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Photosynthetic Efficiency is Higher in Asymmetric Leaves than in Symmetric Leaves of the Same Plant

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Abstract: Symmetry pervades nature, but asymmetry is also rather common. Deviations from genetically programmed symmetry are usually associated with internal or external developmental disturbances and may therefore be related to imperfections in physiological processes. In this study, we test the hypotheses that the photosynthetic efficiency of individual leaves of a plant is negatively related to their asymmetry. We measured chlorophyll fluorescence in leaves of three woody species (*Betula pubescens*, *Populus tremula* and *Salix caprea*) in early and late summer in two localities situated ca. 1000 km apart, and we quantified the asymmetry of these leaves by a multivariate measure based on the relative positions of several landmarks. Contrary to our expectation, we found that the photochemical efficiency of photosystem II was positively correlated with leaf fluctuating asymmetry; this effect was weak but consistent across the studied plant species, localities and seasons. Our finding adds to limited evidence that within-plant variation in leaf asymmetry is associated with variation in leaf physiology. Irrespective of the underlying mechanisms, which remain unknown, the results suggest that trees may benefit even more from their asymmetric leaves, at least in terms of photosynthesis, than they do from their more symmetric leaves.

Keywords: chlorophyll fluorescence; fluctuating asymmetry; insect herbivory; leaf shape; specific leaf area

1. Introduction

Trees, in contrast to highly integrated organisms like animals, can be viewed as conglomerates ('populations') of partially autonomous and repetitive multicellular subunits called modules [1]. This modular structure buffers a tree against aging and disturbances, while also enhancing the resource foraging efficiency through active environmental screening and growth in favourable directions [2–4]. The end result is a significant increase in the performance of the entire plant in terms of fitness-related traits [5]. Biologists have paid appreciable attention to the ubiquitous within-plant heterogeneity in the morphology, chemistry, growth rates and reproduction of individual modules (leaves and shoots) and its ecological and evolutionary significance, particularly with respect to photosynthesis [6] and plant–herbivore interactions [7–10]. Nevertheless, the mechanisms driving within-plant variation in leaf characteristics remain insufficiently understood.

One plastic trait that varies strongly within plant individuals is leaf shape, and especially leaf bilateral asymmetry. Increases in fluctuating asymmetry (FA; the subtle non-directional deviations from symmetry in organs that are otherwise symmetric) are thought to arise from internal or external disturbances occurring during the development of an organism (developmental instability: [11,12]). To date, many researchers have concentrated on the search for differences in FA between plant populations that have presumably experienced different levels of environmental stress [13–16]. At the scale of plant individuals and populations, leaf FA has been reported to increase with several environmental stressors, but controlled experiments have often led to inconclusive or 'negative' results that question the suitability of FA as an indicator of stress [15,17–20].

The studies addressing among-plant and among-population variation in FA usually average the measurements conducted from 5–25 leaves of a plant to remove the within-plant variation in FA. However, accumulating evidence suggests that comparisons of plant individuals and populations can be obscured by a very high variation in FA within an individual plant [21–24]. For example, more than 90% of the total variation in leaf FA in birch (*Betula pubescens*) trees was due to differences among the individual leaves of a plant; by contrast, the differences among trees and populations only contributed a few percentage points to this variation [24]. For the interpretation of leaf fluctuating asymmetry at the level of plants or populations, it is necessary to understand how this large within-plant variation is related to individual leaf quality, as a photosynthetic organ for the plant but also as food for insect herbivores [10,25].

Another trait that has also been proposed as an indicator of stress in plants is chlorophyll fluorescence [26,27]. Like FA, chlorophyll fluorescence is generally measured from several plant leaves, while ignoring among-leaf variations, and only the averaged plant values are analysed. The ratio of variable fluorescence (F_v) to the maximum fluorescence (F_m) of dark-adapted leaves is used to determine the photochemical efficiency of photosystem II, and this efficiency decreases in plants subjected to various stressors, including drought, low levels of soil nutrients, defoliation and pollution [15,28]. However, the great variation in chlorophyll fluorescence among individual leaves within a plant [28,29] complicates the comparisons of the photochemical efficiency of photosystem II between plant individuals and populations.

Although both an increase in leaf FA and a decrease in F_v/F_m may result from plant stress, the relationships between these characters remain virtually unknown. Only a handful of studies [15,28,30] have measured both leaf FA and chlorophyll fluorescence from the same plant individuals. Only a single study has explored the correlation between these two parameters and found different relationships between F_v/F_m and leaf FA at the level of plant individuals in different plant species; these relationships ranged from strongly negative to strongly positive correlations [31]. Uncovering the sources of this variation requires an exploration of the coherence between FA and photosynthetic efficiency at the scale of individual leaves within a plant.

The aim of our study was to provide insight into the potential physiological and ecological significance of variation in leaf asymmetry within woody plant individuals. We measured both FA and photochemical efficiency of photosystem II in individual leaves of three different plant species growing in different geographic and climatic conditions. As leaf traits change with leaf age [32], we chose two measurement dates in early and late summer. In addition, we measured leaf size and specific leaf area as two morphological traits which are potentially related to both FA and F_v/F_m. We used these data to test the hypothesis, derived from the theory of developmental stability [11,12], that the photosynthetic efficiency of an individual leaf is negatively related to its FA. In other words, we expected that a more asymmetric individual leaf would be of lower value for a plant in terms of its contribution to the whole plant carbon fixation in photosynthesis when compared to its more symmetric neighbouring leaves.

2. Materials and Methods

2.1. Study Species and Study Sites

Downy birch (*Betula pubescens* Ehrh.), trembling aspen (*Populus tremula* L.) and goat willow (*Salix caprea* L.) are deciduous tree species with a wide distribution in Eurasia. These fast-growing plants, which vigorously colonise open areas after a fire, clear cutting or other types of disturbance, are often used as model species for ecological and environmental research. In terms of leaf FA, downy birch is the most studied species [10,14,16,24,33,34], whereas the FA of the two other species has only been documented along several pollution gradients [15,35].

The present study was conducted in two localities: one in north-western Russia near Apatity (67° 37′ N, 32° 59′ E) and the other in south-western Finland near Turku (60° 32′ N, 22° 21′ E). Apatity is located in northern boreal forests, about 100 km south of the northern tree limit, and the mean July temperature is +13.6 °C. Turku is surrounded by boreo-nemoral forests, and the mean July temperature is +16.5 °C.

2.2. Measurements of Chlorophyll Fluorescence and Leaf Traits

The first measurement was conducted in early summer, when leaves were fully expanded (Turku: 2 June; Apatity: 15 June), and the second in midsummer, before leaves started to become senescent (Turku: 28 July; Apatity: 17 August). On each date, we collected two nearly symmetric (low FA) and two highly asymmetric (high FA) leaves that showed no visible traces of insect feeding from each of the five haphazardly selected individuals of each plant species at each locality, i.e., 240 leaves in total. At the time of sampling, leaf asymmetry was estimated visually; however, for data analysis, we used landmark-based measurements of FA in leaf shape (see below). Leaves were sampled from low-stature (2–7 m tall) trees growing naturally along the forest edge in a relatively benign environment. Study trees within a site were not more than 200 m apart; different sets of trees were used for early and late summer measurements. In birches, we only sampled the leaves growing on so-called short shoots, which represent over 95% of all leaves and are all of the same age. In aspen and willow, the leaves were sampled from the middle parts of shoots, avoiding the oldest (basal) and the youngest (apical) leaves.

A lightweight leaf cuvette was placed on each collected leaf at the time of sampling to assure dark adaptation, and leaves with attached cuvettes were placed in a plastic box with wet filter paper at the bottom to minimise desiccation. The cuvette covered only a small part of the leaf, and leaf parts outside the cuvette were exposed to natural illumination. Chlorophyll fluorescence was measured using a portable pulse-amplitude modulated chlorophyll fluorometer (Junior-PAM, Heinz Walz GmbH, Effeltrich, Germany). The index measured was the ratio of the variable to maximum fluorescence ($F_\mathrm{v}/F_\mathrm{m}$) obtained under the artificial light treatment. Three measurements were carried out in different places of the lamina of each leaf at an interval between measurements (15 min) sufficient for dark adaptation, and averaged per leaf. All measurements within a site were performed under a similar temperature and illumination.

After the measurements, the leaves were sealed in plastic bags, transported to the laboratory and scanned at a 600 dpi resolution. The obtained images were then used to determine the leaf size from the number of pixels of each leaf and to measure leaf FA (see below). After scanning, two disks (12 mm in diameter) were punched from each leaf, outside the midrib. These disks were dried at +105 °C for 24 hours and weighed to the nearest 0.1 mg, and their weight was used to calculate the specific leaf area (SLA hereafter).

2.3. Measurements of Asymmetry in Leaf Shape

We used a multivariate landmark-based measure of FA in leaf shape, which was recently demonstrated as more suitable for describing FA in birch leaves than a traditional distance-based FA measure [24]. Leaf shape was quantified using five (*B. pubescens*, *S. caprea*) or seven (*P. tremula*) landmarks; slightly different protocols were developed for these plant species because of differences in their leaf morphology. In all species, two landmarks were the tip and the base of a leaf. As a landmark close to the centre of a leaf, we chose the point where the sixth (*B. pubescens*) or fourth (*P. tremula*) lateral vein diverges from the midrib. As comparable measures on both sides of a leaf, we measured the points where the first two lateral veins reach the leaf margin in *B. pubescens* (Figure 1a). In *P. tremula*, the first two lateral veins approach the leaf margin asymptotically, so we measured the two outermost teeth of a leaf and the points where their supplying veins diverged from the first two lateral veins (Figure 1b). Similar homologous landmarks could not be established in *S. caprea,* since the number of veins varies strongly and because the veins in this species do not reach the leaf margin. We therefore drew a line connecting the tip and base of the leaf, an orthogonal line at the half of this midline, and two lines parallel to the midline at the widest point of the leaf. As landmarks, we then measured the centre of the midrib and the points on the left and right leaf margin most distant from the symmetry axis of the leaf (Figure 1c). The coordinates of landmarks were obtained with ImageJ software [36]. All leaves were measured twice independently, and the measurer was not aware of the results of chlorophyll fluorescence measurements.

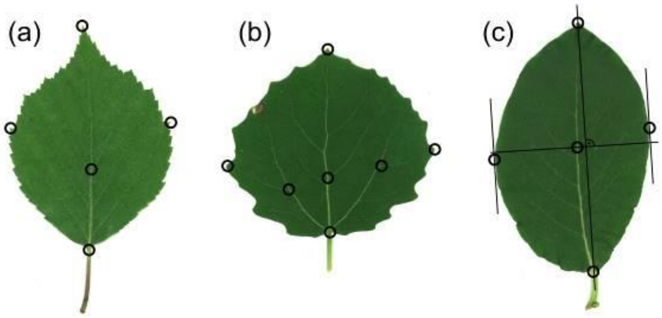

Figure 1. Position of the landmarks (circles) used for the analysis of fluctuating asymmetry in leaves of **(a)** *Betula pubescens*, **(b)** *Populus tremula* and **(c)** *Salix caprea*.

2.4. Statistical Analysis

Leaf shape was analysed separately for each species after Procrustes transformation to correct for differences in size, rotation and location. Procrustes ANOVA was used to test for directional and fluctuating asymmetry, analogously to the use of individual × side ANOVA in the analysis of right-left differences [37]. As a measure of fluctuating asymmetry, a Mahalanobis FA-score was calculated in MorphoJ [38]. This FA-score is based on the asymmetric component of leaf shape corrected for non-isotropic variation and accounts for the measurement error by averaging the two measurements of a leaf.

Differences in FA and in F_v/F_m between leaves of a plant that were at the time of sampling classified as symmetric or asymmetric were analysed with linear mixed models, including the asymmetry class as fixed and the individual tree as a random intercept effect. We explored the sources of variation in leaf size, SLA and leaf-specific values of photosynthetic efficiency using linear mixed models. In these models, plant species, study site and season (early vs. late summer) and their interactions were considered fixed effects, whereas a plant individual (nested within site by season by species combination) was treated as a random intercept effect. FA was used as a covariate, and its effect on F_v/F_m was tested after all other effects had been accounted for (type III sum of squares). We also checked whether the inclusion of two more explanatory variables, log-transformed leaf size and SLA, changed the detected relationships between F_v/F_m and FA. To facilitate accurate F tests of the fixed effects, we adjusted the denominator degrees of freedom using Satterswaithe approximation. All analyses were performed in SPSS version 22.

We illustrated the relationship between leaf FA and leaf photosynthetic efficiency using F_v/F_m values adjusted for all other effects by calculating a model, as described above, which included all effects except the covariate (FA). Adjusted values were calculated by adding the residual values obtained from this model (centred around zero) to the predicted overall F_v/F_m mean value.

3. Results

We found weak evidence for directional asymmetry in the leaves of *B. pubescens*, but very strong evidence for FA in the leaves of all three species (Table 1). The obtained FA scores were much higher in leaves that had been classified as asymmetric (1.53 ± 0.05) during collection than in leaves that had been classified as symmetric (0.92 ± 0.05; $F_{1,179} = 131.7$, $P < 0.001$). FA was weakly related to leaf size in *B. pubescens* ($r = -0.20$, $n = 80$ leaves, $P = 0.083$) and *S. caprea* ($r = 0.22$, $n = 80$, $P = 0.052$), but not in *P. tremula* ($r = -0.08$, $n = 80$, $P = 0.49$). FA was not related to SLA in *B. pubescens* ($r = 0.07$, $n = 80$, $P = 0.53$)

and *P. tremula* ($r = -0.20$, $n = 80$, $P = 0.08$), but decreased with an increase in SLA in *S. caprea* ($r = -0.30$, $n = 80$ leaves, $P = 0.006$).

Table 1. Procrustes ANOVA testing for differences between leaves (Individual), directional asymmetry (Side) and fluctuating asymmetry (Individual × Side) in the shape of leaves of three plant species.

Plant Species	Explanatory Variable	Degrees of Freedom	Mean Sum of Squares	*F*	*P*
Betula pubescens	Individual	237	9.91×10^{-3}	6.74	<0.001
	Side	3	4.38×10^{-3}	2.98	0.032
	Individual × Side	237	1.47×10^{-3}	44.05	<0.001
	Error	480	3.34×10^{-5}		
Salix caprea	Individual	237	4.56×10^{-3}	1.82	<0.001
	Side	3	5.16×10^{-3}	2.06	0.106
	Individual × Side	237	2.50×10^{-3}	55.56	<0.001
	Error	480	4.51×10^{-5}		
Populus tremula	Individual	395	8.78×10^{-3}	3.17	<0.001
	Side	5	1.27×10^{-3}	0.46	0.807
	Individual × Side	395	2.77×10^{-3}	6.24	<0.001
	Error	800	4.44×10^{-4}		

The two morphological leaf traits, leaf size and SLA, strongly differed between plant species, study sites and early and late summer measurements or their interactions (Table 2). The leaf size of two species, *P. tremula* and *S. caprea*, increased from early to late summer in Apatity, but not in Turku (Figure 2a). In contrast, the SLA of all species strongly decreased from early to late summer, but to different degrees (Figure 2b).

Table 2. Sources of variation in leaf size and specific leaf area of woody plant leaves (type III tests of fixed effects). Individual tree was included as a random factor with a variance of 0.0068 ± 0.0021 (log10 leaf size) and 4.91 ± 1.14 (SLA).

Explanatory Variable	Degrees of Freedom	Leaf Size		Specific Leaf Area	
		F	*P*	*F*	*P*
Site	1, 48	19.67	<0.0001	33.83	<0.0001
Season	1, 48	1.04	0.3134	142.20	<0.0001
Species	2, 48	16.12	<0.0001	25.51	<0.0001
Site × Season	1, 48	17.23	0.0001	5.03	0.0296
Species × Site	2, 48	0.28	0.7585	13.11	<0.0001
Species × Season	2, 48	1.42	0.2513	8.09	0.0009
Species × Site × Season	2, 48	8.46	0.0007	1.67	0.1994

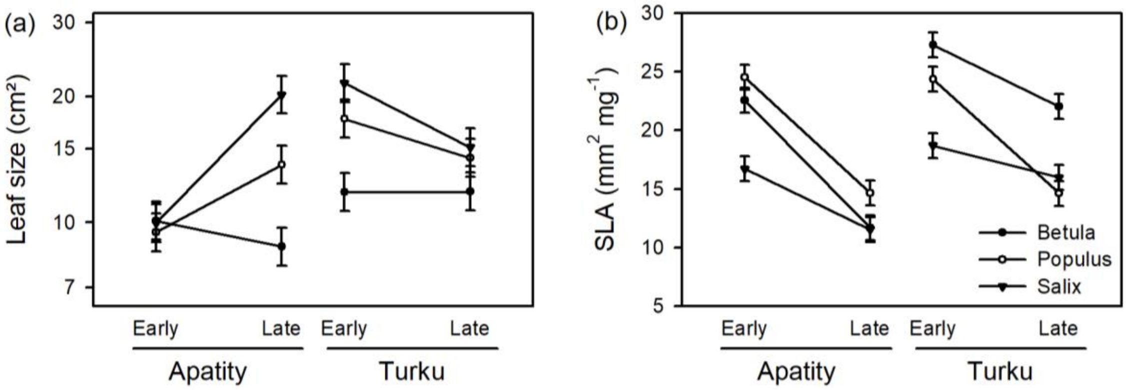

Figure 2. Effects of site (Apatity and Turku), season (early vs. late summer) and species (lines) on **(a)** leaf size and **(b)** specific leaf area (estimated from leaf disks 12 mm in diameter) of the leaves used for analyses of chlorophyll fluorescence and asymmetry.

Photosynthetic efficiency differed between plant species, study sites and early and late summer measurements, and most of the interactive effects were also significant (Table 3). In all three species, the photosynthetic efficiency was much lower at the first measurement date in Apatity (mean ± S.E.: 0.587 ± 0.007) than at the second measurement date (0.825 ± 0.007) or at both dates in Turku (0.804 ± 0.007, and 0.813 ± 0.007, respectively).

Table 3. Sources of variation in the photosynthetic efficiency of woody plant leaves (type III tests of fixed effects). Individual tree was included as a random factor with a variance of 0.00036 ± 0.00014.

Explanatory Variable	Degrees of Freedom	*F*	*P*
Site	1, 48.1	232.56	<0.0001
Season	1, 49.1	333.74	<0.0001
Species	2, 50.5	4.69	0.0135
Site × Season	1, 48.5	288.86	<0.0001
Species × Site	2, 48.1	0.76	0.4753
Species × Season	2, 48.1	8.36	0.0008
Species × Site × Season	2, 49.0	6.56	0.0030
Leaf FA	1, 201.4	6.06	0.0146

The photosynthetic efficiency was higher in leaves that had been classified as asymmetric (0.764 ± 0.014) during collection than in leaves that had been classified as symmetric (0.750 ± 0.014; $F_{1,179} = 10.4$, $P = 0.002$). After accounting for the effects of species, site, season and individual trees, the photosynthetic efficiency of a leaf showed weak but significant increases with an increase in its FA (Table 3, Figure 3). This effect was consistent across species, sites and seasons (interaction between FA and fixed factors: $0.28 < P < 0.56$). When the size of a leaf was included as a covariate, it had no significant effect on F_v/F_m ($F_{1,\,221.3} = 0.74$, $P = 0.39$) and the effect of FA on F_v/F_m did not change ($F_{1,\,199.5} = 5.12$, $P = 0.025$). When SLA was included as a covariate, it was positively related to F_v/F_m ($F_{1,\,145.7} = 5.83$, $P = 0.017$) and the effect of FA on F_v/F_m became even stronger ($F_{1,\,201.9} = 7.38$, $P = 0.007$).

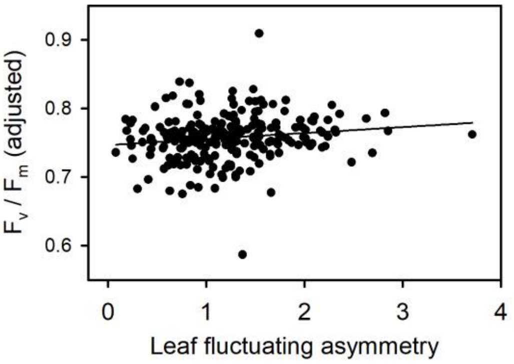

Figure 3. Relationship between the fluctuating asymmetry of a leaf and the maximum efficiency of its photosystem II (F_v/F_m), adjusted for the effects of species, site, measurement date and tree individuals (for the significance of the effect, consult Table 3).

4. Discussion

Our study is the first to demonstrate within-plant correlation between leaf photosynthetic efficiency and leaf FA. The effect detected by us was weak but consistent across the studied plant species, localities and seasons. This finding suggests that leaves showing different levels of FA may contribute differently to whole plant functions, i.e., the leaves may be of different value to a plant.

In theory, the value of a leaf to a plant can be measured as the difference between the carbon it fixes in photosynthesis and the carbon it uses to build leaf tissues and maintain their functioning [39]. However, direct, non-invasive, leaf-specific lifetime measurements of carbon loss and carbon gain are nearly impossible to obtain. Therefore, values of individual leaves within a plant are usually compared indirectly—for example, by studying plant responses to leaf removal [40] or by measuring net photosynthetic rates of individual leaves [41], i.e., by using approaches that are much more labour intensive than chlorophyll fluorescence measurements. In this study, we used the maximal photochemical efficiency (F_v/F_m) as a proxy for the photosynthetic value of a leaf, because the photochemical efficiency is positively correlated with the net photosynthetic rate across a range of experimental conditions [42]. We acknowledge that our proxy does not provide full information on the net photosynthetic rate, and we are aware of the pitfalls and caveats of the chlorophyll fluorescence method (outlined, e.g., in [43]), as well as of studies that failed to find correlations between the maximal photochemical efficiency and net photosynthetic rate [44]. Nevertheless, a lowered F_v/F_m, when it is measured after an appropriate period of dark adaptation, is commonly regarded as a good indicator of a sustained impairment of photochemistry [45]. This index is still widely used in ecological, environmental and agricultural research to quantify stress in plants [42,46,47].

Surprisingly, the relationship between chlorophyll fluorescence and leaf FA was the opposite of what we had expected, as the photochemical efficiency was higher in more asymmetric leaves than in less asymmetric leaves. At the same time, the discovered pattern was consistent with observations by Cornelissen and Stiling [25] regarding higher concentrations of nitrogen in asymmetric leaves than in symmetric leaves of the same plant, because different measures of photosynthesis generally increase with foliar nitrogen concentrations in both among- and within-species comparisons [48–50]. Therefore, a high leaf FA does not signal imperfection in leaf functioning in terms of photosynthesis, as could be suggested from theories that view a high FA as an index of 'low quality' of the individual [11,12].

A mechanistic explanation for the detected pattern is lacking at the moment, because of the acute shortage of information on factors that affect the FA and photosynthetic efficiency of individual leaves. The within-plant variation in FA can be associated with many factors, including the ontogeny of the individual modules, the temporal and spatial micro-environmental variation and the current and previous-year damage by herbivores [22,33,51–53]. However, in our study, the geographic or climatic differences and ontogenetic changes in leaf size and SLA were not responsible for the positive relationship between FA and F_v/F_m, because this relationship was not removed when the two traits were included as covariates. Within-crown module plasticity in trees is primarily linked to irradiance [54], so plastic changes in leaf anatomy could affect photosynthesis through different pathways [55]. In particular, sun leaves are generally smaller and thicker and show higher photosynthetic rates per unit leaf area when compared with shade leaves [56–58]. Leaf FA may increase under shading as a consequence of perturbed growth or as a plastic response aimed at increasing the light uptake [20,59], and the within-crown variation in leaf FA in some tree species is also associated with differences in light exposure [60]. However, the correlation between FA and F_v/F_m discovered by us is unlikely to be driven primarily by micro-environmental variation in irradiance, because this correlation was not explained by either the SLA or the leaf size—two traits that are central to the plant response to light availability [57,58,61].

The observed positive correlation between FA and F_v/F_m may have resulted from their coherent response to the same environmental stressor (e.g. herbivory). While many stresses lead to reductions in the F_v/F_m, the damage by defoliating insects may lead to the overcompensation of photosynthesis and can thereby increase both the leaf FA and F_v/F_m in the damaged leaf, as well as in the neighbouring undamaged leaves [62–64]. Unfortunately, we have only controlled for the absence of insect damage in the leaves selected for our experiment, without considering the damage of their neighbouring leaves, so our suggestions remain speculative. Nevertheless, for an increase in FA to be observed, the damage to neighbouring leaves must have occurred during the sensitive phases of leaf ontogeny [65], i.e., weeks before our first measurements of chlorophyll fluorescence. The photosynthetic overcompensation is generally a short-time process that is detected between several hours to several days after the stress impact [66,67], and it seems surprising that a physiological signal of past stress could be detected weeks or even months after its impact on leaf growth in all three species at both sites. Therefore, although recovery from past stress or even overcompensation of photosynthetic processes is the most plausible interpretation of the finding of a positive correlation between leaf FA and its photosynthetic performance [31], we cannot exclude alternative explanations.

In conclusion, our results suggest that shape FA in plant leaves is positively associated with leaf physiological and/or biochemical traits related to photosynthetic efficiency. The responsible mechanisms may include phenotypic plasticity driven by insect herbivory and/or by micro-environmental variation. Biochemical and nutritional differences between symmetric and asymmetric leaves could result from the differences in photosynthetic efficiency and may explain why leaf-feeding insects use leaf FA as a feeding cue [10,25]. Although the exact mechanisms and consequences remain speculative, our results question the assumption that leaves with greater asymmetry are merely modules of lower value for a plant because they developed under more stressful conditions. In terms of photosynthesis, trees may attain more benefit from their asymmetric leaves than from their nearly symmetric neighbour leaves.

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