



Reduced developmental stability in *Tilia cordata* leaves: effects of disturbed environment

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Abstract

Background and Purpose: Developmental stability (DS) or homeostasis refers to the ability of an individual to produce a consistent phenotype in a given environment. Reduced DS can result from a wide variety of environmentally (or genetically) induced perturbations. The main aim of the presented paper is to highlight the importance of the differences in ability of *Tilia cordata* leaves to buffer their development under contrasting environmental conditions and points to the concept that developmental stability is character specific.

Materials and Methods: Three different techniques were performed in this study: fluctuation asymmetry (FA) and leaf size as integrative measures of environmental stress during leaf developmental processes and within-plant variance in leaf morphology, presented as coefficient of morphological variation (CV). The study tested the hypothesis that the population from a chronically polluted area would express greater developmental instability in leaf traits. Two bilateral, linear dimensions on each leaf: leaf width (LW) and lobe length (LL) were analyzed.

Results: The three different measures of developmental stability all showed a trend for *T. cordata* leaves in the polluted area to be developmentally less stable than leaves from the reference area. Leaves in the reference area were significantly larger compared with those from the polluted site. Although leaves tend to be larger on the outside of a tree's crown, the pattern found here was the reverse. Both, outside and inside leaves from the polluted area had significantly higher FAs than leaves from the same position sampled in the reference area for both traits. Within-tree variance assessed as CV showed that LL was a more variable measure than LW. Moreover, the data suggest that LL is under more selective pressure to adapt to current environmental conditions than LW.

Conclusions: Obtained data suggest that *T. cordata* leaves may represent a reliable indicator for developmental stability evaluation studies based on an assay using a combination of end-points. Furthermore, my results highlighted the differences in ability of leaf morphometric characters to buffer their development under contrasting environmental conditions.

INTRODUCTION

Developmental stability or homeostasis refers to the ability of an individual to produce a consistent phenotype in a given environment (1). By studying developmental stability it might be possible to assess the synergetic effects of toxic compounds or interactions between

pollutants and other stressful factors (2), which are either difficult or impossible to study by other means.

Reduced developmental stability can result from a wide variety of environmentally (or genetically) induced perturbations (3, 4, 5).

Fluctuating asymmetry or FA (5), as random differences in the development of both sides of a bilaterally symmetrical character (6), has been proposed as an indicator of environmental as well as genetic stress (3, 7, 8, 9). Stress is considered to be a significant and lasting deviation from favorable conditions that leads to abnormal demands and destabilization of vital processes (10). As stress during development may influence developmental precision, FA has been proposed as a potentially useful tool for monitoring stress levels in natural populations (1). Thus FA has been used to estimate developmental instability (DI), inability of a bilateral organ or organism to buffer its development against disturbances and to produce a predetermined phenotype (11). However, the main drawback of the use of FA as a diagnostic tool is the difficulty in discriminating the genetic from the environmental components producing FA in the field (12). Although developmental instability, measured as fluctuating asymmetry, is expected to be positively related to stress and negatively to fitness, empirical evidence is often lacking or contradictory when patterns are compared at population level (13). Also, Parsons (8) has suggested that only extreme stressors, which are to be expected in marginal or disturbed environments, can increase FA under field conditions (may lead to detectable FA alterations in organisms).

Besides the FA there are other indicators of developmental instability such as the frequency of asymmetric traits, the frequency of phenodeviants, the within-individual variance and the coefficient of variation (CV) or the relative variation in size of a trait, both among and within populations (11). In this century, investigators delving into the nature of species have collected data on the variance of vegetative and floral characters in both natural populations and common gardens (14, 15). Because the plant body consists of repeated nodal units, a variety of within plant measures of variation can be used to estimate stability (1, 16, 17). Studies using within-plant variance as a measure of stability suggest that variation in developmental stability exists between lines and populations (18). Paxman (16) found differences in stability among several lines of tobacco in leaf and flower traits. However, care must be taken that the units used to estimate variance are indeed developmentally comparable and not confounded by the effects of programmed developmental changes along the shoot (16) or by environmental changes over time (18). Under the accepted theory, if there was strong developmental stability within the organisms' growth processes, then morphological variance would be small. Hence, this had led authors to predict that when developmental processes are highly stabilized, the coefficient of variation is small (11). Therefore, the presence of developmental stress is expected to positively relate to the levels of FA and the phenotypic co-

efficient of variation for analyzed morphological traits. In support of this, (19) and (20) found a positive relationship between fluctuating asymmetry and the coefficient of variation in *Drosophila* and *Plantago major* L. respectively. However, there are sometimes (occasionally) problems in applying and interpreting CV, especially when testing for genetic differences among populations, for example, if trying (when attempting) to elucidate the relationship between heterozygosity and developmental stability. If the genes under investigation determine the expression of the trait that is being assessed in terms of developmental stability, then it may be expected that morphological variance will rise, rather than fall, with increased heterozygosity (21, 22, 23). In this case a stability index of morphological variance could only be applied if genes involved did not directly influence the development of the trait (24).

In the present study, developmental stability in two populations of the small-leaved lime *Tilia cordata* Mill. (Tiliaceae), under different environmental conditions, was estimated using an assay based on a combination of end-points. Three different techniques were performed in this study (leaf size and FA as integrative measures of environmental stress during leaf developmental processes and within-plant variance in leaf morphology presented as coefficient of morphological variation). The study tested the hypothesis that the population from a chronically polluted area would express greater developmental instability in leaf traits.

MATERIALS AND METHODS

Study areas

Two sites were selected as sampling areas in Serbia. The Pančevo site is a site close to a large industrial settlement (estate/zone). The industrial area (covers 290 hectares) is the site of a large petrochemical complex and fuel storage site in Serbia and includes an ammonia plant (»Azotara«, founded in 1962), a factory for chemical fertilizers (»HIP Petrohemija«, founded in 1975), and a crude oil refinery (»Rafinerija Pančevo«, founded in 1968). The data obtained from a long-term monitoring study of the Pančevo site, based on the UNMIX Version 2.4/MATLAB Version 6.5 (25), at three measured locations (Vojlovica, Starčevo and Vatrogasni dom) showed that in the Pančevo site extremely high concentrations of toxic compounds were detected (26). Also, Pančevo is extremely windy and is exposed to the effects of two kinds of winds (southeastern and northwestern) which directly bring pollutants from the industrial zone. For this kind of investigation, however, it is important to emphasize/stress certain chemical compounds, their metabolites and unwanted by-products because they represent the most dangerous chemical pollutants with long-term negative effects on the environment, human health and living organisms. Results showed that the main contaminants released from »Azotara« are: NH_3 , NH_4^+ , NO_x , SO_x , CO (30). Results also indicated that »Azotara«, together with other sources from the industrial zone have

released SO₂ (9%), benzene (9%), NO₂ (21%) and TNMHC (7%) compared with the total amount of toxic compounds found in the air. An investigation performed in »HIP Petrohemija« showed that the »bio-filter« is one of the most dangerous contaminants of the air (with emissions of benzene of about 64kg h⁻¹ and of xilene of about 34kg h⁻¹). Furthermore, there are other pollutants released in huge concentrations from »HIP Petrohemija« such as: benzene, xilene, hydrocarbons from oil and oil derivatives, S, HS. Interestingly, up to 4.5% in oil derivatives is represented by sulphur. Also, in natural gas, HS is present (ed) in the range 0.86–5.40 mg/L. Moreover, »Rafinerija Pančevo« is the dominant source of environmental contaminants such as volatile organic compounds, CO, SO_x, NO_x, polycyclic aromatic compounds (their metabolic transformations by aquatic and terrestrial organisms into carcinogenic and mutagenic metabolites), benzene, xilene. The factory, therefore, represents the first step in the process of benzene and xilene production. The highest concentrations of benzene were detected late at night and early in the morning, which is positively correlated with the most intensive time of traffic flow. In 2005 the average value for the amount of released benzene in the atmosphere was 5 µg m⁻³ (26). Furthermore, recent papers have emphasized the presence of benzene in the air of urban environments caused by increased traffic flow (27).

Vince village is an area far from any known contamination and is practically closed to traffic, and is therefore used as a control area. The two sites are approximately 150 km apart (Figure 1).



Figure 1. Locations of the sampling sites.

Sampling and statistical treatment

Tiliaceae is a family of trees, shrubs or rarely herbs; it includes 400 woody species, among those 30–40 species of *Tilia*, most of them found in the tropics. Ten species are found in the temperate region of the northern hemisphere. In Europe four species are present: *Tilia cordata* Miller, *Tilia platyphyllos* Scop., *Tilia tomentosa* Moench and *Tilia dasystyla* Stev. (28). The core region for *T. cordata* is central and Eastern Europe (29). In Serbia, *T. cordata* grows in several types of mixed forest, i.e. commonly in the Quercus-Carpinetum forest type. The trees favor good loamy site conditions, but they can also be found on sandy infertile soils. Climatic conditions and human impact have been a serious threat to the distribution of *Tilia* in most European countries (30). The leaves of *T. cordata* are simple, alternatively cordate or orbiculate and the margins are serrate (31).

During July of 2005 leaves were obtained from 7 trees of *T. cordata* from two sites in Serbia. Trees were of similar age/height (≈ 40 years/10m). Twenty-five fully developed leaves with respect to the position of a leaf within the tree's crown (inside, outside), thereby 50 leaves per tree (350 leaves per each site) were analyzed. All leaves were sampled from the same height of approximately 2 m. Leaves were washed, dried between sheets of filter paper, measured and then analyzed. Each analysis was performed on the same leaves.

Two bilateral, linear dimensions on each leaf: (1) leaf width (LW) and (2) lobe length (LL) are presented in Figure 2. Both morphometric traits were measured with a digital caliper (0.01mm accuracy). To avoid measurement errors each measurement of the left and right sides of each leaf was performed twice, during two independent sessions. Additionally, all measurements were performed by the same person (M. V.). Trait size for leaf width and lobe length was calculated as the average value of right (R) and left (L) sides; $(R+L)/2$. Individual signed asymmetry was calculated as $(R-L)$ and absolute asymmetry was calculated as the absolute value of the $|(R-L)|$.



Figure 2. Drawings of *T. cordata* leaf showing lobe length (LL) and leaf width (LW) measurements taken.

To estimate FA values for the bilaterally symmetrical character three indices of (6, 32) were used:

- 1) $FA_1 = \text{mean } |R-L|$;
- 2) $FA_4 = \text{variance } (R-L)$;

3) As a better estimate of the true between sides variance, σ_1^2 was calculated by partitioning measurement error out of the side \times individual mean squares of the two way mixed-model ANOVAs. These ANOVAs were performed on all replicates and involved side (R or L) as a fixed effect, individuals as random one, and their interactions. Additionally, this procedure allowed simultaneous testing for the presence of DA (a significant mean square of the side factor) (32).

Before proceeding with the asymmetry analysis, statistical tests were carried out to detect the features confounding analyses of FA (6, 32), such as measurement error, directional asymmetry (DA) and antisymmetry (AS). Firstly, the accuracy of the measurements was tested by calculating a Pearson correlation coefficient (r^2). Secondly, deviation from normality of the (R-L) distributions was assessed using the Kolmogorov-Smirnov test of normality. These distributions were also tested for significant skewness (g_1) and kurtosis (g_2) according to (33). Thirdly, a one-sample t -test for a departure of the mean of (R-L) from an expected mean of zero was done. Fourthly, to determine if asymmetry increases with leaf size, a simple linear regression of absolute asymmetry on leaf size; $|R-L|$ on $(R+L)/2$, was used (32).

The three-way factorial MANOVAs were used in order to assess the effects of site, position, tree and their interactions on leaf size and absolute asymmetry values of leaf width and lobe length. The Sequential Bonferroni corrections (34) were applied to avoid »false« significant results in the MANOVAs.

Furthermore, FA values (FA_4 and the σ_1^2) are variances, so differences between samples can be detected by comparing the heterogeneity of variances. To detect differences between pairs of samples, F -test (35) was calculated for both FA_4 and σ_1^2 . This test is simply a ratio of the larger over the smaller variance. The significance of this ratio need only be looked up in a statistical table for the appropriate degrees of freedom.

Within-tree variance and coefficient of variation

Univariate ANOVAs were carried out in order to reveal the possible within-tree differences among leaves measured. These ANOVAs of each trait for each site were carried out separately with trees within sites designated as a random effect. Because there were no significant differences among leaves within trees under a given environmental condition (see RESULTS), within-tree size differences as well as coefficients of variation were calculated for each tree separately by site and by trait.

Additionally, in order to assess differences in CVs between sites it was necessary to calculate averages of CVs for each character measured in *T. cordata* populations.

In order to compare the mean values of CVs, Student's t -test was performed.

RESULTS

Preliminary results on leaf size and asymmetry analyses

The Pearson correlation coefficient (r^2), between original and repeated measurements, for each side of each leaf (for left side: $r^2 = 0.999$ and for right side $r^2 = 0.999$) showed that the measurements are reliable.

Descriptive statistics for leaf size and asymmetry in *Tilia cordata* populations are presented in Table 1. All (R-L) distributions were normal (Kolmogorov Smirnov test results: $0.060 < d < 0.071$, $p > 0.20$) and none of them was significantly skewed to the right ($+g_1$) or to the left ($-g_1$). Three out of eight analyzed (R-L) distributions were slightly, but not significantly platykurtic ($-g_2$), whereas the rest were leptokurtic ($+g_2$). Thus, no antisymmetry is present. Results also showed that there was no statistically significant departure of the mean of (R-L) from expected mean of zero (indicating the absence of DA; $-1.819 < t < 1.250$, $0.09 < p < 0.26$). Furthermore, the data showed that there was no significant size-dependence of FA within samples for both traits ($0.550 < p < 0.860$). There was no need to repeat all relevant analyses with relative asymmetry (asymmetries divided by size) or asymmetries calculated from log-transformed distances (no data transformation was necessary).

Results on leaf traits showed that the interaction terms in the two-way ANOVAs were highly significant ($p < 0.001$). This signifies that the between side variation is significantly larger than the measurement error. Thus, tests for FA differences among samples are justified (36). These ANOVA's also showed that there was no significant DA in leaf traits ($0.00479 < (\text{side MS}) < 0.011$; $0.061 < F < 0.825$; $0.375 < p < 0.806$) confirming the results of the t -test. Additionally, the highest levels of σ_1^2 and FA_4 were detected in outside leaves in the Pančevo site for LL, Table 1.

Leaf size and asymmetry

MANOVA's results indicated that statistically significant differences in mean leaf size values were observed between the two sites, two positions, and additionally for the two two-way interactions (position by site and position by tree), Table 2. Inside leaves were larger than outside leaves (t -test' results $t = 2.209$, $p < 0.05$) as well as leaves collected in the reference area compared with leaves from the polluted site (t -test' results $t = 2.345$, $p < 0.05$). Results therefore indicated that the largest leaves were the inside leaves in the reference area.

Moreover, MANOVA's results showed that there were significant differences in asymmetry values for LW and LL between the two sites, two positions, as well as for the two-way interaction (positions by site), Table 3. Univariate ANOVAs' results per trait showed that leaves of *T.*

TABLE 1

Summary statistics for leaf traits (lobe length = LL and leaf width = LW) measured with respect to position of a leaf within a tree's crown (outside, inside) in two *T. cordata* populations.

Site	(R + L) / 2bbbb		IR - LI vs. (R + L) / 2		X ^c	Skew	(R - L)		ANOVA					
	Trait	Position	N	\bar{X}			\pm SD ^a	Slope ^b	F	\pm SE	Kurtosis (g ₂)	\pm SE	Variance ^d	$\sigma_1^2 (\times 10^2)$
Vinci village	LL	outside	175	9.510	± 0.928	0.0071	0.651	-0.017	-1.925	± 0.219	9.698	0.016	0.212	50.560
	LL	inside	175	9.571	± 0.964	0.0039	0.720	-0.001	2.157	± 0.200	11.567	0.014	0.019	2.596
	LW	outside	175	5.144	± 0.695	-0.0050	0.005	-0.014	-0.013	± 0.354	-0.792	0.011	0.123	4.200
	LW	inside	175	5.165	± 0.608	-0.0060	0.002	-0.010	0.093	± 0.536	-0.101	0.001	0.101	4.160
Pančevo	LL	outside	175	7.313	± 0.746	0.0347	0.371	-0.127	-0.015	± 0.219	-0.185	0.253	4.448	60.657
	LL	inside	175	7.354	± 0.710	-0.0064	0.033	-0.179	-1.218	± 0.200	3.068	0.216	3.748	35.416
	LW	outside	175	4.164	± 0.155	-0.0300	0.160	0.073	2.566	± 0.330	0.464	0.055	0.534	43.080
	LW	inside	175	4.168	± 0.166	0.0020	0.000	0.364	1.761	± 0.501	3.038	0.024	0.267	51.110

^a = standard deviation; ^b = regression slope; ^c $\text{IR} - \text{LI} / \text{vs. } (R + L) / 2$ = directional asymmetry values; ^d = index # 4 of (10, 36)

TABLE 2

MANOVA's results obtained for the effects of site, position, tree and their interactions on size values of LW and LL in *T. cordata*

Source of variation	df _{1,2}	Wilks's lambda	p-value
Site	2,671	0.962013	0.040*
Position	2,671	0.939180	0.045*
Tree	12,1342	0.967274	0.342
Position x site	2,671	0.925503	0.018*
Tree x site	12,1342	0.695057	0.237
Position x tree	12,1342	0.862395	0.047*
Site x position x tree	12,1342	0.965118	0.307

*Significant at $p < 0.05$ after sequential Bonferonni correction (38)

cordata of long-term exposure to environmental pollutants had significantly greater absolute FAs compared to those from the reference site (for LL: $F_{(1,672)} = 6.369$, $p = 0.014$, for LW: $F_{(1,672)} = 3.929$, $p = 0.049$). ANOVAs' results also indicated that significant differences in FA values resulted from differences between positions in the effects of the site. Thus, outside leaves in the polluted area were more asymmetrical.

Furthermore, comparisons of the σ_1^2 values indicated that there were significant differences between sites with respect to the leaf position within a tree's crown, for both leaf traits. Both, outside and inside leaves from the polluted area had significantly higher σ_1^2 values than leaves from the same position sampled in the reference area, for both traits (F-test results for LL: $20.981 < F_{(174,174)} < 197.263$, $\alpha = 0.01$; F-test results for LW: $2.941 < F_{(174,174)} < 4.341$, $\alpha = 0.01$).

Concerning differences in FA₄ values between samples, results are similar to those found for σ_1^2 values

TABLE 3

MANOVA's results obtained for the effects of site, position, tree and their interactions on absolute asymmetry values of LW and LL in *T. cordata*.

Source of variation	df _{1,2}	Wilks's lambda	p-value
Site	2,671	0.923366	0.014*
Position	2,671	0.838783	0.015*
Tree	12,1342	0.980222	0.540
Position x site	2,671	0.912140	0.015*
Tree x site	12,1342	0.985703	0.328
Position x tree	12,1342	0.959911	0.236
Site x position x tree	12,1342	0.971164	0.102

*Significant at $p < 0.05$ after sequential Bonferonni correction (38)

(F-test results for LL: $15.429 < F_{(174,174)} < 15.813$, $\alpha = 0.01$; F-test results for LW: $5.00 < F_{(174,174)} < 24.00$, $\alpha = 0.01$).

Within-tree variance and coefficient of variation

There were no significant differences among leaves within trees under given environmental conditions ($MS_{Pan\check{c}evo/LL} = 0.0181$, $F = 2.128$, $p = 0.146$; $MS_{Pan\check{c}evo/LW} = 0.079$, $F = 0.416$, $p = 0.519$; $MS_{vinci/LL} = 0.042$, $F = 0.339$, $p = 0.561$; $MS_{vinci/LW} = 0.210$, $F = 1.454$, $p = 0.229$). Therefore, within-tree variance and the coefficient of variation were justified as measures of developmental instability.

Moreover, within-tree variances and CVs were calculated for each tree separately by site and by trait (Figures 3a, b). Higher CV values in the Pančevo site than in Vinci village were observed for both traits. Coefficients of variation for within-tree size differences in Vinci village for lobe length averaging from 9.18–11.367; in the Vinci site for leaf width averaging from 7.677–9.272; in the Pančevo site for lobe length 11.530–12.978 and in the Pančevo site for leaf width averaging from 8.413–9.539.

Concerning results obtained on differences between sites in the averages of CVs calculated for leaf size measures (Figure 4) there was significant difference between populations for lobe length ($t_{LL} = 2.54$, $p < 0.05$; the contaminated area (Pančevo) had significantly higher CV value for LL than the unpolluted site (Vinci). However, comparison in averages of CVs obtained for leaf width showed that there was no significant difference between sites ($t_{LW} = 1.004$, $p > 0.05$).

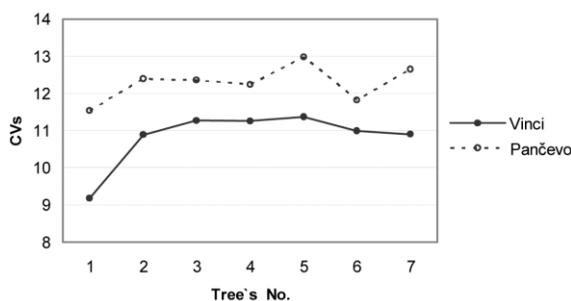


Figure 3a. Averages of coefficients of variation (CVs) for lobe length (LL) measured for each tree separately by site.

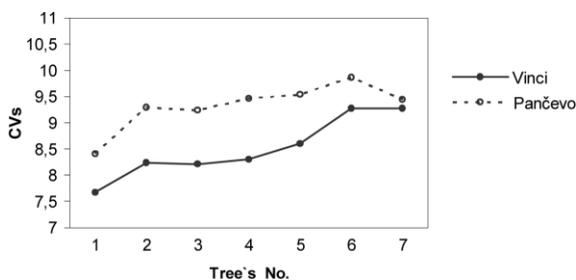


Figure 3b. Averages of coefficient of variation (CVs) for leaf width (LW) measured in each tree separately by site.

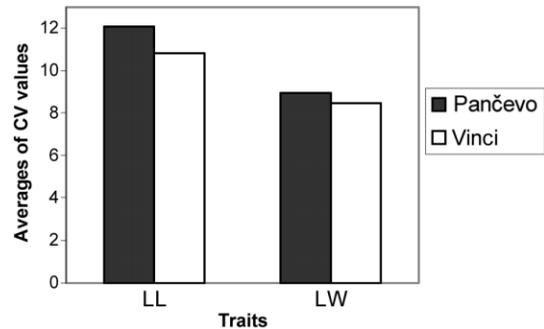


Figure 4. Averages of CVs for lobe length (LL) and leaf width (LW) in *Tilia cordata* leaves from the two sites.

DISCUSSION

One of the most general types of stress experienced by plants is pollution. Pollutants can cause injury to plants, distorting their metabolism, altering their appearance, and lowering their agricultural productivity. All pollutants generate their effects by different mechanisms but most must enter the tissues via the stomata to cause their specific injuries. The injuries are best described by the visible injury or tissue damage symptoms but also plant metabolism tends to be shifted away from the more productivity state. Plants respond to air pollutants similarly to other stresses on several levels: exclusion, tolerance, and repair. The response mechanism depends upon the concentration of the air pollutant, environmental conditions, and the developmental and metabolic state of the plant. Any response is detrimental to plant productivity because it costs the plant metabolic resources. For example, the stomata can close under the pollutant exposure to exclude the pollutant from the interior of the plant, thus preventing damage. However, stomata closure lowers photosynthetic CO₂ fixation and plant productivity will suffer (36).

The three different measures of developmental stability (leaf size and FA as integrative measures of environmental stress during leaf developmental processes and within-plant variance in leaf morphology presented as coefficient of morphological variation) showed a trend for *T. cordata* leaves in the polluted site to be developmentally less stable.

Leaf size varied between sites and within the crown of *Tilia cordata* (Table 2) Significantly larger leaves were observed in the reference area compared with those from the polluted site (Table 1). Therefore, there was a tendency to decrease lobe length and leaf width with pollution. Large leaves usually indicate rapid growth and high quality of habitat, which also suggests low stress. Habitat quality, however, refers to the ability of the environment to provide conditions appropriate for individual and population persistence (37). Thus, the obtained results may be explained by the differences among exposure levels to the negative effects of environmental stressors. These findings are similar to those reported by Jahan and Iqbal (38) performed on *F. bengalensis*, *G. officinale* and *Eu-*

caliptus sp. The leaves that were collected from a polluted area showed reduction in all parameters investigated. In previous years other workers (investigators/studies?) also showed significant reduction in different leaf variables in the polluted environment in comparison with clean (unpolluted) atmosphere. Ninova *et al.* (39) in their study on *Platanus acerifolia* showed changes in leaf blade and petiole size in polluted air. Similarly, leaf anatomy of the above mentioned species also showed reduction in cuticle, epidermis, hypodermis, palisade, parenchyma cells in polluted leaves as compared to leaves collected from an unpolluted area. Significant results were particularly observed in spongy parenchyma and lower epidermis in *F. bengalensis* and *Eucalyptus* sp., respectively. Changes in shape and structure of thin walled mesophyll cells have been widely reported. Mesophyll cells are thin walled and are in direct contact with the environment through stomates. The parenchymatous cells of spongy parenchyma become flattened due to continuous exposure to pollutants. A significant reduction in spongy parenchyma was in the leaves of *F. bengalensis* of a polluted area. Similarly, Iqbal (40) has shown significant reduction in palisade and spongy parenchyma in the leaves of white clover of a polluted population. On the other hand Godzik and Halbawacks (41) have shown fine and irregular cuticular folding on each epidermal cell of both adaxial and abaxial sides of *Aesculus hippocastanum* in the vicinity of air pollution sources.

Leaf size and shape can change significantly within a plant due to developmental age of the plant and the position of the leaf within the plant (heteroblasty) (42). Also, it has been reported that leaves tend to be larger on the outside of a tree's crown, presumably because of great light intensity at this position (43). However, the pattern found here is the reverse; larger leaves are the inside leaves, both within and between sites. The significant position by site effect on leaf size may therefore be explained by the differences in intensity as well as in the availability of the complex mixtures of environmental stressors to affect exposed leaves, indicated that it is possible that pollutants affect leaves differentially (differently).

Additionally, the results of this study revealed that besides leaf size, leaf asymmetry also varies between sites and within the crown of *Tilia cordata* trees (Table 3). Although there are exceptions, more recent work (research) has shown that developmental stability estimated by fluctuating asymmetry is predictable with respect to the habitat quality. Developmental stability measured as fluctuating asymmetry is expected to be positively related to stress and negatively to habitat quality and fitness.

In this study leaves from the polluted area exhibited significantly greater σ_i^2 (FA) values (developmentally are less stable) than leaves from the unpolluted reference area as expected (Table 1). The data presented here are in accordance with those which reported that environmental stresses such as pollution increase asymmetry in plants. For example, Sherhukova (44) estimated developmental stability of small-leaved lime using fluctuating

asymmetry of the leaf traits. The authors concluded that in the industrial, polluted region homeostatic developmental mechanisms are weakened and expressed in the increased degree of leaf asymmetry than in control, reserved regions. Also, Velicković (20) pointed out the significantly higher FA levels (estimated as σ_i^2 values) for leaf width and vein distances within a leaf in *Plantago major* L. in an urban, polluted area than in a control, unpolluted site. Graham *et al.* (1) examined asymmetry of black locust leaves at several distances away from an ammonia production and storage facility in the Ukraine. They found that asymmetry declined with distance. Freeman *et al.* (45) report similar results for a variety of plant populations around chemical production facilities in Russia and the Ukraine. Leaves of soybeans grown under high-voltage transmission lines had greater fluctuation asymmetry than those grown 100 m away (46). Kozlov *et al.* (47) found higher fluctuating asymmetry levels of birch leaves near metal smelters.

In the opinion of the author differences in FAs between sites may also be explained by differences in intensity as well as in availability of the complex mixtures of chemical pollutants, their unwanted by-products and metabolites to affect leaves. Additionally, σ_i^2 means variation in the between-side differences among individuals, thus significant differences in σ_i^2 among sites implies genetic variations among individuals and/or environmental variation in sites among individual plants. A polluted, artificially disturbed, area is often more homogeneous than a natural area and selection due to pollution may reduce genetic diversity (unpublished data). Thus, it is important to consider that the possibility of genetic and/or environmental homogeneity causes this result of σ_i^2 .

Many plants have sun and shade leaves and Mitton (48) suggested that these two kinds of leaves differ in developmental stability. Based on results obtained for the fig (*Ficus carica* L.), Cowart and Graham (43) found that the outside crown is a more stressful environment for leaves than the inner crown and that increased asymmetry of outside leaves probably reflects stress rather than plasticity. Although light intensity is less on the inside, leaves on the outside are subjected to greater cold, heat, ultraviolet light, visible light, desiccation, herbivore load and environmental stresses (49). Thus, outer leaves, because of their position, are more susceptible to the negative effects of complex mixtures of environmental variables, compared to inner leaves. Sakai and Shimamoto (50), however, showed that the effects of position on leaf asymmetry in *Nicotiana tabacum* depended on the particular genotype being examined.

Moreover, a large number of authors have used the degree of morphological variation as an index of developmental homeostasis, particularly for detecting differences among populations (*e.g.* 18, 19, 20, 51, 52, 53).

Certain data from developmental stability analyses in this study are similar to those reported by (20) for the common plantain *P. major*. Both, *P. major* and *T. cordata* leaves from contaminated areas had increased FAs and

within-plant variation for leaf traits than in the control, reference area. The use of data from *P. major* is more of a reference than for direct comparison.

In this study the author found within-tree variation assessed as coefficient of variation in *T. cordata* leaf morphology. Although higher CV values in the Pančevo site than in the Vinci village were observed for both traits (Figures 3a, 3b) as expected; significant differences between populations however were determined for one leaf trait, lobe length (Figure 4). Therefore, lobe length was a more variable measure than leaf width and may be an especially useful character in developmental stability analyses of leaves. Fisher (54) suggested that traits with high variation may be undergoing active evolution or have trivial effects on fitness. (Each individual integrates its response to the total environment, which generates the general response of the species within that environment, there is a band of adaptability of the individual to any environmental condition.). As Maynard-Smith *et al.* (55) speculated stability increases over time as populations become adapted to new environments, and any instability that might be involved in their divergence may have been selected away by now. Hoffmann and Parsons (56) suggest that stressful conditions can lead to rapid evolutionary change by causing intense natural selection. According to Møller and Swaddle (11), adverse environmental conditions may affect the evolutionary process at (a range of) different levels, and tend to facilitate adaptation to these scenarios. Thus, it is possible that lobe length is under more selective pressure to adapt to current environmental conditions than leaf width.

Finally, results suggest that *T. cordata* leaves represent a reliable indicator for developmental stability evaluation studies based on an assay using a combination of end-points. Furthermore, the results of the present study highlight the differences in the ability of leaf morphometric characters to buffer their development under contrasting environmental conditions.

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