Acta Physiol Plant (2013) 35:1925–1932
DOI 10.1007/s11738-013-1231-z

ORIGINAL PAPER

Protein changes and proteolytic degradation in red and white clover plants subjected to waterlogging

Veselin Stoychev · Lyudmila Simova-Stoilova · Irina Vaseva · Anelia Kostadinova · Rosa Nenkova · Urs Feller · K. Demirevska

Received: 28 August 2012 / Revised: 28 January 2013 / Accepted: 30 January 2013 / Published online: 14 February 2013

Abstract Red (*Trifolium pratense* L., cv. "Start") and white clover varieties (*Trifolium repens* L., cv. "Debut" and cv. "Haifa") were waterlogged for 14 days and subsequently recovered for the period of 21 days. Physiological and biochemical responses of the clover varieties were distinctive, which suggested different sensitivity toward flooding. The comparative study of morphological and biochemical parameters such as stem length, leaflet area, dry weight, protein content, protein pattern and proteolytic degradation revealed prominent changes under waterlogging conditions. Protease activity in the stressed plants increased significantly, especially in red clover cv. "Start", which exhibited eightfold higher azocaseinolytic activity compared to the control. Changes in the protein profiles were detected by SDS-PAGE electrophoresis. The specific response of some proteins (Rubisco, Rubisco-binding protein, Rubisco activase, ClpA and ClpP protease subunits) toward the applied stress was assessed by immunoblotting. The results characterized the red clover cultivar "Start" as the most sensitive toward waterlogging, expressing reduced levels of Rubisco large and small subunits, high content of ClpP protease subunits and increased activity of protease isoforms.

Communicated by Z.-L. Zhang.

V. Stoychev (✉) · L. Simova-Stoilova · I. Vaseva · A. Kostadinova · R. Nenkova · K. Demirevska
Plant Stress Molecular Biology Department, Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bldg 21, 1113 Sofia, Bulgaria
e-mail: veselinstoychev@abv.bg

U. Feller
Institute of Plant Sciences and Oeschger Centre for Climate Changes Research (OCCR), University of Bern, Altenbergrain 21, 3013 Bern, Switzerland

Keywords ClpA and ClpP protease subunits · Proteolysis · Rubisco · Red clover (*Trifolium pratense* L.) · White clover (*Trifolium repens* L.) · Waterlogging

Abbreviations

Clp	ATP-dependent protease belonging to serine protease
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
FW	Fresh weight
M	Protein marker
MW	Molecular weight
P	Band with proteolytic activity
PHMB	Para-chloro-mercuribenzoate
PMSF	Phenylmethylsulfonyl fluoride
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RA	Rubisco activase
RBP	Rubisco-binding protein
RLS	Rubisco large subunit
RSS	Rubisco small subunit
RT	Room temperature
RuBP	Ribulose-1,5-bisphosphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis

Introduction

Waterlogging may occur as a result of natural seasonal changes in climate, but often it is a consequence of misbalanced redistribution of water resources caused by anthropogenic activities (Directive 2007/60/EC 2007). Globally, flooding of soils leads to significant reduction of soil quality, which negatively influences the quality of agricultural production. Subsequently this reflects on the

yield of animal protein. As a result, huge economic losses in the livestock sector have been observed (Beha et al. 2002). Recently, waterlogging was established as an important problem for the agricultural practice (Irfan et al. 2010).

Fodder crops like clovers protect the soil humus via the root system and are often used as bioremediators sown to recover areas undergone erosion. As nitrogen-fixing legumes, *Trifolium* species are preferred as crops grown on nitrogen-deficient soil. Their natural habitats are often the areas subjected to flooding. Different members of *Trifolium* family have varying tolerance to waterlogging (Aschi-Smiti et al. 2003).

The most widely cultivated clovers are *Trifolium repens* L. (white clover) and *Trifolium pratense* L. (red clover). Both species are important forage legumes grown mostly in temperate humid regions, and this makes their response to waterlogging a relevant research topic.

Root hypoxia provoked by water excess leads to increased anaerobic fermentation which adversely impacts various morphological and physiological events among which are photosynthesis, energetic metabolism, redox status, programmed cell death, RNA processing, as well as protein synthesis or degradation (Jackson 2003). Proteins are an important part of perception and response to abiotic stress (Hashiguchi et al. 2010). There are scarce data concerning the effect of various environmental stresses on clover plants at biochemical and molecular level (Sanderson et al. 2003; Seker et al. 2003).

The degradation of the most abundant plant protein Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase, R, EC 4.1.1.39) under unfavorable environmental conditions serves as a major source of reserve amino acids for plants subjected to stress (Kingston-Smith et al. 2005). Degradation of Rubisco large subunit (RLS) and accumulation of anaerobic polypeptides in waterlogged tomato plants was observed previously (Ahsan et al. 2007a; Kosová et al. 2011). Rubisco activase (RA) protein, which is localized in chloroplasts (Salvucci et al. 1985) could also be influenced by excessive watering.

Chaperones have essential function in protein homeostasis under normal condition and are highly responsive to various stresses (Wang et al. 2004). Chaperons, including Rubisco binding protein (RBP, also known as chaperonin 60) and Rubisco activase (RA), accumulated under stress conditions (Demirevska et al. 2008a, b) and it was assumed that they interact transiently with other proteins, promoting different processes. Kosová et al. (2011) demonstrated that various stress factors induced changes in proteins involved in oxidative phosphorylation (subunits of F1-ATPase) and also in mitochondrial chaperonin 60, which is responsible for the proper protein folding.

Protein quantity and degradation under stress conditions depend on the degree of proteolysis (Van der Hoorn 2008). Plant genomes encode hundreds of proteases, which belong to various unrelated families. Proteases play key role in plants and contribute to the processing, maturation or degradation of specific sets of proteins in response to developmental or environmental factors (Van der Hoorn 2008). The dynamic changes of protease content and activity are subjected to complex control at various levels, including transcriptional and translational regulation, post-translational processing and activation, and synthesis of specific protease inhibitor proteins (Beers et al. 2004; Van der Hoorn 2008). The response of proteases in clover plants subjected to different kinds of stress was studied previously (Kingston-Smith et al. 2005; Sullivan and Hatfield 2006; Vaseva et al. 2011). It was found that the roots of two clover species (*Trifolium fragiferum* and *Trifolium repens*) were characterized with higher porosity under waterlogging and this was associated with lysogenous breakdown of cortical cells, proving morphological changes caused by stress-induced protease activity (Aschi-Smiti et al. 2003). The genetic potential of plants to sustain stress determines their most powerful tool—adequate and rapid adjustment of the protein profile (Bogeat-Triboulot et al. 2007). Under conditions of hypoxia, the fine tuning of protease activity is among the major factors responsible for the survival of waterlogged plants. The effect of waterlogging on clover plants was insufficiently studied.

The aim of the present work was to access protein profile changes in red and white clover subjected to water excess and subsequent recovery to provide some insights into regulatory mechanisms of autoproteolytic processes in leguminous forage plants subjected to excessive water. Proteolytic degradation of Rubisco, Rubisco-binding protein (chaperonin 60) and Rubisco activase under hypoxia caused by waterlogging were evaluated.

Materials and methods

Plant material and growth conditions

One red (*Trifolium pratense* L, cv. "Start") and two white clover varieties (*Trifolium repens* L, medium leafed cv. "Haifa" and small leafed cv. "Debut") were grown in pots containing 500 g soil; pH 6.5; electrical conductivity 470 μS/cm; gravity $\rho \approx 0.45$; organic matter 25 %; dry matter 35 %) at 25/21 °C day/night temperature, 250 μmol m^{-2} s^{-1} photosynthetically active radiation and 16-h photoperiod. Water content in pots was controlled daily to maintain relative soil humidity of 65 % of the maximum soil moisture capacity by gravimetric measurements.

Waterlogging was imposed on 21-day-old plants (with fully developed first trefoil leaf) for a period of 14 days, followed by a 21-day period of recovery. Stressed plants were flooded to 1 cm above the soil level and this state was monitored twice a day. Control plants were watered daily maintaining optimal soil moisture. All analyses were performed with bulk samples derived from second, third and fourth leaves. Leaf material was frozen in liquid nitrogen and preserved at −80 °C.

Growth parameters

Growth parameters (stem length, leaflet area, and dry weight) were measured at the end of the treatment with excessive amounts of water (35-day-old plants) and after the period of recovery (56-day-old plants). Leaflet area was calculated according to the formula $S = \pi ab$, where a and b are the semi-major and semi-minor axes, respectively. Statistically reliable series of 50 plants were used for measurements.

One gram of fresh leaf material (in three replicates) was dried overnight at 105 °C to determine leaf dry weight (DW).

Protein extracts

Leaf material (0.250 g FW) was homogenized in 1 ml ice-cold 100 mM Tris–HCl buffer (pH 7.5) containing 1 mM EDTA, 1 mM DTT, 0.1 % Triton X-100 and centrifuged at 12,000*g* for 20 min at 4 °C. Supernatant was used to determine the total soluble protein content according to Bradford (1976) using bovine serum albumin as protein standard. The cleared samples were mixed with sample buffer containing 10 % w/v SDS, 10 mM beta-mercaptoethanol, 20 % v/v Glycerol, 0.2 M, Tris–HCl—pH 6.8 and 0.05 % w/v bromophenol blue (Laemmli 1970) in ratio 1:1 and were stored at −20 °C for SDS electrophoresis analyses.

The extracts for determination of total proteolytic activity were prepared from 0.250 g fresh leaf material, which was homogenized in 1 ml ice-cold 100 mM Tris–HCl buffer (pH 7.6) containing 10 mM MgCl, 10 mM β-mercaptoethanol, 2 % Polyclar, 2 mM EDTA and centrifuged at 12,000*g* for 20 min at 4 °C.

SDS-PAGE electrophoresis

Leaf samples with equal protein quantity (10 µg) were separated by 12 % SDS-PAGE with a Mini Protean II Dual Slab Cell (Bio-Rad) and were stained with Coomassie brilliant blue R-250 according to Laemmli (1970).

Immunoblotting

After separation by 12 % SDS-PAGE, proteins were transferred on nitrocellulose membrane (Bio-Rad) as described by Mitsuhashi and Feller (1992) using Trans Blot system (Bio-Rad). The immunoblots were developed with primary antibodies (raised in rabbits) against Rubisco (RLS and RSS), RA, RBP, ClpA and ClpP (Demirevska et al. 2008a). Goat-anti-rabbit-IgG and peroxidase-anti-peroxidase soluble complex were used to enhance the sensitivity of the antigen–antibody reaction as described by Mitsuhashi and Feller (1992). Peroxidase reaction was developed with 4-chloro-alpha-naphthol (Sigma).

Total protease activity

The total protease activity of leaf extracts was determined spectrophotometrically using azocasein as substrate according to Fisher and Feller (1993). The reaction was stopped with 50 % TCA, mixed in ratio 1:1 with 1 N NaOH and the absorbance was measured at 450 nm. One arbitrary unit of azocaseinolytic activity was defined as the amount of enzyme capable to increase A_{450} with 0.01. Azocaseinolytic activity is represented as mg protein h^{-1}.

In-gel staining of proteases

Visualization of proteases after electrophoretic separation in gelatine containing 10 % PAGE was performed according to Beyene et al. (2006). Samples with equal final protein concentration of 10 µg were loaded in each lane. After separation, gels were incubated in 2.5 % Triton X-100, then at 37 °C for 16 h in 25 mM Na_2HPO_4/KH_2PO_4 (pH 6.8) with 5 mM L-cysteine and 0.1 % Triton X-100 for 30 min at room temperature. Areas with protease activities were revealed as clear spots against dark blue background after staining with Coomassie Brilliant Blue R-250.

Statistical analysis

Growth parameters (Figs. 1, 2), total protein content (Fig. 3) and azocaseinolytic activity (Fig. 6) were obtained from three independent experiments and submitted to multifactor ANOVA analysis (MSTAT-C). Mean values were separated as a group using Duncan's Multiple Range Test at significant difference $P \leq 0.05$. Standard deviations are indicated by vertical bars. Different letters above columns indicate significant differences between the groups, whereas the same letters show no significant difference. SDS-PAGE (Fig. 4), immunoblots (Fig. 5) and in-gel staining (Fig. 7) were repeated three times and the obtained results were similar. Representative images are shown in the figures.

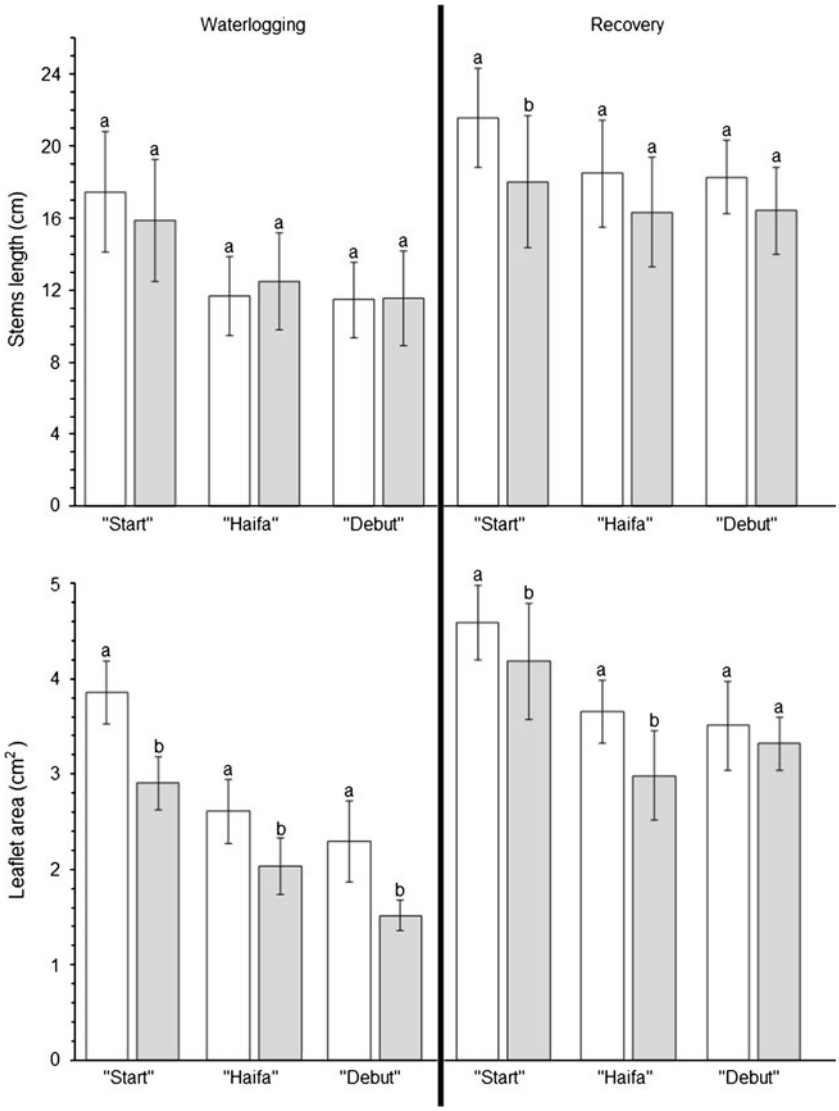

Fig. 1 Stem length and leaflet area of red (*Trifolium pratense* L., cv. "Start") and white (*Trifolium repens* L., cvs. "Haifa" and "Debut") control (*white bars*) and waterlogged (*gray bars*) clover plants. *Error bars* represent standard deviations of three replicates obtained from three independent experiments. *Different letters* above columns indicate significant differences between the groups at $P < 0.05$

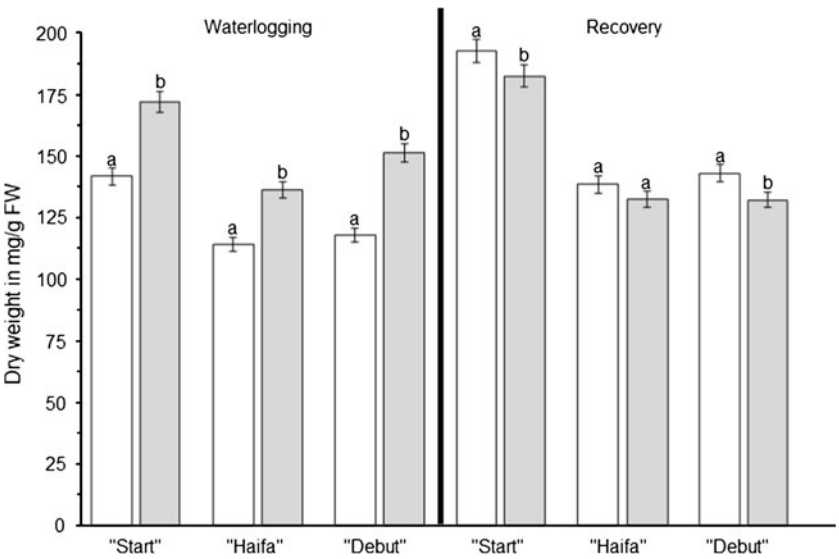

Fig. 2 Dry weight of red (*Trifolium pratense* L., cv. "Start") and white control (*white bars*) and waterlogged (*gray bars*) clover plants (*Trifolium repens* L., cvs. "Haifa" and "Debut"). *Error bars* represent standard deviations of three replicates obtained from three independent experiments. *Different letters* above columns indicate significant differences between the groups at $P < 0.05$

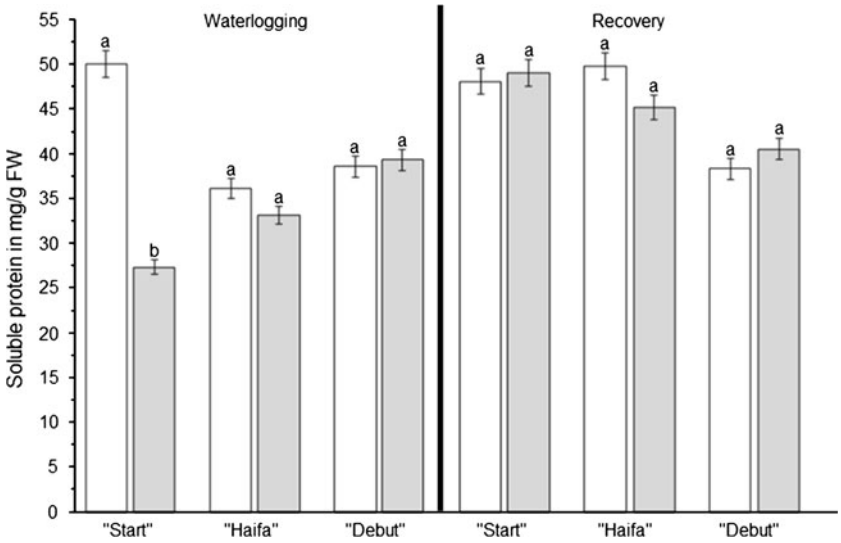

Fig. 3 Total leaf soluble protein of red (*Trifolium pratense* L., cv. "Start") and white control (*white bars*) and waterlogged (*gray bars*) clover plants (*Trifolium repens* L., cvs. "Haifa" and "Debut"). *Error bars* represent standard deviations of three replicates obtained from three independent experiments. *Different letters* above columns indicate significant differences between the groups at $P < 0.05$

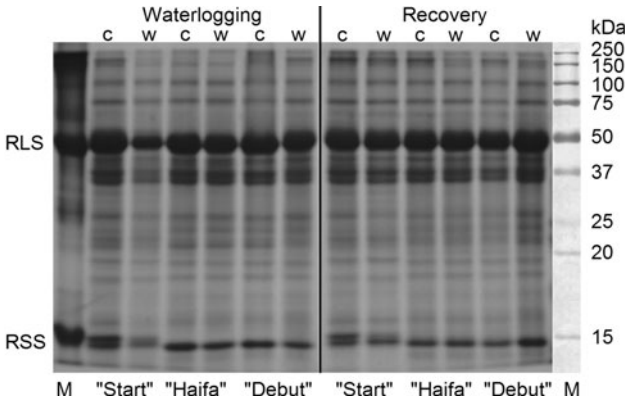

Fig. 4 Leaf soluble protein patterns after 12 % SDS–PAGE of red (*Trifolium pratense* L., cv. "Start") and white (*Trifolium repens* L., cvs. "Haifa" and "Debut") control (*c*) and waterlogged (*w*) clover plants. The first lane was loaded with Rubisco marker (RLS and RSS). "M" is molecular weight marker Precision Plus Protein Prestained Standards Dual Color (BIO-RAD). Samples with 10-μg protein quantity were loaded per lane

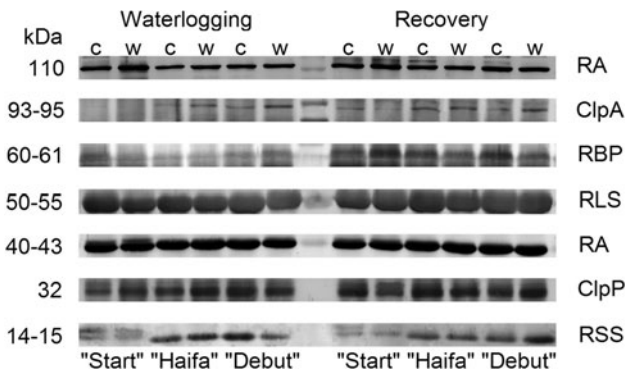

Fig. 5 Immunoblot analysis of extracts from red (*Trifolium pratense* L., cv. "Start") and white (*Trifolium repens* L., cvs. "Haifa" and "Debut") control (*c*) and waterlogged (*w*) clover plants with polyclonal antibodies against RLS, RSS, RA, RBP, ClpA and ClpP. Samples with 10-μg protein quantity were loaded per lane

Results

Biometrics documented only slight reduction of stem length of the waterlogged red clover cultivar "Start" (Fig. 1). The stem length of both white clover varieties "Haifa" and "Debut" remained similar to the controls under treatment with excess water. A slight delay in stem growth of the treated plants was noticed after recovery in all tested varieties. Significant reduction of leaflet area in waterlogged plants from all the varieties was observed (Fig. 1). The leaf area differences between the control and stressed plants were preserved after recovery period.

Increased leaf dry weight was documented in all the plants subjected to waterlogging (Fig. 2). The differences between control and treated samples vary within the range of 19.5–28.4 % and diminished after recovery from stress to 4.2–7.6 %.

Twofold decrease of total leaf soluble protein content in red clover cv. "Start" was observed under waterlogging, while no significant changes between control and treated plants were found in the tested white clover varieties (Fig. 3). After recovery, total leaf soluble protein content in control and recovered plants reached similar levels.

Protein profile changes were documented mainly in the samples derived from the stressed red clover. The sample from *Trifolium pratense* waterlogged plants was distinguishable among the others, since it exhibited decreased intensity of the bands corresponding to RLS and RSS (Fig. 4). SDS-PAGE electrophoresis revealed also two closely migrating bands at position corresponding to RSS only in control and waterlogged red clover samples.

Immunoblot analyses confirmed the presence of two low-intensity bands at RSS position, as well as it visualized two isoforms of RA in red clover (Fig. 5). In general, RA (40–43 kDa) levels were not significantly influenced by the

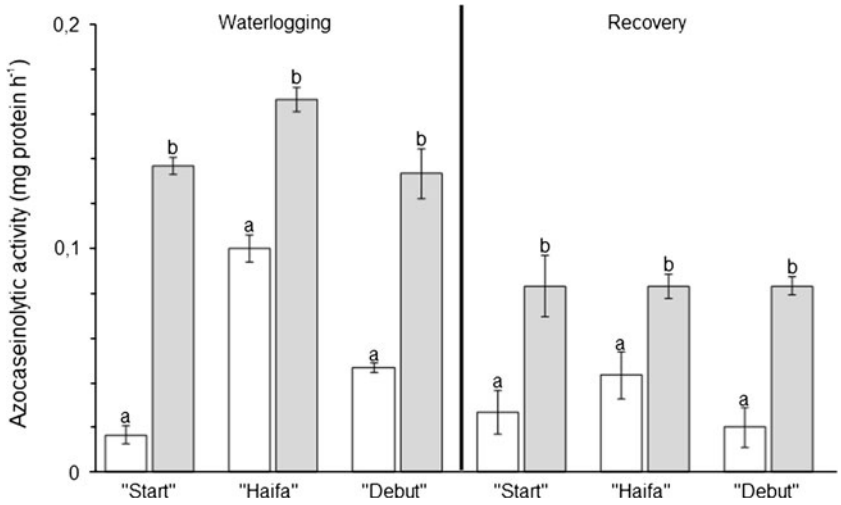

Fig. 6 Azocaseinolytic activity of extracts from red (*Trifolium pratense* L., cv. "Start") and white (*Trifolium repens* L., cvs. "Haifa" and "Debut") control (*white bars*) and waterlogged (*gray bars*) clover plants. *Error bars* represent standard deviations of three replicates obtained from three independent experiments. *Different letters* above columns indicate significant differences between the groups at $P < 0.05$

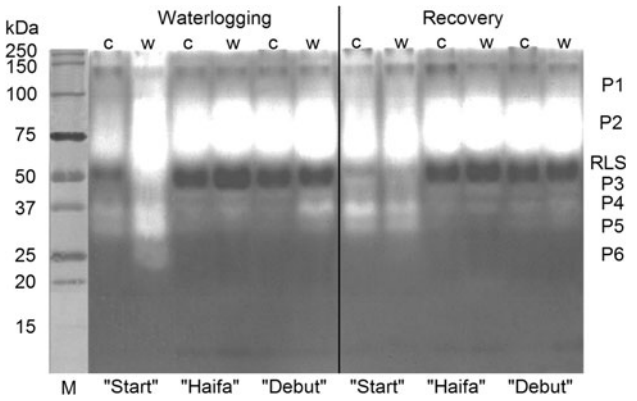

Fig. 7 In-gel staining of proteolytic activity of extracts from red (*Trifolium pratense* L., cv. "Start") and white (*Trifolium repens* L., cvs. "Haifa" and "Debut") control (*c*) and waterlogged (*w*) clover plants. Samples with 10-μg protein quantity were loaded per lane. "M" is molecular weight marker Precision Plus Protein Prestained Standards Dual Color (BIO-RAD)

excessive water and remained similar in control, waterlogged and recovered plants (Fig. 5). However, waterlogging promoted slightly reduced band intensity of RLS and RSS content in cv. "Start" (Fig. 5). Decreased RSS band intensity was documented also in waterlogged small-leafed white clover "Debut". RBP in cv. "Start" was inhibited by the excessive water treatment as well.

ClpP levels in all waterlogged samples, except the ones from the small leafed white clover variety, were higher compared to the controls (Fig. 5). The red clover plants "Start" exhibited also prominent increase of ClpA level under stress conditions. On the other hand, only ClpA seemed to accumulate in cv. "Debut" after flooding. ClpA and ClpP signals diminished after recovery in red cv. "Start" and white clover cv. "Haifa" and remained substantially higher in the other *T. repens* cv. "Debut". A 110 kDa band, which was revealed by immunoblotting with polyclonal antibodies against RA, seemed to be strongly induced by excessive water treatment only in red clover and remained higher after recovery (Fig. 5).

Total azocaseinolytic activity in samples from waterlogged plants increase between 1.667-fold ("white clover cv. Haifa") and 8.2-fold (red clover cv. "Start") (Fig. 6). All recovered plants had higher azocaseinolytic activity but the differences between them and the relevant controls regarding this parameter diminished.

Six bands with proteolytic activity were revealed in red clover after gelatin/SDS PAGE in non-reducing conditions at pH 6.8 and subsequent in-gel staining (Fig. 7). Proteolytic bands in white clover samples were only five. Significant proteolytic activity was observed for protein band P2 (with an approximate MW of 75 kDa) in all samples. Waterlogged red clover plants exhibited the highest proteolytic levels at P4 and P5 zone. Significant P6 activity was documented only in cv. "Start" samples from flooded plants. Waterlogging seemed to affect most significantly P4 activity in white clovers as well. Flooded cv. "Debut" plants expressed higher P4 activity compared to cv. "Haifa". After recovery, P2, P3, P4 and P5 proteolytic activities of cv. "Start" samples were similar without any activity documented in P6 zone. The 56-day-old controls and the recovered from stress red clovers (cv. "Start") had diminished amounts of RLS. After recovery, the controls and the stressed white clover plants did not exhibit differences of proteolytic activity in all revealed bands.

Discussion

Waterlogging affects biochemical, morphological and physiological parameters of various plant species (Ahsan et al. 2007b; Rijke et al. 2005; Simova-Stoilova et al. 2012; Colmer and Voesenek 2009). In the present study, we

focused on the changes of protein composition and proteolytic degradation in flooded red and white clovers. Previously published data demonstrated that waterlogging altered protein patterns, mainly characterized with well-established presence of proteins related to anaerobic conditions and increased proteolytic activity (Feller et al. 2008; Bailey-Serres and Voesenek 2008). According to Ahsan et al. (2007b), the varying content of some proteins in response to waterlogging could be related to the negative effect which this kind of stress has on plant growth and development. The measured high leaf dry weight of waterlogged plants (Fig. 2) may be linked to altered water balance and to the secondary drought stress developed in flooded crops.

Under the present experimental conditions, stem length (Fig. 1) and total soluble protein content (Fig. 3) were negatively influenced only in the waterlogged red clover plants, which corresponded well to the most prominent changes observed in cv. "Start" protein patterns. In an experiment with waterlogged tomato roots, authors have observed distinct changes in total protein profile where 16 proteins were up- and 13 proteins were down-regulated (Ahsan et al. 2007b). It has been documented that the most affected protein by different abiotic stress factors was the large subunit of Rubisco (Kokubun et al. 2002; Demirevska et al. 2008a; Feller et al. 2008; Nakano et al. 2010; Vassileva et al. 2011). The results obtained in the present study are consistent with these previously published data. SDS-PAGE separation and immunoblotting confirmed that the levels of RLS in red clover cv. "Start" declined under waterlogging (Figs. 4, 5) and were not influenced significantly in white clover varieties. This could be an indication for better tolerance of *Trifolium repens* toward excessive water stress. Strongly affected by the treatment, 110 kDa band, revealed with anti-RA polyclonal antibodies in red clover, could serve as an additional candidate-marker for the level of waterlogging stress in the sensitive *Trifolium pratense* varieties.

Diminution of some important proteins under abiotic stress could be a result of proteolytic degradation. Disturbed nutrition and water relation, impeded root–shoot transport, and nutrient starvation are capable to provoke proteolysis activation which could mobilize internal nutrient resources. As a readily mobilized nitrogen store, Rubisco could be a source of amino acids and other metabolites under stress conditions. The total proteolytic activity increased more than eightfold in waterlogged red clover, indicating its high sensitivity toward this kind of stress. *Trifolium pratense* cv. "Start" exhibited the highest degree of RLS proteolysis as well. Lower proteolytic activity was documented in the more tolerant white clover cultivars "Haifa" and "Debut". In an earlier study with the same red and white clover varieties, Vaseva et al. (2012) found that *Trifolium repens* cvs. "Haifa" and "Debut" were more tolerant also toward water deprivation. Hence, it appears that under excessive watering or water shortage, white clovers sustain more successfully the negative physiological consequences than the red clover variaty "Start".

The high intensity of ClpA and ClpP immunosignals in waterlogged cv. "Start" samples indicated changes in the plastidial Clp system and suggested alterations in the plastidial ATP-dependent proteolysis in this rather sensitive red clover.

It could be concluded that changes in total soluble protein content and protein pattern induced by proteolytic degradation is an important indicator for waterlogging resistance of white clover. Experimental observations clearly indicate the higher sensitivity of red clover cv. "Start" toward waterlogging compared to white clover varieties "Haifa" and "Debut". The mechanisms which determine the varying sensitivity toward waterlogging remain a subject of future in-depth research applying different molecular biology approaches.

Author contribution Veselin Stoychev: PhD student, literature references on the problem, measurement of growth parameters, leaf protein extraction, determination of total soluble protein, SDS-PAGE of soluble proteins, determination of total proteolytic activity, statistics, writing the draft of the manuscript, figures.

Lyudmila Simova-Stoilova: Associate Professor, proteolytic analysis, revision of the manuscript.

IrinaVaseva: Assistant Professor, revision of the manuscript, text editing.

Anelia Kostadinova: Biologist, responsible for growing of the plants under control and stress conditions, leaf protein extractions.

Rosa Nenkova: Technician, responsible for growing of the plants under control and stressed conditions, leaf protein extractions.

Urs Feller: Professor, raised the antibodies against ClpA and ClpP, provided preliminary findings based on them and was involved in the revision of the manuscript.

Klimentina Demirevska: Professor, supervisor of the PhD student, idea and experimental design, electrophoretic and immunobloting analyses, revision and improvement of the manuscript.

Acknowledgments The authors thank Prof. Ts. Mihovski from the Institute of Mountain Stockbreeding and Agriculture, Troyan, Bulgaria for kindly providing cv. "Debut" seeds. This study was supported by Bulgarian National Science Foundation (project No. BG051PO001-3.3.06-0025).

Conflict of interest The authors declare that they have no conflict of interest.

References

Ahsan N, Lee DG, Lee SH, Kang KY, Bahk JD, Choi MS, Lee IJ, Renaut J, Lee BH (2007a) A comparative proteomic analysis of tomato leaves in response to waterlogging stress. Physiol Plant 131(4):555–570

Ahsan N, Lee DG, Lee SH, Lee KW, Bahk JD, Lee BH (2007b) A proteomic screen and identification of waterlogging-regulated proteins in tomato roots. Plant Soil 295:37–51

Aschi-Smiti S, Chaibi W, Brouquisse R, Ricard B, Saglio P (2003) Assessment of enzyme induction and aerenchyma formation as mechanisms for flooding tolerance in *Trifolium subterraneum* 'Park'. Ann Bot 91(2):195–204

Bailey-Serres J, Voesenek LACJ (2008) Flooding stress: acclimations and genetic diversity. Annu Rev Plant Biol 59:313–339

Beers EP, Jones AM, Dickerman AW (2004) The S8 serine, C1A cysteine and A1 aspartic protease families in *Arabidopsis*. Phytochem 65:43–58

Beha EM, Theodorou MK, Thomas BJ, Kingston-Smith AH (2002) Grass cells ingested by ruminants undergo autolysis which differs from senescence: implications for grass breeding targets and livestock production. Plant Cell Environ 25:1299–1312

Beyene G, Foyer ChH, Kunert KJ (2006) Two new cysteine proteinases with specific expression patterns in mature and senescent tobacco (*Nicotiana tabacum* L.) leaves. J Exp Bot 57(6):1431–1443

Bogeat-Triboulot MB, Brosche M, Renaut J, Jouve L, Le Thiec D, Fayyaz P, Vinocur B, Witters E, Laukens K, Teichmann T, Altman A, Hausman J-F, Polle A, Kangasjarvi J, Dreyer E (2007) Gradual soil water depletion results in reversible changes of gene expression, protein profiles, ecophysiology, and growth performance in *Populus euphratica*, a poplar growing in arid regions. Plant Physiol 143:876–892

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Colmer TD, Voesenek LACJ (2009) Flooding tolerance: suites of plant traits in variable environments. Funct Plant Biol 36: 665–681

Demirevska K, Simova-Stoilova L, Vassileva V, Feller U (2008a) Rubisco and some chaperone protein responses to water stress and rewatering at early seedling growth of drought sensitive and tolerant wheat varieties. Plant Growth Regul 56(2):97–106

Demirevska K, Simova-Stoilova L, Vassileva V, Vaseva I, Grigorova B, Feller U (2008b) Drought induced leaf protein alterations in sensitive and tolerant wheat varieties. Gen Appl Plant Physiol 34(1–2):79–102

Directive 2007/60/EC of the European parliament and of the council of 23 October 2007 on the assessment and management of flood risks. Off J Eur Union L: 27–34

Feller U, Anders I, Demirevska K (2008) Degradation of Rubisco and other chloroplast proteins under abiotic stress. Gen Appl Plant Physiol 34(1–2):5–18

Fisher A, Feller U (1993) The pattern of peptide hydrolase activities in shoots of field-grown winter wheat during the cold season. Agronomie 13:293–299

Hashiguchi A, Ahsan N, Komatsu S (2010) Proteomics application of crops in the context of climatic changes. Food Res Int 43:1803–1813

Irfan M, Hayat S, Hayat Q, Afroz S, Ahmad A (2010) Physiological and biochemical changes in plants under waterlogging. Protoplasma 241:3–17

Jackson MB (2003) The impact of flooding stress on plants and crops. School of Biological Sciences, University of Bristol, Bristol, pp 1–15

Kingston-Smith AH, Bollard AL, Minchin FR (2005) Stress-induced changes in protease composition are determined by nitrogen supply in non-nodulating white clover. J Exp Bot 56(412): 745–753

Kokubun N, Ishida H, Makino A, Mae T (2002) The degradation of the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase into the 44-kDa fragment in the lysates of chloroplasts incubated in darkness. Plant Cell Physiol 43(11): 1390–1395

Kosová K, Vítámvás P, Prášil IT, Renaut J (2011) Plant proteome changes under abiotic stress—contribution of proteomics studies to understanding plant stress response. J Proteom 74:1301–1322

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 277:680–685

Mitsuhashi W, Feller U (1992) Effects of light and external solutes on the catabolism of nuclear-encoded stromal proteins in intact chloroplasts isolated from pea leaves. Plant Physiol 100: 2100–2105

Nakano R, Ishida H, Kobayashi M, Makino A, Mae T (2010) Biochemical changes associated with in vivo RbcL fragmentation by reactive oxygen species under chilling-light conditions. Plant Biol 12:35–45

Rijke E, Aardenburg L, van Dijk J, Ariese F, Ernst WHO, Gooijer C, Brinkman UATH (2005) Changed isoflavone levels in red clover (*Trifolium pratense* L.) leaves with disturbed root nodulation in response to waterlogging. J Chem Ecol 31(6): 1285–1298

Salvucci ME, Portis AR, Ogren WL (1985) A soluble chloro-plast protein catalyses ribulosebisphosphate carboxylase/oxygenase activation in vivo. Photosynt Res 7:193–201

Sanderson MA, Byers RA, Skinner RH, Elwinger GF (2003) Growth and complexity of white clover stolons in response to biotic and abiotic stress. Crop Sci 43:2197–2205

Seker H, Rowe DE, Brink GE (2003) White clover morphology changes with stress treatments. Crop Sci 43:2218–2225

Simova-Stoilova L, Demirevska K, Kingston-Smith A, Feller U (2012) Involvement of the leaf antioxidant system in the response to soil flooding in two *Trifolium* genotypes differing in their tolerance to waterlogging. Plant Sci 183:43–49

Sullivan ML, Hatfield RD (2006) Polyphenol oxidase and o-diphenols ihibit postharvest proteolysis in red clover and alfalfa. Crop Sci 46:662–670

Van der Hoorn RAL (2008) Plant proteases: from phenotypes to molecular mechanisms. Annu Rev Plant Biol 59:191–223

Vaseva I, Akiscan Y, Demirevska K, Anders I, Feller U (2011) Drought stress tolerance of red and white clover—comparative analysis of some chaperonins and dehydrins. Scient Horticult 130:653–659

Vaseva I, Akiscan Y, Simova-Stoilova L, Kostadinova A, Nenkova R, Anders I, Feller U, Demirevska K (2012) Antioxidant response to drought in red and white clover. Acta Physiol Plant 34(5):1689–1699. doi:10.1007/s11738-012-0964-4

Vassileva V, Demirevska K, Simova-Stoilova L, Petrova T, Tsenov N, Feller U (2011) Winter wheat cultivars under long-term field drought—biochemical and ultrastructural constraints affecting yield. J Agron Crop Sci 198(2):104–117. doi:10.1111/j.1439-037X.2011.00492.x

Wang W, Vinocur B, Shoseyov O, Altman A (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response (Review). Trends Plant Sci 9(5):244–251