BALTIC FORESTRY

http://www.balticforestry.mi.lt
ISSN 1392-1355
eISSN 2029-9230

Baltic Forestry 2020 26(2): 474
Category: research article
https://doi.org/10.46490/BF474

Two galling insects (*Hartigiola annulipes* and *Mikiola fagi*), one host plant (*Fagus sylvatica*) – differences between leaf and gall chemical composition

SEBASTIAN PILICHOWSKI [1]* AND MARIAN J. GIERTYCH [1,2]

[1] *University of Zielona Góra, Faculty of Biological Sciences, Poland*
[2] *Polish Academy of Sciences, Institute of Dendrology; Parkowa 5, 62-035 Kórnik, Poland; giertych@man.poznan.pl*

** Corresponding author: s.pilichowski@wnb.uz.zgora.pl*

Pilichowski, S. and Giertych, M.J. 2020. Two galling insects (*Hartigiola annulipes* and *Mikiola fagi*), one host plant (*Fagus sylvatica*) – differences between leaf and gall chemical composition. *Baltic Forestry* 26(2): article id 474. https://doi.org/10.46490/BF474.

Received 17 March 2020 **Revised** 17 December 2020 **Accepted** 22 December 2020

Abstract

Gall-inducing organisms change the chemical properties of galled organs and host plants due to the development of gall tissues that are supposed to shelter the galler and provide it with nutrients. *Hartigiola annulipes* and *Mikiola fagi* represent the gall midge family (Diptera; Cecidomyiidae). They share a host plant species, the common beech (*Fagus sylvatica*), whose leaves they use for galling. Their galls are single-chambered and occur on the upper side of the leaf blade. The morphologies of their galls are different, but there is a lack of studies comparing the impact of both species on the common host. Therefore, we analysed the total contents of carbon, nitrogen, soluble carbohydrates, starch, soluble phenolics and tannins in galls, galled leaves with removed galls and leaves without galls. Samples were collected in two different forest stands in western Poland (Scots pine forest with artificially planted beech trees and a natural beech forest). The influence of the studied gallers on the leaves is species- and forest-dependent. Perhaps seasonal changes and the level of infestation are also responsible for the chemical changes. The content of soluble carbohydrates in the galls of *H. annulipes* reaches an optimized level and is independent on the forest type. A high infestation level by *H. annulipes* is manifested itself in an elevated content of total soluble phenolics and tannins in leaves, while gall tissues do not accumulate soluble phenolics. The low levels of nitrogen in the gall tissues of both gallers leads to the rejection of the nutrition hypothesis; however, *M. fagi* galls act as sinks for soluble carbohydrates.

Keywords: plant galls, chemistry, gall midge, common beech, biochemistry

Introduction

Plant galls develop because of the activity of herbivorous organisms. The more common view states that the term 'gall' refers to the growths induced by arthropods (Raman 2007). Plant galls, which are abnormally produced organs and the organisms developing within them, constitute large stresses for a whole plant or its modules. Thus, the biochemical and physiological aspects of plant biology are connected to the growth and development of galls. Due to interactions between a host plant and the galling animal, the content of various mineral and organic components may change in infested plant modules (Hartley and Lawton 1992, Hall et al. 2016). Rapp and Kirst (1974) found that the gall tissues induced by *Mikiola fagi* Hartig 1839 (Cecidomyiidae), when compared to the leaf tissues, had lower concentrations of Ca^{2+} and soluble proteins, similar contents of Na^+ (in some cases, however, Na^+ level was higher), K^+ and carbohydrates, and drastically lower levels of chlorophyll *a* and *b*. Approximately 90% of the total chlorophyll and two-thirds of the soluble proteins were in the nutritive tissue. Furthermore, galled leaves had lower chlorophyll contents than non-infested leaves (Rapp and Kirst 1974). In other study, the same authors showed that galls containing larvae become nutrient sinks like the terminal parts of host modules. Kirst and Rapp (1974) traced the transport of CO_2-marked with ^{14}C isotopes into the leaves and found that a few minutes were enough for the isotope to reach the *M. fagi* gall tissue, which proves the influx of carbon to galls (which is subsequently included

in carbohydrates and secondary metabolites). The galls of two *Daphnephila* (Cecidomyiidae) species, galling *Machilus thunbergii* Siebold and Zucc., had lower contents of nitrogen and carbon than the leaves; nonetheless, the C/N ratio was higher in the galls than in the leaves (Huang et al. 2014). Moreover, the gall tissues of both species contained more soluble carbohydrates, and the galls belonging to *D. sueyenae* had more starch than galled and ungalled leaves. Huang et al. (2014) indicated the absence of gas exchange in both *Daphnephila* galls due to a lack of stomata and that the photosynthesis conducted by the gall tissues was insufficient to provide enough nutrients for these tissues and the larvae developing inside a gall. Therefore, the transport of nutrients within a host is forced to supply the gall tissues and the larva.

Larvae developing inside galls compete for nutrients with host organs (Burstein et al. 1994, Dorchin et al. 2006). Plants exhibit various defensive mechanisms against herbivores, including galling arthropods. They can produce secondary metabolites that may have deterrent, repellent and toxic characteristics, which hinder the development and growth of the animal feeding on them (Ibanez et al. 2012), or trigger a hypersensitive reaction (Fernandes et al. 2003, Pilichowski and Giertych 2017). Phenols, including tannins, prevent insects from feeding (Goncalves-Alvim et al. 2004, Ibanez et al. 2012, Moctezuma et al. 2014, Oszmianski et al. 2015). Gallers can affect plant physiology, which manifests as lower concentrations of non-tannin phenolics in galls (Nyman and Julkunen-Tiitto 2000), elevated concentrations of tannins in galls (Nyman and Julkunen-Tiitto 2000) and elevated concentrations of total phenolics in galls (Gupta 2011) and galled leaves (Ferreira et al. 2014). The question is what is the reason for such changes? Are elevated concentrations of phenolics (e.g., flavonoids, tannins) or other secondary metabolites a defensive reaction of plant against the galler or potentially harmful for the galled organ and gall herbivores (Ferreira et al. 2014, Sashirekha 2014)? The chemical properties of galls may change with time depending on the developmental stage (Ferreira et al. 2014). Moreover, the level of nutritive stress of the plant host may have an important influence on the concentration of various compounds.

In addition to the chemical composition, the morphological structure of leaves can also significantly affect the interaction with herbivores (Cornelissen et al. 2004). Specific leaf area (SLA) is commonly used as a factor to determine light conditions; thus, it indirectly shows the quality of the leaf, which may impact the herbivory rate (Poorter et al. 2004, Anil and Parthasarathy 2016, Fellner et al. 2016, Konopka et al. 2016). SLA relates to photosynthesis efficiency and building up the biomass based on the photosynthesis assimilates, as well as with nitrogen investment regarding species-specific ecological and biological traits (Poorter and Evans 1998, Reich et al. 2002, Gulías et al. 2003). Nutritional quality of plant material with higher N concentrations is usually increased in sun-exposed leaves (Fortin and Mauffette, 2002, Levesque et al. 2002). Leaf N concentration of *F. crenata* an *F. sylvatica* decreases as light availability increases (Yamasaki and Kikuzawa 2003, Stiegel et al. 2017). Light increases carbon-based defence compounds of leaves (Dudt and Shure 1994, Crone and Jones 1999, Roberts and Paul 2006). SLA was chosen as a supportive factor potentially indicating differences in leaf biochemistry via varying light conditions between understorey beech trees growing in two different stands.

The aim of this study was to determine the impact of two gall midge species on the chemical properties of their host tree – the common beech (*Fagus sylvatica* L.). The results obtained here were used to answer the following questions:

1. Do galls of *H. annulipes* and *M. fagi* change the chemistry of common beech leaves?
2. Are there chemical differences between galls, galled leaves and non-galled leaves?
3. Do gallers influence the accumulation of soluble phenols and tannins in galled leaves?
4. Do both galling species exhibit different influences on the host organs?
5. Does the impact of the galler species vary depending on the study site?

We hypothesized that the two studied gallers change host-plant biochemistry. We suspected that the influence of *H. annulipes* and *M. fagi* will differ, even though they share a common host and its leaves as the organ being galled, due to their different gall morphology and phenology.

Materials and methods

Insect and plant material

Leaves bearing galls of *H. annulipes* (Hartig 1839) and *M. fagi* (Hartig 1839) (Diptera: Cecidomyiidae) of the common beech, *F. sylvatica,* were collected in September/October from trees growing in the understory (maximal collection height: 3 m) from two locations in Western Poland:

1. a managed Scots pine forest (PF) in Zielona Góra (51°54'0.6" N, 15°29'16.1 E; altitude 120–126 m) consisting primarily of 70-year-old Scots pines, *Pinus sylvestris* L., managed by foresters with beech trees growing in the understory (ca. 14 years old). The soils are poor and of podzol type. High availability of light enables the development of understory plants. The collection was made from artificially planted beech trees.
2. a mixed temperate broad-leaf forest (BF) with limited management, "Las Żarski", (51°36'25.3" N, 15°7'54.7 E; altitude 180–210 m), where the main habitat type is *Luzulo-Fagetum* beech forest dominated by *F. sylvatica* and *Quercus petraea* (Matt.) Liebl. It has a low diversity of plant species growing in the understory due to shading by the dense broad-leaf canopy. The area was formed by a terminal moraine of a

glacier during the Riss glaciation, and the highest point is Żarska Mountain (Góra Żarska), which is 227 m high. The collection was made in a forest with a considerable portion of over 100-year-old beech trees.

Both gall midge species produce one generation annually, which develop in single-chambered galls appearing on the upper surface of the common beech leaves. The main differences between them are observed in the gall morphology and oviposition behaviour. *M. fagi* galls are large, conically shaped, and thick-walled, while *H. annulipes* galls are smaller and have "hairs" on their surface. After overwintering in galls, the insect mate, and then oviposition occurs. *M. fagi* females lay eggs on buds and twigs in late March/early April, and *H. annulipes* females oviposit on freshly developed leaves in April (Rohfritsch 1971, Urban 2000). In 2015 (with *H. annulipes* galls) and 2016 (with *M. fagi* galls), 20 trees per location were randomly chosen regarding their infestation level (in total 40 trees in each year). Each tree had to bear about 20 galls *of M. fagi* or 40 galls of *H. annulipes* to prepare samples for chemical analyses. In a few cases the mass obtained was too small to do all the analyses. From each tree, 20 leaves without galls (LW) and 20 leaves with galls (LG) were collected. Thereafter, in the laboratory, the galls (G) were shed off of the leaf blades and cut into pieces with scissors to remove the larvae inhabiting them. This resulted in the preparation of three types of tissues: leaves without galls (LW), galled leaves with removed galls (LG) and galls without larvae (G). In total, 120 samples (two locations, 20 trees, three types of tissues) were prepared for each gall midge species.

Chemical analyses

The contents of total carbon, total nitrogen, total soluble phenolics, tannins, soluble carbohydrates and starch were analysed in each sample. For the analyses, the samples were dried at 65°C (40°C for tannins) for 72 hours. To determine the nitrogen (N%) and carbon contents (C%), an Elemental Combustion System 4010 CHNS-O analyser (Costech Instruments, Italy/USA) was used. The content of soluble carbohydrates was assessed as described by Haissig and Dickson (1979) and Hansen and Moller (1975) using methanol-chloroform-water extraction and the colour reaction with anthrone and spectrophotometric analysis at $\lambda = 625$ nm. Starch content was measured in the precipitate remaining after the extraction for soluble carbohydrates at $\lambda = 450$ nm after a colour reaction with dianisidine. Both were expressed as percentages of the dry mass.

The phenol content was expressed as μM chlorogenic acid g^{-1} d.m. after the use of Folin Ciocalteu's Phenol Reagent (Sigma F – 9252) and spectrophotometric analysis ($l = 660$ nm) according to Johnson and Schaal (1957), as modified by Singleton and Rossi (1965). Tannin content was determined by extraction with absolute methanol and the application of the colorimetric method and absorption for $l = 500$ nm (Price et al. 1978). Tannins were expressed as μM catechin g^{-1} d.m.

Specific leaf area analyses

SLA analyses were conducted on leaves collected in September. In both stands, five beech trees (maximal tree height: 2.5 m) were randomly chosen, and 20 leaves were collected from each. In total, 100 leaves were collected from each stand. Then, the area of the leaves was measured using ImageJ (version 1.48, Wayne Rasband, National Institutes of Health, USA). The leaves were then dried and weighed in the laboratory using an OHAUS Corp. Adventurer Pro scale model AV2102CM with $d = 0.01$ grams (precision of scale division) to obtain SLA values [cm^2/g], which were compared to find differences in the light conditions between stands.

Statistical analyses

A Student's *t*-test was used to compare SLA means in PF and BF. To obtain the normal distribution of both populations, the SLA values were reversed (1/SLA). To test the normality, the Shapiro-Wilk test was used. After initial testing of residual normality (Shapiro-Wilk test), a two-way analysis of variance (ANOVA) with mixed effects was used to evaluate the influence of location and type of tissue and their interaction (fixed factors) on element and chemical compound concentrations. Tree nested in location was used as a random factor. The results expressed in percent were arcsin-transformed for normality for ANOVA analyses. A Tukey *HSD* test was used to assess significant differences between locations and the type of tissue. The analyses were conducted regarding the galling species. Statistical analyses were performed in JMP® 11.2.0 (SAS Institute 2014).

Results

Nitrogen

The nitrogen content in the galls (G) of both tested species was significantly lower than in the leaf tissues (LW, LG; Figure 1, Tables 1 and 2). However, the presence of a gall in any species did not modify the nitrogen content in the galled leaves (LG) (Tables 1 and 2). The location was significant only in the case of *H. annulipes*, and despite differences in the nitrogen content of the individual research areas, its content in the galls (G) was very similar, which confirms a significant interaction (Table 1 and Figure 1).

Carbon

For *H. annulipes,* there were no significant differences in the carbon content between types of tissue within locations (Figure 2a); however, the location impacted the carbon content (Table 1). Thus, slight but statistically significant differences were observed between locations. The carbon content in samples collected in BF was lower than

in PF, although it was not significantly different between galls (G) and leaves without galls (LW) (Figure 2a). In contrast, the type of tissue and the interaction between the type of tissue and location had an impact on carbon content in *M. fagi* (Table 2). Galls (G) had the lowest carbon content among the types of tissue; galled leaves (LG) from PF had higher carbon content than from BF, while leaves without galls (LW) from both locations did not differ from galled leaves (LG) (Figure 2b).

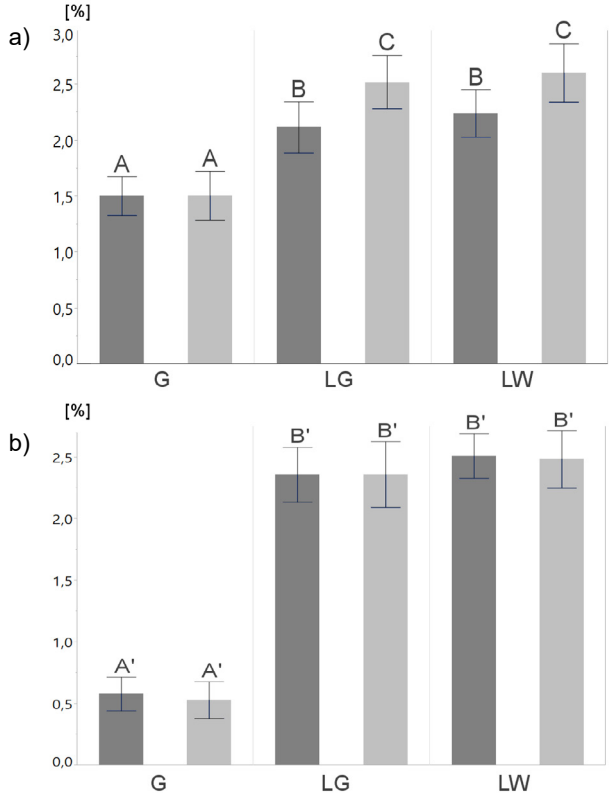

Figure 1. Percentage content of the total nitrogen in three types of plant tissues: galls (G), galled leaves with removed galls (LG) and leaves without galls (LW). Results for the two gall-midge species: *Hartigiola annulipes* (a) and *Mikiola fagi* (b)

The two locations are distinguished by colour: dark grey – PF, light grey – BF. Error bars represent standard deviations. Different letters show significant differences between values.

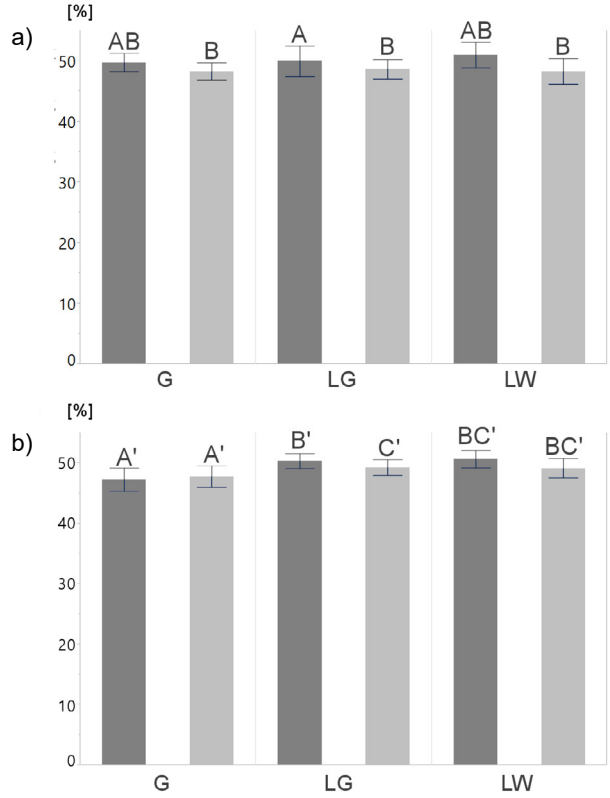

Figure 2. Percentage content of the total carbon in three types of plant tissues: galls (G), galled leaves with removed galls (LG) and leaves without galls (LW). Results for the two gall-midge species: *Hartigiola annulipes* (a) and *Mikiola fagi* (b)

The two locations are distinguished by colour: dark grey – PF, light grey – BF. Error bars represent standard deviations. Different letters show significant differences between values.

Table 1. ANOVA results for nitrogen (N), carbon (C), tannins, soluble phenolic compounds, soluble carbohydrates and starch content for location (type of forest), type of tissue (galled leaves (LG), leaves without galls (LW), and galls (G) of *Hartigiola annulipes*) and their interaction

Hartigiola annulipes	N (%)				C (%)			
Source	*DF*	*DFDen*	*F*	*P*	*DF*	*DFDen*	*F*	*P*
Location	1	36.9	24.34	**<.0001**	1	38.6	23.85	**<.0001**
Type of tissue	2	69.2	231.73	**<.0001**	2	73.9	0.74	0.4812
Location*Type of tissue	2	69.2	7.31	**0.0013**	2	73.9	1.57	0.2158
	Soluble carbohydrates (%)				Starch (%)			
	DF	*DFDen*	*F*	*P*	*DF*	*DFDen*	*F*	*P*
Location	1	38.8	190.36	**<.0001**	1	35.7	0.59	0.4458
Type of tissue	2	71.8	1.88	0.1599	2	70.4	0.58	0.5624
Location*Type of tissue	2	71.8	38.38	**<.0001**	2	70.4	1.58	0.2126
	Tannins (μM g^{-1} d.m.)				Soluble phenols (μM g^{-1} d.m.)			
	DF	*DFDen*	*F*	*P*	*DF*	*DFDen*	*F*	*P*
Location	1	41.3	7.52	**0.0090**	1	69.8	22.00	**<.0001**
Type of tissue	2	74.2	329.48	**<.0001**	2	77.8	2.80	0.0667
Location*Type of tissue	2	74.2	43.23	**<.0001**	2	77.8	2.90	0.0612

Table 2. ANOVA results for nitrogen (N), carbon (C), tannins, soluble phenolic compounds, soluble carbohydrates and starch content for location (type of forest), type of tissue (galled leaves (LG), leaves without galls (LW), and galls (G) of *Mikiola fagi*) and their interaction

Mikiola fagi	N (%)				C (%)			
Source	*DF*	*DFDen*	*F*	*P*	*DF*	*DFDen*	*F*	*P*
Location	1	38	0.51	0.4804	1	38	3.80	0.0588
Type of tissue	2	76	2344.34	**<.0001**	2	76	39.08	**<.0001**
Location*Type of tissue	2	76	0.82	0.4455	2	76	6.27	**0.0030**
	Soluble carbohydrates (%)				Starch (%)			
	DF	*DFDen*	*F*	*P*	*DF*	*DFDen*	*F*	*P*
Location	1	38	2.85	0.0995	1	37.92	2.98	0.0926
Type of tissue	2	76	79.73	**<.0001**	2	75.59	0.99	0.3768
Location*Type of tissue	2	76	1.25	0.2920	2	75.59	1.58	0.2126
	Tannins (μM g^{-1} d.m.)				Soluble phenols (μM g^{-1} d.m.)			
	DF	*DFDen*	*F*	*P*	*DF*	*DFDen*	*F*	*P*
Location	1	38	3.87	0.0564	1	37.66	4.99	**0.0315**
Type of tissue	2	76	38.47	**<.0001**	2	74.87	149.82	**<.0001**
Location*Type of tissue	2	76	2.92	0.0601	2	74.87	0.14	0.8661

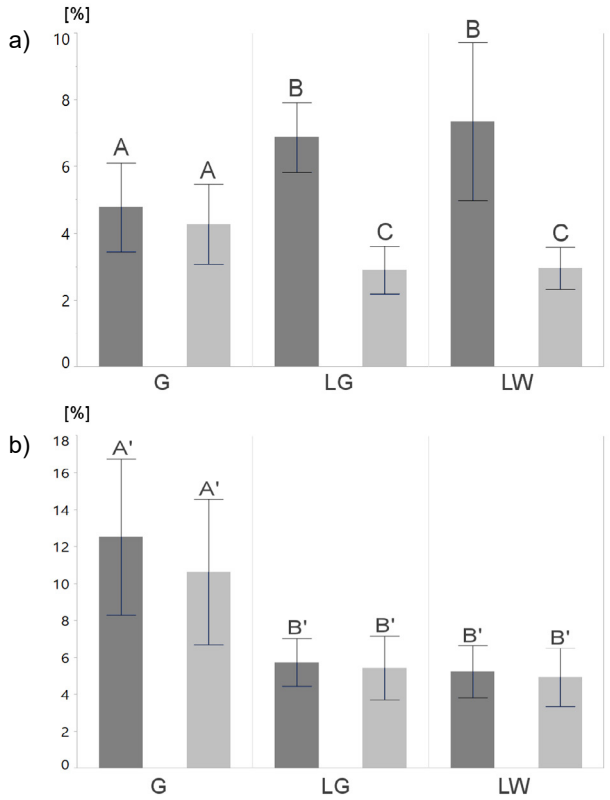

Figure 3. Percentage content of the total soluble carbohydrates in three types of plant tissues: galls (G), galled leaves with removed galls (LG) and leaves without galls (LW). Results for the two gall-midge species: *Hartigiola annulipes* (a) and *Mikiola fagi* (b)

The two locations are distinguished by colour: dark grey – PF, light grey – BF. Error bars represent standard deviations. Different letters show significant differences between values.

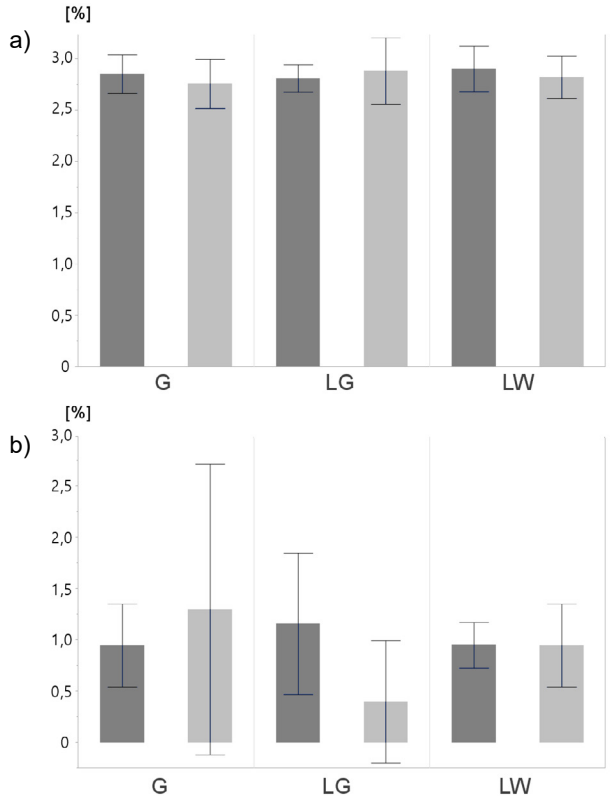

Figure 4. Percentage content of the starch content in three types of plant tissues: galls (G), galled leaves with removed galls (LG) and leaves without galls (LW). Results for the two gall-midge species: *Hartigiola annulipes* (a) and *Mikiola fagi* (b)

The two locations are distinguished by colour: dark grey – PF, light grey – BF. Error bars represent standard deviations. No significant differences were detected in the starch content analysis.

Soluble carbohydrates and starch

The content of soluble carbohydrates in *H. annulipes* galls (G) did not differ between the two locations, although the soluble carbohydrate content in leaves (LW, LG) from BF was significantly lower than in leaves from PF. Their content between galled (LG) and non-galled leaves (LW) did not differ within locations but differed between locations, which confirms a significant interaction (Table 1, Figure 3a). Nonetheless, the soluble carbohydrate content in *M. fagi* samples was influenced solely by the type of tissue (Table 2). *M. fagi* galls (G) were richer in soluble carbohydrates than the leaves (LW, LG), which did not differ between the types of tissue and the locations in which they were collected (Figure 3b).

In both species, we could not find any differences between locations and types of tissues in the starch content (Figure 4, Tables 1 and 2).

Soluble phenols

The content of phenolic compounds in the galls (G) of both species was lower than in the leaves (LW, LG), but significant differences were found only in *M. fagi* (for *H. annulipes* $p = 0.061$). We did not find an effect of galling on changes in the level of phenols in the leaves (LW, LG) (Tables 1 and 2, Figure 5). Significant differences in the content of phenols between locations were revealed mainly in the leaves (LW, LG); however, this did not result in significant differences in the content of phenols in the galls (G) between locations.

Tannins

In the case of *H. annulipes,* all factors influenced the tannin content (Table 1), and only the type of tissue had an impact on the tannin content in *M. fagi* samples (Table 2). *H. annulipes* galls (G) had strongly elevated levels of tannins, which was much higher than in the leaves (LW, LG). Additionally, galls (G) collected in BF had more tannins than those from PF. Moreover, leaves (LW, LG) from PF were richer in tannins than leaves from BF, and galled leaves (LG) had significantly more tannins than non-galled leaves (LW). Leaves (LW, LG) from BF did not differ statistically (Figure 6a). Nonetheless, *M. fagi* galls (G) had the lowest tannin content, which did not differ from leaves

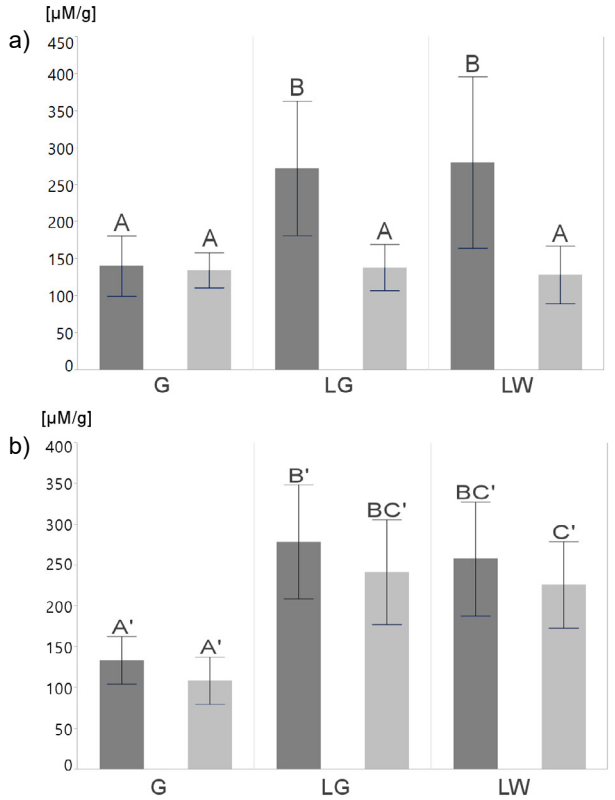

Figure 5. Content of the total soluble phenolics expressed as µM chlorogenic acid g^{-1} d.m. in three types of plant tissues: galls (G), galled leaves with removed galls (LG) and leaves without galls (LW). Results for the two gall-midge species: *Hartigiola annulipes* (a) and *Mikiola fagi* (b)

The two locations are distinguished by colour: dark grey – PF, light grey – BF. Error bars represent standard deviations. Different letters show significant differences between values,

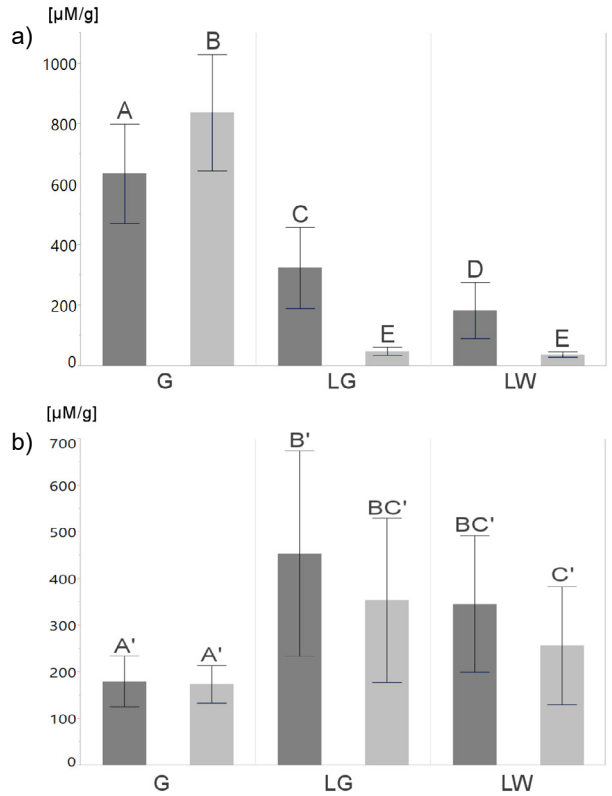

Figure 6. Content of the tannins expressed as µM catechin g^{-1} d.m. in three types of plant tissues: galls (G), galled leaves with removed galls (LG) and leaves without galls (LW). Results for the two gall-midge species: *Hartigiola annulipes* (a) and *Mikiola fagi* (b)

The two locations are distinguished by colour: dark grey – PF, light grey – BF. Error bars represent standard deviations. Different letters show significant differences between values,

without galls (LW) collected in BF. Galled leaves (LG) from PF had the highest tannin content; however, it was not significantly different from galled (LG) and non-galled leaves (LW) from BF and PF, respectively. Furthermore, leaves (LW, LG) did not differ between locations in either type of tissue (Figure 6b).

Specific leaf area

There were no differences in SLA between locations.

Discussion

The stand environmental conditions, including the tree-species combination and chemical properties of soil, may affect various traits of tree organs, nutrient turnover, as well as the spatial distribution and number of galls (Nicolai 1988, Balsberg Påhlsson 1989, Veldtman and McGeoch 2003, Castellanos et al. 2006, Forey et al. 2016, Meyer et al. 2020). Although this study was conducted in two different forest stands, the SLA comparison, which is a useful parameter reflecting light conditions, showed no differences between stands. However, the chemical analyses of leaves (LW, LG) and galls (G) indicated that the carbon, soluble carbohydrate and soluble phenol contents tended to be higher in PF than in BF. We suspect that the light availability would have played essential role in these differences, even though SLA analyses showed no differences. Perhaps, measuring the light penetration through the canopy and the photosynthetically active radiation would provide more complex results in this matter. PF stand is a managed forest dominated by Scots pine (*P. sylvestris*) trees, while BF stand is a mixed broad-leaf forest with a high contribution from the common beech (*F. sylvatica*). Pines have thinner crowns, allowing the trees growing in the understory to obtain lighter, which contrasts with broad-leaved beeches. The SLA analysis did not reflect these expected differences possibly because the morphological structure of the leaves is also influenced by other factors, not solely light (Poorter and De Jong 1999), including stand and leaf age (Beets and Lane 1987), or soil nitrogen content (Zheng et al. 2017). Moreover, some chemical compound contents differ in leaves (LW, LG) between years, which is particularly shown in the case of tannins (Figures 5a and b). Perhaps seasonal climatic conditions impact such changes.

Nitrogen is an important element found in various organic compounds. Gall inducers are expected to be attracted by a high availability of nitrogen in their hosts (the nutrition hypothesis) (Shannon and Brewer 1980, Price et al., 1987, Hartley and Lawton 1992, Goncalves-Alvim et al. 2004). However, there are studies that do not support the nutrition hypothesis (Hartley 1998, Castro et al. 2012). Furthermore, the nitrogen distribution may be correlated with light conditions in various forest types (White and Scott 2006). Leaves without galls (LW) and leaves with removed galls (LG) of *H. annulipes* did not differ within stands, but samples collected in BF (beech forest) had higher nitrogen content than those collected in PF (pine forest). Nonetheless, similar studies performed the following year (in 2016), but concerning *M. fagi*, showed no differences in the nitrogen content between stands. Moreover, leaves with no infestation (LW) by *M. fagi* and galled (LG) by *M. fagi* did not statistically differ and had higher nitrogen content than galls (G), which leads to an assumption that the galler does not significantly impact nitrogen distribution. Nitrogen resources are limited, but interestingly, N% values in *H. annulipes* seem to be optimized in the galls (they reach the same level), independent of the differences between locations. Field observations conducted in western Poland showed that *M. fagi* galls rarely occur, which contrasts with *H. annulipes* galls, which are strongly overrepresented (having even 76 galls on a leaf blade (Pilichowski and Giertych 2018)). *H. annulipes* galls are smaller than *M. fagi* galls, but they possess "hairs" on the surface. Is this morphological difference responsible for such a distinction in nitrogen content? If nitrogen in host organs is limited, it appears that nitrogen must be allocated from the surrounding leaves / shoots to allow for gall induction to reach high levels of galls. Gall inducers can influence and control the nitrogen content of the host organ. The partial removal of galls of some cynipid gallwasps can lead to an increase of nitrogen in galls left on the leaves (Hartley 1998) or may not impact the total nitrogen content in galls (Hartley and Lawton 1992). Comparison of the nitrogen content of both studied gall midge species shows that *M. fagi* galls have less nitrogen than *H. annulipes* galls, at least at the terminal phase of gall development. It may also reflect the quantity of proteins that contain nitrogen, since Rapp and Kirst (1974) showed that *M. fagi* galls (G) had fewer proteins than galled (LG) and non-galled leaves (LW). Additionally, Paclt and Hassler (1967) found that young *M. fagi* galls have lower nitrogen concentrations than leaves. Such findings may be explained by the poor physiological activity of plant galls (Castro et al. 2012, Huang et al. 2014). In contrast to these results, Koyama et al. (2004) showed that galled leaves have elevated concentrations of amino acids (in their study, the levels were five-fold higher than in the non-galled leaves). Thus, the leaves infested by galling aphids might be more attractive to other herbivores.

On the other hand, the measured content of soluble carbohydrates in *H. annulipes* galls (G) remained at the same level, regardless of their concentration in the leaves (which differed between stands). Thus, galls (G) tend to reach an optimal level of soluble carbohydrates, even if it means storing a lower quantity of them. In contrast, the large galls (G) of *M. fagi* exhibited much higher concentrations than beech leaves (LW, LG). In case of soluble carbohydrates, *H. annulipes* may be an attractive sink especially in shaded leaves whose photosynthetic activity is low since the soluble carbohydrates content is optimized as explained above. In case of *M. fagi* galls, they may be much more attractive than leaves to herbivorous insects since they accumulate high concentrations of soluble car-

bohydrates. This is due to a high soluble carbohydrates content modified by stand conditions (Figure 3). Differences between galls and leaves may vary in various months, with galls supplied better during the late summer (Kirst and Rapp 1974). Furthermore, leaves with *M. fagi* galls impose CO_2 influx from non-galled leaves (Kirst and Rapp 1974). Such a mechanism ensures that galled leaves will be supplied with photosynthetic substrates to synthesize carbohydrates. Nonetheless, it is worth mentioning that the starch content between leaves (LW, LG) and galls (G) in both locations and species did not differ significantly. The total carbon content did not differ between *H. annulipes* samples within locations; however, it was slightly higher in PF than in BF. The reason for such differences remains unknown. However, the population genetic variability or the level of infestation (high in PF, low in BF) may play an important role in explaining the differences. Moreover, the carbon content in *M. fagi* galls (G) was significantly lower than in leaves (LW, LG) and was independent of small differences in the carbon content in leaves collected in both locations. Galled leaves (LG) may exhibit slightly elevated carbon contents due to the allocation and influx of CO_2 to support the *M. fagi* galls (Kirst and Rapp 1974).

To date, numerous authors have reported that phenols play important roles in the defensive mechanisms of plants against herbivores (Abrahamson et al. 1991, Giertych et al. 2007, Petrakis et al. 2011, Ferreira et al. 2014). This study showed that leaves, which were galled (LG) by *H. annulipes* and those without galls (LW), had similar concentrations of phenolic compounds (either in PF or BF), while the content of phenolics in galls (G) collected in PF was lower than in corresponding leaves (LW, LG), yet like those from BF. It is important to note that the level of phenols in leaves from BF was significantly lower than in leaves from PF and like the level of phenols in galls from PF. The infestation intensity might be the factor explaining such differences between leaves collected from both locations. In 2015, when samples were collected to investigate chemical host properties concerning *H. annulipes* infestation, their galls were common in PF stand, but rare in BF (usually one gall per galled leaf). Moreover, the infestation level is reflected in the differences between stands, where leaves from PF (high infestation) had much more soluble phenolics than those from BF (low infestation). The results lead to the assumption that there is a threshold of galling tolerance in the host plant, and after it is exceeded, a mass accumulation of soluble phenolics in galled (LG) and non-galled leaves occurs (LW). This phenomenon can indicate a high infestation level and a defensive response to galling. Eventually, since *H. annulipes* galls avoid phenols (likely to protect the larva from the host plant's defensive mechanism), it can be hypothesized that the gall inducer uses (or manipulates) the host plant's defensive means to protect the galled host organ from being eaten by other herbivores. On the other hand, galls with low levels of phenolics might be attractive for herbivores. Nonetheless, in the case of *H. annulipes,* the "hairs" on the surface of maturing galls seem to be enough to deter them. Mechanical barriers play defensive roles in various galling species (Ito and Hijii 2004, Zargaran et al. 2011). Finally, the data obtained from both stands for *M. fagi* samples did not differ greatly. The level of phenolics in leaves (LW, LG) from PF was slightly higher or similar compared to the levels obtained from leaves collected in BF (Figure 5b). The results for samples representing both stands showed that phenolic levels were higher in leaves (LW, LG) than in the galls (G), which corresponds to the result of *H. annulipes* samples. Due to the low infestation level in both locations, it would be risky to suspect that the development of *M. fagi* galls increases the phenolics level at the host level, what occurs in case of infestation by *H. annulipes*. Nonetheless, *M. fagi* galls are thick-walled and larger than *H. annulipes* galls and start to develop earlier in spring (Rohfritsch 1971, Urban 2000). Such morphological and phenological differences may be responsible for the accumulation of soluble phenolics in the leave, even at a low infestation level. Moreover, similarly to *H. annulipes*, *M. fagi* galls do not accumulate phenolics. Possibly the thick walls of *M. fagi* galls ensure a mechanical barrier as defence against herbivores. It seems that incorporating phenolics into the defensive mechanisms of gall tissues may become harmful for gall insects. Forey et al. (2016) showed that total phenolic compounds vary in forest stands depending on the stand composition. This finding can also explain the differences between pine and beech forest stands. As oaks can be negative partners for beeches (Forey et al. 2016), pines might also trigger defensive mechanisms in beech trees. This, however, needs to be investigated.

Tannins are polyphenols that can be tolerated by some herbivores (Barbehenn and Constabel 2011) or become toxic and make host organs less attractive to feed on (Feeny 1970, Goncalves-Alvim et al. 2004). The effect of tannins depends on their chemical structure and composition (Barbehenn and Constabel 2011, Moctezuma et al. 2014). In the case of *H. annulipes* samples, highly galled leaves (LG) from PF had more tannins than the corresponding leaves without galls (LW), while poorly galled leaves from BF (LW, LG) did not differ statistically but accumulated the least tannins among collected samples. Galls (G) from BF had significantly more tannins than galls (G) from PF, but generally galls seem to accumulate tannins. The role of tannins in relationships between insects and plants is diverse. They may act as deterrents and defensive compounds, also as phagostimulators and the role of tannins can be modified by the environment (Bryant et al. 1993, Barbehenn et al. 2009, Cardinal-Aucoin et al. 2009, Barbehenn and Constabel 2011, War et al. 2012). Since *H. annulipes* galls are rarely found damaged by herbivores (authors' observation), we suppose that the tannin accumulation in *H. annulipes* galls makes them deterrent for herbivores, which ensures survival of the gall inducer. In the case of *M. fagi*, results obtained from both locations show that *M. fagi* galls

(G) do not store a considerable number of tannins. However, the galling process impacts the tannin level in galled hosts (Figure 6b), especially at high infestation levels (PF). It seems that the host responds to the galling process by a synthesis of tannins, but at the same time, gall tissues do not accumulate high concentrations of tannins. Since gall inducers are known to be capable of controlling the chemistry of the host (Hartley and Lawton 1992, Hartley 1998, Nyman and Julkunen-Tiitto 2000), we suspect that *M. fagi* prevents the biosynthesis of tannins in gall tissues.

Moctezuma et al. (2014) showed that catechin molecules can be incorporated into the synthesis of tannins. It would be probable that leaves bearing *H. annulipes* galls use catechins to produce tannins to allocate them in galls instead of catechins. Furthermore, some studies (Pascual-Alvarado et al. 2008, Tooker et al. 2008) proposes that gall inducers stimulate the host plant to increase the concentrations of secondary metabolites, which can result in deterring effects against other herbivores. This would be beneficial for both the host plant and the gall insect due to herbivore feeding avoidance on organs rich in phenolics. Phenolics may influence the growth of galls as regulators (Abrahamson et al. 1991, Bedetti et al. 2014, Lingaraj et al. 2015) and then contribute to the chemical defence. Like soluble phenolics, the concentration of carbohydrates in galls can change with time. Abrahamson et al. (1991) report that gall tissues reach the highest level of phenolics at the final stage of development. The study presented here also reports data obtained for galls collected at the peak of development and growth. Isaias et al. (2015) noted that high concentrations of carbohydrates and phenols may relate to the restoration of redox homeostasis in the plant tissues forming galls. Furthermore, phenolic compounds lead to the accumulation of indole acetic acid (IAA), a plant growth regulator (Lingaraj et al. 2015). These results show that chemical compounds that have been treated primarily as nutrients or repellents may turn out to be important chemical factors responsible for gall formation in the future.

In addition, it should be mentioned here that we cannot discard a possibility that collecting material for analyses of two different gall midge species during two following years had no impact on the results. Especially when it goes for obtaining comparative values of the studied chemical compounds in beech leaves due to seasonal changes in herbivory rate or microclimatic conditions.

Conclusions

H. annulipes and *M. fagi* galls modify plant host chemistry on various levels. *H. annulipes* optimizes the level of soluble carbohydrates in gall tissues. Galls of both studied species do not exhibit a strong accumulation of nitrogen and starch, which does not support the nutrition hypothesis in this respect and for these two species of gall-inducers. However, *M. fagi* galls are sinks for soluble carbohydrates. *H. annulipes* and *M. fagi* galls do not accumulate starch and phenols. Nonetheless, during high infestations of *H. annulipes,* the total soluble phenolic contents increase significantly in galled and non-galled leaves. Moreover, high infestations of *H. annulipes* elevate the tannin concentrations in the leaves of the host, mainly in the galled leaves. *M. fagi* galls do not store tannins. However, their presence may slightly increase the total tannin levels in galled leaves. Both gall midge species differ in their influence on host plant chemistry despite being monophagous and sharing the same host and its organ for galling.

Acknowledgements

The study funded by the National Science Centre (Narodowe Centrum Nauki), Poland (Preludium 6, grant number: 2013/11/N/NZ9/00106).

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