Leaf Fluctuating Asymmetry of *Myrtus Communis* L., Affected by Increases in Atmospheric CO$_2$ Concentration: Evidence from a Natural CO$_2$ Spring

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Abstract

We studied leaf fluctuating asymmetry in *Myrtus communis* occurring around the "I Borboi" natural CO$_2$ spring (Tuscany, Italy) and in a nearby control site, in a Mediterranean environment. Developmental instability, measured as leaf fluctuating asymmetry, is expected to be positively related to environmental stress and negatively to habitat quality. A gradiental decline in leaf width and leaf angle fluctuating asymmetry was found along the decreasing CO$_2$ concentration from double to ambient. The correlation is ascribed to the positive effect of elevated CO$_2$ on developmental stability. Probably, the adaptation process of individuals grown in close proximity to the CO$_2$-enriched area took advantage of improved water use and carbon balance of *M. communis* plants.

Keywords: elevated CO$_2$, developmental instability, leaf width asymmetry, leaf angle asymmetry, *Myrtus communis* L.

Abbreviations

FA (fluctuating asymmetry), DS (developmental stability), DI (developmental instability), DA (directional asymmetry), AS (antisymmetry)

Introduction

Developmental stability (DS) is the ability of individual organisms to produce invariant phenotypes under different environmental conditions [1, 2] and its reduction can derive from a number of environmental and genetic perturbations (e.g [3].) Fluctuating asymmetry (FA), directional asymmetry (DA) and antisymmetry (AS) depict asymmetry in plant (and animal) morphological traits [4]. In particular FA, which describes the magnitude of random deviations from perfect symmetry [5-7], offers a unique tool for comparative studies of developmental stability [8]. In plants, FA has been proposed to be an indicator of genetic and environmental stress [9, 10]. Individual and population levels of bilateral FA have been related to several biotic and abiotic stresses, including environmental factors such as nutrition [11], temperature [12], radiation [13, 14], and pollution [15]; as well as genetic factors such as mutation, inbreeding and hybridization [16].

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So far there has been only one study investigating the relationship between increases in CO_2 concentration and variations in leaf symmetry [17], and a positive link was evidenced. The experiment was performed on two oak species in open top chambers, with two contrasting CO_2 concentrations: 350 ppm vs. 700 ppm. As the forest was fire-maintained and trees were 3-5 m in height, it is doubtful that results could be representative of adult individuals. Indeed, most studies have demonstrated that FA may decline during a plant's lifetime [18]. Besides, vegetation is not likely to be subjected to a dramatic increase in atmospheric CO_2 concentrations. Instead, plants are experiencing a gradual increase in CO_2 concentration throughout generations. Because the magnitude of random deviations from the symmetrical bilateral shape of the leaf has been shown to increase as a consequence of herbivory [9, 10] and the long-term effect of elevated CO_2 concentration on distribution of herbivores is not known, we excluded damaged leaves from further analyses.

To our knowledge, no studies have been conducted on FA of plant populations in natural CO_2 degassing vents. Natural CO_2 springs offer the opportunity to study long-term response of entire plant communities to elevated CO_2 [19]. We tested the hypothesis that a relationship exists between increasing CO_2 concentration (from ambient to elevated) and gradually declining leaf FA. We took advantages of a shrub species *Myrtus communis* L. growing under elevated CO_2 concentration in an otherwise natural Mediterranean-type ecosystem [20], and in nearby homologous conditions except for ambient CO_2 concentration. The main benefits of natural CO_2 springs are the long history of high CO_2 concentration experienced by plants, and the occurrence of natural climatic cycles and biotic interactions, including competition and herbivory.

The ultimate aim of this work was to estimate the degree of DS in *M. communis* according to the value of leaf FA in ambient condition vs. long-term elevated CO_2 concentration. We expected that elevated CO_2 concentration might favorably affect leaf development and hence decrease deviations from the bilateral symmetry of *M. communis* leaves.

Materials and Methods

Study Site

The study site, a natural CO_2 vent called "I Borboi" is situated near Lajatico (Pisa, Tuscany, Italy, 43°26' N, 10°42' E). The vegetation is a coppiced Mediterranean woodland dominated by *Quercus ilex* L., including *Q. cerris* L., *Fraxinus ornus* L., *Q. pubescens* Willd., and *Arbutus unedo* L., and a shrub layer with *M. communis*, *Erica arborea* L., *Juniperus communis* L., *Cistus salvifolius* L., *Cytisus scoparius* L., and *Smilax aspera* L., as coexisting species [20-22]. The CO_2 vent is located on the north facing slope (20%) of a hill about 200 m above sea level. Bubbly CO_2 is emitted from several vents located along a seasonal creek, generating a permanent gradient in CO_2 concentration from ambient levels (about 360-380 ppm) outside the CO_2-enriched area, up to a few percent CO_2 very close to the main vent. The enriched area extends over an area of about 0.7 ha [22]. Some H_2S is present in the emitted gas, but its concentration never exceeds 0.04 µmol mol^{-1}. The climate is Mediterranean, with cool winters and hot dry summers, precipitation being mainly concentrated in autumn and spring periods.

Data Collection

Since *M. communis* was present across the entire CO_2 concentration gradient, this species was chosen to test our hypothesis. Crosswise the CO_2 concentration gradient, a total of six sampling plots was set, starting near the vent (2 m away, in order to avoid eddies of very high concentration). Every 15 m, one sampling plot of approximately 5 m^2 was placed, each containing at least three *M. communis* individuals. Individuals of similar size were selected for sampling, and samples were taken from crown portions with similar sun exposure. On each plot, about 80 randomly selected leaves were collected at a height of 1-1.5 m above ground. Damaged leaves were excluded. At least 50 leaves from each plot were dried and stored for further analysis. In the control plot, far off the main vent, we collected 100 leaves, 80 of which were stored, after exclusion of damaged exemplars. The six plots were located along decreasing CO_2 concentrations (Table 1). A detailed description of the spatial variation of the CO_2 concentration around the degassing vent can be found in the work of Tognetti et al. [22]. In addition, CO_2 concentration also was measured during leaf sampling with an infrared gas analyzer (EGM-1, PPSystem, Hitchin, UK). The unit we used for CO_2 concentration was the old "ppm," which means µmol CO_2 / mol air, according to SI.

Table 1. Main features of the six sampling points across a CO_2 concentration gradient at the "I Borboi" natural CO_2 spring.

Sampling plot	Mean concentration of CO_2 (ppm)	Distance from CO_2 source (m)	Relative height above CO_2 source (m)
1	570	2	0.4
2	530	18	2.3
3	490	34	3.9
4	450	50	6.5
5	410	66	8.1
6	370	82	10.5

Measurements and Data Analysis

Selected leaves were dried like herbarium specimens (dried between filter paper sheets). Scanning was done using a digital camera (Eurocam, Euromex, The Netherlands) connected to a personal computer. To quantify FA, widths of all leaves were measured on both right and left lamina side, from leaf edge to midrib, at the widest part of the lamina (in general the middle point of the leaf, as indicated in Fig. 1) All measurements were processed using the TPS Dig program [23].

Statistical analyses were carried out in the 374 undamaged (not grazed, not visibly attacked by herbivores or pathogens) fully developed leaves. Repeated measurements were carried out on 79 leaves from plot 6. A mixed-model ANOVA was first used for estimating asymmetry relative to measurement error (individual interaction x side), DA and AS, following the approach of Palmer and Strobeck [5]. We tested the accuracy of measurements by calculating a Pearson correlation coefficient (r^2) between original and repeated measurements for each side. Then, deviation from normality of (R-L) distributions was assessed using the Kolmogorov-Smirnov (K-S)-test of normality. A one-sample t-test for departure of the mean of (R-L) from an expected mean of zero was also performed, while AS was analyzed using measures of platykurtism and departures from normality with one-sample (K-S)-test. Each type of asymmetry in morphological traits is characterized by a different combination of the mean and variance of the distribution of (R-L) differences [5], where R and L represent the measurement of right and left sides of bilaterally symmetrical traits. DA is characterized by a normally distributed (R-L) where the mean departs significantly from zero. AS is associated with a bimodal distribution of (R-L) about a mean of zero or, more subtly, as a broad peaked unimodal (platykurtic) distribution.

The FA of leaves was calculated according to Palmer and Strobeck [5]: $FA_4 = var(R–L)$, where var is the variance, and R and L are the right and the left side, respectively; σ_i^2 was calculated by partitioning measurement error out of the R or L side x individual mean squares of ANOVA results. These ANOVAs were performed on all replicates, and involved side (R or L) as a fixed effect, individuals as a random one, and their interaction. The correlation between concentration of CO_2 and level of FA and length of leaves were tested using the Pearson correlation coefficient.

Results

The between–individual variation in no DA of leaf width and angle was significantly larger than that of the measurement error. On the other hand, the degree of asymmetry was larger than the degree of measurement error. We did detect evidence of DA; mean asymmetry in each trait was not significantly different from zero with a leptokurtic distribution, compatible with FA, though incompatible with AS.

We found a significant and negative correlation between CO_2 concentration and leaf FA; r^2 between CO_2 concentration and FA of leaf width was 0.85 ($P = 0.032$), while r^2 between CO_2 concentration and FA of leaf angle was 0.75 ($P = 0.089$) (Table 2, Fig. 2). That means that with increased CO_2 concentration the leaf FA decreased. If FA is considered as a measure of DI, it could be stated that the DI was lower under elevated CO_2 than in control plot.

The correlation between CO_2 concentration and leaf length was positive, but not significant ($r^2 = 0.63$, $P = 0.177$). There was no correlation between the leaf length and FA of leaf width ($r^2 = 0.08$, $P = 0.137$), while the correlation between the leaf length and FA of leaf angle was very low but significant ($r^2 = 0.16$, $P = 0.001$) (Fig. 3).
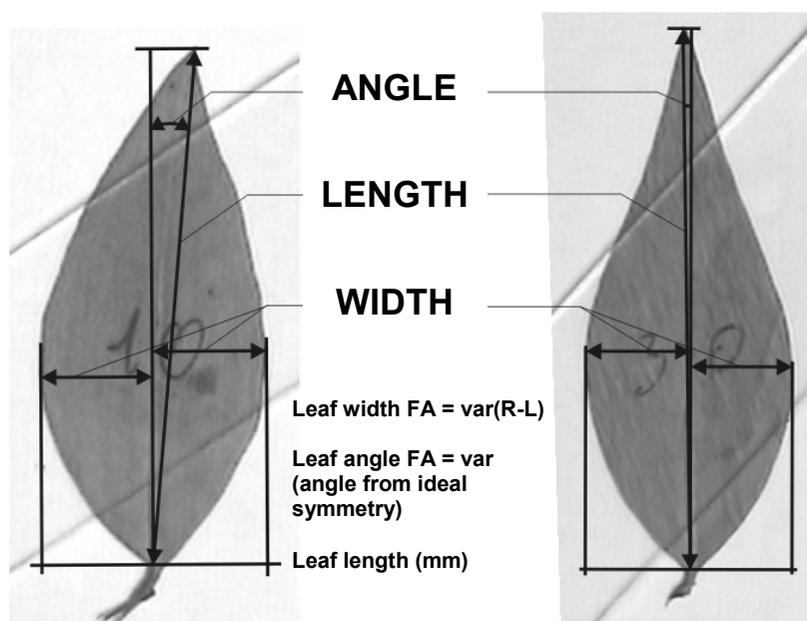

Fig. 1. Schematic representation (not to scale) of measurements used to define FA in *M. communis*, showing angle, length and width of the lamina. The FA is characterized by a different combination of mean and variance of the distribution of (R-L) differences, where R and L represent the measurement of right and left sides of bilaterally symmetrical traits.
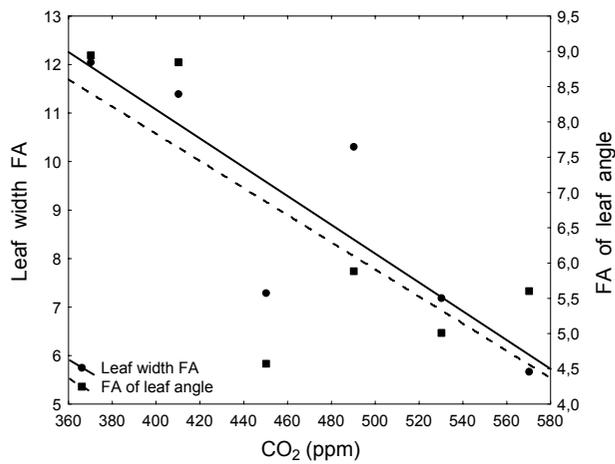
Fig. 2. Correlations between CO_2 concentration and leaf width FA and FA of leaf angle. See text for details and coefficients.
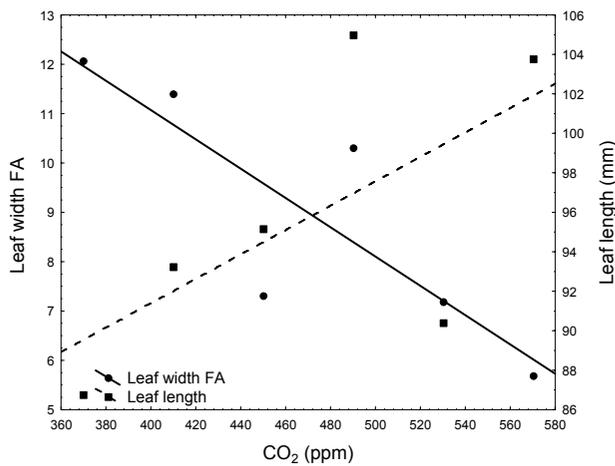

Fig. 3. Correlations between CO_2 concentration and leaf width FA and leaf length. Each point of the leaf length represents the mean for samples on single plot along the CO_2 gradient. See text for details.

It was confirmed that under elevated CO_2 leaves possibly grow longer (bigger); but leaf length and leaf FA appeared to be independent covariations.

Discussion

Leaf asymmetry estimates can be affected by many environmental factors, which need to be considered during sampling to avoid biased interpretation of results [12]. The importance of sample size has also been evidenced [24]. To use FA as a measure of DI in leaves, it is necessary to account for size dependence, measurement error and departures from ideal leaf FA [5, 25]. Plants are modular, with a number of repeated structures, so that an individual can be considered as a meta-population of leaves, and numerous measurements can be performed on each unit [26]. Microclimate conditions may not affect leaf FA, as in Pistacia lentiscus growing on north and south facing slopes [2], while differences within the crown in Ficus carica were ascribed to microclimate variation [14]. Plants used in our study have been growing on the same slope, at a distance of a few hundred meters from each other, and with similar microclimatic conditions (light and rainfall); no relevant differences in soil nutrients were evidenced in the study area as well [21].

A clear effect of elevated CO_2 on FA was evidenced in *M. communis* leaves; leaf FA appeared to decrease progressively, as CO_2 concentration increased. Our results confirm what was obtained by Cornelissen et al. [17] on *Quercus myrtifolia* and *Quercus geminata* (in a short-term experiment). Cornelissen et al. [17] evidenced a lower nitrogen concentration in leaves of *Quercus* species grown under elevated CO_2 concentration, which is consistent with results on this *M. communis* population at "I Borboi" [27]. This behaviour is considered common in plants grown under elevated CO_2 concentration, and may be related to downward regulation of photosynthetic enzymes in *M. communis* grown under elevated CO_2 concentration, as hypothesized by Tognetti et al. [20, 22] after analyzing carbon isotope composition in leaves. In *M. communis*, the higher carbon availability may have reduced the negative impact of competition, as evidenced, in the same CO_2 spring, for *Q. ilex* [28]. In several experiments, different woody species from

Table 2. Values of FA variance for leaf width and angle in *M. communis* at the "I Borboi" natural CO_2 spring.

Plots (number of leaves)	FA - variance of leaf width	FA - variance of leaf angle	Mean (min-max) of leaf length (mm)
1 (60)	5.68	5.61	103.8 (79.1-130.7)
2 (54)	7.19	5.02	90.4 (64.3-119.5)
3 (61)	10.31	5.90	105.0 (60.6-129.8)
4 (60)	7.31	4.58	95.1 (30.3-114.3)
5 (60)	11.40	8.86	93.2 (31.9-116.7)
6 (79)	12.07	8.95	86.8 (59.6-102.7)

natural CO_2 springs have been reported to display higher photosynthetic rates than those from control sites, in spite of downward regulation [29, 30], and both the suboptimal nature of atmospheric CO_2 concentration and the homeostatic nature of plants are well documented. Growth promotion is among the effects of elevated CO_2 concentration, and an increased leaf FA has been attributed to quicker and enhanced growth [11, 31]. Yet in *Quercus* species Cornelissen et al. [17] observed a reduction in leaf FA under elevated CO_2 concentration in spite of an increase in leaf area. However, this behavior is not common to all Mediterranean woody species; the long-term exposure of forest trees to elevated CO_2 concentration may even result in a reduction of leafiness [28, 32], suggesting that the growth strategy of Mediterranean sclerophylls prioritizes water saving over carbon uptake.

Water stress has been reported to increase DI both in herbaceous plants and in shrub species [33, 34]. Nevertheless, an analysis of FA in *Q. ilex* leaves from areas characterized by different precipitation regimes [35] showed that plants living in stressful sites were more symmetrical; leaves from non-stressful sites were prone to FA in drought years. Conditions experimented by plants may play a major role in leaf FA response to stress [12]. The reduction of transpiration in *M. communis* under elevated CO_2 concentration, due to reduced stomatal opening and density [20], may lead to enhanced water saving and explain the decrease in leaf FA under elevated CO_2 concentration [17]. *Myrtus communis,* grown under elevated CO_2 concentration at "I Borboi," showed a reduction in xylem embolism and an increase in hydraulic efficiency [36]. Therefore, improved water relations could represent a good explanation for the observed reduction in leaf FA.

Short-term CO_2 enrichment experiments have often shown a decrease in the concentration of plant nutrients [37], which could further explain FA reduction in *M. communis* leaves under elevated CO_2 concentration at "I Borboi." Peñuelas et al. [37] found a lower concentration of most microelements in *M. communis* under elevated CO_2 concentration at "I Borboi" than at the control site; only Mg, Mn and S increased - the last one probably in consequence of the H_2S emitted by the CO_2 spring. Leaf FA has been shown to be positively associated with environmental stress such as pollution load [38]. However, a direct effect of the exposure to low H_2S concentration on FA reduction, although possible, seems unlikely, as well as other effects on leaf morphology. Different nutrient economy and improved water relations in consequence of CO_2 enrichment, therefore, can be considered as key factors for FA reduction.

Several authors have evidenced a relationship between increasing leaf FA and decreasing defense compounds, which, together with higher nitrogen contents, make asymmetric leaves more palatable. Peñuelas et al. [27] have shown that the concentration of phenolics in the same *M. communis* population was not influenced by the distance from the main CO_2 degassing vent and thereafter by changing CO_2 concentration. These results contradict carbon resource-sink hypotheses and may be ascribed to complex environmental conditions, uneven resource availabilities, photosynthetic down regulation and homeostatic organism nature. Yet, the response seems to be species specific [32, 27] and suggests caution in attributing changed structural compounds, and consequential implications for plant-herbivore interaction, to long-term elevated CO_2 concentration in natural conditions. The ability of individuals to cope with current environmental conditions, possibly reflected in leaf asymmetry, may be affected by the evolutionary process [8, 4]. Adaptation and selection could explain the tendency towards decreased leaf FA in plants from the CO_2 spring relative to ambient conditions; the more symmetrical leaves under long-term elevated CO_2 concentration were more developmentally stable in these conditions.

In conclusion, FA of *M. communis* leaves might represent an environmental index for Mediterranean-type ecosystems. At the natural CO_2 spring "I Borboi," a reduction in leaf FA was ascribed to the positive effect of elevated CO_2 concentration on developmental stability, affecting water use and carbon balance of plants. Consequences of long-term elevated CO_2 concentration on plant-herbivore interaction and litter decomposition, which are relevant to global carbon budget, would merit further investigation.

Acknowledgments

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