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PHYSIOLOGICAL REACTION OF *Phalaenopsis* × hybridum 'Innocence' ON *Pseudococcus longispinus* (Targoni Tozetti) FEEDING

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Abstract. The physiological response of *Phalaenopsis* × hybridum 'Innocence' to biotic stress caused by *Pseudococcus longispinus* feeding was investigated. The condition of the cytoplasmic membranes expressed by a value of electrolyte outflow (E_L) and TBARS and the activity of antioxidative system enzymes: catalase and peroxidase, and the amount of non-enzymatic antioxidant – proline, were determined. The changes in all the analyzed physiological parameters depended on the duration of the pest feeding. The outflow of electrolytes, TBARS content and catalase activity was the highest in the first period of the experiment (after 24-hour of mealybug feeding). Significant increase of peroxidase activity and proline content was noted after 7 days of insects feeding. The values of all analyzed parameters (except E_L) demonstrated a decreasing tendency after 14 days of *P. longispinus* feeding. The observed reaction of *P.* hybridum 'Innocence' testifies to mechanisms triggered with the aim of neutralizing the effects of biotic stress and enabling the normal functioning of the cells in the orchid plants colonized by longtailed mealybug.

Key words: longtailed mealybug, moth orchid, biotic stress, TBARS, electrolyte outflow, antioxidants

INTRODUCTION

Insects feeding on ornamental plants are a source of stress which negatively affects their condition and decorative value. Strong stress in particular is induced by insects with sap-sucking mode of feeding. They insert stylets into the phloem and thus damage plant tissues causing disorders of the electric potential of cytoplasmic membranes, cell hydration and metabolite transport. In response to biotic stress, the plant induces physiological-biochemical defense reactions [Sempruch 2008]. One of the first plant reactions to pest attack is increased production of reactive oxygen species (ROS) such

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as $O_2\bullet^-$, H_2O_2, $^1O_2$, $HO_2\bullet^-$, OH•, ROOH, ROO•, RO• which are highly reactive and destroy lipids, proteins, carbohydrates, DNA and finally cause cells death [Gill and Tuteja 2010]. Among these reactive oxygen species a special role plays hydrogen peroxide, which on one hand shows direct toxicity to pathogens and herbivores and can prevent plants from pathogens invading through wound sites [Orozco-Cárdenas and Ryan 1999]. On the other hand, it acts 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.

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) [Gill and Tuteja 2010, Łukasik et al. 2012]. Hu et al. [2009], Gulsen et al. [2010] and Golan et al. [2013] have pointed that peroxidase play an important role in plant's response to biotic stress. They suggest that increased peroxidase levels in specific plants compartments may enhance the plant's ability to tolerate insect feeding and/or play a critical role in the plant's defense system. The proposed functions of peroxidases in plants include lignification, suberization, somatic embryogenesis, auxin metabolism, wound healing [Hiraga et al. 2001, Allison and Shultz 2004, Passardi et al. 2005]. Peroxidases are involved also in plant defence against biotic and abiotic factors.

Orchids, especially those of the *Phalaenopsis* variety, are one of the most often purchased ornamental pot plants. Their economic significance in gardening production is still increasing due to the development of efficient reproduction methods from meristems and the cultivation of new low-maintenance varieties [Griesbach 2002]. Orchids are the most readily and abundantly attacked by polyphagous mealybugs, including *Pseudococcus longispinus* (Targioni Tozzetti), which feeds mainly on their underground parts. It is difficult to detect the presence of this pest on orchids in the initial phase of its feeding due to the very small body size of first instar larvae (crawlers). Second and third instar larvae, in turn, exhibit a tendency to hide in various plant hollows or under pots. The behavior of this pest means that it is noticed on the plants in high abundance, when the range of its harmfulness is considerable (autor's own unpublished study) and its chemical control is less effective. It should be emphasized that the presence on the mealybug body of a cover made of waxy filamentous secretion additionally hampers chemical control of this pest in later feeding periods. The need for the search for pro-ecological methods to control polyphagous *P. longispinus* is justified by the constantly increasing popularity of orchids and the common occurrence of this scale insect. This study is focused on the recognition of orchids physiological defense reactions to *P. longispinus* feeding and constitutes a significant addition to knowledge concerning natural mechanisms of plant resistance to pest feeding, which are increasingly being used in breeding plant resistance.

The purpose of the present study was to determine the role of oxidative stress in the defense response of orchid *Phalaenopsis* × hybridum 'Innocence' to infestation of *Pseudococcus longispinus* (Targioni Tozzetti) (Hemiptera: Coccoidea: Pseudococcidae). The degree of cellular damage was estimated on the basis of electroltyte leakage measurement and the level of thiobarbituric acid reactive substances (TBARS) – a product of lipid peroxidation. As well as the changes in level of proline and in activity

of antioxidant enzymes, POD and CAT were assessed. The results obtained could also serve as an important biochemical markers for plant resistance against sap-sucking insects.

MATERIALS AND METHODS

Plants and insects material. The orchids chosen for analysis were purchased from JMP Flowers Gardening Enterprise in Stężyca, where they were sold as so called intermediate products (plants without inflorescence shoots). The experiment was conducted in the laboratory of the Department of Entomology, University of Life Sciences in Lublin. The plants were grown in plastic transparent pots of a diameter of 12 cm, filled with coarse pine bark bedding and were situated in a cultivation chamber on textile subirrigation mats (Polprotex) covered with black agrofabric. Care practices only included once a week plants flooding with tap water. The experiment included orchids (after a 4-week adaptation period) in a phase of seven fully developed leaves, without inflorescence shoots, which were colonized with *P. longispinus* individuals derived from laboratory breeding conducted on *Phalaenopsis* × hybridum 'Innocence' for 6 months preceding the experiment. Five young females or third instar larvae of *P. longispinus* were transferred onto each plant using a bristle brush. The number of *P. longispinus* individuals on the orchids did not change for the whole period of the experiment due to the long pre-reproduction period of this species. The sample constituted of 3 plants, on which the insects were feeding for 24 hours, 7 days and 14 days, respectively, and the control plants (without the pest). The experiment was conducted in three replications for each combination. The same experimental conditions were applied in the cultivation chambers for all the plants used during this study (temperature 27 ±1°C; humidity 50 ±5%, photoperiod L:D = 16:8).

Physiological analysis. 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 condition of the cytoplasmic membranes was expressed by a value of electrolyte outflow (E_L) and thiobarbituric acid reactive substances content (TBARS). In addition, the activity of antioxidative system enzymes, i.e. catalase and peroxidase, and the amount of non-enzymatic antioxidant – proline, were determined. These are the parameters which are used the most often in the examination of physiological plant reaction during insects feeding [Golan et al. 2013, Mai et al. 2013].

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 hour, after which the first electroconductivity measurement was made (K_1). The plant material was then boiled at 100°C (15 min). After another 24 hour of shaking, electroconductivity was measured again to determine total electrolyte content (K_2). Electrolyte leakage is expressed as a percentage of its total content in the tissue, according to the formula: $E_L = (K_1/K_2)\ 100\%$.

The level of membrane lipid peroxidation was assessed by determining thiobarbituric acid reactive substances (TBARS) content according to Heath and Packer [1968]. Crushed plant material (0.2 g) was homogenised 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 TBARS concentration in a sample was calculated using the molar absorbance coefficient, which for TBARS is 155 nM^{-1} cm^{-1}, and expressed as nanomoles per 1 g fresh weight.

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 × g for 10 min at 4°C. The supernatant thus obtained was used for further procedures.

Peroxidase activity towards guaiacol 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. Peroxidase activity towards guaiacol was determined using the absorbance coefficient for this enzyme, which is 26.6 mM cm^{-1}. The result was converted to peroxidase activity per fresh weight, expressed as U × mg^{-1} fresh weight.

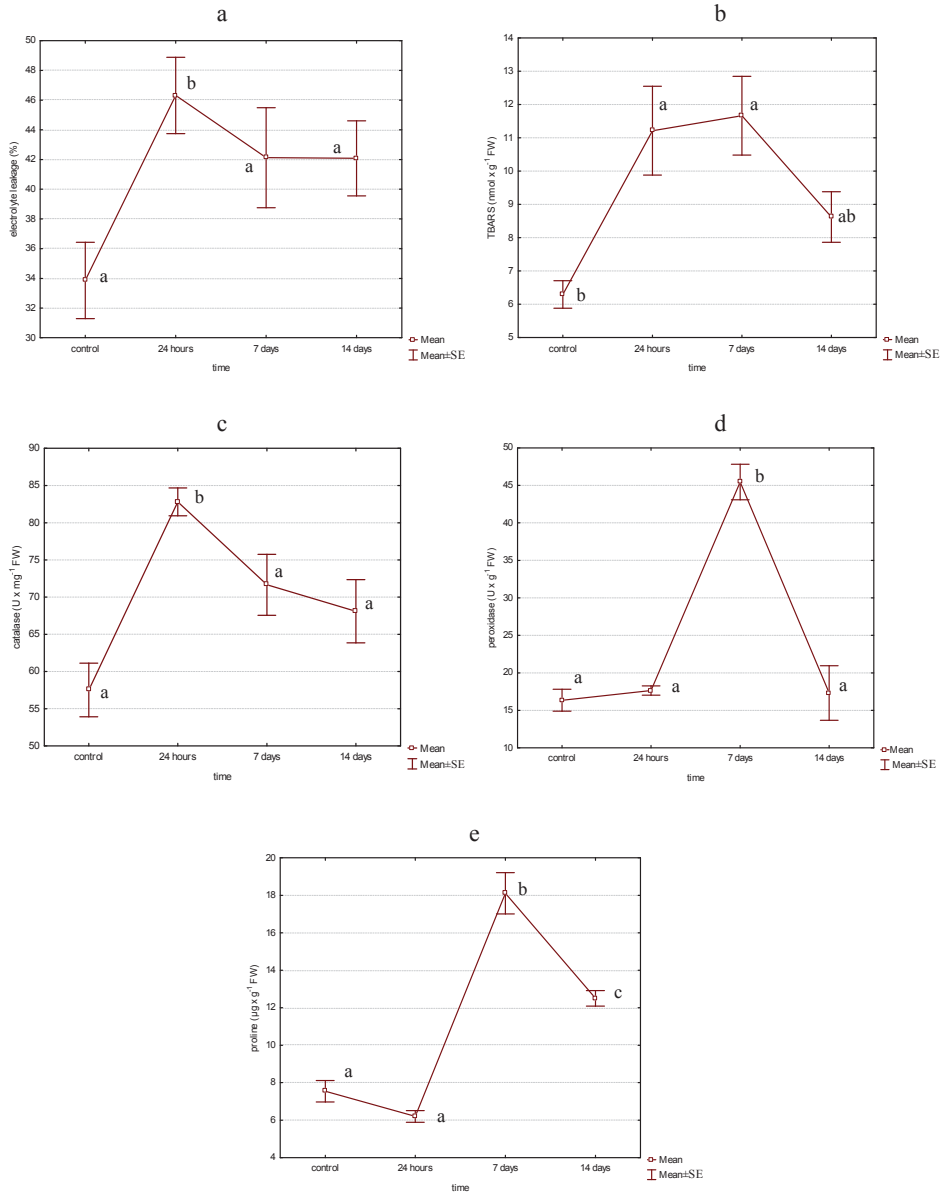
Catalase activity was determined as described by Chance and Meahly [1955] and modified by Wiloch et al. [1999]. The reaction mixture contained 2 cm^3 50 mM K-phosphorus buffer, pH 7.0, 0.2 cm^3 H_2O_2 and 0.1 cm^3 enzymatic extract. Extinction was measured for 3 min using a Cecil CE 9500 spectrophotometer 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^{-1} fresh weight.

To determine free proline level, 0.5 g of leaf samples from each group were homogenized in 3% (w/v) sulphosalycylic acid and then homogenate filtered through filtered paper [Bates et al. 1973]. Mixture was heated at 100°C for 1 hour in water bath after addition of acid ninhydrin and glacial acetic acid. Reaction was then stopped by ice bath. The mixture was extracted with toluene and the absorbance of fraction with toluene aspired from liquid phase was read at 520 nm. Proline concentration was determined using calibration curve expressed as μg proline g^{-1} FW.

Experimental data were verified statistically with Statistica 9 (StatSoft, Poland). The results were analyzed using ANOVA analysis of variance. Significant differences between means were determined by Tukey test at the significance level of $\alpha = 0.05$.

RESULTS

The influence of *P. longispinus* feeding on the state of cell membranes. The results of the analysis of *P.* × hybridum 'Innocence' leaves suggest differentiated plant



Means marked with the same letter do not differ significantly at α = 0.05

Fig. 1. Changes of physiological parameters in the leaves of *Phalaenopsis* × hybridum 'Innocence' under the influence of *Pseudococcus longispinus* feeding: a) electrolyte leakage, b) thiobarbituric acid reactive substances (TBARS) content, c) catalase activity, d) peroxidase activity towards guaiacol, e) free proline content

physiological reaction to biotic stress, depending on the duration of *P. longispinus* females feeding (fig. 1). The highest changes in cytoplasmic membrane stability of orchid leaves were observed after a 24 hour period of *P. longispinus* feeding. Statistically significant increases ($p < 0.05$) in electrolyte outflow and thiobarbituric acid reactive substances content, of 36.8% and 78.2%, respectively, were noted at this time compared to the results derived from control plant measurements (figs 1a, b). An insignificant decrease in the E_L parameter ($p > 0.05$) value and a slight increase in TBARS content were noted during the analysis performed after 7 days of insects feeding compared to the measurements performed after 24 hours of the experiment. The values of the analyzed parameters measured after 14 days from plants colonization with mealybugs were lower with respect to the values of the parameters analyzed at the two previous times (after 24 hours and 7 days of *P. longispinus* feeding). It was found decrease in TBARS content (of over 35%, $p > 0.05$) compared to its content in the plants on which the insects were feeding for 7 days (fig. 1b).

Activity of antioxidants in orchid tissues in response to *P. longispinus* feeding. The highest activity of catalase in orchids leaves was noted after 24 hours of *P. longispinus* feeding (an activity increase of 43.9% compared to this enzyme activity in the control plants, $p < 0.05$). A decrease in this enzyme activity was observed at the other measurement times (after 7 and 14 days of mealybug feeding) (fig. 1c).

Peroxidase activity towards guaiacol in orchid leaves increased insignificantly during the first term of the experiment compared to the plants without *P. longispinus* feeding. High activity of this enzyme was only noted after 7 days of insects feeding. At this time, the activity of peroxidase was subject to a nearly 3-fold increase compared to the activity in the control plants; it was also higher with respect to the activity in the plants after 14 days of mealybug feeding (fig. 1d).

Initially, proline content in *P.* × hybridum 'Innocence' leaves was subject to a slight decrease (analysis performed after 24 hours from colonization) compared to the control plants. The highest content of the analyzed protein was noted in the leaves during the measurement conducted after 7 days of insects feeding (a nearly 2.5-fold increase compared to the control plants). The 14 day period of scale insects presence on the orchids resulted in a significant decrease in proline content ($p < 0.05$), however, its level was higher compared to the control plants, and the plants after 24 hours of scale insect feeding (fig. 1e).

DISCUSSION

Destructive ROS activity results in damage to plant cytoplasmic membranes. Severe damage to cytoplasmic membranes is caused by an excess of and rapid increase in H_2O_2. The plant is not able to immobilize an excess of reactive oxygen species generated during biotic stress, therefore, peroxidation of membrane lipids is often observed [Gill and Tuteja 2010, Aslanturk et al. 2011]. This leads to a decrease in cytoplasmic membrane hydration, their increased permeability for those substances which normally infiltrate specific channels, as well as damage to proteins included in them. It also results in the inactivation of receptors, enzymes and ionic channels [Gill and Tuteja

2010]. A measure of the changes in cytoplasmic membranes of plant cells affected by insects feeding is, inter alia, the level of electrolyte outflow resulting from the damage occurring and the level of TBARS content.

As a result of analysis, it was demonstrated that the level of electrolyte outflow and thiobarbituric acid reactive substances content were subject to changes depending on the duration of mealybug feeding, and they reached high values as soon as after 24 hours of pest presence on the plant. An increase in MDA content was also noted by Khattab and Khattab [2005] in the leaves of eucalyptus colonized by gall-forming psyllid. Mai et al [2013] recorded progressive increase in TBARS content in *Pisum sativum* leaves in the duration of pea aphid infestation. Also, Golan et al. [2013] demonstrated that *Coccus hesperidum* feeding caused a significant increase in the content of malondialdehyde in fern leaves abundantly colonized by the insects.

The amount of reactive oxygen species, such as anion superoxide ($O_2^{\bullet -}$) or hydrogen peroxide (H_2O_2), in plant tissues is subject to a very quick increase during insect feeding [Bolwell et al. 2002, Imbiscuso et al. 2009]. The antioxidative systems of the plant, including enzymatic antioxidants: superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), peroxidase (POD), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione S-transferases (GST), glutathione peroxidase (GPX), are mobilized as a response to an enhanced synthesis of reactive oxygen species damaging peroxisomes, mitochondria or chloroplasts [Maffei et al. 2007, Gill and Tuteja 2010]. The present study also involved analysis of the activity of catalase and peroxidase. Catalase is an enzyme essential in the process of ROS detoxication in plants, and it is characterized by the highest efficiency in the process of H_2O_2 transformation into H_2O and O_2 [Gill and Tuteja 2010]. CAT scavenges H_2O_2 generated during mitochondrial electron transport, beta-oxidation of the fatty acids [Yang and Poovaiah 2002]. The highest activity of this enzyme in orchid leaves was noted after 24 hours of *P. longispinus* feeding, while after 7 and 14 days of the experiment a slight decrease of its activity was observed. In lima bean, gene activation of CAT reaches the highest activity 6 h after aphid feeding [Maffei et al. 2006]. On the other hand, Mohase and van der Westhuizen [2002] demonstrated that Russian wheat aphid infestation inhibited catalase activity in wheat plants. A different activity course was noted in the case of POD. The highest activity of this antioxidant was demonstrated in plants subjected to the activity of biotic stress for 7 days. The activity of this enzyme in plants examined after a shorter (24 hours) or longer (14 days) feeding period was lower and close to the activity in the control plants. An increase in peroxidase activity was also noted by Mohase and van der Westhuizen [2002] and Moloi and van der Westhuizen [2006] in wheat colonized by Russian wheat aphids, as well as by He et al. [2011] in chrysanthemum infested by *Macrosiphoniella sanborni* (Gill.) aphids. Golan et al. [2013] demonstrated that the activity of peroxidase was many-fold higher in fern leaves colonized by sparse *C. hesperidum* individuals, compared to plants abundantly colonized by this insect, in which the activity of this enzyme was similar to that in the control plants. Activity elevation of POD, an enzyme involved in oxidative signal transduction probably controls the cellular H_2O_2 concentration. It is noteworthy that the role of POD is somewhat arbitrary, as this enzyme is involved in defense-related events that occur in the extracellular matrix. These include the strengthening of

plant cell walls by lignification and the formation of intermolecular cross-links as well as suberin formation [Mehdy 1994, He et al. 2011], where it functions as a defense enzyme more than as an antioxidant enzyme.

Proline is a non-enzymatic antioxidant essential for primary metabolism. It presumably plays a role in plant adaptation to unfavorable conditions by an attenuating of unprofitable ROS activity [Szabados and Savouré 2009, Chen and Dickman 2005]. In this study, the changes in proline content in orchid leaves were similar to the changes in peroxidase activity toward guaiacol in the examined periods of *P. longispinus* feeding. The highest proline content was observed in the leaves during measurement performed after 7 days of insect feeding. The content of the examined protein decreased significantly after 14 days, and its level was higher compared to the content in the control plants and in the plants after 24 hours scale insects feeding. A similar tendency, characterized initially by either a lack or a small increase in free proline content, then its rapid accumulation and finally a decrease in this amino acid level, is observed in the case of the activity of various abiotic factors [Gibon et al. 2000, Choudhary et al. 2005]. An increase in proline content was also observed by El-Akkad [2004] in *Populus nigra* leaves colonized by *Pemphigus populi* aphids.

CONCLUSIONS

The changes in physiological parameters (electrolyte outflow, thiobarbituric acid reactive substances content, proline amount, catalase and peroxidase activity) revealed the occurrence of oxidative responses in *P.* × hybridum 'Innocence' leaves induced by *P. longispinus* feeding. The values of parameters determining cytoplasmic membrane condition reached high values during the initial period of mealybugs feeding. In the group of antioxidants, the highest activity of catalase was noted during the initial period whereas, peroxidase activity and proline content was the highest after one week of scale insects feeding. The values of all analyzed parameters (except E_L) demonstrated a decreasing tendency after 14 days of insects feeding. The observed reaction of *P.* hybridum 'Innocence' testifies to mechanisms triggered with the aim of neutralizing the effects of biotic stress and enabling the normal functioning of the cells in the orchid plants colonized by longtailed mealybug.

Induction of *P.* × hybridum 'Innocence' induced resistance could already be observed with a small number of *P. longispinus* individuals.

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FIZJOLOGICZNA REAKCJA STORCZYKA *Phalaenopsis* × hybridum ‘Innocence’ NA ŻEROWANIE CZERWCA *Pseudococcus longispinus* (Targoni Tozetti)

Streszczenie. Badano fizjologiczną reakcję *P.* × hybridum ‘Innocence’ na stres biotyczny wywołany żerowaniem *P. longispinus*. Określono stan błon cytoplazmatycznych wyrażony wartością wypływu elektrolitów (E_L) i zawartością substancji reagujących z kwasem tiobarbiturowym (TBARS), a także aktywność enzymów systemu antyoksydacyjnego: katalazy i peroksydazy, oraz ilość nieenzymatycznego antyoksydanta – proliny. Zmiany w wartościach wszystkich analizowanych parametrów zależały od długości żerowania szkodnika. Wartość E_L, zawartość TBARS oraz aktywność katalazy były najwyższe w pierwszym terminie eksperymentu (24-godzinne żerowanie czerwca). W przypadku aktywności peroksydazy i zawartości proliny istotny wzrost notowano dopiero po 7 dniach

żerowania. Po 14 dniach od zasiedlenia storczyków wartości wszystkich analizowanych parametrów (wyjątek E_L) wykazywały wyraźną tendencję spadkową. Obserwowana reakcja *P*. hybridum 'Innocence' świadczy o uruchomieniu przez roślinę mechanizmów, których zadaniem jest neutralizacja skutków stresu biotycznego i umożliwienie komórkom powrotu do normalnego funkcjonowania.

Słowa kluczowe: wełnowiec szklarniowy, storczyki, stres biotyczny, TBARS, wypływ elektrolitów, antyoksydanty

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