Kernel Weight; TW, Test Weight; UPP, SDS-Unextractable Polymeric Proteins.

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been reported in many studies (see Finlay et al., 2007 for overview). In most of these studies gluten quality was analysed by rheological methods or by baking tests, and large variation in gluten quality due to both E, G, and G*E have been documented. In several studies, E is shown to be the main cause of variation in wheat quality, whereas the variation caused by G*E was of less importance (Finlay et al., 2007). The temperature during grain filling is among the environmental factors found to affect gluten quality. In Scandinavia, weaker gluten quality is reported in the seasons having cooler and wetter weather (Johansson and Svensson, 1998; Moldestad et al., 2011; Uhlen et al., 2004). Moldestad et al. (2011) found the temperature during grain filling to be the weather parameter that was most strongly associated with gluten quality, and reported lower resistance to stretching of the gluten dough when the mean daily temperature drops below 17–18 °C. Several researchers have performed experiments in controlled climate chambers and analysed gluten quality and composition (Johansson et al., 2005; Malik et al., 2013, 2011; Randall and Moss, 1990; Uhlen et al., 1998). Some of these studies showed effects on gluten polymer structure and found increased UPP with increasing temperature (Malik et al., 2013, 2011; Uhlen et al., 1998), whereas in other studies, no consistent differences were reported (Johansson et al., 2005). Recently, Moldestad et al. (2014) investigated the effects of temperature during grain filling on gluten quality in growth tunnels where a temperature gradient was established in the longitudinal direction, and found increased UPP and gluten strength with increasing temperatures. However, another study performed in tunnels mimicking cool/wet and warm/dry growth conditions (Georget et al., 2008) could not document differences in gluten quality due to these weather conditions. Thus, contrasting results may reflect complex relationships between the growth temperature and the gluten quality. In a recent review, Johansson et al. (2013) suggests how several environmental factors such as temperature, nutrient availability and the duration of grain filling may involve a number of interacting biochemical mechanisms of relevance for the gluten polymer structure. Still, there are needs for further confirmation of the effects on gluten quality of suggested environmental factors as well as an increased understanding of their mechanisms.

It is generally experienced that higher protein content as well as stronger gluten quality is obtained for spring wheat from the USA compared to wheat grown in Western Europe. The different weather conditions in these regions are believed to be a main factor causing these quality differences. However, few investigations have tried to compare the impacts of different weather conditions in such mega-environments to gluten quality parameters. The present study characterizes gluten from a set of twelve wheat varieties from Norway and Minnesota, USA grown in field trials at different locations in both countries. The aim was to 1) reveal the effects of different climates on gluten quality, 2) compare the gluten quality potential of the Norwegian varieties with the expected superior North American Hard Red Spring (HRS) wheat varieties, and 3) explore the possibility of using varieties of genetically strong gluten to obtain satisfactory quality in regions with a cooler and wetter climate.

2. Materials and methods

2.1. Field experiments

Twelve spring wheat varieties, including nine varieties adapted to Norwegian/Scandinavian growth conditions and three HRS varieties from Minnesota, USA (Supplementary Table 1), were grown in field trials at several locations during the seasons 2009–2011. All varieties possessed strong gluten and the high molecular weight glutenin subunits (HMW-GS) 5 + 10 encoded by *Glu-D1*. The varieties from Minnesota were selected to be representatives for the HRS quality. The field trials were located at four research farms in the southeast of Norway and were run from 2009 to 2011, at Vollebekk (59.660468, 10.781989), Bjørke (60.80276, 11.20403), Rød (59.34387, 10.89505) and Apelsvoll (60.70024, 10.86952), and at three locations in Minnesota, USA in 2011, at St. Paul (44.98958, 93.17923), Crookston (47.818558, 96.613451) and Morris (45.592758, 95.873911). A replicated complete block design with two replicates was used. The amount of fertiliser used at sowing was optimised for each location. The varieties from Minnesota were very susceptible to lodging when grown in Norway, and they were supported by nylon nettings stretched across the plots to avoid this. The experiments in Norway were treated with fungicides sufficient to control diseases with the potential to destroy grain quality.

The phenological development stages heading (Zadoks 49) and yellow ripeness were recorded for each plot at Vollebekk and Apelsvoll, whereas the phenological data was estimated based on calculations of day-degrees for the locations Bjørke and Rød. Heading (Zadoks 49) was recorded in the experiments in Minnesota. Weather data was collected from weather stations located close to the fields. Mean daily temperatures and sum of precipitation during the grain filling period was calculated for each location. Supplementary Table 2 summarises sowing dates, dates for heading and yellow ripening and the weather parameters for all environments.

The experiments were harvested plot-wise with an experimental plot combine. Samples were dried below 15% moisture and cleaned. The experiments at Rød, Bjørke and Apelsvoll in 2011 suffered from severe sprouting, and were excluded from further analyses.

2.2. Physical grain analyses and milling

Thousand kernel weight (TKW) and test weight (TW) were determined for all samples. Wholemeal flour was milled on a Laboratory Mill 3100 (Perten Instruments AB, Huddinge, Sweden) using a screen of 0.8 mm. Samples of 50 g were milled from each variety and replicated for all locations.

2.3. Analyses of whole-meal flour

Falling Number (FN) was determined for all samples using a Falling Number 1800 (Perten Instruments AB, Huddinge, Sweden). Sodium dodecyl sulphate sedimentation volume (SDS) was determined according to the AACC method 56–70 (AACC 2000). Protein content was determined by near infrared (NIR) reflectance spectroscopy using a Perten Inframatic 9200 (Perten Instruments AB, Huddinge, Sweden).

2.4. Gluten micro-extension test

Gluten micro-extension tests were performed as described by Moldestad et al. (2011) using the SMS/Kieffer Dough and Gluten Extensibility Rig (Kieffer et al., 1998) for the TA.XT *plus* Texture Analyser (Stable Micro Systems, Godalming, UK). Gluten was prepared from wholemeal in a Glutomatic 2100 (Perten Instruments AB, Huddinge, Sweden) by using a 2% NaCl solution to remove salt soluble components. The dough was mixed for 1 min before 10 min of washing. To remove starch and bran particles, two different filters were used in the process. An 88 μm sieve was changed after 2 min and replaced by an 840 μm sieve. To remove excess water, the gluten dough was centrifuged in a custom-made centrifuge mould at 3000 g for 10 min at 20 °C (Beckmann TJ-25 (Rotor TS-5.1–500). Subsequently, it was pressed in the standard Teflon mould and

rested for 45 min at 30 °C before analysis with the Kieffer-rig. The parameters resistance to extension (Rmax) and extensibility (Ext) were recorded from the extensograms according to Kieffer et al. (1998). The analysis was performed only on samples having a falling number above 200.

2.5. Statistical analysis

ANOVA was performed on combined data from all years and locations using the GLM procedure in Minitab 16 (Minitab Ltd., Coventry, UK). All field trials (location*year) were considered different environments, and included in ANOVA as a random variable. The model Response = environment + variety + environment*variety was used. Tukey test was used for comparisons of the means, and $LSD_{95\%}$ values were calculated. Principal component analysis (PCA), which is a multivariate approach designed for multicorrelated data, was carried out using The Unscrambler v 10 Z.1 (CAMO Software AS, Oslo, Norway) on the quality data from the grain, flour and gluten dough analysis. This method is meant to give an overview of the data, to reveal which properties are related, and to find the properties most important in distinguishing between samples (Martens and Martens, 2001). Finlay–Wilkinnson regressions (Finlay and Wilkinnson, 1963) for the Rmax were calculated for the varieties against the environment (location*year) mean.

3. Results

The PCA score plot (Fig. 1A) shows how the different years and locations differ from each other in quality. The first two principal components explain 61% (PC1 explained 38%, PC2 explained 23%) of the variation in the dataset analysed. There is a clear difference between the locations in Minnesota compared to the locations in Norway. The loading plot (Fig. 1B) shows that the protein content, TKW and FN span out the variation in the data set along the first principal component. Ext and Rmax span out the variation along the second principal component. Hence, the viscoelastic properties of gluten measured by the Kieffer-Rig varied independently of the protein content. The samples from Minnesota had lower TKWs compared to the samples from Norway. Within the locations in Minnesota, the samples from St. Paul differ from the two other locations by having higher Rmax and lower protein content. The samples from Vollebekk, Norway in 2011 differ from the other samples grown in Norway by having higher Rmax.

The locations in Minnesota had mean daily temperatures of 21.5–24.2 °C during grain filling, whereas this varied from 14.3 to 16.9 °C for the Norwegian locations (Supplementary Table 2). The accumulated precipitation during grain filling was low in Crookston with only 57 mm. Frequent precipitation during grain filling was seen in the locations in Norway and at Morris, and total precipitation for the period varied between 143 mm (Bjørke 2010) to 264 mm (Apelsvoll 2010). At St. Paul, 118 mm of a total precipitation during grain filling of 253 mm was recorded in one day, approximately mid-way in the grain filling period.

The environment averages for the quality parameters are shown in Table 1. The samples harvested in the Minnesota locations had higher protein contents, lower TKWs and TWs, and higher FNs than the samples harvested in Norway. The gluten quality, measured by SDS, Rmax and Ext, showed overlapping location means between the environments in Minnesota and in Norway. The highest Rmax was obtained in the samples from St. Paul, which also had the lowest extensibility. Large variation in Rmax was found between the locations in both mega-environments. Among the Norwegian locations, Bjørke in 2009 had very low Rmax, while Vollebekk in 2011 had high Rmax. Among locations in Minnesota, St.Paul obtained high Rmax values whereas lower values were found at Croockstone and Morris.

Table 2 shows the yield and quality parameters for the two groups of varieties, the Norwegian varieties and the HRS varieties, when grown both in Norway and Minnesota. The grain development was good for both variety groups when grown in Norway, as seen from the high TWs and TKWs. All varieties produced smaller grains in the Minnesota environments, but the difference for the HRS varieties was only half compared to the reduction in TKW for the Norwegian varieties when grown in Minnesota. While the TWs for the HRS varieties were about the same when grown in Norway or Minnesota, the Norwegian varieties had very low TWs when grown in Minnesota. Thus, the HRS varieties produced somewhat larger and well-filled grains in Norway compared to when grown in Minnesota, while the Norwegian varieties produced small and shrivelled grains when grown in Minnesota compared to when they were grown in Norway. Mean grain yield for the HRS varieties was slightly, but not significantly higher when grown in Norway compared to when grown in Minnesota. The Norwegian varieties out yielded the HRS varieties when grown in Norway, and vice versa. Low TKWs could explain most of the yield decreases of the Norwegian varieties compared to the HRSs when grown in

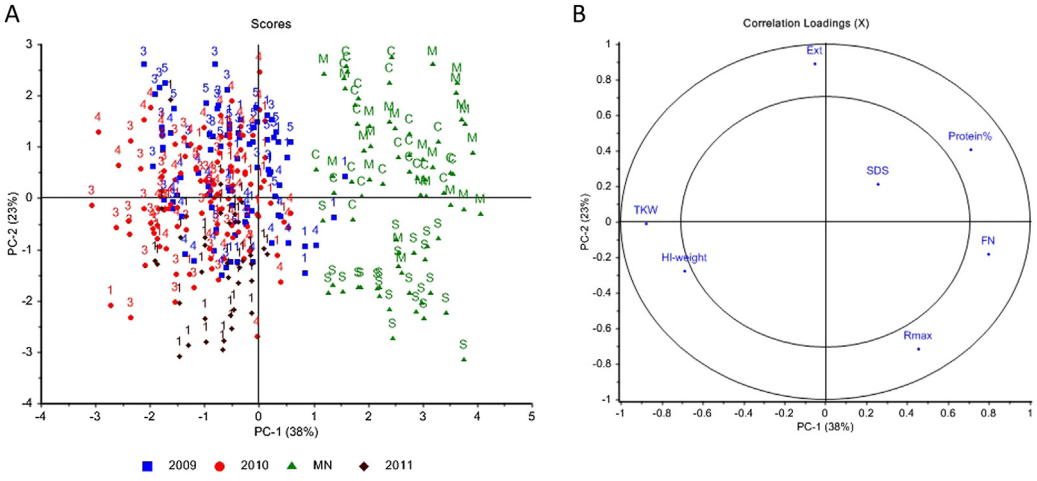

Fig. 1. Biplots of the scores (A) and loadings (B) for PC1 and PC2 from the PCA analysis. The growth seasons in Norway are visualised by different colors and the locations by numbers (1 = Vollebekk, 3 = Bjørke, 4 = Rød, 5 = Apelsvoll). The locations in Minnesota 2011 are visualized by letters (S = St. Paul, C = Crookstone, M = Morris). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Quality analyses of grain, flour and gluten dough obtained at the 11 locations, average of varieties and replicates.

		TW, Kg/hl[1]	TKW, g[1]	FN, s	Protein, %[2]	SDS, ml	Rmax, N	Ext, mm
Norway	Vollebekk 2009	75.1	36.2	316	13.7	77	0.60	116.9
	Bjørke 2009	79.0	34.8	293	12.3	78	0.41	148.4
	Rød 2009	80.2	35.3	307	15.1	73	0.68	123.4
	Apelsvoll 2009	77.5	36.2	238	14.7	89	0.57	135.2
	Vollebekk 2010	75.5	36.7	204	13.6	76	0.54	101.8
	Bjørke 2010	82.5	38.5	267	13.0	77	0.58	111.8
	Rød 2010	79.9	39.7	256	14.5	81	0.75	122.0
	Vollebekk 2011	80.4	37.6	262	12.3	80	0.92	101.5
Minnesota	Morris 2011	73.3	25.3	415	17.5	81	0.68	131.0
	St. Paul 2011	75.4	25.8	440	15.0	71	1.00	95.2
	Crookston 2011	75.5	26.9	399	17.1	85	0.71	141.5
	$LSD_{95\%}$	1.5	2.5	97	0.6	3.5	0.1	12.6
	P value	>0.0001	>0.0001	>0.0001	>0.0001	>0.0001	>0.0001	>0.0001

[1] Given as is.
[2] Given on dry weight basis.

Minnesota. In addition, the number of grains produced per m^2 was also reduced when the Norwegian varieties were grown in Minnesota. Higher TKW could not compensate for the lower grain number per m^2 when the HRS varieties were moved from Minnesota to Norway. Protein contents of 12–13%, typical for the Norwegian spring wheat, were achieved for the Norwegian varieties when grown in Norway, whereas higher protein contents were achieved for both groups when grown in Minnesota as well as for the HRS varieties grown in Norway. SDS was higher for the Norwegian varieties than the HRS varieties in both environments. A significant difference, however, was only found for the Norwegian environments. Interestingly, SDS values were similar for the Norwegian varieties between the environments, even though the protein contents were much higher in samples grown in Minnesota. Both the Norwegian varieties and the HRS varieties achieved higher Rmax when grown in Minnesota. The HRS varieties had higher Ext than the Norwegian varieties when grown in Norway, whereas no significant difference was found between the variety groups when grown in Minnesota.

For both mega-environments, highly significant differences in the gluten quality parameters Rmax and SDS were found for variety ($p < 0.001$) and for the variety*environment interaction ($p < 0.001$). Ext varied less between varieties and significant differences were found only for the Norwegian environments. Table 3 shows the variety means of Rmax, Ext and SDS from both mega-environments. When grown in Minnesota, the varieties Bastian, Bajass-5, Bjarne and Quarna obtained Rmax values similar to Sabin, the strongest of the HRS varieties when grown in this mega-environment, whereas Zebra and Demonstrant showed lower values. In the Norwegian environments, Bajass-5 and Berserk obtained the highest Rmax values, and were significantly higher than the HRS varieties Sabin and Tom. Demonstrant, Bjarne and Bastian had similar Rmax values to Sabin, whereas Zebra had lower values. Highly significant differences between varieties were found for SDS for both mega-environments ($p < 0.001$), and high values were found for Bastian, Berserk, Bajass-5 and Bjarne.

Highly significant environment*variety interactions were found for Rmax, both within environments in Norway and Minnesota, as well as in the combined analyses. To explore differences between varieties in the stability of the gluten quality across environments, regressions between Rmax of the variety and the Rmax field experiment mean were calculated (Fig. 2). The calculations showed that the Norwegian variety Berserk differed from the other varieties by having higher Rmax in the environments where the Rmax means were low, giving a low b-value of the linear regression equation for Berserk.

4. Discussion

By including varieties from Minnesota in Norwegian field trials, and vice versa, challenges might appear due to lack of agronomic adaptation. Registrations in the field trials in Norway showed that the varieties from Minnesota were quite similar to the Norwegian varieties in phenological development. Both heading dates and dates for yellow ripeness were within the range of the Norwegian varieties. Varietal differences in disease resistance to prevalent pathogens in the two mega environments was expected, but disease infestations were avoided as fungicides were applied in the Norwegian experiments. At the sites in Minnesota, no severe disease infestations were established in 2011. An obvious difference between the variety groups was the long and weak straw of the varieties from Minnesota when grown in Norway. Severe lodging was however prevented by supporting the HRS plots with nylon nettings.

Table 2
Yield and quality parameters presented as averages of Norwegian (N) varieties grown in Norway (N), HRS varieties grown in Norway, Norwegian varieties grown in Minnesota (MN) and HRS varieties grown in Minnesota. Different letters given after the means indicate significant differences at the P < 0.05 according to Tukey's test.

	TW, kg/hl[1]	TKW, g[1]	Yield, kg/ha[2]	No. grain/m^2	FN, s	Protein, %[3]	SDS, ml	Rmax, N	Ext, mm
N varieties in N (n = 110)	79.4 a	36.4 a	5353 a	14832 a	305 b	12.8 b	81.4 a	0.616 b	126.7 b
HRS varieties in N (n = 28)	79.7 a	37.4 a	3680 b	9944 c	247 c	17.0 a	73.3 b	0.523 b	139.2 a
N varieties in MN (n = 48)	73.2 b	24.4 c	2748 c	11272 bc	423 a	16.4 a	81.2 a	0.778 a	125.3 b
HRS varieties in MN (n = 12)	79.9 a	31.5 b	4027 b	12994 ab	436 a	17.2 a	76.3 ab	0.843 a	120.2 b
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.011

[1] Give as is.
[2] Given as 15% moisture.
[3] Given on dry weight basis.

Table 3
Protein content, Rmax, Ext and SDS of the varieties grown in Norway (N) and in Minnesota (MN).

		Protein content (%)		Rmax (N)		Ext (mm)		SDS (ml)	
		N	MN	N	MN	N	MN	N	MN
Norwegian	Basjass-5	12.7	16.3	0.76	0.99	113	111	91	88
	Bastian	12.9	16.2	0.59	0.86	137	128	86	86
	Berserk	12.2	16.8	0.79	0.74	110	127	81	90
	Bjarne	12.1	16.1	0.61	0.87	108	116	78	86
	Demonstrant	11.1	15.8	0.61	0.63	109	128	65	73
	Quarna	13.2	17.7	0.66	0.93	127	114	82	77
	Zebra	11.5	15.3	0.47	0.63	121	134	67	69
HRS	Sabin	14.1	17.0	0.70	0.89	124	115	68	71
	Tom	14.9	17.4	0.49	0.80	140	126	70	82
	$LSD_{95\%}$	0.5	1.2	0.05	0.21	13	22	3	4

The Norwegian varieties out-yielded the HRS varieties when grown in Norway, as well as the HRS varieties out-yielded the Norwegian ones when grown in Minnesota. These results could be expected due to their adaptation to the local environment. Nevertheless, normal grain development of HRS varieties was observed when they were grown in Norway whereas the Norwegian varieties had improper grain filling when they were grown in Minnesota (as both TKWs and TWs were considerably low). Mean day temperature during grain filling in Minnesota was 4.5–7.5 °C higher than the highest temperature recorded during grain filling among the Norwegian environments. It is previously reported that higher temperatures during grain filling reduce grain weight by shortening the duration of grain filling (Sofield et al., 1977). Since starch is a major storage component of endosperm, reduction in starch accumulation as is observed at higher temperatures (Hurkman et al., 2003; Altenback et al., 2003) attribute to lower grain weight when grown at higher temperatures. Increased temperatures are found to influence the accumulation of proteins less (Altenback et al., 2003; DuPont et al., 2006), thus the protein content is expected to increase due to decreased dry matter at higher temperatures. Hence, differences in grain weight and protein content between the two mega-environments were considered to relate to temperature differences between the two. However, the poorer grain filling of the Norwegian varieties when grown in Minnesota compared to the local HRS varieties indicate a poorer adaptation to high temperatures during grain filling.

It is well known that HRS wheat varieties from USA possess high gluten strength and are among the stronger wheats worldwide. The present results showed, however, that the Norwegian varieties Bajass-5, Berserk, Bastian and Bjarne had similar or even slightly higher Rmax values compared to the best HRS variety Sabin when grown in Minnesota and Norway, respectively. Bastian was released in Norway in 1989 as a strong gluten cultivar, and was a result of a long-term breeding strategy to improve bread-making quality of Norwegian wheats. The cultivars Bjarne, Berserk and Bajass-5 are all progenies from crosses with Bastian. Our results revealed that these Norwegian varieties possess high genetic potential to produce wheat with strong gluten similar to the HRS varieties when grown in an optimal environment. All varieties included in this study were having HMW-GS alleles giving high gluten scores according to Payne et al. (1984), including the 5 + 10 subunit encoded by the Glu-1D loci. Hence, the results further suggest that these varieties might have differences in allelic composition of other gluten proteins (LMW-GS and gliadins) giving high gluten strength. Hence, further studies should be conducted to explore the genetic background of these varieties.

The results from this study showed that the Rmax of the varieties was highly dependent on environmental conditions. Although Norwegian varieties were obviously adapted to the Norwegian environment as higher TKWs and yield were observed when they were grown in Norway, the gluten quality was generally stronger when they were grown in Minnesota. The HRS varieties dealt with the Norwegian environment better than vice versa as TKWs, TWs

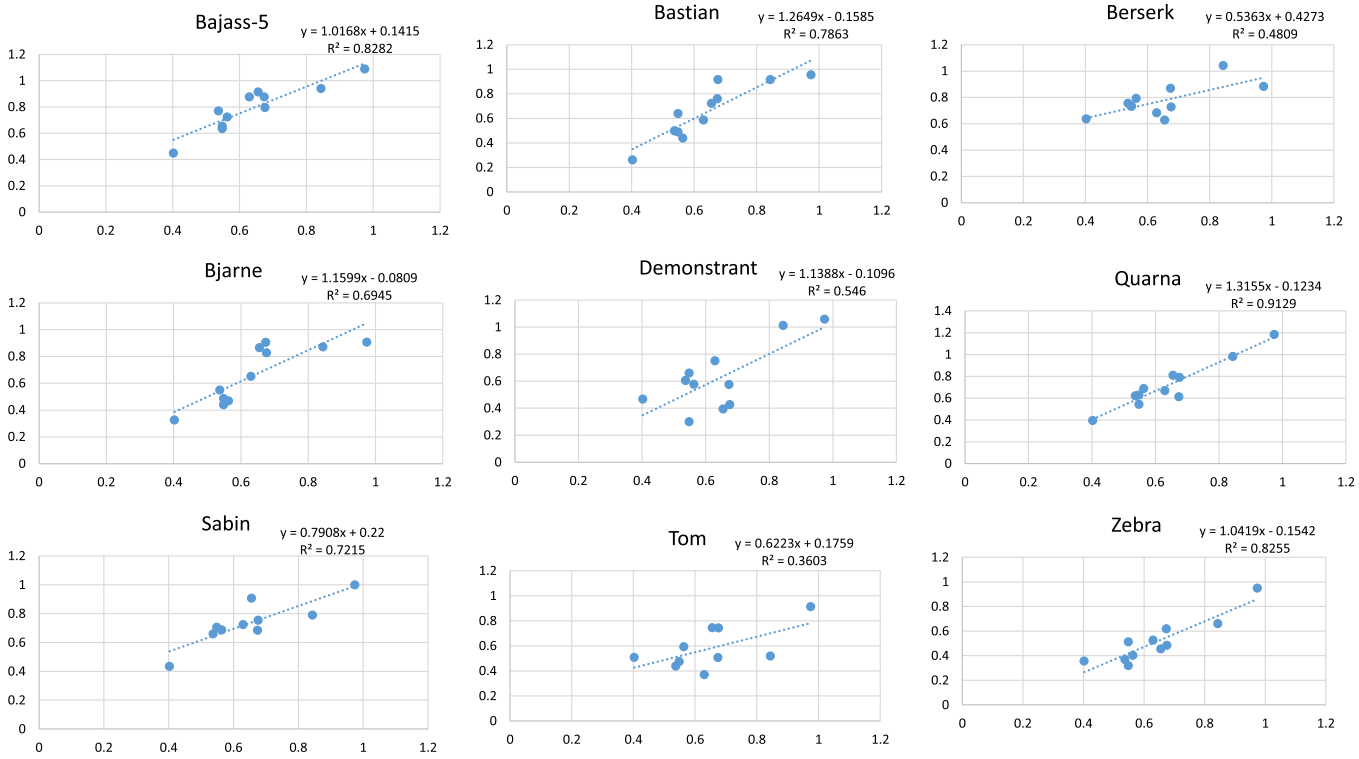

Fig. 2. Plot of Rmax for the actual variety against the Rmax location mean for Norwegian and HRS varieties.

and yield were similar to values obtained from the Minnesota environment, while gluten quality was negatively influenced by the Norwegian environment. These results may partly agree with other investigations showing increased gluten strength with increasing temperatures (Johansson and Svensson, 1998; Malik et al., 2013, 2011; Moldestad et al., 2011; Randall and Moss, 1990; Uhlen et al., 2004). In the present study, the highest Rmax values were obtained at St. Paul, the location with the highest temperature during grain filling. Similar and even lower Rmax levels were however found for the environments Morris and Crookston in Minnesota to some of the environments in Norway, having considerably lower temperatures during grain filling. Furthermore, Rmax for Bjørke in 2009 was much lower than that for Apelsvoll in 2009, even though both locations had approximately equally low temperature and high precipitation during grain filling. Hence, consistent relationships between the weather parameters and gluten quality could not be revealed. Obviously, other factors than the recorded weather parameters are causing the large variation in Rmax.

The results obtained in this study are similar to those reported by Moldestad et al. (2011) in finding very low Rmax values in some Norwegian environments subjected to lower temperature and high precipitation during grain filling. For these environments, the low Rmax values were not reflected in a lower SDS. The SDS location means varied less, whereas a consistent variation between varieties was found. The SDS measures differences linked to solubility properties of the gluten proteins in flours, whereas the Kieffer extensibility test measures the viscoelastic properties of gluten after mixing and resting. As shown by Weegels et al. (1996), changes in the glutenin aggregates occur during mixing which can be observed as an increase in their extractability and a decrease in amount of glutenin macropolymers (GMP). This is followed by an increase in GMP during resting, indicating that a re-assembly of the glutenin aggregates occur in this phase. Thus, SDS and Rmax measures different properties of the proteins as they occur in flours or in a rested dough, respectively. In general, positive relationships are found between the amount of GMP in the flour (affecting SDS) and the amount of GMP in a rested dough (affecting Rmax). The apparent discrepancy between SDS and Rmax seen in this study can indicate that some environmental factors linked to the locations having low Rmax values may hinder a normal re-assembly of the gluten network during resting.

One such factor could be infestations by *Fusarium* species (spp.), as it is reported that proteases from *Fusarium* spp. in infected grains have the ability to degrade gluten proteins (Gartner et al., 2008; Nightingale et al., 1999; Wang et al., 2005). Several *Fusarium* spp. are commonly infecting Norwegian wheat fields (Bernhoft et al., 2013), and the infestation was prevalent during the seasons 2009–2011. Koga et al. (2012) reported severe gluten protein degradation in winter wheat from Norwegian fields in 2011 having extremely low Rmax values. The proteases derived from *Fusarium* spp. was suggested as the most plausible explanation for protein degradation in their study. Hence, infestations by *Fusarium* spp. could be one likely explanation for the extremely low Rmax values found in some environments in Norway. More research is however needed to unravel the possible negative consequences of *Fusarium* infestation on the gluten quality. Furthermore, these results suggested that mechanisms affecting both synthesis and polymerisation of gluten proteins during grain development as well as those factors that might cause deleterious gluten protein degradation needs to be considered to understand environmental impacts on gluten quality.

Significant environment*variety interactions were found for Rmax. For the Norwegian environments, these were mainly caused by different ranking of the varieties in environments resulting in low Rmax values compared to environments resulting in moderate and high Rmax values. The variety Berserk differed from the others by having high Rmax values also in the environments with low Rmax mean. This was also seen from the lower b-value of the Finlay–Wilkinson regression, indicating a higher stability in gluten strength across environments. These results are in line with others who have reported variation in stability among varieties in breadmaking or gluten quality, as measured by either baking tests or other gluten quality tests (Johansson et al., 1999). However, the genetic basis for the variation in stability of the different quality parameters is scarcely understood. Also in this investigation, more research is needed, both to confirm an increased tolerance in Berserk towards environments, causing a weaker gluten, and to unravel the genetic mechanisms. If confirmed in new experiments, Berserk may represent a very important genetic source in breeding for both increased stability of increased gluten strength, which is of overall importance for the baking industry.

The results from the present study revealed that relationships between environmental factors and gluten quality were complex. The temperature during grain filling affected grain weight and protein concentration. Although higher Rmax means were obtained in Minnesota, no consistent effects of temperatures on the viscoelastic property of gluten were documented. This result may be in line with those of Johansson et al. (2013), who recently concluded that the temperature is not among the most important factor affecting the polymerisation of gluten proteins during grain filling. Instead, they proposed that short cultivar-determined plant development times give weak or unstable gluten. These relationships could not be confirmed in this study as the early maturing variety Bastian as well as the newer varieties originated from crosses with Bastian were having the higher Rmax values. Our results suggests that the most important factors to obtain superior gluten quality is the genetic background providing strong gluten as well as the ability to exhibit stable gluten quality over diverse environments. Berserk was identified as one promising candidate showing both strong gluten and more stable gluten when grown in different environments. Further detailed studies are needed to unravel genetic factors associated with the stability of gluten quality.

5. Conclusions

The main differences in quality traits between samples grown in Norway and Minnesota were found for protein content, TKW and TW, whereas for Rmax, large variation in Rmax was found between locations within both mega-environments. Wheat grown in Minnesota appeared to have stronger gluten quality, however consistent relationships between Rmax on gluten and the temperature during grain filling could not be documented. The results suggest that the weakening effect of low temperatures, as found at some locations in Norway, are caused by other environmental factors that relate to lower temperatures.

Our study revealed that Norwegian varieties possess high potential to produce wheat with strong gluten, and that wheat of strong gluten quality can be produced in cooler climates as experienced in Norway. The variety Berserk showed higher stability in Rmax as it obtained higher values in the environments with low average Rmax. Berserk may represent a very important genetic source in breeding for both increased stability of increased gluten strength, which is of overall importance for the baking industry.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcs.2015.01.004.

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Paper V

Investigating environmental factors that cause extreme gluten quality deficiency in winter wheat

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Abstract

Field trials with Norwegian winter wheat were conducted in several locations between 2005 and 2013. There is a large variation in gluten quality, measured as maximum resistance to extension of gluten (R_{max}), between and within the seasons. Moreover, extremely low R_{max} was observed in wheat grown in several locations in 2007 and 2011. Therefore, two cultivars and four locations within the 2011 season showing contrasting results were selected, and the composition and the size distribution of gluten proteins were characterized. The results revealed that the proportion of large glutenin polymers decreased in mature grain samples with extremely weak gluten. Moreover, re-polymerization of large glutenin polymers did not take place in gluten prepared from these samples during the resting period. Investigation of gluten proteins in an *in vitro* system indicated protease activities in these samples, as a drastic degradation of gluten proteins, including HMW-GS, was observed after incubation. The origin of proteases is still unconfirmed, however, fungal proteases seem to be a major candidate as a high proportion of grain in these samples was infected by *Fusarium* spp. A genotypic difference was found between the two cultivars and one of them had higher resistance against the factors influencing gluten quality in negative way.

Keywords: Wheat quality, gluten proteins, protein degradation, protease, *Fusarium*

Introduction

Gluten quality varies largely depending on genotypes and environment. Genetic variations, particularly the allelic variation of gluten proteins among genotypes are causing a variation in gluten quality, with well established relationships between allelic variations in high molecular weight subunit composition and dough strength (Payne, 1987; Shewry et al., 1992). On the other hand, environmental factors such as temperature, water availability and nutrient availability, particularly Nitrogen (N) and Sulfur (S), vary in fields and a variation in such environmental factors during the growing season causes changes in gluten quality (reviewed by Altenbach (2012)).

Gluten proteins are accumulated in the wheat endosperm during grain filling, and the composition and the size distribution of these proteins determine the gluten quality. Gluten proteins comprise of glutenins and gliadins. Glutenins are polymeric proteins formed by high molecular weight-glutenin subunits (HMW-GS) and low molecular weight-glutenin subunits (LMW-GS). Although the proportion of HMW-GS in total gluten proteins is small, their contribution to breadmaking quality is among the largest by forming large glutenin polymers, also being classified as the SDS-unextractable polymeric proteins (UPP). Assembling of glutenin polymers to UPP takes place during the desiccation/maturation phase at the end of seed development (Carceller and Aussenac, 1999) and the proportion of UPP in total polymeric proteins (%UPP) is positively correlated with dough strength (Gupta et al., 1993). Gliadins are a group of monomeric proteins responsible for dough viscosity or extensibility. Hence, the ratio of glutenin to gliadin also influence the viscoelastic properties of gluten (Uthayakumaran et al., 2000).

Field trials with spring and winter wheat cultivars in several locations in the southeast of Norway have been conducted since 2005 and the viscoelastic properties of gluten have been systematically analysed. Moldestad et al. (2011) observed a large variation in the viscoelastic properties of gluten prepared from spring wheat grown in several locations between 2005 and 2008. They found the relationship between the temperature during grain filling and gluten strength. Besides, extremely weak gluten was observed in both spring and winter wheat samples obtained from this field trails. Such a serious quality deficiency in gluten proteins was observed only when weather conditions were with low temperatures and high precipitation during the growing season. However, their occurrence was inconsistent and unexpected. Wheat grown in some locations within a season had acceptable or good gluten quality, although weather conditions were similar to the locations where wheat with extremely weak gluten was observed. The factors causing this serious quality deficiency must have been caused by environmental factors that associated with the locations, however it is still uncertain which factor is causing this phenomenon. These samples with extremely weak gluten met the requirements for food such as protein content, falling number and test weight. The grains are most likely purchased as wheat for breadmaking at grain delivery because purchase of cultivars for breadmaking in Norway is based on their division into five classes (class 1-5) rather than the direct measurement of quality at the purchase. Such wheat with extremely low protein quality probably cause unstable protein quality in wheat

purchased for breadmaking, and therefore, poses an extra challenge for everyone involved in the value chain from wheat production to breeding and research.

The aim of this study was to characterize the gluten proteins in quality deficient samples, and further investigate possible factors causing this problem. Materials from the 2011 season were suitable for this purpose of study by providing samples with both good and extremely poor gluten quality. Two cultivars and four field locations from the 2011 season, showing contrasting quality, were selected and the viscoelastic properties of gluten as well as the composition of gluten proteins were analyzed. Moreover, possible factors that cause this type of quality loss were investigated.

Material and Methods:

Wheat samples

The wheat samples analyzed for gluten quality were selected from field trials of winter wheat carried out during the period 2005-2013 including four to six cultivars and several locations each season (Supplemental Table a). The field trials were laid out as a randomized block experiment with two replicates. Field locations including both experimental stations and local farms were spread in the southeastern part of Norway which is the main wheat production area in the country. Fertilization and application of herbicide/fungicide at each site were done according to the management practice of the specific field. The plots were harvested at maturity and plotwise samples were dried and prepared for the quality analyses. To eliminate the samples with pre-harvest sprouting (PHS) damage, only the wheat samples with falling numbers above 200, which is the limit for grading wheat for breadmaking in Norway, were included in this study.

Extremely poor gluten quality were observed in some field trials in the 2011 season, therefore, two cultivars Finans and Olivin from four different locations (Apelsvoll=Field 1, Buskerud=Field 2, Graminor Rød=Field 3 and Graminor Bjørke=Field 4) showing large quality differences were chosen for further more detailed analyses of gluten proteins.

Grain and flour analysis

Protein content of whole grain was determined by near infrared transmission (NIT) using Foss Infratec™ 1241 Grain Analyzer (FOSS Tecator AB, Höganes, Sweden). 1000 grain weight (TGW) was measured and wholemeal flour was milled on a Laboratory Mill 3100 (Perten Instruments AB, Huddinge, Sweden) using a screen of 0.8 mm. Samples of 200 g were milled from two replicates of each cultivar. Falling Number (FN) was determined using a Falling Number 1800 (Perten Instruments AB, Huddinge, Sweden). α-amylase activity was measured by the Ceralpha method (AACC; Method 22-02.01) according to manufacture procedure (Megazyme, Irland).

Kieffer Dough and Gluten Extensibility Rig

Viscoelastic properties of gluten were determined with the SMS/Kieffer Dough and Gluten Extensibility Rig (Kieffer et al., 1998) as described by Moldestad et al. (2011). Gluten was prepared by Glutomatic 2100 (Perten AB, Huddinge, Sweden). 10 g wholemeal flour and a 2 % (w/v) NaCl solution were mixed for 1 min prior to 10 min washing with same solution for removing starch, bran particles and other salt soluble components. The gluten was centrifuged in a special centrifuge mold in a swing-out rotor (Rotor TS-5.1-500) at 2600 g for 10 min at 20 °C (Beckman TJ-25 centrifuge, USA). The gluten was then placed in the standard mold and incubated for 45 min at 30 °C before further analysis with the Kieffer-rig extensograph. The maximum resistance to extension (R_{max}) and extensibility (Ext) was recorded by the TA.XT plus Texture Analyzer (Stable Micro Systems, Godalming, UK).

Analysis of protein degradation

Total gluten proteins were extracted with 50 % (v/v) propan-1-ol and 4.5 % (w/v) DTT from 20 mg wholemeal flour at room temperature by Precellys ®24 homogenizer (Bertin Technologies, France). A homogenization was carried out twice for 20 s at 5500 rpm speed with 10 s pause between the homogenization steps. After a centrifugation for 15 min at 3700 g, a supernatant from each sample was transferred to a new Eppendorf tube and freeze dried (gluten proteins). Remaining proteins were extracted from the flour pellet by adding 62.5 mM Tris-HCl, 2 % (w/v) sodium dodecyl sulfate (SDS), 10 % (v/v) glycerol, 0.005 % (w/v) Bromphenol Blue and 1.5 % (w/v) DTT buffer (pH 6.8). The homogenization and centrifugation were carried out as described above and the supernatant was recovered in a new Eppendorf tube (residue fraction). Freeze-dried gluten proteins were dissolved in the residue fraction and incubated for 4 h and 16 h at 30 °C. An incubation duration was chosen according to the results of Eggert et al. (2011) showing a severe degradation of HMW-GS after 4 h incubation in an in vitro model system. For the control samples, re-suspended protein samples were immediately heated at 60 °C for 15 min before 16 h incubation. Cysteine protease is one of the major proteases that are activated under seed germination, hence a cysteine protease inhibitor, trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64) (Sigma-Aldrich, USA), was added to the mixture as a final concentration of 10 μM and incubated for 16 h.

Incubated gluten proteins were separated on NuPAGE®NOVEX 4-12% Bis-tris Mini Gels (Invitrogen AS, USA) by SDS-PAGE. The condition of electrophoresis was 40 V for 20 min, and then voltage increased to 140 V and continued for 105 min. The gels were stained for 3 h with 40 % (v/v) Methanol, 10 % (w/v) Trichloroacetic acid (TCA) and 0.1 % (w/v) Coomassie Brilliant Blue R-250 and destained for 18 h with 10% (w/v) TCA. The gels were scanned with an Epson perfection 4990 PHOTO scanner and the relative amount of HMW-GS and total proteins was quantified with ImageQuant TL software (GH Healthcare life science, UK)

Size exclusion Fast performance liquid Chromatography (SE-FPLC)

Flour samples were extracted sequentially to obtain two extracts (SDS-extractable and SDS-unextractable) according to Morel et al. (2000) with modifications described by Tronsmo et al. (2002). 240 mg wholemeal flour was extracted by 30 mL of 1 % (w/v) SDS, 0.1 M sodium phosphate extraction buffer (pH 6.9) for 80 min at 60 °C with continuous stirring. The SDS-extractable protein fraction was obtained by centrifuging the sample at 37,000 g for 30 min at 20 °C (Beckman L-80 Ultracentrifuge, Rotor 50.2Ti, USA). The SDS-unextractable protein fraction was extracted further by adding 30 mL extraction buffer to the flour residue followed by 3 min sonication (with 70% automatic amplitude compensation) by a Sonics VC130 (Sonics and Materials, Newton, CT 06470 - 1614, USA). Each fraction was filtered (Millex-HV 0.45 μm, Millipore, Ireland) and separated on a Superose ®12HR 10/30 column connected to an ÄKTA SE-FPLC (GE Healthcare Life science, UK) with 0.1% SDS, 0.08 M NaCl, 0.05 M Sodium phosphate elution buffer (pH 6.9).

Gluten proteins were also extracted from gluten prepared by Glutomatic 2100 and analyzed with SE-FPLC. Immediately after the 45 min rest at 30 °C, the gluten was frozen in liquid nitrogen and freeze dried. Freeze dried gluten was ground with a mortar to fine powder and 35 mg of each sample was used for extraction.

The SDS-unextractable fraction gives one main peak (F1*) which consists of the large polymeric proteins, and SDS-extractable fraction gives four main peaks (F1 to F4). The proportion of each peak (%F1* and %F1-%F4) were calculated as a percentage of total area of the two chromatograms (SDS-extractable and SDS-unextractable fractions). The proportion of SDS-unextractable polymeric proteins in total polymeric proteins (%UPP) was calculated as [F1*/(F1*+F1)×100] and the ratio of monomeric to polymeric proteins was calculated as [(F3+F4)/(F1*+F1+F2)].

Mycological analysis

Mycological analysis of *Fusarium* spp. and *Microdochium nivale* was done by Kimen Seed Laboratory AS (Ås, Norway) according to the International Seed Testing Association (I.S.T.A. 2014). 100 grains were placed on potato dextrose agar plates and incubated for 7 days at 20 °C under 12 h Near Ultra Violet light and 12 h darkness. After 7 days incubation, the evaluation of each grain was done by identifying mold by growth and color of mycelium, spores, pycnidia and perithecia.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA), a pairwise comparison was carried out by Tukey's test and simple linear regressions were performed by R using R commander modified at Norwegian University of Life Sciences (Ås, Norway).

Results

Viscoelastic properties of gluten

Table 1 shows average R_{max} of cultivars grown in each field between the 2005 and 2013 seasons and had FN higher than 200. There was a large variation in average R_{max} between the fields within and between the seasons. Wheat grown in the 2005, 2006 and 2013 seasons had generally higher R_{max} values, while they were considerably low in wheat grown in the 2007 and 2011 seasons. The 2007 and 2011 seasons were characterized by relatively lower temperatures with frequent precipitation compared to season 2005, 2006 and 2013 (Supplemental Figure a). In the 2007 season, average R_{max} values of each location were close to or lower than 0.4 N in all fields except for Field 3 that had a value close to 0.7 N. Considerably low average R_{max} values were observed in four out of five locations in the 2011 season indicating that gluten from these samples had hardly any resistance to extension, while Field 2 from the same season had an average R_{max} of 0.6 N (Table 1). The results showed that samples from several locations in the 2011 season had remarkable quality deficiencies, and were therefore chosen for further analyses in the present study.

Extension curves of Finans and Olivin showed in Figure 1 demonstrate contrasting R_{max} and Ext between the samples grow in Fields 2 and 3. Notably, the extension curve of Finans grown in Field 3 is considerably smaller than that of Finans grown in Field 2. Gluten prepared from two cultivars grown in Field 2 showed high R_{max} values for both cultivars, indicating strong gluten (Table 2 and Figure 1). On the other hand, the samples grown in the other three fields resulted in much lower R_{max} than samples grown in Field 2 (Table 2). Especially, Finans grown in these three fields had extremely low R_{max}. The values were close to 0.1 N in samples from Fields 1 and 4, and even lower value of 0.05 N was observed in Finans grown in Field 3 (Table 2 and Figure 1). Moreover, Finans gown in Fields 1 and 3 had very low Ext. Olivin grown in these three fields had higher R_{max} ranging from 0.35-0.46 N compared to Finans. This value is much lower than the value obtained in this cultivar grown in Field 2 (Table 2 and Figure 1), but it was similar to the average R_{max} values that were commonly found from winter wheat grown in Norway between 2007 and 2012 (Table 1). Furthermore, extensibility in Olivin grown in Field 3 was much higher than Olivin grown in Field 2 (Table 2 and Figure 1).

Size distribution of gluten proteins

The size distribution of gluten proteins was analyzed by SE-FPLC to investigate the proportion of large glutenin polymers and the ratio of monomeric to polymeric proteins in flour and in gluten (Table 2). The result showed normal $\%UPP_{(flour)}$ in both cultivars grown in Field 2. These results correspond well with the high R_{max} values observed for these samples. Samples from the other three fields had significantly lower $\%UPP_{(flour)}$ than samples from Field 2, and the lowest %UPP value was observed in samples grown in Field 3. Low %UPP was caused by both significantly lower %F1* and significantly higher %F1 in samples grown in Fields 1, 3 and 4 compared to Field 2. The lowest %F1* was observed

in samples grown in Field 3 and the values were almost half of %F1* observed in samples from Field 2. Significantly higher %F4 was also observed for samples grown in Field 3. Olivin had significantly higher $\%UPP_{(flour)}$ than Finans ($p<0.001$) grown in Fields 1,3 and 4.

Gluten proteins were also extracted from the gluten prepared by Glutomatic 2100 followed by 45 min resting, and separated by SE-FPLC. The level of $\%UPP_{(gluten)}$ was much lower than the values observed in the flour. Among the four fields, gluten prepared from samples grown in Field 2 had significantly higher $\%UPP_{(gluten)}$ than the samples from the other three fields (Table 2). The proportions of $UPP_{(gluten)}$ were less than 5% in gluten prepared from samples grown in Field 1,3 and 4, and no differences in $\%UPP_{(gluten)}$ were found between the two cultivars grown in these fields (Table 2).

Gluten protein degradation

Gluten proteins extracted from flour were dissolved in the residue fraction from the corresponding sample and incubated for four and 16 h. Proteins were then separated by SDS-PAGE and HMW-GS were quantified. Figure 2(a) displays the electrophoretograms of incubated proteins from both Finans and Olivin grown in Field 3. It is clear that both glutenins and gliadins are degraded after incubation. No degradation of gluten proteins was observed when the residue fraction was heated at 60 °C prior to incubation (data not shown). Figure 2(b) showed the proportion of HMW-GS left in the incubated samples, calculated as [total HMW-GS in the incubated sample / total HMW-GS in the control sample]. Gluten proteins were degraded in all samples after incubation. A severe degradation of gluten proteins was observed especially in the samples grown in Field 3. After 4 h incubation, approximately 20% of HMW-GS were degraded in Olivin, while 40% were degraded in Finans grown at the other three fields. No inhibitor effect was observed using cysteine protease inhibitor (E-64) (data not shown).

α-amylase activity

FN and α-amylase activity (Table 2) were analyzed to investigate pre-harvest sprouting (PHS) damage. The samples grown in Field 2 had the highest FN and the lowest α-amylase activity. The FN in the samples grown in Fields 3 and 4 was over 250 s and they showed moderate α-amylase activity. The samples from Field 1 showed the lowest FN together with the highest α-amylase activity close to 0.4 U/g flour.

Mycological analysis

Mycological analysis for *Fusarium* spp. and *M. nivale* was carried out to get an overview of fungal infestation in grain samples (Figure 3). Although typical symptoms of fungal infection, such as chalky white to pink color on the surface of grain, were not observed from grain appearance, a high proportion of grains grown in Fields 1 and 3 were infested by fungi. *Fusarium* spp. infestation dominated in grain samples from Field 3, while 42 % of grains grown in Field 1 was infested by *M. nivale*. The grains from

Field 4 were partly infested by both *M. nivale* and *Fusarium* spp. The lowest proportion of grains was infested by fungi in samples from Field 2.

Discussion:

Field trials with Norwegian winter wheat carried out between 2005 and 2013 showed a large variation in gluten strength. Higher average R_{max} values were observed in the 2005, 2006 and 2013 seasons during this study. These seasons were characterized by a warmer weather during grain filling in July with average temperatures around 18-19 °C, combined with considerably less precipitation (Supplemental Figure a). The result is in agreement with the field trials conducted with spring wheat cultivars between 2005 and 2008 which showed the large variation in R_{max}, primarily caused by weather conditions (Moldestad et al., 2011). Moldestad et al. (2011) observed higher R_{max} values in warm and dry seasons in 2005 and 2006 compared to cool and wet seasons in 2007 and 2008. The seasons between 2007 and 2012 were characterized by lower temperatures, high and frequent precipitation during July, and the frequent precipitation continued in August covering the maturation and harvesting period (Supplemental Figure a). Therefore, the R_{max} values obtained in these seasons were expected to be lower compared to the warm and dry seasons according to the earlier studies (Johansson and Svensson, 1998; Moldestad et al., 2011). Besides, the 2007 and 2011 seasons were special for two reasons. First, a considerably low average R_{max} (< 0.3 N) was observed in several fields. Such a low average R_{max} could not be explained only by weather condition since the weather conditions during grain filling were relatively similar between the 2007 and 2012 seasons, except considerably higher precipitation was recorded in the 2007 season. Second, samples with better quality were observed in other fields within the seasons. The field locations were relatively closer to each other and thus the temperature between the fields were similar within the seasons (Supplemental Figure a). Since a winter wheat resumes growth in early spring, and flowering time are mainly determined by the temperature, plant development as well as grain filling were presumed to occur simultaneously between the locations within the seasons in Norway. Hence, other biotic and/or abiotic factors prevalent with cooler and wet weather during these two seasons were suspected to cause this quality deficiency. Thus, gluten proteins from two cultivars grown in four fields in the 2011 season were characterized and possible factors that causing this quality deficiency were further investigated.

From our experience, gluten prepared from spring wheat cultivars grown in Norway under favorable weather conditions generally give R_{max} values of over 0.7-0.8 N and Ext ranges from 100 to 140 mm. Since gluten strength of winter wheat is generally weaker than that of spring wheat, the R_{max} values obtained from two cultivars grown in Field 2 were close to maximum, hence they had good breadmaking quality. By contrast, analysis of gluten prepared from Olivin grown in Field 3 resulted in less elastic and highly extensible gluten indicating the alteration of viscoelastic properties. Even though gluten was formed from Finans grown in Fields 1, 3 and 4, the results imply an almost complete loss of

its breadmaking properties. A high positive correlation between R_{max} and %UPP (r=0.92) clearly showed that samples with weak gluten had a lower proportion of large glutenin polymers in flour. This was primarily caused by a decrease in the proportion of SDS-unextractable polymeric proteins (%F1*) as well as an increase in SDS-extractable polymeric proteins (%F1). Moreover the proportion of monomeric proteins (%F4) increased in samples from these three fields. The results indicated that molecular weight of gluten proteins, especially large glutenin polymers, decreased in samples from Fields 1, 3 and 4. Moreover, re-polymerization of large glutenin polymers did not take place in gluten prepared from these samples after resting for 45 min.

Gluten proteins extracted from samples with extremely poor protein quality were severely degraded when they were incubated with the residue fraction, while such degradation of proteins were not observed when samples were heated at 60 °C prior to incubation. Our results resembled to the observation made by Morel et al. (2000). They showed that large polymeric proteins decreased after incubation (over 19 hours) when proteins were extracted from proteolytically-active samples at ambient temperature, while they did not observe the protein degradation when protein extraction was carried out at 60 °C. Our results, therefore, indicated protease activity in samples with extremely poor protein quality. These high protease activities are probably the cause of low %UPP in gluten prepared from these samples.

Further investigations were carried out to explore whether the observed protein degradation was caused by endogenous or exogenous proteases. Possible endogenous proteases can be caused by PHS. Lorenz et al. (1983) reported that higher α-amylase activities during seed germination are often associated with high protease activities, and that could affect the structure and the functionality of gluten. All the samples analyzed had FN higher than 250 s in wholemeal and it did not indicate severe PHS damages in these samples. However, α-amylase activities moderately increased in the samples grown in Field 1. The samples harvested at Fields 3 and 4 also showed slight increase in α-amylase activities. Earlier studies showed that FN between 200-250 s in white flour is a critical range for PHS damage which corresponds to the α-amylase activity of 0.3-0.4 U/g flour (McCleary and Sheehan, 1987; Moot and Every, 1990). Consequently, endogenous protease activities might increase to some extent in the samples with elevated α-amylase activities. Cysteine protease is the typical endogenous protease induced under seed germination (Bottari et al., 1996; Poulle and Jones, 1988). However, cysteine protease inhibitor (E-64) hardly prevented protease activities in our samples. From these results, it is hard to conclude that proteases caused by PHS are highly activated or/and causing the protein degradation in our samples. Even so, further research is required to investigate the association between α-amylase and protease activities under the germination process in Norwegian wheat cultivars. Wheat cultivars grown in Norway have been selected by FN due to PHS problems, changes in protease activity associated with PHS that was induced by environmental conditions are hardly investigated.

It has been reported that exogenous proteases derived from *Fusarium* spp. reduce breadmaking quality drastically (Dexter et al., 1996; Nightingale et al., 1999; Papouskova et al., 2011; Wang et al., 2005). Previously, *Fusarium* head blight (FHB) was not the major problem for cereal production in Norway. However, the number of cereal grains infected with *Fusarium* spp. has increased more than twofold over the last decade compared to the three previous decades. *F. avenaceum*, *F. graminearum*, *F. culmorum* and some *Michrodochium* spp. are currently the most common species causing problems in cereal production in the country (Bernhoft et al., 2013). Even though typical symptoms of *Fusarium* infection were not observed from the grain appearance, a relatively high proportion of grain was infected by *Fusarim* spp and *M. nivale*, especially in samples grown in Fields 1 and 3. Frequent and continuous rainfall during the maturation and harvesting was recorded in the 2011 season and this weather was probably favorable for fungi development. It has been reported that *Fusarium* infested samples have lower grain weight because of the shriveled characteristic of the grain (Bechtel et al., 1985; Dexter et al., 1996; Wang et al., 2005). Samples grown in Field 3 which had the highest *Fusarium* infestation showed significantly lower TGW than samples grown in Field 2. A high proportion of infected grains with *Fusarium* may coincide with high protease activity, substantial changes in the composition of gluten proteins and reduced TGW in samples grown in Field 3. Eggert et al. (2011) observed 97% of HMW-GS was digested after 4 h incubation with protease derived from *F. graminearum*. The observed protein degradation in this study, at least in samples grown in Field 3, could be caused by protease derived from *Fusarium* spp. The samples grown in Field 1 had higher *M. nivale* infestation than *Fusarium* spp. To our knowledge, it has not been reported yet that proteases from *M. nivale* may degrade gluten proteins. However, as *M. nivale* is closely related to the Fusarium genus, and was earlier classified as *Fusarium* spp., it cannot be excluded that *M. nivale* may form proteases affecting the gluten proteins. Further studies investigating how *M. nivale* influence the grain morphology and gluten quality are required. Pekkarinen et al. (2002) and Pekkarinen and Jones (2002) isolated and purified two types of alkaline proteinase; trypsine-like proteinase and subtilisin-like proteinase from *F. culmorum*. Activities of these two proteinases were not inhibited by E-64. Thus, it is probably reasonable to consider that proteases dominating in our samples are not cysteine proteases but other types of protease derived from *Fusarium*.

Our results indicated that the molecular weights of gluten proteins, including large polymeric proteins, decreased in mature grain samples with poor gluten quality. Because the first step of protein extraction for SE-FPLC analysis was carried out at 60 °C for 80 min, proteases presented in our materials were supposed to be inactivated (Morel et al., 2000; Pekkarinen and Jones, 2002; Pekkarinen et al., 2002). The assembly of large glutenin polymers during the maturation and desiccation phase might have been disturbed by the proteases so that the proportion of large polymers in the grain might not reach their full potential. Alternatively, the large glutenin polymers might have been degraded by protease activities after their formation was completed. This is supported by Bechtel et al. (1985) and Nightingale

et al. (1999) who demonstrated the absence of the protein matrix in the most severely infected area of the endosperm. The protein matrix was assumed to be degraded by fungal proteases sometime after deposition. Wang et al. (2005) and Eggert et al. (2010) showed that the glutenin fraction strongly decreased, while gliadins slightly increased in *Fusarium* infected samples. They also found that HMW-GS, the major contributor to form large glutenin polymers, were affected most severely among the glutenins. Eggert et al. (2011) also concluded that proteases derived from *F. graminearum* have higher preference for degrading the glutenins than gliadins, and HMW-GS were the most preferable sub-fractions. This could be an explanation for the extremely low %UPP observed in the gluten prepared from our samples with poor gluten quality. Overall, proteases derived from *Fusarium* spp. seem to have negative effects on the composition of gluten proteins and the assembly of glutenin polymers during the seed maturation and desiccation as well as the formation of gluten network during the dough preparation. Further systematic investigations are expected to confirm the origin of protease and also whether proteases derived from *Fusaium* spp. caused the extremely poor gluten quality in our samples.

Interestingly, Olivin seems to withstand unfavorable conditions having better gluten quality compared to Finans. Our results showed that Olivin has significantly higher gluten resistance, higher %UPP and a lesser degree of HMW-GS degradation than Finans when the cultivars grown in Fields 1, 3 and 4 were compared. It is of great interest for both researchers and breeders to understand the mechanisms and find the responsible genes that contribute to differences in tolerance to unfavorable conditions.

Conclusions

Extremely poor gluten quality observed in several locations in the 2007 and 2011 season indicated almost complete loss of breadmaking quality. Analysis of gluten proteins in selected samples in the 2011 season showed that the proportion of large glutenin polymers highly decreased in the samples with extremely weak gluten strength. Furthermore, characterization of gluten proteins indicated high protease activities in these samples. The origin of the proteases remains unclear, however, exogenous proteases derived from *Fusarium* spp. seem to play a key role for protein degradation, and thus causing severe quality deficiency. Interestingly, genotypic differences were found as Olivin had better ability to resist the factors that causing the gluten quality deficiency compared to Finans.

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Table 1. Viscoelastic properties of gluten recorded as the maximum resistance to extension (R_{max}) and the extensibility (Ext) measured by the Kieffer Dough and Gluten Extensibility Rig. The data are averages over cultivars and two replicates for each location from 2005 to 2013. Same field number in different year indicates the same location. Ave.:Average. S.D.: Standard deviation

Year	Field nr.	R_{max} (N)		Ext (mm)	
		Ave.	S.D.	Ave.	S.D.
2005	1	0.54	0.08	160.7	12.97
	2	0.63	0.11	144.6	22.92
	5	0.64	0.25	151.6	28.07
	6	0.58	0.01	147.5	10.86
	7	0.78	0.18	123.8	29.67
	8	0.69	0.10	139.6	27.04
	9	0.57	0.08	144.6	17.82
2006	2	0.65	0.10	147.2	14.51
	6	0.56	0.07	162.2	17.00
	7	0.65	0.07	160.6	12.49
	8	0.71	0.09	144.4	10.35
2007	1	0.35	0.08	156.0	23.36
	2	0.34	0.08	145.3	12.86
	3	0.68	0.11	148.6	8.26
	4	0.41	0.08	162.5	8.50
	5	0.32	0.12	167.3	13.89
	6	0.40	0.11	158.9	17.84
	7	0.38	0.09	159.2	12.49
	8	0.20	0.09	188.9	16.05
	9	0.32	0.05	170.8	16.29
2008	1	0.38	0.09	168.6	15.15
	3	0.59	0.11	140.4	5.98
	4	0.53	0.07	149.5	21.74
2009	1	0.36	0.11	107.6	29.21
	2	0.43	0.15	139.0	9.29
	4	0.56	0.11	141.6	8.48
2010	1	0.43	0.07	122.4	9.13
	2	0.49	0.07	135.4	10.54
	3	0.53	0.08	149.2	4.96
	6	0.51	0.06	150.3	6.76
	8	0.53	0.07	154.0	4.37
	9	0.52	0.06	151.3	4.29
2011	1	0.22	0.16	73.8	37.26
	2	0.59	0.15	102.4	8.99
	3	0.14	0.11	102.9	34.24
	4	0.22	0.12	108.8	17.89
	5	0.09	0.07	79.2	33.99
2012	3	0.47	0.47	97.5	28.49
	4	0.40	0.15	109.0	11.87
	5	0.39	0.12	104.5	17.24
	10	0.28	0.13	102.8	23.69
2013	1	0.74	0.11	123.8	9.05
	4	0.51	0.15	145.0	11.24
	5	0.90	0.15	110.4	12.90
	6	0.78	0.19	130.0	14.86
	11	0.57	0.14	122.3	9.71

Table 2. Protein content (%PC), thousand grain weight (TGW), falling number (FN), α-amylase activity, the maximum resistance to extension (R_{max}) and extensibility (Ext) of gluten, the proportion of each peak from SE-FPLC (%F1*-%F4), the proportion of SDS-unextractable polymeric proteins in total polymeric proteins (%UPP) and the ratio of monomeric to polymeric protein (Mon:Pol) of flour and gluten of Finans and Olivin grown in four locations in 2011. Significance level analyzed by ANOVA were shown in the bottom of the table.

Field nr.	Cultiver	Protein (%)	TGW (g)	FN (s)	α-amylase activity (CU/gram)	R_{max} (N)	Ext (mm)	Flour							Gluten	
								%F1*	%F1	%F2	%F3	%F4	%UPP	Mon:Pol	%UPP	Mon:Pol
1	Finans	9.4	43.7	250	0.379	0.13	69.20	10.03	23.10	8.63	16.64	27.78	30.45	1.06	4.88	0.89
	Olivin	10.1	41.3	268	0.404	0.46	127.99	11.05	22.11	7.33	17.44	26.28	33.36	1.08	4.51	0.87
2	Finans	11.9	39.6	374	0.167	0.71	106.33	14.08	18.41	7.45	16.06	26.99	43.32	1.08	13.13	0.96
	Olivin	11.8	38.3	407	0.207	0.69	114.56	14.64	19.15	7.15	16.32	25.87	43.36	1.03	7.71	0.87
3	Finans	12.7	37.0	286	0.262	0.05	80.67	6.64	23.92	8.05	15.16	30.41	21.80	1.18	3.13	0.98
	Olivin	14.1	34.5	357	0.330	0.35	147.36	9.78	22.97	8.18	14.88	29.24	29.96	1.08	3.91	0.90
4	Finans	12.1	46.8	306	0.256	0.14	133.60	9.55	22.69	7.19	16.36	27.73	29.60	1.12	3.54	0.94
	Olivin	12.4	44.5	285	0.316	0.38	127.86	11.41	22.70	7.18	16.08	26.55	33.43	1.03	4.07	0.85
Cultivar (C)		**	*	n.s.	n.s.	***	***	***	n.s.	n.s.	n.s.	n.s.	***	**	n.s.	***
Field (F)		***	***	***	**	***	n.s.	***	***	*	n.s.	*	***	*	**	*
C*F		*	n.s.	n.s.	n.s.	*	*	*	n.s.	n.s.	n.s.	n.s.	**	n.s.	n.s.	n.s

***. **. * indicate significance at 0.1, 1 and 5 %, respectively and n.s. showed not significant.

[a] analyzed by SMS Kieffer Dough and Gluten Extensibility Rig

[b] analyzed by SE-FPL

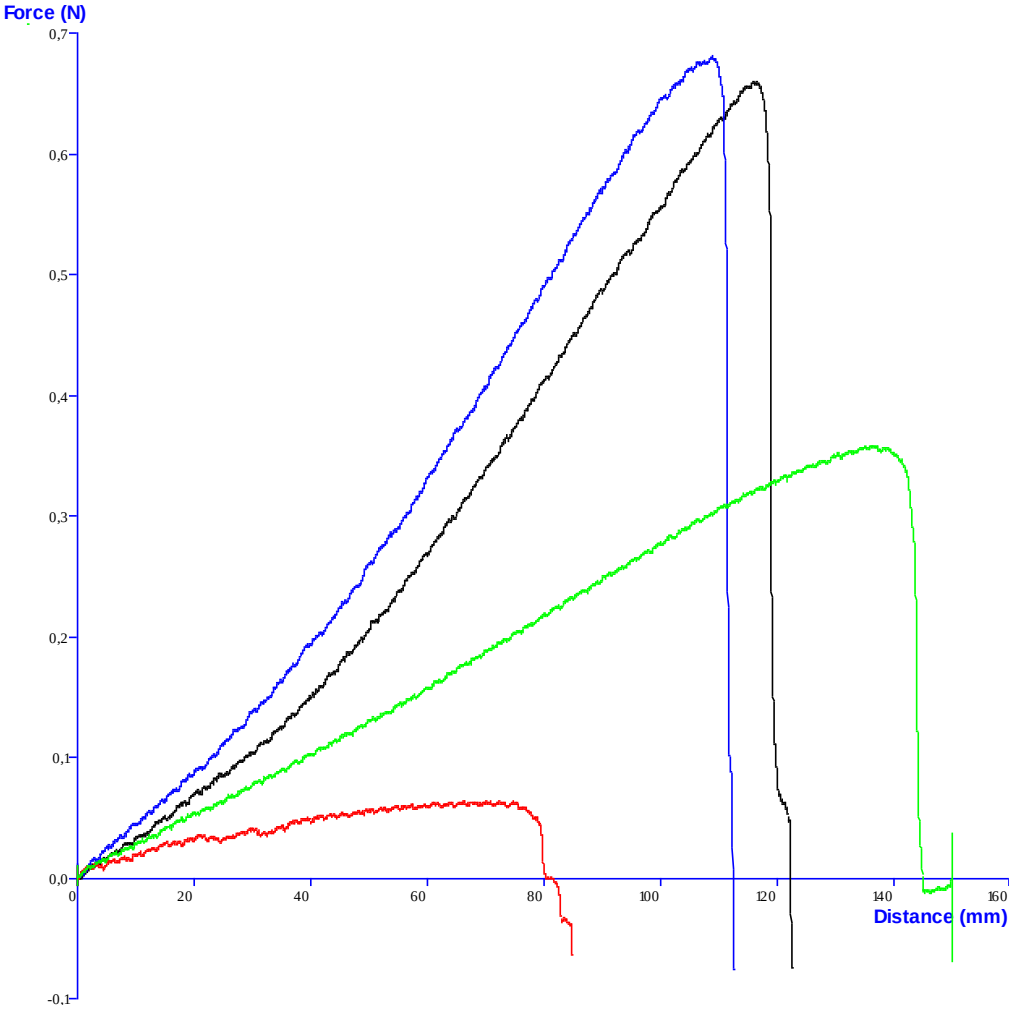
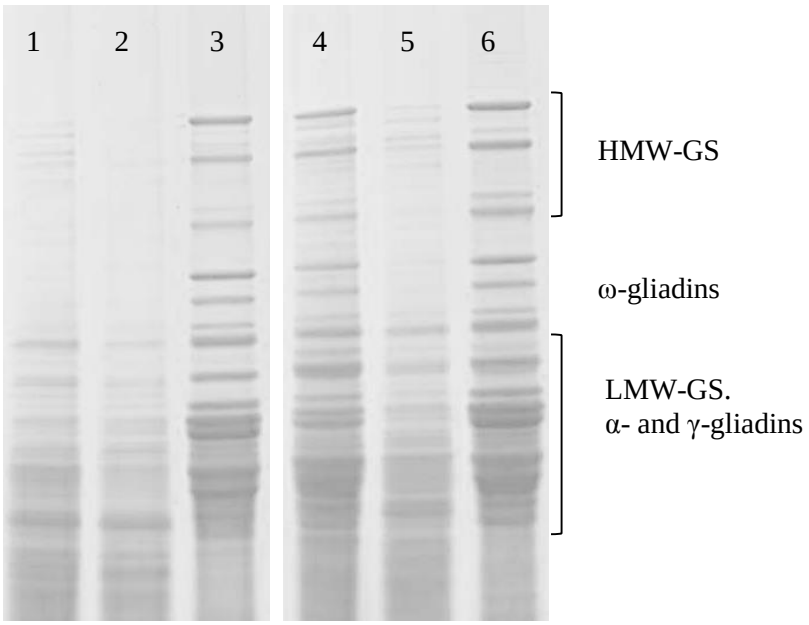


Figure 1. Extension curves obtained by Kieffer Extensibility Rig. — (blue) Finans and — (black) Olivin grown in Field 2, and — (red) Finans and — (green) Olivin grown in Field 3 in the 2011 season.

(A)



(B)

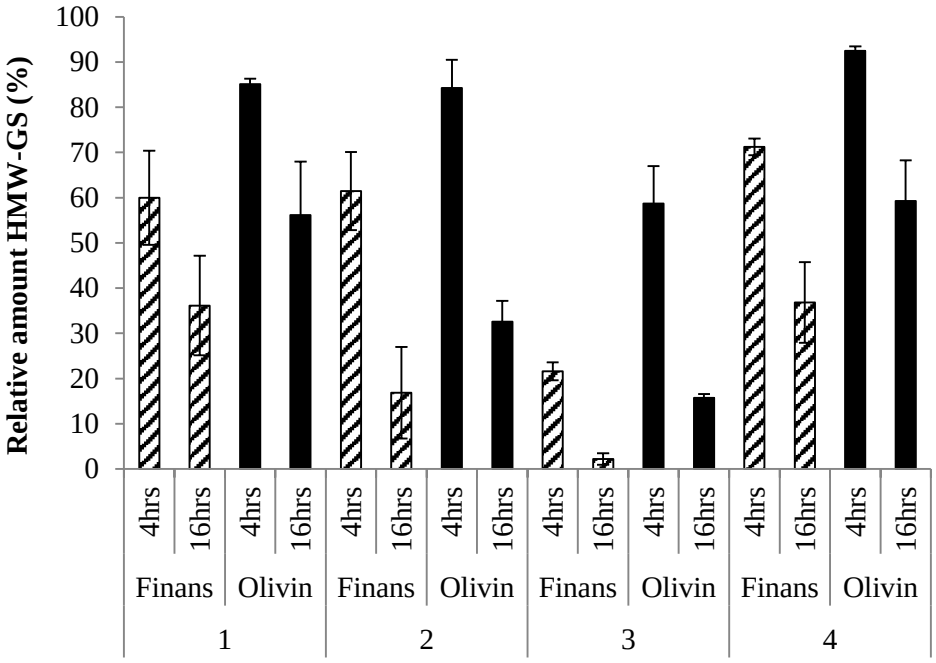


Figure 2. SDS-PAGE (4-12%) analysis of incubated gluten proteins extracted from Finans (1-3) and Olivin (4-6) grown at Field 3 in the 2011 season (A). Control samples were heated at 60 °C for 10 min prior to 16 hours incubation at 30 °C. 1. 4: 4 hours incubation. 2. 5: 16 hours incubation. 3. 6: control. The proportion of HMW-GS remained in the samples after incubation (B). Relative amount of HMW-GSs were calculated by dividing the total HMW-GS in the incubated sample (lane 1-2 and 4-5 in A) by total HMW-GS in the control sample (lane 3 and 6 in A). Bars show standard deviations of two replicates.

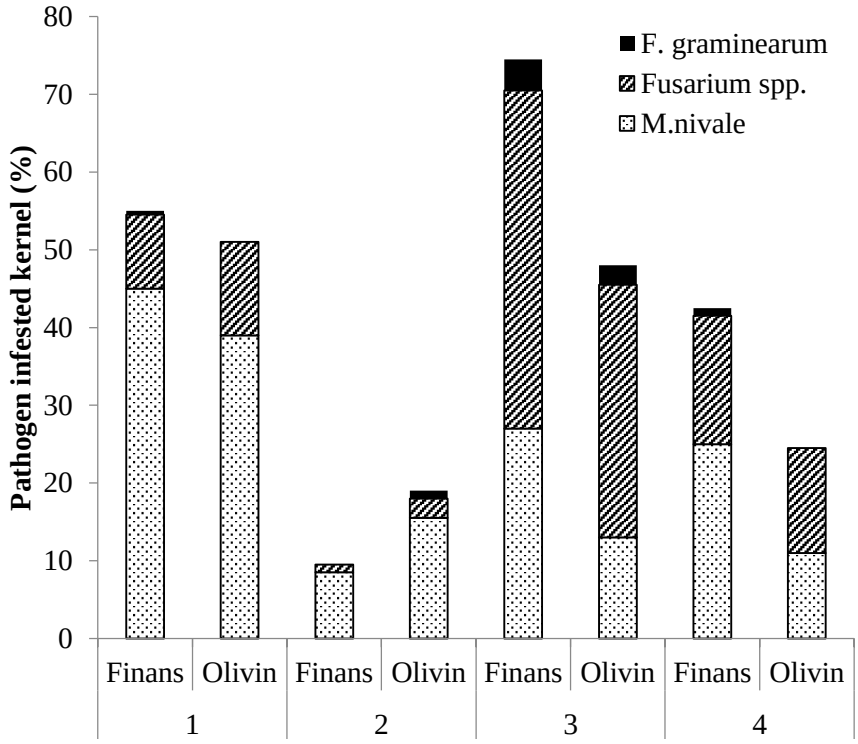
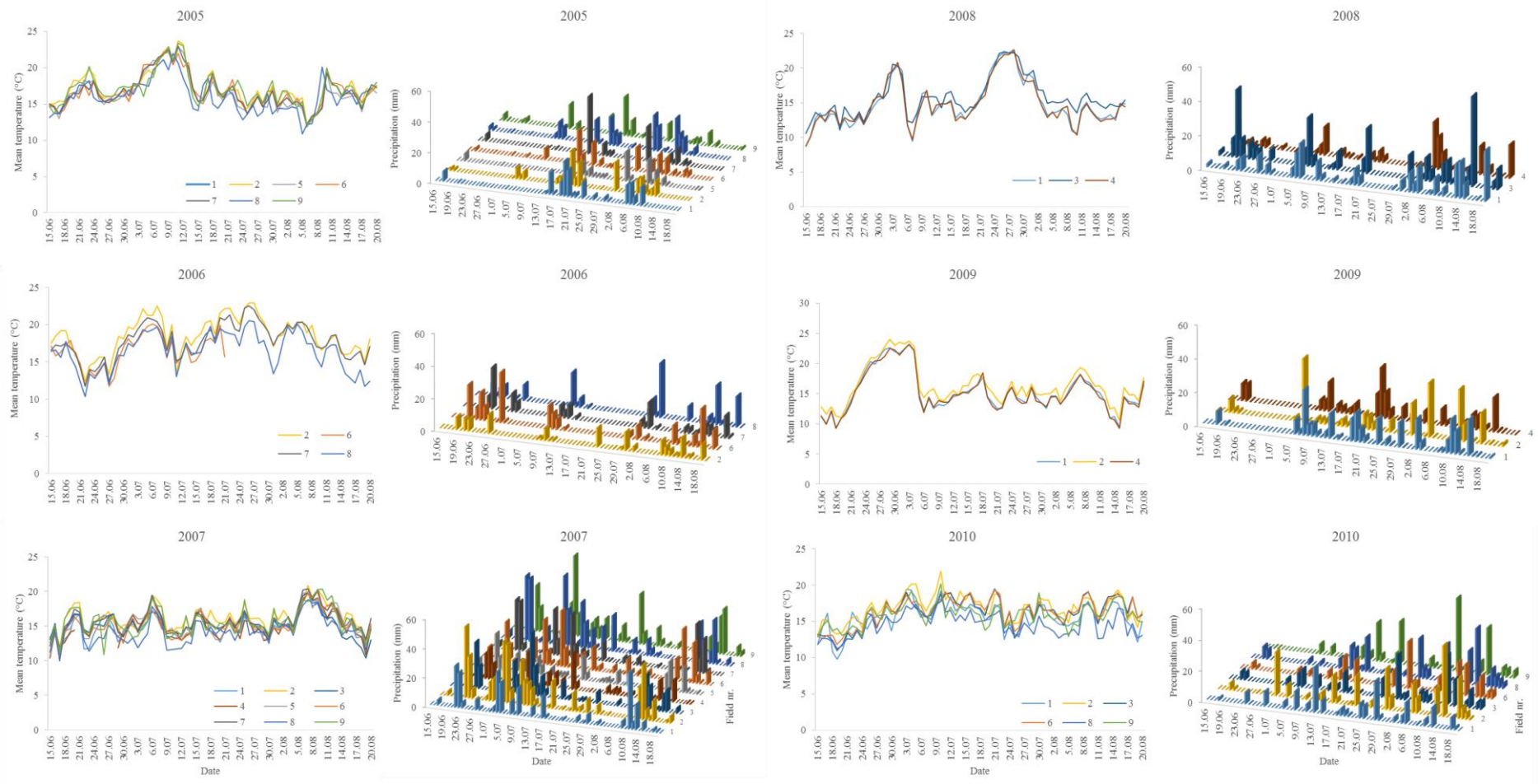


Figure 3. The proportion of fungal infested grain (%) in Finans and Olivin grown at four fields in the 2011 season.

Supplemental Table a. Number of field sites, number and name of cultivars used for field trials of winter wheat from 2005 to 2013

Year	Number of field sites	Number of cultivars	Cultivars
2005	7	4	Bjørke, Magnifikk, Mjølner and Olivin
2006	4	4	Bjørke, Magnifikk, Mjølner and Olivin
2007	9	4	Bjørke, Magnifikk, Mjølner and Olivin
2008	3	4	Bjørke, Magnifikk, Mjølner and Olivin
2009	3	4	Bjørke, Magnifikk, Mjølner and Olivin
2010	6	4	Bjørke, Magnifikk, Mjølner and Olivin
2011	5	5	Ellvis, Finans, Magnifikk, Mjølner and Olivin
2012	4	5	Ellvis, Finans, Magnifikk, Mjølner and Olivin
2013	6	6	Ellvis, Finans, Kuban, Magnifikk, Mjølner and Olivin



Supplemental Figure a. Mean temperature and precipitation from 15. June to 20. August (from ear emergence to maturation) in several locations 2005-2013. Field number in both mean temperature and precipitation figure from each year indicates the same location. Same field number in different year indicates the same location, and they are corresponding to Field numbers given in Table 1.

Supplemental Figure a. continues.