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Leaf variegation is associated with reduced herbivore damage in *Hydrophyllum virginianum*

Brandon E. Campitelli, Ivana Stehlik, and John R. Stinchcombe

Abstract: Leaf variegation refers to local regions of the upper surface of a leaf having reduced or obstructed chlorophyll, which results in whitish spots. These lighter spots may compromise the photosynthetic efficiency of a leaf, and many competing hypotheses have been put forward to explain why this patterning may be adaptive. It has been suggested that variegation is either an adaptive response to environmental conditions or a defence mechanism against herbivore damage. To test whether leaf variegation reduces herbivore damage, we first assessed the frequency of variegated and nonvariegated leaves in natural populations of the plant *Hydrophyllum virginianum* L., and second, measured herbivore damage to both variegated and nonvariegated leaves. We found that variegated leaves were present at high frequencies within natural populations (6%–31%) and that nonvariegated leaves sustained nearly twice the amount of damage by comparison with variegated leaves. Therefore, leaf variegation appears to be beneficial by reducing herbivore damage to leaves. These data are consistent with the fundamental prediction of the herbivory hypothesis for the benefits of leaf variegation.

Key words: herbivory, variegation, plant defence, optimal defense theory.

Résumé : La variégation foliaire se rapporte à des régions locales de la surface adaxiale d'une feuille montrant une réduction ou une obstruction de la chlorophylle, ce qui se traduit par des taches blanchâtres. Ces taches plus claires peuvent compromettre l'efficacité photosynthétique d'une feuille, et l'on a soulevé diverses hypothèses pour expliquer pourquoi ce patron pourrait être adaptatif. On a suggéré que la variégation constitue soit une réaction adaptative aux conditions environnementales ou soit un mécanisme de défense contre l'herbivorie. Afin de vérifier si la variégation réduit les dommages par herbivorie, les auteurs ont d'abord évalué la fréquence des feuilles avec ou sans variégation, dans des populations naturelles de l'*Hydrophyllum virginianum* L., et ont ensuite mesuré le dommage par herbivorie, chez les feuilles avec ou sans variégation. Ils ont constaté que les populations naturelles comportent une forte proportion de feuilles avec variégation (de 6 à 31%) et que les feuilles sans variégation subissent presque deux fois plus de dommages que les feuilles avec variégation. Conséquemment la variégation foliaire apparaît bénéfique en réduisant les dommages causés aux feuilles par herbivorie. Ces données concordent avec la prédiction fondamentale de l'hypothèse de l'herbivorie concernant les bénéfices de la variégation foliaire.

Mots-clés : herbivorie, variégation, défense des plantes, théorie de défense optimale.

[Traduit par la Rédaction]

Introduction

The diversity in leaf shape, size, colour, and morphology is typically explained as adaptation to abiotic conditions plants face while assimilating carbon and minimizing water loss (e.g., Givnish and Vermeij 1976; Givnish 1979). For example, the contrasting morphology of sun and shade leaves, with smaller, thicker sun leaves and larger, thinner shade leaves, is interpreted as a function of different light availabilities and risks of water loss in sunny and shady environments (Jurik 1986; Lambers et al. 1998). While such environmental influences are of clear importance in determining leaf morphology and physiology, several studies have suggested that herbivores may also play a selective role in determining leaf morphology and development (Rausher 1978; Gilbert 1979; Williams and Gilbert 1981; Smith 1986; Niemela and Tuomi 1987; Givnish 1990; Brown et al.1991).

The sessile nature of plants and the ubiquity of insect herbivores have lead to the evolution of several features to reduce herbivore damage (Schaefer and Rolshausen 2006). These features include secondary compounds such as gluco-

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sinolates (Velasco et al. 2007), cyanogenic glycosides (Alonso-Amelot and Oliveros-Bastidas 2005), alkaloids (McKey 1974; Adler 2000), and other secondary compounds (Cooper-Driver 1990), as well as physical defenses such as trichomes (Ågren and Schemske 1994; Mauricio and Rausher 1997), spines (Young et al. 2003), and thorns (Gomez and Zamora 2002). Another mechanism of plant defense that has received comparatively less attention is the reduction of herbivory through changes in morphology of leaves in response to visually searching herbivores (Rausher 1978; Gilbert 1979; Williams and Gilbert 1981; Smith 1986; Givnish 1990; Brown et al. 1991). For example, if herbivores form search images, this can lead to divergence in leaf shape among species (Rausher 1978), or if they avoid ovipositing on plants that appear to have eggs on them, this can lead to the evolution of egg mimics on leaves (Gilbert 1979; Williams and Gilbert 1981). These and other studies (Gilbert 1982; Niemela and Tuomi 1987; Mackay and Jones 1989; Rivero-Lynch et al. 1996; Campitelli et al. 2008) suggest that visual and shape variation in leaves can significantly reduce herbivory.

Here we examine the potential for leaf variegation to reduce herbivore damage in Virginia waterleaf, *Hydrophyllum virginianum* L. (a species that Givnish (1990) specifically suggested for this question). As an understory herb of deciduous forests in mid-western and eastern North America, *H. virginianum* possesses two basic leaf morphologies. Some leaves have whitish speckles, whereas others are uniformly green. This phenomenon is referred to as leaf variegation. Leaf variegation is the result of a decrease, deficiency, or obstruction of chlorophyll in a confined portion of the exterior leaf that faces upwards (Givnish 1990). The masking of chlorophyll suggests that leaf variegation may compromise photosynthetic efficiency compared with a nonvariegated leaf (Smith 1986; Givnish 1990; Sadof et al. 2003). This raises the question: if leaf variegation potentially reduces photosynthetic capacity, does it provide any benefits?

Leaf variegation may provide benefits if it reduces the amount of herbivory that a plant suffers — even if variegation evolved for unrelated reasons, or is a passive response to abiotic conditions — provided that herbivore damage reduces plant fitness. Givnish (1990) critically analyzed and discounted several potential environmental factors, such as temperature or adaptation to partial shade, which could lead to variegation, adding further support to the herbivory hypothesis. Several mechanisms have been posited for how variegation could reduce herbivory, including mimicking leaf damage (Smith 1986) or providing camouflage against dappled forest understory backgrounds early in the growing season, when bright conditions under open canopies in deciduous forests are likely to reduce or eliminate the negative effect of variegation on photosynthesis (Givnish 1990). Variegation could also be involved in signalling herbivores about levels of chemical defence (Hamilton and Brown 2001; but see Schaefer and Rolshausen 2007), or exposing insect herbivores to higher levels of predation by disrupting crypsis (Lev-Yadun et al. 2004). Despite differing emphases of these diverse hypotheses, they all converge on one fundamental prediction: leaf variegation should reduce herbivore damage.

Variation is present both within individual plants and populations of *H. virginianum*, and as such, variegated and nonvariegated leaves share the same habitat and herbivore population. These factors make it an ideal species to test for differences in herbivore damage among variegated and nonvariegated leaves (Givnish 1990), because such a comparison will not be confounded by differences in species-specific phylogenetic histories and by habitat-specific differences in microclimate, plant density, habitat preferences, or herbivore species composition (see e.g., Smith 1986 and Givnish 1990). We tested the fundamental prediction of all herbivory hypotheses of the benefits of leaf variegation: variegated leaves suffer less herbivory than nonvariegated leaves. Specifically, we sought to answer two questions. (*i*) What is the relative frequency of variegated and nonvariegated leaves in forest understory populations of *Hydrophyllum virginianum* in southern Ontario? (*ii*) What are the relative amounts of herbivory on variegated and nonvariegated leaves?

Materials and methods

Study species

Hydrophyllum virginianum (Hydrophyllaceae) is a small, clonal perennial, herbaceous understory plant commonly found in patches of different sizes (100–500 individuals). Patches were normally no larger than 4–5 m^2. *Hydrophyllum virginianum* is primarily found under full canopy cover within mature forests and occasionally in forest gaps or near forest edges. Leaf colouration pattern in *H. virginianum* is variable: most leaves are entirely green, while a small percentage of leaves are variegated (contain whitish spots; see Fig. 1). Although Morgan (1971) and Givnish (1990) noted that variegated leaves are “spring-active,” senesce prior to canopy closure, and are replaced by nonvariegated leaves, we found numerous plants with variegated leaves after canopy closure (see below). On larger plants, we occasionally found these two leaf types on the same individual, with variegated leaves always found closer to the forest floor (i.e., first leaves emerging from the plant). We observed many small coleopteran species and a number of species of unidentified lepidopteran larvae feeding on plants in the field (see supplementary data,[2] Fig. S1). We detected no evidence of vertebrate damage, and accordingly focus on insect herbivory.

Sampling details and measurements

We sampled populations of *H. virginianum* at the Koffler Scientific Reserve at Jokers Hill (King Township, Ont.). Joker’s Hill is a 350 ha field station operated by the University of Toronto, approximately 50 km north of Toronto, Ontario (44°03′N, 79°29′W, 300 m a.s.l.; www.zoo.utoronto.ca/jokershill/jh.html). We surveyed populations in the under-

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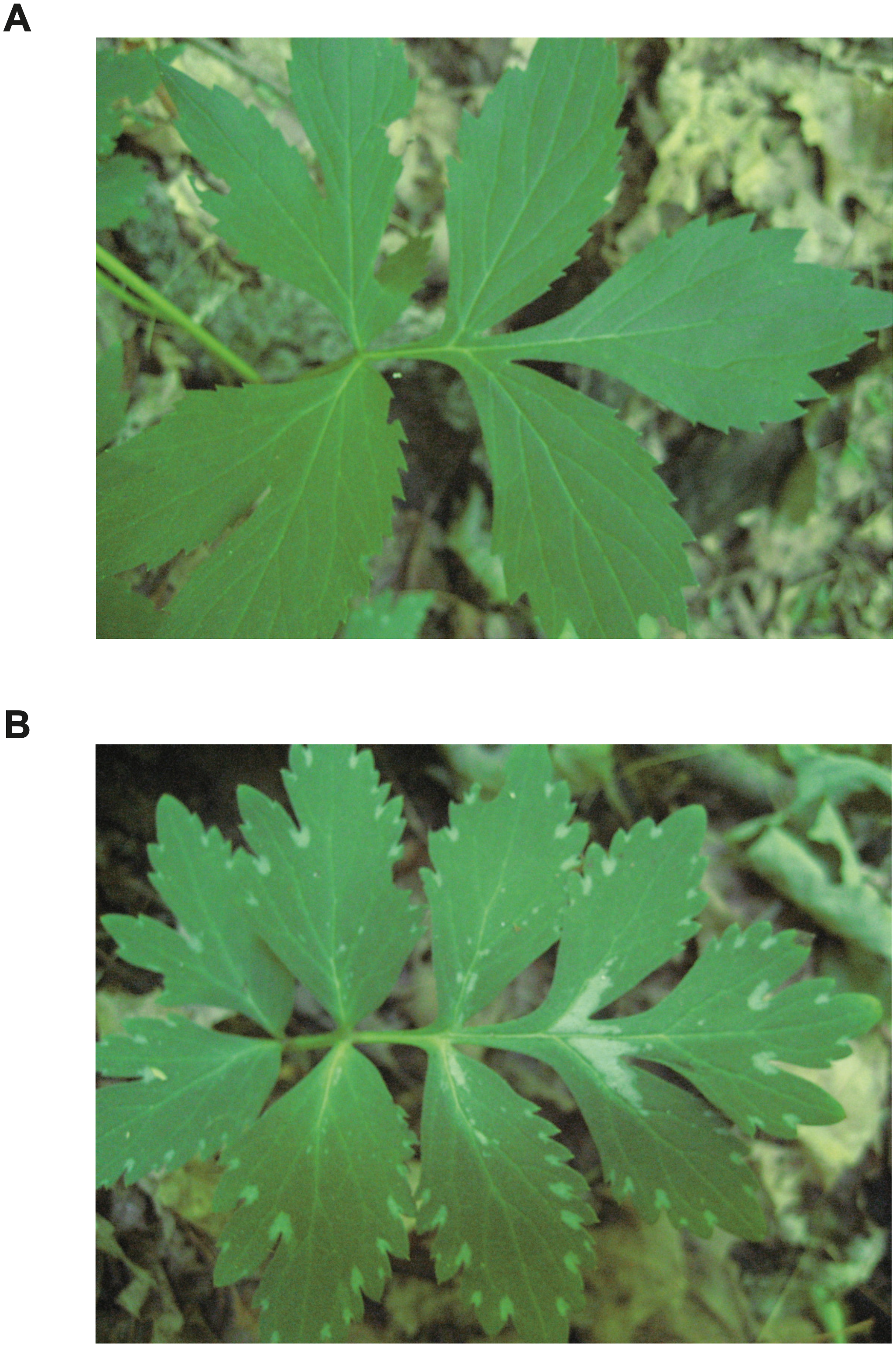
[2] Supplementary data for this article are available on the journal Web site (http://botany.nrc.ca) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Building M-55, 1200 Montreal Road, Ottawa, ON K1A 0R6, Canada. DUD 3721. For more information on obtaining material refer to http://cisti-icist.nrc-cnrc.gc.ca/irm/unpub_e.shtml.

Fig. 1. Comparison of nonvariegated and variegated leaves in *Hydrophyllum virginianum*. (A) Example of an nonvariegated leaf. (B) Example of a variegated leaf. Note the amount of white spots on the leaf in (B).



story of mature mixed forests across the property, covering as much of the reserve as possible. We sampled approximately 10 variegated and 10 nonvariegated plants in 20 populations to estimate herbivore damage on the different leaf types (total N = 394 sampled plants). Although a few plants had both phenotypes present, we only sampled variegated leaves from plants that had exclusively variegated leaves on them. Nineteen populations were under full canopy cover, while one was in a forest gap. As an index of the phenological stage, for 15 populations we also recorded the relative frequency of variegated and nonvariegated plants. Sampling took place from 4 June 2006 to 18 June 2006, 3–4 weeks after canopy closure.

Within a patch, 10 variegated plants were chosen randomly; we chose variegated plants first because they were relatively limiting. To ensure that we sampled nonvariegated plants in an unbiased manner that would also control for microhabitat and microclimatological differences, nonvariegated plants were always sampled by taking the closest plant due north of a variegated plant previously chosen. Pairs of plants were always directly adjacent to one another (<50 cm separation), and were sampled at the same time. From each plant, we harvested the most recently fully expanded leaf, and measured the amount of surface area removed by herbivores with a transparent sheet of acetate grid paper with resolution of 0.125 cm^2, as well as leaf length (see below). Because larger leaves have more surface area to be damaged by herbivores, we converted our estimates of leaf damage to the proportion of leaf area damaged by dividing them by the estimated total leaf surface area.

The pinnately compound leaf shape of *H. virginianum* made an accurate estimation of total leaf surface area in the field difficult. To address this, we used our estimates of leaf length to predict total leaf surface area with linear regression. We first harvested an additional 50 leaves of each phenotype of varying sizes. Using these leaves, we measured leaf length as we did in the field, as well as the total surface area of the leaf using the same gridded acetate sheets we had used to estimate herbivore damage. We then used a linear regression to predict surface area from leaf length for each leaf phenotype. Leaf length was a strong predictor of leaf surface area for both variegated (square root of the surface area = 0.4773 × length + 1.987, $r^2 = 0.7138$) and nonvariegated leaves (square root of the surface area = 0.5069 × length + 1.8044, $r^2 = 0.7911$).

Statistical analyses

To describe frequency variation of the leaf types, and hence the phenological stage of populations, we simply calculated the proportion of variegated individuals in 15 populations. We compared both the total surface area of leaves, and the mean proportion of surface area damaged, between variegated and nonvariegated leaves using a general linear model. Independent variables in the model included population, leaf variegation (variegated/nonvariegated), and the interaction between population and variegation (variegated/nonvariegated). Our initial analyses suggested non-normality of the residuals, so we verified the *P*-values of hypothesis tests with randomization tests. In the randomization tests, we preserved the population identity of individuals, but randomly assigned data to the two different leaf morphs. Because analysis of 10 000 randomizations did not differ from our original analyses, we report traditional *F*-statistics, standard errors, and *P*-values. All statistical analyses were performed with SAS version 9.1.3 (SAS Institute Inc., Cary, N.C.).

Results

Frequency of variegation across populations

The frequency of the two leaf phenotypes was measured for 15 of the 20 populations sampled. The frequency of variegated plants within these populations ranged from 6.7% up to 31.3% with a median of 10.7% (Fig. 2).

Population variation in leaf size and herbivore damage

We found significant variation among populations in leaf size and the proportion of leaf area damaged by herbivores (Table 1). Size of the most recently fully expanded leaf varied more than 2-fold across all populations [minimum population mean = 12.41 ± 1.62 cm^2, maximum population mean = 27.76 ± 1.53 cm^2; least-squared means (lsmeans) ± 1 standard error (SE)], while the proportion of leaf area damaged by herbivores varied more than 8-fold across the 20 populations (minimum population mean = 1.41% ± 1.9%; maximum population mean = 11.5% ± 1.9%; lsmeans ± 1 SE).

Leaf size and herbivore damage in variegated and nonvariegated plants

We found that nonvariegated leaves were significantly larger and had a significantly higher percentage of leaf area damaged than variegated leaves ($F_{1,354} = 25.86$, $P < 0.0001$ and $F_{1,354} = 16.55$, $P < 0.0001$ for surface area and proportion of leaf area damaged; Figs. 3A and 3B). For nonvariegated leaves, the average size of the most recently fully expanded leaf was 22.44 ± 0.49 cm^2, with a mean proportion of leaf area damaged of 8.0% ± 0.6% (lsmeans ± 1 SE). In contrast, for variegated leaves, the average size of the most recently fully expanded leaf was 18.91 ± 0.49 cm^2 and the mean proportion of leaf area damaged was 4.6% ± 0.6 .% (lsmean ± 1 SE). These data indicate that although nonvariegated leaves are somewhat smaller (16% smaller on average), they suffered 44% less herbivore damage, indicating that differences in leaf size between variegated and nonvariegated leaves cannot account for differences in the proportion of leaf area damaged. We failed to detect a significant interaction between leaf variegation and population for either mean leaf size ($F_{19,\ 354} = 0.85$, $P = 0.64$) or the mean proportion of leaf area damaged ($F_{19,\ 354} = 1.03$, $P = 0.42$), suggesting that there is no significant variation in these patterns across populations.

Discussion

Our survey of natural populations of *H. virginianum* suggests considerable variation in the frequency of variegated and nonvariegated leaves and a clear pattern of reduced herbivory on variegated leaves compared with nonvariegated leaves. These results are robust to population variation in the level of herbivory and the phenological stage of the populations (e.g., Table 1*b*). Taken together, these data suggest that if herbivory has deleterious effects on plant fitness, as is often observed (e.g., Marquis 1992), leaf variegation may provide a benefit to plants by reducing herbivory. Below, we first discuss the implications of leaf variegation for herbivory, and the costs and benefits to protecting early season leaves. We subsequently outline some of the limitations of our approach, and conclusions and potential future directions suggested by our study.

Implications of leaf variegation for herbivory

In our study, we find support for the major prediction of

Fig. 2. The frequency of variegated leaves in different populations of *H. virginianum*. The *x* axis represents the different populations measured where population number was arbitrarily designated after they were placed in increasing order. Note the large range (6.7% to 31.3%). The open data point is the median frequency of variegation, while the bars represent the 20th and 80th percentiles of the data.

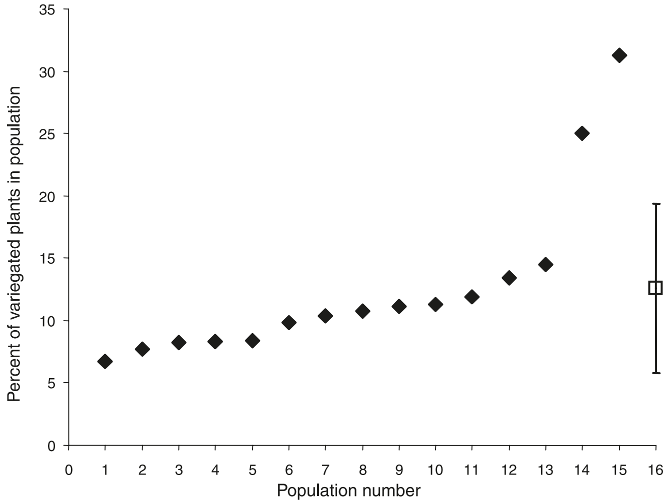
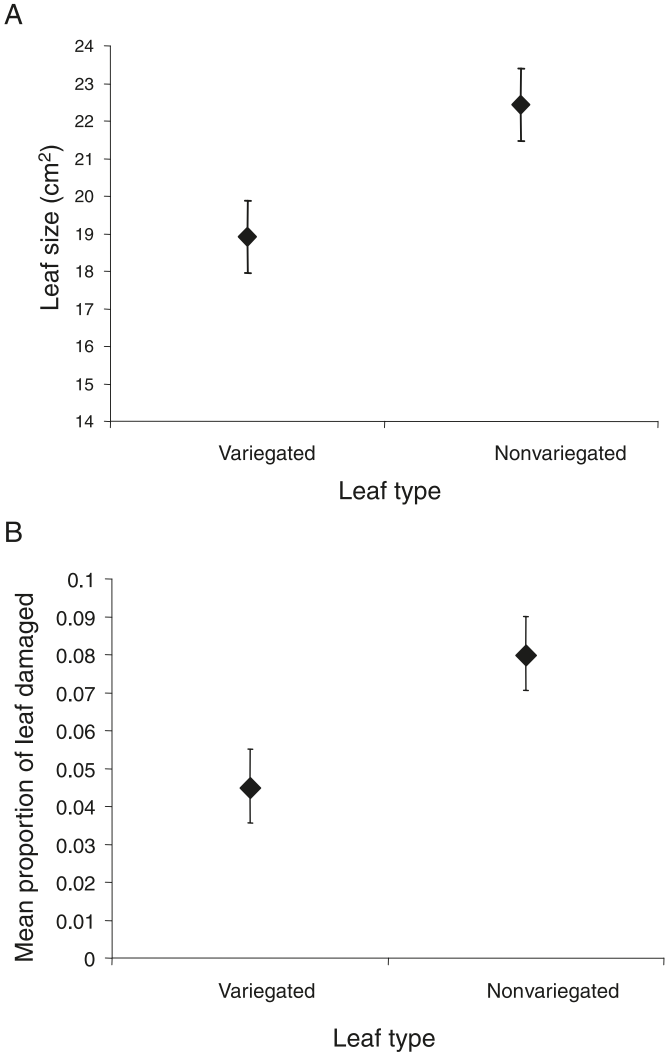


Table 1. Two-way ANOVAs for leaf size and proportion of leaf area damaged in variegated and nonvariegated leaves of *H. virginianum*.

Source	df	F	P
Leaf size (cm^2)			
Population	19	6.34	<0.0001
Leaf variegation	1	25.86	<0.0001
Population × leaf variegation	19	0.85	0.6413
Error	354		
Proportion of leaf area damaged			
Population	19	1.91	0.0125
Leaf variegation	1	16.55	<0.0001
Population × leaf variegation	19	1.03	0.4216
Error	354		

Fig. 3. (A) The mean size of nonvariegated and variegated leaves of *H. virginianum*. Nonvariegated leaves are significantly larger than the variegated leaves ($P < 0.0001$). (B) The mean proportion of leaf damage against either variegated or nonvariegated leaves. Variegated and nonvariegated leaves sustained significantly different amounts of damage ($P < 0.0001$). For both panels, data points represent least-square means ± 1 SE.



the herbivory hypothesis: variegated leaves received roughly half the amount of herbivore damage compared with nonvariegated leaves. Although our study did not address the evolutionary or ecological causes of variegation in *H. virginianum*, the potential relationship between leaf colouration and variegation and herbivore damage has been well documented in the literature, with conflicting evidence (e.g., Smith 1986; Givnish 1990; Brown et al. 1991; Hamilton and Brown 2001; Lev-Yadun et al. 2004). Thus, although leaf variegation may compromise photosynthesis, as suggested by Smith (1986), Givnish (1990), and Sadof et al. (2003), our study suggests early season leaf variegation may provide benefits via reduced herbivory.

Our results are consistent with previous work by Smith (1986) on a leaf colour polymorphism in *Byttneria aculeate*, in which it was shown that variegated leaves received less herbivore damage than plain leaves. However, because individuals in Smith's (1986) study with alternate leaf morphs were found in different microhabitats, the differences in levels of herbivory might not be a function of leaf variegation. Individuals with variegated leaves were much more common in drought-stressed habitats that received high levels of sunlight, suggesting that variation in variegation may be either a plastic response, or microenvironmental differentiation. Additionally, herbivores may forage on *B. aculeata* in preferred habitats (i.e., shady habitats dominated by plain morphs) rather than foraging on preferred morphs, a key distinction that prohibits the conclusion that colour morph per se was what affected levels of herbivory. Our sampling design minimized these types of confounding factors, suggesting that herbivory differences between variegated and nonvariegated leaves of *H. virginianum* were unlikely to be due to confounding environmental factors.

More generally, our results are consistent with a growing pool of evidence that suggests visual aspects of leaf morphology (as opposed to physical aspects such as spines, trichomes, and other well known physical defences) can reduce levels of herbivory. These characteristics may span from leaf colouration (Smith 1986; Givnish 1990; Allen et al. 2003), leaf shape (Rausher 1978; Niemela and Tuomi 1987; Mackay and Jones 1989; Rivero-Lynch et al. 1996), leaf size (Rivero-Lynch et al. 1996), and specialized structures such as egg mimics (Gilbert 1979; Williams and Gilbert 1981).

Costs and benefits of protecting early season leaves

While it seems that a plant would benefit from reducing herbivory, are there costs associated with variegation? We found that variegated leaves were significantly smaller than nonvariegated leaves, which could reflect slower growth. A more likely explanation, however, is that variegated leaves are smaller "sun leaves" produced prior to canopy closure, while nonvariegated leaves are larger "shade leaves" produced after canopy closure. At the whole plant level, variegation is thought to reduce photosynthetic efficiency due to the presance of fewer photosynthetic pigments, thus potentially limiting growth (Smith 1986; Givnish 1990), although Givnish (1990) did point out that this may not be the case in brightly lit conditions. There is little supporting evidence in the literature that variegation restricts carbon gain, with the exception of work by Sadof and colleagues (Sadof and Raupp 1991; Yang and Sadof 1995; Sadof et al. 2003); they found that variegated individuals of *Euonymus japonica* (Sadof and Raupp 1991) and *Coleus blumei* (Yang and Sadof 1995; Sadof et al. 2003) had smaller, yet more abundant leaves than did individuals of the same species with nonvariegated leaves. Nonvariegated leaves had higher photosynthetic and growth rates (Yang and Sadof 1995). Interestingly, Sadof et al. (2003) found that variegated plants were more susceptible to attack by phloem feeders, which they hypothesized was due to reduced assimilation of defensive compounds because of compromised photosynthesis.

Variegation appears to provide the benefit of reduced herbivory in early season leaves, which are subsequently shed during development by *H. virginianum,* exactly as first predicted by Givnish (1990). According to optimal defense theory (e.g., McKey 1974; Ohnmeiss and Baldwin 2000; Pavia et al. 2002; Strauss et al. 2004), it would be in the best interest of the plant to protect young leaves, since these are nutrient rich and are often of high fitness value to the plant (Ohnmeiss and Baldwin 2000; cf. Stinchcombe 2002). The comparatively high levels of nitrogen in young leaves in *H. virginianum* and other spring- or summer-active forest herbs makes them especially attractive to herbivores (Givnish 1990). For this reason, young leaves are often better defended than older leaves (e.g., McKey 1974; Givnish 1990; Zangerl and Bazzaz 1992; Ohnmeiss and Baldwin 2000), although this pattern is not universal (e.g., Feeny 1970; Raupp and Denno 1983; Cronin and Hay 1996; Campitelli et al., unpublished data,). In the *H. virginianum* plants we surveyed, variegated leaves were older than nonvariegated leaves (which are flushed later in sequence so are therefore younger). Thus optimal defense theory would predict that nonvariegated leaves (i.e., younger leaves) would be better chemically defended than variegated leaves (i.e., older leaves produced earlier in the season), and presumably suffer less damage. In short, optimal defense theory would predict the opposite pattern to what we observed in *H. virginianum*.

Limitations of the study

Despite the striking results of our analyses, it is worth considering these data in light of the limitations imposed by our survey design. First, our study was observational, and as such provides weaker evidence than would an experiment in which variegated and nonvariegated leaves (or plants of similar age) were placed in randomized locations. Although variegated and nonvariegated leaves were sampled closely together (always less than 50 cm apart, often less than 10 cm apart), we cannot formally exclude microenvironmental differences in light, water, nutrients, and other abiotic or biotic factors between variegated and nonvariegated *H. virginianum* leaves. We consider this possibility unlikely, however. Second, although we detected a 2-fold reduction in herbivore damage between variegated and nonvariegated leaves, we have no direct evidence that reducing herbivore damage to early season leaves enhances fitness in *H. virginianum*. Obtaining this evidence would be quite challenging in a long-lived, clonal perennial, but we do note that increased herbivory is generally detrimental to plant fitness in other species (Marquis 1992), and that variegation is likely to have little or no negative impact on photosynthesis and thus plant growth and fitness under brightly lit spring conditions (Givnish 1990).

Variegated and nonvariegated leaves in *H. virginianum* are produced in sequence, and thus exposed to different phenologies of weather, environmental conditions, and herbivore load. Because variegated leaves are produced early in the season, it is likely that they have been exposed to herbivores for a longer period of time than nonvariegated leaves, and hence would be predicted to suffer more herbivory. Nonetheless if early season leaves are better defended than later season leaves (e.g., with secondary chemicals), the differences we have detected may reflect phenological transitions in plant defence that are unrelated to variegation (see above). Future studies that measure secondary chemistry and the performance of generalist insect herbivores on variegated vs. nonvariegated leaves in *H. virginianum* would be helpful in addressing these issues.

Conclusions and future directions

Our results suggest that leaf variegation reduces herbivory to early season leaves in *H. virginianum*. Future work to determine the consequences of herbivory for growth and reproduction in *H. virginianum,* as well as the nutrient and chemical defense properties of variegated and nonvariegated leaves will clarify the exact role of variegation as an antiherbivore defense.

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