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BIOCHEMICAL MARKERS OF OXIDATIVE STRESS IN TRITICALE SEEDLINGS EXPOSED TO CEREAL APHIDS

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In this study, we examined whether and to what extent oxidative stress is induced in seedlings of two winter triticale (*Triticosecale* Wittm.) varieties (susceptible Tornado and resistant Witon) in response to infestation by the cereal grain aphid (*Sitobion avenae* L.) and bird-cherry-oat aphid (*Rhopalosiphum padi* L.). We compared the level of hydrogen peroxide (H_2O_2) and lipid peroxidation products as well as markers of protein damage (protein-bound thiol and carbonyl groups). The studied parameters were measured at 6, 24, 48 and 96 h post-initial aphid infestation compared to the non-infested control seedlings. Our studies indicated that the cereal aphid feeding evoked oxidative stress in the triticale seedlings. Cereal aphid feeding increased the H_2O_2 level in triticale tissues, with maximum levels observed at 24 and 48 h post-infestation. Triticale infestation with aphids also increased lipid peroxidation products in triticale seedlings, with the maximal levels at 48 or 96 h post-infestation. Further, there was a reduction in protein thiol content and an increase in protein carbonyl content in the triticale seedlings after infestation with female aphids. Stronger triticale macromolecule damages were evoked by the oligophagous aphid *R. padi*. There was a more substantial protein thiol content reduction in the resistant Witon cultivar and higher accumulation of protein-bound carbonyls in the tissues of the susceptible Tornado cultivar. The changes were proportional to the aphid population and the time of aphid attack. These findings indicate that the defensive strategies against cereal aphid (*S. avenae* and *R. padi*) infestation were stimulated in triticale Tornado and Witon seedlings. Our results explain some aspects and broaden the current knowledge of regulatory mechanisms in plant-aphid interactions.

Keywords: hydrogen peroxide, protein thiols, protein carbonyls, *Rhopalosiphum padi*, *Sitobion avenae*, triticale

INTRODUCTION

All aerobic organisms are subjected to generation of reactive oxygen species (ROS) as metabolic products. ROS are highly reactive chemical molecules with one or more unpaired electrons. The most common ROS include superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\cdot}OH$). ROS are always formed by the inevitable leakage of electrons onto O_2 from the electron transport activities of chloroplasts, mitochondria and plasma membranes or as a byproduct of metabolic pathways localized in different cellular compartments (Sharma et al., 2012). ROS differ in biochemical properties. H_2O_2 is relatively stable, and its concentration in plant tissues is the highest in comparison to the other ROS. The $^{\cdot}OH$ is the most reactive species and is produced from hydroperoxides through the Fenton reaction (Rinalducci et al., 2008). Different ROS react with disparate substrates: H_2O_2 reacts with all types of macromolecules, whereas $O_2^{\cdot-}$ reacts primarily with protein Fe-S centers (Requena et al., 2001; Rinalducci et al., 2008). Under physiological conditions, the ROS concentration in plant tissues is stable and maintained by a system composed of non-enzymatic antioxidants and antioxidant enzymes (Maffei et al., 2006). However, environmental stresses as well as pathogen and herbivore attacks lead to a rapid ROS accumulation ("oxidative burst"; Orozco-Cardenas and Ryan, 1999; Powell et al., 2006). Enhanced ROS production is harmful to plants, as it can cause lipid peroxidation, protein oxidation and DNA modifications (Kuźniak and Urbanek, 2000; Verma and Dubey, 2003, Sharma et al., 2012). The ROS-induced damages to cellular compounds lead to altered intrinsic membrane properties, loss of enzyme

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activity, protein crosslinking and protein synthesis inhibition, all of which result in cell death (Sharma et al., 2012). Proteins are the most abundant cellular ROS targets; they make up 68% of the oxidized molecules in the cell (Rinalducci et al., 2008). In proteins, amino acids that contain thiol groups and sulphur are the most susceptible sites for ROS action (Verma and Dubey, 2003; Davies, 2005). The cysteine thiol group can be oxidized to disulfide, sulphenic acid, sulphinic acid and sulphonic acid. Most of these modifications are reversible, with the exception of sulphonic acid (Rinalducci et al., 2008; Boguszewska-Mańkowska et al., 2015). In contrast, protein carbonylation is an irreversible process, where lysine, arginine, proline and threonine side-chains can be converted to reactive aldehyde or ketone groups, an alteration that causes protein inactivation, crosslinking or breakdown (Davies, 2005; Rinalducci et al., 2008). Additionally, protein carbonylation can result from an indirect mechanism that involves hydroxyl-radical-mediated lipid oxidation (Yuan et al., 2007; Grimsrud et al., 2008). The level of lipid peroxidation has been used as a marker of ROS-mediated damage to cell membranes under abiotic and biotic stresses (Sharma et al., 2012). In lipid peroxidation, polyunsaturated fatty acids (PUFAs) of lipids are chemically damaged by ROS, a process that forms lipoperoxides. The lipoperoxides decompose to form reactive carbonyl compounds, including aldehydes (malondialdehyde – MDA) and hydroxyalkenals, which bind free amino groups of amino acids in proteins (Anjum et al., 2015). ROS-mediated peroxidation of polyunsaturated fatty acids leads to chain breakage and increases membrane fluidity and permeability (Sharma et al., 2012).

Aphids are serious pests worldwide. In Poland, two cereal aphid species dominate on cereals: the monophagous *Sitobion avenae* (F.) and the oligophagous *Rhopalosiphum padi* (L.) (Korbas, 2007). *R. padi* arrives first in the season, whereas *S. avenae* usually arrives later, when plants are in the late tillering or stem-elongation stage (Gianoli, 2000). *S. avenae* and *R. padi* are vectors of barley yellow dwarf virus (BYDV), which is responsible for diseases of cereals. The saliva and the injury caused by aphids induce local and systemic ROS production in the phloem of the host (Moran and Thompson, 2001; Zhu-Salzman et al., 2004; Divol et al., 2005). Oligogalacturonides released from plant cell wall polysaccharides by aphid salivary enzymes cause linolenic acid degradation, which together with systemin, oligogalacturonic acid and chitosan, trigger the synthesis of H_2O_2 and other ROS (Gatehouse, 2002; Orozco-Cardenas and Ryan, 1999). At low or moderate concentrations, ROS act as second messengers in intracellular signaling cascades that mediate several responses in plant cells, whereas at high concentrations, they damage biomolecule structures (Mittler, 2002; Torres et al., 2002; Miller et al., 2008; Sharma et al., 2012). There are numerous studies concerning the role of ROS in plant responses to the abiotic and biotic factors (herbicides, salinity, heavy metals and fungal and viral infections), but studies relating to biochemical markers in the cereals stressed by aphids are still rare. Although the oxidative responses of plants to aphids are mentioned in several plant-aphid interactions, no comparable studies have been reported for triticale, an important agricultural crop. Little is known about the role of oxidative stress in the triticale seedling defense responses to cereal aphid infestation, a significant knowledge gap since these aphids are the most important crop pests in Poland. Aphid feeding can begin at germination and last until harvest. Thus, the purpose of the current work was to compare the levels of H_2O_2 and lipid peroxidation products as well as protein damage markers (protein-bound thiols and carbonyl groups) in two varieties of winter triticale seedlings (Witon and Tornado), which differ in susceptibility to cereal aphids (Witon and Tornado), infested with two cereal aphid species: *S. avenae* and *R. padi*.

MATERIALS AND METHODS

APHIDS

Experiments were conducted using wingless females (*apterae*) of the grain aphid *S. avenae* F. and the bird cherry-oat aphid *R. padi* L. The aphids were reared on wheat seedlings (*Triticum aestivum* L.) cv. Tonacja in an environmental chamber (21°C, 16-h light:8-h dark photoperiod, 70% relative humidity).

PLANTS

Two cultivars of winter triticale (*Triticosecale* Wittm.) that differ in susceptibility to cereal aphids were used. The Witon genotype was previously classified as resistant, whereas Tornado is susceptible to cereal aphid infestation (Sempruch et al., 2009). The seedlings were grown in an environmental chamber in plastic pots (10 × 10 cm, one seedling per pot) filled with medium nutrient fine structure compost with sand.

INFESTATION PROCEDURE

Each nine-day-old triticale seedling was colonized with 30 or 60 aphids. Aphid individuals were carefully transferred to seedlings with a fine paintbrush. Larvae and winged aphid adults were monitored through all experiments. The number

of wingless aphid adults was constant, because two times a day any borne larvae were removed. The control seedling groups were not infested with insects. The triticale seedlings infested with aphids and the non-infested (control) plants were isolated in gauze-covered plastic cylinders. The levels of H_2O_2, lipid peroxidation products, protein thiols (PT) and carbonyl groups in the triticale seedling leaves were determined 6, 24, 48 and 96 h after the initial insect infestation.

H_2O_2 ASSAY

The content of H_2O_2 was determined according to Zhou et al. (2006). The method is based on the peroxidase-catalyzed reaction of 4-aminoantipyrine and phenol with H_2O_2. One g leaf tissue was ground in 5 ml 5% trichloroacetic acid (TCA) with 50 mg active charcoal at 0°C. The homogenate was centrifuged for 15 min at 15,000 *g*. The supernatant was collected, neutralized with 17 M NH_4OH to pH 7 and used for the H_2O_2 assay. The reaction mixture consisted of 1 ml reagent (4 mM 4-aminoantipyrine, 24 mM phenol and 0.4 U/ml peroxidase dissolved in 0.1 M phosphate-buffer pH 7.0) and 1 ml plant homogenate. After homogenate addition, the reaction mixture was incubated at 25°C for 10 min, and the absorbance was measured at 510 nm against a blank, which contained 1 ml distilled water instead of the plant homogenate. The hydrogen peroxide content was calculated from a calibration curve prepared for this standard (Sigma-Aldrich, Poland) and is expressed in nmol/g fresh weight.

MDA ASSAY

The MDA content was estimated according to the method of Heath and Packer (1968). Five-hundred mg triticale seedlings were homogenized in 6 ml 0.1% TCA and centrifuged for 15 min at 15,000 *g*. One ml supernatant was mixed with 4 ml 0.5% thiobarbituric acid (TBA; Sigma-Aldrich, Poland) in 20% TCA and then placed in a boiling water bath for 30 min. After cooling, the mixtures were centrifuged at 10,000 *g*, and the absorbance of supernatants was measured at 535 and 600 nm. MDA concentration was estimated by subtracting the non-specific absorbance at 600 nm from the absorbance at 532 nm, using an extinction coefficient of 156 $mM^{-1}cm^{-1}$, and expressed as nmol per fresh weight.

PT DETERMINATION

The total thiol and PT contents were determined according to de Kok and Kuiper (1986), with minor modifications. Five-hundred mg fresh seedling leaves were homogenized in ice-cold 0.2M Tris-HCl buffer (pH 7.4). The extracts were centrifuged at 20,000 *g* for 10 min at 4°C. The supernatant was used to assay total thiols and non-protein thiols. To determine total thiols, 0.5 ml supernatant was mixed with 1 ml 0.2 mM Tris-HCl (pH 8.2) and 0.1 ml 0.01 M DTNB (Sigma-Aldrich, Poland). The reaction mixture was incubated for 15 min at 30°C. The yellow color that developed was measured at 415 nm. A correction was made for the absorbance of the incubation mixture in the absence DTNB (replaced with distilled water) and in the absence of supernatant (replaced with 0.2M Tris-HCl buffer pH 7.4) To determine non-protein thiol content, the supernatant was deproteinized by incubating in a water bath at 100°C for 5 min and centrifuged at 20,000 *g* for 10 min at 4°C. The non-protein thiol content was determined in a manner similar to that for the total thiols. The PT content was calculated by subtracting the content of non-protein thiols from the total thiols. Total and protein sulfhydryl groups were calculated using the extinction coefficient 13,600 $M^{-1}cm^{-1}$. The PT content was expressed as nmol per mg protein. The protein content in the studied plant extracts was determined using the method by Bradford (1976).

DETERMINATION OF PROTEIN-BOUND CARBONYLS

The content of protein-bound carbonyl was determined according to Levine et al. (1994). Six-hundred mg fresh triticale seedling leaves were homogenized in ice-cold 50 mM sodium phosphate buffer (pH 7.4) that contained 1 mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, Poland). The homogenates were centrifuged at 6000 *g* for 10 min at 4°C. The supernatants were incubated on ice with 1% (w/v) streptomycin sulfate for 15 min and centrifuged at 6000 *g* for 10 min to remove the nucleic acid. Two-hundred µl nucleic-acid-free supernatants were mixed with 800 µl 10 mM 2,4-dinitrophenyl hydrazine (DNPH; Sigma-Aldrich, Poland) in 2.5 M HCl. The blank samples were incubated in 2.5 M HCl. After 1 h incubation at room temperature (in the dark), 1 ml 20% (w/v) TCA was added, samples were incubated on ice for 5 min and then centrifuged at 10,000 *g* for 10 min. The pellets were resuspended in 1 ml ethanol:ethyl acetate (1:1) and centrifuged at 10,000 *g* for 10 min. This procedure was repeated three times. The clean pellets were dissolved in 6 M guanidine hydrochloride in 20 mM potassium phosphate buffer (pH 2.3) and centrifuged at 10,000 *g* for 10 min. The absorbance was measured at 375 nm. Protein recovery was estimated for each sample by measuring the absorbance at 280 nm. The carbonyl group content was calculated using a molar absorption coefficient for aliphatic hydrazones (22,000 $M^{-1}cm^{-1}$) and expressed as nmol carbonyl per mg protein.

STATISTICAL ANALYSIS

The differences in the H_2O_2, lipid peroxidation products, PT and protein-bound carbonyl contents in aphid-challenged triticale plants were examined with a general linear model (GLM) followed by the post hoc Tukey's honestly significant difference (HSD) test. In each GLM model, four factors were used: the time of feeding, the number of aphids, the aphid species and the triticale cultivar. The response variables were: H_2O_2 content, lipid peroxidation products, PT and protein-bound carbonyls. All statistical analyses were provided by Statistica 10.0 (StatSoft, 2012).

RESULTS

GLM analysis showed that the H_2O_2 content was dependent on several study factors (GLM: $F_{7,136} = 5.87$; $P < 0.001$; $R^2 = 0.19$). The significant factors were: the number of aphids (GLM: $F_{2,136} = 10.12$; $P < 0.001$) and the triticale cultivar (GLM: $F_{1,136} = 18.53$; $P < 0.001$; Table 1). Both Tornado and Witon cv. seedlings treated with higher aphid numbers showed proportionally greater H_2O_2 accumulation (Fig. 1). Witon tissues generated more H_2O_2 than the stressed Tornado seedlings. In Witon seedlings, there was remarkable H_2O_2 release. The highest concentration was reached 24 and 48 h post-infestation (hpi) with 60 *S. avenae* or *R. padi*, with values 2.29- and 2.72-fold higher than at 0 h, respectively. By 96 hpi, H_2O_2 decreased to lower levels (Fig. 1). There was a similar trend in Tornado seedlings, but the H_2O_2 level in plants stressed by *R. padi* was comparable for the two studied aphid densities (30 and 60 aphids per plant) at all periods of infestation. In control plants, H_2O_2 remained low throughout cultivation. In most cases, the differences in H_2O_2 concentration in aphid-infested variants and the control plants were significant (Fig. 1).

The content of lipid peroxidation products were dependent on all study factors (GLM: $F_{7,136} = 59.71$; $P < 0.001$; $R^2 = 0.74$): the time of feeding (GLM: $F_{3,136} = 34.45$; $P < 0.001$), the number of aphids (GLM: $F_{2,136} = 39.42$; $P < 0.001$), the aphid species (GLM: $F_{1,136} = 23.17$; $P < 0.001$) and the triticale cultivar (GLM: $F_{1,136} = 212.59$; $P < 0.001$; Table 1). *S. avenae* feeding on Tornado cv. did not affect the MDA content relative to non-stressed control plants, with the exception of 60 aphids per plant at 48 h (Fig. 2). Different results were obtained for *R. padi*, where MDA increased in Tornado plants stressed by 60 aphids per plant at most infestation periods (24, 48 and 96 h). However, the MDA content was comparable in the seedlings infested with 30 and 60 aphids (Fig. 2).

TABLE 1. Statistical results of the GLM for hydrogen peroxide content, lipid peroxidation products, protein thiols and protein-bound carbonyls in aphid-challenged triticale plants.

Parameter	Degrees freedom (df)	F	P-value
Hydrogen peroxide			
Time of feeding	3	0.759	= 0.518
Number of aphids	2	10.126	< 0.001
Aphid species	1	0.001	= 0.974
Triticale cultivar	1	18.526	< 0.001
Lipid peroxidation products			
Time of feeding	3	34.447	< 0.001
Number of aphids	2	39.423	< 0.001
Aphid species	1	23.171	< 0.001
Triticale cultivar	1	212.596	< 0.001
Protein thiols			
Time of feeding	3	20.273	< 0.001
Number of aphids	2	81.000	< 0.001
Aphid species	1	26.942	< 0.001
Triticale cultivar	1	0.009	= 0.923
Protein-bound carbonyls			
Time of feeding	3	21.61	< 0.001
Number of aphids	2	31.88	< 0.001
Aphid species	1	12.64	< 0.001
Triticale cultivar	1	27.64	< 0.001

In the case of Witon cv., the initial period of cereal aphid colonization (6 h) did not change the MDA concentration in infested compared to non-infested plants (Fig. 2). Prolonged aphid feeding evoked a significant elevation in MDA content in Witon seedlings, but there were no alterations observed in plants stressed by 30 *S. avenae* females at 24 h. The highest MDA concentration was observed at the end of aphid infestation (48 and 96 h). At these time points, MDA was higher in Witon seedlings colonized by 60 aphid females (compared to infestation by 30 individuals). Between the tested triticale cultivars, Witon plants exhibited greater MDA elevation in response to aphid infestation. With regards to aphid species,

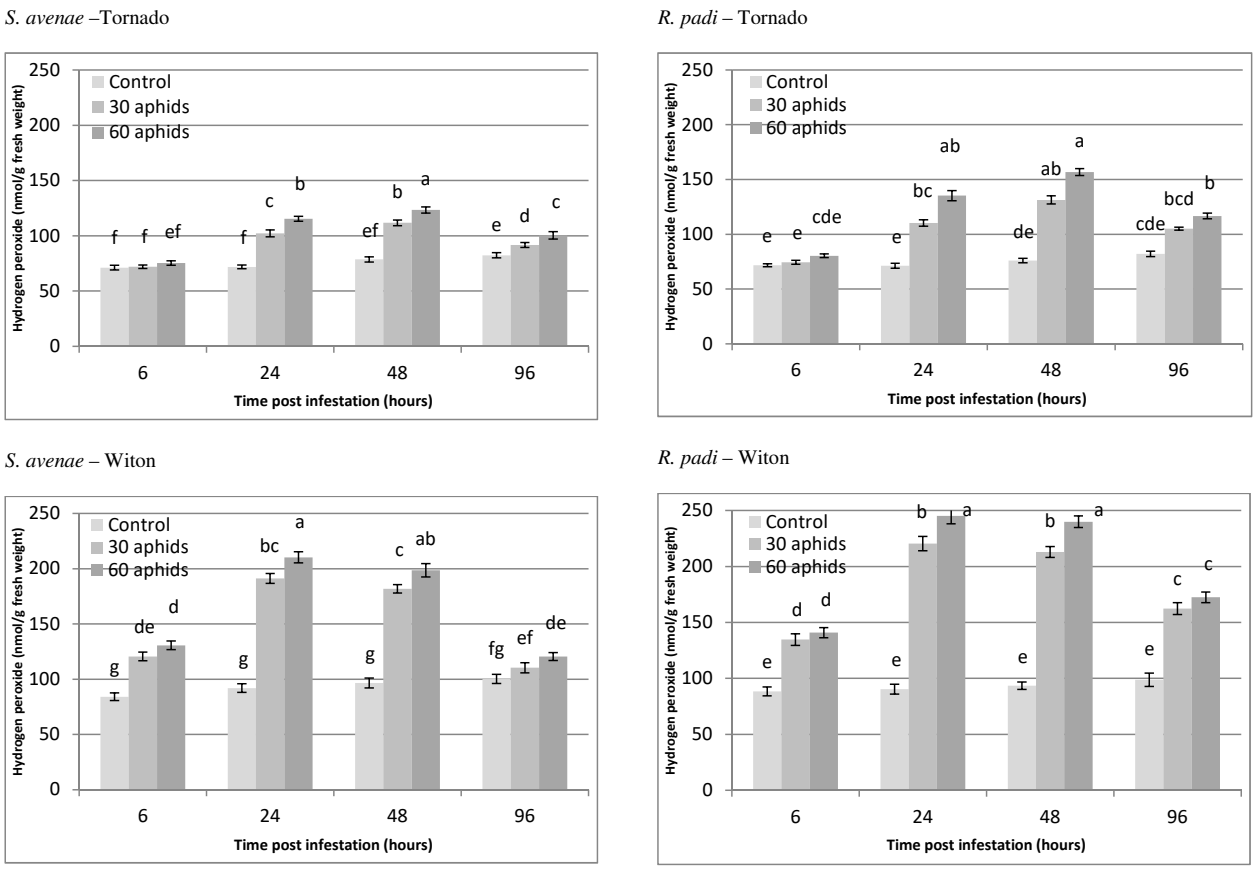

Fig. 1. Influence of the tested cereal aphids on hydrogen peroxide levels (nmol/g fresh weight) in triticale (Tornado and Witon cv.) seedlings. Values represent the mean ± standard deviation (SD) from three independent experiments. The different letters above the SD bars indicate significant differences according to Tukey's test ($P \leq 0.05$).

R. padi affected lipid damage in triticale seedlings more strongly than *S. avenae* (Fig. 2).

The PT content was dependent on three study factors (GLM: $F_{7,136} = 35.68$; $P < 0.001$; $R^2 = 0.63$): the time of feeding (GLM: $F_{3,136} = 20.27$; $P < 0.001$), the number of aphids (GLM: $F_{2,136} = 81.00$; $P < 0.001$) and the aphid species (GLM: $F_{1,136} = 26.94$; $P < 0.001$; Table 1). The initial aphid feeding (6 and 24 h) on Tornado seedlings was accompanied by decreased PT content only in plants treated with 60 *R. padi* adults at 24 h (Fig. 3). Over the next two time periods of infestation (48 and 96 h), there was a significant depletion in the PT content in Tornado cv. seedlings. The intensity of the decrements was comparable at these time periods. PT depletion was dependent on the aphid density only for Tornado seedlings infested with *R. padi* (Fig. 3). A similar tendency was observed for Witon cv., but the PT decrease in plants stressed by *S. avenae* occurred earlier and was dependent on aphid density at almost all studied infestation periods (24, 48 and 96 h). The strongest PT depletion was noted for Witon cv. stressed by *R. padi* (Fig. 3).

The content of protein-bound carbonyl groups was dependent on all study factors (GLM: $F_{7,136} = 24.12$; $P < 0.001$; $R^2 = 0.53$): the time of feeding (GLM: $F_{3,136} = 21.61$; $P < 0.001$), the number of aphids (GLM: $F_{2,136} = 31.88$; $P < 0.001$), the aphid species (GLM: $F_{1,136} = 12.64$; $P < 0.001$) and the triticale cultivar (GLM: $F_{1,136} = 27.64$; $P < 0.001$) (Table 1). The protein-bound carbonyl groups in Tornado cv. tissues remained unaffected after *S. avenae* colonization, with the exception of a higher density of aphids (60 per plant) that increased protein carbonyl content after 96 h (Fig. 4). Tornado seedlings responded differently to *R. padi* infestation: the protein carbonyl increased after almost all infestation periods (24, 48 and 96 h) in relation

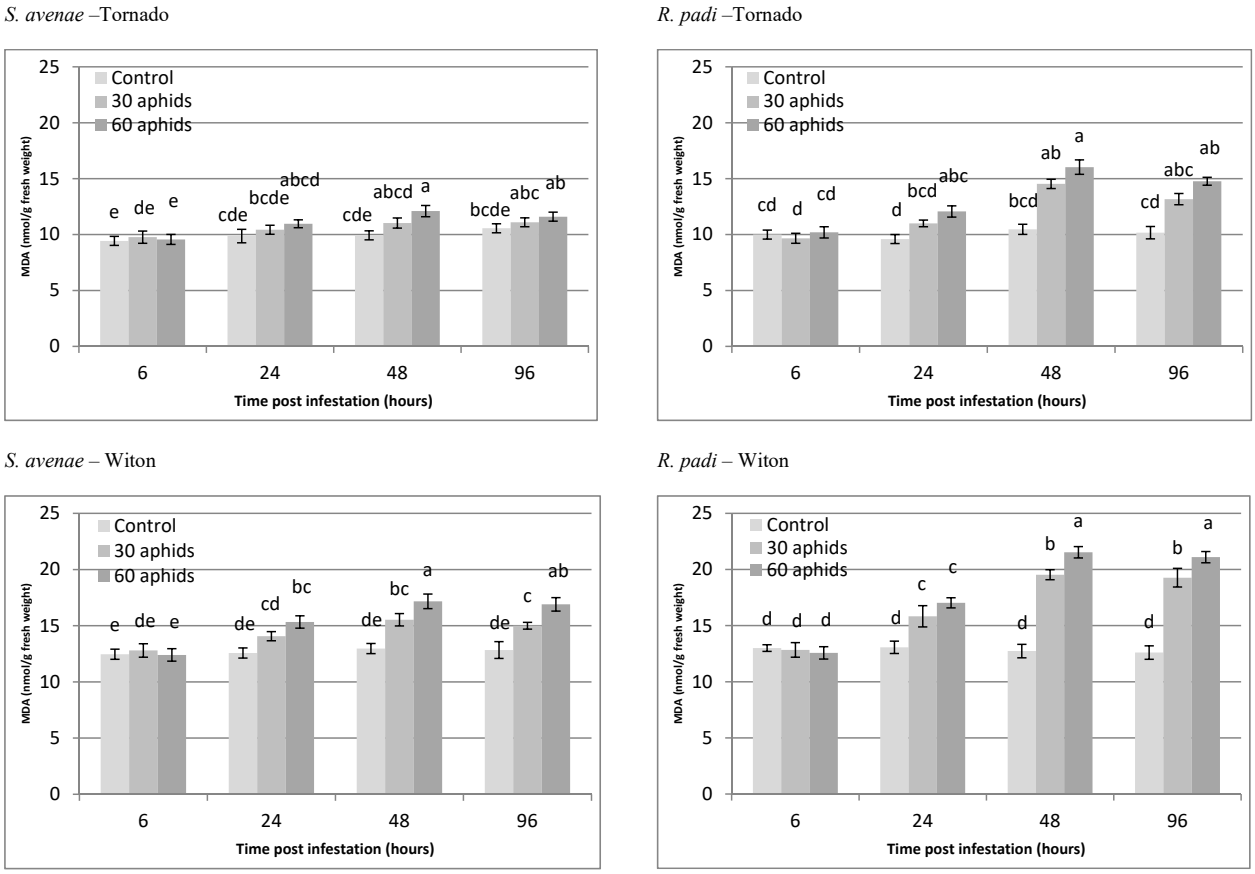

Fig. 2. Influence of the tested cereal aphids on MDA content (nmol/g fresh weight) in the triticale (Tornado and Witon cv.) seedlings. Values represent mean ± standard deviation (SD) from three independent experiments. The different letters above the SD bars indicate significant differences according to Tukey's test ($P \leq 0.05$).

to non-stressed plants. In most cases, Witon cv. colonization by cereal aphids did not alter protein carbonyl content compared to control plants, but the higher *R. padi* number evoked protein carbonyl depletion at 96 hpi. The strongest protein carbonyl content elevations occurred in Tornado cv. stressed by *R. padi* (Fig. 4).

DISCUSSION

Aphid herbivory induces biochemical and physiological changes in host plants that include production of ROS such as O_2^- or H_2O_2 (Moloi and van der Westhuizen, 2006; Mai et al., 2013; Sytykiewicz, 2015; Łukasik et al., 2017). Plant defense mechanisms play a crucial role in protection from H_2O_2, since this ROS is highly stable, has a relatively long half-life, lower cytotoxicity compared to other ROS and can penetrate membranes (Liu et al., 2010; Kärkönen and Kuchitsu, 2014). In the current work, cereal aphid feeding increased H_2O_2 generation in triticale tissue, reaching maximal levels at 24 and 48 hpi. In the case of the resistant cultivar (Witon), we noted earlier and stronger H_2O_2 elevations compared to the susceptible cultivar (Tornado). A similar tendency was observed by Sytykiewicz (2015), where *R. padi* and *S. avenae* infestations of *Zea mays* L. triggered more substantial H_2O_2 content increases in the seedlings of Ambrozja (moderate resistant) and Touran (highly resistant) compared to Tasty Sweet (susceptible). Although many reports demonstrated changes in H_2O_2 concentration under biotic stress factors, studies that relate the role of this molecule in host plant – arthropod (especially sucking-piercing insects) interactions are still rare. Moloi and van der Westhuizen (2006) elucidated that Russian wheat aphid (*Diuraphis noxia* Mordvilko) feeding on wheat leads to significant

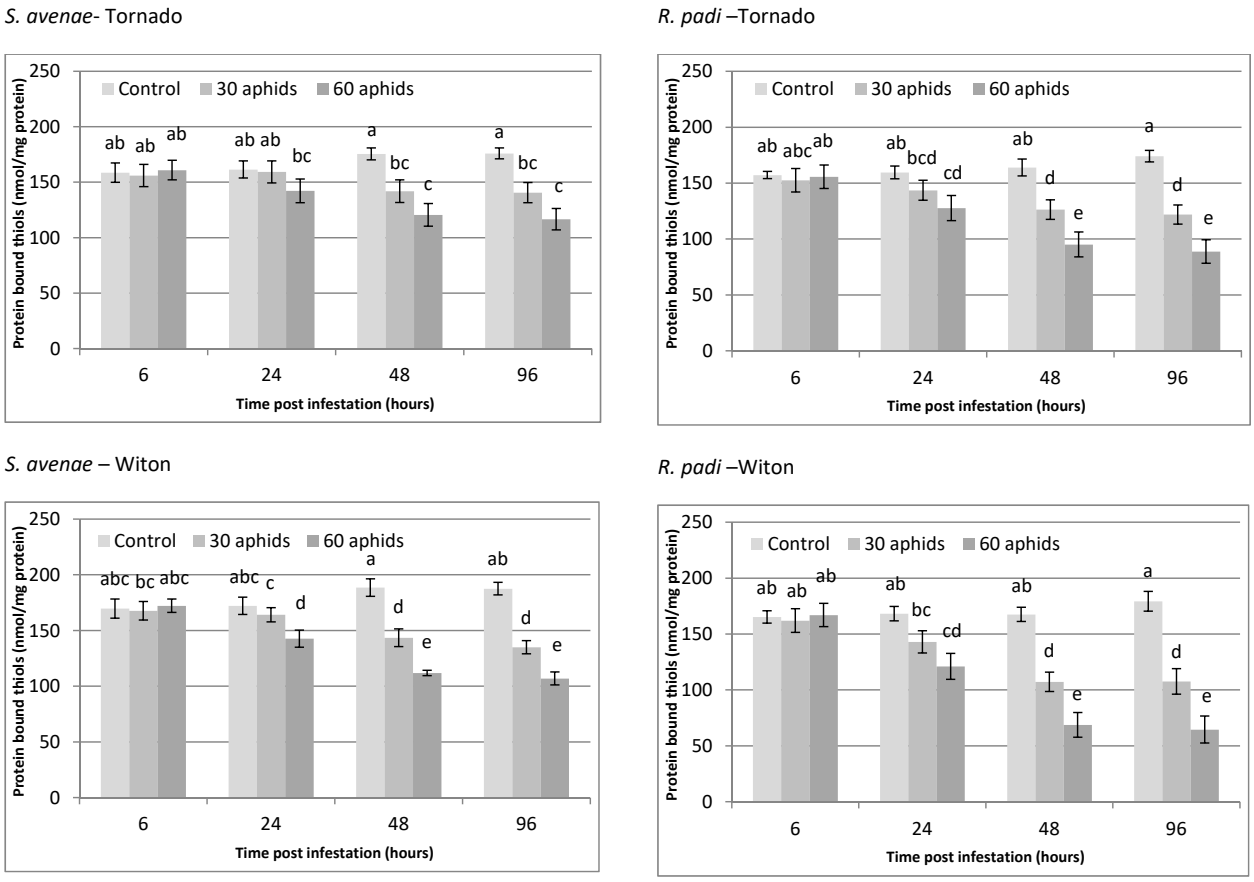

Fig. 3. Influence of the tested cereal aphids on protein thiol levels (nmol/mg protein) in triticale (Tornado and Witon cv.) seedlings. Values represent mean ± standard deviation (SD) from three independent experiments. The different letters above the SD bars indicate significant differences according to Tukey's test ($P \leq 0.05$).

H_2O_2 increases. The accumulation and level of H_2O_2 peaked 3-6 hpi. Mai et al. (2013) observed substantial H_2O_2 overproduction in pea (*Pisum sativum* L.) seedlings after infestation by the pea aphid (*Acyrtosiphon pisum* Harris), with the highest H_2O_2 level 24 hpi. The greenbug *Schizaphis graminum* (Rondani) feeding induces H_2O_2 generation and total soluble peroxidase activity in barley 20 min post-infestation (Argandona et al., 2001). Kerchev et al. (2012) stated that 48 h feeding by the peach-potato aphid (*Myzus persicae* Sulzer) increases H_2O_2 content 2-fold in potato (*Solanum tuberosum* L.) plants compared to uninfested controls. Łukasik et al. (2017) noted that the H_2O_2 content in *Fabaceae* plants (pea, vetch and broad bean) increased after *A. pisum* herbivory and reached its maximal level at 6 hpi. According to these authors, the highest H_2O_2 accumulation was observed in the pea plants, which were characterized by the strongest catalase inhibition. Other results were obtained by Kuśnierczyk et al. (2008), where cabbage aphid (*Brevicoryne brassicae* L.) feeding did not affect H_2O_2 content in *Arabidopsis thaliana* L. ecotype Landsberg *erecta* (Ler).

Excess H_2O_2 is detrimental to plants, since it can cause protein oxidation, membrane lipid peroxidation and damage the reaction center of chloroplasts (Hu et al., 2009). Lipid peroxidation is a complex process that involves lipid radical formation, oxygen uptake, a rearrangement of the double bonds in unsaturated lipids and membrane lipid destruction, with the production of a variety of breakdown products, including alcohols, ketones, alkanes, aldehydes and ethers (Dianzani and Barrera, 2008). In contrast to ROS, aldehydes are highly stable, diffuse out of the cell and attack targets far from their production site (Repetto et al., 2012). One of the indicators of lipid damage, MDA, is the end-product of lipid peroxidation that

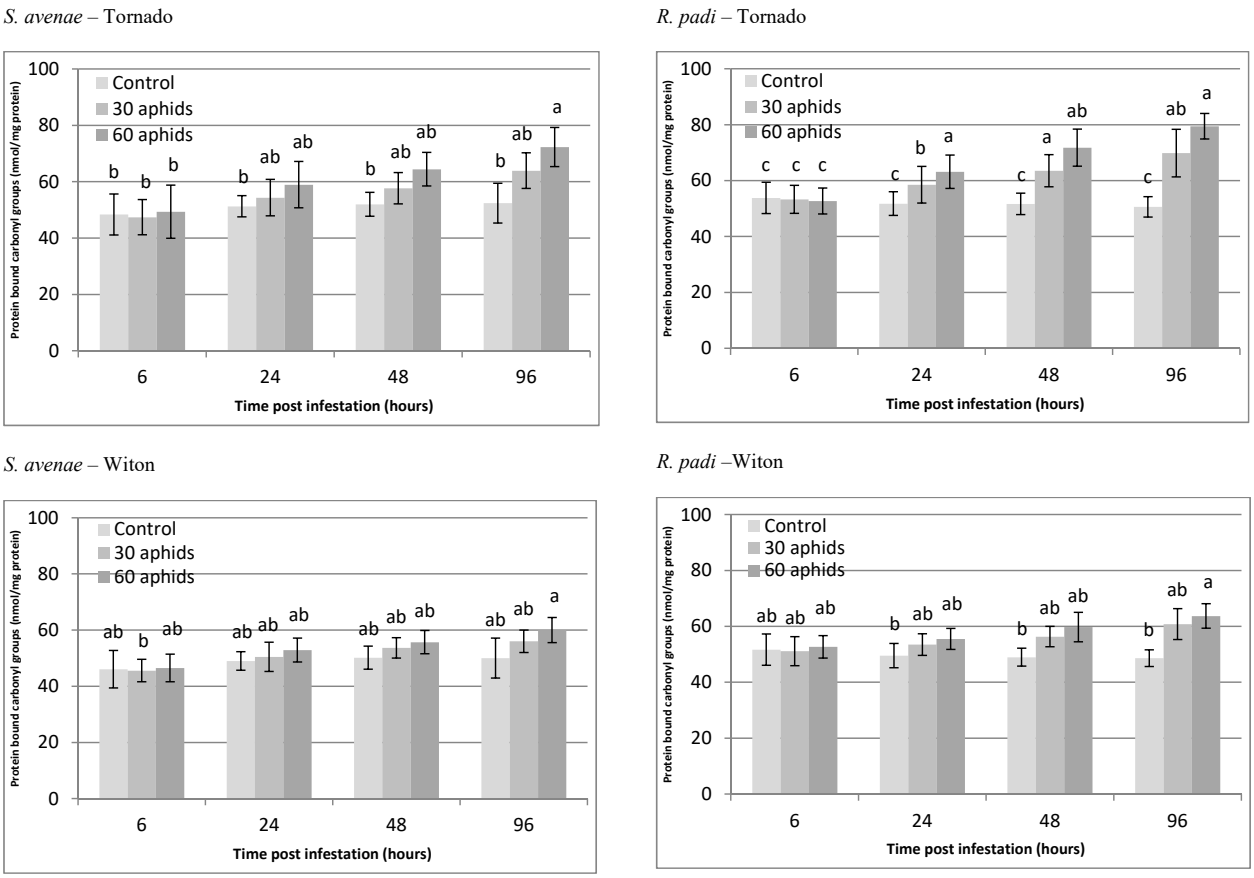

Fig. 4. Influence of the tested cereal aphids on protein-bound carbonyl group levels (nmol/mg protein) in the triticale (Tornado and Witon cv.) seedlings. Values represent means ± standard deviation (SD) from three independent experiments. The different letters above the SD bars indicate significant differences according to Tukey's test ($P \leq 0.05$).

causes protein damage by addition reactions with lysine amino groups, cysteine sulfhydryl groups and histidine imidazole groups (Esterbauer, 1996). There are very few studies that relate lipid peroxidation in plants stressed by sucking-piercing insects. In the current work, we demonstrated that cereal aphid feeding increased MDA content in triticale seedlings, with maximal levels at 48 or 96 hpi. Furthermore, the resistant cultivar (Witon) accumulated more MDA than the susceptible cultivar (Tornado). This finding is in line with the results obtained by Berner and van der Westhuizen (2015), where lipid peroxidation is significantly increased in resistant wheat after 12-h *D. noxia* infestation and continually elevated up to 96 hpi. According to these authors, the MDA increase was 2-fold higher in the infested compared to susceptible wheat cultivars. The opposite findings were reported by Wei et al. (2007), who revealed higher MDA contents in the leaves of alfalfa varieties susceptible to *Aphis medicaginis* Koch compared with the resistant varieties. Additionally, the MDA content rose in both susceptible and resistant varieties during aphid infestation. A small but progressive increase in lipid peroxidation occurs in *P. sativum* (L.) seedlings after *A. pisum* attack, and the strongest lipid damages manifest 72 hpi (Mai et al., 2013). Gall-forming pisyllid infestation of *Eucalyptus* leaves significantly increases lipid peroxidation, measured as the MDA content (Khattab and Khattab, 2005). Similar results were obtained by Khattab (2007), who reported lipid peroxidation induction in cabbage (*Brassica olereacea* L. var. *capitata*) infested with the phloem-sucking aphid *B. brassicae*. The herbivory by the phloem-feeding threecornered alfalfa hoppers (*Spissistilus festinus* Say) elevates lipid peroxidation products and lipoxygenase activity in soybean plants (Felton et al., 1994).

Protein oxidation is one of the earliest plant responses to stressors; it is often used as an oxidative stress marker (Anjum et al., 2005). Thiol

groups and sulphur-containing amino acids are very susceptible to oxidation by ROS and they are the most commonly modified (Rinalducci et al., 2008; Sharma et al., 2012). A decline in PT reflects the oxidation of protein sulfhydryl groups and is an oxidative stress indicator (Ramakrishna and Rao, 2012). There are myriad reports concerning PT oxidation under abiotic stress, but little is known about this protein modification in plants exposed to biotic stress factors. Our study demonstrated more substantial PT content reduction in the resistant Witon cultivar infested with adult cereal aphids. An opposite tendency was noted by Bhoomika et al. (2014), where aluminium (Al) treatment reduced PT content in Al-sensitive rice cultivars, whereas the protein sulfhydryl levels in Al-tolerant cultivar seedlings remained unchanged. The authors speculated that the Al-mediated ROS production and oxidative stress induction are greater in the seedlings of Al-sensitive rather than Al-tolerant cultivars. PT depletion is observed in many plants subjected to abiotic stress factors. For example, protein sulfhydryls are significantly depleted (by approximately 19%) in radish (*Raphanus sativus* L.) seedlings treated with zinc (Ramakrishna and Rao, 2012). Salt stress also decreases PT content in an embryogenic suspension culture of *Dactylis glomerata* L. (Zagorchev et al., 2014). Dehydration significantly depletes PT content in *T. aestivum* L. seedlings; sensitive seedlings exhibit lower PT levels than tolerant ones (Gietler et al., 2016). However, the increase of PT groups occurs in *Z. mays* with increasing copper levels in the growth media (Aly and Mohamed, 2012). Baisakhi et al. (2003) noted a PT increase in a tolerant clone of *Chloris barbata* Swartz exposed to cadmium stress. A similar tendency was observed by Kaur et al. (2018), where earthworm supplementation to cadmium-treated soils increases the PT content in *Brassica juncea* L. plants. Isoproturon-treated maize plants have a higher protein sulfhydryl content than non-treated plants (Nemat Alla and Hassan, 2007). The changes in PT concentrations in plants seem to depend on the type of stress factor and intensity of oxidative stress. Some stressors (e.g., metals and herbicides) may induce PT synthesis, whereas biotic factors (e.g., herbivores) deplete these compounds.

Another important oxidative damage to proteins is the formation of carbonyl derivatives. Protein carbonylation is an irreversible process. It is used as a good indicator of oxidative stress, since the formation of protein carbonyls requires more stringent oxidation conditions than thiol oxidation (Davies, 2005; Møller et al., 2007; Juszczuk et al., 2008). We noted increased protein carbonyl content in the triticale seedlings after female *S. avenae* and *R. padi* infestation. Moreover, there was a higher accumulation of protein-bound carbonyls in the tissues of the susceptible cultivar (Tornado). Similarly, drought-sensitive *Oryza sativa* L. seedlings subjected to water-deficit treatments are characterized by higher increases in protein carbonyl content compared to the tolerant cultivar (Pyngrope et al., 2013). Roychoudhury et al. (2012) proved that carbonylated derivative formation under cadmium stress is greater in salt-sensitive indica rice (IR-29) than in the salt-tolerant variety (Nonabokra). Carbonyl group levels rise upon dehydration in the *Triticum aestivum* L. seedlings, and the protein carbonyl level elevations are higher in sensitive compared to tolerant seedlings (Gietler et al., 2016). Bhoomika et al. (2014) observed an increase in protein carbonyls after exposure of Al-sensitive rice cultivar to Al, but not in the Al-tolerant cultivar. Protein-bound carbonyl content increases in *Z. mays* varieties (Deccan and Sartaj) under chromium stress; there are lower carbonylated protein levels and less ROS accumulation in the Deccan compared to the Sartaj cultivar (Maiti et al., 2012). The protein carbonyl content in Al-sensitive maize plants (S1587-17) increases with elevated Al concentration, but there are no changes in the tolerant variety (Cat100-6; Boscolo et al., 2003). There are not many reports concerning protein carbonylation in plants under the influence of biotic stressors. Exposing maize seedlings (Bosman cv.) to spider mite infestation increases protein carbonyl content and concomitantly reduces catalase, ascorbate oxidase and polyphenol oxidase activities. However, the combination of two stressors (mite feeding and drought) decreases the amount of carbonylated proteins despite the increased activity of all antioxidant enzymes except catalase (Dworak et al., 2016). The authors supposed that protein carbonylation is not directly linked to oxidative stress based on the assessment of antioxidant enzyme activities, but rather it may be the result of a diminished capacity for oxidized protein removal or increased protein susceptibility to oxidative attack.

The results of our study demonstrated greater H_2O_2 accumulation and more substantial protein and lipid damage occurred in triticale seedlings infested with the bird cherry-oat aphid. A similar tendency was noted by Sytykiewicz et al. (2014) and Sytykiewicz (2015), where *R. padi* feeding increased O_2^- and H_2O_2 amounts more substantially than *S. avenae* infestation. Sytykiewicz (2015) noted that the differences in the impact of cereal aphids on the antioxidant status of maize plants might be linked with the chemical composition of aphid saliva, specific routes of stylet penetration or distinct modes of feeding in plant tissues. Łukasik et al. (2012) reported that *R. padi* infestation depletes ascorbate content and induces ascorbate peroxidase activity more strongly in triticale seedlings when compared to

S. avenae infestation. The maize plants stressed by *R. padi* are characterized by more significant decreases in the total antioxidant capacity towards 1,1-diphenyl-2-picnylhydrazyl (DPPH) compared to plants infested with *S. avenae* (Sytykiewicz, 2014). Moreover, the differences in H_2O_2 content and the extent of macromolecule damages in the triticale seedlings stressed by the studied aphid species may be due to the range of host plants. The life-cycle of the oligophagous *R. padi* shows host alternation that involves seasonal migrations between primary hosts (woody plants) and secondary ones (herbaceous plants), whereas the monophagous *S. avenae* life cycle is associated with grasses and cereals (Halarewicz and Gabryś, 2012). Will et al. (2009) postulated that aphid species host range and biotypes are largely determined by the ability of infested plant systems to perceive the salivary proteinaceous elicitors.

CONCLUSION

In summary, the performed studies indicated that cereal aphid feeding evoked oxidative stress in triticale seedlings. This phenomenon was evidenced by elevated H_2O_2 and lipid peroxidation products as well as protein oxidation induction. Moreover, we found differences in the level of oxidative stress markers in triticale exposed to cereal aphid herbivory. The stronger *R. padi*-induced damages of triticale macromolecule suggest that this species more strongly affects the oxidative stress of Tornado and Witon cv.

AUTHORS' CONTRIBUTIONS

Conceived and designed the experiments: IŁ. Performed the experiments: IŁ, SG. Data statistical analysis: SG. Wrote the paper: IŁ, SG. Both authors read and approved the final manuscript.

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