EFFECT OF HOST PLANTS ON BIOCHEMICAL MARKERS OF OXIDATIVE STRESS WITHIN TISSUES OF PEA APHID

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Abstract: The effect of some species of the *Fabaceae* family (the pea *Pisum sativum* L., broad bean *Vicia faba* L. and vetch *V. sativa* L.) on biochemical markers of oxidative stress within tissues of the pea aphid, *Acyrthosiphon pisum* Harris (Homoptera, Aphididae), has been studied. The highest concentration of superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and lipid peroxidation products thiobarbituric acid reactive substances (TBARS) was recorded for morphs that fed on the broad bean. The opposite tendency was observed for the level of total thiols, that were the highest for insects reared on the pea. Among the studied aphid morphs, the highest concentration of superoxide anion and hydrogen peroxide was noted for the wingless females whereas TBARS content was the highest within the winged migrant tissues. Different results were obtained for the total thiols, where *apterae* as well as migrants reared on the tested plants, had comparable content of these compounds. Our experiments indicate that oxidative stress may play an important role in interactions between the pea aphid and their host plants.

Key words: *Acyrthosiphon pisum*, *Fabaceae*, reactive oxygen species, oxidative stress

INTRODUCTION

Aphid herbivory induces biochemical and physiological changes in host plants that include the generation of reactive oxygen species (ROS) such as superoxide anion radical (O_2^-) and hydrogen peroxide (H_2O_2) (Kuźniak and Urbanek 2000; Mittler 2002). Oligogalacturonides released from plant cell wall polysaccharides by aphid salivary enzymes activate the degradation of linolenic acid, which together with systemin, oligogalacturonic acid, and chitosan, trigger the synthesis of hydrogen peroxide (Gatehouse 2002) and other ROS (Orozco-Cardenas and Ryan 1999). Additionally, plants produce pro-oxidant allelochemicals that upon photochemical or biochemical activation may generate ROS (Krishnan *et al.* 2007). ROS react with macromolecules such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins and lipids causing alterations within their structures. In insects, lipid peroxidation is potentially very harmful since lipids are not only components of cell membranes, but also play an important role in developmental and reproductive physiology (Downer 1986).

The oxidative status of the host plants plays an important role in the success of insects. High ROS concentration damages the absorption of ingested nutrients and can cause oxidative damage to the midgut cells (Bi and Felton 1995). Thus, the balance between the generation and elimination of ROS is one of the factors determining the performance of insects on the plants (Krishnan and Sehnal 2006). In biological systems subjected to oxidative stress, a number of different reactions occur resulting in products that may be characteristic of oxidative damage. The biochemical markers of oxidative stress include the depletion of non-enzymatic antioxidants, elevated levels of ROS and the presence of thiobarbituric acid reactive substances (TBARS) associated with lipid peroxidation (Summers and Felton 1994). Most of the previous research related to oxidative stress within herbivores has mainly focused on leaf-chewing insects, but little is known about the role of this process in the adaptation of sucking-percing to their host plants. The purpose of the study was to determine the indices of oxidative stress within the pea aphid, *Acyrthosiphon pisum* Harris (Homoptera, Aphididae) fed on *Fabaceae* plants. The oxidative stress was assessed with generation of the superoxide anion, hydrogen peroxide, content of total and protein thiols (total –SH), and the lipid peroxidation products (TBARS).

MATERIALS AND METHODS

Aphids

Experiments were conducted on various morphs of the pea aphid *Acyrthosiphon pisum* L. such as *apterae, alatae, larvae*. The aphids were reared on seedlings of *Fabaceae* plants in an environmental chamber [21°C, L16 : D8 photoperiod, and 70% RH (relative humidity)].

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Plants

Three species of *Fabaceae* were used in the experiments: the pea *Pisum sativum* L. var. Tulipan, broad bean *Vicia faba* L. var Start and vetch *Vicia sativa* L. var. Jaga. Seed samples were bought in the Horticultural Plant Breeding Seed Production and Nursery in Ożarów Mazowiecki (administrated from Warsaw, Poland). Seed samples were germinated in a climate chamber, which was kept at 21±1°C, L16 : D8 photoperiod, and 70% RH. The seedlings were grown in plastic pots (10x10 cm, 5 seedlings per pot) filled with fine garden soil, commonly used for greenhouse experiments.

Preparation of aphid homogenates

Batches of 100 collected aphids were placed in 50 mM K-phosphate buffer pH 7.0 (for speroxide anion, hydrogen peroxide, total and protein thiols assay) or in 1% phosphoric acid (for TBARS assay), and then homogenized for 5 min at 0°C. The homogenates were filtered through two layers of cheesecloth and centrifuged at 3,000 *g* for 15 min. The pellets were discarded and the supernatants were used for the assay of studied oxidative stress markers.

Hydrogen peroxide assay

The concentration of hydrogen peroxide was determined according to Green and Hill (1984), based on the reaction of 4-aminoantipyrine and phenol with H_2O_2, catalysed by peroxidase. In this manner the coloured product (chinonimin) was formed that was determined spectrophotometrically. The reaction mixture consisted of 1 ml of reagent and contained 4 mM of 4-aminoantipyrine, 24 mM of phenol, 0.4 U/ml of peroxidase dissolved in 0.1 M phosphate-buffer pH 7.0 and 0.3 ml of aphid homogenates. After the addition of homogenates, the reaction mixture was incubated at 25°C for 10 min and the absorbance was measured at 510 nm against the blank containing 0.3 ml of distilled water, instead of the aphid homogenate. The hydrogen peroxide content was calculated from a calibration curve prepared for this standard and expressed in nmol per mg protein.

Superoxide assay

Superoxide content was assayed in accordance with the method of Green and Hill (1984) based on reduction of nitroblue tetrazolium (NBT). The reaction mixture consisted of 0.5 ml of crude homogenate of aphids and 0.5 ml of 0.4 mM NBT in 0.2 M phosphate buffer pH 7.8. The increase in absorbance at 490 nm was monitored against the blank contained 0.5 ml of crude homogenate of aphids and 0.5 ml of 0.2 M phosphate buffer pH 7.8. The reducing activity of NBT by the aphid homogenates was expressed as ΔA_{490}/min/mg protein.

Total thiols assay

Total content of total thiol groups (sulfhydryls) within the aphid homogenates was determined with Ellman reagent [5,5′-dithiobis-(2-nitrobenzoic acid)], that formed colour complexes with –SH groups (Riener *et al.* 2005). The assay was carried out as follows: 0.1 ml of the aphid homogenates were mixed with 0.8 ml of 0.2 M phosphate buffer pH 8.2 and 0.1 ml of 6 mM Ellman reagent and incubated for 10 min at room temperature. After the incubation time, the absorbance of the samples was measured at 412 nm against the blank containing 0.1 ml of 0.2 M phosphate buffer pH 7.0 instead of the aphid homogenate. The concentration of the total thiols was calculated from the calibration curve prepared for glutathione and expressed in nmol per mg protein.

TBARS assay

The content of the thiobarbituric acid reactive substances (TBARS) was measured by modification of the Halliwell and Gutteridge (1990) method. The sample contained 1 ml of the aphid homogenate, 1 ml of 37% thiobarbituric acid (TBA) in 0.25 M HCl, and 1 ml of 15% trichloroacetic acid (TCA). At the same time, two controls were prepared. The first one contained 1 ml of distilled water instead of the aphid homogenate, and in the second one TBA was replaced with 1 ml of distilled water. All the mixtures were then placed in a boiling water bath for 60 min. After cooling, the mixtures were centrifuged and the absorbance of supernatants was measured at 535 nm against the first control. The value obtained for the second control was subtracted from absorbance reading for the sample. The concentration of TBARS was calculated using an extinction coefficient of 156 mmol/l/cm.

Protein assays

The protein content in the studied aphid supernatants was determined using the method given by Bradford (1976).

Statistics

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

RESULTS AND DISCUSSION

Our experiments showed that essential differences in the content of ROS within the pea aphid were related to *apterae* morphs. The highest O_2^- and H_2O_2 concentration was recorded for insects reared on the broad bean and the lowest for those fed on the pea. The winged adults (*alatae*) on the broad bean and vetch possessed a comparable level of H_2O_2 (Table 1). These differences may be associated with diversity of defense mechanisms of host plants against the aphids. Hussein *et al.* (2006) found that growth and reproduction of *Spodoptera littoralis* (Boisduval) was reduced after potato plants were substituted for the semi-artificial diet. This diet switch caused a rise of ROS as well as an elevation of the antioxidant enzymes within the tissues of *S. littoralis* (Krishnan and Kodrik 2006). Krishnan *et al*. (2007) proposed that intensive ROS generation within *Leptinotarsa decemlineata* (Say) tissues was linked to metabolism of ingested plant allelochemicals. The diapausing adults of *L. decemlineata* contained no detectable O_2^- and 10-fold lower content of peroxides than reproducing ones. These findings were probably as-

Table 1. The content of O_2^- (ΔA_{490}/min/mg protein) and H_2O_2 (nmol/mg protein) within tissues of various morphs of the pea aphid fed on *Fabaceae* plants.

Host plant	Aphid morphs	O_2^-	H_2O_2
Pea	apterae	0.21±0.02 c	31.73±0.22 c
	alatae	0.16±0.02 d	30.22±0.39 d
	larvae	0.07±0.01 f	19.67±0.29 g
Broad bean	apterae	0.30±0.01 a	39.84±0.82 a
	alatae	0.24±0.02 bc	34.92±0.29 b
	larvae	0.11±0.02 e	23.81±0.43 e
Vetch	apterae	0.26±0.01 b	35.28±0.52 b
	alatae	0.20±0.02 cd	35.15±0.73 b
	larvae	0.10±0.02 ef	21.11±0.21 f

Data are presented as the mean ±SD; n = 4. Values in columns not followed by the same letter are significantly different at the level of $p \leq 0.05$ (Duncan's test)

Table 2. The content of TBARS (nmol/mg protein) and total thiols (nmol/mg protein) within tissues of various morphs of the pea aphid fed on *Fabaceae* plants

Host plant	Aphid morphs	TBARS	Total thiols
Pea	apterae	0.34±0.02 de	130.86±2.31 a
	alatae	0.40±0.04 bc	129.19±1.48 a
	larvae	0.25±0.03 f	125.48±2.87 bc
Broad bean	apterae	0.42±0.02 ab	120.22±4.29 cd
	alatae	0.45±0.03 a	118.50±2.26 d
	larvae	0.31±0.02 e	100.19±7.80 e
Vetch	apterae	0.38±0.01 c	126.54±1.13 bc
	alatae	0.42±0.02 ab	125.79±2.17 bc
	larvae	0.27±0.01 f	118.71±1.62 d

Data are presented as the mean ±SD; n = 4. Values in columns not followed by the same letter are significantly different at the level of $p \leq 0.05$ (Duncan's test)

sociated with the absence of feeding and food processing (Krishnan *et al.* 2007). The consumption of tannic acid in the diet caused a 70-fold increase of O_2^- within the foregut of *S. littoralis* (Krishnan and Sehnal 2006). Our earlier studies showed that the cereal aphids exposed to the dietary phenolic compounds had a significantly higher level of hydrogen peroxide than the control insects (Łukasik *et al.* 2009). The concentration of H_2O_2 within the tissues of the cereal aphids varied according to their dependence on the triticale cultivar, on which they were reared (Łukasik *et al.* 2008). Thus, the oxidative status of herbivores responds to the quantitative composition and pro-oxidant content of their host plant.

Among the studied aphid morphs, the highest content of O_2^- and H_2O_2 was noted for the wingless females and the lowest for *larvae*. Only *apterae* and *alatae* reared on the vetch exhibited a similar level of H_2O_2 (Table 1). It is in agreement with our earlier studies where pea aphid *larvae* had a higher activity of enzymes which removed H_2O_2, than the activity of wingless *apterae* (Łukasik *et al*. 2011). Different results were obtained for morphs of cereal aphids, in which the H_2O_2 concentration was the highest within migrants (Łukasik and Goławska 2008; Łukasik *et al.* 2009). On other hand, adult *L. decemlineata* had a lower level of ROS and a higher antioxidant potential than the larval stages (Krishnan *et al.* 2007). These results point at stage specifity in the mechanisms regulating oxidative stress.

The highest content of TBARS was noted for morphs reared on broad bean. Compared to the others host plants, only *apterae* adults fed on vetch had a higher level of lipid peroxidation than those fed on pea (Table 2). Lipid damage is the main factor of free-radical toxicity. Summers and Felton (1994) noted that larvae of *Helicoverpa zea* (Boddie) exposed to the dietary phenolic acids had a higher level of lipid peroxidation products than the control insects. Phenolic compounds that have two ionizable hydroxyl groups at the aromatic ring showed the strongest effect of TBARS accumulation. This structural feature conditioned the ability of phenolics to generate ROS (Summers and Felton 1994). The studies on oxidative stress within cereal aphids showed that exposure of cereal aphids to o-dihydroxyphenols, especially chlorogenic acid, caused TBARS to increase in their tissues (Łukasik *et al.* 2009). It is likely, that the highest intensity of lipid damages within aphids fed on broad bean were caused by chemical compounds in this host.

Clear differences were found in the TBARS content within tissues of aphid morphs. The highest level of TBARS was recorded for winged females (*alatae*) and the lowest for *larvae*. Migrants and *apterae* morphs fed on broad bean, however, possessed comparable levels of TBARS (Table 2). These results are in accordance with our previous studies on two species of cereal aphids – monophagous grain aphid *S. avenae* and oligophagous bird cherry aphid *R. padi* (Łukasik *et al.* 2009). Sohal and Allen (1986) proved that flying morphs consumed more oxygen

than wingless adults and this feature was associated with the increased generation of ROS. This „oxidative burst" may lead to an accumulation of lipid hydroperoxides producing reactive aldehydes and ketones called TBARS. On other hand, larval stages of the pea aphid seem to posses more effective antioxidant mechanisms with regard to the low level of markers of oxidative stress such as O_2^-, H_2O_2 and TBARS. The results of our earlier studies showed that pea aphid *larvae* had a higher activity of enzymes which removed H_2O_2, than other morphs (Łukasik *et al*. 2011).

The highest content of total thiols (non-protein and protein) was noted for morphs reared on the pea. The *larvae* fed on this host plant had a similar –SH level as wingless females and migrants fed on vetch. Among the tested host plants, all studied morphs reared on the broad bean were characterized as having the lowest concentration of total thiols (Table 2). The depletion of thiol compounds is one of the biochemical markers of oxidative stress. It was earlier demonstrated that *larvae* of *H. zea* exposed to the *o*-dihydroxyphenols had a higher level of protein disulfides. This information indicates oxidation of thiol groups (Summers and Felton 1994). The experiments conducted on the cereal aphids showed that *o*-dihydroxyphenols and quinones caused a decrease of protein thiols within their tissues (Łukasik and Leszczyński 2005). The depletion of protein in –SH groups may have functional consequences as enzyme inactivations, as a decrease in antioxidant capacity, and as loss of certain specific functions (Shacter 2000). The results of our earlier investigations pointed out that dietary phenolic compounds affected the content of the main non-protein thiol, glutathione (GSH), within tisssues of *S. avenae* and *R. padi* (Łukasik 2006). Petrić-Mataruga *et al*. (1997) suggest that the regulation of the level of GSH in herbivores may be one of the forms of adaptation to oxidative stress. Lack of sufficient GSH concentration may lead to protein damage and depletion of ascorbate (Summers and Felton 1993).

Among the studied morphs, the contents of total thiols were comparable for *apterae* females and winged migrants, whereas larval stages had a slightly lower level of thiol compounds. This dependence concerned all studied host plants (Table 2). It is in agreement with the results of our earlier studies where wingless females of pea aphid had a higher content of non-protein thiol – GSH than *larvae* (Łukasik *et al.* 2011). However, the studies related to the level of total thiols within tisues of cereal aphid morphs showed the opposite pattern (Łukasik *et al.* 2009).

The results presented here demonstrated that the host plants can affect the oxidative balance of the pea aphid. The intensity of oxidative stress within aphid tissues may be conditioned by specific chemical factors which occur in the hosts.

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