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Environmental factors associated with glutenin polymer assembly during grain maturation

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A B S T R A C T

This study aims to understand the environmental factors, focusing on rain and fungal infection, affecting the assembly of glutenin polymers during grain maturation. Spring wheat was grown in the field and grains were sampled from 50% grain moisture until maturity. Grain moisture content, protein content, size of glutenin polymers, the presence of proteases, and the amount of DNA from common wheat pathogenic fungi were analysed.

Rain influenced the rate of grain desiccation that occurred parallel to the rate of glutenin polymer assembly. Rapid desiccation contributed to faster glutenin polymer assembly than gradual desiccation. Severe reduction in the glutenin polymer size coincided with increased grain moisture due to rain. Furthermore, increased fungal DNA followed by presence of gluten-degrading proteases was observed in the grain after humid conditions. The presence of gluten-degrading proteases was presumably involved in reducing the size of glutenin polymers in grain.

Our study gave new insight into how environmental conditions could be associated with the assembly of glutenin polymers during grain maturation. The results suggest that rain and/or fungal proteases play an important role in reducing the molecular size of glutenin polymers.

1. Introduction

Gluten proteins are storage proteins in wheat synthesized in the starchy endosperm cells during grain filling. They consist of polymeric glutenins and monomeric gliadins that contribute to the elasticity and viscosity of gluten, respectively. The polymeric glutenins comprise of high molecular weight-glutenin subunits (HMW-GS) and low molecular weight-glutenin subunits (LMW-GS). The initial assembly of glutenin subunits into glutenin polymers occurs shortly after synthesis, while a rapid increase in molecular weight of glutenin polymers occurs at the end of grain development (Carceller and Aussenac, 2001; Daniel and Triboi, 2002; Shewry et al., 2009). Shewry et al. (2009) suggested that grain desiccation is the driving force behind this assembly of glutenin polymers. Glutenin polymers are linked by intermolecular disulphide bonds and also interact through hydrogen bonds. (Shewry and Tatham, 1997; Belton, 1999). The viscoelastic properties of dough are associated with the proportion of the largest glutenin polymers, known as sodium dodecyl sulphate- (SDS) unextractable polymeric proteins (UPP) (Gupta et al., 1993). Genetic factors such as the ratio of HMW-GS to LMW-GS and the allelic variations of both HMW-GS and LMW-GS affect the molecular size of glutenin polymers (Gupta et al., 1995, Gupta and Macritchie, 1994) and thus the viscoelastic properties of gluten.

Environmental factors also influence the assembly of glutenin polymers. Daniel and Triboi (2002) showed that drought, but not temperature, caused an early onset of the rapid insolubilization of gluten proteins. Carceller and Aussenac (2001) reported that the desiccation rate influenced the rate of glutenin insolubilization, while it did not affect the proportion of the insoluble polymers at maturity. Johansson et al. (2008) observed that a slight increase in grain moisture content (GMC) due to rain led to a decrease in the proportion of SDS-unextractable monomeric proteins but not the proportion of SDS-unextractable polymeric proteins. Moreover, Malik et al. (2011) reported that %UPP was affected by interaction effects of genotype, nitrogen and temperature, and not by one single factor. Environmental factors that affect water status in grain have been reported to influence the assembly of glutenin polymers. However, it is still not well documented how environmental factors such as rain naturally occurring during grain maturation influence the assembly and the proportion of

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glutenin polymers in mature grain. In our previous study we observed that %UPP was considerably reduced in grain from fields exposed to frequent rain during grain maturation. The analysis of such grain samples indicated presence of fungal proteases that are able to degrade gluten proteins, causing reduction in the size of glutenin polymers (Koga et al., 2016). Dexter et al. (1996) observed reduced levels of glutenin in *Fusarium*-damaged kernels compared to healthy kernels, and Eggert et al. (2011) reported that fungal proteases secreted by *F. graminearum* were a plausible explanation for a reduced amount of glutenins in grain.

The present study aims to understand environmental factors that influence the assembly of glutenin polymers during grain maturation under field conditions. We specifically focus on how rain and infection of pathogenic fungi influence the assembly of glutenin polymers during grain maturation and the size of glutenin polymers in the harvested grain. We also discuss how these factors may interact and influence the assembly of glutenin polymers, hence causing undesirable variation in breadmaking quality.

2. Materials and methods

2.1. Wheat samples

2.1.1. Grain samples from non-inoculated field

The spring wheat cultivar, Bjarne (HMW-GS; 1Ax2*, 1Bx6+1By8, 1Dx5+1Dy10) was grown in the field with two replicates at Apelsvoll experimental station, NIBIO, Kapp, and at Vollebekk experimental station, the Norwegian University of Life Sciences, Ås in 2015 and 2016. Date of sowing, anthesis, first sampling and final harvest at maturity are presented in Table 1. The size of the plots was 1.5 m × 8 m. Fertilization of 100 kg nitrogen per ha in a compound NPK fertilizer (YaraMila® Fullgjødsel® 22-3-10, Yara Norge AS, Oslo, Norway) was applied at sowing and 40 kg N/ha was supplementary applied as calcium nitrate (YaraLiva® Kalksalpeter™, Yara Norge AS) at flag leaf development, Zadoks growth stage (Z) 37–39 (Zadoks et al., 1974). Herbicide treatment was performed according to normal practice. The plots were treated with Proline (Bayer, Leverkusen, Germany) against *Fusarium* head blight at anthesis in both locations and seasons. At Vollebekk, the fungicide, Delaro (Bayer) was applied to control leaf diseases prior to anthesis in both seasons. At Apelsvoll, plants were treated with insecticide, Sumi Alpha (PHILAGRO, Pretoria, South Africa) at Z30 in 2015, and with fungicide, Comet Pro (BASF, Ludwigshafen, Germany) against leaf diseases at anthesis in 2016. Our previous study showed that the acquisition of physiological maturity occurred when the GMC reached about 40%, followed by onset of the assembly of glutenin polymers (Koga et al., 2017). Therefore, the sampling of immature spikes was started when the GMC was around 50%. Thirteen spikes from parts of each plot (1.5 m × 3 m) were randomly chosen and harvested with 3–4 days interval until maturity. Three of the spikes were used to determine GMC at each harvest. The remaining spikes were freeze-dried and grain was collected from the spikes by hand (referred as immature grain/-sample). At maturity, the remaining areas of each plot (1.5 m × 5 m) were harvested with a plot combine and grain was dried in a drying cabinet at 30 °C until GMC was below 14% (referred as mature grain/sample).

2.1.2. Grain samples from inoculated field

Mature grain of Bjarne and both mature and immature grain of the spring wheat cultivar Bastian (HMW-GS; 1Ax2*, 1Bx13+1By16, 1Dx5+1Dy10) from a field inoculated with *F. graminearum* were kindly provided by Dr. Morten Lillemo of the Norwegian University of Life Sciences in 2016. The immature grain was used to investigate the effect of fungal infection on the assembly of glutenin polymers during grain maturation. The field was sown at Vollebekk research station and date of sowing, anthesis, and harvest are presented in Table 1. Field inoculation with *F. graminearum* was carried out according to the method described by Tekle et al. (2018). Briefly, grain spawn (infected oat kernels) was prepared with four *F. graminearum* isolates (200726, 200838, 101177 and 101023). The spawn inoculum was dispersed in the field at a density rate of 10 g/m^2 at Z32/33. For optimal germination of ascospores, the field was mist irrigated twice for 9 min per h in the evening after spawn application and four times during anthesis. Immature spikes were harvested from three field plots, and the harvest method and determination of GMC were the same as from non-inoculated field trials as described above. Immature grain was prepared as described above (referred to as *Fusarium*-infected sample). Mature grain from this field was analysed for protein content (%), falling number and %UPP.

2.2. Weather data

Mean daily and hourly temperature, rain and relative humidity (RH) during the period of grain sampling were obtained from the Agricultural Meteorological Service (https://lmt.nibio.no/) for each location in 2015 and 2016. Mean daily temperatures were used to calculate degree-days after anthesis (ddaa). Hourly weather data during grain sampling is available in Supplementary Figure I.

2.3. Milling

Due to the small sample size, grain samples collected from immature spikes were milled into wholemeal flour on an ultra-centrifugal mill ZM 100 (Retsch GmbH & Co. KG, Haan, Germany). Mature grain was cleaned and milled into wholemeal flour on a Laboratory mill 3100 (Perten Instruments AB, Hägersten, Sweden).

2.4. Flour analysis

Falling Number was determined for wholemeal flour of mature grain with a Falling Number 1800 (Perten Instruments AB). Protein content of mature grain was determined by near infrared reflectance using a Perten Inframatic 9200 (Perten Instruments AB).

2.5. The proportion of glutenin polymers

Both immature and mature samples were analysed with a size

Table 1
Sowing, anthesis and harvest date for non-inoculated and inoculated fields at Apelsvoll and Vollebekk in 2015 and 2016. Protein content, falling number and the proportion of SDS-unextractable polymeric proteins (%UPP) in mature grain from the last harvest. Numbers are average of two biological replicates for Bjarne and three biological replicates for Bastian. ddaa, degree-days after anthesis.

Year	Field	Cultivar	Sowing date	Anthesis date	First sampling date (ddaa)	Harvest date	Protein content (%)	Falling number (s)	%UPP
2015	Apelsvoll	Bjarne	28. Apr	13. Jul	17. Aug (506)	15. Sep	13.1	270	29.7
	Vollebekk	Bjarne	20. Apr	6. Jul	3. Aug (413)	31. Aug	12.7	432	23.0
2016	Apelsvoll	Bjarne	28. Apr	5. Jul	8. Aug (553)	5. Sep	13.0	402	36.4
	Vollebekk	Bjarne	18. May	19. Jul	26. Aug (618)	21. Sep	12.8	426	47.4
	Vollebekk +Fus[a]	Bjarne	11. May	3. Jul	–	5. Sep	12.5	288	8.2
	Vollebekk +Fus[a]	Bastian	11. May	4. Jul	3. Aug (505)	5. Sep	11.0	n.a.	9.0

[a] +Fus indicates that grain samples were from the field inoculated with *F. graminearum*.

exclusion-fast performance liquid chromatography (SE-FPLC). Two protein fractions, SDS-extractable and SDS-unextractable proteins, were sequentially extracted from flour samples according to Morel et al. (2000) with some modification described by Tronsmo et al. (2002). The protein fractions were filtered through Millex-HV 0.45 μm syringe filters (Merck Millipore, Darmstadt, Germany) and separated on a Superose™ 12 10/300 GL (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) connected to an ÄKTA SE-FPLC (GE Healthcare Bio-Sciences AB) with an elution buffer [0.05 M sodium phosphate, pH 6.9, 0.08 M NaCl and 0.1% (w/v) SDS]. The flow rate was 0.4 mL/min and length of elution was 1.5- and 1-column volume for SDS-extractable and SDS-unextractable proteins, respectively. The effluent was monitored by UV-absorbance at 210 nm.

Four main peaks (F1-F4) are obtained from the SDS-extractable fraction. The first two peaks (F1-F2) and two following peaks (F3-F4) consist of polymers and monomers, respectively, with some overlap. The SDS-unextractable fraction reveals one main peak (F1*) that consists of large polymers. The proportion of each peak was calculated as [each peak/sum of all peaks]. The proportion of SDS-unextractable polymeric proteins in total polymeric proteins (%UPP) was calculated as [F1*/(F1*+F1) × 100] according to Macritchie and Gupta (1993).

2.6. Analysis of protease activities

Protease activities in both immature and mature grain were analysed with zymography. Salt-soluble proteins were extracted from 200 mg wholemeal flour by adding 1 mL 100 mM sodium phosphate buffer (pH 7.0). The sample mixture was homogenized twice for 15 s at 6600 rpm speed with 30 s pause between agitation by a Precellys 24 (Bertin Technology, France) and centrifuged at 16 000*g* for 20 min at 4 °C. The supernatant was recovered, and protein concentration was measured by the Lowry protein assay (Bio-Rad Laboratories, Inc, CA, USA). The protein extracts were stored at −80 °C.

Zymogram gels were made as follows: stacking gel [4% (w/v) acryl/bis-acrylamide (37.5:1), 125 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate (APS), 0.1% (v/v) tetramethylethylenediamine (TEMED)], separating gel [12.5% (w/v) acryl/bis-acrylamide (37.5:1), 375 mM Tris-HCl (pH 8.8), 0.05% (w/v) APS and 0.05% (v/v) TEMED] with either gluten [final concentration, 2 mg/mL, 0.5% (w/v) SDS and 2.5 mM Tris(2-carboxyethyl)phosphine] or gelatine [final concentration, 1 mg/mL] as substrates. Proteins (20 μg) were separated at 100 V for 4 h or 3 h 15 min on zymogram gels containing gluten or gelatine, respectively in a standard SDS-PAGE electrophoresis buffer [25 mM Tris, 192 mM glycine, 0.1% SDS]. The gels were renatured with 2.5% (w/v) Triton X-100 for 15 min × 2 and wash with 50 mM Tris-HCl (pH 7.6) for 15 min prior to incubation with 50 mM Tris-HCl (pH 7.6), 5 mM $CaCl_2$ and 0.2 M NaCl overnight at 37 °C. After incubation, the zymogram gels were stained with 0.1% (w/v) coomassie brilliant blue R-250, 50% (v/v) methanol and 7% (v/v) acetic acid for 1 h and destained with 20% (v/v) methanol and 7% (v/v) acetic acid until the stacking gels became clear. Gels were scanned with an Epson Perfection 4990 PHOTO scanner.

2.7. Analysis of fungal DNA content

The amount of fungal DNA was analysed to investigate the naturally occurring infection by a selection of plant pathogenic fungi. Four harvest points from the non-inoculated fields were selected according to the results of zymography. Total genomic DNA was extracted from 100 mg flour using FastDNA SPIN Kit for Soil (MP Biomedicals, Ohio, USA) according to the manufacturers' description. The DNA was diluted 1:9 with distilled water and analysed with quantitative PCR (qPCR) to determine the absolute amount of DNA of the following fungi: *Fusarium avenaceum*, *Fusarium culmorum*, *F. graminearum*, *Fusarium poae, Microdochium majus*, *Microdochium nivale* and *Parastagonospora nodorum* (Supplementary Table I). The amount of plant DNA was also determined (Supplementary Table I).

qPCR was performed in at total volume of 25 μL that consisted of 4 μL 10-fold diluted genomic DNA, 300 nM of each primer, 100 nM of each probe, and 1 × SsoAdvanced™ Universal Probes Supermix (Bio-Rad) or 1 × Sso Advanced Universal SYBR® Green Supermix (Bio-Rad), in a C1000TouchTerm Cycler combined with a CFX96TMReal-TimeSystem (Bio-Rad). The conditions for amplification were 95 °C for 3 min, followed by 45 cycles of 95 °C for 10 s and 60 °C for 30 s. An additional melting curve analysis was added for the SYBR assays. Standard DNA from the different fungi was obtained by growing pure cultures on potato dextrose broth (PDB) for 5–7 days in the dark at 20 °C and 80 rpm. The cultures were filtrated, and the mycelium was frozen to −20 °C and freeze-dried. Genomic DNA was extracted from freeze-dried mycelium using CTAB-extraction method as described in Edwards et al. (2012) or using the DNeasy Plant Maxi Kit (Qiagen, Venlo, Netherland) according to manufacturers' instructions. The concentration of standard DNA was determined using Qubit dsDNA HS Assay Kit (ThermoFisher Scientific). For absolute quantification of DNA from fungi, a series of five dilutions in the range 1–4000 pg of standard DNA was used. For the absolute quantification of plant DNA, the serial dilution contained plant DNA in the range 0.008–32 ng. The amount of fungal DNA was normalized against the amount of plant DNA as described in Divon et al. (2012) and the fungal content is presented as pg fungal DNA per μg plant DNA (pg/μg).

3. Results

3.1. The effect of weather on grain moisture content during grain maturation

Weather conditions during grain maturation for the field trials are shown in Supplementary Figure I. Weather conditions during grain maturation in Norway are usually characterized by lower temperature and more humid conditions with considerable fluctuation compared to other wheat growing areas. Both temperature and rain during grain maturation had a great influence on GMC when grain passed physiological maturity (about 40%). Relatively heavy rain combined with low temperature (10–12 °C) at Apelsvoll between 723 and 766 ddaa led to a clear increase in GMC in 2015 (Fig. 1A). Less but frequent rain during grain maturation hindered a rapid desiccation of grain at Apelsvoll in 2016 (Fig. 1C). Grain desiccated rapidly during a period with relatively high temperature (15–18 °C) without rain at Vollebekk, 579–725 ddaa and 768–820 ddaa in 2015 and 2016, respectively (Fig. 1B and D). However, relatively heavy rain between 741 and 788 ddaa caused a rapid increase in GMC from 18.7 to 34.4% at Vollebekk in 2015 (Fig. 1B).

3.2. The assembly of glutenin polymers

In general, the proportion of SDS-unextractable polymers in total proteins (%F1*) increased throughout the sampling period. On the other hand, the proportion of SDS-extractable polymers (%F1) increased to physiological maturity at around 40% GMC and decreased thereafter (Table 2). Consequently, %UPP increased when GMC decreased with some exceptions (Fig. 1). Notably, %UPP did not increase during the period of rain from 723 to 766 ddaa, while it increased again after 776 ddaa at Apelsvoll in 2015 (Fig. 1A). The desiccation of grain took more time in this field and %UPP was 29.7% at maturity (Table 1). Grain desiccated slowly between 718 and 862 ddaa, and the increase in %UPP was corresponding to the slow grain desiccation at Apelsvoll in 2016 (Fig. 1C and Supplementary Figure IC). At Vollebekk, %UPP increased quickly with rapid grain desiccation between 549 and 725 ddaa and between 768 and 820 ddaa in 2015 and 2016, respectively (Fig. 1B and D). Grain reached the lowest GMC (<20%) and the highest %UPP at 725 ddaa, however, %UPP decreased dramatically when GMC increased again due to rain between 741 and 788 ddaa at Vollebekk in 2015

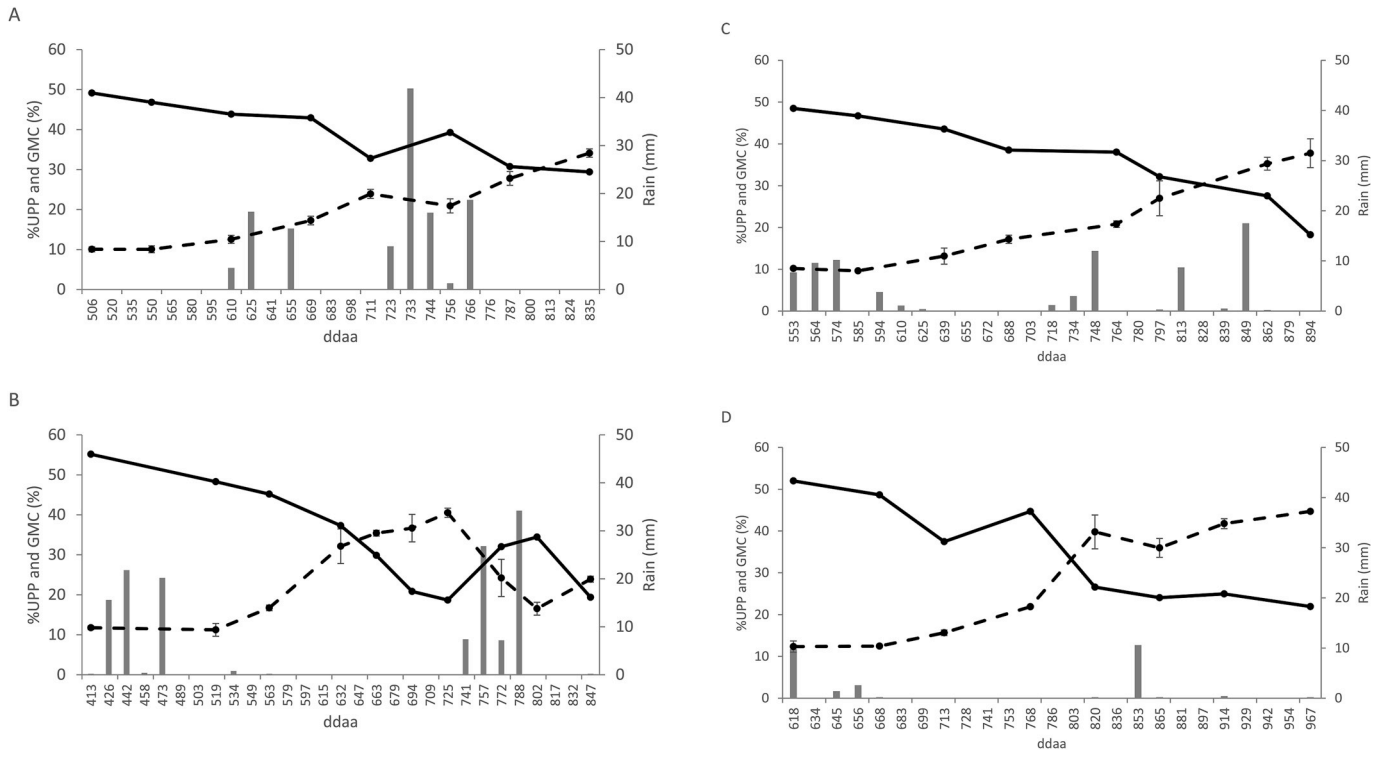

Fig. 1. Grain moisture content; GMC (solid line), and the proportion of SDS-unextractable polymeric proteins; %UPP (dashed line) in immature grain sampled from Apelsvoll (A and C) and Vollebekk (B and D) in 2015 (A and B) and 2016 (C and D). Rainfall data (gray bar) was collected from the closest weather station to each field location. Error bar shows standard deviation of two biological replicates. ddaa, degree-days after anthesis.

(Fig. 1B). When the proportions of FPLC fractions were compared, %F1* decreased and %F1 increased simultaneously without considerable changes in other fractions during this period (Table 2). Grain desiccation occurring after this period led to increase %UPP again, while the value was much lower in mature grain than the highest value obtained at 725 ddaa at Vollebekk in 2015 (Fig. 1B and Table 1).

3.3. The presence of proteases

The presence of proteases was assessed by measuring protease activities using zymography with gels containing either gluten or gelatine as substrate. Clear bands appeared at the uppermost part of the zymogram for grain harvested at the beginning of the sampling period (Fig. 2A–H). The protease activity (intensity of the bands) decreased gradually and intensity was lowest at 711 ddaa (Apelsvoll) and 663–772 ddaa (Vollebekk) in 2015, and 764 ddaa (Apelsvoll) and 820 ddaa (Vollebekk) in 2016. According to Young and Gallie (2000), biosynthetic processes cease and metabolic activities in grain decrease towards physiological maturity, partly due to water loss, and thereafter, the endosperm cells undergo programmed cell death. Therefore, proteases observed in our samples at the beginning of the sampling period were considered as plant origin, as they decreased with grain desiccation.

Clear bands at the uppermost part reappeared in the zymogram of sample harvested after 756 ddaa and 802 ddaa at Apelsvoll and Vollebekk, respectively, in 2015 (Fig. 2A, B, E, and F). This coincided with the appearance of a new band at about 20 kDa (20 kDa-band) which was not observed at the previous harvests in the zymogram with gelatine for the same samples (Fig. 2E and F). The same band appeared in grain sampled after a period of rain at Apelsvoll (797–862 ddaa) and at the beginning of sampling (668 ddaa) at Vollebekk in 2016 (Fig. 2G and H).

3.4. Fungal growth

Four harvest points were chosen according to the results of zymography (Fig. 2) and the amount of fungal DNA for seven pathogenic fungi commonly observed in Norwegian wheat was measured to investigate fungal infection of grain samples. *Parastagonospora nodorum*, and particularly *M. majus*, dominated, as both species were present at both locations and seasons (Table 3). *Microdochium nivale* was also observed to some extent in all fields, but at much lower levels than *P. nodorum* and *M. majus*. The four other fungi were less common. The levels of fungal DNA were generally low in grain sampled at earlier harvest points, i.e. until 669 ddaa (Apelsvoll) and 725 ddaa (Vollebekk) in 2015, and 688 ddaa at Apelsvoll in 2016 (Table 3). Thereafter, the levels increased, often after a couple of days of rain or periods of high RH (Fig. 1 and Supplementary Figure. I). In 2016, the level of fungal DNA was already relatively high at the first harvest (618 ddaa), and it increased dramatically between 668 ddaa and 820 ddaa at Vollebekk (Table 3).

3.5. The assembly of glutenin polymers in Fusarium-inoculated field

Mature grain of both Bjarne and Bastian harvested from the *Fusarium*-inoculated field had considerably low %UPP (Table 1). To investigate the assembly of glutenin polymers in fungal infected grain, immature grain was sampled from *F. graminearum* inoculated field trials. Frequent rain at the beginning of sampling led to a slow desiccation of grain, while GMC decreased rapidly in the middle of August (643–737 ddaa) and the lowest value (17.6%) was reached at 737 ddaa (Fig. 3). Moisture content increased again to up to 35% after rain between 752 and 849 ddaa (Fig. 3). The proportion of total glutenin polymers, % (F1*+F1), consisted of 35–40% of the total proteins, however, %UPP was consistently low throughout the grain maturation period (Fig. 3). The results showed that the assembly of glutenin polymers did not take place, despite glutenins being synthesized in *Fusarium*-infected samples. The presence of proteases was also investigated with zymography (Fig. 2I and J). The intensity of bands was not very high in samples harvested at 505 ddaa, while it increased in samples harvested after 582 ddaa. Thereafter, thick bands were observed throughout the sampling

Table 2
The proportion of FPLC fractions. F1*, SDS-unextractable polymers; F1 and F2, SDS-extractable polymers; F3 and F4, SDS-extractable monomers; and F5, small monomers (albumins and globulins). ddaa, degree-days after anthesis.

Year	Location	ddaa	%F1*	%F1	%F2	%F3	%F4	%F5
2015	Apelsvoll	506	1.99	17.77	16.72	27.56	26.93	9.03
		550	2.15	19.32	16.12	26.97	26.77	8.67
		610	3.43	23.83	14.83	24.24	25.81	7.86
		669	5.24	25.11	13.08	22.65	25.82	8.11
		711	7.43	23.66	12.73	22.98	25.58	7.62
		756	6.54	24.74	12.30	23.00	26.07	7.35
		787	9.07	23.57	11.58	22.39	25.96	7.43
		835	11.33	21.88	11.45	22.30	26.35	6.69
	Vollebekk	413	2.05	15.38	16.46	29.55	27.30	9.26
		519	2.44	19.30	16.51	25.78	28.18	7.80
		563	4.62	22.99	14.52	23.74	27.01	7.12
		632	10.08	21.26	13.14	22.15	26.89	6.49
		663	11.37	20.72	12.59	22.67	25.88	6.77
		694	11.81	20.34	12.10	22.54	25.92	7.29
		725	13.07	19.19	12.07	22.74	25.55	7.38
		772	7.70	24.05	12.24	22.77	25.98	7.25
		802	5.18	26.18	12.79	22.89	26.23	6.73
		847	7.97	25.35	11.70	22.46	25.97	6.54
2016	Apelsvoll	553	1.65	14.56	17.75	29.20	27.25	9.59
		585	1.75	16.49	17.12	28.17	26.97	9.50
		639	3.23	21.33	15.46	24.94	26.84	8.21
		688	4.76	22.93	14.04	23.33	26.61	8.33
		764	6.18	23.52	13.27	22.65	26.96	7.42
		797	8.28	22.36	12.68	22.56	26.89	7.24
		862	11.45	21.02	12.15	22.24	26.40	6.74
		894	12.48	20.56	11.89	21.94	26.21	6.90
	Vollebekk	618	2.02	14.33	17.94	30.44	26.19	9.08
		668	2.12	14.90	17.37	29.49	27.56	8.56
		713	3.65	19.62	15.00	25.91	26.76	9.07
		768	5.09	18.17	15.58	25.98	27.32	7.86
		820	12.70	19.13	12.21	22.31	25.92	7.74
		865	10.85	19.32	13.00	23.12	25.99	7.71
		914	12.80	17.85	12.38	22.77	26.33	7.87
		967	14.34	17.73	12.12	22.51	25.85	7.45

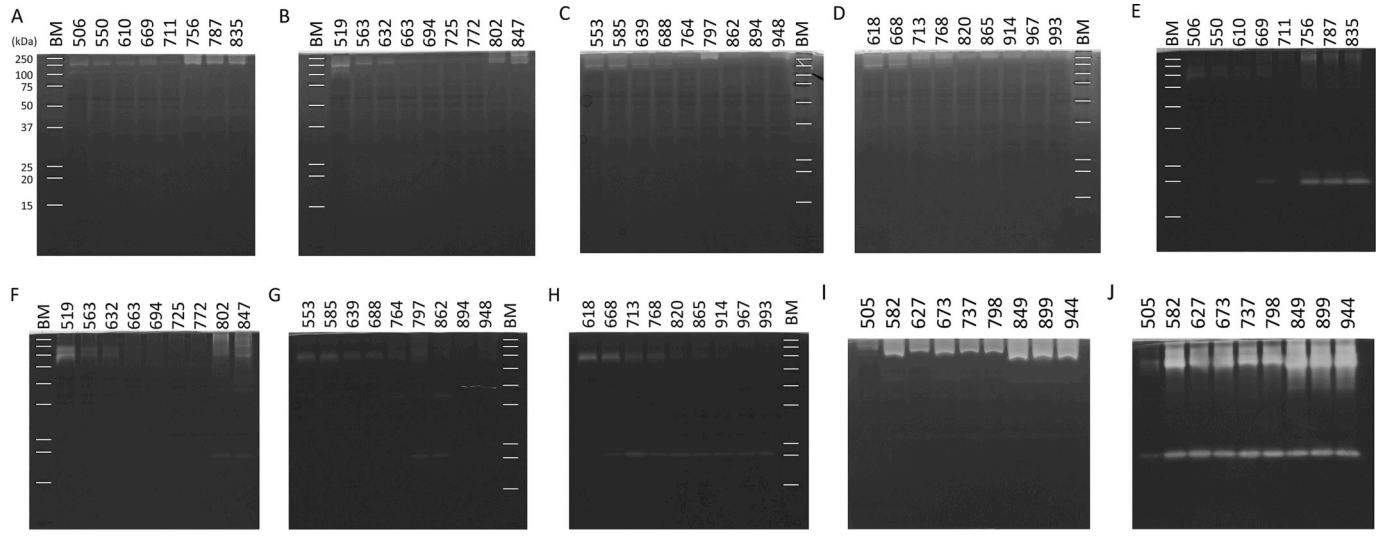

Fig. 2. Protease activities in immature grain of Bjarne sampled at Apelsvoll (A, C, E and G) and Vollebekk (B, D, F and H) in 2015 (A, B, E and F) and 2016 (C, D, G and H). Protease activities in immature grain of Bastian sampled from filed inoculated with *Fusarium graminearum* in 2016 (I and J). Zymography was carried out with gluten (A-D and I) and gelatin (E-H and J) substrates. Numbers over zymogram gels show the degree-days after anthesis of grain at sampling. BM; Bench marker.

period and the intensity of bands increased even more in grain harvested after 849 ddaa. The 20 kDa-band was observed in all *Fusarium*-infected samples in the gelatine zymogram (Fig. 2J).

4. Discussion

4.1. The association between rain and glutenin polymer assembly

Our results showed that rain had great influence on GMC when it had dropped below 40%. Similar to the study of Shewry et al. (2009), our

Table 3
Fungal DNA (pg per μg plant DNA) measured with species specific qPCR during grain maturation at Apelsvoll and Vollebekk in 2015 and 2016.

Year	Field	ddaa	*F. ave*		*F. cul*		*F. gra*		*F. poa*		*M. maj*		*M. niv*		*P. nod*	
			Mean	S.D.*	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
2015	Apelsvoll	506	3	5	0	0	0	0	1	1	13	18	0	0	21	4
		669	7	9	0	0	0	0	1	1	78	4	3	2	32	8
		756	14	17	0	0	0	0	10	6	820	321	35	1	167	13
		835	207	152	0	0	0	0	35	19	2729	1125	144	48	773	366
	Vollebekk	413	4	2	0	0	0	0	1	2	54	50	1	2	59	19
		632	4	3	12	16	0	0	1	1	38	50	0	0	70	45
		725	6	8	0	0	0	0	3	4	194	188	1	1	177	72
		802	7	10	0	1	0	0	1	0	326	381	2	1	882	24
2016	Apelsvoll	553	0	0	0	0	0	0	0	0	3	4	0	0	15	6
		688	0	0	17	24	0	0	0	0	30	23	0	0	10	3
		862	0	0	0	0	0	0	1	0	407	138	4	6	609	276
		894	0	0	0	0	0	0	7	6	436	328	7	2	441	83
	Vollebekk	618	0	0	0	0	0	0	0	0	595	208	0	0	20	18
		668	0	0	0	0	0	0	1	1	624	349	0	0	45	37
		820	0	0	0	0	8	12	0	0	11629	3756	20	27	218	179
		967	0	0	0	0	4	6	0	0	11634	1827	55	77	497	120

Abbreviation; ddaa, degree-days after anthesis; *F. ave*, *Fusarium avenaceum*; *F. cul*, *F. culmorum*; *F. gra*, *F. graminearum*; *F. poa*, *F. poae; M.maj, Microdochium majus; M. niv, M. nivale; P. nod, Parastagonospora nodorum, S.D.*,* standard deviation of two biological replicates.

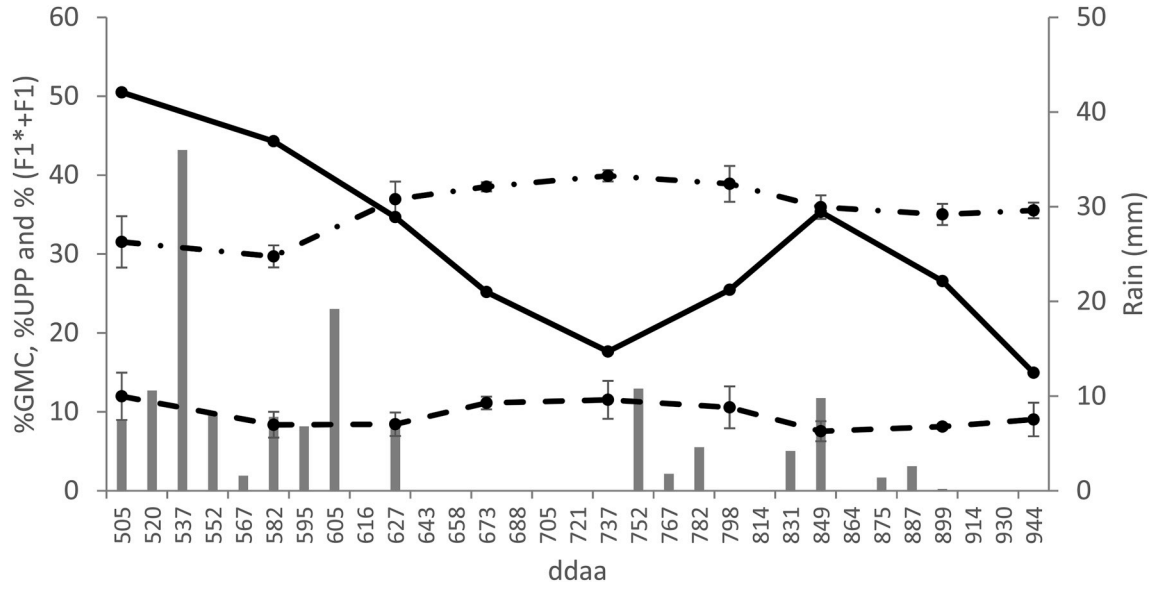

Fig. 3. Grain moisture content; %GMC (solid line), the proportion of SDS-unextractable polymeric proteins; %UPP (dashed line) and the proportion of polymeric proteins in total proteins; %(F1*+F1) (dash-dotted line) in immature grain (spring wheat cv. Bastian) sampled from fields artificially inoculated with *Fusarium graminearum* at Vollebekk in 2016. Rainfall data (gray bar) was collected from the closest weather station. Error bar shows standard deviation of three biological replicates. ddaa, degree-days after anthesis.

results also suggested that the assembly of glutenin polymers was driven by desiccation. Slow grain desiccation led to slow assembly of glutenin polymers and vice versa. Our results support the findings of Carceller and Aussenac (2001) who also demonstrated that the speed of glutenin insolubilization was parallel to the desiccation rate. Our results from Apelsvoll in 2015 demonstrated that the assembly of glutenin polymers was interrupted if rain hindered grain desiccation. Moreover, samples from Vollebekk in 2015 showed a reduction in %UPP. The results from this field trial indicated that large glutenin polymers were disassembled to smaller polymers when GMC increased after a period of rain (741–788 ddaa). Increased GMC may be linked to the process reducing the size of glutenin polymers. According to Belton (1999), hydration of proteins causes loss of intra- and inter-molecular hydrogen bonds. Loss of inter-chain hydrogen bonds in glutenin polymers will not directly affect the size of polymers, however, the structure of the glutenin polymers will probably become loosened. Gobin et al. (1996) reported that conditioning of dried grain led to a reduction of disulphide bonds in storage proteins and suggested the involvement of thioredoxin. It was previously shown that thioredoxin was involved in reducing disulphide bonds at the beginning of the germination process (Lozano et al., 1996; Kobrehel et al., 1992). Hence, it can be hypothesized that an increase in GMC may alter the structure of proteins as well as biological status of the endosperm cells, such as activation of thioredoxin and related enzymes, and this can in turn cause a reduction of the disulphide bonds of the glutenin polymers.

4.2. Assembly of glutenin polymers in grain from F. graminearum infected plants

The assembly of glutenin polymers did not take place in *Fusarium*-infected samples during grain maturation. Moreover, these samples possessed gluten-degrading proteases throughout the whole grain maturation period. Our results indicated that *F. graminearum* secreted gluten-degrading proteases, thus the molecular size of the glutenin

polymers remained small. *In vitro* degradation of gluten proteins, particularly of the HMW sub-fraction, by proteases secreted by *F. graminearum* has also been reported previously (Nightingale et al., 1999; Eggert et al., 2011). Not only proteases from *F. graminearum* but also from several other *Fusarium* spp. have been reported to degrade gluten proteins (Wang et al., 2005; Pekkarinen et al., 2000). Therefore, proteases secreted by pathogenic fungi could be one of the factors interrupting polymer assembly or reducing glutenin polymers.

4.3. The interaction between rain and fungal infection and their association with glutenin polymer assembly

Samples from Vollebekk in 2015 had high %UPP and almost no proteases when GMC dropped below 20% at 725 ddaa. However, relatively heavy rain (741–788 ddaa) coincided with a sharp decrease in % UPP. The levels of fungal DNA as well as gluten-degrading proteases increased in the grain sometime after the rain started. The gluten-degrading proteases were considered as fungal proteases because their activity increased with increasing fungal DNA and the 20 kDa-band appeared in the gelatine zymogram. This band was consistently observed in *Fusarium*-infected samples. The results indicated that the size of glutenin polymers was reduced by increased GMC due to rain and/or fungal proteases. A reduction in %UPP was already observed at 772 ddaa, while the proteases appeared at a detectable level in the zymogram first at 802 ddaa. This observation suggested that an increase in GMC due to rain directly triggered a reduction in molecular size of glutenin polymers. Fungal proteases may also be involved in degrading glutenin polymers after their emergence (802 ddaa). Glutenin polymers were reassembled during re-desiccation, yet %UPP remained lower than the value reached before the rain. The results indicated that increased GMC and fungal proteases caused changes in the grain which limit the reassembly of glutenin polymers.

Grain samples were infected by several fungi and the period of rain (723–766 ddaa) was followed by an increase in fungal DNA, particularly *M. majus* and *P. nodorum* at Apelsvoll in 2015. Similar changes in the levels of fungal DNA and co-occurring gluten-degrading proteases were observed after a period of rain at Apelsvoll in 2016, although the overall levels of rain, fungal DNA and gluten-degrading proteases were lower than in 2015. In both seasons, mature grain from Apelsvoll had lower % UPP than the highest %UPP obtained at Vollebekk which is presumably close to the potential of this cultivar. We suspect that a combination of slow grain desiccation due to humid conditions and the presence of fungal proteases might create an environment in the endosperm cells unfavourable for forming disulphide bonds of glutenin polymers. Blandino and Reyneri (2009) suggested a negative impact of *Microdochium* spp. infection on dough strength. However, information on whether *Microdochium* spp. and *P. nodorum* secrete gluten-degrading proteases is limited. Several environmental factors, such as nitrogen and sulfur levels and temperature, can affect the composition of gluten proteins and thus the size of glutenin polymers (Southan and Macritchie, 1999). The difference in %UPP observed between Vollebekk and Apelsvoll may, to some extent, be caused by different environmental conditions, such as soil type affecting nitrogen and sulfur availability in addition to weather conditions.

Grain sampled at Vollebekk in 2016 had a relatively higher level of *M. majus* DNA at the first harvest compared to other locations and/or seasons. The level of *M. majus* DNA increased considerably even though there was less rain during grain maturation (668–820 ddaa). Despite little rain, high RH combined with relatively low temperatures (Supplementary Figure ID) probably contributed to the development of *M. majus*. The 20 kDa-band appeared in the zymogram in grain sampled at the second harvest (668 ddaa) at Vollebekk in 2016, which corresponded well with the increase of fungal DNA. Analysis of the samples resulted in higher %UPP after rapid assembly concomitant with the rapid grain desiccation between 768 and 820 ddaa. The reason for high %UPP despite high amount of fungal DNA in the samples is not clear. Gluten-degrading proteases in these field samples did not increase although fungal DNA increased considerably. Relatively low levels of gluten-degrading proteases in the samples might not be detrimental for the polymer assembly. The ability to secret gluten-degrading proteases might vary both within and between fungal species and might depend on the environment. Rain at 853 ddaa did not seem to have a large effect on GMC nor %UPP. Johansson et al. (2008) also showed that small changes in GMC did not reduce %UPP. These results may disagree with Gobin et al. (1996) who observed a reduction of disulphide bonds after conditioning of dried grain.

4.4. Future prospects

Our study showed a relationship between humid weather conditions during grain maturation and reduced glutenin polymer size. We hypothesize that increased GMC caused by frequent/heavy rain during the later stage of grain maturation leads to chemical or physiological changes in grain, i.e., activation of thioredoxin, which are involved in the reduction of disulphide bonds in glutenin polymers. Secreted fungal proteases are other factors facilitating diminishing the polymer size as pathogenic fungi thrive under humid conditions. Pathogenic fungi are assumed to secret gluten-degrading proteases for nitrogen acquisition from infected grain. Incidence of poor gluten quality caused by the factors discussed here is prominent not only under the Norwegian/Nordic weather conditions characterized by frequent rain and high humidity during grain maturation, but also in other areas with similar weather conditions. Moreover, climate change may increase wheat production areas subjected to humid conditions in the future. Hence, further research is required to reveal biotic and abiotic factors affecting glutenin polymer assembly and to understand biological mechanisms of how glutenin polymers are reduced under humid weather conditions.

5. Conclusion

Our study showed that the assembly of glutenin polymers follows grain desiccation. Increase in GMC due to weather conditions during grain desiccation caused interruption or disassembly of the glutenin polymers. The assembly of glutenin polymers continued again during the following desiccation, yet the polymer size did not reach its potential. Fungal DNA increased under humid conditions in grain from both naturally and artificially infected fields. Gluten-degrading proteases were present in grain with high level of fungal DNA, and thus presumed to be of fungal origin. Our results strongly indicate that frequent rain during grain desiccation reduces glutenin polymer size, either as an effect of increased GMC disturbing the polymer assembly, or by the fungal proteases.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcs.2019.102865.

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