

## Water stress and abscisic acid treatments induce the CAM pathway in the epiphytic fern *Vittaria lineata* (L.) Smith

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### Abstract

Among various epiphytic ferns found in the Brazilian Atlantic Forest, we studied *Vittaria lineata* (L.) Smith (Polypodiopsida, Pteridaceae). Anatomical characterization of the leaf was carried out by light microscopy, fluorescence microscopy, and scanning electron microscopy. *V. lineata* possesses succulent leaves with two longitudinal furrows on the abaxial surface. We observed abundant stomata inside the furrows, glandular trichomes, paraphyses, and sporangia. We examined malate concentrations in leaves, relative water content (RWC), photosynthetic pigments, and chlorophyll (Chl) *a* fluorescence in control, water-deficient, and abscisic acid (ABA)-treated plants. Plants subjected to drought stress (DS) and treated by exogenous ABA showed significant increase in the malate concentration, demonstrating nocturnal acidification. These findings suggest that *V. lineata* could change its mode of carbon fixation from C<sub>3</sub> to the CAM pathway in response to drought. No significant changes in RWC were observed among treatments. Moreover, although plants subjected to stress treatments showed a significant decline in the contents of Chl *a* and *b*, the concentrations of carotenoids were stable. Photosynthetic parameters obtained from rapid light curves showed a significant decrease after DS and ABA treatments.

*Additional key words:* chlorophyll fluorescence; malate; morphoanatomy; photosynthetic pathway; pigments.

### Introduction

Ferns are extensively distributed worldwide. These plants have developed remarkable adaptations in extreme environments, being found in widely different habitats (Rathinasabapathi 2006). Many species are cosmopolitan and preferentially live in the tropical regions of the world, especially in humid and shady forests (Rathinasabapathi 2006). Prado (2003) found approximately 13,000 species of ferns and lycophytes worldwide, out of which more than 1,200 belong to Lycophyta and about 11,500 to Monilophyta (Pryer *et al.* 2004).

Ferns are a very important group of plants which show well diversified ecological aspects. They have adapted to

both land and water. They are climbers, thrive amid rocks, and they can be also epiphytes or hemiepiphytes, ranging from tiny plants of a few millimeters to long, arborescent forms reaching 20 m (Tryon and Tryon 1982). The epiphytes comprise approximately 10% of the vascular flora (Kress 1986). They are found almost exclusively in tropical forests, where they make up to 25% of species (Nieder *et al.* 2001). According to Madison (1977), epiphytic plants do not have direct connections with the ground. They only use phorophytes as a support, not as a source of nutrients. In an ecological context, epiphytism is an interaction between plants in which the epiphytic

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**Abbreviations:** *A* – scaling constant for the height of the light curve; ABA – abscisic acid; AL – actinic light; Car – carotenoids; DM – dry mass; DS – drought stress; ETR – electron transport rate; ETR<sub>max</sub> – maximum electron transport rate; FM – fresh mass; F<sub>0</sub> – minimum fluorescence; F<sub>m</sub> – maximum fluorescence; F<sub>v</sub> – variable fluorescence; F<sub>m</sub>' – minimum fluorescence of a light-adapted leaf; F' – fluorescence yield of a light-adapted leaf; FLM – fluorescence microscopy; F<sub>v</sub>/F<sub>m</sub> – maximum photochemical efficiency of PSII; I – irradiance; I<sub>opt</sub> – optimal irradiance for the saturation of photosynthesis; k<sub>w</sub> – scaling constant for the x-axis of the light curve; LM – light microscopy; OAA – oxaloacetic acid; PAM – pulse-amplitude modulation; *P* – photosynthesis; PEG – polyethylene glycol; PEPC – phosphoenolpyruvate carboxylase; RLC – rapid light curve; RWC – relative water content; SEM – scanning electron microscopy; SL – saturating light pulses; TM – turgid mass; Φ<sub>PSII</sub> – actual photochemical efficiency of PSII.

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plant is dependent only on the substrate supplied by the host plant (phorophyte); otherwise, they obtain nutrients directly from atmospheric moisture, without emitting haustorium structures (Bennett 1986). *Vittaria lineata* (L.) Smith (Pteridaceae) is an epiphytic fern found in the Brazilian Atlantic Forest. It is an exclusively epiphytic species without petioles and presenting laminar leaf (10–50 cm), simple, pendulous; linear sori is in a row between the rachis and the margin and monolet spores (Tryon and Tryon 1982, Smith *et al.* 2006). Known popularly as "shoe cord fern", this species grows preferentially in damp woods as epiphytes on tree trunks or in the canopy (Tryon and Tryon 1982). Based on its beauty and hardiness, it is an ornamental plant widely used in gardens.

The epiphytism favors the capture of light radiation, but limits the availability of water. The CAM pathway is one of the most important adaptations of some epiphytic ferns to water deficit. It enables CO<sub>2</sub> assimilation at increased water deficiency and high irradiance stress (Benzing 1986, 1990; Kluge *et al.* 1989). In plants that show the CAM pathway, CO<sub>2</sub> enters the mesophyll leaf tissue through open stomata predominantly at night and is fixed in the reaction catalyzed by phosphoenolpyruvate carboxylase (PEPC) in the cytosol. The oxaloacetate (OAA) resulting from PEPC activity is reduced to malic acid, which is accumulated in the vacuole at nighttime (Lüttge 1993, Nimmo 2000).

In the Polypodiaceae, the CAM pathway was found in five species of *Pyrrosia* and in *Platyserium bifurcatum* (Hew and Wong 1974, Wong and Hew 1976, Winter *et al.*

## Materials and methods

**Plant collection:** Plants of *Vittaria lineata* (L.) Smith were collected from the natural habitat in the Environmental Conservation Unit "Desterro" (UCAD), located in the northwestern part of the island of Florianópolis, Santa Catarina, Brazil (27°31'50"S, 48°30'44"W, an area of 495 ha, which is about 1% of the area of the island). The unit is managed by the Federal University of Santa Catarina (UFSC), Florianópolis, Santa Catarina, SC, Brazil.

**Plant acclimation:** Plants were transported to the greenhouse of the Botany Department of the Federal University of Santa Catarina, where they grew for a minimum of six months. The plants were placed in brackets on the walls. During the testing period, the daily temperature of the greenhouse varied between 20–30°C, and at night, it was kept around 15°C. The relative humidity was monitored and ranged between 60–80%. The PPFD inside the greenhouse alternated between 43 and 85 μmol(photon) m<sup>-2</sup> s<sup>-1</sup>. Irradiance was measured at the mid-region of the plants with an *LI-190* sensor (*LI-COR Instruments*, USA) connected to the *LI-250* light meter (*LI-COR Instruments*, USA) placed close to plants. The plants were watered daily with distilled water to retain high humidity. Once a week,

1983, Sinclair 1984, Ravensberg and Hennipmam 1986, Rut *et al.* 2008). Increase in titratable acidity was found in *Dictymia* J. Sm (Griffiths 1989) and *V. lineata* (Carter and Martin 1994). Recently, Martin *et al.* (2005) showed some evidence of the CAM pathway in *Vittaria flexuosa* Fée and *Anetium citrifolium* (L.) Splitg., but not in *V. lineata*. On the other hand, Martin *et al.* (2005) suggest the CAM pathway for *V. flexuosa* and *A. citrifolium*, which may be also a sporadic phenomenon in *V. lineata*, thus explaining the disparity between the findings of Martin *et al.* (2005) and those of Carter and Martin (1994). However, the titratable acidity may not be enough to establish the presence of the CAM pathway because substances other than malate may influence the pH of the samples. Zotz and Ziegler (1997) studied the occurrence of CAM in different vascular epiphytes in a central Panamanian forest via <sup>13</sup>C isotope and they verified C<sub>3</sub> metabolism in *V. lineata*.

CAM was initially verified in the epiphytic fern *Vittaria lineata* (L.) Smith by Carter and Martin (1994), but the presence of this pathway in *V. lineata* has never been confirmed (Zotz and Ziegler 1997, Martin *et al.* 2005). We hypothesized that *V. lineata* could change its mode of carbon fixation from C<sub>3</sub> to the CAM pathway in response to water deficit and exogenous application of ABA. Therefore, this study aimed to analyze the effects of water deficit and ABA application on relative water content (RWC), photosynthetic pigments, rapid light curves, and fluctuations in the malate contents in leaves in order to evaluate the physiological adaptations of *V. lineata* to stress.

they were irrigated with Hoagland's solution (20%, v/v) (Hoagland and Arnon 1938). After the acclimation period of at least six months, the plants were divided into three groups, each subjected to a different condition: (1) control plants irrigated daily for two weeks, (2) plants subjected to DS for a period of one week (without irrigation), and (3) plants irrigated 5 times a week with a solution of 10 μM ABA for a period of 15 d.

**Structural analysis of leaves:** From the leaves *in vivo*, we cut freehand cross sections, both longitudinal and paradermic, with a razor blade, using polystyrene as support material. These sections were placed on a glass slide with water and covered with a cover slip. Histochemical staining was carried out using *Lugol's* solution for identification of starch (Jensen 1962), FeCl<sub>3</sub> for phenolic compounds (Johansen 1940), Sudan IV for lipophilic substances (Gerlach 1984), and acidified phloroglucinol for lignin (Costa 1982). The stomata count was performed in triplicate in the median region of the abaxial surface. The stomata count was done with a 20× objective of a *Leica DM2500* optical microscope (LM) on images captured with a digital camera (*Leica DFC295*, *Leica*, Germany).

Structural analysis of the leaves was also performed on samples fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2), washed in the same buffer, dehydrated in ethanol series, and preserved in ethanol 70% (v/v) (Ruzin 1999). Some samples were infiltrated in a mixture of polyethylene glycol (PEG) 1500 and 70% ethanol (1:1) for 24 h in an oven at 60°C. They were again incubated in an oven for 24 h in pure PEG 1500. Following this incubation, the samples were embedded in PEG 1500. Other samples were infiltrated in methacrylate (*Jung's Historesin*, Leica, Wetzlar, Germany). The samples preserved in ethanol 70% (v/v) were dehydrated in ethanol series up to 96% (v/v) and processed according to the manufacturer's instructions. The samples were embedded in PEG and methacrylate, and afterwards, they were sectioned by rotary microtome (*Leica RM 2125 RT*; Leica, Wetzlar, Germany) and stained with Astra blue (Bukatsh 1972) or toluidine blue (O'Brien *et al.* 1965) and safranin (Kraus and Arduin 1997).

The samples were examined under LM (*Leica DM2500*; Leica, Wetzlar, Germany), and images were captured with an attached digital camera (*Leica DFC295*, Leica, Wetzlar, Germany).

For structural analysis of the leaves *in vivo* by fluorescence microscopy (FLM), we used an *Olympus BX41* fluorescence microscope (*Olympus*, Tokyo, Japan) with a mercury-vapor lamp as the light source (*HBO 100*) and blue color filter *U-MWU2* (excitation: 330–385 nm, emission: 420 nm), red color filter *U-MWG2* (excitation: 510–550 nm, emission: 590 nm), green color filter *U-MWB2* (excitation: 460–490 nm, emission: 520 nm), and triple filter (excitation: 330–385 nm, 460–490 nm, 510–550 nm; emission: 420 nm, 520 nm, 590 nm). The images were captured with a *QColor 3C* digital camera (*Q-imaging*, Surrey, BC, Canada).

**Ultrastructural analysis of leaves:** The prepared leaf samples were subjected to total dehydration in ethanol series and kept in ethyl ether for 48 h at 20°C. Afterwards, the sample containers were opened and kept in laminar flow to favor complete evaporation of the ether. The samples were then placed on aluminum brackets with the aid of double-sided carbon tape. Leaf samples were metalized with 30 nm of gold in a *Bal-Tec CED 030* metalizer (*Bal-Tec AG*, Balzers, *Liechtenstein*). Scanning electron microscope (SEM) *JEOL JSM - 6390LV* (*JEOL*, Tokyo, Japan) was used to analyze the samples.

**Analysis of malate content:** Malate contents were evaluated *via* enzymatic analyses according to Möllering (1985) in order to verify the difference in malate concentration ( $\Delta$  malate) between night and day. Three samples (1.0 g each) were taken from fresh leaf at 6:00 and 18:00 h from each treatment. They were immediately immersed in liquid nitrogen (–192°C) to stop all enzymatic reactions until analysis was carried out. For the extraction, samples were homogenized in 10 ml of distilled water at

98°C, kept in water bath at 98°C for 10 min, and subsequently centrifuged at  $3,500 \times g$  for 10 min. The precipitate was discarded and the extracts kept at room temperature. Aliquots of 100  $\mu$ L were used to quantify the malate through the *K-LMALL* enzymatic kit (*Megazyme International Ireland Limited*, County Wicklow, Ireland). The detection of malic acid requires two enzymatic reactions. The first reaction is catalyzed by L-malate dehydrogenase, whereby the malic acid is oxidized to OAA by  $\text{NAD}^+$ . However, since the reaction balance is favorable to malic acid and  $\text{NAD}^+$ , a further reaction is needed to keep the NADH product, and this is achieved by conversion of OAA to L-aspartate and  $\alpha$ -ketoglutarate (2-oxoglutarate) in the presence of a large excess of L-glutamate by glutamate oxaloacetate transaminase. The amount of NADH formed in the reaction described above corresponds stoichiometrically to the amount of malic acid. Thus, NADH may be measured by the increase in absorbance. The analyses were performed spectrophotometrically (*SP-220*, *Biospectro*, Brazil) at 340 nm, in triplicate, and the concentration of malate was expressed in  $\mu\text{mol g}^{-1}$  (dry mass, DM).

**Determination of relative water content (RWC):** After each treatment, leaf discs (for each replicate  $n = 3$ ) of 1.0 g of fresh mass (FM) were cut and placed in flasks containing distilled water for 180 min under the irradiance of  $20 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$  at  $25 \pm 2^\circ\text{C}$  to obtain the turgid mass (TM). Then, the leaf segments were dried in an oven at 60°C for 24 h and subsequently weighed to obtain DM. The RWC values of fully expanded, mature leaves were expressed by the equation:

$$\text{RWC} = [(\text{FM} - \text{DM}) / (\text{TM} - \text{DM})] \times 100 \text{ (Barrs 1962).}$$

**Photosynthetic parameters by Chl *a* fluorescence:** Chl fluorescence was evaluated using a pulse amplitude-modulated fluorometer (*Diving-PAM*, *Walz*, Effeltrich, Germany), equipped with an optic fiber 5.5 mm in diameter and a blue diode (470 nm) as the light source. During the testing period, the recorded temperature was  $25 \pm 2^\circ\text{C}$ . The analyses were carried out between 10:00 and 12:00 h. All plants were acclimated in the dark for 25 min before the application of the first pulse of saturating light. The experiments were performed in triplicate. ( $n = 3$ ). Ten rapid curves were obtained for each replicate. The minimum fluorescence ( $F_0$ ) was obtained by submitting samples to a measured modulated light [ $0.1 \mu\text{mol}(\text{photons}) \text{m}^{-2}\text{s}^{-1}$ ], and the maximum fluorescence ( $F_m$ ) in the dark-acclimated samples was obtained just after applying a SL [ $9,000 \mu\text{mol} \text{m}^{-2} \text{s}^{-1}$ ]. Using the option "Rapid Light Curve" (RLC) in the *Diving-PAM* software, the whole light curves were obtained by applying a series of eight 0.3s saturating light pulses (SL,  $9,000 \mu\text{mol}(\text{photons}) \text{m}^{-2}\text{s}^{-1}$ ), each one, followed by exposure to crescent actinic light (AL) [from 2 to  $2250 \mu\text{mol}(\text{photons}) \text{m}^{-2} \text{s}^{-1}$ ]. The parameters, *i.e.*, electron transport rate (ETR), were calculated using the *WinControl* software (*V.3.18*) (*Walz*,

Germany) according to Genty *et al.* (1989). During the RLC, each SL and AL period produced a light-adapted maximum fluorescence ( $F_m'$ ) and a light-adapted minimum fluorescence ( $F'$ ). The ETR between PSII and PSI was estimated using the following equation:  $ETR = \Phi_{PSII} \times PPF \times 0.5 \times 0.84$ , where  $\Phi_{PSII}$  is the effective photochemical quenching yield of PSII obtained by  $(F_m' - F')/F_m'$ , PPF is the photosynthetic photon flux density during exposure to the actinic light, 0.5 is related to the incident photon between PSII and PSI, and 0.84 is the fraction of incident quanta absorbed by the leaf (Bjorkman and Demmig 1987, Schreiber *et al.* 2004, White and Critchley 1999, Runcie and Durako 2004, Ralph *et al.* 2005).

**Extraction and quantification of photosynthetic pigments:** After determination of the RLC, photosynthetic pigments were analyzed according to Lichtenthaler (1987) using samples of 1.0 g of leaf FM ( $n = 3$ ). For extraction, leaf samples were taken from liquid nitrogen and immediately homogenized in 10 ml of aqueous acetone (80%, v/v) for 5 min, using a mortar. The homogenate was centrifuged for 10 min at  $3,500 \times g$ , and the volume was adjusted to 10 ml. The absorbances at 663, 646, and 470 nm were analyzed with a UV-VIS spectrophotometer (*SP-220, Biospectro*, Brazil).

## Results

**Structure and ultrastructure of leaves:** The leaf of *Vittaria lineata* exhibited the average thickness of 1.05 mm. In the leaf crosssections, we noted the presence of two linear furrows on the abaxial surface (Fig. 1A). Inward, the mesophyll consisted of palisade and spongy parenchyma. Three meristemes were observed in the leaf, one central and two lateral corresponding to the regions, where the furrows were linearly located (Fig. 1A). Stomata were observed only in the furrows on the abaxial surface, showing the characteristic hypostomatic leaf, and occurred at the average concentration of 113 stomata per  $mm^2$ . (Fig. 1B). In the epidermis of the linear furrow, a large number of uniseriate and unbranched paraphyses were seen, in addition to sporangia sori in a single row parallel to each leaf edge (Fig. 1B). Frontal view of the epidermal surface in SEM revealed that the cuticle was smooth, but with wax deposits (Fig. 1C). The FLM image (green excitation: 460–490 nm, emission: 520 nm) confirmed the presence of stomata having a red color as a result of chloroplasts in guard cells (Fig. 1D). *V. lineata* leaves present polycytic type of stomata characterized by a subsidiary cell in a horseshoe shape. The anticlinal walls of the epidermal cells are sinuous (Fig. 1E). In the epidermis of the furrow, glandular trichomes were observed to secrete substances of a lipid nature, as evidenced by positive reaction to Sudan IV (Fig. 1F).

**RWC and malate content:** Statistically insignificant difference in RWC was observed independently of water-deficit

**Statistical analysis:** Data were analyzed by *Excel* and *BioEstat* software. Data were expressed as mean  $\pm$  standard deviation. The analysis of variance (multifactor *ANOVA*) was followed by the mean comparison test (*Tukey 5%*) for data with normal distribution and homoscedasticity (Zar 1996). To quantitatively compare RLCs using parametric statistics, some descriptive parameters were used: maximum electron transport rate ( $ETR_{max}$ ) and optimal irradiance for the saturation of photosynthesis ( $I_{opt}$ ). ETR data were plotted as rapid light curves ( $RLC = ETR$  vs.  $I$ , where  $I$  are the irradiation pulses applied to draft the curves). Both parameters were plotted in a waiting-in-line equation ( $y = x e^{-x}$ ) using an *Excel* macro (*Microsoft Office Excel 2010*) (adapted from Ritchie 2008). The empirical model for ETR vs.  $I$  was first used by Gloag *et al.* (2007), who applied a suitable mathematical model for photosynthesis:  $ETR = A k_w I e^{-k_w I}$  (adapted from Gloag *et al.* 2007 and Ritchie 2008). The equivalent form for this first equation is:  $ETR = ETR_{max} k_w I e^{-k_w I}$ . The  $ETR_{max}$  ( $A$ ) occurs at an irradiance value of  $1/k_w$ , the optimal irradiance for the saturation of photosynthesis ( $I_{opt}$ ). The adjusted values for constants  $A$  (scaling constant for the height of the curve) and  $k_w$  (scaling constant for the axis X) were determined using the equation described by Gloag *et al.* (2007).

treatments and application of ABA compared with the control plants, where the values were kept high (Table 1).

In *V. lineata*, the control plants showed insignificant differences in malate contents between 6:00 and 18:00 h (Fig. 2), presenting a low value of  $\Delta$  malate [ $0.65 \mu mol g^{-1}(DM)$ ]. This result proved no evidence of a CAM pathway in the control plants. On the other hand, when the plants were subjected to DS for seven days and exogenous application of ABA for a period of 15 d, an increase in the concentrations of  $\Delta$  malate was evidenced (Fig. 2). Daily significant differences occurred between the night and day malate concentration in both treatments, showing a nocturnal acidification and revealing an upregulation of the CAM pathway in response to different types of stress. In the leaves of plants subjected to DS and ABA, the daily difference in the concentration of night and day malate ( $\Delta$  malate) was 140 and 187 times greater, respectively. Therefore, plants treated with ABA showed the greatest accumulation of malate during nighttime, indicating a high rate of nocturnal fixation of atmospheric  $CO_2$ .

**Photosynthetic pigments and RLC analysis:** The contents of Chl *a* and total Chl were significantly lower under DS and ABA when compared with the control treatment in leaves of *V. lineata*. Moreover, the content of Chl *b* and carotenoids (Car) after ABA treatment did not differ from the control. Car contents remained stable, even under stress treatments (Fig. 3).

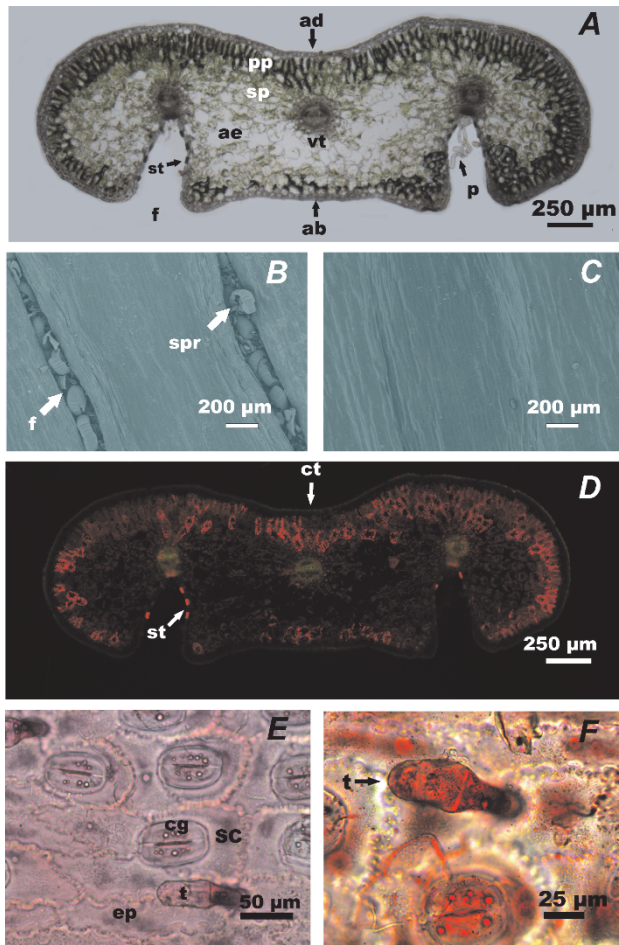


Fig. 1. Cross sections of *Vittaria lineata* leaf in LM showing adaxial epidermis, abaxial epidermis, furrow, mesophyll consisting of parenchyma tending to palisade and spongy parenchyma, paraphyse, stomata, and three vascular tissues of the meristele type (A). SEM image of the adaxial epidermal surface of the leaf: furrow and sporangia inside the furrow indicated by arrows (B). SEM image of the abaxial epidermal surface of the leaf (C). Cross sections of the leaf in FLM (excitation: 460–490 nm, emission: 520 nm) showing stomata inside the furrow (red) and the cuticle (green) indicated by arrows (D). LM image of the epidermal abaxial surface of the leaf after reaction to safranin, showing polycyclic stomata with subsidiary cells in a horseshoe shape and glandular trichome (E). Front view of the inner wall of the furrow showing glandular trichomes with positive reaction to Sudan IV, indicating the presence of lipids (F). ab – abaxial epidermis, ad – adaxial epidermis, ae – aerenchyma, ct – cuticle, epidermal cell, f – furrow, gc – guard cell p – paraphyse, pp – palisade parenchyma, sc – subsidiary cell, sp – spongy parenchyma, st – stomata, spr – sporangia, t – trichome, vt – vascular tissue of the meristele type.

Table 1. The changes of the relative water content (RWC) in leaves of *Vittaria lineata* under different treatments: control, drought stress (DS, 7 d), and 10  $\mu$ M abscisic acid (ABA) (15 d). Lowercase letters indicate the groups differentiated by ANOVA followed by Tukey's test,  $p > 0.05$  ( $n = 3$ ).

Treatment	RWC [%]
Control	92.36 $\pm$ 0.25 <sup>a</sup>
DS	91.69 $\pm$ 0.48 <sup>a</sup>
ABA	91.45 $\pm$ 0.44 <sup>a</sup>

In *V. lineata*, DS and ABA caused a reduction of 19.4 and 29.1% in  $I_{opt}$ , respectively (Table 2). A decline in the  $ETR_{max}$  under DS treatments (26.2%) and ABA (19.4%)

were also observed (Table 2). Rapid light curves in Fig. 4 show decrease in ETR of plants under DS and ABA treatment.

## Discussion

The succulent leaf has been described as a basic feature of plants that assimilate carbon fixation through CAM metabolism (Neales and Hew 1975). *Vittaria lineata* presents succulent leaves with two longitudinal furrows on the abaxial surface, where abundant stomata are located in a protected area, which possibly represents a microclimate that is intermediate to the internal and external environment of the leaf blade. Such a stomata location allows the creation of a humid microclimate within the longitudinal furrows to maintain water during recurring periods of drought. The epidermal cells show sinuous anticlinal walls, with thick cuticle. Hietz and Briones (1998) reported that these features are commonly found in epiphytic ferns and are among attributes responsible for maintaining water during recurring periods of drought. *Vittaria lineata* leaves show a parenchyma tending to the palisade, a spongy parenchyma characterized by cells

with large vacuoles and an aerenchyma. *Sensu lato*, aerenchyma is simply parenchymatous tissue with a large volume of intercellular space (Drew *et al.* 2000). The presence of aerenchyma in leaves of xerophytic species of the *Selaginella* was verified almost a century ago by Uphof (1920). In this paper, we propose that the aerenchyma in *V. lineata* could hold water vapor and accumulate  $CO_2$  from nocturnal respiration for photosynthetic activity during the day. In *V. lineata* leaves, stomata are located only in the furrows, which also have paraphyses, trichomes and sporangia. The presence of other structures in the epidermis of the furrows could hinder the stomata  $CO_2$  uptake. Actually, the mechanisms for  $CO_2$  accumulation in aerenchyma occur in plants living in habitats with stressful environments. In the aquatic plant *Lobelia dortmanna* L. (Campanulaceae), Pedersen and Sand-Jensen (1992) show that the concentration of  $CO_2$  in its lacunae increases

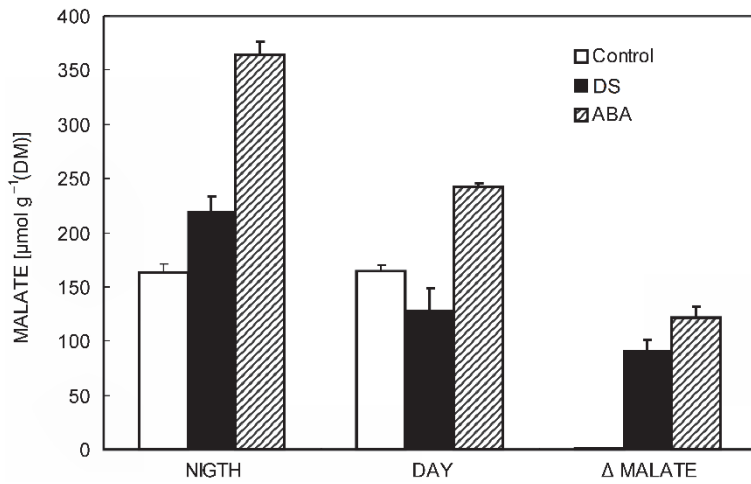


Fig. 2. Daily fluctuation (6:00–18:00 h) in malate concentration and diurnal malate fluctuations ( $\Delta$  malate) in leaves of *Vittaria lineata* under different treatments: control, drought stress (DS, 7 d), and ABA-treated leaves (15 d) (10  $\mu$ M). Lowercase letters indicate the groups differentiated by ANOVA followed by Tukey's test,  $p > 0.05$  ( $n = 3$ ). ABA – abscisic acid; DM – dry mass; DS – drought stress.

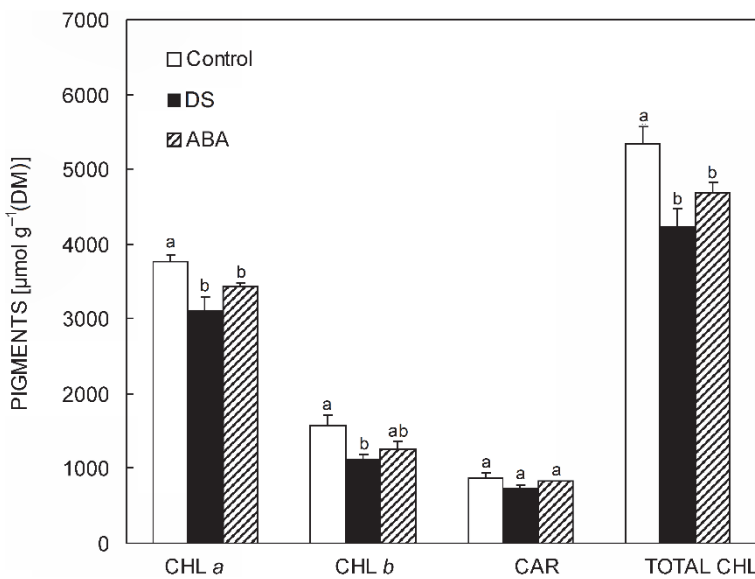


Fig. 3. Chlorophyll (Chl) a, Chl b, total Chl, and carotenoid (Car) contents in *Vittaria lineata* leaves under different treatments. Mean  $\pm$  SD ( $n = 3$ ). Lowercase letters indicate the groups differentiated by ANOVA followed by Tukey's test,  $p > 0.05$  ( $n = 3$ ). ABA – abscisic acid; DM – dry mass; DS – drought stress.

Table 2. Rapid light curve (RLC) parameters plotted as electron transport rate (ETR) vs. irradiance (I) in sporophytes of *Vittaria lineata* under different treatments. Means followed by the same letter are not significantly different. Lowercase letters indicate the groups differentiated by ANOVA followed by Tukey's test,  $p > 0.05$  ( $n = 3$ ).  $A$  – scaling constant for the height of the light curve;  $ETR_{max}$  – maximum electron transport rate;  $I_{opt}$  – optimal irradiance;  $k_w$  – scaling constant for the x-axis of the light curve.

Parameter	Control	Water deficit	ABA
$I_{opt}$ [ $\mu$ mol(photon) $m^{-2} s^{-1}$ ]	1.653 $\pm$ 59 <sup>a</sup>	1.332 $\pm$ 83 <sup>b</sup>	1.172 $\pm$ 23 <sup>b</sup>
$ETR_{max}$ [ $\mu$ mol(electron) $m^{-2} s^{-1}$ ]	97 $\pm$ 4 <sup>a</sup>	72 $\pm$ 6 <sup>b</sup>	78 $\pm$ 4 <sup>b</sup>
$k_w$	0.0006 $\pm$ 0.0001	0.0007 $\pm$ 0.0001	0.0008 $\pm$ 0.0001
$A$	259 $\pm$ 14	190 $\pm$ 22	220 $\pm$ 19
Correlation coefficient $r$	0.98	0.99	0.97
$n$ samples/curves	3/30	3/30	3/30

23 times of ambient atmospheric concentration, ranging from about 0.3% during the day to about 0.7% at night. Similar results were obtained from *Typha latifolia* L. (Typhaceae) (Constable *et al.* 1992), where the gas in the leaf aerenchyma ranged from ambient  $CO_2$  contents around noon to about 0.6% of  $CO_2$  (18 times higher than ambient) in the early morning.

The responses of plants to DS are highly complex.

Early responses usually help the plant to survive, while it acclimates, by the accumulation of certain new metabolites. They endow the plant with the structural capabilities required to improve its function under DS (Pinhero *et al.* 1997). In the present paper, we verified the increase in  $\Delta$  malate, when plants of *V. lineata* were subjected to water depletion and exogenous application of ABA. Therefore, both ABA and DS induced CAM pathway in



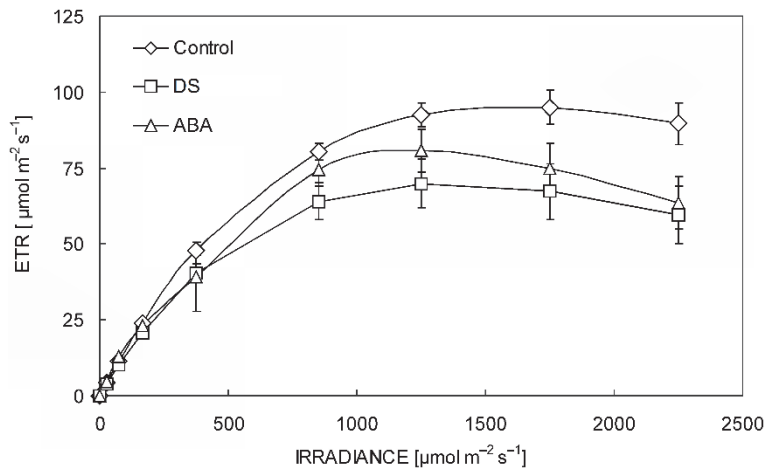


Fig. 4. Rapid light curve (RLC) plotted as ETR vs. irradiance in sporophytes of *Vittaria lineata* under different treatments: Mean  $\pm$  SD ( $n = 3$ ). Lowercase letters indicate the groups differentiated by ANOVA followed by Tukey's test,  $p > 0.05$  ( $n = 3$ ). ABA – abscisic acid; DS – drought stress.

*V. lineata*. The accumulation of malate at nighttime is a characteristic of the CAM pathway (Chu *et al.* 1990, Cushman and Borland 2002, Rut *et al.* 2008, Freschi *et al.* 2010). Thus, our data suggest that *V. lineata* seemed to change its mode of carbon fixation from  $C_3$  to the CAM pathway in response to DS and exogenous application of ABA. This response was observed earlier by Chu *et al.* (1990) who observed that the facultative halophyte *Mesembryanthemum crystallinum* shifts its mode of carbon assimilation from the  $C_3$  pathway to CAM in response to DS and ABA. Plants exhibiting this behavior are referred to as facultative CAM in contrast to obligatory CAM, where the metabolism is not dependent on environmental or developmental factors (Chu *et al.* 1990). According to Horton (1971), exogenous ABA initiates stomata closure in leaves of angiosperms. In contrast, Brodribb and McAdam (2011) and McAdam and Brodribb (2012) showed that lycophyte and fern stomata lack key responses to ABA and to the epidermal cell turgor. Brodribb and McAdam (2011) observed that the stomata of the fern species, *Pteridium esculentum*, showed no response to high xylem ABA concentration, despite the accumulation of high contents of ABA in the leaves.

In *V. lineata*, DS and exogenous application of ABA did not alter the RWC when compared with the control plants. Both treatments led to a decrease in the contents of Chls, as well as  $\text{ETR}_{\text{max}}$ ,  $I_{\text{opt}}$ , and, consequently, the RLC of *V. lineata* leaves. The decrease in Chl content in plants under stress has been previously described in angiosperm species (Ashraf *et al.* 1994, Jaing *et al.* 1994) and epiphytic ferns in a Mexican rainforest, *i.e.*, *Polypodium plebeium* Schltdl. & Cham., *Elaphoglossum petiolatum* (Sw.) Urban, *Phlebodium areolatum* (Humb. and Bonpl. ex Willd.) J. Sm., and *Asplenium cuspidatum* Lam. (Tausz *et al.* 2001). In barley leaves, ABA inhibited the photosynthetic process, as evidenced by lower values of  $F_v/F_m$

(N'Soukpoé-Kossi *et al.* 1999). According to Maxwell and Johnson (2000), the decrease of RLC under stress indicates photoinhibition of PSII. Drought stress is known to inhibit photosynthetic activity in tissues as a consequence of an imbalance between light capture and its utilization; downregulation of PSII activity results in an imbalance between the generation and utilization of electrons (Peltzer *et al.* 2002). From the data obtained in our study, we suggest that DS and application of exogenous ABA negatively regulated photosynthesis or the rate of electron transport in leaves of *V. lineata*. No significant differences between treatments were observed in Car contents in leaves of *V. lineata* after DS and ABA treatment. Tausz *et al.* (2001), also observed that the amount of Car was not altered by different treatments of water restriction in relation to the control treatment in most ferns. Car are part of ROS detoxification in plants under DS. However, the degree, to which the activities and amounts of antioxidant enzymes increase under DS, varies among plants, depend-

ing on the species, the development, and the metabolic state of the plant, as well as the duration and intensity of the stress (Reddy *et al.* 2004).

In the present paper, we observed an interesting leaf anatomy in *V. lineata*. It could maintain leaf water balance and probably allow the plant to accumulate nocturnal  $\text{CO}_2$  in aerenchyma as a strategy to compensate for the restriction in stomata number and localization, which could limit  $\text{CO}_2$  assimilation. Our data demonstrated that *V. lineata* could change its mode of carbon fixation from  $C_3$  to the CAM pathway in response to DS and exogenous application of ABA. We suggest that controversial data on photosynthetic pathway of *V. lineata* (Carter and Martin 1994, Zotz and Ziegler 1997, Zotz 2004, Martin *et al.* 2005) could be attributed to the presence of the facultative CAM pathway induced by DS and ABA.

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