CHANGES IN CHLOROPHYLL \(\alpha\) FLUORESCENCE AND PIGMENTS COMPOSITION IN OAK LEAVES WITH GALLS OF TWO CYNIPID SPECIES (Hymenoptera, Cynipidae)

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ABSTRACT

Gall-inducing insects may cause multiple physiological changes in host plants, such as the loss of photosynthetic pigments and reduced photosynthetic capacity. However, the direction of these changes is usually insect species-dependent. Therefore, the objective of this research was to characterize the indirect effects of galls induced by asexual generation of *Neuroterus numismalis* (Fourc.) and *N. quercus-baccarum* L. on photosynthesis by comparing changes in photosynthetic and photoprotective pigments and chlorophyll \(\alpha\) fluorescence in foliar tissue with and without galls in naturally growing pedunculate oak trees (*Quercus robur* L.). The presence of galls of both Cynipidae species caused a significant decrease of chlorophyll \(\alpha\), \(b\) and carotenoids contents. Moreover, photosynthetic parameters (\(F_{0}\), \(F_{m}\), \(F_{v}/F_{m}\), \(Y\), qP, qN) were significantly decreased. These results provide valuable information for diagnosing the oak infections using a non-invasive method, such as chlorophyll \(\alpha\) fluorescence and predicting the effect of infections on photosynthetic productivity.

Key words: gall wasps, photosynthetic pigments, chlorophyll fluorescence, carotenoids, anthocyanins

INTRODUCTION

Galls are the result of specific interactions between gall-inducing herbivores and their host plants. They are formed entirely from plant tissues as the response of plant cells to specific stimuli. Plants translocate metabolites and phytohormones, triggering an abnormal cell growth and division and altering the tissue differentiation, resulting in the formation of distinct gall morphotypes [Isaias et al. 2015]. Galls are considered a new organ with specialized tissues with typical features and functions, where larvae find food and shelter [Stone et al. 2002]. Although the mechanisms of gall induction are not fully understood and depend on taxa that cause them, it is known that gall formation modifies foliar processes; these include plant organ structure [Oliveira et al. 2011], nutrient synthesis [Kot et al. 2018], photosynthetic pigment concentration [Gailite at al. 2005], photosynthetic and transpiration efficiencies [Haiden et al. 2012, Kmieć et al. 2018] or oxidative enzyme activities [Gailite et al. 2005, Carneiro and Isaias 2011] eliciting the plant responses.

Cynipidae includes more than 1300 species of gall inducers that attack mainly oak trees [Melika and Abrahamson 2002]. Moreover, galls induced by these species are the archetype for nutritive galls with...
a concentric zone formed by true nutritive tissues that nourish the larva. Nutritive tissues are surrounded by sclerified layers (parenchymatous cortices) that confer mechanical protection [Oliveira et al. 2016]. Galls induced by asexual generation (♀♀) of Neuroterus numisimalis (Fourc.) and N. quercusbaccarum L., which are the subject of this study, are an example of this group of galling insects.

Most of the previous studies on gall-inducing cynipids focused on their distribution [Mete and Demirsoy 2012], morphology and anatomy of galls [Kovácsné-Koncz et al. 2011], life cycles of particular species and their natural enemies [Bird et al. 2013], spatial distribution of galls in forest canopy [Kampichler and Teschner 2002], the inquilines of oak galls [Katlím and Azmaz 2015], cytological changes [Harper et al. 2004] and the total content of carbohydrates, nitrogen and phenols in gall tissues [Hartley 1998]. No studies have been conducted on photosynthetic characteristics of gall-inducing wasps, except for unspecified species of cynipid wasp on Quercus velutina [Aldea et al. 2006], and it is commonly known that changes of photosynthetic indices and the content of assimilation pigments are the most frequent directly tested parameters to assess the impact of insects on plants [Leszczyński 2001].

Photosynthesis is a multistep biochemical process that occurs when the light-harvesting antenna complexes (LHCs) absorb light energy and transfer it to the reaction centers (RCs) of photosystems I or II (PSI or PSII) [Gururani et al. 2015]. An almost exclusive photoactive chromophore of all reaction centers is chlorophyll a (Chl a), a key photoactive pigment, which converts and stores incoming excitation energy in the form of oxidation-reduction potential [Roháček et al. 2008]. In turn, Chl a fluorescence is the light emitted by Chl a molecules, which provides information on electron energy transformation in photosynthetic systems [Dinç et al. 2012, Belyaeva et al. 2016]. Chl a fluorescence is one of the main methods to investigate the function of PSI and the effect of different environmental stresses on photosynthesis [Kalaji et al. 2012]. The loss of chlorophyll content occurs as an effect of a stress, because chloroplasts are able to produce strong oxidants responsible for the oxidation of pigments as well as proteins and lipids of thylakoid membranes [Guidi and Degl’Innocenti 2012]. Stress conditions also change the relative proportions of absorbed light energy that are used for photosynthesis and chlorophyll fluorescence induction [RoháČek et al. 2008]. Carotenoids (Car) that transfer the excitation energy to Chl b also participate in light-harvesting reactions [Młodzińska 2009]. They act as quenchers of triplet chlorophyll and singlet oxygen that protects PSII reaction center (RC) from photooxidative damage [Merzlyak et al. 2003, Młodzińska 2009]. Photoprotection seems to be the main role of carotenoids, nevertheless they are also recognized as stabilizers of pigment-protein complexes of the photosynthetic apparatus [Solovchenko 2010] as well as signalling precursors during plant development under abiotic and biotic stresses [Ashraf and Harris 2013]. Anthocyanins (Anth) also belong to the group of ‘stress-pigments’, the function of which is to absorb excess sunlight. Accumulation of anthocyanins in plant tissues makes them less susceptible to photoinhibition, and increases their PSII efficiency [Solovchenko 2010].

Although gall-inducing Cynipidae are known to affect many physiological and biochemical parameters in host plants, photosynthetic functioning in these species have not been extensively studied. In this study, we attempted to determine the indirect effects of galls induced by asexual generation of N. numismaticus and N. quercusbaccarum on photosynthesis, as estimated by Chl a fluorescence in naturally growing pedunculate oak trees (Quercus robur L.). We focused on changes in foliar tissues with galls compared to non-infested ones to better characterize similarities and differences in (i) the amount of photosynthetic and (ii) photoprotective pigments, as well as (iii) Chl a fluorescence intensity. These results provide useful information in verifying the stress tolerance of host plant to oak gall-wasps.

MATERIAL AND METHODS

Study site and sampling. The effect of galls induced by asexual generation (♀♀) of N. numismaticus and N. quercusbaccarum on photosynthesis was measured in the beginning of September on Q. robur trees naturally growing in field plantings located along a trail 15 km from Lublin (Poland) to the southwest (51.239°N, 22.379°E). Individual trees for each of the two types of fully developed galls were marked and samples of intact leaves and leaves with galls
were randomly selected for measurements. Galls of each species were counted and only leaves with 25–30 galls were included. The measurements were taken on 20 leaves (10 leaves for each sample) for each gall-inducing Cynipid species. Chl a fluorescence was measured in the field conditions and subsequently the same leaves were detached with scissors and brought to the laboratory in plastic bags within 1 h after collection. In the laboratory, galls were cut off with a scalpel and materials were categorized for each gall-inducing species as: control leaves (leaves without galls) and leaves with removed galls. Thus prepared plant material was used for analysis.

**Chlorophyll a fluorescence.** Chlorophyll a fluorescence was measured with a PAM-2000 fluorimeter (Walz GmbH, Germany) using the saturation pulse method [Schreiber 2004]. The measurement was performed before 11 a.m. to avoid the midday depression of photosynthesis. The leaves were dark adapted for about 20 min before the measurement. The minimum (F₀) and maximum (Fₘ) fluorescence was measured after dark adaptation when all PSII reaction centers were open. Then, light sufficient to drive photosynthesis was applied, and the ground fluorescence (Fₐ) was measured under steady state conditions after 10 minutes. The maximum fluorescence (Fₘ') was determined after steady state conditions by applying pulses of the saturated white light every 60 seconds when actinic light was on. The maximum quantum yield of photosystem II (PSII) was obtained according to the formula: 

\[\frac{F_v}{F_m} = \frac{(F_m - F_0)/F_m}{(F_v - F_0)/F_v}\]

Calculation of photochemical quenching (qP) and non-photochemical quenching (qN) requires the F₀ parameter, which was obtained after a pulse of dark red light applied to previously light-adapted leaves. The qP and qN parameters were calculated according to the equations: 

\[qP = \frac{(1 - (F_m - F_0)/(F_m - F_v))}{(1 - (F_m - F_0)/(F_m - F_v))}\]

\[qN = \frac{(F_m - F_0)/F_m}{(F_m - F_0)/F_m}\]

**Assays of the content of photosynthetic and photoprotective pigments.** The samples (0.5 g) of non-galled leaves and leaves with galls were extracted in 80% acetone. The content of pigments (chlorophyll a, b and carotenoids) was measured according to the method described by Lichtenthaler and Well-burn [1983]. Absorbance was measured at three wavelengths (λ): 470 nm (Car), 646 nm (Chl b) and 663 nm (Chl a), using a CE 9500 Cecil spectrophotometer. The concentration of individual pigments was estimated by the following formula and expressed as mg/g of fresh weight:

\[C_{Chl\ a} = 12.21 \times A_{663} - 2.81 \times A_{646}\]

\[C_{Chl\ b} = 20.13 \times A_{646} - 5.03 \times A_{663}\]

\[C_{Car} = (1000 \times A_{470} - 3.27 \times C_{Chl\ a} - 104 \times C_{Chl\ b})/227,\]

where: A₂ – absorbance value for wave length λ.

For anthocyanin determination, 1 g sample of fresh leaf was taken and extracted for 4 h in 10 ml of 0.1% HCl–MeOH at room temperature. The extracts were measured using a spectrophotometer (Cecil CE 9500) at 530 and 657 nm. The A formula = (A₅₃₀ – 0.25 A₆₅₇) was employed to compensate for the contribution of chlorophyll and its degraded products to the absorption at 530 nm. Anthocyanin contents was expressed in milligrams of Cyanidin-3-glucoside equivalent per 1 g of fresh weight. Anthocyanidin content was calculated according to the formula of Rabino and Mancinelli [1986]:

\[[\text{Absorbance} \times 449.2 \times \text{dilution factor}] / [29.600 \times \text{sample weight (g)}],\]

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

**Statistical analysis.** All data were presented as means (±) with standard errors values (±SE) for 10 (chlorophyll fluorescence) and 3 (pigment contents) replicates. They were analyzed using Statistica for Windows v. 13.1 (Statistica StatSoft Inc. 2016); p < 0.05 was used as the threshold of significance. Student’s t-test was used for analysis due to the normal distribution of all data.

**RESULTS AND DISCUSSION**

Gall-inducing species have the ability to alter morphological, physiological and biochemical host-plant traits. These changes include the decrease in net photosynthesis, with PSII damage and reduction in
pigment contents [Castro et al. 2012]. Nevertheless, it seems that the direction and intensity of these changes are not univocal and usually dependent on the insect species [Gailite et al. 2005].

In our research, the pattern of chlorophyll a and b and total chlorophyll contents was similar for individual gall wasp species (Figs. 1, 2). In general, the average contents of these pigments were significantly lower in leaves with galls when compared to control leaves. The lower photosynthetic pigment contents in leaves with galls of different herbivores are common [Gailite et al. 2005, Oliveira et al. 2011, Samsone et al. 2011, Castro et al. 2012, Patankar et al. 2013, Huang et al. 2014b, 2015, Mukherjee et al. 2016]. This can be explained by accelerated degradation of the pigment or disruption of its biosynthesis [Terletskaya et al. 2017]. On the other hand, the low content of photosynthetic pigments in leaves with galls results from the fact that leaves are drained from photo-assimilates during gall formation, and the galls themselves act as sinks for photoassimilates [Carneiro et al. 2014]. The leaves with galls of N. numismalis were characterized by an extremely high decrease of Chl a and b contents. They were reduced by 61.39% and 65.65%, respectively, when compared to non-infested leaves. A higher reduction of Chl b indicated chlorophyll destruction [Jason et al. 2004]. Additionally, the increased chlorophyll ab ratio indicated that the Chl b damage caused by the presence of N. numismalis galls was much higher than that of Chl a. In this respect, Bogard [1976] showed that Chl b was produced from Chl a, therefore, it was possible that galls affected the transformation of Chl a to Chl b. In turn, values of these pigments in the leaves with N. quercusbaccarum galls decreased by only 13.66% and 10.82%, respectively.

Carotenoids are known to play an important role as accessory pigments in photosynthesis as well as antioxidants, the production of which serves as a reactive oxygen species (ROS) scavenging mechanism in chloroplasts [Havaux 2013]. In this study, the content of carotenoids was significantly higher in the leaves without galls. Nevertheless, the presence of N. numismalis galls resulted in a higher reduction (more than 2-fold) in the content of this pigment when compared to the average control value (Fig. 1). Huang et al. [2015] and Kmieć et al. [2018] also showed lower values of carotenoid contents in the leaves with galls.

![Fig. 1. Photosynthetic pigments concentration in oak leaves without and with galls of Neuroterus numismalis (Fourc.); * values significant at p < 0.05 by Student's t-test (n = 3 ±SE)](https://czasopisma.up.lublin.pl/index.php/asphc)
in ae) on their host trees, respectively. Fixed level of carotenoids is dependent on the metabolic balance between their biosynthesis, degradation and storage [Hannoufa and Hossain 2012]. Synthesis of Car as well as Chl a and b decreases during the stress conditions due to the destruction of pigment protein complexes that protect the photosynthetic apparatus or oxidative damage of chloroplast lipids and proteins [Mibe et al. 2017].

The ratio of chlorophyll alb was variable and depended on gall-inducing species (Fig. 3). It was significantly higher in leaves with N. numismalis galls when compared to those without galls. The increase in the Chl alb ratios suggested that Chl a was degraded relatively slower than Chl b [Yang et al. 2003]. In turn, the presence of N. quercusbaccarum galls resulted in lower value compared to the control. According to Yüzbaşioglu et al. [2017], the reduction of the chlorophyll alb ratio may be explained by the enlargement of PS II antenna complexes. A similar trend to the chlorophyll alb ratio was found for Car/total Chl ratio (Fig. 3). The ratio of chlorophylls and carotenoids varies in different environmental conditions and can be adjusted within genetically programmed limits in response to different stresses [Gitelson et al. 2017]. According to Barry and Newnham [2012], short-term stress usually affects the carotenoid metabolism, while medium- and long-term stress causes a decrease in chlorophyll content.

Anthocyanin accumulation is induced in plants by various unfavorable environmental conditions and often correlates with enhanced stress tolerance [Ramakrishna and Ravishankar 2011]. Anthocyanins function as ROS scavengers, photoprotectants and stress signals during stresses [Kovinich et al. 2015]. The production and accumulation of anthocyanins, as a direct defense against the attack of herbivores, may either kill or retard their development [War et al. 2012]. The presence of galls of both Cynipidae species caused the increase in anthocyanin concentrations when compared to control leaves. However, only leaves with N. numismalis galls showed significant changes in anthocyanin content, as almost a 3-fold increase was observed (Fig. 1). Previous works have shown that the over-accumulation of anthocyanins under stress was correlated with the chlorophyll status [Karageorgou and Manetas 2006, Patankar et al. 2013]. It delayed the chlorophyll loss by maintaining the photosynthetic apparatus function and preventing the photosynthetic machinery damage [Misyura et al. 2013].

![Fig. 2. Photosynthetic pigments concentration in oak leaves without and with galls of Neuroterus quercusbaccarum L.; * values significant at p < 0.05 by Student’s t-test (n = 3 ±SE)](https://czasopisma.up.lublin.pl/index.php/asphc)
Reduction in the quantum yield of PSII can be associated with chlorophyll degradation [Melo et al. 2017]. Insect feeding, as a biotic stress, is known to alter Chl a fluorescence kinetics, but the underlying mechanisms are still not fully elucidated. Some studies have shown a significant inhibitory effect of insect feeding on PSII activity [Kmieć et al. 2016, Golan et al. 2015], whereas other reports have found no significant effect on the structure and function of PSII [Gutsche et al. 2009, Huang et al. 2014a, 2015].

The fluorescence increases from the initial fluorescence intensity (F₀) to the maximum intensity (Fₘ) under saturating light conditions. In our study, leaves with galls of both cynipid species showed significant decreases of F₀ and Fₘ when compared to control leaves (Figs. 4, 5). These findings are consistent with previous study [Hsu et al. 2015, Kmieć et al. 2018] and confirm that the drop in F₀ and Fₘ parameter values reveals the stress induced by arthropods feeding. F₀ measures the stability of the light harvesting complex, and its decrease is interpreted as the participation of the pigment apparatus in down-regulating photosynthesis [Giersch and Krause 1991]. In turn, reduced values of maximum fluorescence (Fₘ) and variable fluorescence (Fᵥ) determined by the Fᵥ = Fₘ – F₀ equation, lead to a decrease in the maximum quantum yield of photosystem II (Fᵥ/Fₘ) [Vassilev and Manolov 1999]. This was reflected in our study. The corresponding decrease of Fᵥ/Fₘ ratios in leaves with N. numismalis and N. quercusbaccarum galls were significantly reduced by 34% and 20%, respectively (Figs. 4, 5). It indicated that the galling process affected the PSII efficiency of host leaves through physical damages to the reaction centers in photosystem [Huang et al. 2014b]. It was reported that the presence of galls of midge belonging to the genus Cecidomyia on Carys glabra leaves, cynipid wasps on Quercus velutina, eriophyid mites on Ulmus alata [Aldea et al. 2006], Bystracoccus mataybae (Eriococcidae) on Matayba guianensis [Oliveira et al. 2017] as well as galling aphids on Ulmus trees [Kmieć et al. 2018] significantly decreased the Fᵥ/Fₘ ratios. It confirms that this indicator can be used to estimate the functional damage of plants [Guidi and Degl’Innocenti 2012]. Chlorophyll fluorescence is correlated with galls number, as it often declines as gall numbers increases [Huang et al. 2014b, Kmieć et al. 2018]. The Fᵥ/Fₘ values are close to 0.83 for most...
non-stressed plant species [Kalaji et al. 2012]. The $F_0/F_m$ values for control plants presented here and in the literature [Patankar et al. 2013, Huang et al. 2014b, 2015, Oliveira et al. 2017] were below this threshold. It suggests that other environmental stresses affect the inhibition of PSII damage repair in trees.

The presence of *Neuroterus numismalis* and *N. quercusbaccarum* galls resulted in a significant reduction of the effective quantum yield of photosystem II photochemistry ($Y$) (Figs. 4, 5). Decreases in $Y$ values are associated with increases of excitation energy quenching in the PSII antennae and indicate the efficiency of the absorbed photon conversion into chemical products [Malkin and Niyogi 2000].

Fluorescence quenching parameters, such as photochemical fluorescence quenching ($q_P$) and non-photochemical quenching ($q_N$) are commonly used to evaluate the photosynthetic efficiency of plants exposed to insect feeding [Golan et al. 2015, Kmiec et al. 2016]. Photochemical fluorescence quenching is associated with photosynthetic activity of electron transport from PSII, while non-photochemical quenching is a protective response of photosynthetic apparatus to excess light [Shangguan et al. 2000].

![Fig. 4. Changes in parameters of chlorophyll $a$ fluorescence in oak leaves without and with galls of *Neuroterus numismalis* (Fourc.); * values significant at $p < 0.05$ by Student’s t-test ($n = 10 \pm SE$)](https://czasopisma.up.lublin.pl/index.php/asphc)

![Fig. 5. Changes in parameters of chlorophyll $a$ fluorescence in oak leaves without and with galls of *Neuroterus quercusbaccarum* L.; * values significant at $p < 0.05$ by Student’s t-test ($n = 10 \pm SE$)](https://czasopisma.up.lublin.pl/index.php/asphc)
According to Juneau et al. [2005], qN is much more sensitive indicator of stress response than qP. In our study, we found that the presence of galls of both cynipid species reduced the photochemical quenching (qP) and stimulated the non-photochemical quenching (qN) in oak leaves. A decrease in the qP values was about 30% in leaves with galls of both cynipid species (Figs. 4, 5). While the increase of the qN value in leaves with N. numismalis galls was higher than in those with N. quercusbaccarum galls. A trend similar to our findings was found in the gallung aphidelm model [Kmieć et al. 2018].

CONCLUSIONS

In conclusion, the presence of galls induced by asexual generation of N. numismalis and N. quercusbaccarum on Q. robur trees affected the photosynthetic and photoprotective pigments as well as Chl a fluorescence. Leaves with galls of both Cynipidae species were characterized by significantly lower values of Chl a, b and carotenoid contents. Furthermore, photosynthetic parameters (F_0, F_m, F_v/F_m, Y, qP, qN) were significantly decreased. This indicated that N. numismalis and N. quercusbaccarum infestations had a significant impact on physiology, biochemistry, morphology and anatomy of oak leaves. The reduction of chlorophyll fluorescence parameters, such as F_v/F_m, Y, qP and qN showed that the thylakoid membrane was severely damaged, similarly as light–harvesting and electron transport components in this membrane. The results also suggest that chlorophyll fluorescence can be widely applied in studies on naturally growing plants as an early diagnostic measure of stress in plants caused by adverse environmental conditions, especially insect pests.

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REFERENCES


