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 avenueae F. (grain aphid) or oligophagous Rhopalosiphum padi L. (bird cherry-oat aphid). Secondly, 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.

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, Zea 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 (O2-) to oxygen (O2) and hydrogen peroxide (H2O2). 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 isoforms 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–
humidity of 65\% controlled chamber at 22\°C. Plants were grown in a climate-controlled plant growth chamber described above. To date, there are no available studies regarding the regulation of sod genes and 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 antioxidative 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 \mu 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-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. The bioassays were performed on leaves of 14-day-old seedlings. Adult wingless aphids were used in the experiments. Aphids 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. 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 cm³ 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 mm³ of the supernatant and 365 mm³ 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 (%) = \( \frac{A_0 - A_s}{A_0} \times 100 \) (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, R.NaSe-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, A260/280 and A260/230 ratios were calculated to estimate RNA integrity and purity. Only RNA samples of high quality (A260/280>2.0 and A260/230>1.8) were selected for further analysis.

cDNA synthesis

Purified total RNA (1 \mu g) 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 evaluated 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 as 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 mm³ and consisted of 10 mm³ 2× TaqMan Fast Universal PCR Master Mix, 1 mm³ 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<0.05).
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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 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). 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 antioxidative capacity.
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

| 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±1.4b | 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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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   | ≤0.0001   |
| 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 3.4-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 seedlings and a 160% increase in Ambrozja plants 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.

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 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<0.05).

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Ambrozja seedlings than in controls. The increases in 85% higher in Tasty Sweet seedlings and 5–330% higher in the colonized and control maize seedlings was smaller than at 24 seedling, and 80–130% increase (at 40–60 per seedling) in increase in expression at 40–60 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).

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

| Parameter | Df | F-value sod2 gene | P-value sod2 gene | F-value sod3.4 gene | P-value 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 |

Of, degree of freedom; values of P ≤ 0.05 were considered statistically significant. doi:10.1371/journal.pone.0094847.t004

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 plants, respectively). The factorial ANOVA confirmed the significant effects of four tested parameters and their interactions on the relative sod9.4 gene expression within maize seedlings (Table 4).

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, whereas infestation with 60 S. avenae per plant led to 13 and 16% increases in Tasty Sweet and Ambrozja seedlings, respectively. 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).

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.

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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≤0.05).

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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≤0.05).

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between Ambrozja plants infested with 60 *R. padi* sodB and other experimental variants (Figure 4).

Colonization reduced post-hoc investigated parameters and their interactions on the transcriptional activity of sodB gene in maize seedlings (Table 5). However, the post-hoc analysis (Tukey’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-elicted 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 the host systems have been extensively studied over the last decade [69–76]. In Poland, four aphid species have been identified on the host systems.

### 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 |
|-----------------------------------|----|---------|---------|---------|---------|
| 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 ≤ 0.05 were considered statistically significant.

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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], phytopathogenic 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 (fod1 and fod2) 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

**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<0.05).

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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 composition of salivary secretions are likely the main factors of starch degradation in colonized T. aestivum. 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 R. padi ROS generation in host plants. In support of this model, extensive composition of salivary secretions are likely the main factors of starch degradation in colonized T. aestivum. 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.

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Expression of Superoxide Dismutase Genes in Maize

**Expression Assays**

1. **TaqMan® Gene Expression Assays** were designed and prepared by Life Technologies (Poland).

**Table S2 A. List of Z. mays genes quantified using Custom TaqMan® Gene Expression Assays**

**Table S2 B. Sequences of primers designed for amplification of sod9 and gapdh genes**

**Table S2 C. Sequences of Custom TaqMan® Gene Expression Assays**

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.
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