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Leaf shape variation as a potential biomarker of soil pollution

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ABSTRACT

Halophytic plants play a fundamental role in salt marshes, influencing their structure, dynamics, and cycling of nutrients and minerals. These plants have the ability to retain metals in the soil, or absorb and retain them in underground structures, or transport them to their aerial structures. Here we aim to study shape variation in the leaves of *Cressa truxillensis* inhabiting the salt marsh of San Antonio Oeste, according to their proximity to a source of metals in the soil. A gradient of bioavailability of metal was observed in the soil, decreasing from the site closest to the source to the most distant point, where Zn was the most abundant metal followed by Pb and Cu. We used landmark-based geometric morphometric tools to study leaf shape variation. We observed more oval leaf growth on the farthest point of the pollutant's source, and lanceolate shape close to it. No significant among-site size differences were found. Collectively, these results suggest that the stress conditions associated with the soil metals' concentration generate changes in the leaf shape of *Cressa truxilensis*. Considering that this species has not been extensively analyzed, this study establishes a baseline and supports the use of the leaf as an early biomarker of stress by contamination in plants associated with marshes.

1. Introduction

In salt marshes affected by pollution, soils and plants play an important role due to their ability to hold the pollutants, hence altering their dynamics in the environment (Almeida et al., 2011; Duarte et al., 2010; Hung and Chmura, 2007). In this regard, plants interact with these elements modifying their bioavailability, absorbing pollutants from the soil and distributing them in their tissues, both accumulating them in the roots and rhizomes or in stems and leaves (Burke et al., 2000; Duarte et al., 2010; Idaszkin et al., 2014, 2017c; Reboreda and Caçador, 2007b). Like other halophytes, plants inhabiting in salt marshes are adapted to extreme conditions of flooding, and soil anoxia and high salinities. Furthermore, occupation of contaminated soils has been reported as well (Duarte et al., 2010; Idaszkin et al., 2011, 2015, 2017c; Redondo-Gómez, 2013). However, when pollutants such as heavy metals are in excess they could be toxic for the plants, having deleterious effects. In this sense, plants could display diverse strategies to counteract the soil metal excess, either limiting the uptake or transporting of the metal, or through internal tolerance mechanisms (Ashraf et al., 2010). Some of the most common signs that exhibit plants facing metal toxicity are decrease in growth and biomass production, alterations of the metabolism, activation of the antioxidant system, senescence, and morphological changes (in root, stem and/or leaves) (Kabata-Pendias, 2011; Nagajyoti et al., 2010).

Biochemical, physiological and morphological responses produced in organisms growing under stressful conditions, such as soil metal excess, can be considered biomarkers of stress. Currently, as opposed to the quantification of accumulated pollutants in soils and tissues, biomakers are considered more useful in environmental studies, being that they provide information on the potential effect of these substances on the health of organisms. Furthermore, these have the advantage of evidencing early symptoms of the damages caused by the contaminants, and therefore they can be used as an early sign of the presence of contaminants in the environment. Within the more common biomarkers used as a response in plants exposed to heavy metals are variations in the content of photosynthetic pigments, phytochelatins and non-protein thiols, free proline, phenolic acids and antioxidant enzyme activities (Ferrat et al., 2003; Keltjens and van Beusichem, 1998; Kirbag Zengin and Kirbag, 2007; Monni et al., 2001). Also, the use of these techniques could enable rapid, continuous, and low-cost monitoring protocols of the pollution's deleterious effects on communities, being the search of an overcoming method to be applicable in environmental quality researches a fundamental challenge.

On the other hand, an alternative way to evaluate the pollution

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effects in the organism shape is through the application of geometric morphometric methods (GMM), which allows the detection of subtle morphological variations (Márquez et al., 2017). This method allows the study of shape and size variations with a high level of detail (Adams et al., 2004; Bookstein, 1991). GMM, unlike the conventional linear-distance based morphometrics, enables a proper separation between size and shape variation (Bookstein, 1997). Another advantage is the preservation of the geometric information throughout the statistical analyses, which allow the detailed and graphic visualization of both the magnitude and the direction of the morphometric changes (Zelditch et al., 2012). In the last two decades the use of GMM has increased in zoological and botanical studies (Klingenberg et al., 2012; Viscosi et al., 2009; Viscosi and Cardini, 2011). Some of these assess the relationship between variations in shape of some structure and the pollution of their environment, with most of the previous research being performed on animals (Márquez et al., 2011, 2017; Primost et al., 2016). However, main plant studies using GMM are focused on determining patterns of variation in form at the inter- or intra-specific level, on structures such as seeds (Chemisquy et al., 2009), floral organs (Shipunov and Bateman, 2005), and leaves (Iwata et al., 2002; Jensen et al., 2002; Vieira et al., 2014). For example, GMM was used to classify and discriminate varieties of Orchids, based on variations in shape of floral pieces (sepals, petals and labellum) (Dalayap et al., 2011). Regarding pollution studies, Vujić et al. (2015) evaluated pollution impact on the petal shape of *Iris pumila* flowers using GMM, finding that plants growing on a site exposed to polluted air present a smaller and more rounded shape than plants from a site without contamination.

Previous studies evidence a gradient in soil metals content within the salt marsh surrounding the San Antonio Bay (Patagonia, Argentina) (Idaszkin et al., 2015, 2017a, 2017b, 2017c). This salt marsh is inhabited by the cord grasses of the genus *Spartina* and other halophytes such as *Sarcocornia perennis, Limonium brasiliense,* and *Cressa truxillensis*. In particular, *C. truxillensis* Kunth (Convolvulaceae) is a native perennial halophyte widely distributed in the American continent. It has small pubescent leaves (3–12 * 1.5–4 mm) with an elliptic to lanceolate form. It was precisely the shape of its leaves which motivated its selection as the subject of this study, considering that their size and their arrangement in a two-dimensional plane allow the application of geometric morphometrics techniques in 2D. Therefore, the main goal of this study was to evaluate the relationship between shape variations on leaves of *C. truxillensis* plants and the metal bioavailables in soils in the San Antonio Oeste salt marsh, which will allow postulating the use of a potential response as a biomarker of soil stress pollution.

2. Materials and methods

2.1. Sampling

We worked in San Antonio salt marsh, located surrounding the San Antonio Bay (40°44′S, 54°68′W), in a Natural Protected Area (Río Negro, Argentina, Fig. 1). Sampling sites comprised three sites within the salt marsh adjacent to the main tidal channel (sites called “A”, “B” and “C”), site A is located in the topographically higher area of the salt marsh and receives the surface runoff from the mining deposits drainage. Site B is located near the above site but in a lower topographic sector of the salt marsh, whereas site C is located in the same channel as sites 1 and 2, but in an external sector of the salt marsh with more marked tidal influence (Fig. 1).

Table 1
Number of leaves collected at each site (n), their length (between 1 and 2 landmarks; mean (SD)) and width (between semilandmarks 6–11; mean (SD)) per site.

Sites	n	Length (mm)	Width (mm)
A	133	6.45 (0.90)	3.10 (1.07)
B	116	6.65 (0.89)	3.34 (1.16)
C	97	6.60 (0.73)	3.44 (1.10)

In order to study the size and shape attributes on leaves of *Cressa truxillensis*, plant samples were collected during December 2015 within each sample site. From these plants, branches with flowers (Table 1) from which the fully deployed leaf was separated immediately below the last flower (bracts) were randomly collected. A total of 346 leaves were obtained. In order to avoid the loss of turgor and consequently any type of modification of the leaf shape, each sheet was digitized in situ using a conventional Epson perfection v37 scanner, obtaining the image of the adaxial side of the leaf.

2.2. Soil bioavailable metals

In order to determine the soil bioavailable metal concentrations, at these three sites of the salt marsh, thirty soil samples (five per site) 15-cm-diameter and 15-cm-depth were collected at low tide. The soil samples were dried at 80 °C until constant weight and sieved through a 2 mm mesh to remove large stones and dead plant material. Then, to extract the labile or potentially bioavailable metals, 1 g of dried and sieved soil was used to make a cold extraction with 25 ml of 0.5 N HCl (Agemian and Chau, 1976). Copper (Cu), lead (Pb), and zinc (Zn), in both matrixes were then measured by inductively coupled plasma (ICP-AES) spectroscopy (Shimadzu 9000). In all cases, the average uncertainty of metal ion determination was < 2%. All extractions were carried out in duplicate and blanks were processed as the samples. Results were reported on a dry weight. Reagents of analytical grade were used for the blanks and for calibration curves. Quality assurance of soils was done through analysis of standard reference freshwater sediment CNS392-050. The recovery in soil was > 87% for all measured metals.

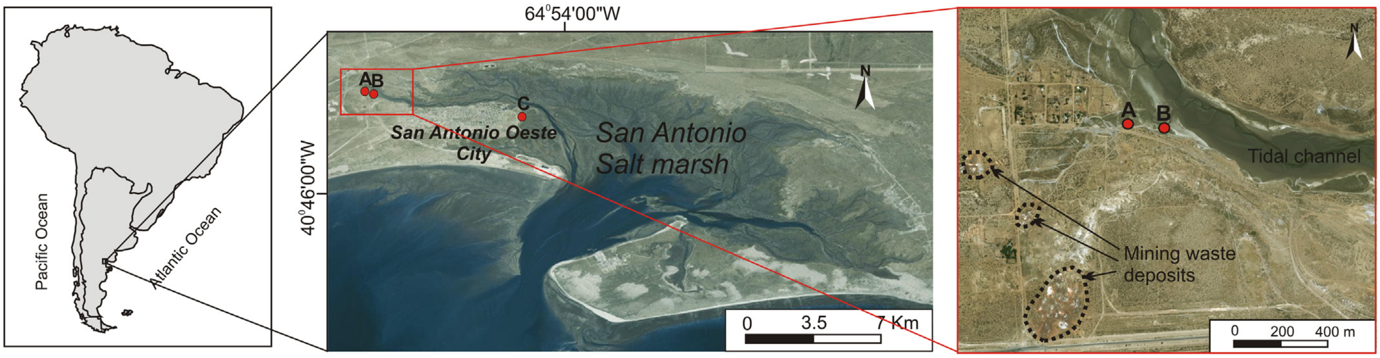

Fig. 1. Location map showing the study area and the sampling sites.

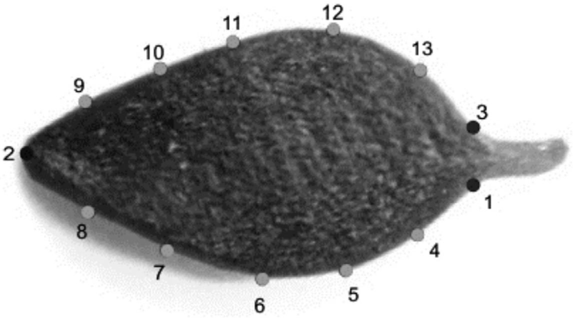

Fig. 2. Landmarks (black dots) and semi-landmarks (grey dots) configurations used to capture the contour of the leaf of *Cressa truxillensis*.

Table 2
Descriptive summary of landmarks and semilandmarks used to capture the contour of each leaf.

Landmark number	Location and description
1	Change of right curvature between petiole and limbus
2	Leaf apex
3	Change of left curvature between petiole and limbus
Semilandmarks number	Location and description
4–8	*semilandmarks* between *landmarks 1* and *2*
9–13	*semilandmarks* between *landmarks* 2 and 3

2.3. Leaf shape and size capture

The overall leaf shapes were captured by defining a configuration of 3 landmarks and 10 semi-landmarks (Fig. 2, Table 2). All leaves were digitized by one observer (MPP) using the TPSdig2 module of the TPS series (Rohlf, 2015). All semi-landmarks were allowed to slide along the outline profile of the leaf using the TPSrelw module, according to the minimum bending energy criterion (Gunz et al., 2005). Since the leaf presents object symmetry (symmetric in themselves), we adjusted the aligned coordinate of the shape following the recommendations of Mardia et al. (2000) and Klingenberg et al. (2002). Subsequently, the landmark configurations were superimposed by Generalized Procrustes Analysis with reflection (Dryden and Mardia, 1998; Klingenberg et al., 2002). Centroid size (CS) was calculated as a proxy to size (Zelditch et al., 2012), and it is the square root of the sum of the squared distances from the landmarks to the centroid which they define.

2.4. Statistical analysis

One-way Analysis of Variance (ANOVA) was used to evaluate differences in soil bioavailable metal concentrations between sites. For significant results ($p < 0.05$) the parametric Tukey test was applied (Zar, 1999). Before the analyses, data were tested for normality with the Shapiro-Wilk test and for homogeneity of variance with the Levene test. To correct for nonnormality and heterogeneity of variance, the Cu content variable was ln (X)-transformed.

To assess whether size (CS) was significantly different among sites, we calculated a One-Way ANOVA using Infostat software (Di Rienzo et al., 2009). The presence of allometry (the shape change exclusively explained by variations in size) was tested by a multivariate regression analysis between leaf shape scores as a dependent variable (aligned Procrustes coordinates) and size as an independent variable (CS), using MorphoJ v1.06d (Klingenberg, 2011).

To study the magnitude and direction of leaf shape variation, a principal component analysis (PCA) of the variance-covariance matrix (Zelditch et al., 2012) was done. To test for differences among sites, we performed a MANOVA test in Infostat software (Di Rienzo et al., 2009). Then we have computed among-site Mahalanobis distances. Also, to display axes of among-site maximum discrimination in leaf shape, we performed a Canonical Variate Analysis (CVA).

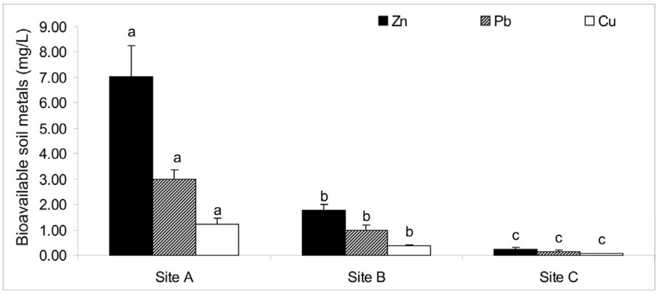

Fig. 3. Bioavailable soil metals in San Antonio Oeste Salt Marsh (mean (SD), n = 5). Different letters indicate statistic significance ($p < 0.05$) among sites in Tukey test.

3. Results

3.1. Soil bioavailable metals

As expected, bioavailability of the soil metal studied was statically higher in site A followed by site B and then by C. In addition, in all sites Zn was the most abundant metal, then Pb and, Cu, in lower concentration (Fig. 3).

3.2. Leaf shape and size capture

The size of the leaf did not differ significantly among sites ($p = 0.12$). However, there is a significant effect of allometry: the multivariate regression of the leaf shape onto centroid size was statistically significant ($p < 0.0001$), and accounted for 10.34% of the total shape variation. In consequence, subsequent analysis was done on the regression residuals, considered here as a new, allometry-free shape variable. The first two principal component (PC) scores accounted for 91.1% of the total leaf shape variation. PC1 (83.2%) was related with the leaf slenderness (Fig. 3). For negative values, leaves were narrowly elliptic shaped, with acute bases and tips; while for the positive values, leaves presented oval shapes. The leaf shape representations in negative values of PC2 were associated with the bottom curved and tapered at the petiole, while obovate shapes are represented in the positive values of this second PC. The leaf shapes from the three sites were mostly overlapped. However, leaves from site A (polluted) are represented all across the total range of variation of PC1 (Fig. 4).

The MANOVA analysis performed on the first eleven PCs indicated a highly significant among-site leaf shape differentiation (Wilk's $\lambda = 11.21$, $F = 0.53$, d.f. 22 and 666, $p < 0.0001$) and the Mahalanobis distance was shorter between site A and B, in relation to site C. Concerning the discriminant analysis, the most conspicuous trait separating shapes along the CV1 (88% of the variance) was the limbo expansion, along with the shortening plane of petiole-limbo towards the negative values (Fig. 5). The cross-validated classification showed that leaf shape is correct in the 82.5% of cases on unpolluted sites (C).

4. Discussion

Nowadays, environmental pollution is an ecological issue which is worryingly increasing. In this sense, it's essential to find tools that allow us to identify early deleterious effects in the biota health. Particularly, different biomarkers showed to be successful in monitoring programs for environmental quality, because they can offer biologically relevant information on the potential impact of contaminants on organismal health. In plants, biochemical and physiological measurable responses are most frequently used, such as photosynthetic pigment content, antioxidant enzyme activities or secondary metabolite synthesis (Bonnet

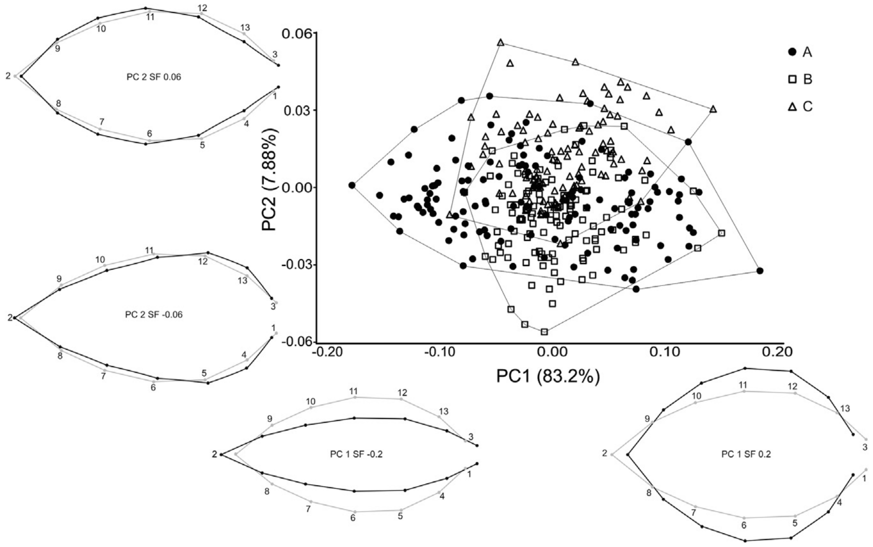

Fig. 4. Convex hulls representing variation along the first two principal components of the leaf *Cressa truxillensis*. The wireframe graphs represent the transformation and displacement vector from the overall mean shape (light grey outlines and grey dots) to the positive and negative extreme shape (black outlines and black dots). Scale factor (SF) is the magnitude of leaf shape change onto the PC score direction. Percentage explained variance for each axis is in parentheses. Site A, solid circle dots, B empty square marks and C empty triangle marks.

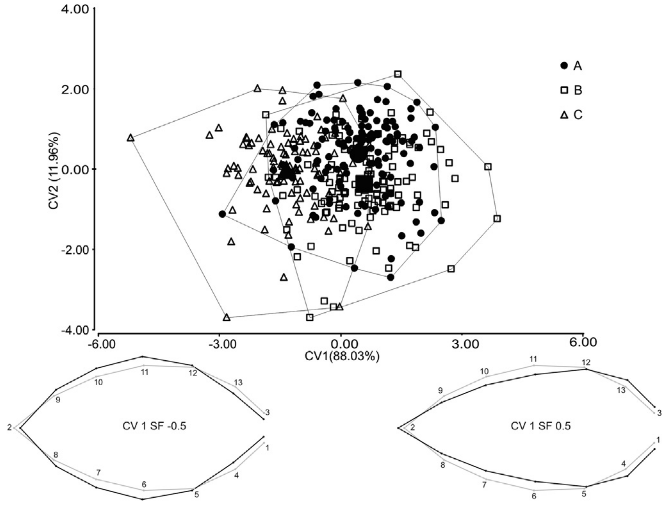

Fig. 5. Maximum variation in leaf shape of the *Cressa truxillensis* along the first and second canonical axes with convex hulls. Centroid shapes of each group are indicated with large and solid symbols. The wireframe graphs represent the transformation and displacement vector from the overall mean shape (light grey outlines and grey dots) to the positive and negative extreme shape (black outlines and black dots) on the canonical variate (CV1) axis. Site A, solid circle dots, B empty square marks and C empty triangular marks.

et al., 2000; Duarte et al., 2013; Gajić et al., 2009; Mendelssohn et al., 2001). However, morphological variations are also applied, mainly throughout the classic morphometric methods, such as length, width and weight (Ambo-Rappe et al., 2011; Bonnet et al., 2000; among others). In the sample studied here, we found a significant degree of variation in leaves shape of *Cressa turxillensis,* according to the gradient of soil heavy metal bioavailability in San Antonio Oeste salt marsh. This result allows us to propose the use of the leaf shape as an early biomarker of stress by soil pollution in salt marsh plants.

The leaf size of *C. truxillensis* was not affected by the soil heavy metal content gradient in the studied salt marsh. In contrast, previous works focused on the impact of air pollution on the morphology of *Iris pumila* standards (petals) by geometric morphometric methods (GMM) founded that these structures were shorter and wider in the polluted area, in relation to the unpolluted environment (Vujić et al., 2015). On the other hand, a study using classic morphometric methods and focusing on different plant structures, such as leaves and floral pieces (sepals, petals, stamens and carpels), indicated that the size of the studied structure decreases in places that present some degree of disturbance or stress, such as aerial contamination by different compounds such as nitrogen oxides (NOx), small solid particles (PM10) and sulfur oxides (SOx) (Syed et al., 2008). In addition, Vujić et al. (2016) applied GMM to compare the leaf shape of the herb *Mercurialis perennis* between highly disturbed sites by trampling, and a site without such disturbance. The authors stated that leaves from the disturbed site were smaller, postulating this as an adaptive response, since a smaller leaf size decreases the probability of being trampled. In another study based on classic morphometric methods, Velîčković (2010) compared leaves of the species *Tilia cordata* from two Serbian sites, one of the sites close to a large industrial settlement area, with chemical (fertilizer and ammonium production) and petrochemical (petroleum refinery) industries, and the other site without harmful substances. Their results showed that the leaves are smaller in the contaminated area, indicating slow growth and environmental degradation.

On the other hand, the greater shape variance of site A observed across the first PC would suggest a reduced canalization of the leaf shape, perhaps as a response to high pollution. Furthermore, significant among-site differences at the individual variation level were observed. This result suggests a correspondence between the changes in the leaf shape associated with the contaminant factor. In agreement with the statistical analysis, the visualization of the shape variation by the CVA (classified *a priori* by site) showed that there is a change in the leaf mean shapes among the three sampling sites. In addition, in the CV1 axis the most polluted sites (i.e. A and B) are represented by lanceolated leaves, whereas the site with the lowest soil metal concentration (C) had rather globose leaves. An opposite pattern of change was found by Vujić et al., (2015, 2016) in floral structures of *Iris pumilla* and leaves of *Mercurialis perennis*, respectively.

Halophyte species, as *C. truxillensis*, could present different mechanisms conferred throughout the evolution to deal with soil hypersalinization, which make them easily adaptable and plastic to different conditions of environmental stress (Flowers and Colmer, 2008). Some of these adaptations are ion compartmentalization, osmotic adjustment, succulence, selective ion uptake and transport, development of antioxidant responses, salt-secreting glands in the leaves, stomata almost exclusively on adaxial leaf surfaces, among others (Lokhande and Suprasanna, 2012; Maricle et al., 2009). It was even, observed that some salt marsh species make curling leaves, as a response to deal with high soil content to avoid water loss by reducing transpiration (Maricle et al., 2009). All these mechanism are considered an advantage to avoid high soil heavy metal bioavailability, as well (Lutts and Lefèvre, 2015). Even the elongation of the leaves from the polluted site (A) could indicate a slight curling, but this is not detectable with the GMM protocol we have used, since folding leaves narrower at the base would be easier than folding broad-based or globular leaves.

Although the concentrations of metals in the leaves of *C. truxillensis* are unknown, the soil metal concentrations in San Antonio salt marsh are well know, as well as the fact that the metal concentrations in the dominant plant species (*Spartina densiflora* and *Sarcocornia perennis*) are lower than in the soils (Idaszkin et al., 2017a, 2017c). In both plant species, the soil bioaccumulation and translocation factors are mostly less than 1, especially in the polluted sites (Idaszkin et al., 2017a, 2017c). However, it is know that soil heavy metal increase is an important stressful factor for plants, since it induces plants to make an effort to avoid their absorption, for example changing the physical and chemical soil features to affect metal mobility, speciation, bioavailability and in consequence their potential toxicity (e.g. increasing the pH in the rizosphere or increasing oxygenation, transporting oxygen through the aerenchyma to the rizosphere zone) (Greger, 2004; Maricle and Lee, 2002; Reboreda and Caçador, 2007a, 2007b). Therefore, we emphasize that the study of the relationship between the bioavailability of metals in soil and the variation in the shape of the leaves is relevant in environmental and impact assessment studies, and it is in accordance to our goal of evaluating the potential use of the leaf shape as a biomarker for soil heavy metal pollution. However, more research on this issue needs to be undertaken to a better understanding of the association between leaf shape and soil pollution, being that leaf shape could derive from the complex interaction of several factors.

5. Conclusions

This research represents one of the few studies on this plant species, and is a pioneer in the application of GMM in the issue of changes in leaf shape salt marsh plants.

Although leaf sizes of *C. truxillensis* do not change in relation to the soil pollution stress, the leaf shape presented among-site changes. The leaves from the more polluted site had lanceolate leaves, while leaves from the cleaner site had an oval shape.

The use of GMM as a useful, practical and low-cost tool is emphasized in the evaluation of small differences independently of the variations in size within the field of botany, strengthening the use of leaf shape variations as a potential biomarker of stress on plants growing on contaminated soil.

Further studies should be delineated in order to evaluate the relationship between the leaf shape variation and other known stress biomarkers, such as the antioxidant enzyme activities, photosynthetic rate and pigment concentration (chlorophylls and carotenoids) as a way to validate the leaf shape as a biomarker, and evaluate how it is associated with the health of the organisms studied.

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