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Effects of *Acyrtosiphon pisum* (Harris) infestation on the hydrogen peroxide content and activity of antioxidant enzymes in *Fabaceae* plants

I. Łukasik*, A. Kornacka[1], S. Goławska, H. Sytykiewicz, I. Sprawka, A. Wójcicka
Department of Biochemistry and Molecular Biology
University of Natural Sciences and Humanities in Siedlce,
Prusa 12, 08-110 Siedlce, Poland
E. Mail: iluk_71@tlen.pl

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ABSTRACT

The H_2O_2 content increased in *Fabaceae* (pea, vetch and broad bean) plants after aphid herbivory and reached the highest level at 6 h post-infestation. Prolonged aphid feeding decreased the H_2O_2 content, but it was still higher in infested plants than in control ones. The maximum increase of H_2O_2 concentration was noted in *A. pisum* pea plants. *A. pisum* feeding reduced the CAT activity in infested plants. CAT was inhibited until 48 h after infestation in pea and vetch and until 24 h in broad bean. The pea plants drastically inhibited the CAT. Initial feeding of *A. pisum* (1 h, 2 h) slightly increased the APX activity in seedlings of pea and broad bean. Thus the changes in enzyme activity were dependent on plant species. The strongest inhibition and induction of APX was noted in tissues of broad bean. Our results indicated that H_2O_2 and H_2O_2-scavenging enzymes may play a significant role in the defence mechanisms of *Fabaceae* plants towards the pea aphid.

Keywords: *Acyrtosiphon pisum*, antioxidant enzymes, ascorbate peroxidase, catalase, hydrogen peroxide, legumes, pea, pea aphid, *Pisum sativum*, reactive oxygen species (ROS).

INTRODUCTION

The oxidative stress resulting, from the production of reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$), is one of many plant responses to both biotic and abiotic stresses. Under normal physiological conditions, the concentration of ROS is stable and maintained by low molecular weight antioxidants and antioxidant enzymes (18). However under the biotic and abiotic stresses, the rapid accumulation of ROS ("oxidative burst") occurs (26,27). The starting molecule for production of many reactive oxidants (free radicals, singlet oxygen, halogenes) is superoxide (4). Dismutation of superoxide leads to formation of H_2O_2, one of the major ROS in plant defences against stress factors. H_2O_2 may directly inhibit the growth of plant pathogens and leads to cross-linking of cell wall proteins. It is also involved in programmed plant cell death and induction of systemic acquired resistance (SAR) (14).

On the other hand, ROS can damage the proteins, lipids and DNA, thus plants must maintain balance between the generation of ROS for defence and activity of ROS-scavenging enzymes to avoid the oxidative biodegradation of tissues (28). The first line of plant defence is superoxide dismutase (SOD), which catalyzes the dismutation of O_2^- to H_2O_2. Catalase (CAT) and ascorbate peroxidase (APX) enzymes utilize the H_2O_2. CAT

*Corresponding authors, [1]Laboratory of Physiopathology, The Witold Stefański Institute of Parasitology Polish Academy of Science, Twarda 51/55, 00-818 Warszawa, Poland.

reduces the H_2O_2 to H_2O and oxygen. APX reduces the H_2O_2 and generates dehydroascorbate (DHA). Then DHA is converted by dehydroascorbate reductase (DHAR) to ascorbate (ASA).

It is known that reactive oxygen species (ROS) play major role in plant defence against the pathogens, but their defence against insects is still preliminary and inconsistent (16). The sucking-piercing insects, including aphids, induce the same defence signalling pathways that are activated by pathogens [fungi, viruses and bacteria] (30). The increase in H_2O_2 concentration in resistant plants was observed after infestation by Russian wheat aphid (*Diuraphis noxia*) (Mordvilko) (22). Feeding of *Schizaphis graminum* (Rondani) on barley enhanced the generation of H_2O_2 (3). The feeding of aphids also modulated the activity of enzymes participating in both generation and neutralization of ROS (13,19,22,23,31,32). However, the studies on the oxidative response of *Fabaceae* to aphids are still rare (19,25). In this study, we report changes in H_2O_2 content and activities of antioxidant enzymes (CAT and APX) in *Fabaceae* plant tissues infested by the pea aphid *Acyrtosiphon pisum* (Harris).

MATERIAL AND METHODS

Experiments were conducted on wingless females (*Apterae*) of the pea aphid *A. pisum*. The aphids were reared on seedlings of the pea *Pisum sativum* L. var. Tulipan in an environmental chamber (21°C, Light 16: Dark 8 h photoperiod and 70% R.H.). The seedlings were grown in plastic plastic pots (15 x 15 cm,) filled with medium nutrient fine structure compost with sand.

Three species of *Fabaceae* plants were chosen for experiments: (i). Pea (*Pisum sativum* L.) var. Tulipan, (ii). Broad bean (*Vicia faba* L.) var. Start and (iii). vetch (*Vicia sativa* L.) var. Jaga. These plants were cultivated in climate chamber (21 °C, L16:D8 photoperiod and 70% R.H.). The seedlings were grown in plastic pots (10 x 10 cm) filled with medium nutrient fine structure compost with sand until 10-days . Eight seedling of each test plant were planted per pot as per treatments.

I. Infestation method

Ten-day-old seedlings of *Fabaceae* were infested each with 30 aphids (8-12 days-old). . Control plants were left un-infested. After the aphids had fed on seedlings for 1, 2, 4, 24, 48 and 72 h, control and infested plants were taken for assays of H_2O_2 content and activities of antioxidant enzymes. The influence of aphid infestation on the H_2O_2 level and activities of the enzymes in *Fabaceae* plants was expressed in percentage of control (100% = non-infested plants).

II. H_2O_2 assay

The content of H_2O_2 was determined according to Zhou *et al*. (31). The method is based on the peroxidase-catalyzed reaction of 4-aminoantipyrine and phenol with H_2O_2. One g of leaf tissues was grounded in 5 ml of 5% trichloroacetic acid (TCA) with 50 mg active charcoal at 0°C. The homogenate was centrifuged for 15 min at 15000 *x g*. Supernatant was collected, neutralized by 17 M NH_4OH to pH 7 and used for H_2O_2 assay. The reaction mixture consisted of 1 ml of reagent (4 mM of 4-aminoantipyrine, 24 mM of phenol and 0.4 U/ml of peroxidase dissolved in 0.1 M phosphate-buffer [pH 7.0]) and 1 ml of plant homogenate. After addition of homogenate, the reaction mixture was incubated at 25°C for 10 min and the absorbance was measured at 510 nm against a blank (1 ml of

distilled water instead of the plant homogenate). The hydrogen peroxide content was calculated from a calibration curve prepared for this standard and was expressed in µmol/g fresh weight.

III. CAT assay

CAT activity was measured as described by Aebi (1). The 0.1 g of leaf tissues was grounded in 10 ml of 50 mM K-phosphate buffer (pH 7) at 0°C and the homogenate was centrifuged for 15 min at 15000 *x g*. Supernatant was collected and used for CAT assay. The 0.5 ml plant extract was added to 0.5 ml of 30 mM H_2O_2 and the disappearance of hydrogen peroxide was measured at 240 nm during 3 min at 30 s intervals. Activity of the catalase was expressed as µmol of decomposed H_2O_2 /min/g fresh weight.

IV. APX assay

APX activity was determined according to Asada (4). The 0.2 g of leaf tissues was grounded in 10 ml of 50 mM K-phosphate buffer (pH 7) at 0°C. The homogenate was centrifuged for 15 min at 15000 *x g*, supernatant was collected and used for APX assay. The reaction mixture consisted of 0.75 ml of plant extract and 0.25 ml of 50 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 and boiled samples served as control. APX activity was expressed as m mol of ascorbate oxidized/min/g fresh weight.

RESULTS AND DISCUSSION

The involvement of ROS in plant responses to pathogen attack has been intensively studied (20), but studies relating to their role in plant-herbivores interactions is still preliminary. The mechanisms of plant defence against herbivory attack are correlated with the mode of herbivore feeding and the degree of tissue damage. Chewing herbivores activate wound-response pathways, whereas sucking-piercing insects, including aphids, cause less tissue damage and activate defence-response pathways induced by pathogen attack (30). The saliva and the injury caused by aphids induce a production of ROS in the phloem of plant tissues (8,24,32). Among ROS the crucial role in plant defence mechanisms is of H_2O_2, which is stable and easily diffuses across cell membranes (14).

The exposure of *Fabaceae* plants to adults of *A. pisum* caused increase in H_2O_2 content. The level of H_2O_2 in infested plants was dependent on time of aphid feeding. At the start of experiment, H_2O_2 concentration was slightly increased, then rose up and 4 h after aphid infestation, reached nearly 150 % of H_2O_2 level in control plants. The highest contents of H_2O_2 in seedlings of pea and vetch were noted 6 h after aphid feeding, whereas, the infested seedlings of broad bean exhibited similar level of H_2O_2 after 4 h as well as 6 h after aphid feeding. Six h after infestation the content of H_2O_2 gradually decreased and after 72 h reached the level noted at the beginning of experiment. Comparing he tested host plants, the strongest increase in H_2O_2 concentration was observed in seedlings of pea and the lowest in the broad bean (Figure 1).

The increase in H_2O_2 content after herbivory attack has been reported for many host plants. For example, infestation by the Russian wheat aphid, *D. noxia* significantly induced the accumulation of H_2O_2 in wheat and the level of H_2O_2 peaked 3-6 h after infestation (22). Mai *et al*. (19) observed the enhanced production of H_2O_2 in pea seedlings leaves after infestation of *A. pisum* and the H_2O_2 reached the highest concentration 24 h after the

infestation. *S. graminum* induced H_2O_2 content and total soluble peroxidase activity in barley with the maximum level of H_2O_2 after 20 min and the maximum activity of peroxidase 30 min after infestation (3). Such higher generation of H_2O_2 after infestation by aphids is an evidence of induction of defence mechanisms in host plants. In barley infested by *S. graminum*, the strongest generation of H_2O_2 occurred about 10 min before the maximum activity of soluble peroxidase. It suggests that induction of peroxidase activity results from the H_2O_2 burst and triggers plant responses to aphid attack (3). In our study the period of extensive production of H_2O_2 was similar in all studied host plants, although the strongest generation of H_2O_2 was observed in pea tissues. However, the non-infested broad bean exhibited the highest level of H_2O_2 and this tendency was maintained in post-infestation.

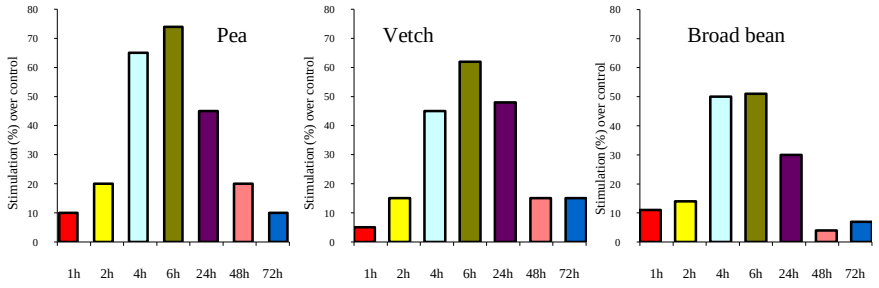

Figure 1. Effects of aphid infestation on H_2O_2 content in *Fabaceae* seedlings

Excess H_2O_2 is harmful to plants, as it can cause membrane lipid peroxidation and damage the reaction center of chloroplasts (12). Therefore, in plant tissues some enzymes (CAT and APX) are present that reduce the H_2O_2 concentration of CAT is inefficient in removing the low concentrations of H_2O_2, hence, plants possess an alternative mechanism for the removal of peroxides (the ascorbate-recycling system), consists of ascorbate (ASA), APX and dehydroascorbic acid reductase (DHAR). First, hydrogen peroxide is reduced by APX, generating dehydroascorbic acid (DHA) and then it is converted to ASA by the GSH dependent enzyme DHAR (5).

Initial feeding of *A. pisum* on the pea seedlings caused a slight reduction in CAT activity in their tissues. The prolonged feeding (4,6,24,48 h) resulted in significant depletion of CAT activity in infested plants, with the lowest activity of CAT 6 h after infestation. Continued aphid feeding on pea (72 h) caused a slight induction of CAT in infested plants in comparison to non-infested ones. Similar tendency was observed for vetch, although the induction of CAT in relation to control plants was observed 48 h after aphid feeding on tested plants. Activity of CAT in seedlings of broad bean infested by *A. pisum* initially slightly decreased than control plants. Continued aphid feeding (4, 6 h) significantly decreased the CAT activity in comparison to previous period of experiments. Prolonged feeding of *A. pisum* (24 h) caused the significant induction of CAT activity. After this period, enzyme activity decreased and was comparable with non-infested seedlings of broad bean. Among tested host plants, the strongest inhibition of CAT was noted in tissues of pea and the strongest induction in tissues of broad bean (Figure 2).

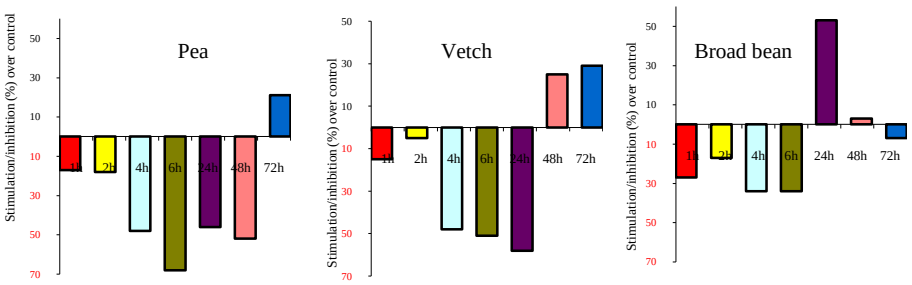

Figure 2. Effects of aphid infestation on CAT activity in *Fabaceae* seedlings

The results of previous studies indicated that CAT activity was reduced as result of plant responses to infestation by sucking insects (13,21,28,32). Mohase and van der Westhuizen (21) observed CAT inhibition in resistant and susceptible wheat cultivars during Russian wheat aphid (RWA) *D. noxia* infestation. However, in resistant plants inhibition occurred earlier (24 h post-infestation) and it was stronger than in susceptible ones. Other results were obtained by Mai *et al*. (19), where CAT activity in pea seedling was enhanced by *A. pisum* infestation with strongest induction at 48 h post-infestation. The depletion of CAT activity may be associated with the biosynthesis of salicylic acid (SA) - one of the key components in the signal transduction pathway leading to plant resistance to biotic stress. CAT was identified as SA-binding protein in tobacco leaves (7). Mittler (20) noted that the plants increase the ROS generation concomitantly with depletion of antioxidant enzymes as a response to biotic stress, but this mechanism is short-term due to toxicity of ROS excess. CAT activity was induced in response plants against chewing insects, that cause extensive damage of plants. Rani and Jyothsna (28) observed the increase of CAT activity in rice infested by yellow stem borer [*Scirpophaga incertulas* (Walker)] and leaf roller [*Cnaphalocrosis medinalis* (Guenée)]. CAT activity was also enhanced in poplar leaves in response to *Clostera anachoreta* larvae (12). However, Bi and Felton (6) noted the depletion of antioxidants, such as ascorbic acid, nonprotein thiols and catalase in soybean wounded by *Helicoverpa zea (*Boddie) feeding. The differences in CAT responses to sucking or chewing insects result from type of feeding on host plants. The sucking insects feed from the contents of vascular tissues and minimalize the induction of wound response (28).

H_2O_2 concentration is also regulated by APX, which reduces H_2O_2 using ASA. Initial feeding of aphids caused slight increase in APX activity in the seedlings of pea. However, the inhibition of APX was observed over next hours of the experiment (4, 6 h). Prolonged feeding (24, 48 h) increased the APX activity, then the inhibition of enzyme in infested seedlings was observed again. Different results were obtained in vetch, where APX activity was initially inhibited in plants infested by *A. pisum*. After 24 h of aphid feeding, APX activity in infested plants was approximately 30% higher than in control ones. Prolonged feeding of aphids (48, 72 h) didn't significantly affect the APX activity in tested seedlings. When *A. pisum* females were transferred to seedlings of broad bean, the induction of APX was observed just 1 h after infestation. However, prolonged feeding (4, 6, 24, 48 h) of aphids caused decrease in APX activity in infested plants in comparison to

non-infested ones. At the end of experiment (72 h) the induction of APX was observed and it was similar to that obtained 1 h after infestation. Among tested host plants, the strongest inhibition as well as the strongest induction of APX was noted within tissues of broad bean (Figure 3).

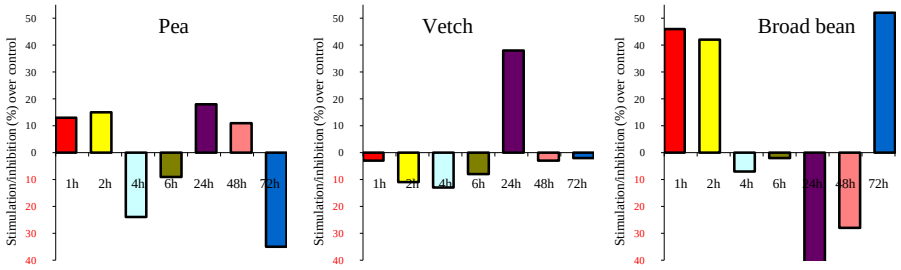

Figure 3. Effects of aphid infestation on APX activity in *Fabaceae* seedlings

The changes in APX activity were probably associated with the level of H_2O_2 in host plants, since APX is induced at lower concentrations of H_2O_2, when CAT exhibits lower substrate affinity. He *et al*. (11) observed a rapid increase of APX activity in the more resistant cultivars of chrysanthemum infested by *Macrosiphoniella sanborni* (Gillette). The activity of APX in poplar leaves damaged by *C. anachoreta* showed a sharp increase at 0.5 h, then a decrease at 2 h and a slight increase at 6 h. This tendency was opposite to the accumulation of H_2O_2 in wounded leaves (12). The studies conducted on triticale infested by cereal aphid *Sitobion avenae* (F.) and *Rhopalosiphum padi* (L.) showed the strongest induction of APX 72 h after infestation (17). Bi and Felton (6) noted the decrease of antioxidants (CAT, ASA, thiols) in soybean after *H. zea* feeding, but APX activity increased 1.5-folds. In our experiment the changes in APX activity during infestation by pea aphid were dependent on plant species, e.g. for vetch no inhibition of APX was noted, while, for pea and broad bean significant depletion of APX was observed after 72 h and 24 h respectively. The feeding of *Brevicoryne brassicae* (L.) on cabbage leaves reduced the activities of antioxidant enzymes (SOD, CAT, APX) and the strongest inhibition was noted for APX (13). The modulation of APX activity after infestation by *A. pisum* suggests that this enzyme plays an important role in defence mechanisms of *Fabaceae* plants.

The studied plant species differed significantly in H_2O_2 level and activity of antioxidant enzymes before infestation of *A. pisum*. The non-infested broad bean was characterized by the highest content of H_2O_2 and the lowest CAT and APX activity, while pea exhibited opposite pattern (Fig. 1–3). These differences might determine the intensity of induction or inhibition studied oxidative stress markers in *Fabaceae* plants. Goławska (9) proved that the most suitable host plant for *A. pisum is* pea. The aphids fed on pea showed higher fecundity and survival than those performed on broad bean, alfalfa or clover.

CONCLUSIONS

The infestation by *A. pisum* increased the production of H_2O_2 and resulted in oxidative stress in *Fabaceae* plant tissues. Fast modulation of the studied enzyme activities after herbivory allows us to state that of H_2O_2-scavenging enzymes play an important role in early responses of *Fabaceae* to the aphid feeding.

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