Plants with purple abaxial leaves: A repository of metrics from stomata distribution.

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Abstract

Plants with purple abaxial leaf surfaces are very common in nature but the ecophysiological aspects of this phenotype are not well known. We observed here that the purple color of the abaxial tegument in living leaves make stomata completely visible. Based in it we measured the relations between stomatic density, celular area and distance between stomata pairs and observed a general log-normal trend line between density x celular area and density x interstomatic distance. These data shows that measures of stomatic distances at purple abaxial leaves are able to be a sensor to environmental changes of living purple plants. In future ecophysiological inferences will be established from the information brought by the measurements of the distance between stomata in purple plants.

Introduction

The purple color of lower abaxial leaf surfaces is commonly observed in deeply-shaded understorey plants, especially in the tropical regions. However the physiological role to pigmentation remains unclear [13, 15]. The pigments responsible for the coloring are known by anthocyanins, it can be found in plant species across a broad range of habitats, especially common in understorey plants of the tropics [10, 17]. The color of the abaxial surface may be transient or permanent depending of the environmental conditions and plant specie. Although the distribution of the color from abaxial leaves among tropical taxa are widespread, it is not known the physiological roles of this phenotype. Some studies regarding the ecophysiological relationships involved in the foliar anthocyanin in upper adaxial surface have demonstrated that pigmentation play a rule in light-attenuation, protecting underlying cells from photo-inhibition through the absorption of high energy blue-green wavelengths [8] Photoprotection have also been implicated in the physiological roles played by anthocyanins in plants in which exposed abaxial leaf surfaces are vulnerable to high-incident light [14]. Some studies have observed that the purple abaxial leaf surfaces appeared to reflect more red light than green surfaces [18]. Therefore anthocyanin pigments may function to reflect adaxially-transmitted red light back into the mesophyll, in order to capture red photons in environments where light is limiting. This hypothesis is known as back-scatter propagation, it has not yet received a complete experimental validation but could explain the color of leaves in understorey plants. It is known that the abaxial surface of leaves in some species of purple plants presents a high concentration of green stomatal cells which generates an interesting contrast between the color of the floor of the epidermis (purple) and the stomata (green), making this cells completely visible in studies of density and distribution pattern on the

leaf epidermis of the extant plants [13]. The stomatic density varies according to the environmental light exposure [11], then the color of the epidermis may also influence the distribution of stomata. Stomatal morphology, distribution and behaviour respond to a spectrum of signals, from intracellular signalling to global climatic change [12]. We observed in this work that in plants with purple abaxial leaves, the stomata are completely visible on the extant leaves due to the contrast between the color of the green stomata and the color of the floor, which is purple. This made it possible to propose strategies for measuring the distribution of stomata in the abaxial epidermis. Our approach was based on measuring the distance between pairs of stomata and compiling these measurements into a quadratic matrix of distances. Furthermore, the measurement of stomatal density as a function of the average stomatal area and interstomatic distances showed a general relationship as observed for stomatal density in function of guard cell length, which fitted with previous mathematical simulations of stomatal gaseous exchange models, indicating that the average relationship has virtually the same trend to one in which changes in density and size are exactly compensatory in terms of CO_2 and water vapour exchange [7, 12]. However the logarithmic decay between stomatal density as a function of the distance between stomata that we observed in this work has not been demonstrated, especially in purple plants. Additionally we induced an environmental change in the growth environment of the plant *Ctenanthe openheimiana* that were exposed to different photoperiods and measured the interstomatic distances, we observed the range of the distance changes; the distance measurements varied among plants when exposed to each condition and were able to discern the groups of plants that were subjected to a specific condition. We illustrated these changes directly in microscopic images of the abaxial surface of the plant.

Our results show that the phenotypic plasticity of stomatal density can be probed by measurements of the distance between stomata in purple plants. Furthermore, it is shown that general mathematical relationships, extending to all plants with stomata, such as stomatal density $\times$ area and density $\times$ distance between stomata can be accurately inferred from measurements in plants with purple abaxial leaves. Therefore, ecophysiological inferences regarding environmental changes can also be probed from the phenotypic plasticity of stomatal distribution in plants with purple abaxial leaves, which makes these plants excellent sensors of environmental change.

1 Materials and Methods

1.1 *Ctenanthe oppenheimiana* crop and light acclimation

Two group of sprouts from the 20 *Ctenanthe oppenheimiana* plants with length sizes ranging 5 to 8 cm were planted in standard Carolina plant growth substrate from Carolina soil from Brazil. The plants were acclimated at a plant controlled growth chamber, Eletrolab EL212/4G-RS, with continuous 55% relative humidity, photon flux from $100\mu mol/m^2 \times s$, temperature $25^o c$ and light/dark photoperiods from 24 and 4 hours per day during a continuos period of 60 days.

1.2 Optic microscopy

Sections of extant leaves of the plants *Tradescantia pallida*, *Tradescantia zebrina*, *Callisia repens*, *Tradescantia minima*, *Ctenanthe oppenheimiana* and *Oxalis atropurpurea* were mounted on slides, covered with fresh water and covered with coverslips. Sections of 0.5 cm^2 from the medial third of leaves of the plants *Tradescantia pallida*, *Tradescantia zebrina* and *Ctenanthe oppenheimiana* were hydrated and subjected to optical microscopy after a maximum period of 60 minutes after excision. Whole leaves of the plants *Callisia*

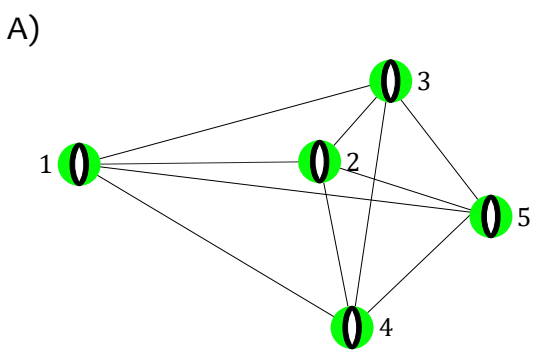
repens and separated lobes of the clover *Oxalis atropurpurea* were used for microscopy. The leaves were excised from the plants and mounted directly on slides with the abaxial surface facing upwards, covered with fresh water and covered with a coverslip. The microscopes used were the Zeiss Axio-Lab A1 and the Zeiss discovery V20 stereo microscope coupled with the Axiocam ERc5s and Icc1 cameras under the management of the Zeiss Axiovision software. The magnifications used varied among the plants. Thus, for Tradescantia pallida, Tradescantia zebrina, Tradescantia minima and Callisia repens, the magnifications 15× were used in the stereomicroscope and 50×, 100×, 200× and 400× in the Axio-Lab A1 microscope. For the plants *Ctenante oppenheimiana*, *Oxalis atropurpurea*, the magnifications of 50×, 100×, 200× and 400× obtained with the objectives of the AxioLab A1 microscope were used.

1.3 Measure of distances between stomata

The microscopic images were processed using Matlab image processing toolbox. The stomates were identified manually in the images, their centroids (x, y) were identified through an algorithm which found the central coordinates of each one of the manually marked dots. The distance between each pair of points in the image is given by the following equation, where d is the distance between two centroids (i,j) of the stomata.

$$d(i,j) = \sqrt{(x_i - x_j)^2 + (y_i - y_j)^2}. \tag{1}$$

Images with magnifications of 100× were used. A distance matrix between each pair of centroid stomata at image is calculated as illustrated in Fig. 4.



B)

	1	2	3	4	5
1	0	d_{12}	d_{13}	d_{14}	d_{15}
2	d_{21}	0	d_{23}	d_{24}	d_{25}
3	d_{31}	d_{32}	0	d_{34}	d_{35}
4	d_{41}	d_{42}	d_{43}	0	d_{45}
5	d_{51}	d_{52}	d_{53}	d_{54}	0

Figure 1. A) Illustration of five stomates (green) connected with each other by lines. B) Distance matrix of each stomata pair. The matrix indexes represent each stomata, and $d(i, j)$ is the distance between each pair of stomata found in A).

2 Results

2.1 Plant microscopy

Samples of microscopic images of six plant species with purple abaxial leaf are shown in the following figure. The optical microscopy images obtained from *Tradescantia pallida*, *Tradescantia zebrina*, *Callisia repens*, *Tradescantia minima*, *Ctenanthe oppenheimiana* and *Oxalis atropurpurea* show green stomata in extant leaves.

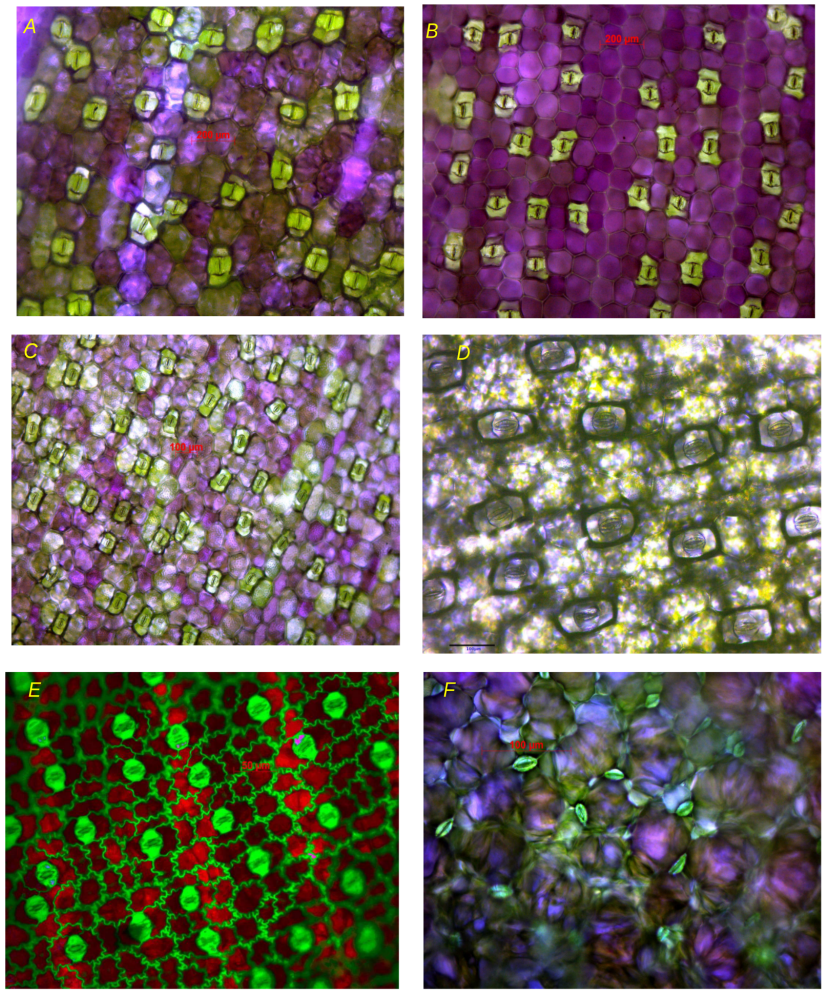


Figure 2. Optical microscopy images shown green stomata distributed over the abaxial surface of leaves. A) Image from *Tradescantia pallida* magnified 50× B) Image from *Tradescantia zebrina* magnified 50× C) Image from *Callisia repens* magnified 50× D) Image from *Tradescantia minima* magnified 100×. E) Image from *Ctenanthe oppenheimiana*, magnified 200× F) Image from *Oxalis atropurpurea* magnified 200×

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The microscopic magnification values used to collect the images of the Figure 2, give the dimension of the size of the stomata in the studied species. The length of the stomata from *Tradescantia pallida*, the largest among all the plants studied, is on the order of more than 10× the stomata of the plant *Oxalis atropurpurea*, the smallest.

Although all plants have the pavement color contrasting with the color of the stomata, the color of the abaxial face has plasticity, especially in the specie *Oxalis atropurpurea* and *Tradescantia minima* in which they varied from green to purple according to environmental variations [5].

2.2 Morphometrics

Depending on the species and the environmental conditions stomata range in size from about 10 to 80μm in length and occur at densities between 5 and 1,000 mm^{-2} of epidermis [19].

In spite of this wide variability there is a strong and general relationship between density and size for different plant groups (grasses and non-grasses)and fossil leaves. [12]. Simulations with a stomatal model of gaseous exchange indicate that the average relationship has virtually the same trend to one in which changes in density and size are exactly compensatory in terms of CO_2 and water vapour exchange [7, 12]. We observed here a similar trend to relation stomata density × stomatic area in 6 plants with purple abaxial leaves. The graph in figure 3 illustrate the trend that show a log-normal fitting similar to observed in [7, 12].

The distance measurements of interstomatic distances according illustrated at figure 1 quantify how the plasticity of the leaf can be measured from the distribution of these cells in the epidermis. The measurements of the mean distances between neighboring stomata × stomatal densities can be evidenced in the graphs of Figure 4, which show the dispersion of the density values as a function of the mean distance between the stomata neighbors for the six species of purple plants.

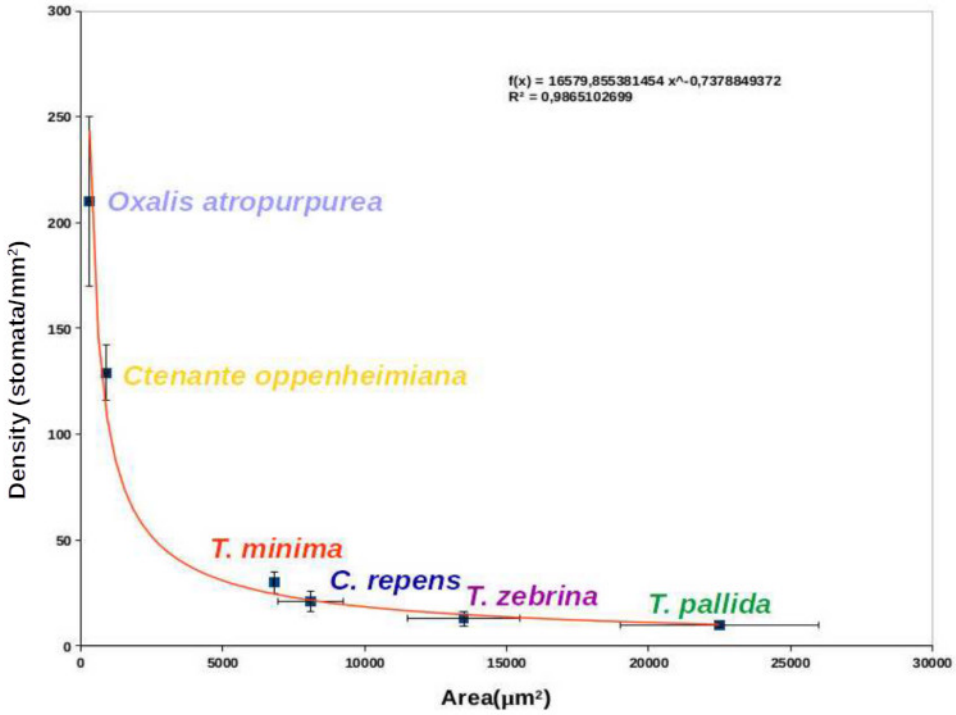

Figure 3. The graph shows the inverse relationship between stomata density and stomatic area. The plant *T. pallida* is the one with the largest stomata area and the lowest average density. Species indicated by name at each point in the graph. The solid curve is log-normal, y $= 16579.8 \times x^{-0.73}$, $r^2 = 0.98$. The error bars show the standard deviations of the measures calculated from n = 18 images of 6 plants per species.

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The results shown in Figure 4 indicate that the mean distances between neighboring stomata are inversely proportional to their stomatic densitiesand vary linearly in each species independently. These measurements are the direct evidence of plasticity as regards the variation of stomata distribution on leaf epidermis. When transposing the mean values of the measurements indicated in the graphs shown above, for a single graph that quantifies the relation mean distance × mean density to 6 plant species studied here, it can be observed the similar log-normal trend line as observed in graph from figure 3 (see figure 5).

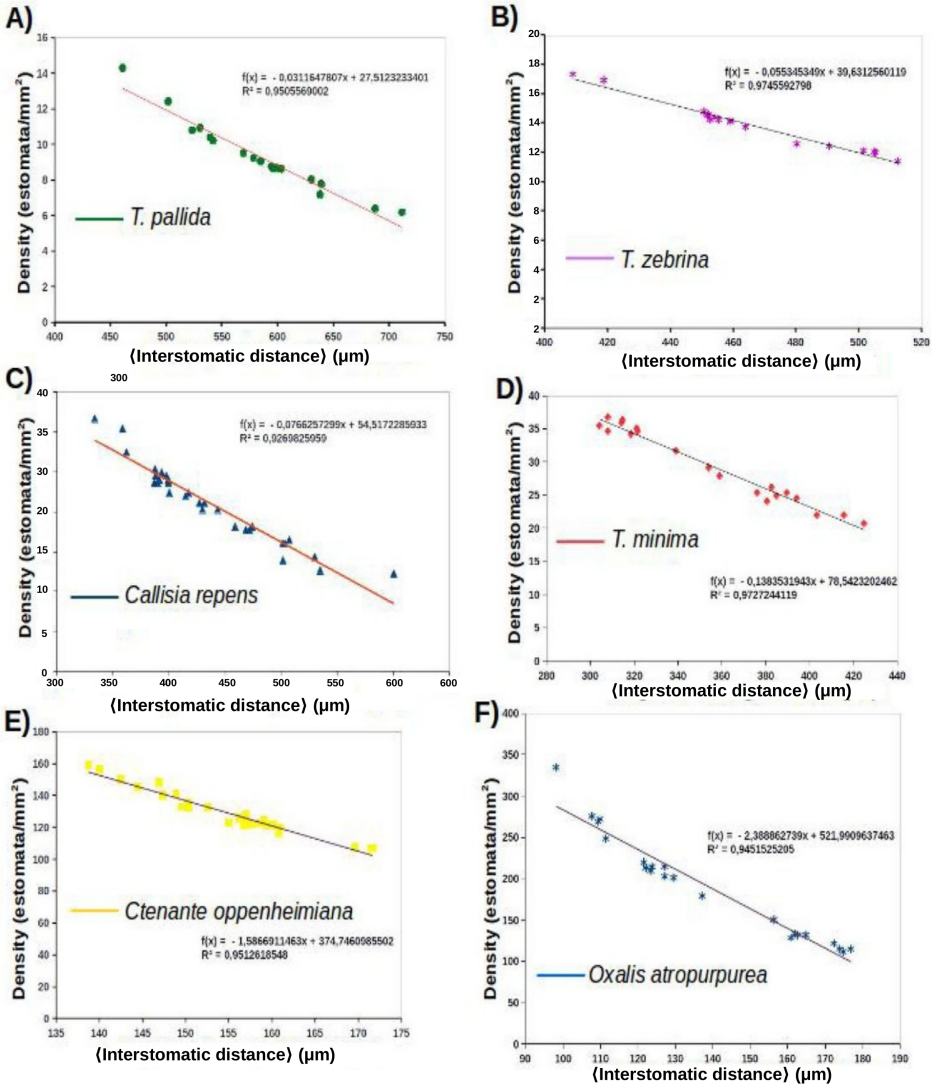

Figure 4. Dispersion of the mean distance values between neighboring stomata as a function of the mean stomatal density for six plants, plots from top to bottom: A) *T. pallida* B) *Tradescantia zebrina* C) *Callisia repens.* D) *T. minima* E) Ctenanthe oppenheimiana. F) *Oxalis atropurpurea.*

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The results shown here indicate that the interstomatic distances quantifies the dispersion of the stomata and precisely measures its distribution on the leaf. Plants with a purple abaxial surface exhibit a natural color contrast with stomata, which are usually green due to the large amount of chlorophyll and chloroplasts present within the guard cell. Therefore the measurement of distance between stomata in purple abaxial surface plants seems to be a powerful strategy for observation of changes in the physiology of plants generated by changes in the environment. Thus ecophysiological aspects in plants will be approached more accurately from the estimation in distribution of stomata on the leaves. Thus the strategy of stomata morphometry in purple plants showed here may

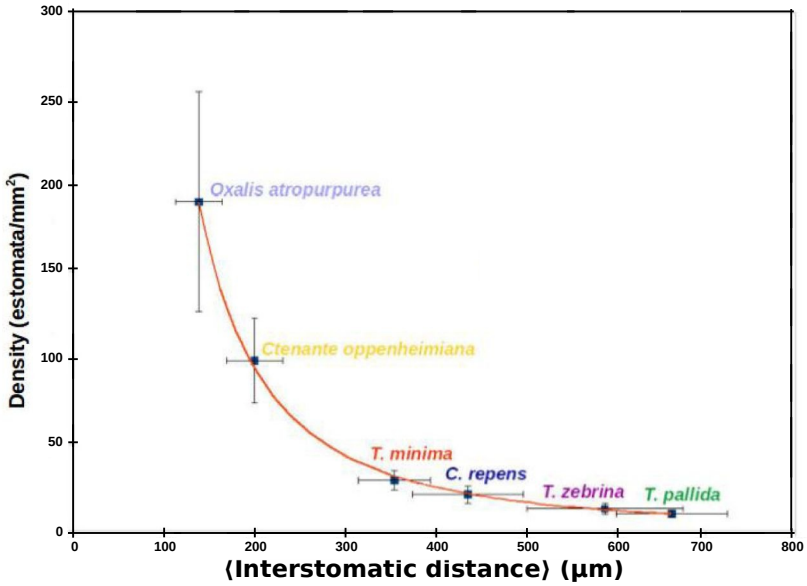

Figure 5. Dispersion of the mean interstomatic distance × mean stomatic density for *Tradescantia pallida*, *Tradescantia zebrina*, *Callisia repens*, *Tradescantia minima*, *Ctenanthe oppenheimiana* and *Oxalis atropurpurea*. The solid curve is log-normal, $y = 2260026.3x^{-1.90}$, $r^2 = 0.99$ The error bars indicate the standard deviation of the measurements in relation to the mean values of distances between stomata and stomatal densities by species. (N = 5 plants per species, 3 images per plant)

provide valuable answers to the understanding of how plants respond to climate change.

3 Discussion

The stomata are modified cells responsible to plant gas exchange, consequently play fundamental roles in photosynthesis and carbon assimilation by plants. Consequently, they are fundamental in the flows of carbon and water on planet Earth [9, 12, 16]. Therefore, quantitative information on the morphology of stomata in the leaf with data on relationships between leaf stomatic density, stomata size and the environmental plasticity of these characters are important probes to understand how plants perceive and feel environmental changes. We observed in this work that microscopic images of purple abaxial epidermis of living plants show stomata that are completely visible and discernible from the rest of the epidermis due to the contrast between the purple colors of the epidermis and the green stomata (see figure 2). The contrast green/purple is very striking in the plant of the species *Ctenanthe oppenheimiana* (see figure 2 E)). It allowed us to count the distribution of these cells, determine their size and measure the distance between them in the microscopic images of 6 plants with purple abaxial leaves. The measurements revealed similar morphological relationships (see figure 3) to those already observed in previous works [7, 12], which identify an inverse log-normal relationship between stomatal density and stomatal size as a general law applicable to a large number of plants including fossil plants. These studies also show that stomatal

density is highly variable when plants grow in different concentrations of CO_2 and that this characteristic is striking in plants grown in different geological eras on Earth, especially during the Phanerozoic, when the concentrations of CO_2 in the atmosphere are different. The measurements in living plants with purple abaxial epidermis made here show a correlation coefficient with a log-normal curve much higher than that observed in previous studies ($R^2 > 0.9$) (See figure 3). This probably occurs because few plants were used here, with stomatal density values with larger intervals between them. Furthermore the measurements were made in living plants and without histological processing of the leaf. These data confirm the simulations with a stomatal model of gas exchange made in previous studies [7] and confirm the inverse relationship between stomatal density and stomatal size as a log-normal relationship. We also propose, in this work, a method to measure stomatal plasticity by measuring the distance between stomata present in a purple abaxial epidermis. The compilation of the distance measurements between stomata through a quadratic matrix (see figure 1) proved to be a powerful tool for measuring the plasticity of stomatal distribution in the leaf. This is confirmed by the inverse log-normal relationship found between the mean distance between stomata and mean stomatal density among different plant species (see figure 5), similar to that observed in figure 3 of this work and for the relationship between mean stomatal size and mean stomatal density made in previous works [7, 12]. It is interesting to note that this relationship is linear for measurements made in a single species as evidenced in figure 4, and logarithmic when observed between different species (see figure 5). These results show that the variation in stomatal density within a same species does not make the relationship between density and distance between stomata logarithmic, which leads us to assume that the extent of larger variations in stomatal density values between species is what makes this relationship logarithmic. However, a larger number of measurements in plants of the same species subject to large environmental variations are required to confirm this assumption. Together, our results show that measuring the distance between neighboring stomata in purple plants is a robust strategy to predict changes in stomatal distribution in the face of any environmental factors that alter this distribution. At the same time, the clarity of stomatal distribution in microscopic images of abaxial epidermis of living purple plants shows that these plants are a repository of metrics of this distribution.

4 Supporting Information

4.1 Abaxial leaf images of purple plants

Purple plants of species not known had their collection sites referenced and a photograph of the whole plant was collected. The leaves of each plant were analyzed by optical microscopy. The unknown purple plants were named with the codes USP1, USP2, USP3, USP4, USP5 for proper assignment. Cuttings of 0.5 cm^2 of the leaves of each plant were put in slides, with the abaxial surface facing up, covered with water, over covered with coverslips, and submitted to optic microscopy at microscope Axio-Lab A1-Zeiss at magnifications 50× and 100×.

Geographical coordinates, microscopic and whole plant images are available at the following web addresses:

Table 1. List of database web addresses for microscopic and full purple plant images

Plant sample	web database	reference
USP1	`https://doi.org/10.7910/DVN/VYMSUX`	[4]
USP2	`https://doi.org/10.7910/DVN/DW3SNJ`	[3]
USP3	`https://doi.org/10.7910/DVN/V8GZYZ`	[6]
USP4	`https://doi.org/10.7910/DVN/CJCVZS`	[2]
USP5	`https://doi.org/10.7910/DVN/6GYAGO`	[1]

5 Acknowledgments

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