Physiological and Biochemical Responses of Fern *Nephrolepis biserrata* (Sw.) Schott. to *Coccus hesperidum* L. Infestation

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We examined the effects of feeding by the polyphagous insect *Coccus hesperidum* on its host plant *Nephrolepis biserrata* under different intensities of infestation. As an effect of scale insect feeding there were significant changes in the values of parameters reflecting the state of cell membranes. *N. biserrata* plants reacted to the biotic stress by increasing guaiacol peroxidase activity and decreasing catalase activity. Our data show that these processes play key roles in plant tolerance mechanisms, here the fern's response to insect feeding. The observed complex reaction of *N. biserrata* testifies to actively proceeding, complex and very often contrasting mechanisms triggered with the aim of neutralizing the effects of biotic stress and enabling normal cell functioning in plants attacked by scale insects.

Key words: Biotic stress, electrolyte leakage, malondialdehyde, antioxidant enzymes, guaiacol peroxidase, catalase.

INTRODUCTION

During the whole vegetation period, plants are under the threat of constant attack by pathogens such as viruses, bacteria, fungi and herbivorous insects. One species of honeydew-producing scale insect commonly observed on ornamental plants cultivated under cover is *Coccus hesperidum* L. It is a cosmopolitan, typically polyphagous species noted on a number of host plants and one of the most dangerous pests of citrus plants in California, Texas and South Africa (Ben-Dov, 1993). This soft brown scale is one of the most troublesome species of greenhouse scale insects occurring in Poland (Dziedzicka, 1990). Like other soft brown scales, it settles on the leaves, the green parts of shoots and on fruits (Copland and Ibrahim, 1985). Feeding by this pest brings about yellowing and falling of leaves, and reduces fructification and plant vigor. The feeding and honeydew production of scale insects are biotic stress factors which affect the plants' physiological activity, growth and productivity. A common response of plant tissues to various stress factors,

including biotic stress, is disturbance of the balance between the production of reactive oxygen species (ROS) and their removal by antioxidative systems called sweepers or catchers of free radicals. The cause of this phenomenon is overproduction of ROS, known as oxidative stress. One of the plant's first reactions to feeding by insects is increased activity of antioxidative systems which sweep reactive forms of oxygen and prevent oxidative stress in plant cells. Those systems include enzymatic antioxidants like superoxide dismutase (SOD), which catalyzes dismutation of O_2^- to H_2O_2 and O_2. Hydrogen peroxide plays different roles in plant resistance. H_2O_2 shows direct toxicity to pathogens and herbivores and can prevent pathogens from invading plants through wound sites (Orozoco-Cardenas and Ryan, 1999). It acts also as a secondary signal molecule inducing the expression of defense genes (Vranová et al., 2002). A high concentration of H_2O_2 may be toxic to the host plant as well as to the insect, so a herbivore attack can stimulate enzymes scavenging H_2O_2. Hydrogen peroxide can also cause membrane lipid peroxidation and damage the reac-

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tion center of chloroplasts. Excess hydrogen peroxide is reduced by peroxidase (POD, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11) and catalase (CAT, EC 1.11.1.6) (Felton et al., 1994a,b; Argandona et al., 2001; Łukasik et al., 2012). Because CAT is inefficient at removing low concentrations of H_2O_2, the main mechanism for scavenging ROS is based on POD and APX. POD also catalyzes synthesis of lignin and suberin as well as accelerating the formation of covalent bonds between lignin and other polymers of the cell wall, which strengthen its structure and increases its mechanical resistance to an insect's attack (Ingham et al., 1998; Maffei et al., 2007). The plant response to biotic stress induced by herbivorous insects is still poorly understood; little is known about the effect of feeding by scale insects on the plants' physiological parameters.

Here we examined the effect of different intensities of *Coccus hesperidum* feeding on the state of cell membranes and the activity of selected antioxidative enzymes in *Nephrolepis biserrata* (Sw.) Schott. plants.

MATERIAL AND METHODS

PLANTS AND INSECTS

The experiments were carried out in the laboratory of the Department of Entomology, University of Life Sciences in Lublin (Poland). The studied material consisted of two-year-old *Nephrolepis biserrata* (Sw.) Schott. plants measuring 50–60 cm, grown in pots (15 cm diam) filled with a standard horticultural substrate, at 20–22°C and 65–70% RH (monitored with a hygrothermograph) under a 14 h photoperiod. *Coccus hesperidum* (superfamily Coccoidea), a polyphagous pest of greenhouse ornamental plants, was selected for artificial colonization of the plants. The fern plants were divided into three groups of ten plants each: uninfested control plants, plants infested by 10 *C. hesperidum* first-instar nymphs (crawlers) per plant, and plants colonized by 100 crawlers per plant. The crawlers were transferred from the rearing laboratory to the plants with a thin wooden spatula. Plant extracts were analyzed 6 months after artificial infestation, when the insect populations had grown. The material for analysis consisted of three groups (ten plants each) divided according to the mean number of insects per fern plant: control, uninfested plants; series 1, plants infested with 1–10 scale insects per plant; and series 2, plants with more than 100 insects per plant.

STATE OF CELL MEMBRANES

The physiological state of the plants was analyzed in the laboratory of the Department of Plant Physiology of the University of Life Sciences in Lublin. The state of leaf cell membranes was checked in plants of each series by determining electrolyte leakage (E_L) from leaves according to the method described by Kościelniak (1993), using an Elmetron CC-317 microcomputer conductometer. Ten rings (0.9 cm diam) were cut with a cork borer from leaves of each series, then covered with 20 cm^3 redistilled water and shaken at room temperature for 24 h, after which the first electroconductivity measurement was made. The plant material was then boiled at 100°C (15 min). After another 24 h of shaking, electroconductivity was measured again to determine total electrolyte content. Electrolyte leakage is expressed as a percentage of its total content in the tissue.

The level of membrane lipid peroxidation was assessed by determining malondialdehyde (MDA) content according to Heath and Packer (1968). Crushed plant material (0.2 g) was homogenized in 0.1 M potassium phosphate buffer, pH 7.0, then centrifuged at 12,000 × g for 20 min. Next, 0.5 cm^3 of the homogenate was added to 2 cm^3 20% trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid (TBA) and incubated for 30 min in a water bath at 95°C. After incubation the samples were quickly cooled and centrifuged again at 10,000 × g for 10 min. Absorbance was measured at 532 and 600 nm with a Cecil CE 9500 spectrophotometer. The malondialdehyde concentration in a sample was calculated using the molar absorbance coefficient, which for MDA is 155 nM^{-1} cm^{-1}, and expressed as nanomoles per 1 g dry weight.

ANTIOXIDATIVE ENZYME ACTIVITY

Preparation of enzymatic extract

Leaves (0.2 g) were homogenized in a mortar in 0.05 mol dm^{-3} phosphorus buffer, pH 7.0, at 4°C. The homogenate was then centrifuged at 10,000 x g for 10 min at 4°C. The supernatant thus obtained was used for further procedures.

Determination of guaiacol peroxidase activity

Guaiacol peroxidase activity was measured following the method given by Małolepsza et al. (1994). The reaction mixture contained 0.5 cm^3 0.05 mol dm^{-3} phosphorus buffer, pH 5.6, 0.5 cm^3 0.02 mol dm^{-3} guaiacol, 0.5 cm^3 0.06 mol dm^{-3} H_2O_2 and 0.5 cm^3 enzymatic extract. Extinction was measured at 1 min intervals for 4 min with a Cecil CE 9500 spectrophotometer at 480 nm. Guaiacol peroxidase activity was determined using the absorbance coefficient for guaiacol peroxidase, which is 26.6 mM cm^{-1}. The result was converted to guaiacol peroxidase activity per fresh weight, expressed as U/mg fresh weight.

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Determination of catalase activity

Catalase activity was determined as described by Chance and Meahly (1955) and modified by Wiloch et al. (1999). The reaction mixture contained 2 cm^3 50 mM K-phosphorus buffer, pH 7.0, 0.2 cm^3 H_2O_2 and 0.1 cm^3 enzymatic extract. Extinction was measured for 3 min using a Cecil CE 9500 spectrophotometer reading the initial and final results at 240 nm. Catalase activity was determined using the absorbance coefficient, which for catalase is 0.036 mM cm^{-1}. The result was converted to catalase activity per fresh weight, expressed as U/mg fresh weight.

STATISTICAL ANALYSIS

All statistical analyses employed Statistica 9.1 (StatSoft). One-way ANOVA with Tukey's simultaneous test was applied at p = 0.05 and p = 0.01. All analyses were done in five replicates for each plant series and physiological parameter.

RESULTS

STATE OF CELL MEMBRANES

Electrolyte leakage (parameter E_L) differed between *N. biserrata* control plants and insect-infested plants (Fig. 1). Among the latter, E_L differed significantly between highly infested and less infested plants: it was highest (18.16%) for leaves from series 2, the group with more scale insects on the leaves.

The malondialdehyde content of *N. biserrata* leaves (parameter MDA) differed significantly between control plants and the plants from series 2 (Fig. 2). MDA also differed significantly between series 2 (group with >100 insects/plant) and series 1 (1–10 insects/plant). It did not differ significantly between the control and series 1. MDA was highest (11.69 nmol g^{-1} fresh weight) in leaves from series 2.

GUAIACOL PEROXIDASE ACTIVITY

The presence of *C. hesperidum* on *N. biserrata* plants altered the activity of the examined enzymes of the antioxidative system. The only significant difference in guaiacol peroxidase activity versus the control was for samples from series 1: 0.5 nmol g^{-1} fresh weight, ~17 times higher than in the control. POD activity in plants bearing more than 100 scale insects per plant (series 2) decreased to 0.02 nmol g^{-1} fresh weight, not significantly different from the control (Fig. 3).
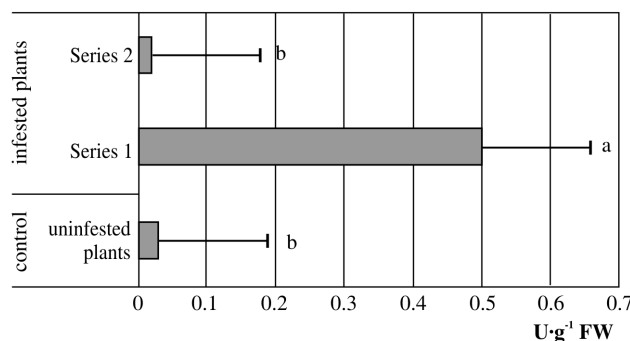
CATALASE ACTIVITY

Catalase activity in leaves changed in an insect-density-dependent manner. It was highest in uninfested
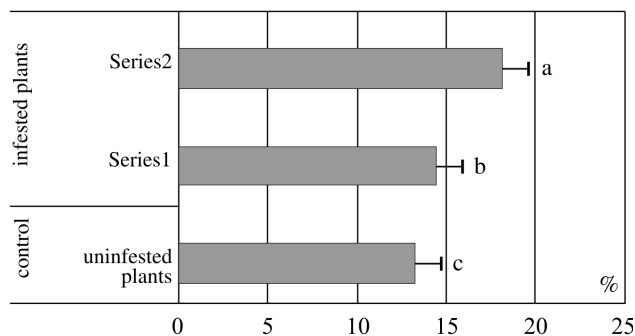
Fig. 1. State of *N. biserrata* leaf cell membranes as determined by electrolyte leakage under different intensities of *C. hesperidum* feeding (= insect density). Means bearing the same letter do not differ significantly at p = 0.01 (Tukey test).
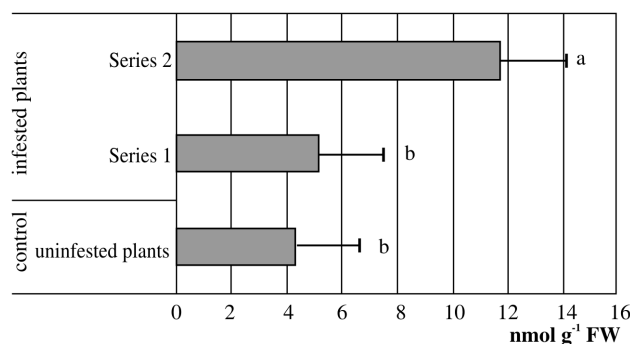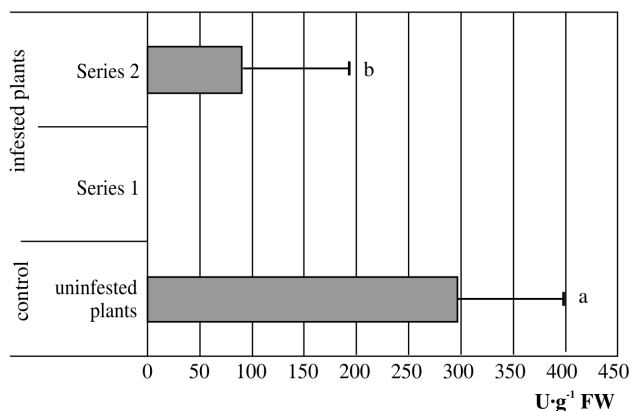

Fig. 2. State of *N. biserrata* leaf cell membranes as determined by malondialdehyde content under different intensities of *C. hesperidum* feeding (= insect density). Means bearing the same letter do not differ significantly at p = 0.05 (Tukey test).

Fig. 3. Peroxidase activity in *N. biserrata* leaves under different intensities of *C. hesperidum* feeding (= insect density). Means bearing the same letter do not differ significantly at p = 0.01 (Tukey test).

control plants (296.44 nmol g^{-1} fresh weight), significantly lower in the plants from series 2 (92 nmol g^{-1} fresh weight), and completely absent in the samples from series 1 (Fig. 4).

Fig. 4. Catalase activity in *N. biserrata* leaves under different intensities of *C. hesperidum* feeding (= insect density). Means bearing the same letter do not differ significantly at p = 0.01 (Tukey test).

DISCUSSION

Insects and diseases present potential biotic stresses to host plants. Plants challenged by insects respond through changes in the composition and physical properties of the cell wall and through biosynthesis of secondary metabolites.

Oxidative stress is a disturbance of the balance between the intensity of oxidative processes that induce the formation of reactive oxygen species (ROS) and the antioxidative defense system. Toxic products of the oxidation reaction have a cytostatic effect on the cell, damaging the cell membranes and leading to cell death *via* apoptosis or necrosis. Balanced cell metabolism is maintained by antioxidative enzymes such as superoxide dismutase, catalase, peroxidase, ascorbate peroxidase, glutathione S-transferase and polyphenol oxidase (Hu et al., 2009; He et al., 2011) or vitamins E, C and A. These molecules facilitate removal of excess ROS from cells. One effect of oxidative stress following mechanical injury to plant tissues by feeding insects is lipid peroxidation, which consists in destabilization and breakup of lipids with simultaneous production of aldehydes and alcohols. In plant cells it is manifested mainly in damaged plasma membranes (cell membranes, chloroplast and mitochondrial membranes) leading to changes in their structure and physical state. We found significant changes in the values of parameters reflecting the state of cell membranes under insect feeding (electrolyte leakage, malondialdehyde content). The plants' response to the biotic stress differed depending on the intensity of colonization by *C. hesperidum*. Analysis of electrolyte leakage showed a stronger reaction to biotic stress in leaves of heavily infested plants. Lightly infested plants had significantly higher E_L than con-

trol plants, and significantly lower E_L than massively insect-colonized plants

Feeding by scale insects also significantly raised the malondialdehyde content of fern leaves. Aslanturk et al. (2011) noted increased MDA under stress caused by a gall-forming psyllid on eucalyptus trees; the rate of MDA synthesis increased with the duration of stress. Insect herbivory of *Eucalyptus* brought about a significant increase in lipid peroxidation, measured as malondialdehyde, as compared with healthy trees. Biotic and abiotic stresses stimulate the production of active oxygen and consequently lipid peroxidation of cell macromolecules (Baker and Orlandi, 1996; Aslanturk et al., 2011). Increased lipid peroxidation may be due to the inability of antioxidants to capture all the active oxygen species induced by this biotic stress (Aslanturk et al., 2011).

Biotic stress causes a decrease in the content or quality of nutritive substances in plants, which directly affects the demographic parameters of the insects feeding on them (Duffey and Felton, 1991; Felton et al., 1994a; Duffey and Stout, 1996). The production and destruction of reactive oxygen species (ROS) in plants play a key role in plants' tolerance mechanisms (Hildebrand et al., 1986; Heng-Moss et al., 2004). The major task of oxidative enzymes (peroxidase and catalase) is catalysis and reduction of by-products of oxygen metabolism, to prevent injury to plant cells (Hildebrand et al., 1986; Bernays et Chapman, 1994; Felton et al., 1994a,b; Stout et al., 1999; Ni et al., 2001; Allison and Schultz, 2004; Heng-Moss et al., 2004). Biotic stress caused by insect feeding can activate defense mechanisms in plants, including increased peroxidase activity. These enzymes occur in different places in plant cells and fulfill a number of functions in them. For example, they contribute to degeneration and detoxification of ROS, lignification and strengthening of the cell wall, and catabolism of auxins (Dowd and Lagrimini, 1997; Hiraga et al., 2001; Welinder et al., 2002; Allison and Schultz, 2004). Mittler et al. (1999) observed increased peroxidase levels in plants under pathogen attack as a direct response to increased ROS. Heng-Moss et al. (2004) reported changes in peroxidase content, which in resistant plants remained higher in hemipteran-colonized plants than in the control; control plants responded to insect feeding with lower or nonsignificant changes in peroxidase activity. In our study, guaiacol peroxidase activity increased significantly in plants under scale insect colonization. The increase was observed in series 1 (light infestation), where peroxidase activity was 17 times higher than in the control. In the same variant, catalase activity was completely absent. In plants strongly colonized by scale insects (>100 insects/plant), on the other hand, both peroxidase and catalase activity dropped below

the control levels. Heng-Moss et al. (2004) analyzed changes in catalase activity in buffalograss under feeding by a hemipteran (chinch bug); it dropped in plants subjected to feeding by this phytophagous species as compared to the level in control plants. Unlike peroxidase, catalase is localized mainly to the peroxisomes of photosynthetic plant cells, and is the marker enzyme for them. It takes an active part in neutralization of hydrogen peroxide and (primarily) superoxide to water (van Den Munckhof, 1996). The joint effect of catalase and peroxidase helps to ease the effect of ROS in plants invaded by insects. In our study the *N. biserrata* plants reacted to the biotic stress of scale insect feeding by increasing the activity of guaiacol peroxidase in the group of lightly infested plants (series 1). On the other hand, catalase activity decreased under that stress. The heavily infested plants (series 2) showed a very strong response to scale insect feeding: an almost 17-fold drop in peroxidase activity versus the control, and no catalase activity. Hildebrand et al. (1986) suggested that an increase of peroxidase activity allows the plant to detoxicate peroxides, reducing tissue damage. Peroxidase takes part in lignification of cell walls, so increased activity of this enzyme would be expected to contribute to *N. biserrata* plants' resistance to feeding by insects. The complexity of the response in *N. biserrata* testifies to actively proceeding, complicated and very often contrasting mechanisms triggered with the aim of neutralizing the effects of biotic stress and enabling normal cell functioning in plants attacked by scale insects. Literature data (Hildebrand et al., 1986; Heng-Moss et al., 2004) indicate that these processes play key roles in plant tolerance mechanisms, and our findings on the response of *N. biserrata* to *C. hesperidum* feeding add more confirmation.

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