On the relationship between fluctuating asymmetry, sunlight exposure, leaf damage and flower set in *Miconia fallax* (Melastomataceae)

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Abstract: We examined how different levels of sunlight exposure influenced the leaf fluctuating asymmetry (FA, a measure of stress) and fitness in *Miconia fallax* (Melastomataceae), and whether FA was related to leaf necrosis and fitness. The results revealed that plants receiving both direct and lateral sunlight all day long had the lowest level of FA compared to plants receiving less light. Contrary to our expectations, FA was not related to leaf damage/necrosis. Sunlight exposure was not related to fitness, but the relationship between FA and flower set was negative and significant. Leaf damage was also not related to flower set. According to the FA analysis, *M. fallax* was sensitive to sunlight levels, which is expected given that this species has pioneering characteristics. Our study shows that FA can both be used to anticipate changes in plant fitness, and also as a tool to assess stress in plants growing in different conditions.

Key words: Brazilian Savanna, developmental instability, environmental stress, leaf morphometry, leaf necrosis.

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Introduction

In nature, plants are susceptible to several factors that may affect their development and performance (Belsky *et al.* 1993; Krishnan *et al.* 2000; Lentz & Cipollini Jr. 1998; Tamire & Mengistou 2014). For instance, the damage provoked by herbivore insects usually induces serious changes in plants, influencing fitness parameters, such as the production of leaves and fruits (Ananthakrishnan 1993; Fernandes *et al.* 1993). Usually high levels of leaf damage are negatively related to plant fitness, causing the plant to perish in extreme cases (Furmann *et al.* 2005; Karban & Strauss 1993).

The environment also plays a key role in plant performance because climate and habitat conditions generally control the distribution and occur- rence of plants, as well as their growth rates and inter-specific interactions (Cuevas-Reyes *et al.* 2011; Kelly & Goulden 2008; Raz *et al.* 2011; Singh *et al.* 2001). In this context, plants growing in stressful conditions (i.e., located in a microhabitat that does not fulfill their requirements) may present changes in metabolism, physiology and development (Alves-Silva & Del-Claro 2013; Corne- lissen & Stiling 2011; Choudhury *et al.* 2014; but see Hódar 2002).

Developmental Instability (DI) reflects the inability of a population to develop under un- favourable environmental conditions (Markow 1995), and one common measure to estimate the DI of plants is the Fluctuating Asymmetry analy- sis (hereafter "FA") (Møller 1997; Palmer & Strobeck 1986; Palmer & Strobeck 1992). This technique is based on the small and random

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departures from the perfect symmetry in otherwise bilaterally symmetrical structures (e.g., leaves). Stressful conditions affect plant homeostasis, and paired organs grow with conspicuous differences in bilateral symmetry (Parsons 1992). For instance, FA has been associated with pollution (Kozlov *et al.* 1996), shade conditions (Puerta-Piñero *et al.* 2008), soil characteristics (Cornelissen & Stiling 2011) and differences in plant nutrients (Santos *et al.* 2013), among other variables (Møller & Shykoff 1999). In all these examples, plants developing in unfavourable habitats presented high FA levels, indicating conditions of stress. Adverse habitat conditions alter both the FA and the plant productivity (Jan *et al.* 2013). Nonetheless, few studies assess the interaction effects of habitat conditions, FA, plant damage (by herbivores, parasites or pathogens) and plant fitness (Costa *et al.* 2012). In fact one issue that remains scarcely studied is the influence of FA on plant fitness. In a review by Møller (1997), the data suggested a negative relationship between asymmetry and fitness (see also Møller 1999), but other studies failed to find a strong relationship between these variables (Clarke 1998; Lens *et al.* 2002). Nonetheless, the problem with these studies is that they are based mostly on animal populations; for instance, few data in Møller (1997) describe the relationship between fecundity and FA in plants, while most examples are based on animals. According to Clarke (1995), the use of FA to anticipate animal fitness is paramount in conservation ecology, because it is a biological monitoring system that can predict changes in the performance of animal populations. However, there is still a gap in the knowledge of how well FA can also be applied to plant ecology (Andalo *et al.* 2000).

To address these issues, we examined the interaction effects of habitat conditions on leaf FA and its relationship with leaf damage and flower set in *Miconia fallax* DC. (Melastomataceae). *Miconia fallax* is a common shrub in the Cerrado biome (Brazilian savanna) where it is regarded as a pioneer given its high occurrence on the edges and disturbed sites, and the high amount of flowers and fruits produced (Alves-Silva 2012; Maruyama *et al.* 2007). In this context, it is expected that light exposure might be a limiting factor for *M. fallax* growth and development. In a recent paper, Alves-Silva (2012) showed that *M. fallax* individuals growing in the shade had higher levels of FA compared to plants growing on the edges. As described above, FA can sometimes be

negatively related to plant fitness, but so far this relationship has been not examined in *M. fallax*. A negative relationship between FA and plant productivity might suggest that FA does affect the fitness in *M. fallax*, and that it can be further used to anticipate decrements in plant reproductive outputs.

In the present study, we first investigated how different sunlight conditions affected leaf FA, leaf damage and the production of flowers. Then, we examined whether FA was related to leaf damage and the production of flowers. We hypothesized that plants receiving total sunlight (both lateral and direct sunlight) would (i) present less FA levels and (ii) have more flowers compared to plants receiving less light. We also hypothesized a (iii) negative relationship between FA and leaf damage and (iv) FA and flower set. Any appreciation of the understanding of the main factors influencing plant stress, development and productivity can be a tool for the advance of FA studies and the effect of stress on plant development and fitness.

Materials and methods

Study site

The fieldwork was carried out in a cerrado reserve (230 ha) in Uberlândia city, Brazil (18º 59' S - 48º 18' W). The area is characterized by a fair amount of grasses (< 1 m in height) and a diverse community of shrubs including Malpighiaceae, Myrtaceae and Fabaceae, all of which are typically < 2 m in height. The temperature in the region varies little, peaking at ~25 ºC in February and ~20 ºC in June. Rainfall, however, occurs predominately (90 %) from October to mid-April (Laboratory of Climatology, UFU, Brazil). Soils are hydromorphic and acidic with large amounts of aluminum and potassium. For more details on the vegetation and climate at the area, see Cardoso & Lomônaco (2003).

Plant species

Miconia fallax is an ornithocorous shrub species that is common place at the study area (Maruyama *et al.* 2007). Shrubs are multi-branched and the plant rarely exceeds 1.8 m in height. Leaves are smooth-margined, coriaceous and reflexive. The color ranges from green to brown/beige on the adaxial and abaxial sides, respectively. Leaf venation is acrodromous with a pair of secondary veins converging toward the apex

(Reis *et al.* 2004). In some years leaves can be heavily parasitized by nematode galls, but these herbivores do not have an influence on leaf FA (Alves-Silva 2012). *Miconia fallax* flowers are white and grow in inflorescences located at the apex of branches. Flowering is massive and lasts from three to four weeks, in general, taking place in September and October.

Sampling design

Miconia fallax sampling was conducted in September 2013 when leaves were mature and completely expanded, and blooming was commonplace in all individual plants. A total of 36 *M. fallax* individuals were tagged in an area of ~10 ha. The abundance of flowers at the occasion was assessed in the most preeminent inflorescence from each individual plant, which corresponded to the most apical inflorescence (Fig. 1A).
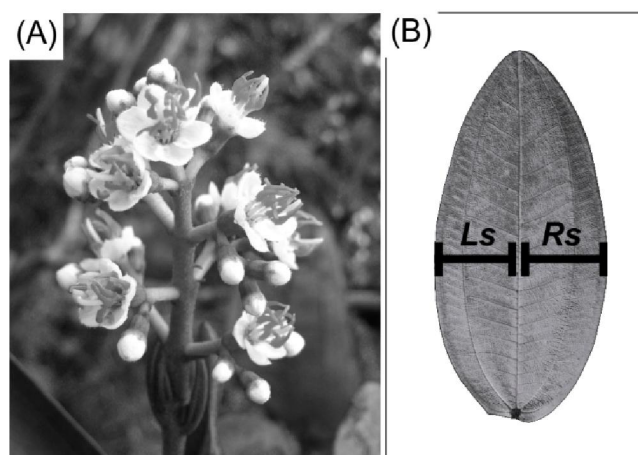

Fig. 1. (A) The most apical inflorescence of a *Miconia fallax* shrub in a neotropical savanna and (B) how measurements of leaf fluctuating asymmetry were performed. *Ls* - left side, *Rs* - right side. Flower width = 15 mm; Leaf width = 60 mm.

In the field, we also established different categories for *M. fallax* crown illumination based on the incidence of sunlight, adapted from Clark & Clark (1992). The category of *'vertical sunlight'* was assigned to plants surrounded by other trees, receiving only direct sunlight from above during the midday. The *'lateral sunlight'* rank was given to plants shaded by large trees, thus, receiving only lateral light part of the day; and *'total sunlight'* was considered for plants receiving both direct and lateral light all day long. No individual

plant was totally shaded at the area, so such a category could not be assessed.

The ten most apical leaves were collected from each plant, accounting for 360 leaves. With this methodology, we were able to sample leaves that had been exposed to the same environmental conditions at the individual level (Møller 1995), thus, our results could be examined without ambiguity. This would not be possible if we had sampled leaves from different branches (in the same individual plant) as the micro-environmental conditions might be different.

Leaves from each individual plant were given an identification code, placed in a cool box (~ 20 ºC) and taken to the laboratory where they were examined for the presence of necrosis along the blade and variations in side morphometry (FA analysis). Leaves were placed under a section of transparent glass and photographed. Pictures were transferred to a computer where both leaf necrosis marks (quantity), necrosis area (mm² and %) and FA measurements were assessed with the use of the Image J software. In *M. fallax*, leaf necrosis can have several origins, including herbivory and pathogens (*pers. obs.*). For the sake of clarity, in this study we did not discriminate between causes of leaf damage, but rather considered them altogether to ease the analyses (Alves-Silva & Del-Claro 2013).

Fluctuating asymmetry analyses

To assess leaf FA, we measured the widths of all leaves (N = 360 leaves) on both the right (*Rs*) and the left sides (*Ls*), from the leaf edge to the midrib (main/primary vein), at the middle point of the leaf (Fig. 1B). To test the accuracy of the measurements previously performed, a subsample of 50 leaves was remeasured in both the *Rs* and *Ls*. This type of approach is mandatory in FA studies as it may indicate whether measurements were conducted with sufficient precision to discard errors (Santos *et al.* 2013). Hence, the Index of Repeatability (IR) followed by two-factor analysis of variance (ANOVA) was used to determine whether the between-sides variation was significantly larger than the measurement error (see Cornelissen & Stiling 2011; Cuevas-Reyes *et al.* 2011). The significance of the interaction (individual* leaf side) was greater than expected by measurement error in *Rs* - *Ls* (IR = 98 %; $F_{1,49}$ = 9.9710; P < 0.0001) indicating that leaves were measured with appropriate precision (Alves-Silva & Del-Claro 2013; Santos *et al.* 2013).

FA, together with Directional Asymmetry and Antisymmetry, is one of three types of asymmetry, and in ecological studies it is important to determine whether the study subject really presents FA, i.e., random and small differences in *Rs* minus *Ls* with a mean equal to zero (Palmer & Strobeck 1986). Thus, to discard other types of asymmetry, the Directional Asymmetry, which depicts significant differences between leaf sides (*Rs* minus *Ls*) was checked with a Student's *t*-test, by testing that the average *Rs* minus *Ls* value did not differ from zero. Antisymmetry (significant differences in the *Rs* minus *Ls* distribution from the normal curve) was examined with a histogram chart (Rowe *et al.* 1997) followed by a normality test.

Miconia fallax leaf measurements had a mean not different from zero in *Rs* minus *Ls* ($t = 0.7929$, df = 359, $P > 0.05$), thus Directional Asymmetry was discarded. The data also fit the normal curve (bell-shaped distribution, Lilliefors' normality test $P > 0.05$), thus rejecting Antisymmetry. Taken together, the data fit purely FA and not other types of asymmetry. To conclude, FA dependence on leaf size was tested through linear regression using the absolute difference of the *Rs* minus *Ls* measurements (Woods *et al.* 1998), but results were not significant ($F_{1,48} = 1.1456$, $r^2 = 0.0233$, $P > 0.05$). Therefore, FA in our study was assessed as the mean difference between the right and left sides {i.e., FA=[$\Sigma | (Rs - Ls) | / N$]} (Palmer & Strobeck 1986).

Statistical analyses

Quantitative data is presented as the mean ± SE. The relationship between FA and sunlight classes was examined with a Kruskal-Wallis test, as original data did not fit the assumptions of normality (Lilliefors' normality test, $P < 0.05$). The influence of leaf damage on FA was examined with Pearson correlations, and we took into account different measures of leaf damage, including the abundance of necrosis marks per leaf, the necrosis area (mm^2) and the percentage necrosis per leaf. The relationship between flower set and sunlight exposure was examined with a one-way ANOVA test. A general linear model test was used to verify whether flower set was related to FA and leaf necrosis (mm^2). In this test, we used only one measure of leaf damage (leaf necrosis area, mm^2). All statistical tests followed Zar (1984) and procedures were performed using Graphpad Prism and Systat softwares.

Results

The number of necrosis marks in *M. fallax* leaves varied from 0 to 19 (4.08 ± 0.29, N = 1459 necrosis marks), while the necrosis area per leaf ranged between 0 to 236 mm^2 (30.22 ± 2.67 mm^2), filling on average less than 2 % (1.27 ± 0.11 %, range = 0 - 3.54 %) of the leaf blade (N = 360 leaves examined). We found a significant difference in FA according to *M. fallax* light exposure, as plants receiving sunlight all day long had the lowest level of leaf FA ($H = 15.3529$, df = 3, $P < 0.001$) (Fig. 2). Contrary to our expectations, none of the three measures of leaf damage were even closely related to FA: necrosis marks per leaf ($r = 0.2043$, $P = 0.2319$, N = 36), leaf necrosis area ($r = 0.2188$, $P = 0.1997$, N = 36), and percentage necrosis per leaf ($r = 0.1199$, $P = 0.4860$, N = 36) (Fig. 3).
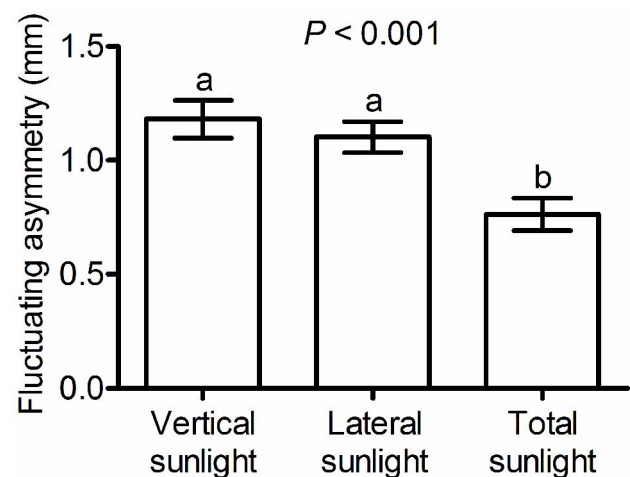

Fig. 2. Levels of fluctuating asymmetry (mean and SE) according to light exposure categories in *Miconia fallax*, showing that FA was significantly influenced by light incidence. Lowercase letters upon bars indicate statistically significant differences (Kruskal-Wallis test, $P < 0.001$).

The abundance of flowers per *M. fallax* inflorescence ranged between 9 and 211 (83.89 ± 7.31 flowers per shrub, N = 36 shrubs examined). The abundance of flowers was slightly higher in plants receiving total sunlight compared to plants exposed to lateral and direct sunlight, but differences were not statistically significant ($F_{2,33} = 0.7833$; $P > 0.05$) (Fig. 4). According to the GLM test, flower set in *M. fallax* was significantly and negatively related to FA. Leaf necrosis was also negatively related to flower set, but the relation-
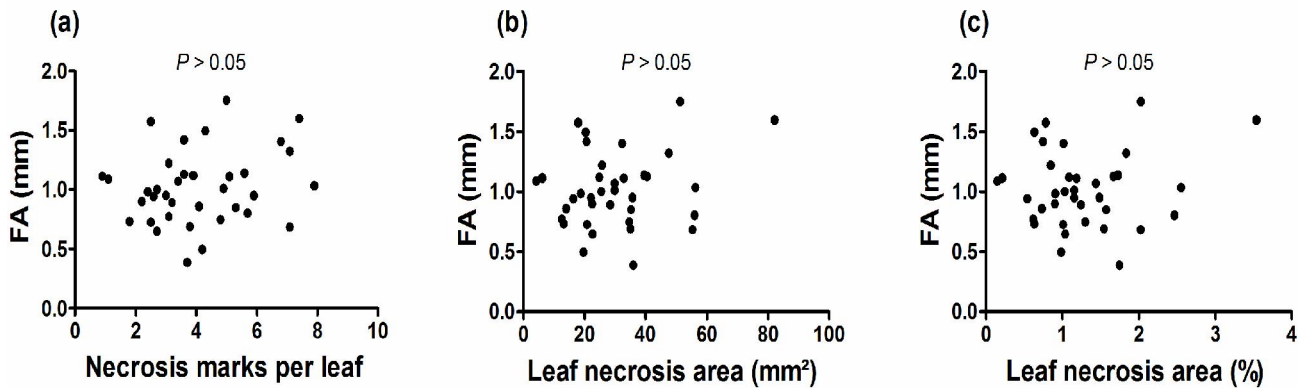
Fig. 3. Relationship between leaf fluctuating asymmetry (FA, mm) and different measures of leaf damage, revealing that leaf FA had no influence on leaf necrosis in *Miconia fallax* (Pearson correlation tests, $P > 0.05$ in all cases).

Table 1. Coefficients and analysis of variance of the relationship among *Miconia fallax* flower set, plant height (m), leaf necrosis area (mm²) and fluctuating asymmetry (FA, mm). Flower set was related only to FA. Std. Error - standard error; MS - mean squares; df - degrees of freedom.

Variables	Coefficient	Std. Error	*t*-value	*P*-value
Constant (flowers)	206.5612	51.4659	4.0136	0.0003
Main factors				
FA	-110.1243	46.1875	-2.3843	0.0232
Leaf necrosis	-1.5472	1.4249	-1.0858	0.2857
Interaction effects				
FA * leaf necrosis	1.1320	1.1639	0.9726	0.3380
Analysis of Variance				
	MS	*F*-Ratio	*R²*	*P*-value
Regression (*df* = 3,32)	6604.9988	4.4401	0.2939	0.0102

ship was not statistically significant (Table 1, Fig. 5). The interaction effect '*FA*leaf necrosis*' was also shown to not influence *M. fallax* flower set.
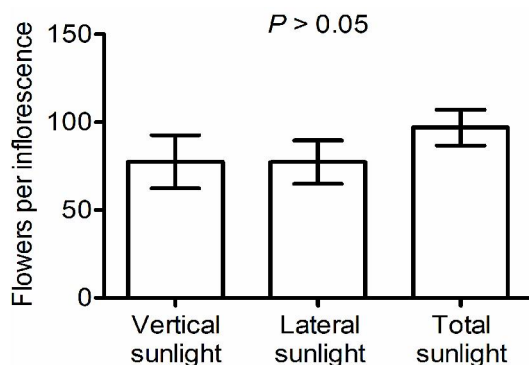

Fig. 4. Abundance of flowers per inflorescence (mean and SE) in *Miconia fallax* according to sunlight categories. Plants receiving full sunlight had more flowers compared to the other categories, but differences were not statistically significant (ANOVA tests, $P > 0.05$).

Discussion

The genus *Miconia* contains several species labelled as pioneers based on their preference for sunny sites and high recruitment in areas recovering from disturbance, where they grow faster and produce more fruits (Fontes 1999; Miyanishi & Kelleman 1986; Pascarella *et al.* 2007; Tabarelli & Mantovani 1999). In the present study, sunlight exposure was responsible for changes in *M. fallax* FA and productivity. For instance, FA levels were significantly lower in plants receiving sunlight all day long, and flower set was also higher (although not significant) in comparison to the other plant groups receiving less light.

Light exposure can also promote other increments in plants (Marques *et al.* 2000). For instance, Dalling *et al.* (2001) observed that gap size (and consequently more light exposure) increased several characteristics of the pioneer
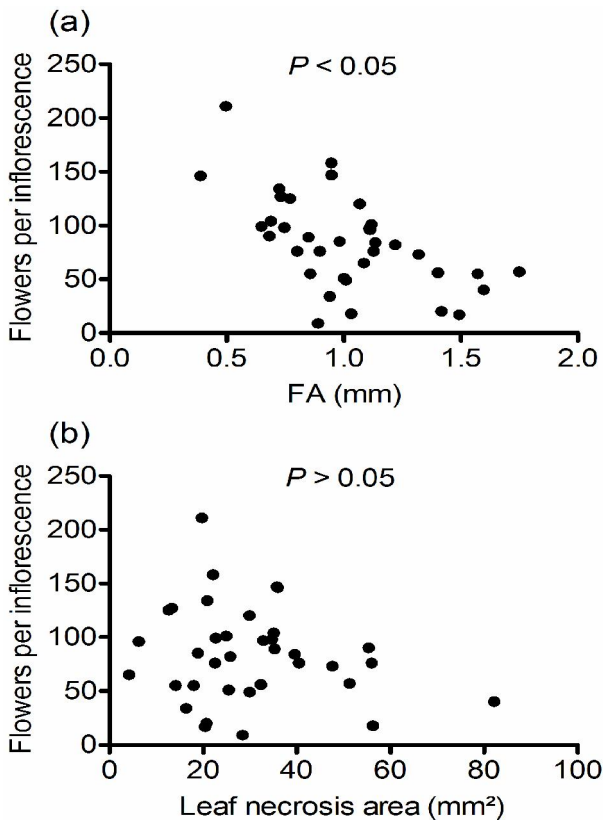
Fig. 5. Relationship between *Miconia fallax* flower set and (a) fluctuating asymmetry (FA, mm) and (b) leaf necrosis area (mm²). Results were significant only in the former case (see results in Table 1), indicating a negative relationship between flower set and FA.

tree *Miconia argentea* (Sw.) DC, such as leaf area, seedling mass, growth rate and photosynthesis. In the study by Lomônaco & Reis (2007), the authors showed that *M. fallax* growing in shaded areas (Cerradão) presented heavier fruits with larger seeds; in contrast plants in the open cerrado produced more fruits but with low fruit and seed mass. These results indicate a strong trade-off between seed size and number and suggest that in shaded spots *M. fallax* invested more in quality as an adaptation to maximize seedling growth in a shady habitat. Both studies by Dalling *et al.* (2001) and Lomônaco & Reis (2007) reveal the importance of sunlight for pioneer *Miconia* and, thus, its lack may negatively influence plant performance and increase stress levels.

The influence of light/shade on FA has been poorly explored. Møller (1995) affirms that light conditions during plant development might be a direct cause of increased leaf asymmetry (see also Alves-Silva 2012). Such an approach was demonstrated by Puerta-Piñero *et al.* (2008) by showing that shade was responsible for severe decrements in seedling (*Quercus pyrenaica*: Fagaceae) performance, and FA was also higher in shaded plants compared to plants exposed to sunlight. In contrast to these studies, the FA levels in the neotropical shrub species *Banisteriopsis campestris* (A. Juss.) (Malpighiaceae) growing in sunny sites was higher and had a correlational effect with the production of extrafloral nectar (Alves-Silva & Del-Claro 2013). In both cases, FA was used to predict changes in plant development and could be used as a tool to measure environmental stress, but previous information on the natural history of species was essential to establish relevant hypotheses.

Contrary to our expectations, leaf damage (necrosis marks, necrosis area and percentage of necrosis per leaf) was not significantly related to FA. The relationship between herbivory and FA is still controversial; in some cases herbivores themselves may provoke FA, while in others they may seek the more asymmetrical leaves (Cornelissen & Stiling 2005; Møller 1995). This situation is yet more complicated if we take into account that some plant species may compensate for herbivory by buffering the deleterious effects of tissue damage (McNaughton 1983), and in these cases both damaged and healthy leaves do not present differences in FA levels (Alves-Silva 2012; Berteaux *et al.* 2007).

The influence of plant damage on fitness depends on several factors, especially on the extent and the structure that is being damaged (Amsberry & Maron 2006; Maron 1998). The lack of relationship between leaf damage and flower set in *M. fallax* may indicate that the plant can tolerate leaf damage and invest in reproductive structures, but we observed that the extension (mm²) of the leaf blade affected by necrosis was low, on average only 2 %. Strauss & Agrawal (1999) discuss the fact that tolerance may vary from plant to plant, as some species can support up to 25 % of leaf area loss without incurring significant losses in fitness (Rosenthal & Kotanen 1994).

FA was negatively related to *M. fallax* fitness, indicating that shrubs under stress suffered significant decrements in flower set, but this result must be treated with caution, because FA is a non-specific stress indicator (Martel *et al.* 1999), which can be boosted by a wide range of environmental factors (Møller & Shykoff 1999). In our study, FA was caused by differences in sunlight, but sunlight

exposure itself did not significantly influence *M. fallax* fitness, but rather leaf FA. This measure of stress was negatively and significantly related to the fitness parameter. According to Diaz *et al.* (2004), FA indicates the levels of maladaptation of a population to certain stressful conditions during development, and thus FA should be related to fitness. In our study, poor quality plants presented higher leaf FA, which is evidence that the plants were under stress (Cornelissen & Stiling 2011). In such a condition, plants can present changes in the physiological and metabolic processes, which in turn can affect the development, growth and the production of some compounds (Alves-Silva & Del-Claro 2013; Lempa *et al.* 2000). Furthermore, stressed plants are expected to have low investment on fecundity (see Díaz *et al.* 2004).

Our results add important evidence that FA negatively affects plant fitness, thus counterbalancing the studies reporting no evidence of FA on plant performance (Andalo *et al.* 2000; Siikamäki *et al.* 2002). The relationship between FA and plant fitness in still not completely understood, as in some plants FA indeed negatively affects fitness parameters while in other it does not (Lens *et al.* 2002; Møller 1997). This occurs because plant fitness can be measured by several structures such as buds, flowers, fruits and seeds. Each one of these structures can be affected in a different way by plant environmental stress (Andalo *et al.* 2000; Clarke 1995; Leung & Forbes 1997; Siikamäki *et al.* 2002).

We conclude that in the case of *M. fallax*, FA was a useful tool to examine the extent of plant stress and its effects on flower set, used as a measure of plant fitness. Upcoming studies especially in the neotropics (where there is a lack of FA research) are needed before generalizations can be made on the relationship between environmental/biotic stresses, FA and an organism's fitness.

Acknowledgements

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