Bulletin of Entomological Research (2020) **110**, 34–43
doi:10.1017/S0007485319000221

Effect of *Neuroterus quercusbaccarum* (L.) galls on physiological and biochemical response of *Quercus robur* leaves

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Abstract

Gall formation is associated with multiple changes in plant cells, which still requires a better understanding. In this study, galls caused by sexual generation (♀♂) of *Neuroterus quercusbaccarum* (L.) (Hymenoptera: Cynipidae) on pedunculate oak trees (*Quercus robur* L.) were used as a model. Cytoplasmic membrane condition, concentration of hydrogen peroxide (H_2O_2), the activity of antioxidant enzymes and amino acid decarboxylase as well as chlorophyll fluorescence parameters were determined. Changes in physiological and biochemical parameters were analyzed in foliar tissues with galls and gall tissues themselves and compared to control. The presence of galls on oak leaves caused an increase of lipid peroxidation level. A significant decline in H_2O_2 and TBARS content with the reduction of guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) activity were observed in gall tissues. The activity amino acid decarboxylase, i.e., LDC, ODC and TyDC varied between samples, which may affect the content of amino acids. The presence of *N. quercusbaccarum* galls caused an insignificant increase of the chlorophylls, carotenoids and anthocyanin contents, while the content of pigments and their ratios in gall tissues was extremely low. Moreover, photosynthetic parameters (F_0, F_m, F_v/F_m, Y, q_P) were significantly decreased. Data generated in this study indicate that the development of *N. quercusbaccarum* galls on pedunculate oak leaves has a negative effect on host plant related to the disruption of cell membrane integrity, disturbance of photosynthesis and reduction of the antioxidant potential of the host plant.

Keywords: gall wasp, cytoplasmic membrane condition, H_2O_2 content, antioxidant enzymes, amino acid decarboxylase, chlorophyll fluorescence

(Accepted 13 March 2019; First published online 13 June 2019)

Introduction

Galls are the result of mutual interactions between gall-inducing herbivores and their host plants (Patra *et al.*, 2010; Samsone *et al.*, 2012; Jiang *et al.*, 2018). They are formed entirely from the plant tissues and this process involves the enlargement and proliferation of plant cells (Oliveira *et al.*, 2016). Their structures are determined by the gall-inducing species, even on the same host plant (Hartley, 1998). Oak gall wasps (Hymenoptera: Cynipidae) appear to be most specialized among gall-inducers. They have complex cyclic parthenogenetic life cycles and the ability to induce not only species-dependent, but even generation-dependent galls on oaks and other Fagaceae (Stone *et al.*, 2002). An example of such a model is *Neuroterus quercusbaccarum* (L.), which inhabits oak trees – *Quercus robur* (L.), *Q. pubescens* Willd. and *Q. petrea*

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(Matt.) Liebl. – throughout Europe, North Africa and Asia Minor (Kierych, 1979). *N. quercusbaccarum* is heterogonic, with sexual (♀♂) and agamic (∞) generation per year. These two generations differ from each other in galls structure and morphology of adult wasps. Females of agamic generation are flying from January to May. They lay eggs on the developing leaves or into flower buds of male catkins (Kovácsné-Koncz *et al.*, 2011). A spherical and smooth-surfaced currant gall forms around the developing larva. Galls are approximately 4 mm in diameter, one-chambered and single in catkins or below the leaves, green at first, then with a red mark above when mature in May and June. The larvae pupate in galls on a tree; females and males of sexual generation emerge from May to July. Fertilized females are laying eggs on oak leaves. A spangle gall develops around the hatched larvae. Galls are single-chambered, in the shape of a disc with a rising center, yellow with red hairs. They have a diameter of about 5 mm; they occur from July to October beneath the leaves and mature in August. The larva pupates in the gall on the ground in winter (Kampichler & Teschner, 2002).

Gall formation is associated with multiple changes in plant cells, including morphological features, nutrient concentration, metabolic signaling, photosynthetic capacity or oxidative enzyme activities (Oliveira *et al.*, 2011; Giron *et al.*, 2016; Kmieć *et al.*, 2018; Kot & Rubinowska, 2018). The earliest response of plants to galling stimuli have been often associated with increased production of reactive oxygen species (ROS) (Isaias & Oliveira, 2012), which may induce detrimental oxidation of macromolecules including nucleic acids, proteins and lipids, leading to cellular toxicity (Bela *et al.*, 2015). On the other hand, ROS are also produced as a natural by-product of the plant metabolism and have a crucial role in cell signaling, which may determine the extent of gall tissue alterations (Isaias & Oliveira, 2012; Carneiro *et al.*, 2014). ROS include hydroxyl radical (OH•), superoxide anion radical ($O_2^{\bullet -}$), perhydroxyl radical (HO_2•), alkoxyl radical (RO•), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). H_2O_2 has an important role in plants as a signaling molecule in defense response due to its relatively stable form of oxygen and the ability to diffuse freely (Maffei *et al.*, 2007). Nevertheless, excessive production of H_2O_2 in plant cells leads to oxidative stress and programed cell death (Quan *et al.*, 2008). The accumulation of ROS increases lipid peroxidation (LPO) in biological membranes affecting their structure, which decreases membrane fluidity, increases permeability and loss of enzymatic activity (Gill & Tuteja, 2010). High ROS production is buffered by several non-enzymatic (e.g., ascorbic acid, glutathione, tocopherol and carotenoids) and enzymatic (e.g., peroxidases, dismutase, catalase, polyphenol oxidase) mechanisms. This cellular detoxification system protects plant cells from oxidative damage by converting ROS into less toxic products in the cell (Gill & Tuteja, 2010; Pandey *et al.*, 2017). Among enzymatic components, peroxidases play a key role in the suppression of toxic H_2O_2 (Caverzan *et al.*, 2012).

It is known that galling insects induce local accumulation of nutrients in plant tissues (Giron *et al.*, 2016; Kot *et al.*, 2018*a*). This also applies to amino acids, thus changes in the metabolism of these compounds may be an important part of biochemical plant responses to signals associated with the presence of gall-inducing species. Few published studies (Sempruch *et al.*, 2013; 2014; Kmieć *et al.*, 2018), regarding insect–plant interactions have suggested that the degradation of amino acids as nutrients for herbivores may be connected with the state of amino acid decarboxylases. They are key enzymes of plant amines biosynthesis and ornithine decarboxylase (ODC; EC 4.1.1.17) and lysine decarboxylase (LDC; EC 4.1.1.18) play a crucial role in this process. However, their activity depends on both plant and herbivore species, their density and duration of infestation (Sempruch *et al.*, 2012*b*). In turn, tyrosine decarboxylase (TyDC; EC 4.1.1.25) participate in the biosynthesis of aromatic monoamines and other classes of defensive plant compounds (Miller-Fleming *et al.*, 2015).

Changes in the content of assimilation pigments and photosynthetic activity are a common mechanism to assess the impact of insects on plants. Insect feeding, as a biotic stress, causes a loss of chlorophyll content, because chloroplasts during stress are able to produce strong oxidants responsible for the oxidation of pigments (Guidi & Degl'Innocenti, 2012). In turn, galls show a high demand for assimilates; however, certain gall-inducing species have the ability to stimulate the rate of photosynthesis, while others reduce it, which probably depends on the tolerance of plant species to insect feeding (Nabity *et al.*, 2009; Haiden *et al.*, 2012). The analysis of chlorophyll a fluorescence is one of the most widely used methods of investigating changes in the photosynthetic apparatus and photosynthetic efficiency in higher plants (Kalaji *et al.*, 2012).

Interactions between gall-inducing Cynipidae and oaks as their host plants seem to be a model system for studying gall insect–host plant interactions due to their high specialization. Cynipidae gall wasps are highly monophagous and their galls are the archetype of highly specialized nutritive tissues that form discrete microhabitats. It will be useful to carry out physiological and biochemical studies, which will allow clarifying some key events of oak–Cynipidae interactions to better understand host plant responses after the attack of gall-inducing Cynipidae. The present study used galls caused by sexual generation (♀♂) of *N. quercusbaccarum* on pedunculate oak trees (*Q. robur* L.) as a model. We analyzed the enhancement of selected physiological and biochemical parameters in foliar tissues with galls and gall tissues themselves and compared it to non-infested tissues. We aimed to answer the following questions: can galls alter the fluidity of cell membranes? Can galls induce oxidative stress and modulate plant's reaction to it? How galling process affects photosynthesis and pigment contents? We determined cytoplasmic membrane condition, H_2O_2 content, the activity of antioxidant enzymes and amino acid decarboxylase as well as chlorophyll fluorescence parameters to answer these questions.

Methods and materials

Study site and sampling

The study site was located in Lublin (Poland) (22°34′E, 51°14′N), where the climate is typical for a humid continental climate with cold, damp winters and warm summers. Plant material was collected in May from pedunculate oak trees (*Q. robur* L.), which were part of urban green areas. The trees were about 15–20 years old and 5–8 m tall. Individual trees with fully developed galls induced by sexual generation (♀♂) of *N. quercusbaccarum* (L.) were marked ($n = 10$). Samples of leaves with visible, fully developed galls and accessible at arm's length were randomly collected, however leaves with only one gall were included. Leaves located at a similar site on the shoots and a similar canopy position just like leaves with galls were used as a control. Ten leaves with galls and ten non-galled leaves were collected from each tree, so each sample consisted of 100 leaves. Leaves were cut off

https://doi.org/10.1017/S0007485319000221 Published online by Cambridge University Press

with scissors and brought in plastic bags to the laboratory within 1 h after collection. In the laboratory, galls were cut off with a scalpel and sectioned to remove larvae, and the plant material was categorized as follows: control leaves (leaves without galls), leaves with removed galls and galls. Leaves and galls in the sample were cut into small pieces and mixed to be more representative. Such plant material was weighed and used directly for physiological analysis (H_2O_2 assay, E_L assay, LPO, GPX and APX activity, photosynthetic and photoprotective pigment contents). The material used to assess amino acid decarboxylase activities was frozen and stored at −80 °C until analysis. Physiological and biochemical assays were made in three biological replicates ($n = 3$).

The effect of *N. quercusbaccarum* galls on photosynthesis was evaluated by chlorophyll a fluorescence measurements. It was determined in field conditions on 40 leaves-20 intact leaves as control and 20 leaves with fully developed galls. The same leaves were subsequently detached for pigment analysis after chlorophyll a fluorescence measurements.

Fluorescence measurement

Fluorescence measurements were conducted using the saturation pulse method with a PAM-2000 fluorometer (Walz GmbH, Germany) (Schreiber, 2004). They were taken before 11 am, after adaptation in the dark for about 20 min. The minimum (F_0) fluorescence was measured after dark adaptation. Subsequently, a flash of light sufficient to drive photosynthesis was applied and the maximum fluorescence (F_m) was measured. After 10 min the substrate fluorescence (F_s) was determined under steady-state conditions. The maximum chlorophyll fluorescence ($F_{m'}$) was measured by applying pulses of the saturated white light every 60 s when actinic light was on.

The maximum quantum yield of photosystem II (PSII) was determined using the following formula:

$$F_v/F_m = (F_m - F_0)/F_m, \quad (1)$$

where F_v (variable fluorescence) is equal to the increase in fluorescence induced by the saturation pulse. The effective quantum yield (Y) of PSII photochemistry was determined as

$$\Delta F/F'_m = F'_m - (F_s/F'_m) \quad (2)$$

Fluorescence quenching parameters, such as q_P (photochemical quenching) and q_N (non-photochemical quenching) were calculated according to the equations:

$$q_P = [(F'_m - F_s)/(F'_m - F'_0)] \quad (3)$$

and

$$q_N = [(F_m - F'_m)/(F'_m - F'_0)] \quad (4)$$

q_P and q_N requires the F_0' parameter, which was obtained after a dark red light pulse applied to previously light-adapted leaves

Laboratory assay

H_2O_2 content

The procedure of Jena & Choudhuri (1981) was followed to determine H_2O_2 content. Briefly, 0.5 g of plant material was ground in 3 ml of phosphorus buffer (50 mM, pH 6.5) at 4 °C. Then, the mixture was centrifuged at 6000 × *g* for 25 min. Next, 1.5 ml of the supernatant was added to 0.5 ml TiO_2 in 20% (v/v) H_2SO_4 and centrifuged again at 6000 × *g* for 15 min at room temperature. The absorbance of the supernatant was measured at 410 nm using a spectrophotometer (Cecil CE 9500, UK). H_2O_2 content was calculated using the molar absorbance coefficient (0.28 μM^{-1} cm^{-1}) and expressed as nanomoles per 1 g fresh weight.

Electrolyte leakage (E_L) assay

It was measured according to the method of Kościelniak (1993) using an Elmetron CC-317 microcomputer conductometer. Ten leaf rings of 0.9 cm diameter were cut with a cork borer from each sample, and subsequently transferred to 20 cm^3 of deionized water and incubated at room temperature on a rotary shaker for 24 h. The initial electrical conductivity ($K1$) then was measured. The samples were autoclaved at 100 °C for 15 min. Final conductivity of the solution was measured ($K2$) after 24 h of shaking. Electrolyte leakage was calculated using the following formula:

$$E_L\ (\%) = (K1/K2) \times 100.$$

Membrane lipid peroxidation

Its level was measured as the amount of thiobarbituric acid reactive substances (TBARS), according to Heath & Packer (1968). Plant tissues (0.2 g) were homogenized in 0.1 M potassium phosphate buffer (pH 7.0) and then the homogenate was centrifuged at 12,000 × *g* for 20 min at room temperature. The supernatant (0.5 cm^3) was mixed with 2 cm^3 of 20% trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid (TBA). The mixture was incubated at 95 °C for 30 min, and then the samples were quickly quenched. Another centrifugation was carried out at 10,000 × *g* for 10 min. The absorbance was measured at 532 and 600 nm using a spectrophotometer (Cecil CE 9500, UK). The TBARS content was calculated using the molar absorbance coefficient (155 nM^{-1} cm^{-1}) and expressed as nanomoles per 1 g fresh weight.

Peroxidase activity

For guaiacol peroxidase (GPX, EC 1.11.1.7) and ascorbate peroxidase (APX, EC 1.11.1.11) activities, 0.2 g of each sample was homogenized in 0.05 mol dm^{-3} phosphate buffer (pH 7.0) containing 0.2 mol dm^{-3} EDTA and 2% PVP at 4 °C. Then, the homogenates were centrifuged for 10 min (10,000 × *g*, 4 °C) and immediately used for analyses. GPX activity was measured as described by Małolepsza *et al.* (1994). The reaction mixture contained 0.5 cm^3 of 0.05 mol dm^{-3} phosphate buffer (pH 5.6), 0.5 cm^3 of 0.02 mol dm^{-3} guaiacol, 0.5 cm^3 of 0.06 mol dm^{-3} H_2O_2 and 0.5 cm^3 of enzyme extract. The variation in absorbance was measured at 480 nm for 4 min, at 1 min intervals using a spectrophotometer (Cecil CE 9500, UK). GPX activity was calculated using the absorbance coefficient for this enzyme (26.6 mM cm^{-1}) and expressed as the change in peroxidase activity per fresh weight (U mg^{-1} FW).

APX activity was determined according to the method of Nakano & Asada (1981). The reaction mixture contained 1.8 ml 0.1 M phosphorus buffer (pH 6.0), 20 µl of 5 mM sodium ascorbate, 100 µl of 1 mM H_2O_2 and 100 µl of enzymatic extract. The absorbance was measured at 290 nm for 5 min, at 1 min intervals with a spectrophotometer (Cecil CE 9500,

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UK). APX activity was calculated using the absorbance coefficient for this enzyme (2800 M^{-1} cm^{-1}) and expressed as the change of peroxidase activity per fresh weight (U mg^{-1} FW).

Amino acid decarboxylases assays

Plant material was homogenized with 0.2 M phosphate buffer (pH 8.2) containing β-mercaptoethanol and ethylenediaminetetraacetic acid (EDTA) for ODC, while Tris-HCl buffer pH 5.6 (0.2 M) was used for the extraction of LDC and 0.5 M acetate buffer (pH 5.6) for TyDC. Subsequently, the obtained extracts were filtered through two layers of cheese-cloth and centrifuged at 18,000 × *g* at 5 °C. The activity of ODC, LDC and TyDC was assayed using a UV-Vis spectrophotometer (Hewlett Packard 8453) according to Ngo *et al*. (1987) and Phan *et al*. (1982, 1983), respectively. Enzyme activities were expressed as μmol of appropriate amine, generated during 1 h of enzymatic reaction by 1 mg of enzymatic protein. The protein content in enzymatic extracts was determined with the method of Lowry *et al*. (1951).

Photosynthetic and photoprotective pigment content

In total, 0.5 g of each sample was extracted in 80% acetone. The procedure of Lichtenthaler & Wellburn (1983) was followed to determine the content of chlorophyll a, b and carotenoids. The absorbance was determined at 470 nm (for Car), 646 nm (for chlorophyll b) and 663 nm (for chlorophyll a) using a spectrophotometer (Cecil CE 9500, UK). The following formulas were used to estimate pigment contents:

$$C_{\text{chl a}} = 12.21 \times A_{663} - 2.81 \times A_{646}$$
$$C_{\text{chl b}} = 20.13 \times A_{646} - 5.03 \times A_{663}$$
$$C_{\text{car}} = (1000 \times A_{470} - 3.27 \times C_{\text{chl a}} - 104 \times C_{\text{chl b}})/227,$$

where A_λ – absorbance value for wale length λ.

The values of pigment contents were expressed as mg/g fresh weight.

To determine anthocyanin contents, 1 g of each sample was taken and extracted for 4 h in 10 ml of 0.1% HCl–MeOH at room temperature. Then, the extracts were measured using a spectrophotometer (Cecil CE 9500, UK) at 530 and 657 nm. The formula: $A = (A_{530} - 0.25A_{657})$ was used to compensate for the contribution of chlorophyll and its degraded products to the absorption at 530 nm. Anthocyanidin content was calculated following the formula of Rabino & Mancinelli (1986): [Absorbance × 449.2 × dilution factor]/[29.600 × Sample Weight (g)],

where 449.2 = molecular weight of Cyanidin-3-glucoside; dilution factor = final volume/initial volume; 29.600 = molar extinction coefficient.

The values of anthocyanin contents were expressed in milligrams of Cyanidin-3-glucoside equivalent per 1 g of fresh weight.

Statistical analysis

All data were presented as means ($\bar{x}$) with standard error values (±SE) and statistical analyses were calculated using Statistica for Windows v. 13.1 (Statistica StatSoft Inc., 2016). The Shapiro–Wilk test was used to verify the normality of the obtained results. The homogeneity of variance was analyzed using test of Levene. One-way ANOVA with the Tukey's simultaneous test (HSD) was used to compare differences in physiological and biochemical parameter contents/activities, as well as photosynthetic and photoprotective pigments concentrations in the samples. Changes in chlorophyll a fluorescence parameters between tissues were examined using the Student t-test or the non-parametric Mann–Whitney *U* test. The significance threshold was set at $p < 0.05$. Physiological and biochemical assays were performed in three independent biological replicates ($n = 3$), while chlorophyll a fluorescence in twenty replicates ($n = 20$).

Results

H_2O_2 content

The presence of *N. quercubaccarum* galls on the oak leaves caused a twofold reduction in the H_2O_2 content compared to control leaves (fig. 1a). The level of this free radical in the galls was the lowest, as a 20-fold difference between gall tissues and control was measured.

The state of cell membranes

E_L measurements from cells were used to estimate the degree of cell membrane damage during the gall formation process of *N. quercusbaccarum* on oak leaves. E_L levels did not differ significantly between control and leaves with galls (fig. 1b). E_L in galls was higher by almost 60% compared to control and leaves with galls.

Lipid peroxidation was expressed by the amount of malondialdehyde (MDA) determined with TBARS measurements. It was considered an indicator of oxidative stress. A significant increase in TBARS content was observed in leaves with *N. quercusbaccarum* galls in comparison to control samples (fig. 1c), as a 21% difference was noted. In turn, a high decrease in TBARS content was recorded in gall tissues; it was reduced by 57.91 and 62.42% when compared to control and leaves with galls, respectively.

Activity of antioxidants

The presence of *N. quercusbaccarum* galls on oak leaves had no effect on GPX activity (fig. 1d). However, this enzyme showed low activity levels in the galls. It was nearly 70% lower compared with control and leaves with galls.

Leaves with galls and galls of *N. quercusbaccarum* were characterized by a lower APX activity compared with control samples, but changes between leaves with galls and control were not significant (fig. 1e). The activity of this enzyme in galls was almost threefold and 2.5-fold lower when compared to control and leaves with galls, respectively.

Changes in the activity of amino acid decarboxylases

Statistical analysis showed significant differences in the activity of amino acid decarboxylases in leaves with galls of *N. quercubaccarum* and galls themselves, with the exception of TyDC (Table 1). Leaves with galls were characterized by an extremely low activity of LDC, which was 4.5-fold and 1.5-fold lower than in control and gall tissues, respectively. Leaves with galls also exhibited lower ODC activity of this enzyme compared to control samples. Whereas, the sharpest enhancement of its activity, 25-fold and 29-fold, was observed in galls of this cynipid species compared to control tissues and leaves with galls, respectively. The pattern of TyDC activity in *N.*

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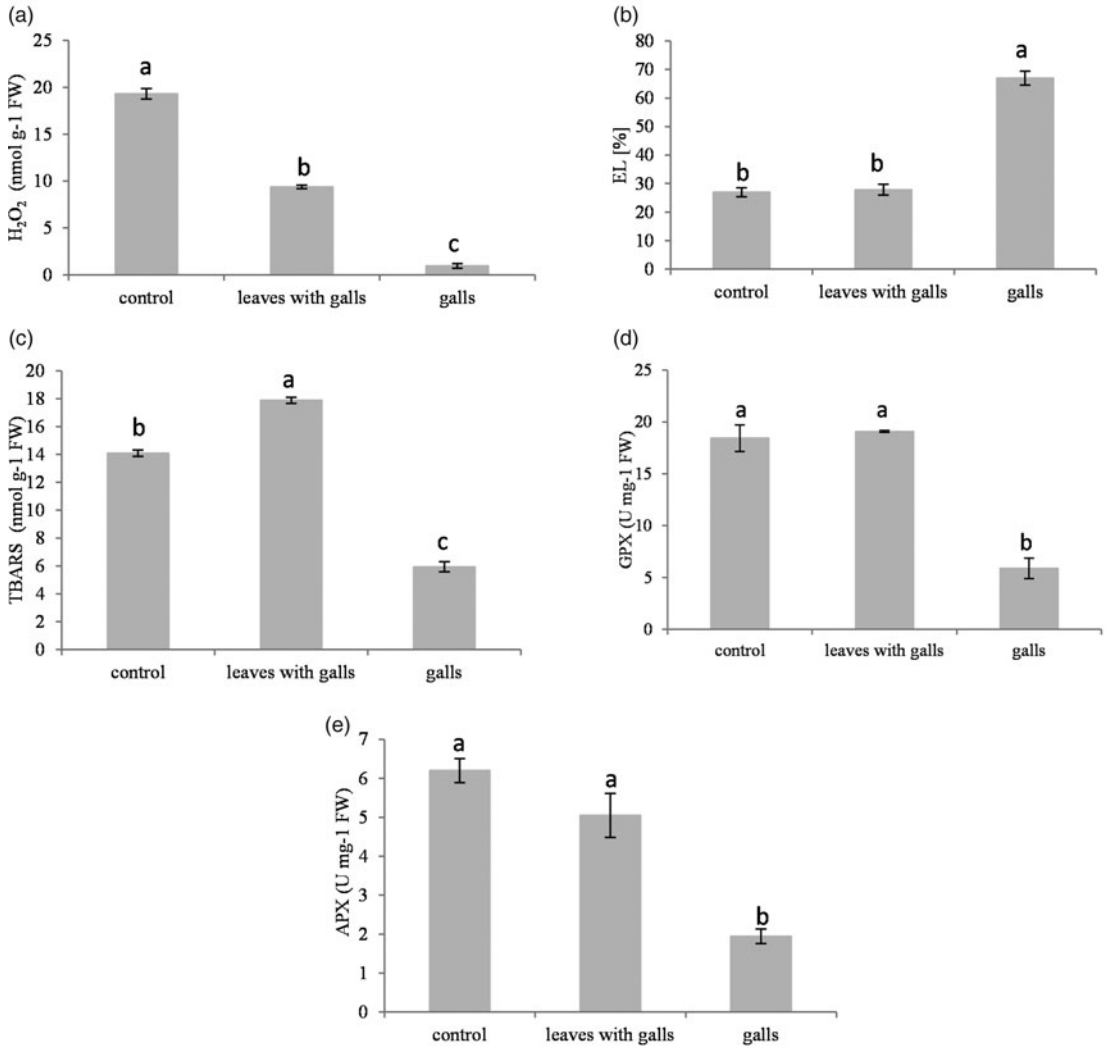

Fig. 1. Changes in the content/activity of hydrogen peroxide (H_2O_2) (A), electrolyte leakage (E_L) (B), thiobarbituric acid reactive substances (TBARS) (C), guaiacol peroxidase (GPX) (D) and ascorbate peroxidase (APX) (E) in the leaves with galls and in galls of *N. quercusbaccarum* (L.) compared to control leaves; values followed by the same letter do not differ significantly at $p < 0.05$.

quercusbaccarum galls and leaves with galls was similar and was characterized by slightly higher values than in control leaves.

Changes in the content of photosynthetic and photoprotective pigments

The average contents of chlorophyll a and b and total chlorophyll in leaves with galls were slightly higher, but not statistically confirmed, when compared to control leaves. In turn, galls of *N. quercusbaccarum* were characterized by extremely low levels of these pigments, which were more than 97% lower than in both, control and leaves with galls (Table 2). The content of carotenoids was lower in leaves with galls as well as in gall tissues, as compared to control samples. However, these changes were significant only between control and galls of *N. quercusbaccarum* (133-fold difference). The chlorophyll a/b ratio was significantly lower in both, leaves with galls and galls of *N. quercubaccarum* when compared to the average control value. Nevertheless, a higher reduction (more than 2.6-fold) was measured in gall tissues. A similar trend to the chlorophyll a/b ratio was found for the carotenoids/total chlorophyll ratio. The carotenoids/total chlorophyll ratio was lower by 43.48 and 38.09% in control and leaves with galls, respectively. The presence of *N. quercubaccraum* galls did not cause any significant changes in anthocyanin concentrations when compared to control leaves. The sharpest, almost 7.7-fold decrease was recorded in galls compared to control as well as leaves with galls.

Fluorescence measurement

The presence of *N. quercusbaccarum* galls resulted in a significant decrease in the values of all photosynthetic activity indices, except for the q_N parameter (Table 3). The initial fluorescence intensity (F_0) and the maximum intensity (F_m)

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Table 1. Changes in the activity of amino acid decarboxylase in oak leaves with galls and gall tissues of *N. quercusbaccarum* (L.); values followed by the same letter do not differ significantly at $p < 0.05$ ($n = 3$).

Amino acid decarboxylase	Sample ($X \pm SE$)		
	Control	Leaves with galls	Galls
LDC (μM, cadaverine × mg^{-1} protein × h^{-1}) $F_{2,6} = 356.6$ $p < 0.001$	7.86 ± 0.14a	1.74 ± 0.16c	2.63 ± 0.21b
TyDC (μM, tyramine × mg^{-1} protein × h^{-1}) $F_{2,6} = 0.578$ $p = 0.59$	4.45 ± 0.29a	5.32 ± 0.66a	5.53 ± 1.08a
ODC (μM, putrescine × mg^{-1} protein × h^{-1}) $F_{2,6} = 3205439.45$ $p < 0.001$	98.31 ± 0.51b	84.61 ± 0.69c	2460.61 ± 1.01a

Table 2. Content of photosynthetic and photoprotective pigments in oak leaves with galls and gall tissues of *N. quercusbaccarum* (L.); values followed by the same letter do not differ significantly at $p < 0.05$ ($n = 3$).

Sample	Chlorophyll (mg g^{-1} FW)			Carotenoids (mg g^{-1} FW)	Chlorophyll a/b ratio	Carotenoids/ chlorophyll a + b	Anthocyanins (mg g^{-1} FW)
	a	B	a + b				
Control	14.82 ± 0.18a	2.88 ± 0.08a	17.70 ± 0.26a	3.99 ± 0.06a	5.15a	0.23a	3.15 ± 0.17a
Leaves with galls	15.43 ± 0.31a	3.22 ± 0.12a	18.65 ± 0.43a	3.93 ± 0.12a	4.79b	0.21a	3.14 ± 0.15a
Galls	0.14 ± 0.01b $F_{2,6} = 1754.9$ $p < 0.001$	0.07 ± 0.01b $F_{2,6} = 420.8$ $p < 0.001$	0.22 ± 0.02b $F_{2,6} = 1286.5$ $p < 0.001$	0.03 ± 0.01b $F_{2,6} = 846.1$ $p < 0.001$	1.96c $F_{2,6} = 556.8$ $p < 0.001$	0.13b $F_{2,6} = 37.0$ $p < 0.001$	0.41 ± 0.29b $F_{2,6} = 54.6$ $p < 0.001$

showed significant decreases (1.2 and 1.3-fold, respectively) when compared to control leaves. In consequence, the reduction of the maximum fluorescence (F_m) values and variable fluorescence (F_v), determined by the $F_v = F_m - F_0$ equation, led to a decrease in the maximum quantum yield of photosystem II (F_v/F_m). In our study, F_v/F_m ratios in leaves with *N. quercubaccarum* galls were significantly reduced, by almost 20%. A subsequent reduction was observed for the maximum quantum yield of PSII values (*Y*), as a 1.4-fold difference between leaves with cynipid galls and control was measured. The values of fluorescence quenching parameters, such as photochemical fluorescence quenching (q_P) and non-photochemical quenching (q_N) showed a varied pattern of changes. We found that the presence of galls significantly reduced the q_P coefficient and stimulated q_N in oak leaves (Table 3). A decrease of 27.75% in q_P values, and an increase of 37.44% of the q_N value was detected in leaves with galls compared to control tissues.

Discussion

Oxidative stress is an important part of plant response following the attack of herbivores. Feeding of *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) increased the rate of lipid peroxidation and •OH radical formation within soybean tissues (Bi & Felton, 1995). These results are consistent with our data, proving an increase in the rate of lipid peroxidation in oak leaves, on which *N. quercusbaccarum* induced galls. Such a reaction can lead to damage of membrane phospholipids, loss of cell membrane integrity and uncontrolled leakage of electrolytes. Moreover, according to Bi *et al.* (1997), oxidative stress caused by *H. zea* in cotton tissues was accompanied by the induction of the activity of certain oxidative enzymes, such as lipoxygenases, peroxidase, diamine oxidase, ascorbate oxidase, and NADH oxidase I, with a simultaneous decrease of such foliar antioxidants as ascorbic acid, total carotenoids and non-protein thiols. On the other hand, the attack of *B. brassicae* increased ascorbic acid level and the rate of lipid peroxidation and reduced the activity of superoxide dismutase (SOD), APX and ascorbate oxidase in cabbage leaves (Khattab, 2007). Ascorbic acid accumulation was also induced in triticale tissues under *Sitobion avenae* (F.) and *Rhopalosiphum padi* (L.) (Hemiptera: Aphididae) attack in the initial stage of infestation (Łukasik *et al.*, 2012). Prolonged aphid feeding resulted in losses of ascorbate. Moreover, this study showed an increase in APX activity in triticale tissues throughout the test period. These data strongly suggested that the mode of oxidative changes caused by herbivorous insects in plant tissues is strictly dependent on the genotype of herbivore and/or host plant and the time of infestation. In our study, a decrease in H_2O_2 levels in leaves with galls and in galls of *N. quercusbaccarum* on pedunculate oak leaves was observed, which was similar to changes induced by *H. zea* in soybean tissues (Bi & Felton, 1995). These results may suggest that this process involves reducing the antioxidant potential of plant tissues by decreasing the level of some antioxidants with low molecular weight, such as carotenoids, and reducing peroxidases activities. Furthermore, it is possible that SOD activity is also inhibited during this phenomenon. The latter enzyme catalyzes the dismutation reaction of superoxide anion radical to oxygen and hydrogen peroxide constituting the primary line of defense (Sytykiewicz, 2014). Thus, a decrease in its activity may at least partly result in lower H_2O_2 level. However, this problem requires further research.

It can be assumed that the direction and intensity of oxidative changes are regulated at the transcriptional level by up-regulation and down-regulation of specific genes. This mechanism has been demonstrated for maize response to the

https://doi.org/10.1017/S0007485319000221 Published online by Cambridge University Press

Table 3. Changes in chlorophyll a fluorescence parameters along a control leaves of *Q. robur* L. and leaves with *N. quercusbaccarum* (L.) galls ($n = 20$).

Parameter	Sample ($X \pm SE$)		Test		
	Control	Leaves with galls	Student's t test t_{38}	Mann–Whitney U	p-value
F_0	0.2326 ± 0.0057	0.1996 ± 0.002	–	21	<0.001
F_m	1.0445 ± 0.0394	0.7886 ± 0.0222	–	18.5	<0.001
F_v/F_m	0.7038 ± 0.0113	0.5667 ± 0.0111	8.62	–	<0.001
Y	0.5059 ± 0.0116	0.3635 ± 0.0214	–	35.5	<0.001
q_P	0.6208 ± 0.0107	0.4485 ± 0.0142	9.67	–	<0.001
q_N	0.1452 ± 0.0096	0.2321 ± 0.0076	–	18	<0.001

feeding of *S. avenae* and *R. padi* (Sytykiewicz, 2014; 2016*a*; 2016*b*; Sytykiewicz *et al.*, 2014) as well as in other host–herbivore systems (Collins *et al.*, 2010; Munner *et al.*, 2018). Transcriptional responses were dependent on plant genotype, insect species and duration of infestation. These conclusions are also consistent with the results of Khattab (2007), who found a decrease in soluble protein content in cabbage tissues during the oxidative response to *B. brassicae* foraging.

The herbivorous insects are often able to induce beneficial changes in the physiology and biochemistry of host plants. For example, aphid feeding often resulted in a local increase in the content of free amino acids, thereby limiting the nutritional value of host plant tissues for these insects (Florencio-Ortiz *et al.*, 2018). Similarly, some gallers induce the over accumulation of amino acids, sugars and other plant nutrients in gall tissues (Giron *et al.*, 2016). Therefore, amino acid transformations can be an important component of biochemical mechanisms regulating galls development. Our results showed that the decarboxylation rate of some amino acids was affected by the development of *N. quercusbaccarum* galls on pedunculate oaks leaves. The response included an increase in ODC activity in galls and inhibition of LDC activity in galls and leaves with galls. Since ODC participates in the degradation of non-protein ornithine, and LDC breaks down essential lysine, both reactions can be beneficial to insects. On the other hand, ODC and LDC are involved in the biosynthesis of plant polyamines and its hydroxycinnamic acid amide derivatives (HCAAs). Kmieć *et al.* (2018) conducted research on galls induced by *Tetraneura ulmi* (L.) (Hemiptera: Eriosomatinae) on Siberian elm leaves, detecting an increasing ODC activity during the initial period of galls formation. The activity of ODC, LDC and TyDC decreased at later stages of this process. Such responses were connected with an increase in the content of some plant amines during the initial period of galls formation and in the fully developed galls. However, these compounds were not found in mature galls. In turn, Subramanyan *et al.* (2015) studied the response of wheat (*Triticum aestivum*) to virulent larvae of *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae). In that study, *M. destructor* induced a strong increase of polyamines (PAs) in wheat tissues and the response was connected with a higher abundance of *Ta-odc*, *Ta-sams* and *Hfr-samdc* transcripts encoding key enzymes of polyamine biosynthesis. The authors concluded that polyamines did not participate in wheat defense mechanisms induced by *M. destructor* larvae, because the response was typical for the susceptible wheat genotype and not the resistant one. However, our earlier studies showed that the excessive accumulation of these compounds under *R. padi* infestation was characteristic of more resistant triticale cultivar (Sempruch *et al.*, 2012*a*). In addition, it was proved that these biomolecules at 1 and 10 mM concentrations disturbed the feeding behavior of bird cherry-oat aphid on triticale seedlings (Sempruch *et al.*, 2016). Therefore, the role of plant amines in their interactions with herbivorous insects is not clear and requires further research.

Recent studies indicated rather small and insignificant differences in the photosynthetic pigment contents (chlorophylls and carotenoids), the ratios of chlorophyll a/b and carotenoids/chlorophylls as well as anthocyanin contents in both the control leaves and in the leaves with galls. In turn, the content of pigments and their ratios in gall tissues was extremely low. Several studies documented a similar pattern in galls induced by *Nothotrioza myrtoidis* Burck (Hemiptera: Psylloidea) on *Psidum myrtoides* (Carneiro *et al.*, 2014) and cecidomyiid galls on *Aspidosperma australe* and *A. spruceanum* (Oliveira *et al.*, 2011). The decrease of assimilatory pigments in galls of Cynipidae species can be explained by the fact that chloroplast location in tissues is usually restricted to external cortical layers (Patra *et al.*, 2010). Nevertheless, chlorophyll loss is also associated with medium- and long-term abiotic and biotic stresses (Barry & Newnham, 2012) and gall formation can be considered to the stress agent. On the other hand, the accumulation of anthocyanins is induced under environmental stresses in plants (Ramakrishna & Ravishankar, 2011). In our experiment, the level of this pigment in gall tissues was low, which did not match the results of Yang *et al.* (2003), who did not detect anthocyanins in infected leaves, while galls contained significant amounts of these pigments.

According to Dorchin *et al.* (2006), gall-inducing species that feed on specialized nutritive tissues (e.g., cynipid wasps) cause less damage to surrounding tissues, thus it is more likely that assimilation rates will increase. However, other findings (Aldea *et al.*, 2006; Kot *et al.*, 2018*b*) and the results presented here showed no evidence to support this hypothesis. Galling process of *N. quercusbaccarum* on oaks leaves caused a decrease of the maximum quantum yield of photosystem II (F_v/F_m), which characterizes the functional state of PSII in dark-adapted leaves. This probably could be caused by physical damages to the reaction centers in the photosystem (Huang *et al.*, 2014*a*). Moreover, chlorophyll fluorescence depends on the number of galls, and it declines with the increasing gall number (Huang *et al.*, 2014*b*; Kmieć *et al.*, 2018). Changes in F_v/F_m values can sometimes be misinterpreted. Vassilev & Manolov (1999) recorded slight changes of F_v/F_m together with a stronger decrease of maximal fluorescence (F_m) values and variable fluorescence ($F_v = F_m - F_0$) values. Therefore, all fluorescence parameters mentioned above should also be analyzed (Huang *et al.*, 2013). In this

https://doi.org/10.1017/S0007485319000221 Published online by Cambridge University Press

study, a significant decrease of F_m and F_0 values was observed, which proved, together with our previous study (Kot *et al.*, 2018*b*), that Cynipidae species that induce galls on leaves exerted a negative effect on photosynthetic rates. The occurrence of *N. quercusbaccarum* galls also resulted in a strong down-regulation of the effective quantum yield of photosystem II photochemistry (*Y*), i.e., the measure of the actual photochemical efficiency of PSII in illuminated leaves (Vassilev & Manolov, 1999). The results of previous studies demonstrated that insect feeding had various effects on this parameter, depending on insect feeding modes or duration of infestation. For example, the presence of aphid galls (Kmieć *et al.*, 2018), midge galls (Nabity *et al.*, 2012), cynipid wasp galls (Kot *et al.*, 2018*b*) as well as aphids on barley (Gutsche *et al.*, 2009) caused a significant reduction of *Y* values. On the other hand, plants infested by scale insects showed increased (Retuerto *et al.*, 2004) or decreased (Kmieć *et al.*, 2016) values of this parameter.

Fluorescence quenching parameters, such as photochemical (q_P) and non-photochemical quenching (q_N) coefficients are commonly used as indicators of plant stress exposed to insect feeding (Gutsche *et al.*, 2009; Golan *et al.*, 2015; Kmieć *et al.*, 2016). However, it seems that q_N is a much more sensitive indicator of a stress response than q_P (Juneau *et al.*, 2005). In our study, the presence of *N. quercusbaccarum* galls on oak leaves reduced q_P and stimulated q_N. This result was consistent with the findings of Kmieć *et al.* (2018). The decrease of ΦPSII and q_P indicates that less of the absorbed photon-energy captured by open PSII reaction centers is used in the photochemical reaction (Yang *et al.*, 2009).

In conclusion, the development of *N. quercusbaccarum* galls on pedunculate oak leaves has a negative effect on host physiology, related to the increase in lipid peroxidation, disruption of cell membrane integrity and disturbance of photosynthesis. During this process, the antioxidant potential of the host plant is also reduced, as a result of a decrease in the content of hydrogen peroxide and low-molecular and enzymatic antioxidants. The activity of ODC and LDC changes in gall tissues, which may affect the content of amino acids and their decarboxylation products – amines.

Acknowledgements

The study was financed by the University of Life Sciences in Lublin (Project no. OKE/DS/2 in 2013–2017) and Siedlce University of Natural Sciences and Humanities (Project no. 245/08/S).

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