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ORIGINAL PAPER

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BIOCHEMICAL ALTERATIONS IN *Ulmus pumila* L. LEAVES INDUCED BY GALLING APHID *Tetraneura ulmi* L.

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ABSTRACT

It is commonly believed that gall inducers have the ability to control and program the host plant growth to their own benefit. The pattern of changes in the contents of reducing sugars, protein and phenolic compounds as well as the activity of chitinase and β-1,3-glucanase in galls, galled and intact leaves of *Ulmus pumila* were investigated during three stages of *Tetraneura ulmi* gall development. High protein accumulation in galls at the initial period of gall formation, increased biosynthesis of total phenolics during galling process, and the highest activity of pathogenesis-related protein at the stage of mature galls were detected. Therefore, it can be suggested that *T. ulmi* can manipulate the biochemical machinery of the galls for its own needs.

Key words: galls, phenolics, proteins, reducing sugars, chitinase, β-1,3-glucanase

INTRODUCTION

Ulmus pumila L., known as Siberian elm, is resistant to Dutch elm disease and phloem necrosis. It is cultivated throughout the Americas, Asia and Europe due to its tolerance to a wide range of growing conditions [Mittempergher and Santini 2004]. Insect galls, as additional sinks, disturb normal growth and development of the host plant [Larson and Whitham 1997]. The dense occurrence of bean-shaped galls formed by *Tetraneura ulmi* (*Hemiptera*: *Eriosomatinae*) was found on different elm species [Urban 2003, Kmieć and Kot 2007, Kmieć et al. 2016]. In the spring, the first instar of *T. ulmi* fundatrix hatches from the egg, migrates to young developing leaves and starts the galling process. Each gall is induced by a single larva. When gall is fully developed, the aphid propagates in the gall by parthenogenetic reproduction. Winged progeny disperses from the cracking gall in June and flies to the secondary host [Urban 2003, Kmieć and Kot 2007]. Only two generations of aphids develop in the gall. However, *T. ulmi* can be considered an important occasional pest of elms [Urban 2003, Kmieć et al. 2016].

The plants' reaction to insect feeding include physiological and biochemical responses. Plants activate a wide range of protective mechanisms associated with induced resistance after herbivores attack. One of the earliest responses is a generation of reactive oxygen species (ROS). The intensity and magnitude of ROS production within plant tissues can elicit the oxidative burst, which in turn affects the nutrient concentration and enzymatic activities [War et al. 2012, Czerniewicz et al. 2017]. Our previous work has revealed that *T. ulmi* galling

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process exposes the host plant to high oxidative stress [Kmieć et al. 2018a]. Defense mechanisms of plants include accumulation of secondary metabolites and activation/synthesis of defense peptides and proteins involving many defense enzymes [War et al. 2012]. Available studies indicate that the plant reacts to aphid infestations by a similar defense as during the pathogen attack, i.e. synthesis of pathogenesis-related (PR) proteins, e.g. chitinases and β-1,3-glucanase and secondary compounds [Krishnaveni et al. 1999, Forslund et al. 2000, Inbar et al. 2003]. The induction of PR proteins has negative effects on pathogens and insect pests. Chitinases and β-1,3-glucanase are capable of hydrolyzing chitin and β-1,3-glucans in fungal cell walls, respectively [Krishnaveni et al. 1999]. Chitinase can also hydrolyze chitin in the insect gut and exoskeleton [Sharma et al. 2011]. The enzymatic activities of β-1,3-glucanase, peroxidase and chitinase were involved in the resistance response induced by aphid, *Diuraphis noxia*, in wheat, and it was a typical hypersensitive reaction (HR). These PR-proteins accumulate in the apoplast, which is known to play an important role in the plant's defense mechanism [Moloi and van der Westhuizen 2006]. Plant phenols, phenolic acids and flavonoids, as derivatives of the shikimic acid and phenylpropanoid pathways, play a major role in host plant resistance to herbivore [Cwalina-Ambroziak et al. 2014]. Quinones formed by the oxidation of phenols inhibit the protein digestion in herbivores and can be directly toxic to insects [War et al. 2012]. Phenolic compounds are known as protective agents against various aphid species due to their influence on insects' growth and feeding [Wójcicka 2010, Czerniewicz et al. 2011].

Different galling species are able to modify the nutritive value in the feeding area through the local increase in the content of amino acids, sugars and other plant nutrients [Giron et al. 2016]. Although galls of sucking insects are regarded as structurally simple and devoid of nutritive cells [Carneiro and Isaias 2015], manipulation in biochemistry of *Pauropsylla depressa* galls involves accumulation of sugars and protein [Dsouza and Ravishankar 2014]. According to Larson and Whitham [1991], galls of *Pemphigus beate* functioned as physiologic sinks drawing in resources from surrounding plant sources. Also, leaf-galling phylloxera *Daktulosphaira vitifoliae* induced assimilation and importation of carbon into the gall [Nabity et al. 2013].

Thus far, little research has been conducted on the relationship between galling aphids and mechanisms of gall formation and functioning. Most of the studies have been conducted in the stage of mature galls [Larson and Whitham 1991, 1997, Inbar et al. 2003, Rehill and Schultz 2003, Koyama et al. 2004, Gailite et al. 2005, Samsone et al. 2012, Kmieć et al. 2018a, b]. The aim of the present study was to determine the contents of reducing sugars, proteins and phenolic compounds as well as the activity of PR proteins in galls, galled and intact leaves during subsequent stages of gall development. The following questions are addressed: Can *T. ulmi* galls be considered the sinks for reducing sugars and protein? Does continuous galling aphid feeding trigger the plant defense mechanisms similar to the HR?

MATERIAL AND METHODS

Plant material. The samples were collected from *U. pumila* trees, which were part of urban green areas in Lublin, Poland (51.24°N, 22.57°S), in three stages of gall development. In the first initial stage of galling, galls were green, about 5 mm in height with one fundatrix larva inside. Galls were green and fully grown in the second stage and fundatrix and its several young offsprings were found inside. The third stage involved the time period just before the gall opening. Galls were yellowish with nymphs in the fourth stadium and migrants inside. One sample accounted for 50−100 leaves (depending on the size of galls) with galls collected from trees within hand's reach. Phenologically similar intact leaves were collected as a control at each sampling point. Galling and intact leaves were brought to the laboratory within 1 h after collection, where they were prepared for biochemical analysis. Firstly, all galls were cut off from the leaves by a scalpel. Subsequently, the aphids were removed from galls using a soft brush. Parts of the leaf blade with visible damage were cut off. There were 4 combinations of the experiment: 1) control (intact) leaves, 2) undamaged part of lamina (without

visible discoloration and corrugation) of galling leaves, 3) damaged part of galling leaf lamina, 4) galls. Plant material was frozen and stored at –80°C until analysis. Part of the material (used to determine the content of phenolic compounds) was lyophilized.

Biochemical analysis. Reducing sugar content was determined by the dinitrosalicylic acid method described by Miller [1959]. The absorbance was measured at 540 nm using a UV/VIS spectrophotometer. The quantity of reducing sugars was calculated from the standard curve using a glucose solution and was expressed as mg g^{-1} fresh weight (FW). Protein content of the extracts was determined according to the method described by Bradford [1976]. The quantity of protein in the supernatant was calculated from the standard curve using a bovine serum albumin solution as standard protein and was expressed as mg g^{-1} fresh weight (FW). Total phenolic content was determined using the Folin-Ciocalteu reagent [Singleton and Rossi 1965] and calculated as gallic acid equivalent (GAE) in mg per g of dry weight (DW). The absorbance was measured spectrophotometrically at λ = 725 nm. The activity of chitinase was determined according to the method described by Boller et al. [1983]. The amount of released N-acetyl-glucosamine was determined by the Miller [1959] method as glucose equivalent (mg ml^{-1}). Chitinase activity was defined as glucose equivalent (μg min^{-1}) and expressed as specific activity in the units of enzyme activity per mg protein (μg min^{-1} mg^{-1} protein). The activity of β-1,3-glucanase was determined according to the method described by Vázquez-Garcidueňas and Leal-Morales and Herrera-Estrella [1998] with some modifications. The amount of liberated reducing sugars was determined by the Miller [1959] method as glucose equivalent (mg ml^{-1}). β-1,3--glucanase activity was defined as glucose equivalent (μg min^{-1}) and expressed as specific activity in the units of enzyme activity per mg protein (μg min^{-1} mg^{-1} protein).

Statistical analysis. Biochemical characteristics of tree leaves changed during the developmental stages from bud break to senescence [Jiang et al. 2006]. Univariate ANOVA was applied to analyze the effect of aphid feeding on the contents of reducing sugars, protein and phenolic compounds as well as chitinase and β-1,3-glucanase activity in galls and different parts of elm leaves at each stage of gall development. The Tukey HSD test was used for multiple comparisons of means. Statistical significance was set at α = 0.05. Biochemical assays were performed in five independent biological replicates (n = 5). Arithmetic means with standard deviation (±SD) are presented in tables and figures. All statistical analyses were performed using Statistica 13.1 [StatSoft, Poland].

RESULTS AND DISCUSSION

The present study revealed that *T. ulmi* infestation induced significant alterations in biochemical activity of the primary host. The plant reaction to galling aphid feeding varied and depended on the gall developmental stage. Available studies of plant-galling insect interactions have described general up-regulated expression of primary metabolism and nutrient transport in the plant, as well as down-regulation of defense-associated processes [Oates et al. 2016]. Increased concentrations of sugars, carbon-containing and nitrogen-containing compounds have been reported for interactions between *D. vitifoliae* and grape [Nabity et al. 2013], *P. betae* and *Populus angustifolia* [Compson et al. 2011]. In our study, galled and intact leaves contained different contents of reducing sugars depending on the stage of gall development (Tab. 1). In the initial stage, the level of sugars was significantly lower in both parts of galled leaves, and especially in galls compared to the control. The reducing sugar contents were still significantly lower in the stages of fully developed and mature galls in the damaged portions of galled leaves and galls in comparison to intact leaves (Tab. 1). Histochemical studies showed that *Cecidomyiidae* and *Hemiptera* galls accumulated reducing sugars [Oliveira et al. 2011, Dsouza and Ravishankar 2014]. On the other hand, our findings are consistent with the results of Arriola et al. [2018], who demonstrated that nutritive cells of globoid galls formed on *Smilax campestris* by gall midge did not contain reserves, such as reducing sugars, lipids and proteins. Khattab and Khattab [2005] presented similar observations of psyllid galls on *Eucalyptus obliqua*.

Table 1. Reducing sugars content (mg g^{-1} FW) in *Ulmus pumila* L. tissues during galling process of *Tetraneura ulmi* L.

Plant tissue	Stage of gall development		
	initial (1st)	fully developed (2nd)	mature (3rd)
Intact leaves	21.06 ±1.5 a	24.40 ±1.8 a	33.37 ±1.5 a
Leaves UP	16.84 ±1.4 b	17.51 ±2.3 ab	30.23 ±4.0 a
Leaves DP	13.26 ±0.7 c	11.99 ±1.2 bc	17.96 ±4.9 b
Galls	8.85 ±0.3 d	8.84 ±0.3 c	20.34 ±2.6 b

Leaves UP – undamaged part of galled leaf lamina, leaves DP – damaged part of galled leaf lamina (with visible discoloration and corrugation)
Means sharing the same letter in the column do not differ significantly at $p \geq 0.05$ (Tukey HSD test)

Table 2. Total soluble protein content (mg g^{-1} FW) in *Ulmus pumila* L. tissues during galling process of *Tetraneura ulmi* L**.**

Plant tissue	Stage of gall development		
	initial (1st)	fully developed (2nd)	mature (3rd)
Intact leaves	2.56 ±0.7 b	4.99 ±2.3 a	5.23 ±1.4 a
Leaves UP	3.86 ±1.2 a	3.95 ±1.3 ab	3.66 ±0.8 ab
Leaves DP	2.22 ±1.2 b	2.56 ±0.6 b	3.84 ±0.4 ab
Galls	4.01 ±0.7 a	3.72 ±0.9 ab	2.79 ±0.8 b

Leaves UP – undamaged part of galled leaf lamina, leaves DP – damaged part of galled leaf lamina (with visible discoloration and corrugation)
Means sharing the same letter in the column do not differ significantly at $p \geq 0.05$ (Tukey HSD test)

Table 3. Total phenols content (mg GAE g^{-1} DW) in *Ulmus pumila* L. tissues during galling process of *Tetraneura ulmi* L.

Plant tissue	Stage of gall development		
	initial (1st)	fully developed (2nd)	mature (3rd)
Intact leaves	14.45 ±0.4 c	9.06 ±0.4 b	11.91 ±0.5 b
Leaves UP	16.38 ±0.8 b	9.68 ±0.3 b	10.74 ±0.8 b
Leaves DP	26.30 ±1.0 a	23.73 ±0.5 a	12.60 ±0.2 b
Galls	26.79 ±0.7 a	36.52 ±1.8 a	83.13 ±1.0 a

Leaves UP – undamaged part of galled leaf lamina, leaves DP – damaged part of galled leaf lamina (with visible discoloration and corrugation)
Means sharing the same letter in the column do not differ significantly at $p \geq 0.05$ (Tukey HSD test)

The total soluble protein content in elm tissues involved in galling process was highly variable (Tab. 2). Protein content was approximately 1.5-fold higher in gall tissues and undamaged parts of galled leaves in comparison to intact leaves at the stage of gall initiation. The level of protein decreased along with gall development, especially in damaged portions of galled leaves and in galls (Tab. 2). Ni et al. [2001] revealed that the increase of protein content in aphid-infested leaves indicated that plants activated enzymatic responses to insect feeding. Our previous study documented elevated catalase and ascorbate peroxidase activities in *T. ulmi* galls and galled leaves in the early stage of gall formation [Kmieć et al. 2018a]. In the initial stage, we also showed an increase in the content of polyamines and enzymes of their biosynthesis, e.g. ornithine decarboxylase and lysine decarboxylase [Kmieć et al. 2018b]. A significant decrease in protein content in galls and galled leaves in the subsequent stages of gall development could be associated with increasing the oxidative stress caused by aphid feeding [Kmieć et al. 2018a]. It has been suggested that aphids can induce changes similar to senescence in leaves by degrading the leaf proteins and taking advantage of their translocation [Giordanengo et al. 2010].

The level of soluble phenols was significantly higher in both parts of galled leaves and in galls as compared to intact leaves in the initial period of gall formation (Tab. 3). In the successive stage, *T. ulmi* galls contained 4-fold higher content of phenolics than the control tissues; their increased level was also detected in damaged portions of galled leaves. Mature galls accumulated 83.13 ±1.0 mg GAE g^{-1} DW, i.e. over 6.6-fold higher than in other analyzed tissues at this stage. GAE content was also 2.3- and 3.1-fold higher in comparison to gall tissues in the second and first stage of their development, respectively (Tab. 3). Aphids enhance nutrition by stimulating the phloem proliferation and increasing the sink strength of the gall [Rehill and Schultz 2003]. They can directly compete for mobilized nutrients with natural plant sinks [Larson and Whitham 1997]. The influx of carbohydrates with phloem sap support the production of plant phenolics, thus galls are likely to accumulate them [Lattanzio et al. 2006, Oates et al. 2016]. It is well known that phenolics have antioxidant properties and constitute one of the most widespread group of defensive compounds against herbivores and microorganisms [Sharma et al. 2012, War et al. 2012, Czerniewicz et al. 2017]. Production of oxidative radicals by peroxidases in association with phenols directly deters insects and/or produces toxins that reduce plant's digestibility [War et al. 2012]. The accumulation of phenolics has been observed in a variety of insect galls and is a general phenomenon [Khattab and Khattab 2005, Isaias et al. 2015, Kot et al. 2018]. Their biosynthesis is considered the galler defense against parasitoids and pathogen infection [Inbar et al. 2003]. Our previous study demonstrated an increase in hydrogen peroxide content at the third stage of gall development [Kmieć et al. 2018a]. Phenolics minimize the effects of the oxidative burst by ROS [Oliveira et al. 2011, Isaias et al. 2015, Czerniewicz et al. 2017], thus their elevated accumulation in galls could be a response to ROS production. Phenolics could also modulate the level of auxins by interacting with auxin oxidases and provoking the cell hypertrophy observed in gall tissues [Carneiro and Isaias 2015, Oliveira et al. 2016]. Previously, we indicated low hydrogen peroxide concentration in *T. ulmi* galls during the initial period of gall formation [Kmieć et al. 2018a], thus high accumulation of total phenols in galls at this stage confirming their developmental role.

Chitinase activity was clearly different at particular stages of gall development (Fig. 1). The highest activity of this enzyme in the initial period of gall formation was detected in intact leaves. Its activity in galled leaves and galls was similar and about 1.4-fold lower compared to the control. In turn, chitinase activity in gall tissues in the stage of fully developed galls was the lowest in comparison to galled (both parts) and intact leaves. In contrast, chitinase activity in mature galls (just before their opening) and undamaged portions of galled leaves was about 2-fold higher as compared to intact leaves (Fig. 1). Chitin is an important structural component of the aphid stylets and exoskeleton. Generally, chitinase degrades chitin and can act as an α-amylase inhibitor and interfere with plant digestion [Binu and Palaniswami 2006, and references therein]. The induction of this enzyme during feeding of *D. noxia* [van der Westhuizen et al. 1998],

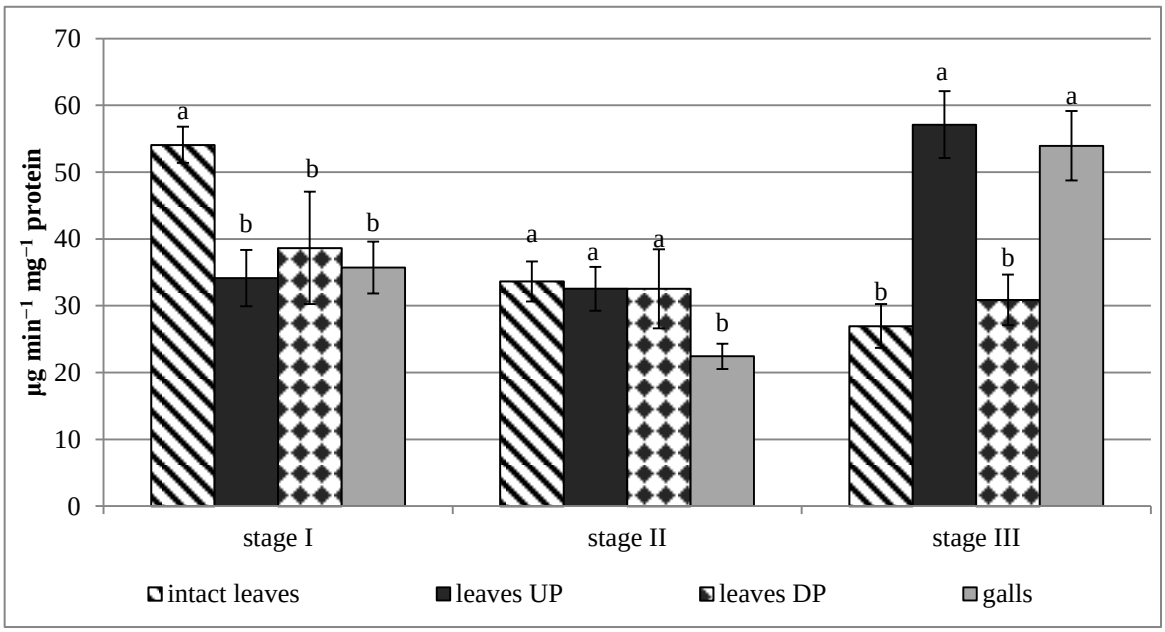

Fig. 1. Chitinase activity in *Ulmus pumila* L. tissues during galling process of *Tetraneura ulmi* L. Leaves UP – undamaged part of galled leaf lamina, leaves DP – damaged part of galled leaf lamina (with visible discoloration and corrugation). Bars sharing the same letter at each stage do not differ significantly at $p \geq 0.05$ (Tukey HSD test)

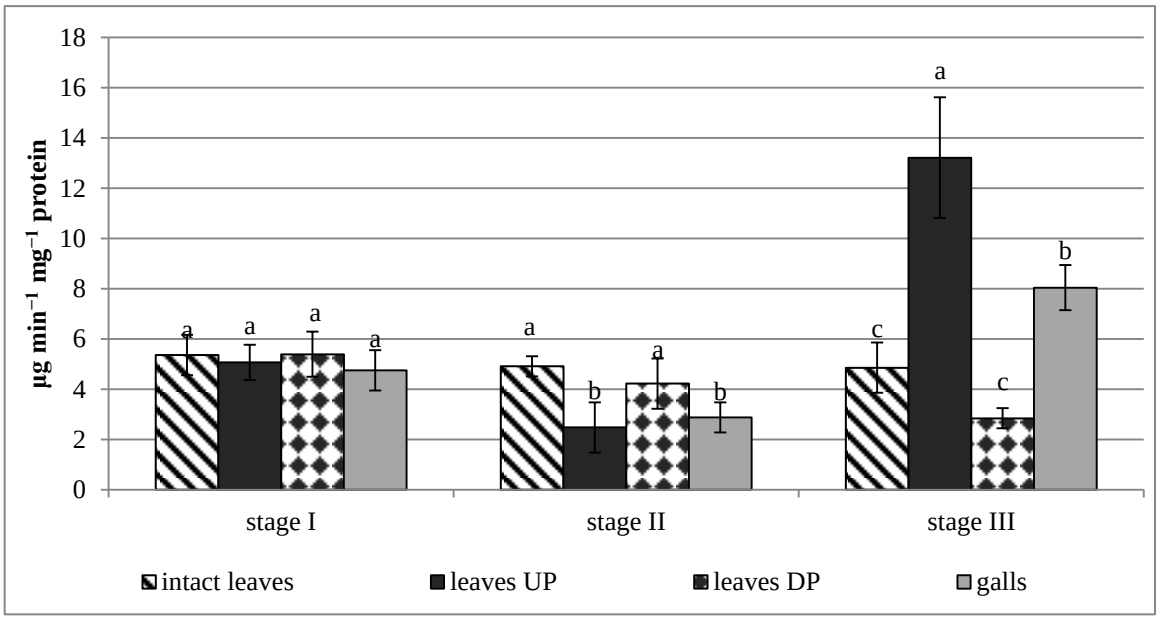

Fig. 2. The β-1,3-glucanase activity in *Ulmus pumila* L. tissues during galling process of *Tetraneura ulmi* L. Leaves UP – undamaged part of galled leaf lamina, leaves DP – damaged part of galled leaf lamina (with visible discoloration and corrugation). Bars sharing the same letter at each stage do not differ significantly at $p \geq 0.05$ (Tukey HSD test)

Rhopalosiphum padi [Forslund et al. 2000] and *Schizaphis graminum* [Krishnaveni et al. 1999] aphids was found. However, there is no clearly defined defense function for chitinase against aphid attack. Aphids put stylets directly in contact with plant cell surfaces during probing and feeding, and core structural components as chitin could be detected by the plant. On the other hand, aphid gel saliva coats the stylets, thereby preventing extracellular perception and/or signaling [Will and van Bel 2006, Morkunas et al. 2011].

The activity of β-1,3-glucanase was dependent on the stage of gall development. The activity of this enzyme in all analyzed elm tissues did not differ significantly in the first stage, when single fundatrix fed in the gall, however, it was the lowest in galls (Fig. 2). In the stage of fully developed galls, β-1,3--glucanase activity in undamaged portions of galled leaves and in the gall tissues was significantly lower compared to intact leaves and damaged portions of galled leaves. A significant increase of its activity was detected in undamaged portions of galled leaves (2.7--fold) and in galls (1.6-fold) in the stage of mature galls in comparison with intact leaves. β-1,3-glucanase activity in those tissues was also higher as compared to damaged portions of galled leaves (Fig. 2). Van der Westhuizen et al. [2002] used the immunogold labelling technique to localize β-1,3--glucanase in wheat infested by *D. noxia*. They found intracellular accumulation of this enzyme in chloroplasts and cell walls of infested resistant plants. Furthermore, denser labelling in cell walls of the vascular bundle cells was observed. Our findings indicated the highest activity of β-1,3-glucanase in galls and undamaged portions of galled leaves only at the last stage of gall development, when they were filled with nymphs and adult migrants. β-1,3-glucanase has the ability to hydrolyze callose, a β-1,3-glucan polymer, which accumulates after injury allowing for continuous flow of nutrients in the phloem [Forslund et al. 2000, Will and van Bel 2006]. It was documented that feeding of different aphid species [Krishnaveni et al. 1999, Inbar et al. 2003], thrips [Binu and Palaniswami 2006] and *Cynipidae* [Kot et al. 2018], induced chitinase and β-1,3-glucanase in host plants. It is possible that the accumulation of phenolic compounds in galls in combination with high activity of PR proteins (e.g. β-1,3-glucanase, chitinase) in the stage of mature galls does not protect the plant but the aphids. Mature galls of *T. ulmi* release emigrants, which are exposed to predation, parasitism and pathogenic infection [Urban 2003]. On the other hand, chitinases and β-1,3-glucanases are involved in cell wall oligosaccharide release. The sugars such as sucrose, glucose, fructose and trehalose can function as messengers in plant signal transduction and dissipation of free radical species during aphids infestation [Morkunas et al. 2011]. Activation of PR proteins associated with high level of phenolics and previously indicated lipid peroxidation and ROS accumulation in tissue of mature galls [Kmieć et al. 2018a] seems to be similar to the plant hypersensitivity reaction in pathogen infection [Lattanzio et al. 2006].

CONCLUSIONS

The results presented in this work show much higher accumulation of protein in galls at the initial period of gall formation, increased biosynthesis of phenolics during the galling process and the highest activity of PR protein at the stage of mature galls. The galls did not accumulate reducing sugars at any developmental stage. Galling activity of *T. ulmi* triggered defense reactions similar to HR, but only in mature galls filled with numerous migrant nymphs. Therefore, it can be suggested that *T. ulmi* could manipulate the biochemical machinery of the host plant for its own needs. Studies on recognition and responses to biotic stress can help to understand the interactions between galling aphids and their host plants, which is the basis of modern plant protection.

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