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Short Communication

Effect of Cereal Aphid Infestation on Ascorbate Content and Ascorbate Peroxidase Activity in Triticale

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Abstract

The aim of the present study was to compare ascorbate (ASA) content and ascorbate peroxidase (APX) activity in seedlings of the less susceptible triticale cultivar Witon and the more susceptible triticale cultivar Tornado after infestation by the grain aphid *Sitobion avenae* (F). and the bird cherry-oat aphid *Rhopalosiphum padi* (L.). The content of ASA in triticale seedlings slightly decreased after 24 hrs of infestation by cereal aphids, but prolonged feeding (48 hrs and 72 hrs) resulted in significant losses in ASA dependent on cultivar and aphid species. The aphid infestation caused an increase in APX activity in triticale tissues throughout the test period. The aphid herbivores induced APX activity to a higher level in a less susceptible cultivar than in a more susceptible one. The oligophagous species *R. padi* caused a higher decrease of ASA and stronger induction of APX activity in tested triticale than the monophagous species *S. avenae*. The experiments carried out indicate that ascorbate and ascorbate peroxidase may play a significant role in the defense mechanism of aphid infested triticale.

Keywords: *Sitobion avenae*, *Rhopalosiphum padi*, ascorbate, ascorbate peroxidase, oxidative stress, winter triticale

Introduction

Phloem-feeding insects induce many alterations in their host plants, including morphological changes, modified resource allocation, and symptoms such as chlorosis, necrosis, and malformation of new growth [1]. In response to a herbivore attack, plants have developed many defense mechanisms, including cell wall modifications, hypersensitive cell death, production of phytoalexins, accumulation of pathogenesis-related (PR) proteins, and plant volatiles [1, 2]. One of the most rapid defense reactions to biotic stress is "oxygen burst," which constitutes the production of reactive oxygen species (ROS), primarily superoxide ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2) [3-6]. H_2O_2 has been proposed to play multiple roles in plant resistance exhibiting direct toxicity toward herbivores, leading to the cross-linking of cell wall proteins and acting as a signal molecule for the induction of defense genes [3, 4, 7-9]. The high concentration of H_2O_2 may be toxic to both phytophagous insects and host plants. Thus a herbivore attack can stimulate the enzymes scavenging H_2O_2. Among those important roles is ascorbate peroxidase (APX), which is presented in all cell compartments and has a high affinity to H_2O_2 [10]. First, H_2O_2 is reduced by APX, generating dehydroascorbate (DHA). DHA is then converted to ASA by the glutathione-dependent enzyme, dehydroascorbate reductase (DHAR) [11].

Aphids cause less tissue damage than chewing insects, since they use a stylet to access the vascular tissues or

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directly on plant cells to feed [12]. The piercing/sucking insects induce the signaling pathway most commonly activated by plant pathogens such as fungus, bacteria, and viruses [13]. There are numerous studies concerning the effect of pathogen attack on the activity of antioxidant enzymes in plants, but little is known about the role of oxidative stress in aphid interactions with host plants [5, 14-17]. In this paper we report changes in the level of ASA and the activity of APX in winter triticale infested by two species of cereal aphids: the grain aphid *Sitobion avenae* (F) and the bird cherry-oat aphid *Rhopalosiphum padi* (L.).

Material and Methods

Plants and Aphids

Two cultivars of winter triticale (Triticosecale Wittm.), characterized by different degrees of susceptibility to cereal aphids were chosen for experiments: Tornado (more susceptible) and Witon (less susceptible). Plants were cultivated in a climate chamber and kept at 21±1ºC and 70% relative humidity (HR) under a 16 h photoperiod. The plants were grown in plastic pots (7 cm × 7 cm × 9 cm) with fine garden soil commonly used for greenhouse experiments, one plant per pot.

The insects came from the aphid stock cultures kept at the University of Natural Sciences and Humanities at Siedlce. The aphids were reared on seedlings of winter wheat cv. Tonacja, in a climatic chamber at 21±1ºC and photoperiod L16:D8. Experiments were conducted on wingless adults (*apterae*) of the grain aphid and the bird cherry-oat aphid.

Infestation Procedure

Nine-day-old triticale seedlings were infested each with 20 or 40 aphids on leaves. Control plants were left noninfested. After the aphids had fed on seedlings for 24, 48, and 72 hours, control and infested plants were taken for assays of ASA content and APX activity. The influence of aphid infestation on the ASA concentration and APX activity in winter triticale was expressed in percentage of control (100% = noninfested plants).

Ascorbate Assay

The concentration of ASA was determined by a spectrophotometric assay [18]. One gram of leaf tissue was ground in an ice-cold mortar using a pestle, and 5 ml of 5% trichloroacetic acid (TCA) was added. The homogenate was centrifuged at 15,000 g for 15 min. The supernatant was used to assay ASA concentration. The reaction mixture contained 0.2 ml of plant homogenate, 0.6 ml of 0.2 M phosphate buffer pH 7.4, 1 ml of 10% TCA, 0.8 ml of 42% H_3PO_4, 0.8 ml of 4% α,α′-dipirydyl, and 0.4 ml of 3% $FeCl_3$. The reaction mixture was incubated at 42ºC for 40 min, and after centrifugation absorbance at 525 nm was measured against a control containing 0.2 ml of 5% TCA instead of plant homogenate. ASA content was calculated from a calibration curve prepared with standard and was expressed in nmol per fresh weight.

APX Assay

APX activity was assayed following the oxidation of ascorbic acid at 290 nm (extinction coefficient 2.8 $mM^{-1} \cdot cm^{-1}$) according to a method of Asada [19]. 0.1 g of leaf tissue was ground in an ice-cold mortar using pestle, and 5 ml of 67 mM K-phosphate buffer pH 7 was added. The homogenate was centrifuged at 15,000 g for 15 min. The supernatant was used to assay APX activity. The reaction mixture consisted of 0.75 ml of plant homogenate and 0.25 ml of 67 mM K-phosphate buffer pH 7 containing 2.5 mM ascorbic acid and 0.2 ml of 30 mM H_2O_2. The decrease in absorbance at 290 nm was monitored for 5 min using a spectrophotometer. Boiled samples served as controls. APX activity was expressed as μmol ascorbate oxidized/min/mg protein.

Protein Assay

The protein content in the studied plant homogenates was determined using the method given by Bradford [20].

Statistics

All data are reported as means ± SD, n = 4, where each replication represents one independent plant homogenate. Data were subjected to a one-way analysis of variance (ANOVA) followed by the Duncan's multiple-range test.

Results

The level of ASA within studied triticale cultivars was slightly affected by aphids after 24 hrs of infestation. Prolonged feeding (48 hrs) resulted in significant losses in ASA, but the level of depletion was dependent on cultivar and aphid species. Feeding by *S. avenae* similarly affected content of ASA in both triticale cultivars and the loss of ASA was independent of a number of aphids. Different results were obtained in the case of *R. padi* infestation, where stronger depletion of ASA was observed in less susceptible Witon cv. The lower ASA content was detected in both cultivars infested with 40 adults of *R. padi*. At 72 hrs following feeding, the decrease of ASA was pretty similar to that obtained for 48 hrs of infestation. Only leaves of Witon cv. infested by 20 adults of *S. avenae* had ASA content comparable with control non-infested plants (Table 1).

The herbivory of triticale seedlings significantly increased APX activity. After 24 hrs of feeding, cereal aphid infestation induced APX activity to a higher level in a less susceptible cultivar (Witon) than in a more susceptible one (Tornado). In the case of Tornado cv., no influence of aphid species or aphid number on APX activity was

Table 1. Changes in ASA concentrations in seedlings of triticale cv. Witon after cereal aphid infestation (control non-infested plants 380±19 μmol/g fresh weight = 100%).

Aphid species	Number of aphids per plant	Time of feeding (h)		
		24	48	72
S. avenae	20	97±2^{a}	80±4^{cd}	96±3^{a}
	40	100±4^{a}	80±3^{cd}	77±3^{d}
R. padi	20	94±5^{ab}	66±3^{e}	70±2^{e}
	40	88±4^{bc}	26±5^{f}	30±4^{f}

Data are presented as mean±SD; n = 4. Values not followed by the same letter are significantly different at level P≤0.05 (Duncan's Test).

Table 2. Changes in ASA concentrations in seedlings of triticale cv. Tornado after cereal aphid infestation (control non-infested plants 280±25 μmol/g fresh weight = 100%).

Aphid species	Number of aphids per plant	Time of feeding (h)		
		24	48	72
S. avenae	20	95±3^{a}	85±4^{bc}	80±3^{bc}
	40	98±3^{a}	85±5^{bc}	80±4^{bc}
R. padi	20	90±4^{ab}	78±3^{c}	76±4^{c}
	40	90±3^{ab}	55±5^{d}	61±4^{d}

Data are presented as mean±SD; n = 4. Values not followed by the same letter are significantly different at level P≤0.05 (Duncan's Test).

Table 3. Changes in APX activity in seedlings of triticale cv. Witon after cereal aphid infestation (control non-infested plants 0.68±0.12 μmol ascorbate oxidized/min/mg protein = 100%).

Aphid species	Number of aphids per plant	Time of feeding (h)		
		24	48	72
S. avenae	20	120±6^{e}	135±6^{d}	138±6^{d}
	40	117±3^{e}	133±4^{d}	170±5^{b}
R. padi	20	137±2^{d}	140±4^{d}	180±9^{b}
	40	121±7^{e}	155±6^{c}	200±6^{a}

Data are presented as mean±SD; n = 4. Values not followed by the same letter are significantly different at level P≤0.05 (Duncan's Test).

observed. The infestation of Witon cv. by various numbers of *S. avenae* females similarly affected APX activity, whereas *R. padi* caused higher induction of APX at 20 aphids per plant. The leaves of Witon cv. infested by fewer aphids exhibit higher APX activity at *R. padi* infestation. In the case of more numbers of insects, no differences between infestation by monophagous and oligophagous species were observed.

Continued aphid feeding on triticale (48 hrs) caused a little stronger induction of APX in comparison to the previous period of the experiment. The level of induction was independent of a number of aphids with the exception of Witon cv. infested by *R. padi* that exhibited higher APX activity at infestation by 40 adults. The response of APX to aphid feeding was greater for plants infested by oligophagous species *R. padi*. The enzyme activity in Witon cv. rose to a higher level than in Tornado.

The prolonged feeding (72 hrs) of aphids on studied triticale cultivars caused the strongest induction of APX. Similar to an earlier period of the experiment (48 hrs), greater APX induction was observed for plants infested by *R. padi,* which had a nearly 2-fold higher level of APX than the control ones. The induction of enzyme was stronger for plants infested by more aphids. The higher level of APX was noted for the less susceptible Witon cultivar.

Discussion

Under physiological conditions, the low level of ROS is maintained by low-molecular-weight antioxidants and scavenging enzymes [21, 22]. The regulation of ROS level is one of the important factors controlling physiology of a plant [12, 23]. Biotic stress factors such as bacteria, fungi, viruses, and even herbivores induce the generation of ROS and affect the level of antioxidants [4, 24-26]. Rapid accumulation of H_2O_2 is an early signaling event after herbivory damage [6, 27]. Aphids also induce the generation of H_2O_2 [5], and ROS are molecules of defense signaling pathways with known involvement in the activation of plant responses to aphid feeding [28, 29]. The high level of H_2O_2 is harmful to plants, so antioxidant enzymes neutralizing H_2O_2 are enhanced after herbivores attack. Because catalase is inefficient at removing low concentrations of H_2O_2, plants evolved alternative mechanisms for scavenging peroxides based on ascorbate and ascorbate peroxidase.

Both chewing and sap-feeding insects influence synthesis and redox cycling of ASA in their host plants, altering their nutritional value and susceptibility to pests [30]. It has been reported that herbivores caused the oxidation and loss

Table 4. Changes in APX activity in seedlings of triticale cv. Tornado after cereal aphid infestation (control non-infested plants 0.28±0.07 μmol ascorbate oxidized/min/mg protein = 100%).

Aphid species	Number of aphids per plant	Time of feeding (h)		
		24	48	72
S. avenae	20	106±6^{f}	116±4^{ef}	121±5de
	40	108±4^{f}	115±5^{ef}	160±7^{c}
R. padi	20	110±3^{f}	128±3^{d}	171±6^{bc}
	40	115±5^{ef}	130±6^{d}	185±5^{a}

Data are presented as mean ± SD; n = 4. Values not followed by the same letter are significantly different at level P≤0.05 (Duncan's Test).

of ASA as well as enhanced the activity and expression of proteins involved in ASA metabolism [30]. In our study, the content of ASA in triticale seedlings decreased after infestation by cereal aphids. Bi and Felton [27] have shown similar results in soybean seeds, where the feeding on *Helicoverpa zea* (Boddie) larvae in soybeans resulted in significant changes in foliar antioxidants. It was manifested by 26% loss of ASA and 104% higher DHA levels compared to control plants [27]. Mechanical wounding is used to mimic damage caused by herbivores. It was earlier reported that wounding and jasmonates influenced ASA accumulation in various plant species [31-33]. However, these factors both enhanced ASA content in *Arabidopsis* foliage, but depressed ASA levels in tomato [33]. This indicates that the ASA regulation mechanism is complex and differs between plant species. Patykowski and Urbanek [25] suggested that ASA was not decisive for tomato resistance, since ASA content changed similarly in less and more susceptible cultivars after infection by *Botrytis cinerea* (Pers.).

ASA protects cells from oxidative damages, participates in the regeneration of vitamin E, and acts as a cofactor for enzymes involved in biosynthesis of flavonoids and phytohormones [30]. Thus the mutants with suppressed ASA levels are hypersensitive to stress conditions [34].

APX reduces H_2O_2 concentration, thus the induction of APX activity in aphid-infested triticale suggests that this enzyme plays an important role in the ROS signaling pathways. A rapid induction of APX activity in the more resistant cultivars of chrysanthemum infested by *Macrosiphoniella sanbourni* (Gillette) indicated that the enzyme is involved in early responses of chrysanthemum to aphid attack [17]. In lima bean herbivores damage caused the increase of H_2O_2 levels accompanied by induction of glutathione peroxidase (GPX), glutathione reductase (GR), peroxidase (POD), APX, and CAT [35]. Hu et al. [6] noted the increase of POD, APX, and CAT activity in poplar leaves after an attack by *Clostera anachoreta* (Denis and Schiffermüller) larvae. APX showed the highest activity at 0.5 h of infestation, but at a longer feeding APX activity in infested leaves was still higher than in the control ones. Bi and Felton [27] observed an accumulation of high-level ROS in soybean, although APX activity increased 1.5-fold after *H. zea* herbivores. This might result from the decreased level of antioxidants such as CAT, ASA, or thiols.

In our experiments the greater induction of APX activity in infested plants was noted for the less susceptible cultivar Witon, especially at prolonged aphid feeding. Electrical penetration graph (EPG) recordings showed that cereal aphids feeding on Witon cv. seedlings were characterized by longer duration of no probing, total pathways, and shorter time of salivation into sieve element and phloem sap ingestion than aphids that fed on Tornado cv. [36]. The grain aphid *S. avenae* and the bird cherry-oat aphid *R. padi* feeding on Witon cv. had a higher content of H_2O_2 than that which occurred on Tornado cv. [37]. Thus less susceptible Witon cv. seems to have a greater potential for triggering oxidative stress within aphid tissues. Moreover, the production of H_2O_2 under aphid infestation was higher in seedlings of Witon cv. than in Tornado, the more susceptible cultivar [Łukasik, unpublished]. The results obtained by Moloi and Westhuizen [5] showed that infestation by *Diuraphis noxia* (Mordvilko) induced the accumulation of H_2O_2 to higher levels in the resistant than susceptible wheat.

A different pattern was recorded for tomato leaves after *B. cinerea* infection, where no significant differences in APX activity between tested cultivars (Perkoz – less susceptible, Corindo – more susceptible) were observed [25]. Similarly, APX activity in cotton plants was not affected by cotton aphid *Aphis gossypii* (Glover) [16].

The results of this study demonstrate that levels of APX induction in triticale grew-up during the experimental period and reached nearly 200% after 72 hrs of aphid infestation. Paranidharan et al. [2] showed that APX activity in rice increased from 1 day after inoculation with *Rhizoctonia solani* (Kühn), and maximum activity was recorded 5 days after inoculation. Next, the enzyme activity started to decline, but significantly higher APX activity was recorded even at 7 days after inoculation.

The oligophagous species *R. padi*, which change hosts between woody plants and grasses, caused higher depletion of ASA and stronger induction of APX activity in tested triticale than the monophagous species *S. avenae*. This suggests that *R. padi* feeding probably caused more damage in comparison to *S. avenae*, but this finding needs to be examined further.

In general, we found that aphid herbivory enhanced defense mechanisms of triticale based on ascorbate and ascorbate peroxidase. Further studies relating to H_2O_2 generation and H_2O_2-scavenging enzymes in the short term are in progress to explain the role of oxidative stress in chemical interactions between cereal aphids and their host plants.

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