

Effects of water stress on the composition and immunoreactive properties of gliadins from two wheat cultivars Nawra and Tonacja

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ABSTRACT

BACKGROUND: Water shortage during wheat vegetation causes changes in the composition of gliadins in grains, which can lead to changes in their immunoreactive properties.

RESULTS: The investigated wheat cultivars exposed to water stress accumulated significantly lower amounts ($P < 0.05$) of gliadins and glutenins in grains. The composition of proteins accumulated in grains was also modified. Water shortage results in a decreased share of $\alpha\beta$ and γ fractions in total gliadins. Grains of wheat cultivated under water stress contain significantly higher ($P < 0.05$) levels of ω -gliadins by 4.5 and 43.3% for Nawra and Tonacja cultivars, respectively. Water stress promotes an increase in the share of P and Q/E residues in gliadins. In protein samples R5 antibodies recognized increased amounts of gliadins matching the QQPFP sequence. Wheat proteins also reacted with IgE antibodies isolated from subjects allergic to gluten.

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CONCLUSION: Cultivation of wheat under conditions of water stress results in the qualitative and quantitative changes of gliadins by increasing their immunoreactivity.

KEYWORDS: water stress, wheat, gliadin, immunoreactivity

INTRODUCTION

Plants grown in the field are exposed to environmental factors affecting their development. These include biotic factors, e.g. plant pathogens and abiotic factors, e.g. water stress, thermal stress and osmotic stress.¹⁻³ Under environmental conditions plants are usually exposed to more than one stressor at the same time, e.g. increased temperature and water shortage. Unfavourable conditions during the cultivation of wheat can deteriorate its technological parameters by changing the quality and content of both starch and storage proteins.⁴

The amount of synthesized protein in grains also depends on the availability of water during wheat vegetative development and the formation of grains. Water deficit during plant development contributes to decreased grain yields, increased total content of protein in grains, and a changed share of high molecular weight subunits (HMW-GS) at the cost of low molecular weight subunits (LMW-GS) of glutenins.^{2,4-7} FLAGELLA *et al.* found an increase in the amount of glutenin macropolymers associated with a general modulation of HMW-GS and LMW-GS when a terminal water stress occurred during grain filling in durum wheat.² Scientific reports generally indicated a higher protein expression and a higher gliadin and gliadin-to-glutenin ratio under drought stress during grain filling.^{8,9} In contrast, PANOZZO *et al.* carried out a field experiment and found no effect of water stress on the gliadin-to-glutenin ratio in kernels.¹⁰ The literature reports

significantly higher glutenin contents in grains of *Triticum aestivum* at maturity when multiple water deficit and high temperature were applied during vegetative and generative growth than single abiotic stress treatments.¹¹ YANG *et al.* found the higher gliadin and glutenin contents at maturity, 52 days post anthesis (dpa) during water stress applied at terminal spikelet than the control. However, the higher glutenin contents were observed at 19 dpa during high temperature stress applied both at the vegetative and generative stages, indicating differences in the response of grain protein fraction to drought and heat stress. Some storage proteins, including α -gliadin, γ -gliadin and LMW-GS, decreased and one LMW-GS increased in abundance in response to water deficits.¹¹ Experiments by KONOPKA *et al.* revealed that grains developing under water stress contain lower amounts of albumins, globulins, γ -gliadins and glutenins in comparison to kernels from wheat not exposed to stress.¹² On the other hand, BEGCY and WALIA observed that typical and novel γ -gliadin were repressed with reduction in gene expression by 40% and 54% for gliadins and glutenins during water stress.¹³ POMPA *et al.* reported that during drought stress in the warm years HMW-GS, α -gliadin and globulins 3 protein were up-regulated with down-regulation of LWM-GS and γ -gliadins in wheat.¹⁴ Two-dimensional gel electrophoresis of durum wheat storage proteins cultivars Ciccio and Svevo also demonstrated an increase in protein accumulation at physiological stage in gliadin region under water deficit.¹⁵

Changes in the composition and quality of wheat storage proteins caused by the response of plants to water shortage may affect the immunoreactive properties of proteins. Processed wheat grain consumed in the form of pasta, bread and other products is the main component of the everyday diet and one of the most common causes of allergy and food intolerance.¹⁶ Environmental stress during grain filling affected the expression of α -gliadins increased their contents during water stress.

GIULIANI *et al.* identified α -gliadin involved in wheat allergy overexpressed at the early-mid stage in durum wheat cultivar Ciccio and at milk, waxy and physiological stage in durum wheat cultivar Svevo. They also identified α -gliadin contained the celiac disease-related T-cell stimulatory epitopes glia- α 9 and glia- α 20.⁸

Major allergens isolated from wheat storage proteins include $\alpha\beta$ -gliadins (Tri a 21.0101), γ -gliadins (Tri a 20), and ω_5 -gliadins (Tri a 19), as well as low- and high-molecular weight subunits of glutenins (Tri a 26; Tri a 36).¹⁷⁻¹⁹ The smallest allergenic peptide able to react with IgE antibodies has the QQQPP amino acid sequence.²⁰ The ω_5 -gliadin fraction (Tri a 19) can also cross-react with proteins from other cereals, e.g. γ_{70} - and γ_{35} -secalin from rye (Sec c 20) and γ_3 -hordein from barley (Hor v 21).²¹ Wheat storage proteins having a toxic effect on subjects with coeliac disease include α -, β -, γ -, ω -gliadins and LMW-glutenins.²²

On the other hand, peptides with a toxic and/or stimulating effect on T lymphocytes are important in the aetiology of coeliac disease. Polypeptide fragments are considered toxic if they can induce *in vitro* and *in vivo* damage to the intestinal mucosa and epithelial cells. In contrast, immunogenic peptides are fragments of polypeptides able to stimulate T cells isolated from the intestinal epithelium or peripheral blood of patients with diagnosed coeliac disease.²³ Sequences of both types of peptides contain a large amount of proline and glutamine and have been isolated from different subunits of α - and γ -gliadins and glutenins.²³⁻²⁶ Toxic effects on subjects with coeliac disease have been demonstrated for such peptides as LGQQQPFPPQQPYQPQPQF, PQPELPYPQPQLPY, and QLQPFQPQLPYQPQPS isolated from α -gliadins.^{25, 26} On the other hand, peptides isolated from gliadins, e.g. α (57-89), α (58-69), α (62-75), γ (60-79), and γ (66-78) and glutenins, e.g. (40-59) and (46-60) are able to stimulate T lymphocytes.^{22,24,26 24,27,28}

There is still a lack of scientific data on influence of drought stress on immunoreactive and allergenic properties of wheat storage proteins. However, Giuliani *et al.* demonstrated changes in the expression of α -gliadins under water deficit which were associated with allergy to wheat or identified as T-cell stimulatory epitopes in celiac disease without immunological analysis of these proteins.⁸ It is therefore important to determine the effect of stress factors present during wheat development and affecting the immunoreactivity of wheat storage proteins. The aim of the study was to determine the effects of water stress during the reproductive phase of two wheat cultivars on the content, quality and immunoreactive properties of gliadins.

EXPERIMENTAL

PLANT MATERIAL

An analysis was carried out on grain samples of two wheat cultivars: Nawra (glutenin allele N-7+8-5+10) and Tonacja (glutenin allele 2*-7+9-2+12) obtained from the Experimental Station of the University of Warmia and Mazury (UWM) in Bałcyny. The selected genotypes have excellent baking quality characteristics and high gluten contents. All chemical reagents were supplied by Sigma-Aldrich (St. Louis, MO USA).

WHEAT CULTIVATION

A two-factor pot experiment was conducted in six replications in the greenhouse of the UWM in Olsztyn. Seeds were treated with a fungicide, Funaben Plus 02WS (Chemical Plant "Organika-Azot" S.A., Jaworzno, Poland) and sown on March 25 with a density of 200 seeds m⁻². Seeds were grown in brown soil and irrigated with distilled water to optimal (i.e. 60-70%) capillary water capacity (cwc). After germination, seed density was standardized at 8 seeds per pot. Temperature were modified during the plant life

cycle from 6/4 °C at sowing to 22/18 °C at physiological maturity. The photon source was a LED Light Source lamp with the main spectrum peak at 670 nm and a second peak at 465 nm lighting 10 and 15 h at sowing and at physiological maturity, respectively. The amount of water was adjusted to ensure two levels of soil moisture: optimal, i.e. 60-70% cwc, and 30-35% cwc, corresponding to drought in the period from wheat flowering to grain harvest. Mineral fertilizers were applied twice, before seed sowing and during plant development (4-5 leaves developed) by adding ammonium nitrate and diammonium phosphate at the rate of 6 and 9 g m⁻², respectively.

FRACTIONATION OF WHEAT STORAGE PROTEINS

Grain samples (250 mg) were milled, suspended in buffer (1 mL, 50 mmol L⁻¹ Tris-HCl pH 7.8; 100 mmol L⁻¹ KCl, 5 mmol L⁻¹ EDTA), mixed (5 min, 21°C), and centrifuged (8 000 g, 15 min, 21°C) to remove albumins and globulins according Bean and Lookhart.²⁹ Proteins soluble in buffered salt solutions were extracted twice. Next, grain samples were washed twice with deionised water (1 mL), mixed (5 min, 21°C) and centrifuged to remove precipitate (2 000 g, 15 min, 21°C). Gliadins were extracted (10 min, 21°C) from samples with ethyl alcohol solution (1 mL, 600 mL L⁻¹) and by centrifuging (10 000 g, 15 min, 21°C). 2-propanol (1 mL, 500 mL L⁻¹) with β-mercaptoethanol (0.1 mL L⁻¹) was added to the precipitate and the extraction of glutenins was performed (60 min, 60°C). The mixture was separated by centrifuging (5 000 g, 15 min, 21°C). Protein samples were used for further analyses.

DETERMINATION OF PROTEIN CONTENT

Protein content in fractionated samples was determined by the Bradford technique (Sigma-Aldrich) following the manufacturer's instructions and using

albumins/globulins, gliadins and glutenins as reference standards (concentrations from 31.25 $\mu\text{g mL}^{-1}$ to 1 mg mL^{-1}).

2D-NUPAGE TWO-DIMENSIONAL ELECTROPHORESIS

SAMPLE PREPARATION

Samples of milled grains (100 mg) were suspended in a mixture of acetone (1 mL), trichloroacetic acid (100 g L^{-1}) and β -mercaptoethanol (0.7 mL L^{-1}), mixed thoroughly and incubated (-30°C , 2 h). Precipitated proteins were separated from the mixture by centrifuging (10 000 g , 20 min, 5°C), suspended in a mixture of acetone (1 mL) and β -mercaptoethanol (0.7 mL L^{-1}) and mixed. The mixture was centrifuged again (10 000 g , 20 min, 5°C) to obtain protein precipitate. The residual acetone mixture was evaporated from the analysed samples in a vacuum evaporator (30°C , 15 min, Rotavapor R-215, Büchi Labortechnik AG, Flawil, Switzerland).

Proteins were suspended in a buffer (950 μL) containing ZOOM 2D Protein Solubilizer 1 (914 μL , 2DPS1, Life Technologies, USA), Tris (3 μL , 1 mol L^{-1}), a mixture of protease inhibitors (5 μL , Sigma, P9599), dithiothreitol (10 μL , 2 mol L^{-1} , DTT), and deionised water (18 μL), and mixed for 15 min according to the manufacturer's instructions. Next, N,N -dimethylacrylamide (5 μL , DMA) was added to alkylate proteins and the sample was mixed for 30 min. The process was terminated by adding DTT (10 μL , 2 mol L^{-1}). Dissolved protein fractions were separated from the precipitate by centrifuging (20 000 g , 20 min, 5°C), and frozen (-30°C).

ISOELECTRIC FOCUSING OF PROTEINS

Protein isoelectric focusing was performed according Akagawa *et al.* with slide modifications.³⁰ Protein samples (10 μL) were suspended in a buffer containing 2DPS1 solubilizer (128 μL , Life Technologies), DTT (0.7 μL , 2 mol L^{-1}), a mixture of

ampholytes (1 μL , ZOOM Carrier Ampholytes, Life Technologies), Bromophenol Blue (0.3 μL , 10 g L^{-1}) and deionised water (14.6 μL). The whole sample was mixed and applied onto a gel strip for isoelectric focusing (linear gradient pH 3-10L, Life Technologies) for rehydration (12 h, 21°C). Gel strips were placed inside a ZOOM IPGRunner apparatus (Life Technologies), and isoelectric focusing was performed at room temperature at the following separation parameters: voltage 175 V for 15 min, 175 V up to 2 kV for 45 min, and 2 kV for 30 min.

MOLECULAR WEIGHT SEPARATION OF PROTEINS IN NUPAGE GEL

After the completed isoelectric focusing of proteins gel strips were buffered (15 min, 21°C) in a sample buffer (10 mL, LDS Sample Buffer with Sample Reducing Agent, 9:1, v/v, Life Technologies) and an alkylating mixture (10 mL, LDS Sample Buffer, Life Technologies, 125 mmol L^{-1} iodoacetamide, 15 min, 21°C). Next, the strips were applied onto polyacrylamide gel (NuPage Novex 4-12% Bis-Tris ZOOM Gels, IPG Well 1.0 mm, Life Technologies), and poured over with agarose solution (500 μL , 5 g L^{-1}). Proteins were separated in an electrophoretic apparatus (XCell6 MultiGel Unit, Life Technologies) using a protein separation buffer (NuPage SDS MES, Life Technologies) at room temperature, voltage 200 V for 40 min. Proteins of molecular weights from 2.5 kDa to 200 kDa (Mark12, Life Technologies) were used as mass standards.³⁰

Gels were stained with SimplyBlue SafeStain (Life Technologies) according to the manufacturer's instructions. Electrophoregrams were automatically analysed with Molecular Imaging Software (Kodak, version 4.0) and Phoretix 2D (NonLinear Dynamics Ltd., version 2004-1440-1). The 2 DE gels were subdivided into four regions on the basis of protein molecular weight: the < 35 kDa low molecular weight region (L < 35), the 35-48 kDa low molecular weight region (L 35-48), the 48-60 kDa

intermediate molecular weight region (I) and the > 60 kDa high molecular weight region (H) according Giuliani *et al.*¹⁵ The expression levels of protein spots were determined by protein abundance (vol%) and analysed using the analysis of variance procedure.

FREE ZONE CAPILLARY ELECTROPHORESIS (FZCE) OF GLIADINS

Fractions of gliadins were analysed using a system for capillary electrophoresis (BioFocus 3000, BioRad, Hercules, CA, USA) with a fitted UV-Vis detector according methods of Bean and Lookhart.³¹ A silica capillary, effective length 24 cm and 50 μm in diameter, was used for the electrophoretic separation of proteins. All chemical reagents and buffers were degassed by centrifuging (10 000 *g*, 2 min, 21°C) before their use for electrophoretic separation. Gliadins were separated in isoelectric buffer (50 mmol L⁻¹ iminodiacetic acid, 200 mL L⁻¹ acetonitrile, 500 mg L⁻¹ hydroxy-propyl-methyl-cellulose) at voltage 20 kV at 45°C for 10 min. Protein samples were electrophoretically injected into the capillary (10 kV, 8 s). Proteins were analysed at wavelength 200 nm using the UV-Vis detector. The qualitative and quantitative compositions of proteins were analysed based on peak migration times using BioFocus Integrator software (BioRad).

SODIUM DODECYL SULFATE CAPILLARY ELECTROPHORESIS (SDS-CE) OF GLIADINS

The molecular weight of gliadins was analyzed by means of a capillary electrophoresis system (BioFocus 3000, BioRad) equipped in a UV-Vis detector and reagents kit CE-SDS Protein Kit (BioRad, 148-4160) according Bean and Lookhart.³² In the experiment, use was made of a standard mixture of proteins (CE-SDS Protein Size Standard, BioRad) with molecular weights of: 14.4, 21.5, 31, 45, 66.2, 97, 116 and 200 kDa and

internal standard (1 mg mL^{-1} , benzoic acid, CE-SDS Internal Reference, BioRad). The analyzed solution of gliadins ($100 \text{ }\mu\text{L}$) was mixed with a buffer ($100 \text{ }\mu\text{L}$, Sample Buffer, BioRad), and supplied with an internal standard ($10 \text{ }\mu\text{L}$) and 2-mercaptoethanol ($5 \text{ }\mu\text{L}$, Sigma, M-7154). The sample was heated for 10 min. at a temp. of $95\text{-}100^{\circ}\text{C}$, cooled and stored at a room temperature. The electrophoretic separation was conducted with the use of a silica capillary with a diameter of $50 \text{ }\mu\text{m}$ and effective length of 24 cm. Before each separation, the capillary was stabilized by the application of the following six-stage rinsing: 0.1 mol L^{-1} NaOH, 300 s.; 0.1 mol L^{-1} HCl, 120 s.; H_2O , 120 s.; separation buffer (CE-SDS Protein Run Buffer, BioRad) 600 s.; H_2O , 0 s.; and H_2O , 0 s.. A sample of gliadins was pressure-injected onto the capillary (40 psi-s) and separated from $-$ to $+$, under the applied voltage of 15.0 kV, at the limit of current strength reaching $50 \text{ }\mu\text{A}$, at a temp. of 20°C , for 12 min. The qualitative composition of gliadins was analyzed at a wavelength of 220 nm using a UV-Vis detector, based on the time of peaks migration, by means of BioFocus Integrator software (BioRad).

PARTIAL AMINO ACID SEQUENCE ANALYSIS OF GLIADINS

Samples of gliadins were isolated according to the previously described procedure. Ethanol was removed from the water-alcohol mixture by evaporation under vacuum (Rotavapor R-215, Büchi Labortechnik AG). Samples of gliadins were frozen (48h, -30°C) and lyophilized (Alpha 1-2LD, Christ, Osterode am Harz, Germany). Lyophilized gliadins (100mg) were suspended in hydrochloric acid solution (2 mL , 6 mol L^{-1} , HCl), sealed in glass vials and hydrolysed (24h, 105°C). Samples of hydrolysed gliadins were cooled, filtered ($0.22 \text{ }\mu\text{m}$, syringe filter Millex GV, Merck Millipore, Darmstadt, Germany) and used for electrophoretic analysis according Warren and Adams.³³

The amino acid composition was analysed using a system for capillary electrophoresis (BioFocus 3000, BioRad) and an indirect UV technique. A silica capillary, effective length 70.4 cm and 50 μm in diameter, was used for the electrophoretic separation of amino acids. All chemical reagents and buffers were degassed by centrifuging (10 000 g, 2 min, 21°C) before their use for electrophoretic separation. Amino acids were separated in a solution of electrolytes (0.5 mmol L⁻¹, myristyltrimethyl-ammonium bromide, MTAB, 10 mmol L⁻¹ benzoate, pH 11.2) at 20 kV and 25°C for 25 min. Samples of hydrolysed proteins were injected under pressure (5 psi·s) into the capillary and analysed at wavelength 225 nm using the UV-Vis detector. The qualitative and quantitative compositions of hydrolysed gliadins were analysed based on peak migration times using BioFocus Integrator software (BioRad).

COMPETITIVE AND SANDWICH R5 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The gliadin contents of wheat samples were determined with commercial kit (Ridascreen Gliadin Competitive R7021, Ridascreen Gliadin R7001, R-Biopharm, Darmstadt, Germany). The test uses the monoclonal antibody R5 which is specific for celiacotoxic amino acid sequence QQPFP that is present in gliadins. The gliadin samples obtained from fractionation procedure were analysed according to manufacturer's instructions.

IMMUNOBLOTTING

The electrophoretic separation of gliadins was carried out in a polyacrylamide gel (12 g L⁻¹, NuPage Novex Bis-Tris Gel, Life Technologies), using run buffer (NuPage MOPS SDS Buffer, Invitrogen) and the XCell SureLock Mini-Cell (Life Technologies) electrophoresis system according to the manufacturer's instructions. The weight

standard (Sigma, M4038) were proteins with molecular weights ranging from 6,5 kDa to 205 kDa. Samples of gliadins (20 μ L) were mixed with buffer (5 μ L NuPage LDS Sample Buffer, Life Technologies), reducing agent (2 μ L NuPage Reducing Agent, Life Technologies), water (13 μ L), thermally-denatured at 70°C for 10 min, and after cooling down were transferred into wells (10 μ L). The separation was performed at the voltage of 200 V, with initial current intensity of 100-115 mA, for 50 min.

The electrophoretically-separated proteins were transferred from the polyacrylamide gel onto a nitrocellulose membrane (NC 2, Serva Electrophoresis GmbH, Heidelberg, Germany) in an MINITRANS apparatus (Kucharczyk TE, Poland) according Towbin *et al.*³⁴ The electrotransfer was run in a Tris-glycine buffer with methanol (0.192 mol L⁻¹ glycine, 0.025 mol L⁻¹ Tris 200 mL L⁻¹ methanol, pH 8.3), applying a current with a strength of 25 mA, for 2 h. Nitrocellulose membrane, containing electrophoretically-separated gliadins was fixed in a TBS-T buffer (0.05 mol L⁻¹ Tris-HCl, 0.5 mol L⁻¹ NaCl, 2 mL L⁻¹ Tween 20, pH 7.0) in order to block free sites binding protein. Next, the membrane was incubated (24 h, 4°C) in serum obtained from patients with symptoms of allergy on gluten. The serum was kindly provided by Department of Immunology and Food Microbiology, Polish Academy of Sciences in Olsztyn, Poland. After triple rinsing in a TBS-T buffer, antigen-antibody complexes were visualized onto the membrane by fixing it in a solution of goat antibodies (IgG) specific to human immunoglobulin E, conjugated with horseradish peroxidase (Sigma, A-9667) at a conjugate dilution of 1:2000. The incubation was run for 2 h at a room temperature. After the reaction of an enzyme with a substrate: 4-chloro-1-naphthol (Sigma, C-6788) in the presence of H₂O₂, bands with a dark navy blue colour were observed to appear. The reaction was stopped by immersing the membranes in distilled water.

STATISTICAL ANALYSES

Statistica (ver 10. StatSoft, Tulusa, OK, USA) software was used to perform one-way analysis of variance (ANOVA) and a Bonferroni's test at 95% confidence level ($P < 0.05$) to identify differences among samples. All data presented are mean values $\pm$ standard deviation of triplicates, obtained from two separate experiments.

RESULTS

CHARACTERISTICS OF WHEAT GRAIN PROTEINS

The aim of the presented experiments was to investigate the effect of water stress on the properties of wheat grain proteins. The 1000-kernel weight for wheat plants grown under conditions of water shortage reduced significantly ($P < 0.05$), from 39.8 ± 1.8 to 32.7 ± 1.9 g for Nawra cultivar and from 36.9 ± 1.8 to 31.9 ± 2.0 g for Tonacja cultivar (Table 1). Soil moisture reduced from 60-70 to 30-35% of capillary water capacity during wheat development resulted in decreased content of protein in grains. A single grain of Nawra wheat grown under water stress contained on average 3.62 mg of protein vs 4.87 mg for the control sample (Table 1). A similar relationship was found for grains of Tonacja wheat, which contained 2.87 and 3.99 mg of protein for drought-stressed grains and for the control ones, respectively.

The synthesis of gliadins and glutenins in Nawra grains was significantly reduced ($P < 0.05$) by 11.9 and 20.8%, respectively (Table 1). The content of gliadins and glutenins in grains of Tonacja also decreased by 29.1 and 13.5%, respectively. Grains of Tonacja wheat grown under water stress also had reduced content of albumin/globulin fractions. For Nawra wheat exposed to water stress no significant differences ($P < 0.05$) in the content of albumins/globulins in grains were found as compared to the control sample (15.5 ± 0.8 mg g⁻¹ vs 16.9 ± 0.4 mg g⁻¹).

ELECTROPHORETIC CHARACTERISTICS OF WHEAT STORAGE PROTEINS

Two-dimensional electrophoresis of protein samples isolated from Nawra wheat grains exposed to water stress demonstrated the presence of 145 proteins of molecular weights from 9.01 to 198.16 kDa and isoelectric points from pI 4.20 to pI 9.72 (Figure 1A). The control sample of wheat proteins contained 131 spots corresponding to 9.01 to 193.53 kDa and isoelectric points from pI 4.20 to pI 9.64 (Figure 1B). The proteome of Tonacja wheat grains contained 183 polypeptides for control samples and 180 polypeptides for drought-stressed grains (Figure 1C,D). The control sample contained proteins with molecular weights from 32.97 to 195.99 kDa and isoelectric points from pI 3.42 to pI 9.66, while proteins from drought-stressed grains had molecular weights from 12.43 to 190.44 kDa, and isoelectric points from pI 3.42 to pI 9.68.

The comparison of electrophoretic images resolving proteins from Nawra wheat grown under water stress and controls demonstrated the presence of 109 polypeptides (75%) of the same molecular weights and values of isoelectric points. Other spots (25%) were polypeptides whose synthesis was inhibited by water stress. However, water stress also induced the synthesis of 38 new protein spots. Samples from control and drought-stressed plants (Tonacja) shared 159 (88%) peptides of the same molecular weights and isoelectric points. However, the synthesis inhibition of 24 polypeptides and induced the synthesis of 21 new proteins was observed in stressed grains.

The 2DE gels showed that the protein abundance is statistically ($P < 0.05$) higher in stressed Nawra wheat grains than in control ones at both L < 35 and L 35-48 gel regions without changes in number of spots (Table 2). In turn, the I gel region from drought-stressed grains was characterised by statistically ($P < 0.05$) lower 25.4% percentage volume than the I region from control sample. However, the number of protein spots at I

region was increased during water stress. The number of spots was increased from 115 for control sample to 132 for stressed Nawra wheat grains without changes in percentage volume of proteins at the H region.

Experiments with Tonacja wheat demonstrated no statistically ($P < 0.05$) differences in the L <35 region, however during water stress the protein expression was 39.5% down regulated at the L 35-48 gel region. Both, the I and the H gel region was characterised by increased protein spots during stress conditions. The protein expression at the I gel region showed statistically ($P < 0.05$) 28.5% lower percentage volume of proteins isolated from stressed grains and statistically ($P < 0.05$) 3.7% higher at the H region.

Comparative analysis of gel zones corresponding to proteins inducing allergy to gluten and coeliac disease showed that in Nawra wheat water stress induced the synthesis of one polypeptide (pI 8.55, MW 47.74 kDa), but also inhibited the synthesis of three other polypeptides (pI 8.35, MW 43.93 kDa; pI 8.42, MW 53.56 kDa; pI 8.76, MW 49.35 kDa) belonging to the L 35-48 and the I gel region. In experiments with Tonacja wheat water stress induced the synthesis of one polypeptide (pI 7.89, MW 55.27 kDa) at the I gel region.

The gliadin fraction was separated using capillary electrophoresis for the accurate analysis of allergenic and triggering off coeliac disease peptides. The development of Tonacja wheat under conditions of water limitation resulted in the synthesis and accumulation of 64.2, 70.1 and 85.1 kDa polypeptides (Figure 2) not found in control grains. Water shortage also inhibited the synthesis of 45.3, 55.1 and 91.0 kDa polypeptides. No changes in the molecular weights of extracted gliadin fractions were observed in grains of Nawra wheat.

A statistically significant reduction ($P < 0.05$) in the accumulation of $\alpha\beta$ - and γ -gliadins was observed in grains from both wheat cultivars grown under conditions of water

stress (Figure 3). In grains from Nawra plants grown under water stress the content of $\alpha\beta$ - and γ -gliadins was reduced by 7.9 and 8.3%, respectively (Table 3). In grains from Tonacja plants grown under water stress the content of $\alpha\beta$ - and γ -gliadins was reduced by 23.3 and 30.2%, respectively. Interestingly, grains from both wheat cultivars grown under water stress contained significantly more ω -gliadins ($P < 0.05$) (Table 3). The content of ω -gliadins increased in Nawra grains by 4.5% and in Tonacja grains by 43.3%.

Partial analysis of gliadin composition demonstrated that reduction in soil moisture from 60-70 to 30-35% of capillary water capacity during wheat vegetation facilitated the increase in P content and total Q and E (Table 3). Gliadins isolated from drought-stressed Nawra grains contained significantly more ($P < 0.05$) proline (16.2 ± 0.6 mol %) than gliadins from the control sample (14.6 ± 0.7 mol %). The content of proline (17.4 ± 0.6 mol %) in gliadins isolated from Tonacja plants grown under water shortage was also significantly higher ($P < 0.05$) than in gliadins from the control sample (14.9 ± 0.7 mol %). Under conditions of water stress both wheat cultivars synthesized gliadins containing significantly higher levels ($P < 0.05$) of glutamine and glutamic acid as compared to plants grown under optimal water supply to soil.

IMMUNOCHEMICAL ANALYSES

Sandwich R5 ELISA demonstrated that the content of gliadins in grain samples from Nawra and Tonacja plants grown under stress was 52208 ± 98 and 49091 ± 62 mg kg⁻¹, respectively (Table 4). The content of gliadins in control samples was significantly lower ($P < 0.05$), and accounted for 50263 ± 56 and 47132 ± 58 mg kg⁻¹ for Nawra and Tonacja cultivars, respectively. Competitive ELISA detecting QQPFP sequences in

gliadins also demonstrated a significantly higher ($P < 0.05$) content of gliadins in grain from wheat plants grown under water stress as compared to control samples.

Experiments demonstrated that grains from wheat plants exposed to water stress accumulate gliadins reacting with antibodies isolated from subjects with an allergy to gluten. IgE antibodies bound with proteins from the zone corresponding to molecular weights from 36 to 55 kDa characteristic of ω -gliadins and from 24 to 36 kDa corresponding to α -, β - and γ -gliadins (Figure 4). Western-blotting demonstrated more intense staining of protein samples in the zone of 24-29 kDa, which indicates a higher content of proteins detected by these antibodies. The analysis also demonstrated the presence of 20-24 kDa proteins in the sample of gliadins isolated from grains of Tonacja plants exposed to water stress and not found in the control sample.

DISCUSSION

Our experiments demonstrated that under water shortage the 1000-kernel weight was about 13.5% lower than that of control grains. The total protein content in grains was also reduced. These findings are consistent with experiments carried out by SINGH *et al.*, who demonstrated that in C-306 wheat water shortage between 8 and 15 days postanthesis inhibited the synthesis of some gliadin and glutenin polypeptides and reduced protein content.⁴ Moreover, FÁBIÁN *et al.* found an increased number and size of protein bodies in the endosperm of drought-tolerant Plainsman V wheat exposed to water stress 5 days after pollination.⁵ However, in the drought-susceptible Cappelle Desprez variety no significant differences in the number of protein bodies were found between control and drought-stressed grains. These findings are also supported by pot experiments demonstrated that flour protein content was significantly increased by drought in wheat cv. Duzi.³⁵ Changes in flour protein composition has been linked to

increase in protein content. Monomeric proteins were found to increase more than polymeric proteins in reaction to stress, causing an increase in flour protein content.³⁶ The SDS soluble and insoluble monomeric proteins in wheat cv. Duzi and cv. Kariega were increased under drought stress.³⁵ Proteomic analysis of wheat cv. Gaocheng 8901 in response to drought stress showed albumin and gliadin contents increased, whereas globulin and glutenin did not change appreciably.³⁷ Our results demonstrated that gliadins and glutenins significantly decrease under drought stress in both Nawra and Tonacja wheat cultivars. However, albumin and globulin content significantly decreased in wheat Tonacja during stress conditions without any changes observed in Nawra wheat. These results are similar to findings by Konopka et al..¹² This suggests that the amount and quality of storage proteins in wheat exposed to water stress mainly depends on wheat variety and the timing of drought during plant development.

In wheat, abiotic stress caused by water deficit stimulates the synthesis of new polypeptides or modifies the content of previously synthesized polypeptides that protect proteins and cell membranes from rupture and damage. These include proteins involved in the synthesis of amino acids and saccharides, metabolism, modification of nucleic acids and proteins, e.g. 1-Cys peroxyredoxin, ATP-synthase, β -glucosidase, thiol reductase and α -amylase/trypsin inhibitors being a part of defence system in plants.^{11,38}

Water limitation also leads to changes in the system regulating the synthesis of polypeptides at the stage of post-translational modification by affecting the phosphorylation and glycation of proteins. Both processes can cause changes in the molecular weight and charge of polypeptides.³⁸

Two-dimensional electrophoresis of protein samples isolated from Nawra and Tonacja wheat grains demonstrated the presence of a large amount of polypeptides of molecular weights from 31 to 55 kDa. This zone, corresponding to wheat storage proteins involved

in the pathogenesis of allergy and coeliac disease, covers polypeptides from α -, β -, γ -gliadins and low-molecular glutenins – LMW-GS (28-39 kDa), as well as $\omega_{1,2}$ - (39-44 kDa) and ω_5 -gliadin fractions (49-55 kDa).^{39,40}

Electropherograms indicated a modified composition of proteins in grains of Nawra and Tonacja wheat grown under conditions of water limitation, and modification also concerns allergenic polypeptides and those trigger off coeliac disease. Water deficit during the vegetation of the investigated wheat cultivars results in changes in the proportions of individual prolamin fractions. Water stress decreases the content of $\alpha\beta$ and γ fractions and increases the ω fraction in the total content of gliadins.

ω_5 -gliadin is the wheat storage protein with the strongest allergenic potential. IgE antibody of allergic subjects prone to exercise-induced anaphylaxis detect amino acid sequences: QQIPQQQ, QQFPQQQ, QQSPEQQ, QQSPQQQ and QQQP, which are characteristic of the primary structure of repeat domains in ω_5 -gliadins and $\omega_{1,2}$ -gliadins.^{22,41} Atopic patients also react to QPQQPFP peptides released from γ - and ω_2 -gliadins. Studies on the amino acid sequences of allergens isolated from wheat storage proteins have also identified epitopes strongly reactive to IgE antibody in the repeat domains of α/β -, γ -, ω_2 - and ω_5 -gliadins.⁴² The epitopes are characterised by a high content of glutamine, proline and phenylalanine, and are located in α/β -gliadin (PQQPY+PQPQFPF), γ -gliadin (PFPQ₁₋₂(PQQ)₁₋₂), ω_2 -gliadin (PFPQ₁₋₂PQQ), LMW-glutenin (PQQPPFS+QQQPVL) and HMW-glutenin (PGQGQQ+GYYPSTP/LQQ).⁴³

Partial amino acid analysis of wheat storage proteins from Nawra and Tonacja cultivars demonstrated an increased content of proline and glutamine/glutamic acid in gliadins due to water stress. Similar observations were made by DEL MORAL *et al.* in field experiments.⁴⁴ Storage proteins of durum wheat grown under water limitation contained increased amounts of proline, glutamine, phenylalanine and other amino acids. It should

be pointed out that the high content of P and Q is characteristic of peptides important in the aetiology of allergy and coeliac disease.^{22,41} Higher content of P and Q/E residues in grains of wheat grown in soil with capillary water capacity reduced to 30-35% increases the reactivity of gliadins with IgE antibodies isolated from allergic patients, as well as with R5 antibodies.

R5 antibodies recognize the QQPFP sequence in prolamins of wheat, rye and barley.⁴⁵ This sequence is also frequently found in γ -gliadins, in the repeat domain containing PFPQQ(Q)PQQ(PQQ) or (Q)QPQQPFP sequences.^{22,46} Moreover, the central region of ω_5 -gliadin and $\omega_{1,2}$ -gliadin contains motifs with the (Q)QQQFP sequence repeated 65 times, and (QP)QQPFP repeated 42 times.²² The R5 antibodies also recognize the homologous sequences LQLQPFP, QLPYP, PQPF and PQPFP.⁴⁵

CONCLUSIONS

The qualitative and quantitative changes in gliadins resulted from water shortage in soil during wheat vegetation. Gliadins isolated from grains of drought-stressed wheat plants demonstrated increasing immunoreactivity with R5 antibodies recognized celiac-toxic peptide fragments and increasing reactivity with IgE antibodies isolated from subjects allergic to gluten. Therefore, it should be considered to use the wheat cultivated under conditions of water stress for other purposes than bakery technology.

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Table 1. The 1000-kernels mass and protein content in wheat grains cv. Tonacja and Nawra cultivated in stress (30-35 % of cwc) and control (60-70 % of cwc) conditions.

Cultivar	Variant	1000 kernels mass (g)	Protein content (mg kernel ⁻¹)	Protein content (mg g ⁻¹ dry mass)		
				albumins/globulins	gliadins	glutenins
Nawra	stress	32.7 ± 1.9 a	3.62	15.5 ± 0.8 a	46.8 ± 0.6 a	48.5 ± 0.4 a
	control	37.8 ± 1.8 b	4.87	16.9 ± 0.4 a	50.9 ± 0.8 b	61.2 ± 0.5 b
Tonacja	stress	31.9 ± 2.0 A	2.87	14.8 ± 0.3 A	36.2 ± 0.8 A	39.0 ± 0.1 A
	control	36.9 ± 1.8 B	3.99	16.7 ± 0.0 B	46.4 ± 0.7 B	45.1 ± 0.2 B

Average values in the column followed by different uppercase or lowercase letters are significantly different (p<0.05, ANOVA, Bonferroni test).

Table 2. Number of spots and percentage volume of the gel regions of wheat grains cv. Tonacja and Nawra cultivated in stress (30-35 % of cwc) and control (60-70 % of cwc) conditions.

Gel region	Nawra				Tonacja					
	control		stress		control		stress			
	Number of spots	Region vol (%)	Number of spots	Region vol (%)	Number of spots	Region vol (%)	Number of spots	Region vol (%)	Number of spots	Region vol (%)
L <35	3	2.39 ± 0.02 a	3	2.51 ± 0.05 b	2	1.62 ± 0.07 A	3	1.62 ± 0.02 A		
L 35-48	4	0.98 ± 0.01 a	4	1.80 ± 0.01 b	2	0.81 ± 0.01 A	2	0.49 ± 0.01 B		
I 48-60	9	8.36 ± 0.03 a	6	6.24 ± 0.05 b	10	10.19 ± 0.06 A	11	7.29 ± 0.03 B		
H >60	115	87.59 ± 0.58 a	132	88.95 ± 0.87 a	169	86.91 ± 0.91 A	164	90.14 ± 0.46 B		

Average values in the line followed by different uppercase or lowercase letters are significantly different ($p < 0.05$, ANOVA, Bonferroni test).

Table 3. The content, fraction composition and partial amino acid composition of gliadins isolated from wheat grains cv. Tonacja and Nawra cultivated in stress (30-35 % of cwc) and control (60-70 % of cwc) conditions.

Cultivar	Variant	Gliadins content (%)	Gliadins fraction composition (%)			Gliadins partial amino acid composition (mol %)	
			$\alpha\beta$	γ	ω	P	Z
Nawra	stress	92.7 $\pm$ 1.3 a	92.1 $\pm$ 1.1 a	91.7 $\pm$ 1.3 a	104.5 $\pm$ 0.9 a	16.2 $\pm$ 0.6 b	33.5 $\pm$ 1.2 b
	control	100.0 $\pm$ 1.2 b	100.0 $\pm$ 1.1 b	100.0 $\pm$ 0.9 b	100.0 $\pm$ 1.0 b	14.6 $\pm$ 0.7 a	30.1 $\pm$ 1.1 a
Tonacja	stress	78.4 $\pm$ 1.3 A	76.7 $\pm$ 1.1 A	69.8 $\pm$ 1.3 A	143.9 $\pm$ 0.9 A	17.4 $\pm$ 0.6 B	36.8 $\pm$ 1.3 B
	control	100.0 $\pm$ 0.8 B	100.0 $\pm$ 0.9 B	100.0 $\pm$ 1.0 B	100.0 $\pm$ 0.9 B	14.9 $\pm$ 0.7 A	30.9 $\pm$ 1.4 A

Average values in the column followed by different uppercase or lowercase letters are significantly different ($p < 0.05$, ANOVA, Bonferroni test).

Table 4. Gliadin content measured by sandwich R5 and competitive R5 ELISA in wheat grains cv. Tonacja and Nawra cultivated in stress (30-35 % of cwc) and control (60-70 % of cwc) conditions.

Cultivar	Variant	Gliadin content (mg kg ⁻¹)	
		Sandwich R5 ELISA	Competitive R5 ELISA
Nawra	stress	52208 ± 98 b	50743 ± 87 b
	control	50263 ± 56 a	47369 ± 72 a
Tonacja	stress	49091 ± 62 B	47134 ± 63 B
	control	47132 ± 58 A	46822 ± 71 A

Average values in the column followed by different uppercase or lowercase letters are significantly different ($p < 0.05$, ANOVA, Bonferroni test).

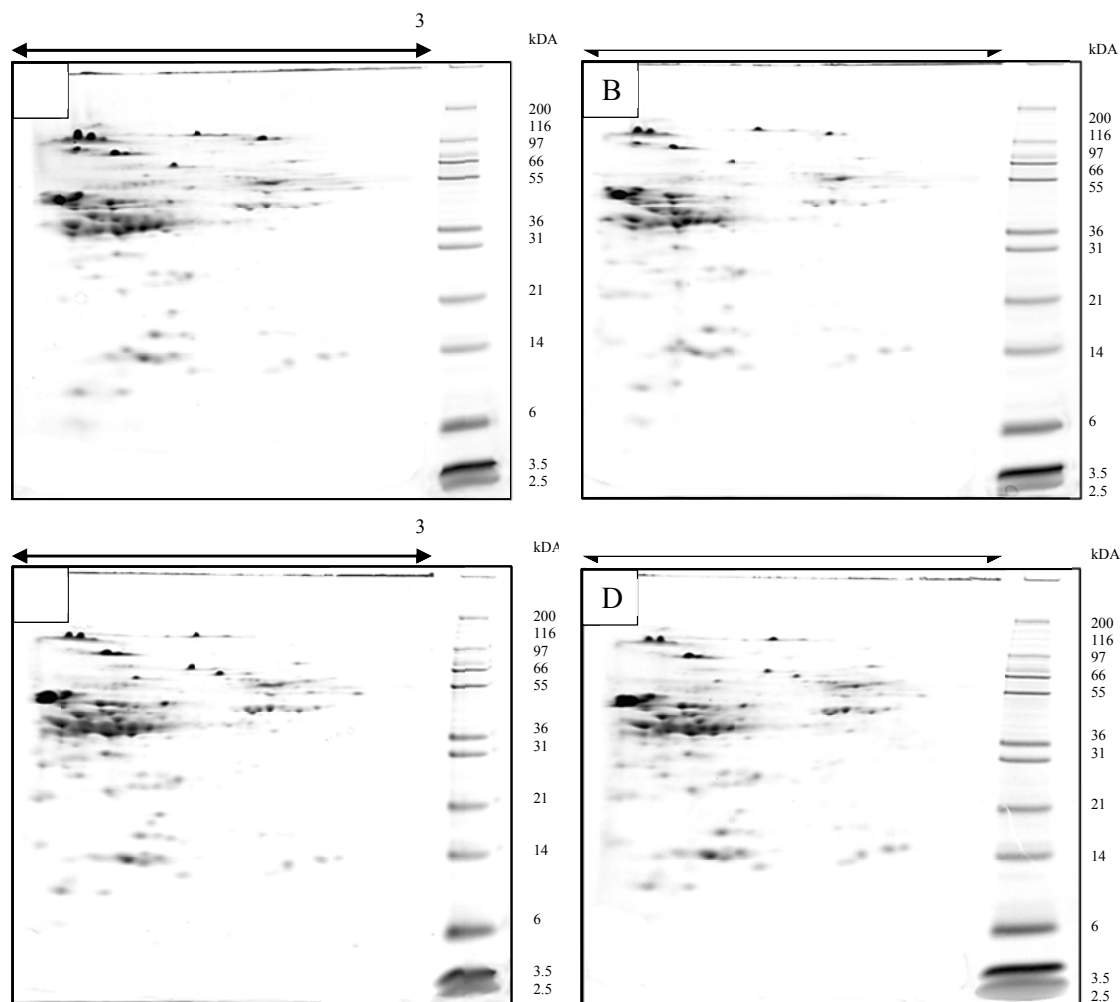


Fig 1. 2D NuPAGE electropherograms of proteins isolated from wheat grains cv. Nawra (A) and Tonacja (C) cultivated in stress conditions (30-35% of cwc) and control wheat grains cv. Nawra (B) and Tonacja (D) (60-70% of cwc).

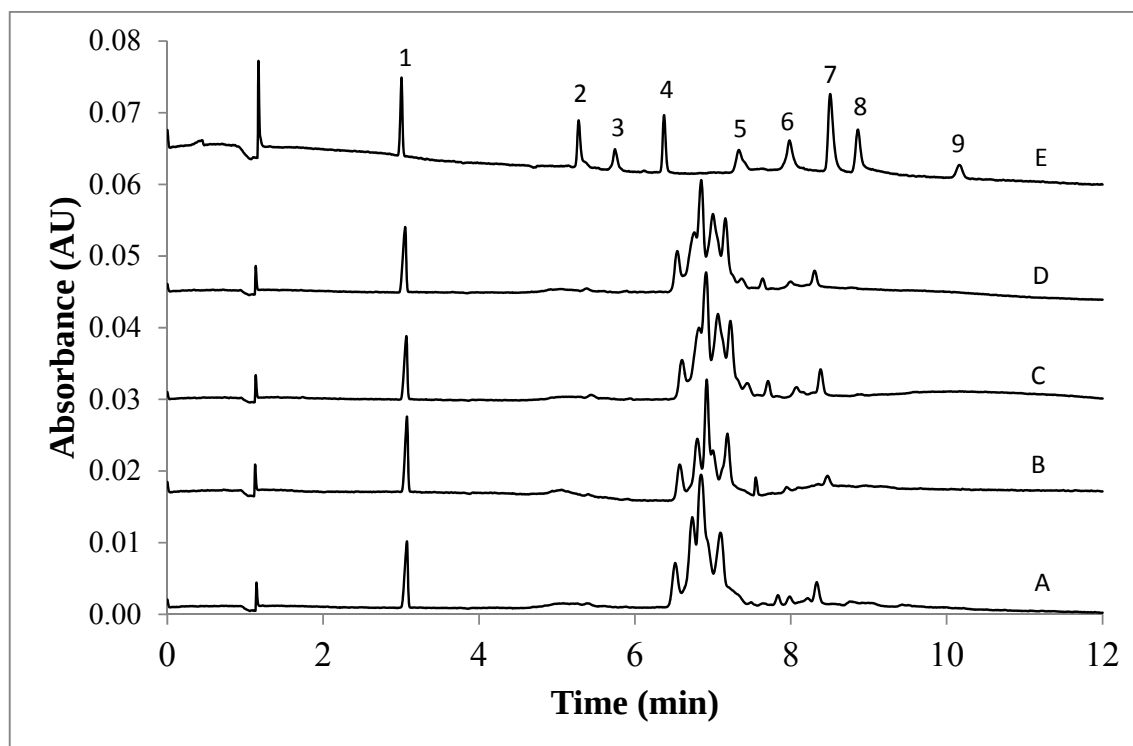


Fig 2. CE-SDS electropherograms of gliadins isolated from wheat grains cv. Tonacja (A) and Nawra (C) cultivated in stress conditions (30-35% of cwc) and control wheat grains cv. Tonacja (B) and Nawra (D) (60-70% of cwc). Molecular mass standard (E) indicating with numbers as follows: 1 – reference standard (benzoic acid), 2 - 14.4 kDa, 3 - 21.5 kDa, 4 – 28.0 kDa, 5 – 45.0 kDa, 6 – 66.2 kDa, 7 – 97.0 kDa, 8 - 116.0 kDa and 9 – 200.0 kDa.

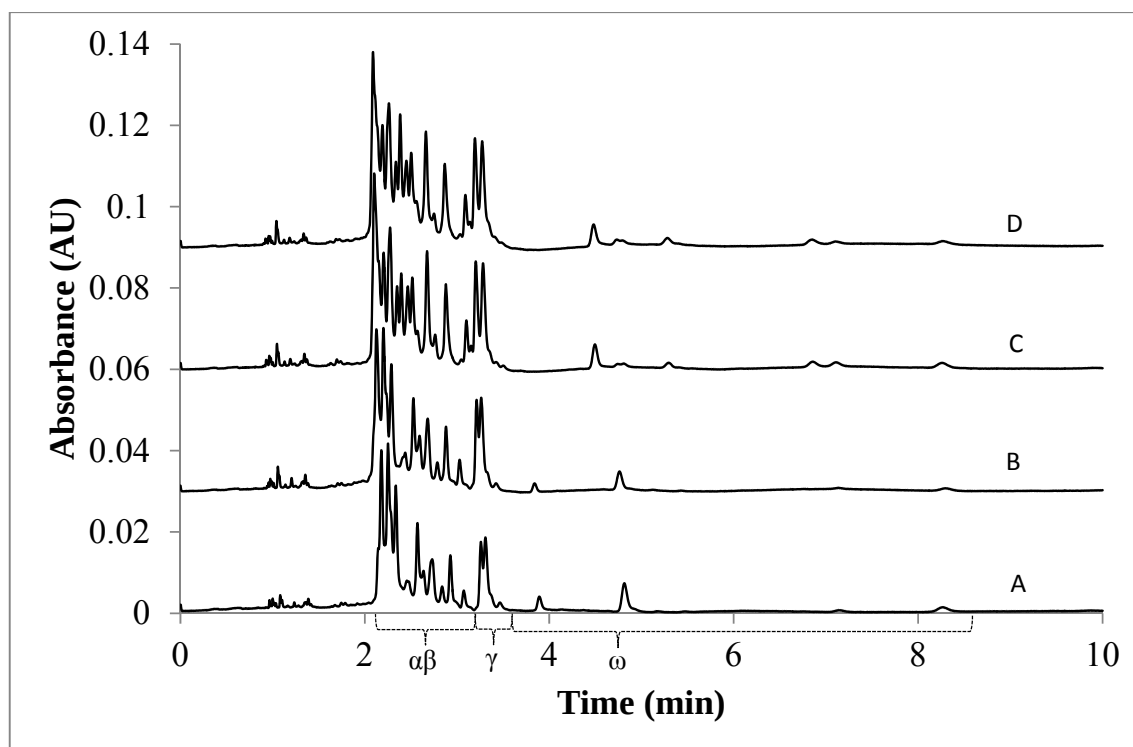


Fig 3. FZCE electropherograms of $\alpha\beta$ -, γ -, ω -gliadins isolated from wheat grains cv. Tonacja (A) and Nawra (C) cultivated in stress conditions (30-35% of cwc) and control wheat grains cv. Tonacja (B) and Nawra (D) (60-70% of cwc).

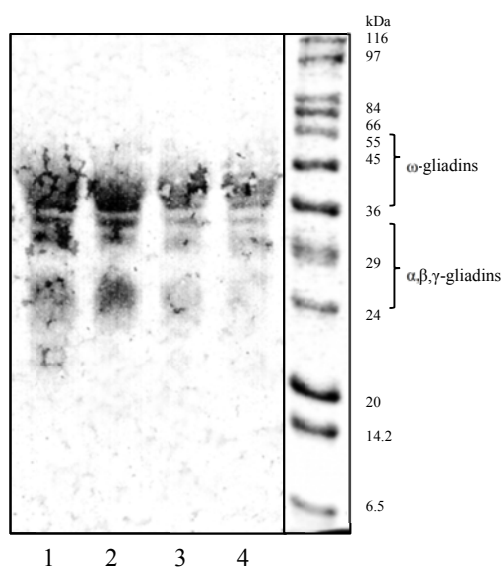


Fig 4. Western blot of gliadin isolated from wheat grains cv. Tonacja (1) and Nawra (3) cultivated in stress conditions (30-35% of cwc) and control wheat grains cv. Tonacja (2) and Nawra (4) (60-70% of cwc).