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Differential Expression of Superoxide Dismutase Genes in Aphid-Stressed Maize (*Zea mays* L.) Seedlings

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Abstract

The aim of this study was to compare the expression patterns of superoxide dismutase genes (*sod2*, *sod3.4*, *sod9* and *sodB*) in seedling leaves of the *Zea mays* L. Tasty Sweet (susceptible) and Ambrozja (relatively resistant) cultivars infested with one of two hemipteran species, namely monophagous *Sitobion avenae* F. (grain aphid) or oligophagous *Rhopalosiphum padi* L. (bird cherry-oat aphid). Secondarily, aphid-elicited alternations in the antioxidative capacity towards DPPH (1,1-diphenyl-2-picrylhydrazyl) radical in insect-stressed plants were evaluated. Comprehensive comparison of expression profiles of the four *sod* genes showed that both insect species evoked significant upregulation of three genes *sod2*, *sod3.4* and *sod9*). However, aphid infestation affected non-significant fluctuations in expression of *sodB* gene in seedlings of both maize genotypes. The highest levels of transcript accumulation occurred at 8 h (*sod2* and *sod3.4*) or 24 h (*sod9*) post-infestation, and aphid-induced changes in the expression of *sod* genes were more dramatic in the Ambrozja cultivar than in the Tasty Sweet variety. Furthermore, bird cherry-oat aphid colonization had a more substantial impact on levels of DPPH radical scavenging activity in infested host seedlings than grain aphid colonization. Additionally, Ambrozja plants infested by either hemipteran species showed markedly lower antioxidative capacity compared with attacked Tasty Sweet plants.

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Introduction

The global production and economic importance of maize (*Zea mays* L.) have steadily increased during the last decade. This increment is likely due to increased worldwide distribution, the adaptability of maize to multifarious environmental conditions, and the introduction of high-yield varieties [1–2]. Maize is a substantial source of raw materials for the pulp and paper industries as well as for fermentation processes in biogas and bioethanol synthesis [1–4]. Additionally, *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 [1–3].

Aphids (Hemiptera, Aphidoidea) are one of the most destructive groups of insects colonizing a large number of maize varieties [5–8]. Infestation of the host plants by these piercing-sucking hemipterans lead to a wide spectrum of deleterious effects, including ultrastructural organ damage, severe depletion of phloem sap constituents, and perturbation of many fundamental physiological processes such as photosynthesis, cellular respiration, growth, and development. Long-term and/or large-scale aphid colonization may result in additional detrimental effects such as large chlorotic lesions, stress-induced premature senescence, apoptosis, and local necrosis [9–17]. Furthermore, these arthropods serve as vectors for a broad range of plant-pathogenic viruses [18–19]. Aphid watery saliva, which is injected into target plant tissues, contains a broad collection of hydrolytic enzymes, metabolic effectors, and toxic compounds that may also stimulate the host to excessive formation of reactive oxygen species (ROS) [20–24]. Circumstantial disturbance of intracellular redox homeostasis in stressed plants may result in diverse cytotoxic effects and initiate the cascade of reactions leading to programmed cell death. Prolonged overproduction of various ROS can lead to peroxidation of membrane lipids and pigments, denaturation of proteins, damage to DNA, and fragmentation of polysaccharides [25–29].

Higher plants have evolved a complex network of antioxidant systems to counteract elevated ROS levels produced in response to unfavorable environmental conditions. This sophisticated machinery encompasses a wide range of lipid- and water-soluble antioxidants (e.g., tocopherols, β-carotene, ubiquinone, ascorbate, glutathione) and antioxidant enzymes such as superoxide dismutases (SODs), catalase, glutathione transferase, glutathione peroxidase, and ascorbate peroxidase [25], [27], [30–36]. SODs are a group of metalloenzymes that constitute the primary line of antioxidative defense by catalyzing the dismutation reaction of superoxide anion radical (O_2^-) to oxygen (O_2) and hydrogen peroxide (H_2O_2). In plants, three types of SODs (Cu/ZnSODs, FeSODs and MnSODs) have been identified, differing in the metal cofactor present within the active site. Cu/ZnSOD isoforms are found in the cytosol, chloroplasts, mitochondria, peroxisomes, and extracellular space, FeSOD isozymes are present in chloroplasts, and MnSODs are localized to mitochondria [25], [37–42]. Significant modulations in SOD activity have been observed in a variety of plants exposed to a broad range of environmental stresses, such as drought [43–47], high or low temperature [48–

50], ultraviolet-B irradiation [51–52], darkness [53], high salinity [54], nitrogen deficiency [55], supplementation with carbohydrates [56], herbicide treatment [57], heavy metal exposure [28], [58–60], magnetic field influence [61], and pathogen infection [62–66].

Although aphid-stimulated physiological and biochemical modulations of a wide spectrum of infested host systems have been extensively studied, the specific effects on the antioxidant machinery remain unclear. To date, there are no available studies regarding the regulation of *sod* genes and antiradical activity against DPPH• in aphid-susceptible and aphid-resistant maize varieties. There is a lack of comparative data regarding the influence of mono- and oligophagous aphid species on the expression patterns of *sod* genes and levels of the antioxidative capacity in colonized *Z. mays* seedlings. It has been hypothesized that susceptible and resistant maize cultivars differ in their transcriptional regulation of *sod* genes and antioxidant activity in response to aphid infestation. Hence, the main purpose of the study was to compare the transcriptional activity of *sod* genes (*sod2*, *sod3.4*, *sod9* and *sodB*) in seedling leaves of the maize Tasty Sweet (susceptible) and Ambrozja (relatively resistant) cultivars colonized by monophagous *Sitobion avenae* F. (grain aphid) or oligophagous *Rhopalosiphum padi* L. (bird cherry-oat aphid). Additionally, it was evaluated whether changes in the relative expression of these *sod* genes and DPPH radical scavenging activity in the stressed maize plants reflect levels of aphid infestation.

Methods

Plant material

Tasty Sweet and Ambrozja *Z. mays* seeds were purchased from local garden supply companies. Plants were grown in a climate controlled chamber at 22±2°C/16±2°C (day/night), relative humidity of 65±5%, light intensity 100 μM m^{-2} s^{-1}, and a long-day photoperiod (light 16 h: dark 8 h). Seedlings were separately planted in round plastic pots (10×9 cm; diameter × height) filled with general-purpose horticultural substrate with no additional fertilization. According to Sytykiewicz et al., Tasty Sweet and Ambrozja maize varieties were classified as aphid-susceptible and aphid-relatively resistant, respectively [8].

Aphids

Apterous parthenogenetic females of the two aphid species were gathered from cereal plants in the Siedlce district (Poland) and reared for 1 year on the seedlings of *Triticum aestivum* L. (Tonacja variety) at the Department of Biochemistry and Molecular Biology, University of Natural Sciences and Humanities, Siedlce. The insects were maintained in the climate controlled plant growth chamber described above. To sustain the aphid populations, new *T. aestivum* plants were added every week, and the old plants were removed after the aphids had settled on the new seedlings. Adult wingless aphids were used in the experiments.

Experimental design

The bioassays were performed on leaves of 14-day-old seedlings of the two maize cultivars that were artificially infested with 10, 20, 40, or 60 adult apterous females aphids per plant. Control plants were not infested with the insects. Transcriptional activity of the SOD isozyme genes (*sod2*, *sod3.4*, *sod9* and *sodB*) and the antioxidative capacity in the leaves of maize seedlings were measured at 1, 2, 4, 8, 24, 48, and 72 h post-initial aphid infestation (hpi). Aphid-stressed and control *Z. mays* seedlings were caged individually in transparent plastic cylinders (20×50 cm; diameter × height) covered with nylon mesh. To terminate each series of experiments, aphids were removed from the infested maize plants, and the leaves were excised and immediately subjected to further analysis.

Assay of DPPH radical scavenging activity

The antioxidative activity of maize extracts towards DPPH• (1,1-diphenyl-2-picrylhydrazyl radical) was determined according to the method of Brand-Williams et al. [67], with minor modifications. Freshly harvested seedling leaves (1.0 g) of *Z. mays* were homogenized with 15 cm^3 of methanol, and next, the samples were vigorously vortexed for 30 min. The cell-free homogenates were filtered through four layers of mesh gauze and centrifuged at 10,000 *g* for 15 min, and after that the pellet was discarded. The reaction mixture consisted of 20 mm^3 of the supernatant and 365 mm^3 of DPPH methanolic solution (0.04%; *w/v*), the control probe and the samples were incubated at room temperature for 30 min. L-ascorbic acid was used as a positive control. The absorbance at 517 nm was measured using an Epoch UV-Vis microplate spectrophotometer (BioTek, USA). The antioxidative activity of the maize extracts was calculated using the following formula: DPPH• scavenging activity (%) = $A_0 - A_S / A_0 \times 100$ (where A_0 is absorbance of the control - DPPH• solution; A_S – absorbance of the tested *Z. mays* samples).

Total RNA isolation and quantification

Total RNA was extracted from the aphid-colonized and control *Z. mays* seedlings. The leaves were harvested and immediately homogenized in liquid nitrogen using a sterile, RNase-free ceramic mortar and pestle. Total RNA was isolated using the Spectrum Plant Total RNA kit (Sigma Aldrich, Poland), and residual genomic DNA was enzymatically hydrolyzed with the On-Column DNase I Digestion Set (Sigma Aldrich, Poland). The purified RNA was quantified using the Epoch UV-Vis microplate spectrophotometer. Additionally, $A_{260/280}$ and $A_{260/230}$ ratios were calculated to estimate RNA integrity and purity. Only RNA samples of high quality ($A_{260/280}>2.0$ and $A_{260/230}>1.8$) were selected for further analysis.

cDNA synthesis

Purified total RNA (1 μg) was used for reverse transcription using the RevertAid Premium First Strand cDNA Synthesis kit (Fermentas, Poland) and oligo$(dT)_{18}$ primers. Two types of negative control reactions were prepared: no template and no reverse transcription.

Quantitative PCR

Transcriptional activity of *sod* genes in the leaves of aphid-infested and control *Z. mays* seedlings was analyzed by quantitative PCR. Expression levels of *sod2*, *sod3.4*, and *sodB* were estimated using gene-specific TaqMan Gene Expression assays (Life Technologies, Poland). Table S1 lists the identification numbers of the assays and reference sequences. The gene encoding glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) was used an the internal control. The expression of *gapdh* and *sod9* genes was quantified with Custom TaqMan Gene Expression assays. Primer sequences and fluorescent probe are shown in Table S2. Expression of the target genes was evaluated in 96-well microplates on the StepOne Plus Real-Time PCR System using the StepOnePlus Software v2.3 (Applied Biosystems, USA). The final PCR reaction volume was 20 mm^3 and consisted of 10 mm^3 2× TaqMan Fast Universal PCR Master Mix, 1 mm^3 20× TaqMan Gene Expression assay mixture (containing a pair of primers and a TaqMan 6-carboxyfluorescein-labeled minor

Table 1. DPPH radical scavenging activity (%) of methanolic extracts prepared from *R. padi*-infested maize seedlings.

Duration of aphid colonization (hpi)	Levels of infestation (number of aphids per seedling)				
	0	10	20	40	60
Ambrozja cultivar					
0	28.2±1.5a	28.2±1.5a	28.2±1.5a	28.2±1.5a	28.2±1.5a
1	28.4±1.6a	28.1±1.4a	28.6±1.7a	28.3±1.5a	27.9±1.4a
2	28.1±1.4a	28.3±1.5a	28.2±1.5a	27.6±1.4a	26.7±1.3ab
4	28.6±1.7a	28.4±1.6a	28.6±1.5a	26.9±1.3b	25.3±1.1b
8	28.8±1.9a	28.8±1.7a	27.7±1.4a	25.2±1.0b	22.4±0.8c
24	29.0±2.1a	27.3±1.4ab	26.8±1.3b	24.0±0.8bc	20.2±0.7c
48	28.3±1.6a	26.8±1.3b	25.2±1.1b	21.4±0.7c	16.3±0.5d
72	28.5±1.7a	25.7±1.1b	24.5±1.0b	18.6±0.6c	12.6±0.3d
Tasty Sweet cultivar					
0	25.6±1.3a	25.6±1.3a	25.6±1.3a	25.6±1.3a	25.6±1.3a
1	25.2±1.2a	25.4±1.2a	25.3±1.2a	25.1±1.1a	25.2±1.2a
2	25.5±1.3a	25.8±1.4a	25.2±1.2a	25.6±1.3a	25.1±1.1a
4	25.3±1.2a	25.1±1.1a	25.6±1.3a	24.9±1.0a	24.6±0.9ab
8	24.9±1.0a	25.0±1.0a	25.1±1.1a	24.7±0.9ab	24.5±0.9ab
24	25.0±1.0a	25.5±1.2a	24.8±1.0a	24.1±0.9ab	23.2±0.8b
48	25.1±1.1a	24.6±1.0a	24.6±0.9a	23.5±0.8b	22.4±0.7b
72	25.7±1.3a	24.7±0.9a	24.0±0.9ab	22.9±0.7b	20.8±0.5bc

Values are presented as the mean ± SD of three replicates; hpi-hours post-initial aphid infestation. Antioxidant capacity of the maize extracts is expressed as the percent inhibition of DPPH radical. The average values in rows denoted by different letters are statistically significant (Tukey's test; $P \leq 0.05$).

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groove binder probe), 4 mm^3 of cDNA, and 5 mm^3 of RNase-free water. Amplification curves were generated under the following thermal parameters: 95°C for 20 s (activation of AmpliTaq Gold DNA Polymerase (Life Technologies, Poland), followed by 40 cycles of 95°C for 1 s and 60°C for 20 s. The comparative C_T ($\Delta\Delta C_T$) method of Livak and Schmittgen [68] was used to calculate the relative expression of each target gene, and the mean values are presented as the fold change ± standard deviation (SD) in the transcript in aphid-infested samples compared with the controls.

Statistical analysis

All data are expressed as the mean ± SD of three independent biological replicates. Significance of differences in the expression of *sod* genes and levels of the antioxidative capacity between the aphid-colonized seedlings of each variety and the relevant control plants was assessed by a factorial analysis of variance (ANOVA). The factorial ANOVA comprised the evaluation of four factors: maize genotype (Ambrozja and Tasty Sweet), aphid species (*R. padi* and *S. avenae*), treatment (10, 20, 40 and 60 aphids per plant), and time post-infestation (0, 1, 2, 4, 8, 24, 48 and 72 hpi). Subsequently, the *post-hoc* analysis was carried out by using the Tukey's test (P-values ≤ 0.05 were considered statistically significant). The obtained results were analyzed using STATISTICA 10 software (StatSoft, Poland).

Results

The influence of aphid colonization on the antioxidative potential of maize seedlings

The performed studies revealed that the investigated aphid species (*R. padi* or *S. avenae*) led to a decrement in levels of the antioxidative capacity towards DPPH radical within the seedling leaves of Ambrozja and Tasty Sweet genotypes in relation to the uninfested plants (Table 1, 2). More severe decline in DPPH radical scavenging activity in the insect-colonized maize seedlings was influenced by the bird cherry-oat aphid feeding when compared to *S. avenae* infestation (e.g. 72-hour feeding of 60 *R. padi* insects evoked 17% and 2% greater decrease in the analysed parameter in Ambrozja and Tasty Sweet plants, respectively, when compared to changes stimulated by the grain aphid). Additionally, seedlings of Ambrozja genotype colonized by the tested hemipteran species characterized by more significant deceleration in levels of the antioxidative potential (e.g. 1–5% and 2–19% greater decrease in plants infested by 10 and 60 aphids, respectively) in comparison with Tasty Sweet variety. Conducted experiments demonstrated that the scale of aphid-triggered depletion in the antioxidant activity in maize seedlings was dependent on duration of aphid exposure and insect density per plant. Exemplarily, the highest infestation level (60 *R. padi* aphids per plant) and the longest duration of insect colonization (72 hpi) resulted in 33 and 14% decrease of the analysed parameter in Ambrozja and Tasty Sweet plants, accordingly, while a lower decline (16 and 12%, respectively) was estimated in the investigated maize cultivars infested by the grain aphid. On the other hand, time-course analysis revealed that the antioxidative capacity in Tasty Sweet seedlings infested by 10 *S. avenae* aphids remained unchanged at different intervals of aphid exposure (1–72 hpi) when compared to the relevant controls, whereas colonization of these plants by the same number of bird cherry-oat aphid individuals led to a slight decline (3–4%) at 48 and 72 hpi, respectively. Ambrozja plants infested by the lowest number of *R. padi* or *S. avenae* aphids (10 per plant) responded a similar decrement (2–9% or 2–6%, respectively) in levels of the

Table 2. DPPH radical scavenging activity (%) of methanolic extracts prepared from *S. avenae*-infested maize seedlings.

Duration of aphid colonization (hpi)	Levels of infestation (number of aphids per seedling)				
	0	10	20	40	60
Ambrozja cultivar					
0	28.2±1.5a	28.2±1.5a	28.2±1.5a	28.2±1.5a	28.2±1.5a
1	28.4±1.6a	28.1±1.4a	28.5±1.7a	28.6±1.7a	28.3±1.6a
2	28.1±1.4a	28.9±2.0a	28.1±1.4a	28.7±1.8a	28.1±1.4a
4	28.6±1.7a	28.2±1.5a	28.7±1.8a	27.5±1.4a	26.9±1.3b
8	28.8±1.9a	28.3±1.6a	28.4±1.6a	27.3±1.4ab	26.5±1.3b
24	29.0±2.1a	28.2±1.5a	28.0±1.4a	26.4±1.3b	26.1±1.2b
48	28.3±1.6a	27.5±1.4a	27.2±1.4ab	24.9±1.0bc	25.0±1.0bc
72	28.5±1.7a	27.0±1.3a	26.8±1.3ab	24.8±1.0ab	23.9±0.8c
Tasty Sweet cultivar					
0	25.6±1.3a	25.6±1.3a	25.6±1.3a	25.6±1.3a	25.6±1.3a
1	25.2±1.2a	25.2±1.2a	25.4±1.2a	25.8±1.3a	25.0±1.1a
2	25.5±1.3a	25.7±1.3a	25.6±1.3a	25.9±1.4a	25.2±1.2a
4	25.3±1.2a	25.2±1.2a	25.0±1.1a	25.4±1.2a	24.8±1.0a
8	24.9±1.0a	25.1±1.1a	24.9±1.0a	24.8±1.1a	24.2±0.9ab
24	25.0±1.0a	25.5±1.3a	24.7±1.0a	24.6±0.9ab	23.3±0.8b
48	25.1±1.1a	25.3±1.2a	24.4±0.9ab	24.3±0.9ab	22.8±0.7b
72	25.7±1.3a	25.2±1.2a	24.2±0.9ab	23.6±0.8b	22.6±0.7bc

Values are presented as the mean ± SD of three replicates; hpi - hours post-initial aphid infestation. Antioxidant capacity of the maize extracts is expressed as the percent inhibition of DPPH radical. The average values in rows denoted by different letters are statistically significant (Tukey's test; P≤0.05).

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antioxidative potential after 8–24 hours of aphid feeding. The factorial analysis of variance evidenced that four tested factors and interactions between these parameters statistically affected levels of the antioxidative capacity in the maize seedlings (Table 3).

Expression profiles of *sod2* in *R. padi*– and *S. avenae*–stressed maize seedlings

Short-term (1 or 2 hpi) feeding by the aphids did not alter *sod2* expression, except in the case of Ambrozja plants infested with 60 *R. padi* at 2 hpi in which there was a 20% increase in transcript

Table 3. Results of the factorial ANOVA of experimental factors (maize genotype, aphid species, treatment = infestation level, time post-infestation) and their interactions influencing the antioxidative activity in maize seedlings.

Parameter	Df	*F*-value	*P*-value
Maize genotype (M)	1	628.45	≤0.001
Aphid species (A)	2	97.6	≤0.001
Treatment (T)	3	45.6	≤0.001
Time post-infestation (TPI)	7	44.7	≤0.001
A × M	2	79.2	≤0.001
A × T	6	18.5	≤0.001
M × T	3	17.1	≤0.001
A × TPI	14	16.7	≤0.001
M × TPI	7	9.2	≤0.001
T × TPI	21	5.4	≤0.001
A × M × T	6	11.0	≤0.001
A × M × TPI	14	9.2	≤0.001
A × T × TPI	42	10.2	≤0.001
M × T × TPI	21	8.1	0.009
A × M × T × TPI	42	5.7	0.035

Df, degree of freedom; values of P≤0.05 were considered statistically significant.

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levels compared with the control (Figure 1). After 4 h of aphid feeding, the *sod2* transcript level increased in both cultivars with both aphid species, ranging from a 1.3-fold increase in Tasty Sweet seedlings infested with 60 *S. avenae* to a 4.6-fold increase in Ambrozja plants infested with 60 *R. padi*. Maximal enhancement of gene expression in aphid-infested seedling leaves in both *Z. mays* cultivars was seen at 8 hpi with 60 aphids per plant (1.5- to 5.4-fold elevation). However, the insect-triggered increase in *sod2* expression was greater in Ambrozja (5.4- and 3.7-fold upregulation by *R. padi* and *S. avenae*, respectively) than in Tasty Sweet plants (2.1- and 1.5-fold upregulation, respectively). The enhanced *sod2* expression in both maize genotypes became less pronounced with prolonged insect colonization (1.3- to 3.5-fold higher expression at 24 hpi, and 1.2- to 3.2-fold increase at 48 hpi, depending on the aphid species and maize cultivar). Consequently, the smallest difference in *sod2* expression between aphid-infested and control plants was seen after long-term aphid infestation (72 hpi). For example, at 72 hpi, 60 *R. padi* aphids stimulated a 28% increase in *sod2* expression in Tasty Sweet plants and a 160% increase in Ambrozja plants, whereas the same number of *S. avenae* aphids increased *sod2* expression by 70% in Tasty Sweet and 15% in Ambrozja plants. The factorial ANOVA testing proved that four experimental parameters and their interactions significantly influenced the transcriptional activity of *sod3.4* gene in *Z. mays* plants (Table 4).

Effect of aphid infestation on the transcriptional activity of *sod3.4* in *Z. mays* tissues

Low and moderate aphid densities (10, 20 and 40 per plant) did not alter *sod3.4* expression at 1 hpi in Tasty Sweet seedlings. In contrast, 60 aphids per plant induced a 12% increase in *sod3.4* expression in *R. padi*–infested Tasty Sweet seedlings and a 5% increase in *S. avenae*–infested Tasty Sweet seedlings at 1 hpi (Figure 2). In the Ambrozja cultivar, the lowest density of either aphid species (10 per plant) did not alter *sod3.4* expression at 1 hpi, but all other aphid treatments led to elevated *sod3.4* expression relative to the control (ranging from a 12% increase in seedlings infested with 60 *S. avenae* per plant to an 86% increase in seedlings infested with 60 *S. avenae* per plant). Furthermore, at 4 hpi *sod3.4* transcript levels were higher in both maize cultivars than at the earlier time points, with the exception of Tasty Sweet seedlings infested with 10 aphids per seedling, for which no change in *sod3.4* expression was observed. The maximal increase in *sod3.4* expression in aphid-stressed maize plants occurred at 8 hpi with 60 aphids per plant (2.3- to 9.4-fold increase relative to control seedlings), and the increase was greater in Ambrozja (6.2- and 9.4-fold increase in *S. avenae*– and *R. padi*–infested seedlings, respectively) than in Tasty Sweet (2.3- and 3.0-fold increase in *S. avenae*– and *R. padi*–infested plants, respectively). Continued feeding of *R. padi* or *S. avenae* on the seedlings (24, 48 and 72 hpi) resulted in progressively lower *sod3.4* expression compared with 8 hpi. For example, 60 *R. padi* aphids per plant caused a 150% increase in *sod3.4* expression in Ambrozja seedlings and a 42% increase in expression in Tasty Sweet plants at 72 hpi. The same density of *S. avenae* resulted in less of an increase in expression

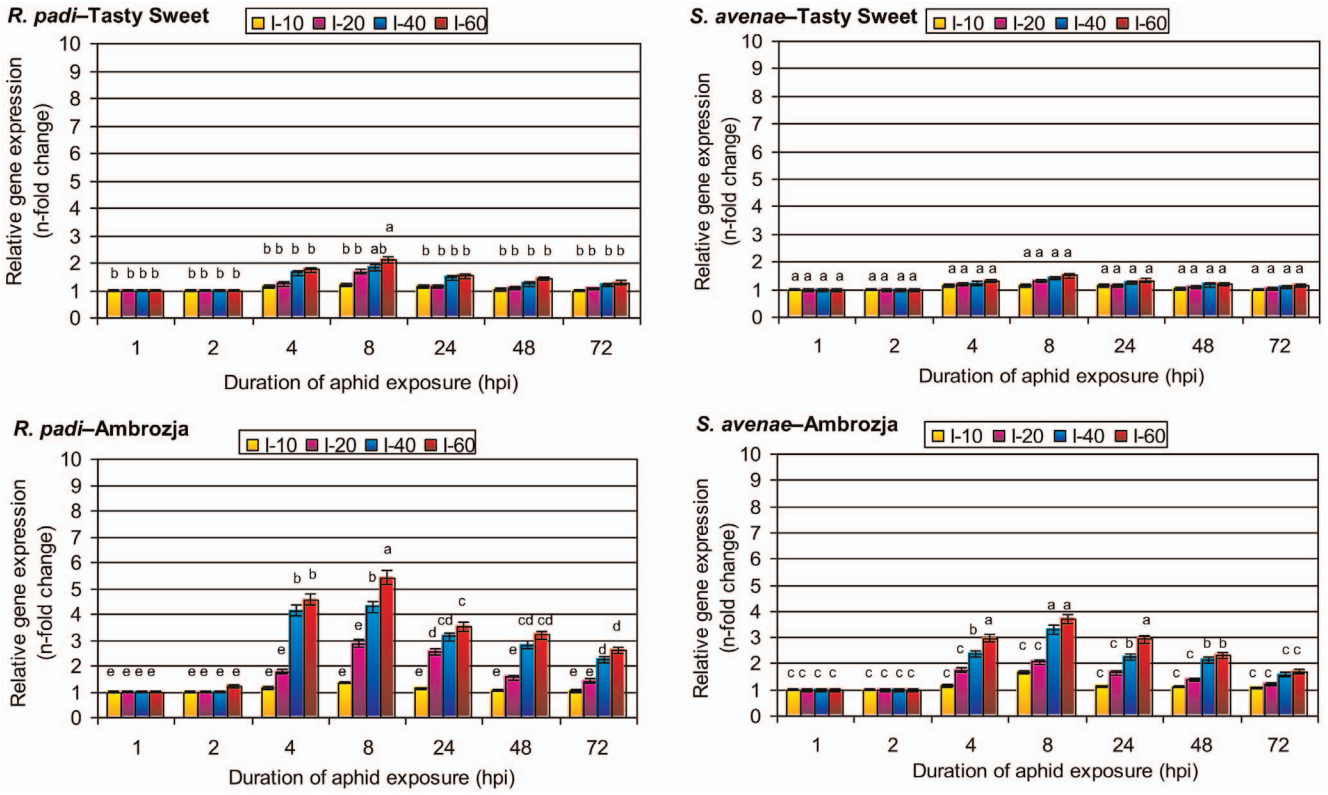

Figure 1. Transcription of *sod2* in aphid-colonized seedlings of Tasty Sweet (susceptible) and Ambrozja (relatively resistant) maize varieties. (I) – infestation level (number of aphids per seedling). Values represent the average fold change in relative gene expression (± SD) in the aphid-infested maize seedlings compared with control (uninfested) plants. For each experimental combination, three independent biological replicates were performed. Different letters indicate statistically significant differences (Tukey's test; $P \leq 0.05$).
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Table 4. Results of the factorial ANOVA of experimental factors (maize genotype, aphid species, treatment = infestation level, time post-infestation) and their interactions influencing the relative expression of *sod2* and *sod3.4* genes in maize seedlings.

Parameter	Df	*F*-value	*P*-value	*F*-value	*P*-value
		***sod2* gene**		***sod3.4* gene**	
Maize genotype (M)	1	48.4	≤0.001	91.8	≤0.001
Aphid species (A)	2	83.9	≤0.001	129.7	≤0.001
Treatment (T)	3	55.0	≤0.001	69.7	≤0.001
Time post-infestation (TPI)	7	33.1	≤0.001	51.4	≤0.001
A × M	2	18.5	≤0.001	28.8	≤0.001
A × T	6	15.0	≤0.001	25.1	≤0.001
M × T	3	7.1	≤0.001	13.8	≤0.001
A × TPI	14	9.6	≤0.001	15.5	≤0.001
M × TPI	7	7.9	≤0.001	21.1	≤0.001
T × TPI	21	5.4	≤0.001	8.4	≤0.001
A × M × T	6	3.5	≤0.001	4.4	≤0.001
A × M × TPI	14	3.7	≤0.001	7.5	≤0.001
A × T × TPI	42	2.6	≤0.001	3.0	≤0.001
M × T × TPI	21	1.9	0.010	5.2	≤0.001
A × M × T × TPI	42	1.8	0.002	2.6	≤0.001

Df, degree of freedom; values of $P \leq 0.05$ were considered statistically significant.
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compared with control plants (21 and 52% in Tasty Sweet and Ambrozja plants, respectively). The factorial ANOVA confirmed the significant effects of four tested parameters and their interactions on the relative *sod3.4* gene expression within maize seedlings (Table 4).

Impact of *R. padi* or *S. avenae* colonization on *sod9* expression in maize plants

At 1 hpi with bird cherry-oat or grain aphids, *sod9* expression in both cultivars did not differ from that measured in control plants (Figure 3). Additionally, colonization with only 10 of either insect species per plant did not alter *sod9* transcription at 2 hpi. Infestation of the maize plants with *R. padi* aphids at 20 per seedling evoked a slight increase in the relative *sod9* expression (6–10%), whereas more *R. padi* aphids (40 or 60 insects per seedling) resulted in a greater upregulation of *sod9*, ranging from a 10% increase in Tasty Sweet plants with 40 aphids per plant to a 90% increase in Ambrozja seedlings with 60 aphids per plant at 2 hpi relative to control plants. Prolonged aphid feeding (4 and 8 hpi) was associated with further augmentation in *sod9* transcription compared with the control, and the highest stimulation of *sod9* expression in the aphid-infested seedlings occurred at 24 hpi, with the Ambrozja cultivar showing a greater increase in the *sod9* transcript (1.4- to 5.3-fold and 1.7- to 8.1-fold increases in *S. avenae*– and *R. padi*–infested seedlings, respectively) than the Tasty Sweet variety (1.1- to 2.2-fold and 1.1- to 2.9-fold increases in *S. avenae*– and *R. padi*–infested plants, respectively). However, at 48 and 72 hpi the difference in transcriptional activity of *sod9* between the colonized and control maize seedlings was smaller than at 24 hpi. At 72 hpi with bird cherry-oat aphids, *sod9* expression was 4–85% higher in Tasty Sweet seedlings and 5–330% higher in Ambrozja seedlings than in controls. The increases in *sod9* expression were also dependent on aphid density, with 15–50% increase in expression at 40–60 *R. padi* aphids per Tasty Sweet seedling, and 80–130% increase (at 40–60 per seedling) in Ambrozja cultivar compared with control plants. However, lower numbers of insects colonizing the Tasty Sweet cultivar (10 *R. padi* and 10–20 *S. avenae* per plant) at 72 hpi did not alter *sod9* expression. The factorial analysis of variance revealed the statistically significant influence of four experimental parameters and their interactions on the amount of *sod9* transcript within the *Z. mays* plants (Table 5).

Aphid-evoked changes in *sodB* expression in leaf tissues of *Z. mays* seedlings

Predation by both aphid species for 1 or 2 h did not affect *sodB* transcript levels in tissues of either *Z. mays* genotype (Figure 4). Similarly, infestation with only 10 *R. padi* or *S. avenae* per plant did not alter *sodB* expression up to 4 hpi. However, higher aphid densities (20, 40 and 60 per plant) slightly enhanced *sodB* expression relative to the control. For example, infestation with 60 *R. padi* per plant led to 17 and 49% increases in the *sodB* expression in Tasty Sweet and Ambrozja plants, respectively, whereas infestation with 60 *S. avenae* per plant led to 13 and 16% increases in Tasty Sweet and Ambrozja seedlings, respectively. After prolonged aphid infestation (8 hpi) similar increases in *sodB* expression were observed, ranging from a 5% increase in Tasty Sweet plants exposed to 10 *S. avenae* per plant to a 76% increase in Ambrozja seedlings exposed to 60 *R. padi* per plant. The largest increases in *sodB* expression occurred at 24 hpi, when 10–60 bird cherry-oat aphids per plant led to 10–62% and 12–127% increases in Tasty Sweet and Ambrozja plants, respectively, relative to the controls. *S. avenae* colonization (10–60 per plant) evoked slightly less of an increase in *sodB* expression, with 7–52% and 9–80% increases in Tasty Sweet and Ambrozja plants, respectively. Relative expression of *sodB* gene was downregulated at 48 and 72 hpi in Tasty Sweet seedlings infested with 40 or 60 *R. padi* per plant or 60 *S. avenae* per plant and in all infested Ambrozja plants, whereas *sodB* transcript amount was unchanged in Tasty Sweet seedlings infested with 10 or 20 *R. padi* per plant or 10, 20, or 40 *S.*

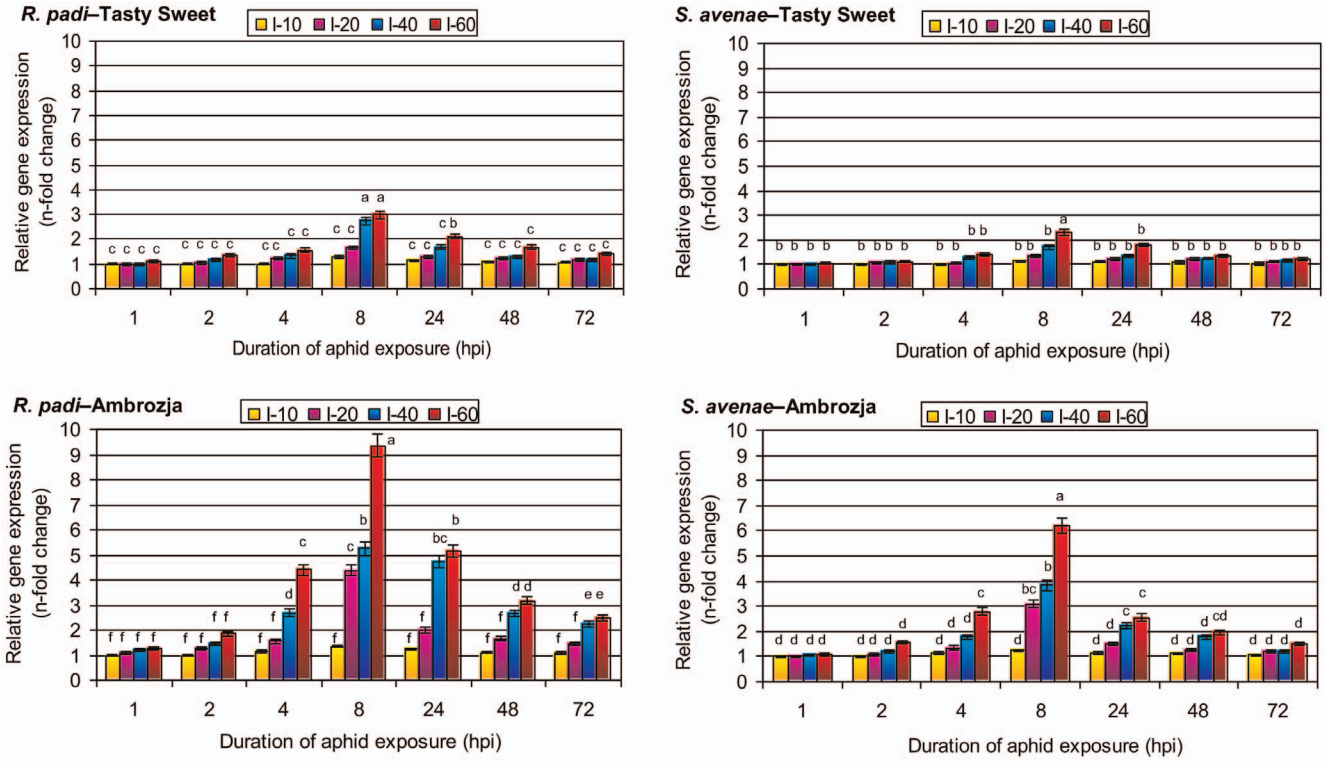

Figure 2. Transcription of *sod3.4* in aphid-colonized seedlings of the Tasty Sweet and Ambrozja maize varieties. (I) – infestation level (number of aphids per seedling). Values represent the average fold changes in relative gene expression (± SD) in the aphid-infested maize seedlings compared with control (non-infested) plants. For each experimental combination, three independent biological replicates were performed. Different letters indicate statistically significant differences (Tukey's test; $P \leq 0.05$).
doi:10.1371/journal.pone.0094847.g002

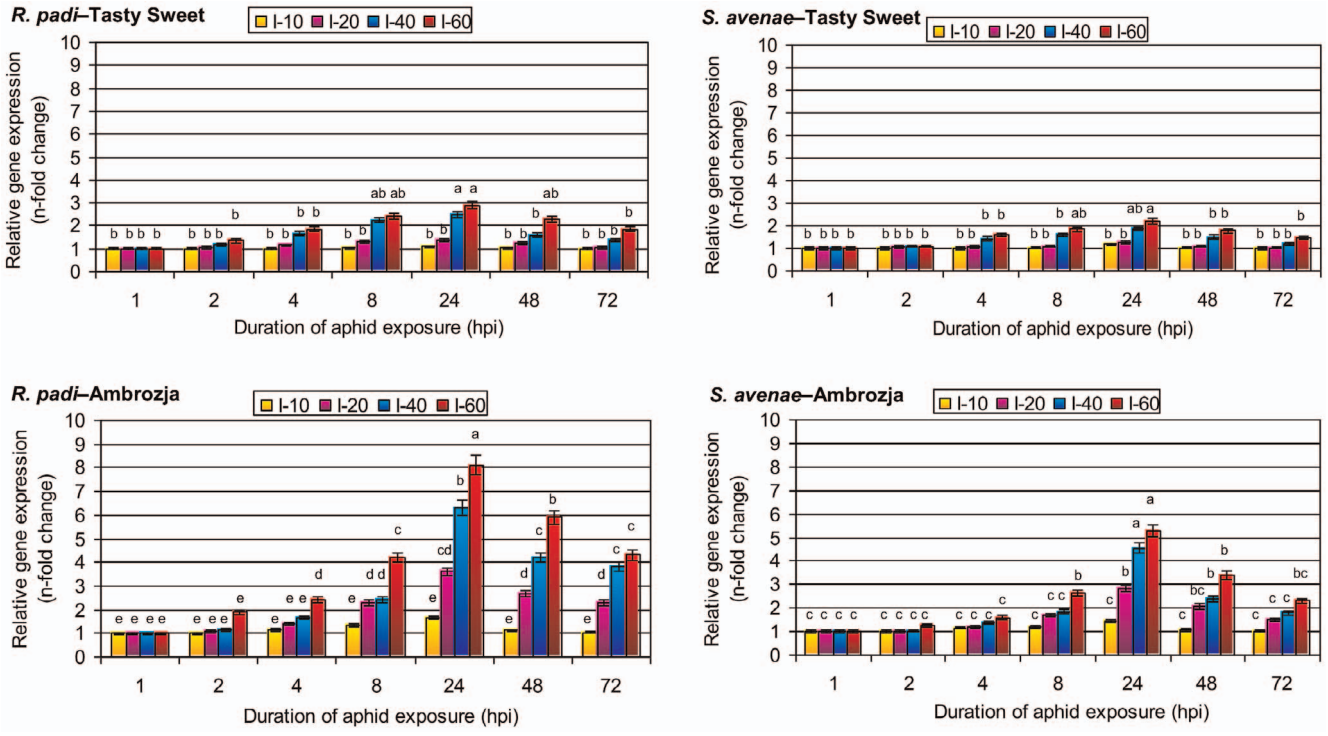

Figure 3. Transcription of *sod9* in aphid-colonized seedling leaves of the Tasty Sweet and Ambrozja maize varieties. (I) – infestation level (number of aphids per seedling). Values represent the average fold changes in relative gene expression (± SD) in the aphid-infested maize seedlings compared with control (non-infested) plants. For each experimental combination, three independent biological replicates were performed. Different letters indicate statistically significant differences (Tukey's test; $P \leq 0.05$).
doi:10.1371/journal.pone.0094847.g003

Table 5. Results of the factorial ANOVA of experimental factors (maize genotype, aphid species, treatment = infestation level, time post-infestation) and their interactions influencing the relative expression of *sod9* and *sodB* genes in maize seedlings.

Parameter	Df	*F*-value	*P*-value	*F*-value	*P*-value
		***sod9* gene**		***sodB* gene**	
Maize genotype (M)	1	1081.0	≤0.001	0.8	0.305
Aphid species (A)	2	1167.5	≤0.001	1.2	0.364
Treatment (T)	3	586.6	≤0.001	1.5	0.201
Time post-infestation (TPI)	7	485.4	≤0.001	0.9	0.508
A × M	2	363.3	≤0.001	1.3	0.275
A × T	6	188.3	≤0.001	1.0	0.416
M × T	3	118.1	≤0.001	0.9	0.434
A × TPI	14	145.3	≤0.001	0.7	0.721
M × TPI	7	191.6	≤0.001	0.6	0.743
T × TPI	21	70.0	≤0.001	1.1	0.367
A × M × T	6	44.0	≤0.001	1.2	0.279
A × M × TPI	14	59.5	≤0.001	1.0	0.401
A × T × TPI	42	21.7	≤0.001	0.8	0.748
M × T × TPI	21	24.9	≤0.001	0.7	0.760
A × M × T × TPI	42	8.7	≤0.001	1.1	0.315

Df, degree of freedom; values of $P \leq 0.05$ were considered statistically significant.
doi:10.1371/journal.pone.0094847.t005

avenae relative to control plants. The greatest suppression of *sodB* expression in tissues of both maize varieties relative to the controls occurred at 72 hpi. Amount of *sodB* transcript in the seedlings colonized with 60 *R. padi* per plant declined by 36 and 49% in the Tasty Sweet and Ambrozja plants, respectively, whereas *S. avenae* colonization reduced *sodB* transcript levels by 28% in the Tasty Sweet seedlings and 44% in Ambrozja seedlings. The factorial ANOVA test did not confirmed any significant effects of four investigated parameters and their interactions on the transcriptional activity of *sodB* gene in maize seedlings (Table 5). However, the *post-hoc* analysis (Tykey's test; P≤0.05) revealed the significant differences in levels of relative expression of *sodB* gene only between Ambrozja plants infested with 60 *R. padi* aphids (24 hpi) and other experimental variants (Figure 4).

Discussion

Harmful effects of the aphid colonization on plant growth and development as well as mechanisms underlying various aphid-elicited physiological and biochemical responses within tissues of the host systems have been extensively studied over the last decade [69–76]. In Poland, four aphid species have been identified on maize crops: *Metopolophium dirhodum* Walk., *Rhopalosiphum maidis* F., *Rhopalosiphum padi* L. and *Sitobion avenae* F. [77–78]. The grain aphid (*S. avenae*) is a monoecious and monophagous hemipteran that colonizes the *Poaceae* plants [79–81], whereas the oligophagous *R. padi* migrates between primary hosts (*Prunus* sp.) and a wide spectrum of secondary hosts (cereals and wild grasses) [70], [82–84]. The present study is the first to demonstrate differences in the expression profiles of several *sod* genes in maize plants having a susceptible or relatively resistant genotype colonized by monophagous or oligophagous aphids, the salivary secretions of which may differ with respect to activity and toxicity—thereby underlying differences in their ability to induce defense responses in the host. The capacity of host systems to maintain redox balance under aphid attack depends on rapid and efficient functioning of complex cellular antioxidative networks. Deciphering the specific strategies involved in sustaining homeostasis in colonized plants may lead to a better understanding of the resistance mechanisms of plants towards these highly deleterious insects.

Recent studies strongly suggest that ROS play a crucial role in complex plant-insect interactions [85–91]. Excessive O_2^- production in plant tissues under adverse conditions may lead to substantial damage to cellular macromolecules (e.g. proteins, lipids, DNA) [27–29]. On the other hand, O_2^- can act as a signaling molecule that triggers ROS-dependent defense systems to allow adaptation to stressors. Mai et al. [24] reported that pea aphid (*Acyrthosiphon pisum*) colonization led to significant insect density- and time-dependent enhancement in the rate of O_2^- and H_2O_2 production in pea (*Pisum sativum*) seedlings. Moloi and van der Westhuizen [20] also demonstrated that Russian wheat aphid (*Diuraphis noxia*) colonization significantly stimulated H_2O_2 accumulation in leaves of the resistant wheat (*T. aestivum*, Tugela line) compared with near-isogenic susceptible plants. These authors postulated that the increased H_2O_2 activates signaling pathways that are responsible for the resistance to *D. noxia*. Similarly, Kerchev et al. [22] observed that potato (*Solanum tuberosum*) leaves attacked by green peach aphids (*Myzus persicae*) have nearly twice the H_2O_2 than uninfested plants. One of the pivotal functions of H_2O_2 in plant tissues is to trigger protein phosphorylation cascades in response to a wide range of environmental stimuli, which leads to widespread induction of stress-related genes [89–90]. The DPPH assay has been widely employed to evaluate the total non-enzymatic antioxidative capacity of a diverse array of plant systems. This analytic procedure is based on reduction of an organic DPPH• (1,1-diphenyl-2-picrylhydrazyl) leading to a gradual decline in the absorbance value in parallel with color changes of the reaction mixture from deep violet to pale yellow [92–94]. The study demonstrated the variability in the extent of the antioxidative capacity in aphid-colonized seedlings of aphid-tolerant and aphid-susceptible maize cultivars. The relatively resistant cultivar had a markedly stronger deceleration of the

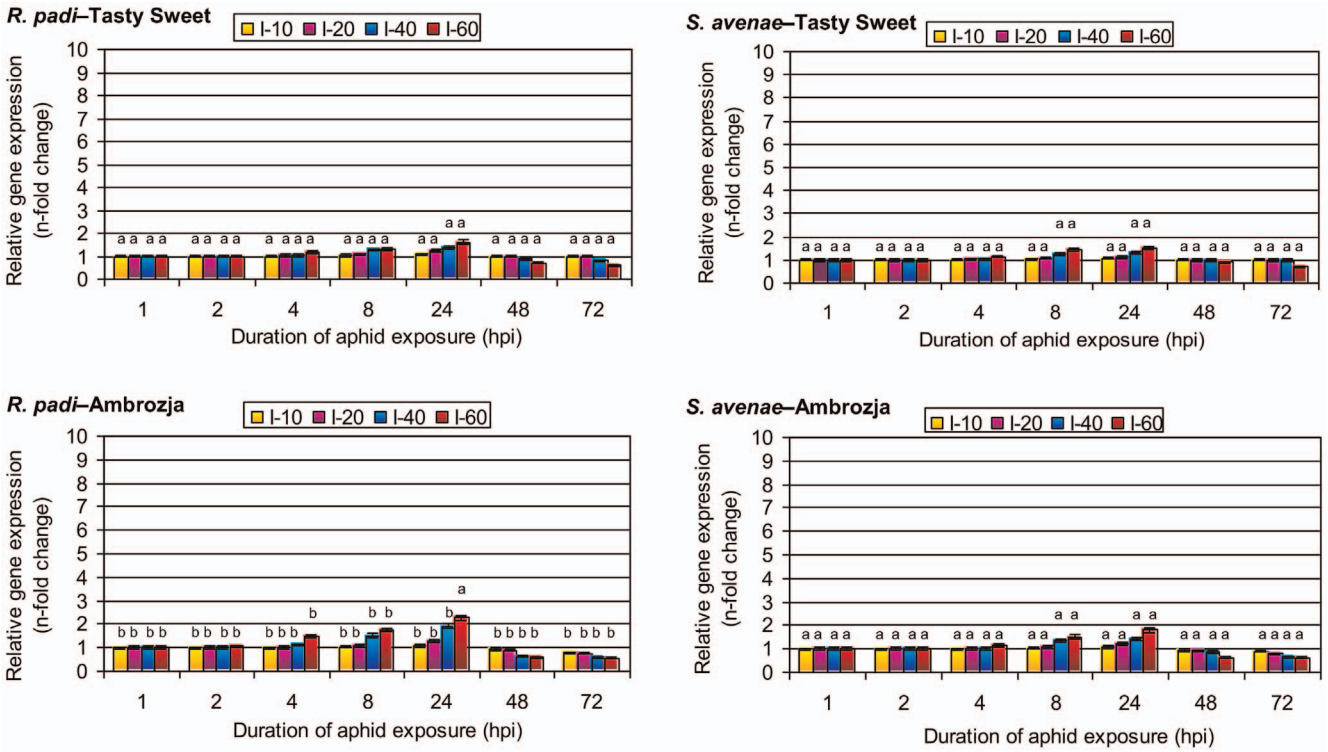

Figure 4. Transcription of *sodB* in aphid-colonized seedling leaves of the Tasty Sweet and Ambrozja maize varieties. (I) – infestation level (number of aphids per seedling). Values represent the average fold changes in relative gene expression (± SD) in the aphid-infested maize seedlings compared with control (non-infested) plants. For each experimental combination, three independent biological replicates were performed. Different letters indicate statistically significant differences (Tukey's test; $P \leq 0.05$).
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scavenging activity of DPPH radicals in response to aphid infestation than the more susceptible variety. Additionally, bird cherry-oat aphid evoked a greater response in the antioxidative activity in maize seedlings than grain aphid, and the changes were proportional to insect abundance and duration of exposure. It should be emphasized that regeneration of the antioxidant pool during a prolonged oxidative burst is progressively less efficient, and therefore the scale of injuries to organelles and cellular compounds is increased under these conditions [25], [31–32]. Similar results were obtained by Xie et al. who reported a significant depletion in the DPPH radical scavenging capacity in salt-stressed seedlings of cotton (var. 99B) when compared to the control plants. However, the additional application of coronatine (COR – chlorosis-eliciting phytotoxin) enhanced levels of salt tolerance by acceleration of the antioxidative potential within the treated seedlings [95]. Demiral et al. also evidenced a profound diminution of the antiradical activity in watery extracts of *Olea europaea* (Gemlik cv.) plants subjected to high salinity [96]. It is important to underline that several other researchers confirmed significant modulations in levels of DPPH• scavenging activity in plants exposed to a broad range of environmental stressors, such as chilling [97–98], drought [99], high temperature [97], pesticide treatment [100], wounding [101], phytopatogenic viruses, bacteria and fungi [102].

Only a few studies have demonstrated significant aphid-elicited modulation of gene expression in tissues of colonized host plants [91], [103–104]. The comparative analyses of *sod* specific expression patterns in the present study revealed that both aphid species tested substantially altered the expression of several *sod* genes in a density- and time-dependent manner. Interestingly, most of the *sod* genes examined were more markedly upregulated in the aphid-relatively resistant Ambrozja plants than in the aphid-susceptible Tasty Sweet plants. It should be noted that both insect species influenced slight fluctuations in expression of *sodB* gene in maize cultivars, however, these modulations were not statistically significant. Kuśnierczyk et al. reported the downregulation of two genes encoding FeSOD (*fsd1* and *fsd2*) in *Arabidopsis thaliana* colonized with cabbage aphids (*Brevicoryne brassicae*) [89]. The same study revealed upregulated expression of four other genes encoding germin-like protein precursors having SOD activity [89]. Kerchev et al. [22] found that the gene encoding a putative cytosolic Cu/ZnSOD was upregulated in *M. persicae*–infested potato plants, whereas the levels of an FeSOD gene transcript gradually declined. Similarly, Dubey et al. demonstrated that the CuSOD 1 gene was upregulated and the FeSOD 3 gene downregulated in leaves of cotton (*Gossypium hirsutum*) infested by cotton aphids (*Aphis gossypii*). These authors postulate that aphids may regulate a similar set of genes to those influenced by plant hormones, microbial infection, or wounding, implying complex crosstalk between the diverse pathways elicited by a broad range of environmental stimuli. Moran et al. [91] also found that the genes encoding cytosolic Cu/ZnSOD1 and a FeSOD were up- and downregulated, respectively, in *A. thaliana* colonized with *M. persicae*. Furthermore, microarray-based analyses of *A. thaliana*–*M. persicae* interactions support the hypothesis that aphid feeding may alter gene expression profiles in a manner similar to wounding or pathogens [91].

In this study, *R. padi* aphids had a greater influence on maize *sod* expression when compared with *S. avenae*. Species-specific differences in the mode of stylet penetration, feeding behaviors, and

composition of salivary secretions are likely the main factors determining the scale of aphid-induced injuries and consequent ROS generation in host plants. In support of this model, extensive injuries have been observed in the parenchyma tissue of *R. padi*–infested *T. aestivum* [105], and bird cherry-oat aphid's mouthpart penetration has been shown to disrupt large areas of the mesophyll in leaf tissues of *Prunus padus* [106]. In contrast, stylet insertion and the phloem-feeding style of the grain aphid appear to result in less damage in colonized *T. aestivum* [107].

Taken together, obtained results suggest that the greater SOD response of Ambrozja seedlings relative to Tasty Sweet seedlings might be an important factor in the ability to alleviate the aphid-stimulated oxidative burst and thus may be the basis of Ambrozja's greater ability to survive infestation. Nevertheless, further comprehensive profiling of stress-associated genes in aphid-infested *Z. mays* plants is required to unravel the molecular mechanisms that regulate the highly complex intracellular antioxidant machinery.

Supporting Information

Table S1 List of the analysed *Z. mays* superoxide dismutase (*sod*) genes quantified using TaqMan® Gene Expression Assays[a)]. [a)] TaqMan® Gene Expression Assays were designed and prepared by Life Technologies (Poland).
(DOC)

Table S2 A. List of *Z. mays* genes quantified using Custom TaqMan® Gene Expression Assays[b)]. [b)] Custom TaqMan® Gene Expression Assay were designed by the author and prepared by Life Technologies (Poland)**. B. Sequences of primers designed for amplification of *sod9* and *gapdh* genes.** F – forward primer, R – reverse primer. **C. Sequences of TaqMan® fluorescent probes designed for amplification of *sod9* and *gapdh* genes.** FAM – 6-carboxyfluorescein, NFQ – 3′–non-fluorescent quencher.
(DOC)

Author Contributions

Conceived and designed the experiments: HS. Performed the experiments: HS. Analyzed the data: HS. Contributed reagents/materials/analysis tools: HS. Wrote the paper: HS.

References

1. Fornalé S, Capellades M, Encina A, Wang K, Irar S, et al. (2012) Altered lignin biosynthesis improves cellulosic bioethanol production in transgenic maize plants down-regulated for cinnamyl alcohol dehydrogenase. Mol Plant 5: 817–830.
2. Herrmann A (2013) Biogas production from maize: current state, challenges and prospects. Agronomic and environmental aspects. Bioenerg Res 6: 372–387.
3. Reddy KR, Henry WB, Seepaul R, Lokhande S, Gajanayake B, et al. (2013) Exogenous application of glycinebetaine facilitates maize (*Zea mays* L.) growth under water deficit conditions. Am J Exp Agr 3: 1–13.
4. Semenčenko VV, Mojović LV, Đukić-Vuković AP, Radosavljević MM, Terzić DR, et al. (2013) Suitability of some selected maize hybrids from Serbia for the production of bioethanol and dried distillers' grains with solubles. J Sci Food Agr 93: 811–818.
5. Strażyński P (2008) Aphid fauna (Hemiptera, Aphidoidea) on maize crops in Wielkopolska – species composition and increase in number. Aphids and Other Homopterous Insects 14: 123–128.
6. Khatoon T, Hussain K, Majeed A, Nawaz K, Nisar MF (2010) Morphological variations in maize (*Zea mays* L.) under different levels of NaCl at germinating stage. World Appl Sci J 8: 1294–1297.
7. Lewis MF, Lorenzana RE, Jung HJG, Bernardo R (2010) Potential for simultaneous improvement of corn grain yield and stover quality for cellulosic ethanol. Crop Sci 50: 516–523.
8. Sytykiewicz H, Czerniewicz P, Sprawka I, Krzyżanowski R (2013) Chlorophyll content of aphid-infested seedling leaves of fifteen maize genotypes. Acta Biol Cracov Bot 55: 51–60.
9. Sprawka I, Goławska S, Czerniewicz P, Sytykiewicz H (2011) Insecticidal action of phytohemagglutinin (PHA) against the grain aphid, *Sitobion avenae*. Pestic Biochem Phys 100: 64–69.
10. Chrzanowski G, Leszczynski B, Czerniewicz P, Sytykiewicz H, Matok H, et al. (2012) Effect of phenolic acids from black currant, sour cherry and walnut on grain aphid (*Sitobion avenae* F.) development. Crop Prot 35: 71–77.
11. Sprawka I, Goławska S, Goławski A, Czerniewicz P, Sytykiewicz H (2012) Antimetabolic effect of phytohemagglutinin to the grain aphid *Sitobion avenae* Fabricius. Acta Biol Hung 63: 342–353.
12. Sempruch C, Leszczyński B, Wójcicka A, Makosz M, Matok H, et al. (2010) Changes in activity of lysine decarboxylase within winter triticale in response to grain aphid feeding. Acta Biol Hung 61: 512–515.
13. Sytykiewicz H, Czerniewicz P, Sprawka I, Goławska S, Chrzanowski G, et al. (2011) Induced proteolysis within the bird cherry leaves evoked by *Rhopalosiphum padi* L. (Hemiptera, Aphidoidea). Acta Biol Hung 62: 316–327.
14. Louis J, Singh V, Shah J (2012) *Arabidopsis thaliana*-aphid interaction. *Arabidopsis* Book 10: e0159. DOI: 10.1199/tab.0159.
15. Goławska S, Łukasik I, Wójcicka A, Sytykiewicz H (2012) Relationship between saponin content in alfalfa and aphid development. Acta Biol Cracov Bot 54: 1–8.
16. Halarewicz A, Gabrys B (2012) Probing behavior of bird cherry-oat aphid *Rhopalosiphum padi* (L.) on native bird cherry *Prunus padus* L. and alien invasive black cherry *Prunus serotina* Erhr. in Europe and the role of cyanogenic glycosides. Arthropod-Plant Inte 6: 497–505.
17. Sytykiewicz H, Szpechcinski A, Czerniewicz P, Sprawka I, Chrzanowski G, et al. (2012) Expression profiling of glutathione transferase (*Gst1*) gene in maize seedlings infested by the bird cherry-oat aphid (*Rhopalosiphum padi* L.). FEBS J 279 (Suppl.1): 70 pp.
18. Stewart LR, Bouchard R, Redinbaugh MG, Meulia T (2012) Complete sequence and development of a full-length infectious clone of an Ohio isolate of maize dwarf mosaic virus (MDMV). Virus Res 165: 219–224.
19. Zielińska L, Trzmiel K, Jeżewska M (2012) Ultrastructural changes in maize leaf cells infected with maize dwarf mosaic virus and sugarcane mosaic virus. Acta Biol Cracov Bot 54: 97–104.
20. Moloi MJ, van der Westhuizen AJ (2006) The reactive oxygen species are involved in resistance responses of wheat to the Russian wheat aphid. J Plant Physiol 163: 1118–1125.
21. Carolan JC, Fitzroy CF, Ashton PD, Douglas AE, Wilkinson TL (2009) The proteome of the pea aphid saliva characterized by LC/MS-MS. Proteomics 9: 2457–2467.
22. Kerchev P, Fenton B, Foyer CH, Hancock RD (2012) Infestation of potato (*Solanum tuberosum* L.) by the peach-potato aphid (*Myzus persicae* Sulzer) alters cellular redox status and is influenced by ascorbate. Plant Cell Environ 35: 430–440.
23. Łukasik I, Goławska S, Wójcicka A (2012) Effect of cereal aphid infestation on ascorbate content and ascorbate peroxidase activity in triticale. Pol J Environ Stud 21: 1937–1941.
24. Mai VC, Bednarski W, Borowiak-Sobkowiak B, Wilkaniec B, Samardakiewicz S, et al. (2013) Oxidative stress in pea seedling leaves in response to *Acyrthosiphon pisum* infestation. Phytochemistry 93: 49–62.
25. Lee YP, Baek K-H, Lee H-S, Kwak S-S, Bang J-W, et al. (2010) Tobacco seeds simultaneously over-expressing Cu/Zn-superoxide dismutase and ascorbate peroxidase display enhanced seed longevity and germination rates under stress conditions. J Exp Bot 61: 2499–2506.
26. Kar RK (2011) Plant responses to water stress: Role of reactive oxygen species. Plant Signal Behav 6: 1741–1745.
27. Ponce de León I, Montesano M (2013) Activation of defense mechanisms against pathogens in mosses and flowering plants. Int J Mol Sci 14: 3178–3200.
28. Rady MM, Osman ASh (2012) Response of growth and antioxidant system of heavy metal-contaminated tomato plants to 24-epibrassinolide. Afr J Agric Res 7: 3249–3254.
29. Rubio-Wilhelmi MM, Sanchez-Rodriguez E, Rosales MA, Begoña B, Rios JJ, et al. (2011) Effect of cytokinins on oxidative stress in tobacco plants under nitrogen deficiency. Environ Exp Bot 72: 167–173.
30. Kumar M, Yadav V, Tuteja N, Johri AK (2009) Antioxidant enzyme activities in maize plants colonized with *Piriformospora indica*. Microbiology 155: 780–790.
31. Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48: 909–930.
32. Foyer CH, Shigeoka S (2011) Understanding oxidative stress and antioxidant functions to enhance photosynthesis. Plant Physiol 155: 93–100.
33. Myouga F, Hosoda C, Umezawa T, Iizumi H, Kuromori T, et al. (2008) A heterocomplex of iron superoxide dismutases defends chloroplast nucleoids against oxidative stress and is essential for chloroplast development in *Arabidopsis*. Plant Cell 20: 3148–3162.
34. Sánchez-Rodríguez E, Rubio-Wilhelmi Mdel M, Blasco B, Leyva R, Romero L, et al. (2012) Antioxidant response resides in the shoot in reciprocal grafts of drought-tolerant and drought-sensitive cultivars in tomato under water stress. Plant Sci 188–189: 89–96.

35. de Carvalho K, de Campos MK, Domingues DS, Pereira LF, Vieira LG (2013) The accumulation of endogenous proline induces changes in gene expression of several antioxidant enzymes in leaves of transgenic Swingle citrumelo. Mol Biol Rep 40: 3269–3279.
36. Sytykiewicz H (2011) Expression patterns of glutathione transferase gene (*GstI*) in maize seedlings under juglone-induced oxidative stress. Int J Mol Sci 12: 7982–7995.
37. Parida AK, Das AB, Mohanty P (2004) Defense potentials to NaCl in a mangrove, *Bruguiera parviflora*: differential changes of isoforms of some antioxidative enzymes. J Plant Physiol 161: 531–542.
38. Rubio MC, Becana M, Sato S, James EK, Tabata S, et al. (2007) Characterization of genomic clones and expression analysis of the three types of superoxide dismutases during nodule development in *Lotus japonicus*. Mol Plant Microbe Interact 20: 262–275.
39. Cohu CM, Abdel-Ghany SE, Gogolin Reynolds KA, Onofrio AM, Bodecker JR, et al. (2009) Copper delivery by the copper chaperone for chloroplast and cytosolic copper/zinc-superoxide dismutases: regulation and unexpected phenotypes in an *Arabidopsis* mutant. Mol Plant 2: 1336–1350.
40. Kukavica B, Mojovic M, Vuccinic Z, Maksimovic V, Takahama U, et al. (2009) Generation of hydroxyl radical in isolated pea root cell wall, and the role of cell wall-bound peroxidase, Mn-SOD and phenolics in their production. Plant Cell Physiol 50: 304–317.
41. Kar RK (2011) Plant responses to water stress: role of reactive oxygen species. Plant Signal Behav 6: 1741–1745.
42. Pilon M, Ravet K, Tapken W (2011) The biogenesis and physiological function of chloroplast superoxide dismutases. Biochim Biophys Acta 1807: 989–998.
43. Islam MR, Hu Y, Mao S, Jia P, Eneji AE, et al. (2011) Effects of water-saving superabsorbent polymer on antioxidant enzyme activities and lipid peroxidation in corn (*Zea mays* L.) under drought stress. J Sci Food Agric 91: 813–819.
44. Benešová M, Holá D, Fischer L, Jedelský PL, Hnilička F, et al. (2012) The physiology and proteomics of drought tolerance in maize: early stomatal closure as a cause of lower tolerance to short-term dehydration? PLoS One 7: e38017. DOI: 10.1371/journal.pone.0038017.
45. Hasheminasab H, Assad MT, Aliakbari A, Sahhafi SR (2012) Influence of drought stress on oxidative damage and antioxidant defense systems in tolerant and susceptible wheat genotypes. J Agr Sci 4: 20–30.
46. Tian Z, Wang F, Zhang W, Liu C, Zhao X (2012) Antioxidant mechanism and lipid peroxidation patterns in leaves and petals of marigold in response to drought stress. Hort Environ Biotechnol 53: 183–192.
47. Zhang W, Tian Z, Pan X, Zhao X, Wang F (2013) Oxidative stress and non-enzymatic antioxidants in leaves of three edible canna cultivars under drought stress. Hort Environ Biotechnol 54: 1–8.
48. Li W, Qi L, Lin X, Chen H, Ma Z, et al. (2009) The expression of manganese superoxide dismutase gene from *Nelumbo nucifera* responds strongly to chilling and oxidative stresses. J Integr Plant Biol 51: 279–286.
49. Jevremović S, Petrić M, Živković S, Trifunović M, Subotić A (2010) Superoxide dismutase activity and isoenzyme profiles in bulbs of snake's head fritillary in response to cold treatment. Arch Biol Sci 62: 553–558.
50. Kayihan C, Eyidogan F, Afsar N, Oktem HA, Yucel M (2012) Cu/Zn superoxide dismutase activity and respective gene expression during cold acclimation and freezing stress in barley cultivars. Biol Plant 56: 693–698.
51. Sánchez-Venegas JR, Dinamarca J, Moraga AG, Gidekel M (2009) Molecular characterization of a cDNA encoding Cu/Zn superoxide dismutase from *Deschampsia antarctica* and its expression regulated by cold and UV stresses. BMC Res Notes 2: 198. DOI:10.1186/1756-0500-2-198.
52. Radyukina NL, Shashukova AV, Makarova SS, Kuznetsov VV (2011) Exogenous proline modifies differential expression of superoxide dismutase genes in UV-B-irradiated *Salvia officinalis* plants. Russ J Plant Physiol 58: 51–59.
53. Camejo D, Martí Mdel C, Nicolás E, Alarcón JJ, Jiménez A, et al. (2007) Response of superoxide dismutase isoenzymes in tomato plants (*Lycopersicon esculentum*) during thermo-acclimation of the photosynthetic apparatus. Physiol Plant 131: 367–377.
54. Rasoulnia A, Bihamta MR, Peyghambari SA, Alizadeh H, Rahnama A (2011) Proteomic response of barley leaves to salinity. Mol Biol Rep 38: 5055–5063.
55. Rubio-Wilhelmi MM, Sanchez-Rodriguez E, Rosales MA, Begoña B, Rios JJ, et al. (2011) Effect of cytokinins on oxidative stress in tobacco plants under nitrogen deficiency. Environ Exp Bot 72: 167–173.
56. Ślesak I, Hałdaś W, Ślesak H (2006) Influence of exogenous carbohydrates of superoxide dismutase activity in *Trifolium repens* L. explants cultured *in vitro*. Acta Biol Cracov Bot 48: 93–98.
57. Qian H, Lu T, Peng X, Han X, Fu Z, et al. (2011) Enantioselective phytotoxicity of the herbicide imazethapyr on the response of the antioxidant system and starch metabolism in *Arabidopsis thaliana*. PLoS One 6:e19451. DOI: 10.1371/journal.pone.0019451.
58. Pawlak S, Firych A, Rymer K, Deckert J (2009) Cu,Zn-superoxide dismutase is differently regulated by cadmium and lead in roots of soybean seedlings. Acta Physiol Plant 31: 741–747.
59. Rodríguez-Serrano M, Romero-Puertas MC, Pazmiño DM, Testillano PS, Risueño MC, et al. (2009) Cellular response of pea plants to cadmium toxicity: cross talk between reactive oxygen species, nitric oxide, and calcium. Plant Physiol 150: 229–243.
60. Navascués J, Pérez-Rontomé C, Sánchez DH, Staudinger C, Wienkoop S, et al. (2012) Oxidative stress is a consequence, not a cause, of aluminum toxicity in the forage legume *Lotus corniculatus*. New Phytol 193: 625–636.
61. Çelik Ö, Büyükuslu N, Atak Ç, Rzakoulieva A (2009) Effects of magnetic field on activity of superoxide dismutase and catalase in *Glycine max* (L.) Merr. roots. Pol J Environ Stud 18: 175–182.
62. Ehsani-Moghaddam B, Charles MT, Carisse O, Khanizadeh S (2006) Superoxide dismutase responses of strawberry cultivars to infection by *Mycosphaerella fragariae*. J Plant Physiol 163: 147–153.
63. Morkunas I, Bednarski W (2008) *Fusarium oxysporum*-induced oxidative stress and antioxidative defenses of yellow lupine embryo axes with different sugar levels. J Plant Physiol 165: 262–277.
64. Morkunas I, Bednarski W, Kopyra M (2008) Defense strategies of pea embryo axes with different levels of sucrose to *Fusarium oxysporum* and *Ascochyta pisi*. Physiol Mol Plant Pathol 72: 167–178.
65. Fang X, Chen W, Xin Y, Zhang H, Yan C, et al. (2012) Proteomic analysis of strawberry leaves infected with *Colletotrichum fragariae*. J Proteomics 75: 4074–4090.
66. Morkunas I, Formela M, Marczak L, Stobiecki M, Bednarski W (2013) The mobilization of defence mechanisms in the early stages of pea seed germination against *Ascochyta pisi*. Protoplasma 250: 63–75.
67. Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. Food Sci Technol 28: 25–30.
68. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25: 402–408.
69. Sempruch C, Horbowicz M, Kosson R, Leszczyński B (2012) Biochemical interactions between triticale (*Triticosecale*; Poaceae) amines and bird cherry-oat aphid (*Rhopalosiphum padi*; Aphididae). Biochem Syst Ecol 40: 162–168.
70. Czerniewicz P, Leszczyński B, Chrzanowski G, Sempruch C, Sytykiewicz H (2011) Effects of host plant phenolics on spring migration of bird cherry-oat aphid (*Rhopalosiphum padi* L.). Allelopathy J 27: 309–316.
71. Sytykiewicz H, Goławska S, Chrzanowski G (2011) Effect of the bird cherry-oat aphid, *Rhopalosiphum padi* L. feeding on phytochemical responses within the bird cherry. Pol J Ecol 59: 329–338.
72. Sempruch C, Marczuk W, Leszczyński B, Czerniewicz P (2013) Participation of amino acid decarboxylases in biochemical interactions between triticale (*Triticosecale*, Poaceae) and bird cherry-oat aphid (*Rhopalosiphum padi*, Aphidideae). Biochem Syst Ecol. DOI: 10.1016/j.bse.2013.10.001 (in press).
73. Sempruch C, Marczuk W, Leszczyński B, Kozak A (2013) Influence of pea aphid (*Acyrthosiphon pisum* Harris) infestation on activity of amino acid decarboxylases within pea (*Pisum sativum* L.) tissues. Acta Biol Cracov Bot 55: 1–6.
74. Goławska S, Krzyżanowski R, Łukasik I (2010) Relationship between aphid infestation and chlorophyll content in Fabaceae species. Acta Biol Cracov Bot 52: 76–80.
75. Sprawka I, Goławska S, Goławski A, Chrzanowski G, Czerniewicz P, et al. (2014) Entomotoxic action of jackbean lectin (Con A) in bird cherry-oat aphid through the effect on insect enzymes. J Plant Interact 9: 425–433.
76. Sprawka I, Goławska S, Parzych T, Goławski A, Czerniewicz P, et al. (2013) Induction of apoptosis in the grain aphid *Sitobion avenae* (Hemiptera: Aphididae) under the influence of phytohaemagglutinin PHA. Appl Entomol Zool 48: 525–532.
77. Pieńkosz A, Leszczyński B, Warzecha R (2005) Podatność kukurydzy na mszyce zbożowe. Prog Plant Prot 45: 989–992.
78. Strażyński P (2008) Aphid fauna (Hemiptera, Aphidoidea) on maize crops in Wielkopolska - species composition and increase in number. Aphids and Other Homopterous Insects 14: 123–128.
79. Gao S, Liu D (2013) Differential performance of *Sitobion avenae* (Hemiptera: Aphididae) clones from wheat and barley with implications for its management through alternative cultural practices. J Econ Entomol 106: 1294–301.
80. Li F, Kong L, Liu Y, Wang H, Chen L, et al. (2013) Response of wheat germplasm to infestation of English grain aphid (Hemiptera: Aphididae). J Econ Entomol 106: 1473–1478.
81. Svobodová E, Trnka M, Dubrovský M, Semerádová D, Eitzinger J, et al. (2013) Determination of areas with the most significant shift in pests' persistence in Europe under climate change. Pest Manag Sci, DOI: 10.1002/ps.3622. [Epub ahead of print].
82. Stoetzel MB, Miller GL (2001) Aerial feeding aphids of corn in the United States with reference to the root-feeding *Aphis maidiradicis* (Homoptera: Aphididae). Florida Entomologist 84: 83–98.
83. Aslan MM, Uygun N (2005) Aphids (Homoptera: Aphididae) of Kahramanmaraş Province, Turkey. Turk J Zool 29: 201–209.
84. Coulette Q, Couty A, Lasue P, Rambaud ZC, Ameline A (2013) Colonization of the biomass energy crop miscanthus by the three aphid species, *Aphis fabae*, *Myzus persicae*, and *Rhopalosiphum padi*. J Econ Entomol 106: 683–689.
85. Cooper WR, Dillwith JW, Puterka GJ (2011) Comparisons of salivary proteins from five aphid (Hemiptera: Aphididae) species. Environ Entomol 40: 151–156.
86. Łukasik I, Goławska S, Wójcicka A, Goławski A (2011) Effect of host plants on antioxidant system of pea aphid *Acyrthosiphon pisum*. B Insectol 64: 153–158.
87. Sytykiewicz H, Sprawka I, Czerniewicz P, Sempruch C, Leszczyński B, et al. (2013) Biochemical characterisation of chlorophyllase from leaves of selected *Prunus* species – A comparative study. Acta Biochim Pol 60: 457–465.
88. Sprawka I, Goławska S, Parzych T, Goławski A, Czerniewicz P, et al. (2014) Mechanism of entomotoxicity of concanavalin A in the bird cherry-oat aphid, *Rhopalosiphum padi*. J Insect Sci (in press).
89. Kuśnierczyk A, Winge P, Jørstad TS, Troczyńska J, Rossiter JT, et al. (2008) Towards global understanding of plant defence against aphids-timing and

dynamics of early *Arabidopsis* defence responses to cabbage aphid (*Brevicoryne brassicae*) attack. Plant Cell Environ 31: 1097–1115.
90. Morkunas I, Mai VC, Gabrys B (2011) Phytohormonal signaling in plant responses to aphid feeding. Acta Physiol Plant 33: 2057–2073.
91. Moran PJ, Cheng Y, Cassell JL, Thompson GA (2002) Gene expression profiling of *Arabidopsis thaliana* in compatible plant-aphid interactions. Arch Insect Biochem Physiol 51: 182–203.
92. Clarke G, Ting KN, Wiart C, Fry J (2013) High correlation of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferric reducing activity potential and total phenolics content indicates redundancy in use of all three assays to screen for antioxidant activity of extracts of plants from the Malaysian rainforest. Antioxidants 2: 1–10.
93. Rahman MM, Habib MR, Hasan MA, Al Amin M, Saha A, et al. (2014) Comparative assessment on in vitro antioxidant activities of ethanol extracts of *Averrhoa bilimbi*, *Gymnema sylvestre* and *Capsicum frutescens*. Pharmacognosy Res 6: 36–41.
94. Asghar MN, Khan IU, Bano N (2011) In vitro antioxidant and radical-scavenging capacities of *Citrullus colocynthes* (L) and *Artemisia absinthium* extracts using promethazine hydrochloride radical cation and contemporary assays. Food Sci Technol Int 17: 481–494.
95. Xie Z, Duan L, Tian X, Wang B, Eneji AE, et al. (2008) Coronatine alleviates salinity stress in cotton by improving the antioxidative defense system and radical-scavenging activity. J Plant Physiol 165: 375–384.
96. Demiral MA, Aktaş Uygun D, Uygun M, Kasirğa E, Karagözler AA (2011) Biochemical response of *Olea europaea* cv. Gemlik to short-term salt stress. Turk J Biol 35: 433–442.
97. Kang HM, Saltveit ME (2002) Antioxidant enzymes and DPPH-radical scavenging activity in chilled and heat-shocked rice (*Oryza sativa* L.) seedlings radicles. J Agric Food Chem 50: 513–518.
98. Kang HM, Saltveit ME (2002) Reduced chilling tolerance in elongating cucumber seedling radicles is related to their reduced antioxidant enzyme and DPPH-radical scavenging activity. Physiol Plant 115: 244–250.
99. Zhu Z, Liang Z, Han R (2009) Saikosaponin accumulate ion and antioxidative protection in drought-stressed *Bupleurum chinense* DCX. Plants Environ. Exp. Bot. 66: 326–333.
100. Krzepiłko A, Zych-Wężk I (2010) Effect of the pesticide Karate 025EC on the antioxidant properties of radish (*Raphanus sativus* L.) seedling extract. Ecol Chem Eng A17: 1629–1634.
101. Boué SM, Shih FF, Shih BY, Daigle KW, Carter-Wientjes CH, et al. (2008) Effect of biotic elicitors on enrichment of antioxidant properties and induced isoflavones in soybean. J Food Sci 73: H43–49.
102. Horsáková J, Sochor J, Krška B (2013) Assessment of antioxidant activity and total polyphenolic compounds of peach varieties infected with the Plum pox virus. Acta Univ Agric Silvic Mendelianae Brun 187: 1693–1701.
103. Barah P, Winge P, Kuśnierczyk A, Tran DH, Bones AM (2013) Molecular signatures in *Arabidopsis thaliana* in response to insect attack and bacterial infection. PLoS One 8: e58987.
104. Kuśnierczyk A, Tran DH, Winge P, Jørstad TS, Reese JC, et al. (2011) Testing the importance of jasmonate signalling in induction of plant defences upon cabbage aphid (*Brevicoryne brassicae*) attack. BMC Genomics 12: 423.
105. Urbańska A, Niraz S (1990) Anatomiczne i biochemiczne aspekty żerowania mszyc zbożowych. Zesz Probl Post Nauk Roln 392: 201–213.
106. Sytykiewicz H (2007) Biochemiczne i anatomiczne aspekty żerowania mszycy czeremchowo-zbożowej (*Rhopalosiphum padi*/L./) na żywicielu pierwotnym. Ph.D. dissertation, Akademia Podlaska, Wydział Rolniczy, Siedlce.
107. Urbańska A (2010) Histochemical analysis of aphid saliva in plant tissue. EJPAU, ser. Biology 13: #26 (http://www.ejpau.media.pl/volume13/issue4/art-26.html).