

A COMPARATIVE EVALUATION OF THE CONSEQUENCES OF *PHYTOPTUS TETRATRICHUS* NALEPA (ACARI: ERIOPHYOIDEA) FEEDING ON THE CONTENT AND TISSUE DISTRIBUTION OF POLYPHENOLIC COMPOUNDS IN LEAVES OF DIFFERENT LINDEN TAXA

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(Received 05 January 2011; accepted 24 March 2011; published online 30 June 2011)

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ABSTRACT — Here we report a comparative study on the content and tissue distribution of polyphenols in leaves of small-leaved linden (*Tilia cordata* Mill.) and silver linden (*Tilia tomentosa* Moench) differentially galled by *Phytoptus tetratrichus* Nalepa (Acari: Eriophyidae). Data showed that at the beginning of the season (early July): (1) the native level of leaf flavonoids (flavonols, anthocyanins, tannins) varied significantly being higher in *T. cordata* than in *T. tomentosa*, (2) eriophyoid mite abundance was markedly lower on leaves of *T. cordata* with 4-fold higher levels of anthocyanins and 6-fold higher levels of tannins than on leaves of *T. tomentosa*, (3) in relation to linden taxa and mite/gall density, the content of flavonoids altered differently: in mite-galled leaves of *T. cordata* both flavonols and tannins accumulated markedly (up to 1% of leaf dry weight), whereas in galled leaves of *T. tomentosa* the increase of tannins and flavonols was much weaker (up to 0.6% of leaf dry weight), (4) due to *P. tetratrichus* feeding, the content of anthocyanins in *T. cordata* leaves decreased, while in *T. tomentosa* it did not change or it increased, depending on gall density. Results obtained suggest that at the beginning of the season *T. cordata* is a less suitable host for *P. tetratrichus* than *T. tomentosa* due to the relatively high amount of native leaf anthocyanins and tannins. Since the most responsive class of leaf flavonoids to *P. tetratrichus* feeding were tannins and flavonols, they are proposed as putative factors negatively affecting the performance of *P. tetratrichus*.

KEYWORDS — gall inducing-eriophyoid mite; leaf galls; *Tilia cordata*; *Tilia tomentosa*; flavonols; anthocyanins; tannins

INTRODUCTION

The Tiliaceae plants grown in natural habitats, city greenery, botanical gardens and nurseries are inhabited by a diverse group of eriophyoid mites (Acari: Eriophyoidea) (Skrzypczynska 1999, Sychała *et al.* 2005, Soika 2006, Soika 2007, Castagnoli *et al.* 2010). Amongst them, gall-inducing species

such as *Eriophyes tiliae* (Pagenstecher 1857), *E. exilis* (Nalepa 1891), *E. nervalis* (Nalepa 1892), *E. leiosoma* (Nalepa 1892), and *Phytoptus tetratrichus* (Nalepa 1891) are the most frequently recorded.

Phytoptus tetratrichus is one of the species that feed on a narrow range of linden taxa. Previous research has revealed that in response to *P. tetratrichus* feeding on leaves of various linden taxa morpho-

logically distinct galls are induced. Leaf-roll galls (e.g. edgerollings) appear along the leaf edges on both small-leaved linden (*Tilia cordata*) and broad-leaved linden (*T. platyphyllos*), whereas warty galls and erineae appear on the upper side and underside of silver linden (*T. tomentosa*) leaves, respectively (Boczek 1961, Soika and Kielkiewicz 2004, Soika 2006). Examination of *P. tetratrichus* protogyne and deutogyne females, collected from the two linden gall types in three consecutive seasons, revealed similarities in morphological characteristics such as the sculpture of the prodorsal shield, the epigynium (genitalia), the number of empodium rays, etc., which play a key role in eriophyoid taxonomy on the level of species (Soika and Kozak *in press*).

We have also previously shown that morphologically distinct galls created by *P. tetratrichus* on leaves of *T. cordata* and *T. tomentosa* are similar in their internal arrangement (Soika and Kielkiewicz 2004). In both cases, gall-mite feeding stimulates growth and induces tissue differentiation resulting in the formation of nutritive tissue layers essential for gall-mite performance. It is worth noting that within each gall type, the numerous cells of the gall are filled with phenolic compounds. The fact that intermediates of phenylpropanoid/flavonoid biosynthetic pathways are implicated in plant-insect interactions (Fenny 1976, Elliger *et al.* 1980, Zucker 1982, Hagerman and Robbins 1987, Rosenthal and Berenbaum 1992, Harborne and Grayer 1994, Ayres *et al.* 1997, Harborne 2000, Harborne and Williams 2000, Ossipov *et al.* 2001, Simmonds 2001, 2003 and refs therein, Schoonhoven *et al.* 2005) suggests that they can also be important in the relationships between host-plant and eriophyoid mites. There is some evidence confirming such a hypothesis (Balasubramanian and Purushothaman 1972 b, Ishaaya and Sternlicht 1969, Larew 1982, Kane *et al.* 1997, Kielkiewicz *et al.* 1997, Shi and Tomczyk 2001, Tomczyk and Boczek 2006, Petanović and Kielkiewicz 2010). Very little is known about the polyphenolic profile within eriophyoid-galled linden leaves, except for the fact that mite feeding may enhance the accumulation of these compounds (Thomsen 1975, 1988, Soika and Kielkiewicz 2004,

Petanović and Kielkiewicz 2010).

Flavonoids are known as important modulators of plant palatability/susceptibility to insect and insect feeding behaviour (Rosenthal and Berenbaum 1992, Bernays and Chapman 1994, Harborne and Grayer 1994, Harborne and Williams 2000, Simmonds 2001, 2003 and refs therein, Schoonhoven *et al.* 2005). There is evidence for the occurrence of a substantial amount of phenylpropanoid/flavonoid biosynthetic pathway metabolites, mainly flavonols (quercetin, quercitrin, isoquercitrin, rutin, kaempferol, myricetin) and phenolic acids (caffeic, *p*-coumaric, chlorogenic) in linden organs (Weglarz *et al.* 2000, Toker *et al.* 2001, Behrens *et al.* 2003, Sroka and Belz 2009). Therefore, we can hypothesize that the occurrence of flavonoid classes such as flavonols, anthocyanins and tannins in leaves of linden plants can contribute to leaf acceptance by *P. tetratrichus* and mediate in linden leaf response during feeding of this eriophyoid.

The objective of the present study was to assess the effect of native i.e. initial) content of 3 classes of flavonoids (i.e. flavonols, anthocyanins, tannins) on the density of eriophyoid mite on leaves of *T. cordata* and *T. tomentosa* and to evaluate the effect of mite feeding on the content of flavonoids in differentially galled leaves of the two linden taxa. Additionally, histochemical analysis of the galled leaf area made it possible to visualise the secondary compounds localisation.

MATERIALS AND METHODS

Small-leaved linden (*T. cordata*) and silver linden (*T. tomentosa*) trees (40 – 50 years) grew at The Botanical Garden (52°13'3.252"N, 21°1'41.34"E) in Warsaw. Trees grew in small groups and both taxa were equally available to *P. tetratrichus*. On July 6th 2006, approximately 4 weeks after the appearance of *P. tetratrichus* (after winter diapause), non-infested (ungalled) and mite-infested (galled) leaves were sampled from *T. cordata* and *T. tomentosa* trees. Collected leaves were comparable in position on the shoots and in the crown. Leaves were immediately brought to the laboratory and divided into 4 groups: 1st — to check the presence in the

density of *P. tetratrichus* in the galls, 2nd — to estimate the galled leaf area, 3rd — to localise phenolics histochemically and 4th — to assess the level of leaf flavonols, anthocyanins and tannins.

Assessment of leaf gall and eriophyoid mite density

To check the number of *P. tetratrichus* individuals within each gall type, the galls were cut from leaves of *T. cordata* and *T. tomentosa* (30 galls from leaves of each species) and examined under stereomicroscope (Olympus SZX9, Japan). Additionally, mite specimens were mounted separately on glass slides using Heinze's medium (Amrine and Manson 1996) and categorized to the species with a phase-contrast microscope (OLYMPUS CX40, Japan). To assess the density of galls on leaf of *T. cordata* and *T. tomentosa*, galls were counted on 25 galled leaves of each taxa.

Galled leaf area estimation and leaf histochemistry analysis

To estimate the magnitude of leaf damage caused by *P. tetratrichus* feeding, 100 galled leaves of *T. cordata* and *T. tomentosa* were measured using the Win DIA3 Image Analysis System (Burwell, Cambridge, England). This system separately analysed the healthy (deep green) and mite-galled (light green or yellowish) leaf areas on the basis of colour difference (Figure 1a–d) and also measured total leaf area. Since mite-infested leaves of *T. cordata* and *T. tomentosa* differed in the number of galls, 3 groups were selected within leaves of each taxa. In the case of *T. cordata*, the first group, called 'the low' counted up to 5 galls/leaf, the second group ('the medium') counted 6 – 10 galls/leaf and the third ('the high') counted 11 – 20 galls/leaf. In the case of *T. tomentosa* there were also 'low', 'medium' and 'high' groups but with a differing gall amount: 1 – 10, 11 – 20, and 21 – 40 galls/leaf, respectively.

To localise hydroxyl groups of phenolics within galls of different development stage, galled-leaf samples (3 × 5 mm) were cut and fixed in CrAF mixture (chromium trioxide, acetic acid, formaldehyde) and embedded in paraffin. Leaf sections were stained with safranin and Fast Green before examination with a light microscope (Olympus BH-2,

Japan) as it was described previously (Soika and Kielkiewicz 2004).

Assessment of leaf flavonols, anthocyanins and tannins

The total content of flavonols, anthocyanins and tannins in linden leaves of each taxa was compared in leaves of 3 treatments: control (intact e.g. ungalled), weakly galled (with a relatively low number of galls, Figure 1a–b), and densely galled (Figure 1c–d). Each treatment consisted of 3 independent (biological) replications. Each replication consisted of 20 – 25 leaves each weighing 1g of fresh weight. All leaf samples were frozen in liquid N and stored frozen until analysed. Simultaneously, the per cent of leaf dry weight (DW) was defined by weighing the leaf samples before and after drying for 3 days at 105°C.

The total flavonol content was determined according to Christ-Müller's method (Polish Pharmacopoeia 2002). Briefly, flavonols were extracted from leaf samples with a mixture of acetone, 25% HCl and 0.5% hexamethylenetetramine (*syn.* methenaminum) (20:2:1; v/v). Absorbance of clear extracts was read at 760 nm (Shimadzu UV-1601 PC, Japan) and the level of flavonols was expressed as quercetin equivalent using a coefficient of $k = 0.875$.

The total level of anthocyanins was measured according to the method of Fuleki and Francis (1968) modified by Elbanowska and Napierala (1989). Leaf samples were extracted with a solution of 1% HCl in metanol (CH₃OH) for 24 h. Differences in the absorbance at 510 nm (Shimadzu UV-1601 PC, Japan) between two buffered leaf extracts showing different colour due to their different acidity (pH = 1.0 and pH = 4.5) was read to assess the level of anthocyanins.

To quantify tannins in plant material is a rather difficult analytical procedure mainly due to structural complexity of tannins (Hagerman and Butler 1994, Reed 1995, Schofield *et al.* 2001, Heil *et al.* 2002, Makkar 2003, Zalacain *et al.* 2003). Since the content of *ortho*-phenolic groups affects the biological activity of tannins (Haslam 1974), the Folin-Ciocalteu colorimetric type of tannin assay has been chosen to be used here. This is a suitable

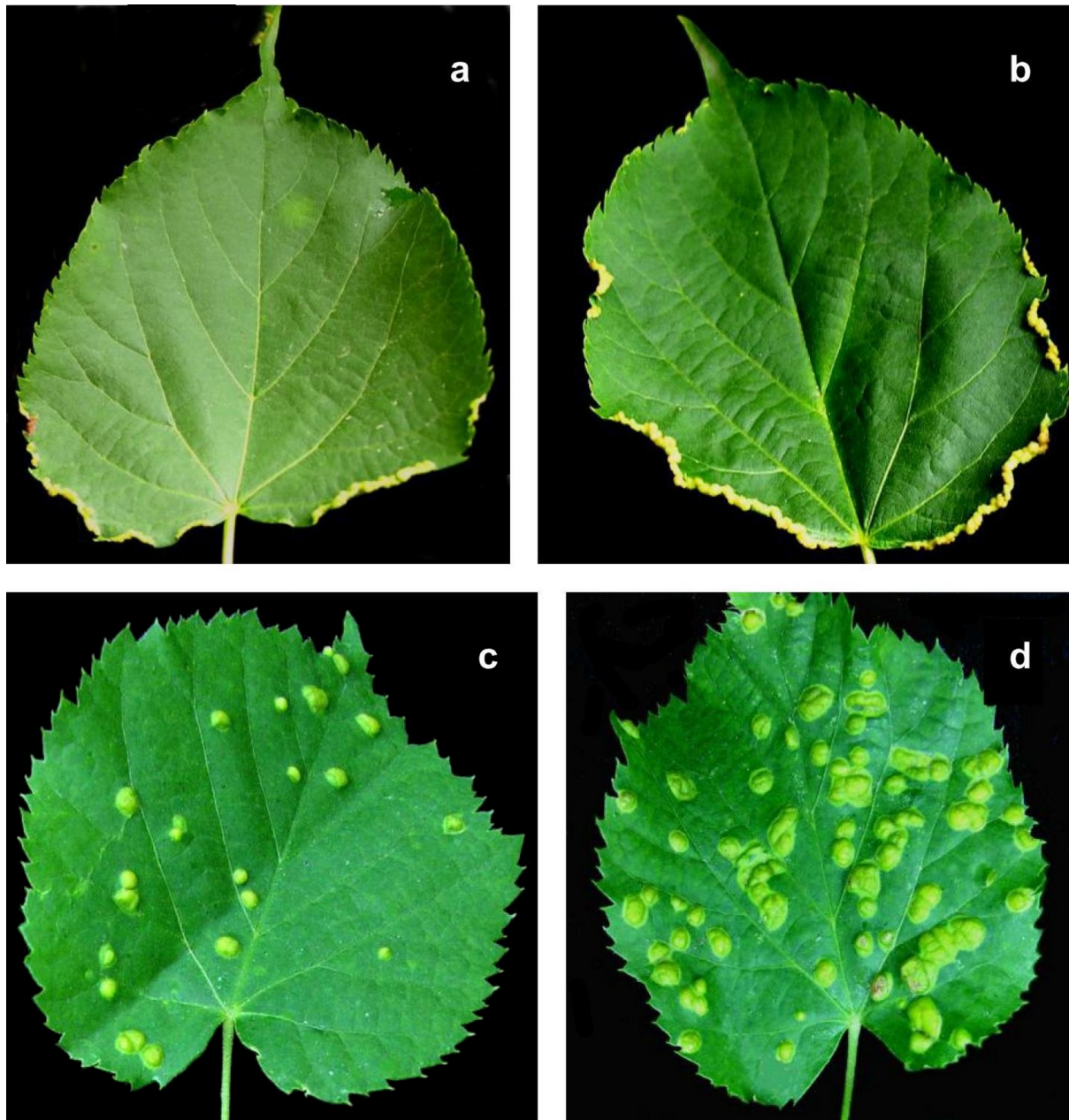


FIGURE 1: The effect of *P. tetratrichus* feeding on linden leaf morphology: edgerollings on *T. cordata* leaves at a low (a) and high (b) density and erineae on *T. tomentosa* leaves at a low (c) and high (d) density visible from the upper side of the leaf blade.

method of estimating the *ortho*-/di/tri-phenolic hydroxyl content of the tannin (Haslam 1974, Reed 1995, Schofield *et al.* 2001). To extract tannins, linden leaf samples were carefully heated with distilled water free of CO₂ for 30 min. in the dark (Polish Pharmacopoeia 2002). The content of released tannins in clear extracts was measured be-

fore and after binding with hide powder using the Folin-Ciocalteu reagent for determination of the total amount of free phenolic hydroxyl groups. For estimation of tannins in leaf samples, pyrogallol (a commercial form of polyphenols) was used as a standard. Absorbance of reaction mixtures was read at 760 nm (Shimadzu UV-1601 PC, Japan). Due

to this analytical procedure the results are not an absolute value, but are pyrogallol equivalents of water soluble tannins.

The total content of flavonols, anthocyanins and tannins was given as a percent of leaf DW.

Statistics

All results presented as % of DW were arcsine square-root transformed prior to the statistical analysis. Means SD of untransformed data are shown in Figure 3 a–c. One-way analysis of variance (ANOVA) was performed on the number of galls and eriophyoid mites on *T. cordata* and *T. tomentosa* leaves. The significance of differences between means was tested by Newman-Keuls test at $P=0.05$. One way analysis of variance (ANOVA) was also applied to examine the differences between galled leaf area on leaves of *T. cordata* and *T. tomentosa*. Comparison of means was made using the Duncan test. Significance was assumed at $P=0.05$.

The differences in the constitutive level of leaf flavonoids, anthocyanins and tannins between linden species were analysed using Student's t-test at $P=0.05$. Finally, two-way analysis of variance (ANOVA) was carried out to determine the effect of linden taxa and gall density on flavonoid, anthocyanin and tannin level. The same dependent variables among treatments (ungalled leaves, low-galled and high-galled leaves) within each species were subjected to one-way ANOVA followed by Tukey's honestly significant difference (HSD) or Kruskal-Wallis tests at $P=0.05$. All data were analysed using Statgraphic Plus Version 4.1.

RESULTS

Leaf gall and eriophyoid mite density

Data comparing the number of galls and *P. tetratrichus* on leaves of *T. cordata* and *T. tomentosa* are shown in Table 1. The differences between the number of galls formed on leaves of *T. cordata* and *T. tomentosa* were statistically significant, however the number of eriophyoids per gall on leaves of *T. cordata* and *T. tomentosa* was similar. Both, the number of galls and mites on *T. cordata* leaves was more than 3-fold lower than on *T. tomentosa* leaves.

Galled leaf area and its histochemistry

At the beginning of July, leaves of *T. cordata* and *T. tomentosa* inhabited by *P. tetratrichus* differed in the type (roll-gall *vs* erineum) and size of leaf damage (Figure 1a–d, Table 2). To quantify the differences in the magnitude of leaf damage, 3 groups of leaves varying in galled leaf area were distinguished (Table 2). In all groups, the galled area of *T. cordata* leaves was 3-fold smaller than the galled area of *T. tomentosa* leaves. This indicates a stronger reduction of photosynthetic active leaf area of galled leaves of *T. tomentosa* than of galled leaves of *T. cordata*.

Histochemical analysis shows that the local thickening (edgerollings and erineum) of the linden leaf blades caused by *P. tetratrichus* feeding resulted from the transformation of mesophyll tissue into new tissue types: hypertrophied parenchyma and nutritive tissues, both consisting of numerous layers (Figure 2a–f). Transverse sections through roll-galls (Figure 2a–d) and erineum (Figure 2e–f) confirm previous data (Soika and Kielkiewicz 2004) showing that within cells of the hypertrophied parenchyma, besides enlarged nuclei, starch grains are clearly visible. Within each fully developed gall type, many cells of the outer layer of nutritive tissues stained deep-red with safranin (Figure 2a–f), which shows the localisation of polyphenolics (Stafford 1988). These secondary compounds with hydroxyl groups (red in colour) were located not only within gall cells but also within the eriophyoid body (Figure 2d).

Leaf flavonols, anthocyanins and tannins

In comparison with the control (ungalled) leaves of *T. tomentosa*, in control leaves of *T. cordata* the total level of flavonols, anthocyanins and tannins was significantly higher: 4% ($t=3.9210$; $P=0.0172$), 4-fold ($t=49.9585$; $P=0.0001$) and 6-fold ($t=35.7992$; $P=0.0001$), respectively (Figure 3 a–c). Analysis of the data using 2-way ANOVA (Table 3) shows a statistically significant effect of treatments (linden taxa and eriophyoid mite) on the content of leaf flavonols, anthocyanins and tannins. In relation to the control (ungalled, intact leaves), the presence of single roll-galls (e.g. a low mite density) on leaves of *T. cordata* resulted in a slight decrease (by 8.5%)

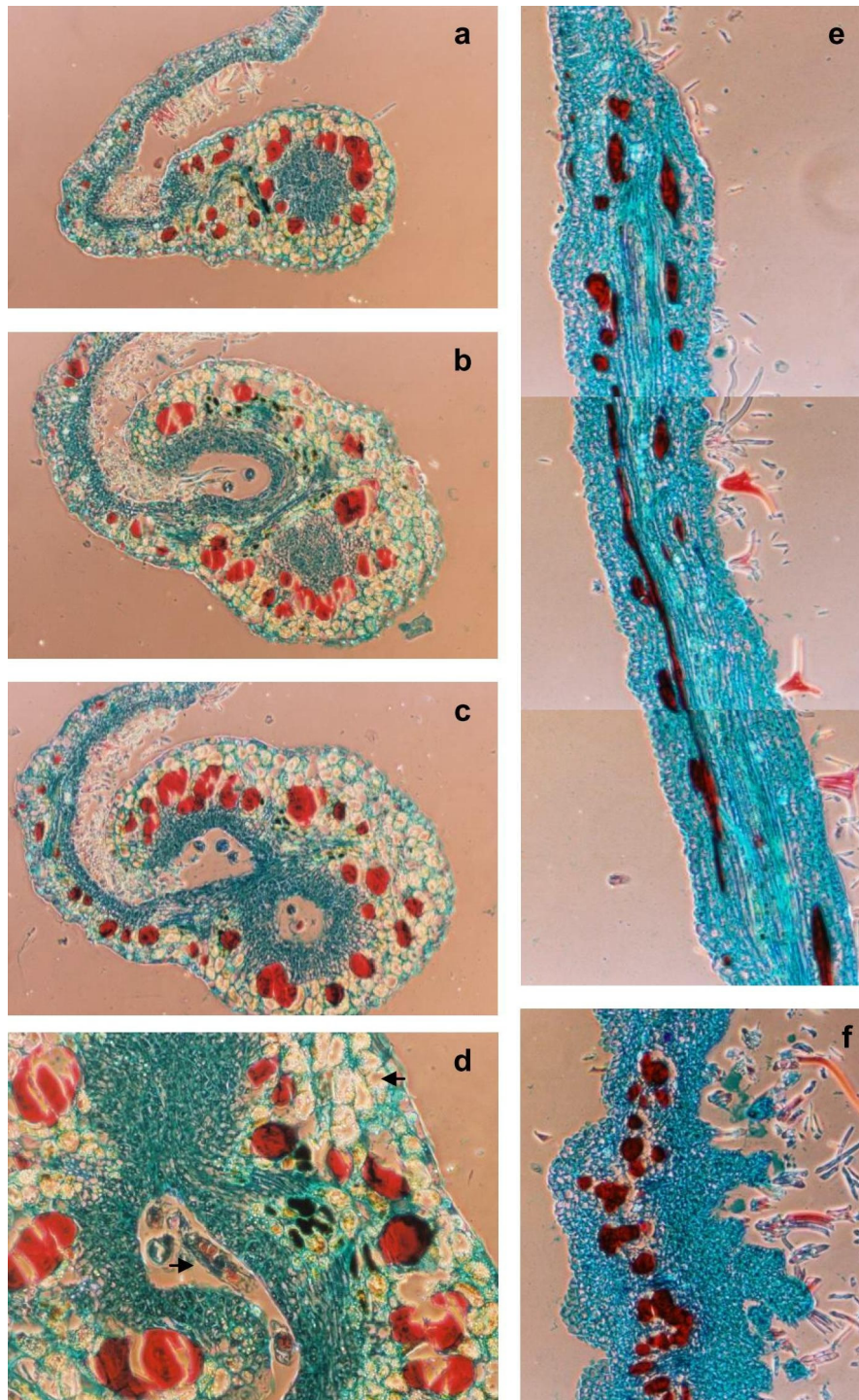


FIGURE 2: The cross-sections through the edgerolling on *T. cordata* (a – d) and erineum on *T. tomenosa* (e-f) leaves at the early phase of development (a-c) and at the more expanded phase (d – f). Phenolics (deep-red in colour) are localised in the cells of the outer layer of the nutritive tissue of both gall types. Starch grains (arrow) dominate within the cells of hypertrophied parenchyma (d). The *P. tetratrichus* specimens are localised within distinguishable cavities of roll-gall (b, c, d). The presence of red coloured deposits within eriophyoid bodies (d, arrow) suggests that phenolics can be sequestered. Magnification: 90x (a, b, c, e, f); 180x (d).

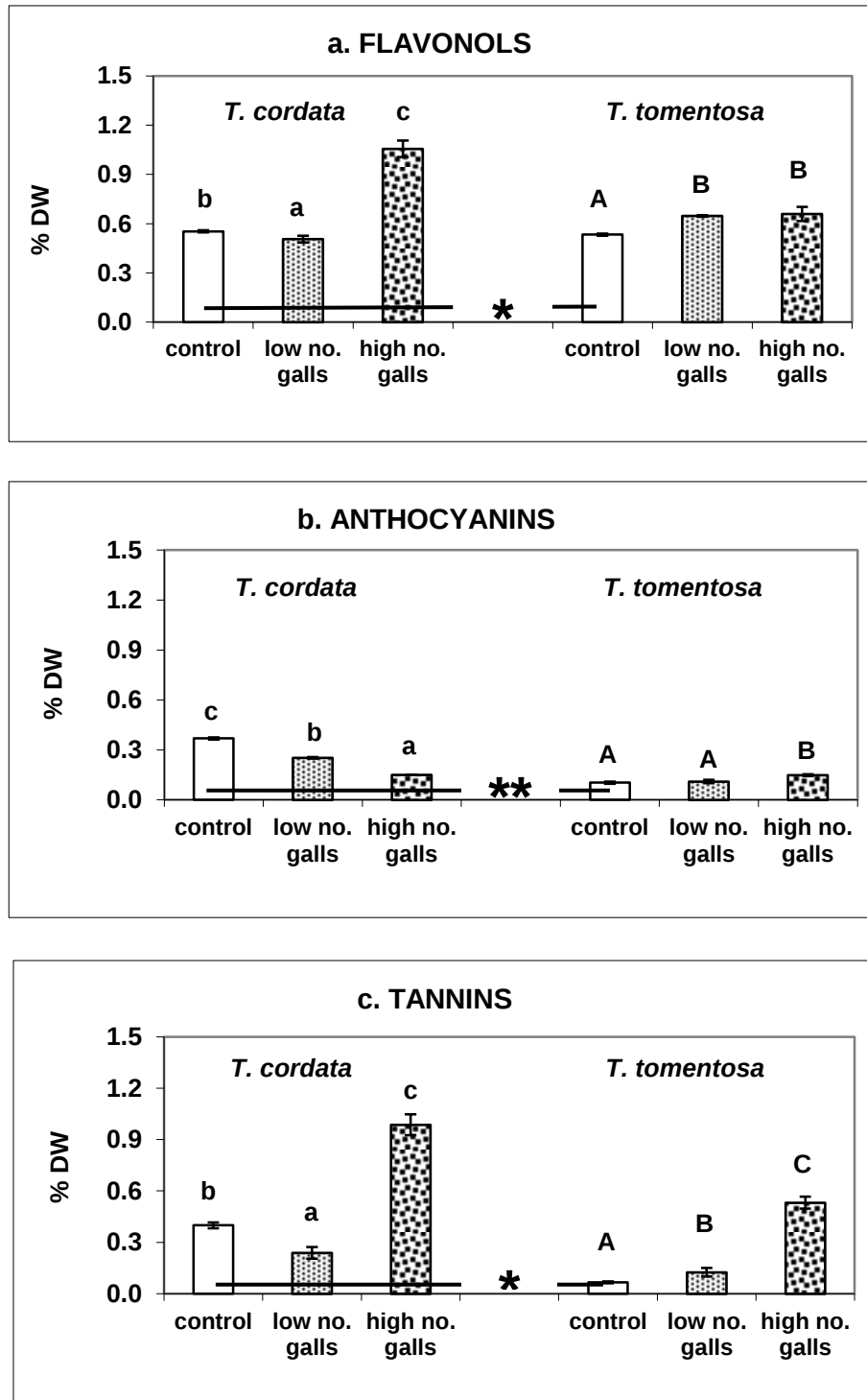


FIGURE 3: The effect of varied gall density (low vs high) on leaves of *T. cordata* and *T. tomentosa* infested by *P. tetratrichus* on the total level of flavonols (a), anthocyanins (b) and tannins (c). Different letters (lower case for *T. cordata* and capital for *T. tomentosa*) above the bars indicate statistically significant differences among treatments (Kruskal-Wallis non-parametric test or Tukey's HSD test, $P=0.05$). The asterisks indicate statistically significant differences among the control treatments for *T. cordata* and *T. tomentosa* (Student's t-test; * – $P<0.05$; ** – $P<0.01$). Means SD of untransformed data are shown.

of flavonol level (Figure 3a). Only when a higher number of roll-galls was presented on the leaves, the flavonols increased 2-fold (Figure 3a). In contrast, in *T. tomentosa* leaves regardless of erineae density (low or high), the flavonol content went up similarly (by 21% and 23%, respectively) compared to the control (Figure 3a).

Relative to the control, the occurrence of both low and high roll-gall densities on *T. cordata* leaves resulted in a decline of anthocyanins by 31 and 60%, respectively (Figure 3b). However, the level of anthocyanins in *T. tomentosa* leaves with a low number of erineae did not change (Figure 3b). Only in leaves with a high number of erineae, the anthocyanins grew by 49% (Figure 3b).

Comparing with the control, the presence of a low number of roll-galls on *T. cordata* leaves resulted in a decrease of tannins by 40%, whereas in leaves with a high number of galls there was a 2.5-fold increase reaching almost 1% of DW (Figure 3c). A low number of erineae on *T. tomentosa* leaves contributed to an increase of tannins by 88%, while the concentration of tannins markedly increased (8-fold) in leaves with a high density of erineae, but never ex-

ceeded 0.6% of DW (Figure 3c).

DISCUSSION

The present study demonstrates that at the beginning of the season (early July), *P. tetratrichus* density was lower on leaves of *T. cordata* than on leaves of *T. tomentosa*. Our previous results have shown that in June and July the density of *P. tetratrichus* on *T. cordata* was about 4-fold lower than on *T. tomentosa* (Soika and Kielkiewicz 2004). In Polish nurseries, in July 2005 leaves of *T. cordata* were least infested by mites in comparison with leaves of *T. platyphyllos* and *T. caucasica* (Soika 2006). However, the most numerous gall former on leaves of *T. cordata* growing in a natural habitat (the Ojców National Park, Poland) was *P. tetratrichus* in 1998, although a year before it was *E. leiosoma* (Skrzypczyńska 1999). Currently, Buchta *et al.* (2006) documented the occurrence of *E. tiliae* on linden trees grown in the same habitat in the urban area of Brno (Czech Republic) and found a lower density of this mite species on leaves of *T. tomentosa* than on leaves of *T. cordata*. The abundance of gall-forming mites and insects is usually affected by a number of physical and chem-

TABLE 1: The mean number of galls per leaf caused by *P. tetratrichus*, mean number of eriophyoid mites within gall and mean number of eriophyoid mites per leaf of *T. cordata* and *T. tomentosa*. Asterisks indicate statistically significant differences between two means by Newman - Keuls test at $P=0.05$.

	<i>T. cordata</i>	<i>T. tomentosa</i>	LSD	Statistics
Number of galls x leaf ⁻¹	2.5	8.3 ***	0.925	$F_{1,4}=56.03$; $P<0.001$
Number of <i>P. tetratrichus</i> x gall ⁻¹	31.2	32.7	2.139	$F_{1,8}=0.02$; $P=0.89$
Number of <i>P. tetratrichus</i> x leaf ⁻¹	78	271.4		

TABLE 2: The comparison of galled area on *T. cordata* and *T. tomentosa* leaves caused by *P. tetratrichus* feeding. Means followed by different letters within the same column are significantly different (Duncan test; $P=0.05$).

Leaf classification (number of galls x leaf ⁻¹)	<i>T. cordata</i>			<i>T. tomentosa</i>		
	Total leaf area (cm ²)	Galled leaf area (cm ²)	% of galled area	Total leaf area (cm ²)	Galled leaf area (cm ²)	% of galled area
Low	29.2 a	0.7 a	2.4	28.8 ab	2.0 a	6.9
Medium	27.1 a	1.6 b	5.9	34.8 b	5.0 b	14.4
High	36.4 a	3.8 c	10.4	22.9 a	8.0 c	34.9

ical traits of the host plant, which are substantial for their performance (Mound 1994, Westphal *et al.* 1996 and refs. therein, Hartley 1998). Since in our study, the trees of both linden taxa were of a similar age and grown in the same habitat and sampled leaves were comparable in position on the shoots and in the crown, it is presumed that variations in the eriophyoid and gall density are the result of host leaf quality.

The fitness of insect-herbivores is usually affected by the interactions between leaf dietary compounds (carbohydrates, proteins, lipids, vitamins etc.) and allelochemicals (Slansky 1992, Hartley 1998). Few studies point out that the ratio of the phenolic to nutritive leaf constituents is an indicator of susceptibility of the host-plant to the eriophyoid mite (Kozłowski 1998, Shi and Tomczyk 2001, Tomczyk and Boczek 2006). The results of the current study reveal a higher initial concentration of flavonoids (flavonols, anthocyanins, tannins) in *T. cordata* than in *T. tomentosa* leaves. This together with our previous study (Soika, personal communication) on higher content of soluble carbohydrates in leaves of *T. cordata* than in leaves of *T. tomentosa* indicates that the secondary compounds could

cause the lower suitability of *T. cordata* leaves for *P. tetratrichus* and result in a lower density of mites and galls produced during the period of early July. Indeed, due to *P. tetratrichus* feeding, fewer galls were formed on leaves of *T. cordata* than on *T. tomentosa*. In both cases (erinea and roll-galls), the occurrence of a high number of starch grains within both nutritive and hypertrophied parenchyma cells suggests that newly formed tissue of galls is metabolically active. This is in accordance with previously reported data on the accumulation of starch and proteins in tissues of galls formed by *E. tiliae* on *T. platyphyllos* Scop. (Thomsen 1975) and on the accumulation of ^{14}C metabolites in galls formed by *E. tiliae* on *T. cordata* (Boczek 1974) referred in Petanović and Kielkiewicz (2010).

Tannins constitute the major class of flavonoid derivatives. Our results show that densely galled leaves of *T. cordata* have a relatively strong capacity to accumulate flavonols and tannins (up to 1% of DW), but not anthocyanins, which can be explained by the fact that the pathway for tannins and anthocyanins is shared. It is also supposed that a relatively high native amount of secondary metabolites in *T. cordata* leaves resulted in their increase in mite-

TABLE 3: The effect of linden taxa (A), gall density (B) and A x B interaction on the total level of leaf flavonols, anthocyanins and tannins.

Source	SS	Df	MS	F-Ratio	P-Value
Main effects					
FLAVONOLS					
A	380.98	1	380.98	194.73	<0.001***
B	2157.63	2	1078.81	551.41	<0.001***
A x B interaction	1526.27	2	763.14	390.06	<0.001***
ANTHOCYANINS					
A	439.06	1	439.06	1653.30	<0.001***
B	87.26	2	42.63	164.29	<0.001***
A x B interaction	259.84	2	129.92	489.22	<0.001***
TANNINS					
A	2345.12	1	2345.12	46.09	<0.001***
B	5986.62	2	2993.31	58.83	<0.001***
A x B interaction	555.76	2	277.88	21.29	<0.001***

infested leaf tissue. In contrast, a relatively lower native level of flavonols and tannins in leaves of *T. tomentosa* resulted in a lower rate of increase of these compounds (up to 0.6% DW) in leaves covered evenly by a high number of erinea. Therefore, it is believed that a relatively high level of tannins and flavonoids in *T. cordata* leaves is a factor contributing to a relatively low potential of *P. tetratrichus*. Similarly, the various rate and level of tannins is proposed as a factor differing response of two European alder species — *Alnus glutinosa* and *A. incana* to *E. laevis* (Kane *et al.* 1997). Although, on leaves of both hosts, adaxial pouch galls were structurally similar, the earlier formation of tanniferous cells in immature galls of *A. incana* delayed the development of *A. incana* galls as compared with the development of *A. glutinosa* galls. This is in agreement with data previously presented by Larew (1982) showing that nutritive tissues of alder galls caused by *E. laevis* accumulate more tannins as the galls mature. These and the present results are good examples of the variable potential of the galled leaf tissues. The occurrence of tannins in host plant organs attacked by other gall-feeders (Larew 1982, Cornell 1983, Taper *et al.* 1986, Taper and Case 1987, Mound 1994, Hartley 1998, 1999, Nyman and Julkunen-Titto 2000, Abrahamson *et al.* 2003, Adams *et al.* 2009) supports our suggestion on the role of these class of flavonoids in the interaction between linden trees and *P. tetratrichus*.

In another study, (Kielkiewicz, personal observation) the increase of tannins was found only in the galls cut out from the linden leaf blade infested by *P. tetratrichus*. Furthermore, the content of flavonols and anthocyanins was much lower in the galls than in the ungalled leaf blade adjacent to the galls. This evidence confirms the local accumulation of tannins within galls and simultaneously suggests that impact of *P. tetratrichus* activity on tannin accumulation goes beyond the galled leaf tissue. Differences in the metabolic capacity of *T. cordata* galls and the leaf blade due to *E. tiliae* feeding over time was previously reported by Czeżuga (1975) and Boczek (1974) referred in Petanović and Kielkiewicz (2010). These and our results are consistent with Hartley's (1998) data that indicates the markedly differing

chemical composition of galled and ungalled surrounding plant tissue, which is caused by insect gall-formers. In this case, the galled tissue contained a higher level of phenolic compounds than the ungalled plant tissue. Furthermore, authors are of the opinion that "gall-formers may produce species-specific and temporally variable changes in the chemical composition of the gall".

The biological function of tannin-rich cells within galls formed by *P. tetratrichus* merits further investigation. However, it is widely accepted that tannins and other phenolic compounds especially located in the outer layer of the galls, protect the gall-formers from external enemies (Cornell 1983, Taper *et al.* 1986, Taper and Case 1987). The only local deposition of tannins within the linden leaf galls suggests that the manipulation of linden host leaf tissues by *P. tetratrichus* may also be self-beneficial.

On the other hand, leaf flavonoid-derived metabolites including tannins are usually considered as anti-herbivore defensive secondary compounds. They have the potential to form complexes with proteins and/or inactivate digestive enzymes and they often act as a pest deterrents or inhibitors (Haslam 1974, Feeny 1976, Elliger *et al.* 1980, Hagerman and Robbins 1987, Appel 1993, Ayres *et al.* 1997, Harborne 2000, Harborne and Williams 2000, Ossipov *et al.* 2001, Simmonds 2001, 2003 and refs therein).

Plant polyphenols have also been reported as antioxidants and/or reducing agents in the digestive tract of some herbivores (Johnson and Felton 2001, Barbehenn *et al.* 2003, Johnson 2005) as well as in plants under biotic/abiotic stresses (Mittler 2002, Mittler *et al.* 2004, Hernandez *et al.* 2009). One of the multiple biological activities of condensed tannins and other *o*-dihydroxyphenolics accumulating within leaves galled by eriophyoids could be protection of auxin from oxidation (Ishaaya and Sternlicht 1969, Balasubramanian and Purshothaman 1972 a,b, Tandom and Arya 1980, Tandom 1985). In this study, the occurrence of phenolics within the body of *P. tetratrichus* suggests that eriophyoids consumed linden leaf polyphenolics. Plant phenolics such as quercetin, catechin or tannic acid show antioxidant activity in the digestive tract fluid and

hemolymph of *Manduca sexta* caterpillars (Johnson 2005). However, it remains open whether compounds derived from linden leaf allelochemicals are metabolized, detoxified and/or utilized by the mite. On the other hand, the phenomenon of sequestration of metabolized/unmetabolized plant secondary compounds is known as common among specialist feeders (Brattstein 1992). Furthermore, Harborne and Grayer (1994) are of the opinion that insect fitness can be improved if the insect is able to sequester flavonoids in its body. Further studies are needed to verify if the sequestration mechanism functions in *P. tetratrichus*.

In the future, a more detailed analysis will be required to further understand the biological activity of flavonoid intermediates or derivatives in *P. tetratrichus* – linden plant interaction.

CONCLUSIONS

We reported here that linden taxa differ in the native level of leaf flavonols, anthocyanins and tannins, and the responsiveness of these 3 classes of flavonoids to *P. tetratrichus* feeding varies in relation to linden taxa and mite/gall density. Generally, at the beginning of the season *T. cordata* is a less suitable host for *P. tetratrichus* than *T. tomentosa* due to the relative richness of anthocyanins, tannins and flavonoids in the leaves. *P. tetratrichus* induces quantitative changes in the content of all leaf flavonoid derivatives analysed here. These changes are conditioned by the density of galls on the linden leaf taxa. It is suggested that only the strong increase of tannins and flavonols in galled leaves of *T. cordata* may decrease palatability/suitability of leaves, which is decisive for the population development of *P. tetratrichus*.

In the future, newly developed analyses need to be performed to establish if and how predominant linden leaf flavonoids influence behaviour and development of *P. tetratrichus* and shape the host linden's response to this eriophyoid species over time. This would be essentially important in the clarification of the biological role of leaf flavonoids (mainly tannins) in the interaction of the *Tilia* host-plant – *P. tetratrichus*.

ACKNOWLEDGMENTS

This work was partially supported by grant No. 0709/p06/2003/52 from the Polish Committee for Scientific Research.

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