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ORIGINAL PAPER

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EFFECT OF THE CEREAL APHID INFESTATION ON THE OXIDATIVE DAMAGES OF PROTEIN IN THE MAIZE (*ZEA MAYS* L.)

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ABSTRACT

We studied the effect of the cereal aphid (the bird cherry-oat aphid *Rhopalosiphum padi* L. and grain aphid *Sitobion avenae* F.) infestations on the oxidative damages of protein in the maize (*Zea mays* L., cultivar 'Touran') seedlings. We found that the content of protein thiols and protein bound carbonyls were dependent from study factors: time of feeding, the number of aphids and species. In relation to uninfested plants (control), prolonged insect (*R. padi* and *S. avenae*) feeding (24–96 h post infestations) was linked to depletion in levels of protein thiols in foliar tissues of maize genotype and accumulation after 96 h post infestations in maize seedlings investigated by higher number of aphids, protein bound carbonyls. Our results indicated that the biotic stress factors, including aphids evoke the oxidation of protein in the maize. The stronger protein damages occurred in the maize seedlings infested with oligophagous *R. padi* females.

Key words: oxidative damages, protein thiols, protein bound carbonyls, maize, aphids

INTRODUCTION

In the natural environment, plants respond to herbivore attack by development of complex direct and undirect defence strategies. The saliva and the injury caused by phloem-feeding insects, including aphids, induce a local and systemic production of reactive oxygen species (ROS) in the phloem of the host [Divol et al. 2005]. It was well documented that the rapid increase in ROS generation called „oxidative burst" is one of the earliest responses of plant to aphid infestation [Mai et al. 2013, Sytykiewicz et al. 2014]. The equilibrium between beneficial and harmful effects of ROS is a very important aspect, because excess ROS is harmfull to plants, as it can cause lipid peroxidation, protein oxidation and modifications of DNA [Sharma et al. 2012]. ROS oxidize all types of cellular components, but proteins are the most abundant targets of ROS, more than DNA and lipids. ROS cause protein carbonylation, modification of covalent bonds, backbone cleavage, protein unfolding and loss of functionality [Rinalducci et al. 2008]. The damages of cellular compounds by ROS lead to altered intrinsic membrane properties, loss of enzyme activity, protein crosslinking, inhibition of protein synthesis, which results in cell death [Sharma et al. 2012].

Z. mays is an important model organism in plant experimental biology, such as studies of the molecular basis of plant-insect interactions, pest resistance mechanisms, and genetic, biochemical and physiological aspects of plant development [Reddy et al. 2013]. In Poland, maize plants are colonized by four 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.) [Krawczyk et al. 2006, Strażyński 2008, Bereś 2015]. The injection of aphid saliva cause alterations in metabolism

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of host plant, leading to the activation of premature senescing process and programmed cell death in plant organs [Anstead et al. 2010]. Aphids also transmit of wide spectrum of plant viruses, including barley yellow dwarf virus (BYDV), beet western yellows virus (BWYV), maize dwarf mosaic virus (MDMV), soybean dwarf virus (SbDV) and sugarcane mosaic virus (SCMV) [Higashi and Bressan 2012, Stewart et al. 2012, Ferriol et al. 2013].

The results of previous experiments revealed that oligophagous bird cherry-oat aphid evoked more significant increase in the amount of ROS and the activity of antioxidant enzymes as well as more substantial depletions in the level of non-enzymatic antioxidants than monophagous grain aphid [Sytykiewicz 2014, 2015, 2016, Sytykiewicz et al. 2014]. Based on these data, the purpose of the current work was to compare the scale of protein damages caused by ROS in the maize seedlings infested with two cereal aphid species – *S. avenae* and *R. padi*. The biochemical studies encompassed assay of two main markers of protein oxidation – protein bound thiols and carbonyl bound groups.

MATERIAL AND METHODS

Plant material. For experiments cultivar of maize Touran (K230) was used. The seeds were provided KWS, Poland. The seeds (1 seed per plate; three replicates) were sowed into plastic pots, 100 mm in diameter, containing a mixture of medium nutrient fine structure soil composed with sand. The plants were grown in a greenhouse (21°C, 70% r.h., and L16:D8), one plant per pot. The pots were regularly watered and no extra fertilizers were added.

Aphids. Females of parthenogenically reproduced bird cherry-oat aphid, *Rhopalosiphum padi* L. and grain aphid, *Sitobion avene* F., were used for the experiments, which were originated from a stock culture kept at the Siedlce University of Natural Sciences and Humanities, Poland. The aphid colonies were maintained on wheat (*Triticum aestivum*), Tonacja for many generations. Aphids were cultured in a growing chamber maintained at 21 ±1°C, L16:D8 photoperiod, and 70% r.h.

Infestation procedure. Leaves of 14-day-old maize seedlings (1 seedling per plate, three replicates) were colonized with 30 or 60 adult wingless *S. avenae* and *R. padi,* respectively females per plant. The control groups of seedlings were not infested with insects. Maize plants infested with aphids and the non-infested (control) plants were isolated in gauze-covered plastic cylinders. The quantified parameters (protein thiols and protein carbonyls) in maize plants were estimated after 3, 6, 24, 48 and 96 h of the continuous aphid infestation.

Chemical analyses

Determination of protein thiols. The contents of total thiol and protein thiol were determined according to Kok and Kuiper [1986] with minor modifications. Fresh seedling leaves of *Z. mays* weighing 600 mg were grinded to a powder in liquid nitrogen and extracted with ice-cold 0.2M Tris-HCl buffer (pH 7.4), using pre-chilled mortars and pestles. The extracts were centrifuged at 20,000 *g* for 10 min at 4°C. Supernatant was used to assay of total thiols and non-protein thiols. To determination of total thiols, 0.5 ml of supernatant was mixed with 1 ml of 0.2 mM Tris-HCl (pH 8.2) and 0.1 ml of 0.01 M DTNB. The reaction mixture was incubated for 15 min at 30°C. The yellow color developed was measured at 415 nm. A correction was made for the absorbance of 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 the content of non-protein thiols, the protein in supernatant was denatured by incubating in a water bath at 100°C for 5 min and centrifuged at 20,000 *g* for 10 min at 4°C. Non-protein thiols content was determined in a manner similar to that for the total thiols. The content of protein thiols was calculated by subtracting the content of non-protein thiols from the total thiols. Total and protein sulfhydryl groups were calculated from the extinction coefficient of 13,600 M^{-1} cm^{-1}. The content of thiols was expressed as nmol per mg protein. The protein content in the plant extracts was determined using the method given by Bradford [1976].

Determination of protein bound carbonyls. The content of protein bound carbonyl was determined according to the method of Levine et al. [1994]. Fresh seedling leaves of *Z. mays* weighing 600 mg were grinded to a powder in liquid nitrogen and extracted with ice-cold 50 mM sodium phosphate buffer

(pH 7.4) containing 1 mM EDTA, using pre-chilled mortars and pestles. 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. An aliquot of 200 µl of nucleic acid-free supernatants were mixed with 800 µl of 10 mM DNPH (2,4-dinitrophenyl hydrazine) 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 of 20% (w/v) TCA was added, samples were incubated on ice for 5 min and centrifuged at 10.000 for 10 min. The pellets were resuspended in 1 ml of ethanol : ethyl acetate (1 : 1) and centrifuged at 10.000 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 for 10 min. The absorbance was measured at 375 nm. Protein recovery was estimated for each sample by measuring the absorption at 280 nm. The carbonyl group content was calculated using a molar absorption coefficient for aliphatic hydrazones of 22.000 M^{-1} cm^{-1} expressed as nmol carbonyl per mg protein.

Statistical analysis. The differences in the content of protein thiols and protein bound carbonyls in aphid-challenged maize plants were carried out by a general linear models (GLM) followed by the post hoc Tukey's HSD test. In each GLM model two fixed factors were used: time of feeding and the number of aphids. The response variables were: the content of protein thiols and protein bound carbonyls. We used the following assumptions: the dependent variables had a normal distribution, time of feeding was divided into 5 categories (3 h, 6 h, 24 h, 48 h, 96 h), and number of aphids into 3 categories (0 aphids, 30 aphids, 60 aphids). The interaction between factors in all GLM was introduced in the parameterization. All statistical analyses were provided by Statistica 10.0 [StatSoft 2012].

RESULTS

The content of protein thiols in *S. avenae* aphid-challenged maize Touran seedlings were dependent from study factors (GLM: $F_{14,30} = 38.57$; $P < 0.001$; $R^2 = 0.92$). The significant factors in analyses were: time of feeding and the number of aphids and also interaction between these factors (Tab. 1). The level of protein bound carbonyls were dependent from study factors, also (GLM: $F_{14,30} = 3.93$, $P < 0.001$, $R^2 = 0.48$). Both study factors were significant (Tab. 1), but interactions between them was not significant (Tab. 1). The content of protein thiols in *R. padi* aphid-challenged maize plants were dependent from study factors (GLM: $F_{14,30} = 50.61$; $P < 0.001$; $R^2 = 0.94$). The significant factors in analyses were: time of feeding, the number of aphids, and interactions between these factors (Tab. 2). The level of protein bound carbonyls were dependent from study factors, also (GLM: $F_{14,30} = 5.99$, $P < 0.001$, $R^2 = 0.61$). Both study factors were significant, and interaction between them too (Tab. 2).

Short-times (3 h and 6 h) infestation of Touran maize seedlings with *S. avenae* and *R. padi* females did not evoke significant alternations in the content of protein thiols (Tabs 3, 4). Similar trends were observed for level of protein thiols in maize tissues exposed to lower abundance of both aphids (30 per plant) at 24 h post infestations. For the other aphids treatments the experiment revealed that the exposure of the seedlings of maize Touran to *S. avenae* and *R. padi* caused significant fluctuations in protein thiols content in comparison to control non-infested plants (Tabs 3, 4). Protein thiols level decreased after 24 h in Touran seedlings infested with 60 both aphids per plant in relation to unifested controls and all the other *S. avenae* and *R. padi* treatments declined the content of protein thiols in the maize seedlings. In general, greater decreases in the content of protein thiols occurred in the maize seedlings exposed to higher abundance of S. *avenae* (60 per plant), in relation to lower infestation level (30 females per plant). Similar trend was also observed for the level of protein thiols in maize tissues infested by *R. padi* (Tabs 3, 4). Among tested aphid species, *R. padi* responded with the greater depletion in protein thiol contents (51% and 66% decline at 96 and 48 h post infestation with 30 and 60 aphids, respectively), while the lower reduction of protein thiols was noted for *S. avenae* (20% relative to non-infested plants after 96 h with 60 aphids per plant) – Tables 3, 4.

Opposite tendency was noted for protein carbonyl groups where until 48 h post infestation early phases of both aphid infestations (3, 6, 24 and 48 h) did not stimulate any changes in the content of protein

Table 1. Statistical results of the GLM of the content of protein thiols and protein bound carbonyls in *S. avenae* aphid-challenged maize plants

Parameter	df	F	P
Protein thiols			
Time of feeding	4	64.56	<0.001
Nb of aphids	2	73.63	<0.001
Time of feeding*No of aphids	8	16.80	<0.001
Protein bound carbonyls			
Time of feeding	4	8.25	< 0.001
Nb of aphids	2	3.71	0.036
Time of feeding*No of aphids	8	1.82	0.112

df – degrees of freedom, F – ANOVA value, P – probability value

Table 2. Statistical results of the GLM of the content of protein thiols and protein bound carbonyls in *R. padi* aphid-challenged maize plants

Parameter	df	F	P
Protein thiols			
Time of feeding	4	25.45	<0.001
Nb of aphids	2	162.75	<0.001
Time of feeding *No of aphids	8	35.15	<0.001
Protein bound carbonyls			
Time of feeding	4	12.27	<0.001
Nb of aphids	2	6.62	0.004
Time of feeding*No of aphids	8	2.70	0.023

df − degrees of freedom, F – ANOVA value, P – probability value

Table 3. Levels of protein thiols (nmol/g fresh weight) in Touran maize seedlings colonized with *S. avenae* (values are mean ±SD)

Time intervals of aphid infestation (h)	Aphid density (per plant)		
	0	30	60
3	174.88 ±5.41de	177.16 ±5.53de	175.70 ±4.31de
6	173.30 ±5.55def	170.45 ±5.61def	171.86 ±4.23def
24	191.50 ±8.91bcd	179.20 ±5.47cde	150.71 ±6.56fg
48	211.57 ±4.58b	161.91 ±6.81ef	134.25 ±6.28g
96	252.60 ±7.25a	201.27 ±7.19bc	202.76 ±8.97b

Mean followed by different letters are different at $P < 0.05$ (Tukey's test)

Table 4. Levels of protein thiols (nmol/g fresh weight) in Touran maize seedlings colonized with *R. padi* (values are mean ±SD)

Time intervals of aphid infestation (h)	Aphid density (per plant)		
	0	30	60
3	175.82 ±10.16cd	172.74 ±9.64cde	179.49 ±8.77cd
6	177.77 ±10.89cd	167.76 ±16.22cde	182.04 ±10.39bcd
24	190.00 ±11.00bc	151.77 ±4.84cdef	94.04 ±5.07gh
48	215.26 ±10.92b	111.97 ±6.28g	73.34 ±5.56h
96	252.36 ±11.15a	123.53 ±5.73fg	125.90 ±5.17fg

Mean followed by different letters are different at $P < 0.05$ (Tukey's test)

Table 5. Levels of protein carbonyls (nmol/ g fresh weight) in Touran maize seedlings colonized with *S. avenae* (values are mean ±SD)

Time intervals of aphid infestation (h)	Aphid density (per plant)		
	0	30	60
3	51.00 ±3.09bc	50.01 ±2.10bc	51.21 ±2.20abc
6	50.47 ±1.59bc	50.52 ±2.56bc	49.62 ±2.55bc
24	48.04 ±1.86c	48.18 ±2.36c	48.26 ±2.11c
48	51.96 ±1.17bc	52.83 ±1.19bc	55.77 ±1.00b
96	50.61 ±1.41bc	51.94 ±1.87bc	58.34 ±1.47a

Mean followed by different letters are different at $P < 0.05$ (Tukey's test)

Table 6. Levels of protein carbonyls (nmol/ g fresh weight) in Touran maize seedlings colonized with *R. padi* (values are mean ± SD)

Time intervals of aphid infestation (h)	Aphid density (per plant)		
	0	30	60
3	49.76 ±1.54c	49.79 ±1.09c	49.46 ±1.82c
6	54.50 ±4.37abc	55.84 ±3.43abc	55.40 ±2.58abc
24	48.44 ±1.18c	46.57 ±4.37c	47.60 ±2.47c
48	52.28 ±2.39bc	53.23 ±2.49abc	61.66 ±3.13ab
96	50.47 ±2.34c	54.39 ±2.99abc	62.32 ±2.01a

Mean followed by different letters are different at $P < 0.05$ (Tukey's test)

CO groups in Touran cultivar (Tabs 5, 6). Prolonged aphid feeding (96 h) evoked a significant elevation of protein carbonyl contents only for higher abundance of aphids (60 per plants). Furthemore, at 96 h post infestation higher increments in protein carbonyl amounts were noted in maize seedlings attacked with 60 *R. padi* females per plant (24% increase in relation to the control) than in seedlings attacked with *S. avenae* (Tabs 5, 6).

DISCUSSION

Abiotic stresses such as drought, chilling, salinity as well as pathogens and herbivores attack evoke accumulation of ROS in plant tissues, leading to disruption of cellular homeostasis [Sharma et al. 2012]. Loss of protein thiol groups is regarded as one of the most instant responses occurring due to oxidative stress [Bhoomika

et al. 2014]. Among protein amino acids the most susceptible for ROS attack are the sulphur containing ones, cysteine and methionine [Rinalducci et al. 2008, Sharma et al. 2012]. The thiol group of cysteine can be oxidized to disulfide, sulphenic acid, sulphinic acid and sulphonic acid. In the current work it has been recorded that the cereal aphids infestation caused the depletion of protein –SH groups in the maize seedlings. Similar results were obtained by Łukasik et al. [2019], where the rose-grass aphid *Methopolophium dirhodum* Walk. infestation significantly limited protein thiols' content in maize cultivars ('Złota Karłowa', 'Ambrozja' and 'Płomyk'). Moreover, the strongest decline in the protein –SH level was observed in 'Płomyk' seedlings (resistant variety) and the lowest was noted in 'Złota Karłowa' plants (susceptible variety) [Łukasik et al. 2019]. Sytykiewicz et al. [2019] demonstrated more substantial reduction of protein thiols' content in resistant maize cultivar ('Waza') infested with *R. padi*. Similar tendency was observed by Łukasik and Goławska [2019] where *R. padi* and *S. avenae* infestations of triticale triggered more substantial protein –SH depletion in the seedlings of 'Witon' (resistant cultivar) compared to 'Tornado' (susceptible cultivar). However, most reports have concerned the oxidation of protein thiols under abiotic stress, but still little is known about this protein modification in plants exposed to biotic stress factors. Gietler et al. [2016] observed a significant decrease in the content of –SH proteins in *Triticum aestivum* L. seedlings under dehydratation. According to these authors, the protein –SH content was reduced to 19% of the control of sensitive seedlings and to 37% of the control tolerant seedlings. Similar tendency was noted by Bhoomika et al. [2014] where aluminium treatment relevantly reduced protein thiols content in Al-sensitive rice cultivar whereas the level of protein sulfhydryls in the seedlings of Al-tolerant cultivar remained unchanged. The salt stress caused the decrease in protein sulfhydryls in embryogenic suspension culture of *Dactylis glomerata* L. [Zagorchev et al. 2014]. However, the increase of protein bound –SH groups was seen in many plants subjected to some types of abiotic stress. Namely, Aly and Mohamed [2012] recorded that the protein thiols content in *Z. mays* was significantly increased with increasing of copper level in the growth media. Thus, the modifications of protein thiol groups in plants seem to be depended on the type of stress factor and intensity of oxidative stress.

In contrast to the modifications of thiol groups, protein carbonylation is irreversible process, in which lysine, arginine, proline and threonine side-chains can be oxidatively converted to reactive aldehyde or ketone groups, causing inactivation, crosslinking or breakdown of proteins [Rinalducci et al. 2008]. Alternatively, protein carbonylation can result from an indirect mechanism involving the hydroxyl radical-mediated oxidation of lipids. Lipid peroxidation products can diffuse across membranes and the reactive aldehyde-containing lipids may covalently modify proteins localized throughout the cell [Grimsrud et al. 2008]. We noted increase of protein carbonyl content in *Z. mays* seedlings after the prolonged feeding (96 h) of cereal aphid females. There are not many reports concerning the protein carbonylation in plants under the influence of biotic stressors. Sytykiewicz et al. [2019] reported an increase in the content of protein carbonyls in maize seedlings after infestation with *R. padi*. According to these authors, higher accumulation of protein-bound carbonyls occurred in tissues of susceptible cultivar ('Złota Karłowa') in comparison to relatively resistant ('Waza'). The similar tendency was noted by Łukasik and Goławska [2019], where the infestation of triticale plants with the cereal aphids was accompanied with stronger protein bound carbonyls' elevation in the tissues of the susceptible cultivar ('Tornado') than in the resistant cultivar ('Witon'). Dworak et al. [2016] noted that the infestation of maize seedlings ('Bosman' cv.) with spider mite caused increase in protein carbonyl content coincided with the reduced CAT, APX and PPO activities in comparison to control plants. 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 (except CAT). Authors speculated that protein carbonylation is not directly linked to oxidative stress based on the level of antioxidant enzyme activities, but may be resulted from diminished capacity of oxidized protein removal or increased protein susceptibility to oxidative attack. The formation of protein bound carbonyls in plants exoposed to abiotic stress factors is well documented. Exposure of maize seedlings to Pb enhanced reactive carbonyl groups (425–512%) during 3–24 h

of treatment [Kaur et al. 2015]. Bhoomika et al. [2014] noted increase of protein carbonyl level after exposure of Al-sensitive rice cultivar to aluminium, but not in the Al-tolerant cultivar. Protein bound carbonyl content increased in *Z. mays* varieties ('Deccan' and 'Sartaj') under chromium stress and the lower level of carbonyl derivatives was noted in 'Deccan' cv., characterized by less accumulation of O_2^- and H_2O_2 than 'Sartaj' [Maiti et al. 2012].

The depletion of protein bound thiols in the maize seedlings attacked by cereal aphids started at 24 h of aphids' feeding and the reduction of –SH protein was the greatest after 48 h of experiment. Sytykiewicz et al. [2014] and Sytykiewicz [2015] revealed the maximal O_2^- formation in the maize seedlings at 4 or 8 h after infestation with cereal aphids, whereas the next studied period (24 h) was associated with the highest rise in H_2O_2 amounts. At his time the greatest decreases in glutathione level in maize seedlings stressed by cereal aphids were noted, but the strongest diminution in content of ascorbate occurred after 96 h of insects' feeding [Sytykiewicz 2016]. Similarly, in our study the prolonged feeding (96 h) evoked the increase in carbonyl groups content in maize seedlings stressed by cereal aphids. Protein carbonylation is used as a good indicator of oxidative stress since the formation of protein carbonyls requires more stringent oxidation conditions than for the oxidation of thiols [Juszczuk et al. 2008].

The results of this study demonstrated that more substantial damages of protein, expressed as protein thiols' depletion and carbonyl groups increase, occurred in maize seedlings infested with oligophagous *R. padi*. It is accordance with results obtained by Sytykiewicz et al. [2014] and Sytykiewicz [2015] where bird cherry-oat infestation led to a greater increase in O_2^- and H_2O_2 amounts than grain aphid attack. Moreover, it has been also identified more substantial depletions in ascorbate and glutathione content as well as greater induction of antioxidant enzymes (ascorbate peroxidase, glutathione reductase) in the maize seedlings colonized by *R. padi*, compared to *S.avenae*-infested plants [Sytykiewicz 2016]. It seems likely that the higher generation of ROS together with the stronger depletion in non-enzymatic antioxidants level in the maize plants attacked by *R. padi*, favors oxidation of protein in infested seedlings. Sytykiewicz [2015] noted that the differences in the impact of cereal aphids on the antioxidant responses of maize might be linked with a diverse chemical composition of aphid saliva, specific routes of stylet penetration or distinct modes of feeding in plant tissues. The maize seedlings infested with *R. padi* showed a more significant decrease in the total antioxidant capacity towards DPPH (1,1-diphenyl-2-picnylhydrazyl) in relation to plants colonized by *S. avenae* [Sytykiewicz 2014]. Furthermore, the differences in damages of proteins in the maize seedlings attacked by two studied aphid species may be due to the range of host plants. The life-cycle of oligophagous *R. padi* shows host alternation involving seasonal migrations between primary hosts (woody plants) and secondary ones (herbaceous plants) remote systematically, whereas the life cycle of monophagous *S. avenae* is associated with grasses and cereals. Greater diversity of host plants indicate that *R. padi* should be better adapted to the chemical composition of plants.

CONCLUSIONS

Our experimental results indicated that the cereal aphids' feeding is associated with the oxidation of protein in maize seedlings, which is evidenced by depletion in the level of protein-bound thiols, as well as by the increase in the protein carbonyls. The stronger damages of maize proteins evoked by *R. padi* indicate that the oligophagous aphids species stronger affected the oxidative status of their host plants.

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