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Biochemical markers of oxidative stress in maize seedlings exposed to rose-grass aphid, *Metopolophium dirhodum*

I. Łukasik and S. Goławska*
Siedlce University of Natural Sciences and Humanities,
Faculty of Exact and Natural Sciences, Institute of Biological Sciences,
Prusa 14, 08-110 Siedlce, Poland
E.Mails: sylwia.golawska@uph.edu.pl, iwona.lukasik@uph.edu.pl

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ABSTRACT

We studied the influence of rose-grass aphid (*Metopolophium dirhodum* L.) infestation on the biochemical markers of oxidative stress in seedlings of two maize (*Zea mays* L.) varieties (relatively resistant Ambrozja and resistant Płomyk). We compared the generation of superoxide anion radicals $O_2^{\cdot-}$, level of hydrogen peroxide (H_2O_2), lipid peroxidation products (MDA) as well as markers of protein damage (protein-bound carbonyl groups). The studied parameters were measured at 24, 48, 72 and 96 h post-initial aphid infestation compared to the non-infested control seedlings. Our studies indicated that the rose-grass aphid feeding evoked oxidative stress in the maize seedlings. Investigated *Z. mays* cultivars exhibited excessive generation of superoxide anion radicals in response to insect treatments. Rose-grass aphid feeding increased the H_2O_2 level in maize tissues with similar levels observed at most periods post-infestation with *M. dirhodum*, also increased lipid peroxidation products with the maximal levels at 48 and 72 h for Ambrozja and 48, 72 and 96 h post-infestation for Płomyk varieties. Further at 48 and 72 h post-initial aphid infestation, there was an increase in protein bound carbonyl groups content in the maize seedlings after infestation with aphids.

Keywords: Biochemical markers, hydrogen peroxide, lipid peroxidation products, maize, *Metopolophium dirhodum*, oxidative stress, protein bound carbonyls, rose-grass aphid, superoxide anion radicals $O_2^{\cdot-}$.

INTRODUCTION

Plants responds to herbivore attack through complex defence strategies [cell wall modification, antixenotic or antibiotic compounds and plant volatiles that repel aphids or attract their natural enemies (16)]. Phloem-feeding insects, including aphids, produce less injuries to plant foliage than chewing insects. So, the piercing-sucking induce the same defence-signalling pathways that are activated by pathogens, such as fungi, viruses and bacteria (52). Aphid feeding may trigger plant signalling pathways driven by jasmonic acid (JA), salicylic acid (SA), ethylene (ET), abscisic acid (ABA), gibberelic acid (GA), reactive oxygen species (ROS) or nitric oxide (NO) that induce the production of chemical defences (16,38,43,50,53). The saliva and the injury caused by aphids induce a local and systemic production of ROS in the plant phloem (10,37,58). The most important ROS include superoxide radical (O_2), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$) and singlet oxygen ($^1O_2$). H_2O_2 plays a key role in plant defence against herbivorous insects, because H_2O_2 can easily diffuse across cell membranes to other compartments and acts as a signalling molecule (8,39).

Zea mays is an important model plant in experimental biology, especially in studies related to the molecular basis of plant-insect interactions, pest resistance mechanisms, and

*Correspondence author

genetic, biochemical and physiological aspects of plant development (14,21,41). In Poland, maize plants are colonized by 4-species of aphids: the rose-grass aphid *Metopolophium dirhodum* (Walk.), the bird cherry-oat aphid *Rhopalosiphum padi* (L.), the corn leaf aphid *Rhopalosiphum maidis* (F.) and the grain aphid *Sitobion avenae* (F.) (2,26,40,45). Aphids transmits wide spectrum of plant viruses viz., barley yellow dwarf virus (BYDV), beet western yellows virus (BWYV), maize dwarf mosaic virus (MDMV), soybean dwarf virus (SbDV) and sugarcane mosaic virus (SCMV) (13,22,34,44,59).

The results of previous experiments revealed that *M. dirhodum* evoked significant changes in the content of thiols, glutathione and activity of GST in the maize plants (31). Based on these data, the purpose of the current work was to determine the changes in the level of biochemical markers of oxidative stress ($O_2^{\cdot-}$, H_2O_2, lipid peroxidation products, carbonyl protein groups) in two varieties of Z. mays (Ambrozja and Płomyk), which differ in susceptibility to *M. dirhodum*.

MATERIALS AND METHODS

I. Aphids

The Experiments were conducted in September-October 2019 at Siedlce University of Natural Sciences and Humanities, Poland, using wingless females (*apterae*) of the rose-grass aphid *M. dirhodum* F. The aphids were obtained from the stock culture, of our University. The aphids were reared on wheat seedlings (*Triticum aestivum* L.) cv. Tonacja in an environmental chamber (21 ºC, 16-h light: 8-h dark photoperiod, 70% relative humidity).

II. Plants

The seeds of two *Z. mays* varieties (Ambrozja and Płomyk) differing in susceptibility to aphids were used. The Ambrozja genotype was previously classified as relatively resistant, whereas Płomyk is resistant to aphid infestation (47,49). The seedlings were grown in an environmental chamber (22±2 ºC/day and 16±2 ºC/night, relative humidity 65±5 %, photoperiod 16L:8D) in plastic pots (10 cm dia; 10 cm height, one seedling per pot) filled with medium nutrient fine structure compost with sand (1000 cm^3 per pot).

III. Infestation procedure

Each 14-day-old maize 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 in all experiments. The control seedling groups were not infested with aphids. The maize seedlings infested with aphids and the non-infested (control) plants were isolated in gauze-covered plastic cylinders. The levels of superoxide anion radical, H_2O_2, lipid peroxidation products, and carbonyl groups in the maize seedling leaves were determined 24, 48, 72 and 96 h after the initial insect infestation.

IV. Superoxide anion radical generation

The production of $O_2^{\cdot-}$ was estimated according to the method of Green and Hill (18), with slight modifications. 0.5 g of the maize seedlings was homogenized in 5 ml of 10 mM phosphate buffer pH 7.8, contained superoxide dismutase inhibitor (1 mM diethyldithiocarbamate). The homogenate was centrifuged at 20000 *g* for 20 min. The obtained supernatant (0.5 ml) was mixed with 0.5 ml of 0.4 mM nitroblue tetrazolium

(NBT) in 10 mM phosphate buffer pH 7.8. The increase in absorbance at 490 nm was monitored against the blank contained 0.5 ml of 0.2 M phosphate buffer pH 7.8 instead of NBT solution. The NBT-reducing activity of plant extracts was expressed as Δ_{A490}/min/mg of protein.

V. H_2O_2 assay

H_2O_2 level was estimated using the method of Zhou *et al.* (61). The seedling tissues (1 g) were homogenized in 5 ml of trichloroacetic acid (TCA) with addition of 50 mg active charcoal in chilled mortars. The homogenates were centrifuged at 15000 *g* for 15 min. Supernatants were adjusted to pH 7.0 using 17 M NH_4OH. 1 ml of supernatant was added to 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). Then the mixture was incubated at 25 °C for 10 min and the absorbance was measured at 510 nm. The H_2O_2 concentration was calculated based on a standard curve and was expressed in nmol/g fresh weight.

VI. Protein-bound carbonyls

Determination of protein carbonyl content was done as per the method of Levine *et al.* (29). Six hundred mg of plant material was homogenized in 50 mM sodium phosphate buffer pH 7.4, with addition of 1 mM ethylenediaminetetraacetic acid (EDTA). After centrifugation at 6000 *g* for 10 min at 4 °C, the supernatant was incubated on ice with 1% streptomycin sulfate for 15 min. Then the supernatant was re-centrifuged at 6000 *g* for 10 min to remove the nucleic acid. 200 µl of obtained supernatant was incubated with 800 µl 10 mM 2,4-dinitrophenyl hydrazine (DNPH) in 2.5 M HCl for 1 h at room temperature (in the dark). The blanks were incubated in 2.5 M HCl alone. After the DNPH reaction, 1 ml 20% TCA was added and samples were incubated for 5 min. Then the samples were centrifuged at 10000 *g* for 10 min. The pellets were washed with an ethanol: ethyl acetate (1: 1) solution three times and the final pellets were dissolved in 6 M guanidine hydrochloride in 20 mM potassium phosphate buffer pH 2.3. After centrifugation at 10000 *g* for 10 min, the absorbance was measured at 375 nm. Concentrations of protein were determined by measuring the absorbance at 280 nm. The carbonyl group content was expressed as nanomoles per mg protein using a molar extinction coefficient 22000 $M^{-1}cm^{-1}$.

VII. MDA assay

The MDA content was determined by the thiobarbituric acid (TBA) reaction (20). The maize seedlings (150 mg) were homogenized with 6 ml 0.1% TCA and centrifuged at 15000 *g* for 15 min. After centrifugation, 1 ml of the supernatant was mixed with 4 ml 0.5% TBA in 20% TCA. The mixture was boiled for 30 min. Thereafter, the mixture was cooling and centrifuged at 10000 *g* for 30 min. MDA concentration was determined from absorbance of supernatants at 535 and 600 nm. MDA content was estimated by subtracting the non-specific absorbance at 600 nm from the absorbance at 532 nm, with molar extinction coefficient equal 155 $mM^{-1}cm^{-1}$.

Statistical analysis

The differences in the superoxide anion radical, H_2O_2, protein-bound carbonyl and lipid peroxidation products contents in aphid-challenged maize 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, three factors were used: the time of feeding, the number of aphids, and the maize cultivar. The response variables were: superoxide

anion radical, H_2O_2 content, lipid peroxidation products, and protein-bound carbonyls. All statistical analyses were provided by Statistica 10.0 (46).

RESULTS AND DISCUSSION

I. Superoxide anion radical level

The rapid increase in ROS generation called „oxidative burst" is one of the earliest responses of plant to aphid infestation (30,32,36,50). Superoxide anion radical is a biologically active molecule and one of the major reactive oxygen species, that may trigger a cascade of events leading to hypersensitive cell death (9). GLM analysis showed that the superoxide anion radical generation ($O_2^{\cdot-}$) was dependent on all study factors (GLM: $F_{6,65} = 63.95$; $P < 0.001$; $R^2 = 0.84$). The significant factors were: the number of aphids (GLM: $F_{2,65} = 121.38$; $P < 0.001$), the time of feeding (GLM: $F_{3,65} = 6.03$; $P < 0.001$) and the maize cultivar (GLM: $F_{1,65} = 122.87$; $P < 0.001$; Table 1).

Table 1. Statistical results of the GLM of the content of superoxide anion radical, hydrogen peroxide, protein bound carbonyls and lipid peroxidation products in aphid-challenged maize plants.

Parameter	df	F	P
Superoxide anion radical			
Time of feeding	3	6.03	= 0.001
Nb of aphids	2	121.38	< 0.001
Maize cultivar	1	122.87	< 0.001
Hydrogen peroxide			
Time of feeding	3	1.68	= 0.180
Nb of aphids	2	78.99	< 0.001
Maize cultivar	1	120.91	< 0.001
Protein bound carbonyls			
Time of feeding	3	10.30	< 0.001
Nb of aphids	2	15.20	< 0.001
Maize cultivar	1	2.17	= 0.146
Lipid peroxidation products			
Time of feeding	3	10.44	< 0.001
Nb of aphids	2	77.29	< 0.001
Maize cultivar	1	1.52	= 0.222

M. dirhodum aphids accelerated the $O_2^{\cdot-}$ production in the colonized Ambrozja and Płomyk maize cultivars compared with the relevant control plants (Fig. 1). Both Ambrozja and Płomyk cv. seedlings treated with higher aphid numbers showed proportionally greater $O_2^{\cdot-}$ accumulation (Fig. 1).

In the current work, the rose-grass aphid feeding increased $O_2^{\cdot-}$ generation in the maize seedlings, reaching maximal levels at 24 and 48 h post-infestation. The resistant variety (Płomyk) showed stronger $O_2^{\cdot-}$ increase than relatively-resistant variety (Ambrozja). The lower initial number of aphid (30 per seedlings) resulted in increments in the superoxide anion radicals content only in the leaves of Płomyk cultivar at 24 h and 48 h post-infestation. Increased number of aphids evoked a significant elevation in $O_2^{\cdot-}$ content in both maize seedlings (Fig. 1). Similar results were obtained by Sytykiewicz *et al.* (50),

where the seedlings of two maize varieties (Ambrozja and Tasty Sweet) colonized with *R. padi* or *S. avenae* aphids responded to an overproduction of O_2^- than non-infested control plants. According to these authors, a stronger increase in O_2^- amounts occurred in the Ambrozja plants (relatively resistant) in relation to Tasty Sweet plants (susceptible). Similar tendency was observed by Mai *et al.* (32) who noted that *Pisum sativum* L. plants infested with the pea aphid (*Acyrthosiphon pisum* Harr.) were characterized by higher contents of O_2^- in relation to the non-stressed control plants. Additionally, the strongest O_2^- generation most significant increase was found at the highest infestation level (30 aphids per seedling). The results obtained by Borowiak-Sobkowiak *et al.* (6) revealed post-infestation enhanced generation of O_2^- in *Asparagus officinalis* L. leaves after colonization by *Brachycorynella asparagi* (Mordv.). Moreover, the strongest O_2^- generation was observed in 96-h leaves of one-month-old plants infested by 30 aphids, the highest density at experiment (6). The studies conducted on seedlings of thuja (*Thuja orientalis* L.) infested with varying populations of *Cinara tujafilina* (Del Guercio) (40 or 80 aphids per plant) showed fluctuations in O_2^- content. The level of O_2^- in *T. orientalis* leaves increased at 24 h post-infestation, to decrease between 24 and 48 h, and to increase and remain high at 72 h, 96 h and 2-weeks feeding by *C. tujafilina* (11). According to these authors, the O_2^- content in 24-h leaves infested with 80 aphids was 1.7 times higher than in the control leaves.

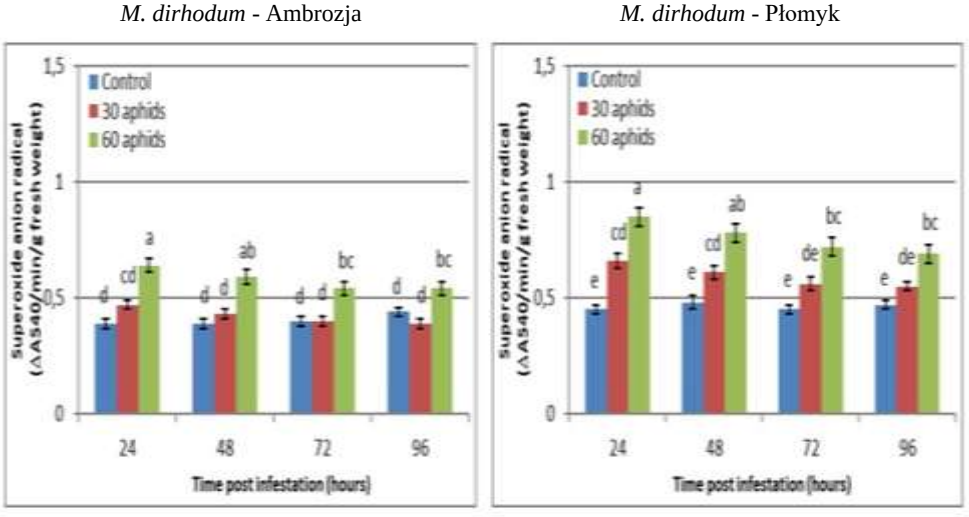


Figure 1. Influence of *M. dirhodum* aphids on levels of superoxide anion radical (ΔA_{540}/min/g fresh weight) in the seedlings of maize (Ambrozja and Płomyk cv.). Values represent means and SD from three independent experiments. The different letters above the SD bars indicate significant differences according to Tukey's test ($P \leq 0.05$).

II. H_2O_2 content

The superoxide anion radical is converted in the dismutation reaction to molecular oxygen (O_2) and less toxic H_2O_2 (50). H_2O_2 plays multiple roles in plant resistance

exhibiting direct toxicity toward herbivores, leading to the cross-linking of cell wall proteins and acting as a signal molecule for the induction of defence genes (35). The exposure of maize plants to *M. dirhodum* caused increase in H_2O_2 content. In our study GLM analysis showed that the H_2O_2 content was dependent on two study factors (GLM: $F_{6,65} = 47.32$; $P < 0.001$; $R^2 = 0.80$). The significant factors were: the number of aphids (GLM: $F_{2,65} = 78.99$; $P < 0.001$) and the maize cultivar (GLM: $F_{1,65} = 120{,}91$; $P < 0.001$; Table 1). Both Ambrozja and Płomyk cv. seedlings treated with higher aphid numbers showed proportionally greater H_2O_2 accumulation (Fig. 2).

M. dirhodum – Ambrozja *M. dirhodum* – Płomyk

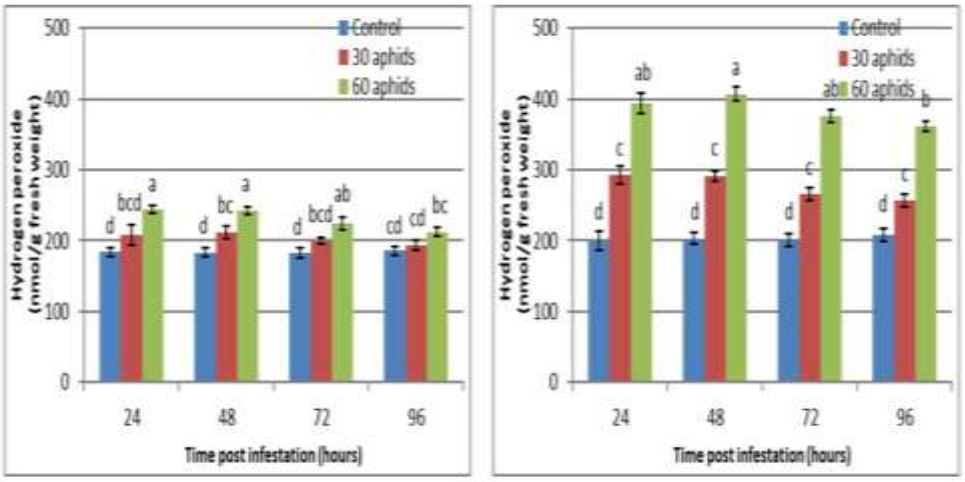


Figure 2. Influence of *M. dirhodum* aphids on levels of hydrogen peroxide (nmol/g fresh weight) in the seedlings of maize (Ambrozja and Płomyk cv.). Values represent means and SD from three independent experiments. The different letters above the SD bars indicate significant differences according to Tukey's test ($P \leq 0.05$).

The level of H_2O_2 in infested plants was independent on time of aphid feeding. Comparing the tested maize varieties, the stronger increase in H_2O_2 content was observed in the seedlings of resistant variety (Płomyk). H_2O_2 level in Ambrozja seedlings stressed by *M. dirhodum* was comparable for the studied aphid densities (30 and 60 aphids per plant) at two periods of infestation (72 and 96h). In the case of Płomyk cultivar, H_2O_2 content was dependent on aphid abundance at all periods of experiment (Fig. 2). Sytykiewicz (48) revealed a progressive elevation in the content of H_2O_2 in the cereal aphid-attacked maize plants until 24 h post infestation. Moreover, the highest increments in the level of H_2O_2 were recorded in the seedlings of resistant maize genotypes in relation to the susceptible ones (48). Kerchev *et al.* (24) observed that 48 h feeding of the peach potato aphid (*Myzus persicae* Sulz.) caused almost 2-fold increase in the H_2O_2 in foliar tissues of potato (*Solanum tuberosum* L.) plants in relation to the uninfested plants. Mai *et al.* (32) demonstrated the increase in the H_2O_2 generation in pea seedlings leaves after infestation of

Acyrtosiphon pisum and the H_2O_2 was maximal after 24 h of infestation. Similarly, strong generation of H_2O_2 at 24 h post infestation in the asparagus aphid-infested leaves of *A. officinalis* was observed (6). Łukasik *et al.* (30) noted that the H_2O_2 amount in *Fabaceae* plants (pea, vetch and broad bean) elevated after *A. pisum* feeding and was maximal at 6 h post infestation. Opposite trend was noted by Kuśnierczyk *et al.* (27), where cabbage aphid (*Brevicoryne brassicae* L.) feeding did not alter H_2O_2 concentration in *Arabidopsis thaliana* L. ecotype Landsberg *erecta* (Ler).

III. MDA content

Excess of ROS may initiate the lipid peroxidation, especially polyunsaturated fatty acid (PUFA) that are most susceptible to the damaging effects of ROS in comparison to fatty acids having one or no double bonds (55). Lipid peroxidation causes decreased membrane fluidity, increases the leakiness of membrane and damage its proteins (55). Among the aldehydes which can be formed as secondary products during lipid peroxidation, malondialdehyde (MDA) has been widely used as a convenient biomarker for lipid peroxidation because of its facile reaction with thiobarbituric acid (TBA) (1). In the current work, the content of lipid peroxidation products were dependent on two study factors (GLM: $F_{6,65}$ = 31,24; $P < 0.001$; $R^2 = 0.72$): the time of feeding (GLM: $F_{3,65}$ = 10.44; $P < 0.001$) and the number of aphids (GLM: $F_{2,65}$ = 77.29; $P < 0.001$) (Table 1). We demonstrated that rose-grass aphid feeding increased MDA content in the maize seedlings, with maximal levels at 48 or 96 h post infestation for Ambrozja cultivar. *M. dirhodum* feeding on Ambrozja cv. affected the MDA content relative to non-stressed control plants, except for 30 aphids per plant at 24 h (Fig. 3).

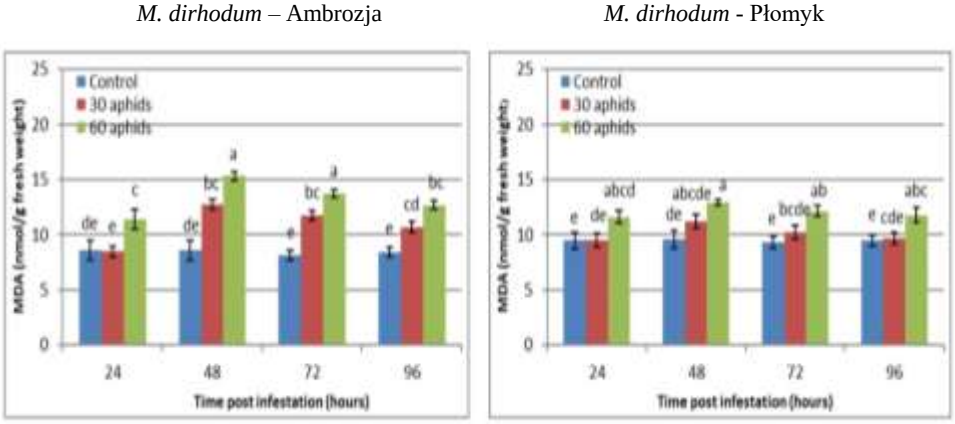


Figure 3. Influence of *M. dirhodum* on MDA content (nmol/g fresh weight) in the seedlings of maize (Ambrozja and Płomyk cv.). Values represent means and SD from three independent experiments. The different letters above the SD bars indicate significant differences according to Tukey's test ($P \leq 0.05$).

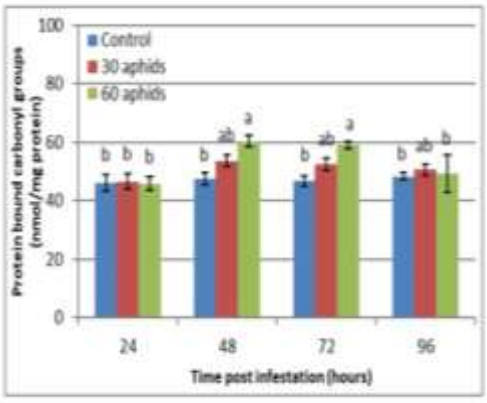
The MDA content was dependent on the aphid density in the Ambrozja seedlings, except for one aphid treatment (96 h) (Fig. 3). In the case of Płomyk cv., the MDA content was comparable in the seedlings infested with 30 and 60 aphids at all periods of aphid

compared to non-infested plants (Fig. 3). Infestation by a higher number of insects (60 per seedlings) evoked a significant elevation in MDA content in Płomyk seedlings, but there were no alterations observed in plants stressed by 30 *M. dirhodum* females in relation to controls at studied cases. Furthermore, the resistant cultivar (Płomyk) accumulated less MDA than the relatively resistant cultivar (Ambrozja). These results are in line with those obtained by Łukasik and Goławska (29), where the triticale infestation with cereal aphids increased lipid peroxidation products in triticale seedlings, with the maximal levels at 48 or 96 h post-infestation. Khattab (25) observed the lipid peroxidation induction in cabbage (*Brassica olereacea* L. var. *capitata*) infested with the phloem-sucking aphid *B. brassicae*. Sytykiewicz *et al.* (51) revealed that MDA content significantly increased in the maize seedlings after *R. padi* infestation and reached the maximum level at 24 h post infestation. According to these authors, the MDA increase was approximately 2-fold higher in Złota Karłowa plants (susceptible cultivar), compared to Waza seedlings (relatively resistant cultivar). Similarly, Wei *et al.* (54) noted the higher MDA content in the leaves of alfalfa varieties susceptible to *Aphis medicaginis* Koch in relation to the resistant varieties. In contrast, Berner and van der Westhuizen (3) reported that the level of lipid peroxidation products significantly increased in resistant wheat after 12-h *Diuraphis noxia* (Mordvilko) infestation and continually elevated up to 96 hpi. Similarly, the resistant triticale cultivar (Witon) stressed by the cereal aphids accumulated more MDA than the infested susceptible cultivar (Tornado) (29).

IV. Protein-bound carbonyls content

The dysfunction of protein metabolism is one of the prime responses to any stress factors (4). Proteins can be damage by direct action of ROS or by secondary damage involving attack by lipid peroxidation products, such as MDA or 4-hydroxy-2-nonenal (HNE) (55). ROS can produce free carbonyl groups by reacting with amino acid side chains of protein molecules, particularly lysine, arginine, histidine, tryptophan and threonine residues (5). Alternatively, protein carbonylation can result from an indirect mechanism involving the hydroxyl radical-mediated oxidation of lipids (9,56). Protein carbonylation is irreversible process and is used as a good indicator of oxidative stress (23).

M. dirhodum – Ambrozja



M. dirhodum – Płomyk

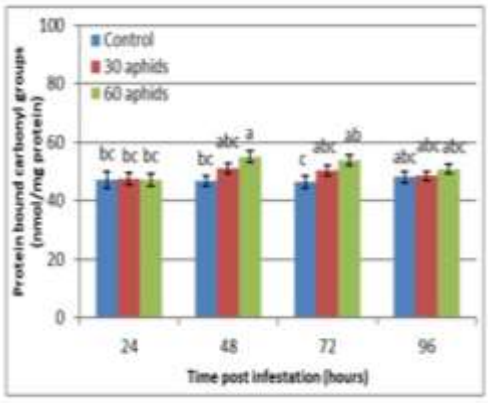


Figure 4. Influence of *M. dirhodum* aphids on levels of protein bound carbonyl groups (nmol/mg protein) in the seedlings of maize (Ambrozja and Płomyk cv.). Values represent means and SD from three independent experiments. The different letters above the SD bars indicate significant differences according to Tukey's test ($P \leq 0.05$).

In our study the content of protein-bound carbonyl groups was dependent on two study factors (GLM: $F_{6,65} = 10.44$; $P < 0.001$; $R^2 = 0.44$): the time of feeding (GLM: $F_{3,65} = 10.30$; $P < 0.001$) and the number of aphids (GLM: $F_{2,65} = 15.20$; $P < 0.001$) (Table 1). The protein-bound carbonyl groups in the tested maize cultivars remained unaffected after *M. dirhodum* colonization, except a higher density of aphids (60 per plant) that increased protein carbonyl content in infested plants after 48 and 72 h, compared to controls (Fig. 4).

Moreover, higher accumulation of protein bound carbonyls occurred in tissues of relatively resistant cultivar (Ambrozja) compared with the resistant cultivar (Płomyk) (Fig. 4). It is in line with results obtained by Sytykiewicz *et al.* (51), where higher accumulation of protein carbonyls occurred in the seedlings of susceptible maize cultivar (Złota Karłowa) stressed by *R. padi*, compared to the relatively resistant cultivar (Waza). Additionaly, the infestation of triticale plants with the cereal aphids was accompanied by stronger protein bound carbonyls' elevation in the tissues of the susceptible cultivar (Tornado) in relation to the resistant cultivar (Witon) (29). There are not many reports concerning the protein carbonylation in plants under biotic stress. The infestation of maize seedlings (Bosman cv.) with spider mite elevated protein carbonyl content coincided with the reduced CAT, APX and PPO activities (12). However, the combination of two stressors - biotic (mite feeding) and abiotic (drought), limited the content of carbonylated proteins despite the increased activity of antioxidant enzymes (12). The formation of protein bound carbonyls in plants exposed to abiotic stress is better documented. The amount of carbonyls in Al-sensitive maize plants (S1587-17 line) increased with an increase of Al concentration, but no changes were observed in the tolerant variety (Cat100-6 line) (7). Gong *et al.* (17) reported an elevated content of carbonyl groups in the leaves of *T. aestivum* under sodium sulfate-caused drought stress. The level of protein bound carbonyl increased in *Z. mays* varieties (Deccan and Sartaj) exposed to chromium and the lower content of carbonyl groups was noted in Deccan cv., characterized by less ROS accumulation than Sartaj (33). Roychoudhury *et al.* (42) reported that the progressive increase in cadmium concentration increased the carbonylated derivatives formation in two rice (*O. sativa*) varieties: salt-sensitive variety of indica rice (IR-29) and salt-tolerant variety (Nonabokra), more effectively in IR-29 variety. The carbonyl groups content increased upon dehydratation in the seedlings of *Triticum aestivum* L. and the elevations in protein bound carbonyls were higher in sensitive seedlings compared to tolerant ones (15).

CONCLUSIONS

The results of our study indicated that the rose-grass aphid feeding is associated with the induction of oxidative stress in maize seedlings, which is evidenced by ROS elevation, as well as by the increase in the content of MDA and protein carbonyls. The aphid-stressed seedlings of resistant maize cultivar (Płomyk) were characterized by less oxidative damages of macromolecules (proteins and lipids) than the seedlings of relative resistant cultivar (Ambrozja).

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