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Responses of wild *Piper* species to drought and rehydration cycles considering stomatal closure as a marker of the alarm phase

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Abstract

Studies that simulate recurrent drought events with subsequent episodes of rehydration better reflect natural conditions and allow visualization of differential acclimatization responses resulting from memory and tolerance mechanisms. *Piper aduncum* and *Piper tuberculatum* were grown in a greenhouse and were subjected to three successive cycles of drought and subsequent rehydration. After suspending irrigation, gas exchanges were measured daily with IRGA. When stomatal conductances close to zero were obtained, the plants were rehydrated and kept irrigated. In *P. tuberculatum*, stomatal conductance was always higher after periods of rehydration compared to the period before the drought, while the transpiration rate was lower only during the drought. The damage to the photosynthetic apparatus was caused by the influence of the interception of the flow of electrons in the transport chain. We came to the conclusion that the dehydrated plants showed an alert signal, which triggered response mechanisms to prevent or deal with the water stress situation.

Keywords: anatomical adaptations; compensatory development; *Piper aduncum*; *Piper tuberculatum*; water deficit.

Highlights

- *Piper tuberculatum* showed greater signs of alarm activation in the face of water stress
- The rehydration cycles provided an increase in the rate of photosynthesis
- Stomatal density increases through drought cycles

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Abbreviations: abE – abaxial epidermis; ABS/RC – energy absorption flux per reaction center; adE – adaxial epidermis; APDM – dry mass of the aerial part; Chl – chlorophyll; C_i – internal CO_2 concentration; CSVBL – cortex stem vascular bundle length; DCSV – diameter of cortex stem vessels; DI_0/RC – energy flux dissipated per reaction center; E – transpiration; EDS – equatorial diameter of stomata; ET_0/RC – energy transport flow per reaction center; g_s – stomatal conductance; ICE – instantaneous carboxylation efficiency; LA – leaf area; LAR – leaf area ratio; LDM – leaf dry mass; LMF – leaf mass fraction; LSMVB – length of stem medulla vascular bundle; LSMX – length of stem medulla xylem; LVBL – leaf vascular bundle length; NL – number of leaves; NRV – number of root vessels; NVMS – number of vessels medulla stem; NXV – number of xylem vessels; PDS – polar diameter of stomata; $PI_{(abs)}$ – performance index for conservation of energy from captured excitons to reduction of intersystem electron acceptors; P_N – photosynthetic rate; PPT – palisade parenchyma thickness; RC/CS_0 – number of active PSII reaction centers per cross sections; RDM – root dry mass; Rep – root epidermis; RI – robustness index; RMF – root mass fraction; R/SR – root/shoot ratio; RS – reducing sugar; RTD – root tissue density; RV – root volume; RVD – root vessel diameter; RXL – root xylem length; SD – stem diameter; Sd – stomatal density; SL – stem length; SMF – stem mass fraction; SPT – spongy parenchyma thickness; SRL – specific root length; TDM – total dry mass; TR_0/RC – energy flow captured by reaction center; TSS – total soluble sugar; WUE – water-use efficiency; XCSL – xylem cortex stem length; φE_0 – quantum efficiency of electron transfer from Q_A^- to the electron transport chain beyond Q_A^-; φP_0 – maximum photochemical quantum yield; Ψ – leaf water potential.

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Introduction

Water deficit is one of the abiotic factors that most affect agricultural production worldwide, restricting growth, carbon allocation, carbohydrate content and allocation, and productivity (Menezes-Silva *et al.* 2017). Drought tolerance from a biological point of view is the individual's ability to survive a stressful event, however, in the agronomic scope, tolerance refers to the maintenance of productive capacity even under stressful conditions (Maggio *et al.* 2018). The main drought survival strategies involve tolerance mechanisms to reduce or repair injuries and prevention strategies, which are dedicated to avoiding harmful effects (Fang and Xiong 2015). Avoidance and tolerance strategies are activated by the perception of water deficit and can act alone or together (Gray *et al.* 2016). Reducing growth under resource constraints is the most widely conserved prevention strategy among plant species (Maggio *et al.* 2018).

Piper species naturally occur in humid forests, mainly in shady environments, particularly in the Atlantic Forest and Amazon (Araújo and Miguel 2011). *P. tuberculatum* is found in diverse environments, such as clearings, understory, pastures, and forests, while *P. aduncum* is commonly found in forest edges and understory, especially in places with soil moisture (Sarnaglia Junior *et al.* 2014).

Under water deficit conditions, only *P. aduncum* has been studied (Pacheco *et al.* 2022). However, plant development and ecophysiological mechanisms have been little explored in terms of tolerance mechanisms for water deficit. Plants of *P. aduncum* grown in pots and subjected to moderate water suppression for eight days had an increase in the contents of photosynthetic pigments and essential oils but did not change the production of dry mass (Pacheco *et al.* 2022). Under field conditions, the cultivation of *P. aduncum* in rainfed conditions provided a 62% reduction in essential oil contents compared to irrigated environments, however, excess water was also harmful (Jacinto *et al.* 2018). In a degraded area subjected to water deficit, *P. aduncum* was considered one of the most sensitive to transplantation due to lower survival and seedling height after 12 months (Oliveira *et al.* 2021b). When in their natural environment, plants are subject to recurrent cycles of drying and rehydration (Braga *et al.* 2022). However, each plant species responds to these stress cycles differently.

Studies that simulate recurrent drought events with subsequent episodes of rehydration impose greater challenges on plant metabolism, better reflect natural conditions, and allow visualization of differential acclimatization responses resulting from memory (Jacques *et al.* 2021). In the essay by Pacheco *et al.* (2022), only a punctual moderate drought event was considered, a factor that may have contributed to the lack of visualization of the effect on dry mass accumulation. On the other hand, in the studies by Jacinto *et al.* (2018) and Oliveira *et al.* (2021b), the tests were carried out in the field, and environments were subject to periods of recurrent drought and rehydration, providing indications that recurrent drought can harm plant development.

The first phase of stress is the alarm or alarm reaction and is initiated by the activation of detection and signaling pathways arising from the disturbance of homeostasis caused by the stressor (Borowitzka 2018). The most observed alarm reaction under water deficit is stomatal closure which can be revealed as the activation of survival mode, where plants exhibit slow and reduced growth and consequent low productivity (Maggio *et al.* 2018). Therefore, the induction of stomatal closure is one of the first mechanisms activated under water deficit to prevent water loss and indicates that the plant has entered the alarm phase (Maggio *et al.* 2018). However, water stress can also cause damage to nonstomatal pathways, such as an imbalance in the electron transport chain or the content of photosynthetic pigments. However, it also directly affects carbon accumulation and carbohydrate synthesis and allocation (Oliveira *et al.* 2021a).

Alarm hypersensitivity will lead to early activation of stress prevention mechanisms and will culminate in greater survival if stress is severe, however, in agricultural environments, drought is usually mild or moderate, and avoiding alarm reactions can increase productivity (Maggio *et al.* 2018). Therefore, knowing how plants behave in the face of episodes of recurrent water deficit, considering stomatal closure as an alarm signal, is important for defining management and genetic improvement strategies that seek less hypersensitive wild species for crossbreeding purposes.

Wild *Piper* species produce metabolites with biological activities of interest to the pharmaceutical and agricultural industries (Salehi *et al.* 2019). Among these species, *Piper aduncum* L. and *Piper tuberculatum* Jacq. stand out due to their insecticidal, acaricidal, antiparasitic, bactericidal, and fungicidal properties (Durofil *et al.* 2021, Moncayo *et al.* 2021). Furthermore, both species are associated with endophytic microorganisms, which has aroused industrial interest (Chithra *et al.* 2014, Oliveira *et al.* 2021b).

The objective of the present study was to evaluate the physiological, morphological, and anatomical responses of two *Piper* species subjected to three cycles of drought and subsequent rehydration, considering stomatal closure as a marker of the alarm phase. Our focus was specifically to evaluate the resilience of plants when subjected to recurrent cycles of water deficit, considering stomatal closure as a marker of the alarm phase. Three hypotheses were tested: (*1*) plants reduce the ability to perceive drought by delaying the alarm at each recurrent drought cycle; (*2*) reduced photosynthesis will be caused by nonstomatal damage; (*3*) growth will be reduced as an avoidance strategy even with no limitations on carbohydrate content.

Materials and methods

Plant material and growing conditions: The experiment was conducted between January and February 2020, at the Linhares Experimental Farm (FEL), of the Capixaba Institute for Research, Technical Assistance and Rural Extension (Incaper), located at 19°25'0.1"S and 40°4'35.3"W, in the municipality of Linhares, in the northern region of the state of Espírito Santo. Two wild species of *Piper* were evaluated (*Piper aduncum* L. and *Piper tuberculatum* Jacq.) propagated by seeds collected from mother plants of the *Active Germplasm Bank* located in FEL.

The seedlings were obtained according to the protocol proposed by Dousseau *et al*. (2011). After 3 months from sowing, seedlings of 5 to 6 cm in height were selected and transplanted into tubes with a capacity of 280 cm^3 filled with commercial organic substrate (*Bioplant*), containing 5 g per tube of slow-release fertilizer (*Osmocote*®, 6 months, formula NPK 15–09–12) and kept in a nursery under 50% shading obtained with standard black screens for another 4 months until they reach 30 cm in length from the shoot.

The transplant was carried out into plastic pots with a capacity of 8 L, filled with a substrate composed of 2.17 kg of limestone, 5 kg of simple super phosphate, 0.33 kg KCl, 58.33 g of FTE, and two bags of 25 kg of chicken manure per m^3 of soil, also adding 5 g of slow-release fertilizer per pot (*Osmocote*®, 8 months, NPK formula 09–15–12). For five months, the plants were acclimatized in a greenhouse with a polyethylene cover and a 30% *Aluminet* screen. Plants were irrigated at field capacity and cultural treatments (fertilization and pest and disease control) were carried out as necessary.

Treatments: Twelve-month-old plants were subjected to three successive cycles of drought and subsequent rehydration. After irrigation was suspended, gas exchange was measured daily with the *LI-COR 6400* infrared – IRGA gas analyzer (*LI-COR Inc*., Lincoln, NE, USA). When stomatal conductances close to zero were obtained, the plants were rehydrated and kept irrigated until the measurements returned to the values of the controls, when the second cycle began, being conducted in the same way for the third cycle. The drying and rehydration time varied within the recurrent water deficit cycles.

The first cycle began on 10 January 2020 and throughout the experimental period, the temperature and humidity of the air inside the greenhouse (*Model 200*, *Spectrum Technologies*, USA) were monitored.

Water potential: Evaluations of leaf water potential (Ψ) were carried out in the two groups of plants (drought and control) before starting the study, T0, and during each period of drought and rehydration of the three cycles, with one plant per plot evaluated. The Ψ_w was measured using the Scholander pressure chamber (Scholander *et al.* 1964) (*Model 1000*, *PMS Instrument Co*., Albany, OR, USA) in fully expanded healthy leaves, collected in the middle third of the plants, in the morning between 05:00 and 6:00 h.

Gas exchange: Evaluations were carried out after the first and second periods of drought after the plants had a conductance close to zero and after rehydration. Gas exchanges were evaluated between 08:00 and 11:00 h, being carried out in fully expanded leaves located at the 2nd or 3rd node from the apex of the branch, two plants per plot were evaluated. A CO_2 injection controller was used to maintain a constant CO_2 concentration of 400 ppm in the chamber of the IRGA *LI-6400*, which has an analysis area of 6 cm^2 and is equipped with a red–blue light source (*6400-02B*) emitted using light-emitting diodes (LEDs). The photosynthetic rate (P_N), transpiration (E) per unit of leaf area, and stomatal conductance to water vapor (g_s) were determined by the infrared gas analyzer in PAR ≥ 1,200 μmol(photon) m^{-2} s^{-1}. In addition, the water-use efficiency (WUE = P_N/E) and instantaneous carboxylation efficiency (ICE = P_N/C_i) were calculated (Machado *et al.* 2005).

Chlorophyll *a* fluorescence: In the third cycle of water deficit (third period of drought and rehydration), evaluations of chlorophyll (Chl) *a* fluorescence were carried out in two plants per plot using a portable fluorometer from *Hansatech*, model *Handy-PEA* (*Hansatech*, UK), according to the recommendations of Strasser *et al.* (2004). Two leaves of each plant were adapted to the dark using leaf clips for 30-min period for complete oxidation of the photosystem. Afterward, a flash of saturating light of 3,000 μmol(photon) m^{-2} s^{-1} was emitted with a duration of 1 s. From the transient fluorescence OJIP, the parameters established by the JIP-test were calculated. The interpretation and normalization of the parameters measured and calculated from this test were by Strasser and Strasser (1995).

Photosynthetic pigments: At the end of the three cycles of drying and rehydration, the chlorophyll and carotenoid contents were quantified, according to the methodology by Arnon (1949) and Rodriguez-Amaya and Kimura (2004), respectively. A leaf was collected from two plants per plot and immediately taken to the Laboratory of Plant Physiology and Post-Harvest at Incaper, where 2- and 3-mm discs were removed for the extraction of chlorophylls and carotenoids, respectively. The discs were weighed and the mass was used in the equations proposed by Arnon (1949) and Rodriguez-Amaya and Kimura (2004). The readings were taken in a spectrophotometer (*Beckman*, *model 640B*) and the pigment contents were expressed in mg g^{-1}.

Carbohydrate allocation and partitioning were evaluated by quantifying reducing sugars, total soluble sugars, and starch in leaves, stems, and roots of plants at the end of the three cycles of drying and rehydration. The plant material was dried in an oven with forced air circulation at 65°C until constant mass, crushed in a *Willey* mill, model *STAR FT-50*, and stored in a freezer at –40°C. The extracts were obtained according to Zanandrea *et al.* (2009), using 0.2 g of plant tissue. For the quantification of total soluble sugars and starch, the anthrone method (Yemm and Willis 1954) was used, with modifications. The reaction volume contained 2 mL of anthrone solution at 0.19% in acid 93.33% sulfuric acid and 1 mL of plant extract and was subjected to 100°C for 3 min. Reducing sugars were quantified according to the protocol described by Miller (1959), using the dinitrosalicylic acid (DNS) method.

Plant development and dry mass allocation: Plant development was evaluated after three cycles of water deficit and rehydration. Five plants per plot were evaluated and the length of the stem (SL) and of the largest root was measured, determined using a tape measure, and expressed in centimeters. Stem diameter (SD) in mm was measured using a digital caliper. The number of leaves (NL) and roots were both counted manually. The root volume (RV) was measured from the displacement of water in a beaker and expressed in cm^3. The leaf area (LA) was determined using a *LI-COR 3100* meter and expressed in cm^2. The allocation of dry mass was evaluated by obtaining the dry mass of leaves (LDM), stems (SDM), roots (RDM), shoots (APDM), and total (TDM), after drying in a forced air circulation oven at 70°C until constant mass, followed by weighing on an analytical scale, with values expressed in g.

Based on these data, the following were calculated: specific leaf area (SLA), specific leaf mass (SLM), leaf mass fraction (LMF), stem mass fraction (SMF), root mass fraction (RMF), root tissue density (RTD), root/shoot ratio (R/SR), leaf area ratio (LAR), specific root length (SRL), and robustness index (RI). The robustness index was obtained by the ratio between SL/SD. The SMF was obtained by dividing the dry mass of the stem by the total dry mass of the plant, expressed in $g\ g^{-1}$, according to Poorter *et al*. (2012). APDM was obtained by adding LDM and SDM and expressed in g. The SRL was obtained by dividing the root length by the root dry mass and the value was expressed in $m\ g^{-1}$, according to Kramer-Walter *et al*. (2016). The RTD was calculated by root dry mass divided by fresh root volume, according to Kramer-Walter *et al*. (2016), and expressed in $g\ cm^{-3}$. RMF was calculated according to Poorter *et al*. (2012), dividing the dry mass of the root by the total dry mass of the plant and expressing the results in $g\ g^{-1}$.

Structural analyzes: Anatomical evaluations were performed at the end of the three drying and rehydration cycles. Completely expanded leaves of the third branch, stem internode fragments between the 5th and 7th node of the largest branch, and root fragments 5 cm above the root cap of five plants in each plot were collected. The tissues were fixed for 48 h in FAA (37% formaldehyde, glacial acetic acid, and 70% ethyl alcohol) and after that time stored in 70% ethyl alcohol.

Cross-sections were made in the stem, root, and midrib region of the leaves. Sections were cleared with sodium hypochlorite and stained with safrablue (9:1 astra blue and safranin). Paradermal sections were made on the abaxial side of the leaves, using the epidermal printing technique, using universal instant adhesive (*Super-Bonder®*) as described in Segatto *et al*. (2004), for stomatal evaluations. All sections were analyzed under a bright field microscope (*Euromex*). The images were captured with a micro camera (*CMEX 5*) and the biometric measurements of the tissues were performed using the *ImageFocus 4* software.

From the paradermal sections of the leaves, the polar (PDS) and equatorial (EDS) diameter of the stomata and the stomatal density (Sd) were evaluated. The thickness of the adaxial (adE) and abaxial (abE) epidermis, the palisade (PPT) and spongy (SPT) parenchyma, the length of the vascular bundle (LVBL), and the number of xylem vessels (NXV) were quantified from the cross-sections of the leaves.

From cross-sections of the stem, the vascular tissues of the cortex (cx) and medulla (me) regions were evaluated. From the cortex, the length of the vascular bundle (CSVBL) and the xylem (XCSL) and the number (NSCV), and the diameter of the vessels (DCSV) were evaluated. From the medulla, vascular bundle (LSMVB) and xylem (LSMX) length and number (NVMS) were evaluated.

From the cross-sections of the roots, the thickness of the epidermis (Rep), the length of the xylem tissue (RXL), and the number (NRV) and the diameter of the vessels (RVD) were quantified.

Statistical analysis and experimental design: The experiment was carried out in a randomized block design, with four replications of ten plants per plot and in a 2 × 2 factorial scheme, with two species in two water conditions (one kept irrigated as control and the other submitted to three cycles of drought followed by rehydration). Statistical analyzes were performed using the *SISVAR version 4.3* statistical program (Ferreira 2011). After analysis of variance, the means were submitted to the *Scott-Knott* group test at a significance level of 5% ($p<0.05$).

Results

Water potential: Plants kept in the control environment did not change leaf water potential, maintaining values close to –0.3 MPA in both species (Fig. 1). The leaf water potential was lower after periods of drought, with values below –1.2 MPA, showing that the plants were under water deficit. The leaf water potential values of the plants before starting the drought cycles (T0) and those that were kept in the control environment did not differ from the plants submitted to the rehydration period, proving that the water status was restored. In *P. aduncum* plants, there were no differences in water potential between periods of drought, even with an increase in days of water suspension, indicating a possible mechanism for maintaining turgor. In *P. tuberculatum*, the leaf water potential was lower after the second drought, corroborating the longer water suspension time.

Gas exchange: At the end of rehydration, the rate of photosynthesis was higher when compared with the beginning of the experiment (T0) and with recurrent drought (Fig. 2*A*), regardless of the evaluated species. No statistical differences were observed between species and environments for intercellular CO_2 concentration (C_i) (Fig. 2*C*). The stomatal conductance (conductance for H_2O) and the transpiration rate were lower in both periods of the drought cycles (dry1 and dry2) for the two evaluated species, however, the behavior differed in the other environments for the species (Fig. 2*B*,*D*). For *P. aduncum*, stomatal conductance and transpiration rate were higher after the second rehydration step (rec2), followed by rehydration 1 (rec1) and T0, which did not

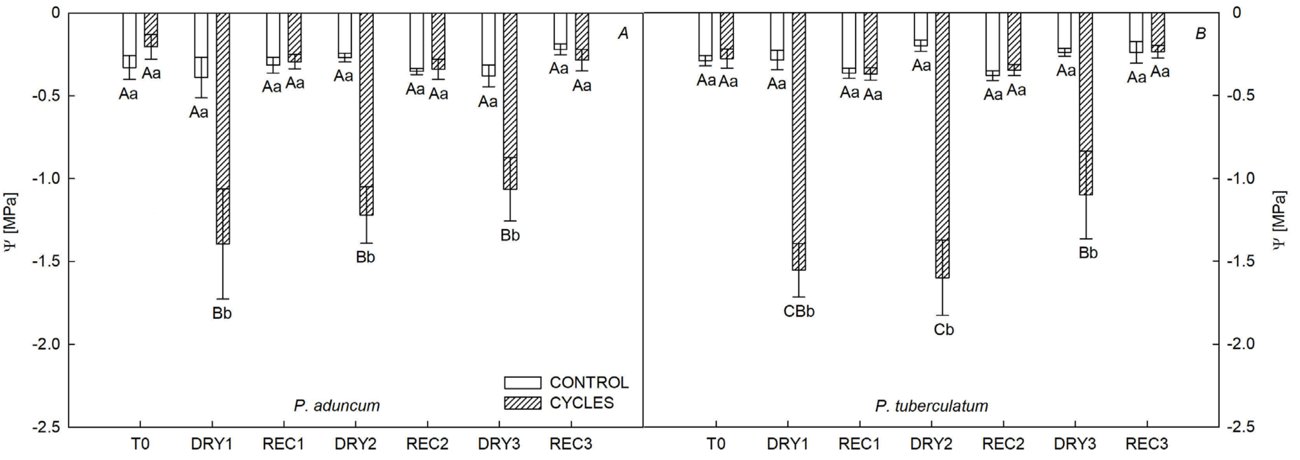

Fig. 1. Leaf water potential (Ψ) measured in the predawn in the wild species of *Piper* (*A* – *P. aduncum* and *B* – *P. tuberculatum*) submitted to three cycles of drought (dry1, 2, and 3) and rehydration (recovery) (rec1, 2, and 3), compared with the control (plants kept irrigated and evaluated throughout the experimental period) and with the values before the drought (T0). Means followed by *the same letter* do not differ from each other by *Tukey*'s test ($p<0.05$), where *capital letters* are used to compare treatments in each environment (control and cycles), while *lowercase letters* compare control with drought cycles in each treatment. *The bar* corresponds to the standard error of the average of four replicates of a plant.

differ from each other. However, the stomatal conductance of *P. tuberculatum* was higher after periods of rehydration compared to T0, while the transpiration rate was lower only in recurrent drought (Fig. 2*B*,*D*).

After periods of drought, there were no differences in stomatal conductance between species, but at T0 and after recovery, *P. tuberculatum* showed higher stomatal conductance compared to *P. aduncum*. The transpiration rate of *P. tuberculatum* was higher at T0 and after the first rehydration, but there were no differences between the species in the other environments.

Water-use efficiency (WUE) was lower in both periods of recurrent drought, regardless of species (Fig. 2*F*). The instantaneous efficiency of carboxylation (ICE) was higher after the rehydration periods, followed by T0 and lower after the dry cycle, regardless of the species (Fig. 2*E*).

Chlorophyll *a* fluorescence: The maximum photochemical quantum yield (φP_0) showed no difference in *P. aduncum* in any of the treatments, however, in *P. tuberculatum*, there was an increment of φP_0 in the control and at the end of the third drought cycle. An exponential increase in φP_0 was observed in *P. aduncum* when compared to *P. tuberculatum* after the drought rehydration cycle (Table 1).

The quantum yield of electron transfer from Q_A^- to the electron transport chain beyond Q_A^- (φE_0) showed an increase in T0, rec3, and dry3 in *P. aduncum* plants compared to *P. tuberculatum* plants (Table 1).

P. aduncum showed no significant difference for the flux of energy absorption per reaction center (ABS/RC) and for the flux of energy captured per reaction center (TR_0/RC) in the three environments (T0, rec3, and dry3). However, in *P. tuberculatum* there was an increase in both ABS/RC and TR_0/RC in plants that underwent the cycles of drought and rehydration (rec3). There was an increase in ABS/RC and TR_0/RC in treatments T0, dry3, and rec3 in *P. tuberculatum* plants compared to *P. aduncum* (Table 1).

P. tuberculatum showed a greater flux of energy dissipated per reaction center (DI_0/RC) in the rec3 treatment plants, followed by T0 and dry3, which did not differ between themselves, while for *P. aduncum*, no significant differences were observed. The DI_0/RC was higher in rec3 in *P. tuberculatum* plants followed by *P. aduncum* (Table 1).

P. aduncum showed higher values in the number of active PSII reaction centers per cross-sections (RC/CS_0) in T0 and dry3, followed by rec3. However, *P. tuberculatum* showed higher values in T0 and dry3, followed by rec3. When the species were compared, *P. aduncum* showed higher values of RC/CS_0 in T0, dry3, and rec3 compared to *P. tuberculatum*. The same pattern previously described was observed for the $PI_{(abs)}$ where *P. aduncum* was superior to *P. tuberculatum* (Table 1).

Photosynthetic pigments: As for chlorophyll (Chl) content, *P. aduncum* showed higher values of Chl *a*, Chl *b*, and total Chl in the control environment. The same was observed when comparing *P. aducum* to *P. tuberculatum* (Fig. 1S*A*–*C*, *supplement*). The total carotenoid contents were higher in the plants of the dry cycles in the plants of *P. tuberculatum* in comparison to *P. aduncum* (Fig. 1S*D*).

Growth: The stem length (SL) increased in both studied species when kept irrigated (control), however, the stem diameter showed a difference only in *P. tuberculatum* where there was an increase in the plants submitted to the three cycles of drought and rehydration (Fig. 2S*A*, *supplement*). *P. aduncum* and *P. tuberculatum* showed an increase in the robustness index (RI) (Fig. 4*E*) and in the stem mass fraction (SMF) in the control plants (Fig. 4*A*).

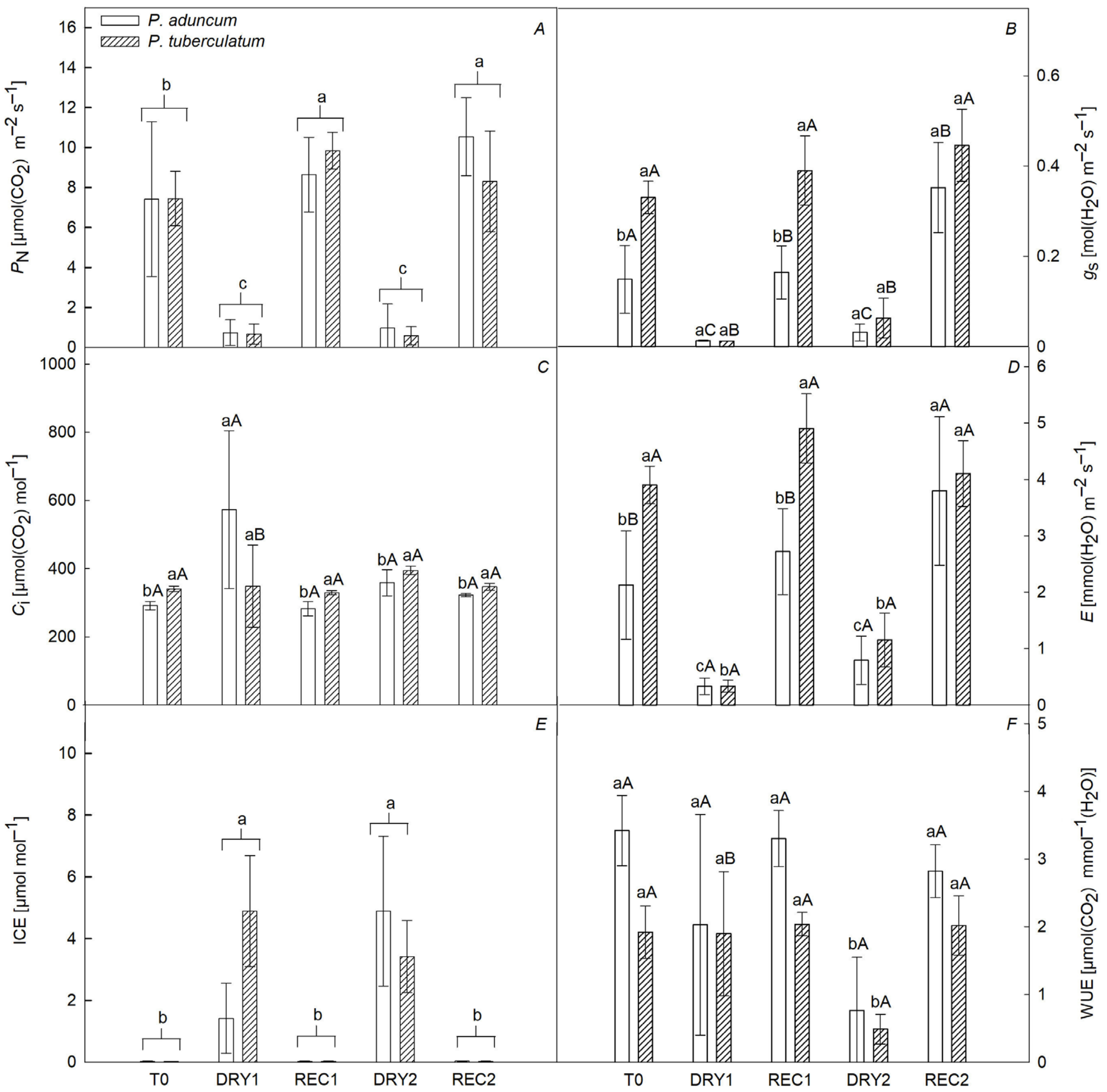

Fig. 2. Gas exchanges in *Piper aduncum* and *Piper tuberculatum* seedlings submitted to two cycles of drought (dry1 and 2) and rehydration (recovery) (rec1 and 2), compared with the control (plants kept irrigated and evaluated throughout the experimental period) and with the values before the drought (T0). Means followed by *the same letter* do not differ from each other by *Tukey*'s test ($p<0.05$), where *capital letters* are used to compare treatments (control and cycles) as a function of each species, while *lowercase letters* compare species with treatments. *The bar* corresponds to the standard error of the average of four replicates of a plant. *A* – photosynthetic rate (P_N), *B* – stomatal conductance (g_s), *C* – internal CO$_2$ concentration (C_i), *D* – transpiration (*E*), *E* – instantaneous carboxylation efficiency (ICE), *F* – water-use efficiency (WUE).

Root volume (RV) and total dry mass were higher in both species evaluated in the irrigated environment. Root tissue density was higher in *P. tuberculatum* after three cycles of drying and rehydration than that in *P. aduncum*. However, the root mass fraction showed a significant increase in control and drought in *P. tuberculatum* compared to *P. aduncum* (Fig. 4*C*). The root/shoot ratio (R/SR) showed an increase in *P. aduncum* in both control and drought compared to *P. tuberculatum* (Fig. 4*D*).

Carbohydrate allocation: As for carbohydrate content, *P. tuberculatum* showed higher contents of reducing sugars (RS) in the leaf control compared to *P. aduncum* (Fig. 3S*A*, *supplement*). Total soluble sugars (TSS) and starch did not show significant differences in the leaves. *P. tuberculatum* had higher contents of RS and TSS in the stem in plants with recurrent drought, *P. aduncum* also had the highest contents of TSS in plants in dry cycles (Fig. 3S*B*). *P. tuberculatum* plants showed higher contents of RS in recurrent and control drought and TSS in drought than *P. aduncum*. However, the roots of both species showed higher starch contents in the control plants compared to the dry cycles. Even in the dry cycles, *P. tuberculatum* showed a higher starch content compared to *P. aduncum* (Fig. 3S*C*).

Table 1. Photochemical parameters derived from the analysis of the JIP-test in *Piper aduncum* and *Piper tuberculatum* plants subjected to three cycles of drought and rehydration (recovery), compared with the control (plants kept irrigated and evaluated throughout the experimental period) and with the values before the drought (T0). Means followed by *the same letter* do not differ from each other by *Tukey*'s test ($p<0.05$), where *capital letters* are used to compare treatments (control and cycles) as a function of each species, while *lowercase letters* compare species with treatments. Maximum photochemical quantum efficiency (φP_0), quantum efficiency of electron transfer from Q_A^- to the electron transport chain beyond Q_A^- (φE_0), energy absorption flux by reaction center (ABS/RC), energy flux captured per reaction center (TR_0/RC), energy transport flux per reaction center (ET_0/RC), energy dissipated flux per reaction center (DI_0/RC), number of active PSII reaction centers per cross-section (RC/CS_0), and performance index for energy conservation from captured excitons to the reduction of intersystem electron acceptors [$PI_{(abs)}$].

Species	Water regime	$\varphi(P_0)$	$\varphi(E_0)$	ABS/RC	TR_0/RC	ET_0/RC	DI_0/RC	RC/CS_0	$PI_{(abs)}$
Piper aduncum	T0	0.742^{aA}	0.378^{aA}	2.988^{aB}	2.207^{aB}	1.119^{aA}	0.773^{aB}	234.437^{aA}	10.724^{bA}
	Rec3	0.746^{aA}	0.411^{aA}	3.003^{aB}	2.232^{aB}	1.213^{aA}	0.771^{aB}	204.116^{bA}	13.761^{aA}
	Dry3	0.758^{aA}	0.402^{aA}	2.893^{aB}	2.174^{aB}	1.131^{aA}	0.719^{aA}	243.248^{aA}	14.601^{aA}
Piper tuberculatum	T0	0.703^{aB}	0.295^{aB}	3.887^{bA}	2.705^{bA}	1.122^{aA}	1.175^{bA}	168.634^{aB}	5.0905^{aB}
	Rec3	0.645^{bB}	0.269^{aB}	4.657^{aA}	2.921^{aA}	1.184^{aA}	1.737^{aA}	152.153^{bB}	3.8700^{aB}
	Dry3	0.723^{aA}	0.308^{aB}	3.545^{bA}	2.546^{bA}	1.071^{aA}	0.998^{bA}	184.429^{aB}	6.386^{aB}

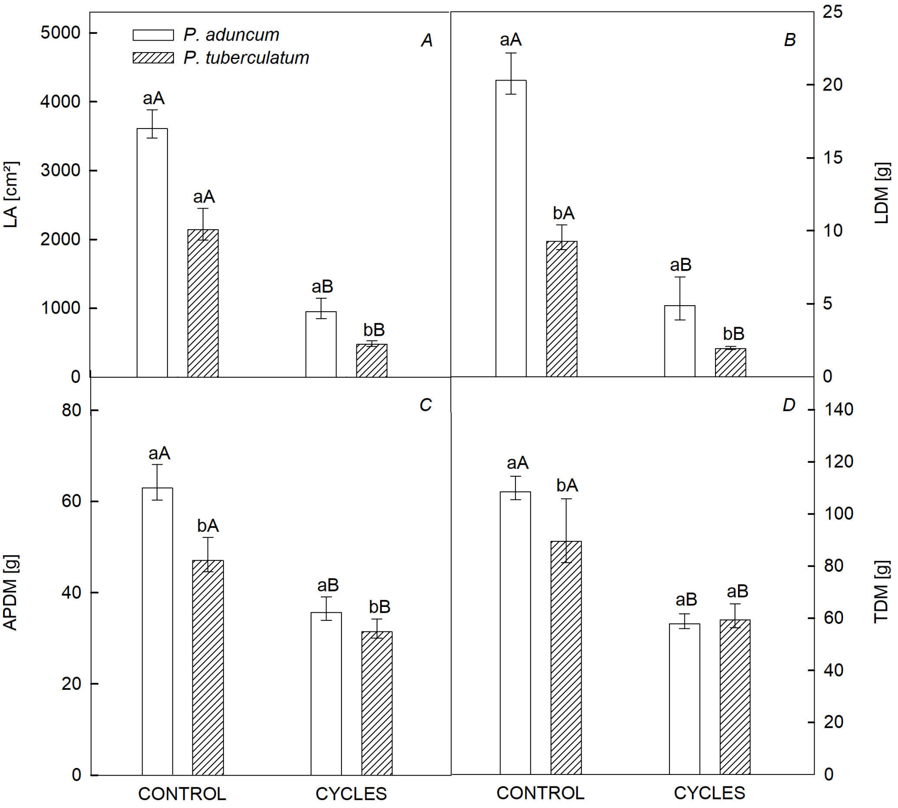

Fig. 3. Characterization of growth parameters in *Piper aduncum* and *Piper tuberculatum* plants submitted to three cycles of drought and rehydration (recovery), compared with the control (plants kept irrigated and evaluated throughout the experimental period). Means followed by *the same letter* do not differ from each other by *Tukey*'s test ($p<0.05$), where *capital letters* are used to compare species in each treatment (control and cycles) while *lowercase letters* compare treatments (control and cycles) in the function of each species. *The bar* corresponds to the standard error of the average of four replicates of a plant. *A* – total leaf area (LA), *B* – leaf dry mass (LDM), *C* – dry mass of the aerial part (APDM), *D* – total dry mass (TDM).

Anatomy: As for the leaf anatomical structures, the polar diameter of the stomata (PDS) of *P. tuberculatum* was higher in the control compared to the drought cycles (Fig. 5*A*). However, the equatorial diameter (EDS) was higher in control and drought in *P. aduncum* compared to *P. tuberculatum* (Fig. 5*C*). The equatorial diameter in *P. tuberculatum* increased in the control, followed by the drought plants. Inversely proportional to the polar and equatorial diameter of the stomata, the stomatal density (SD) was higher in *P. tuberculatum* in both cultivation

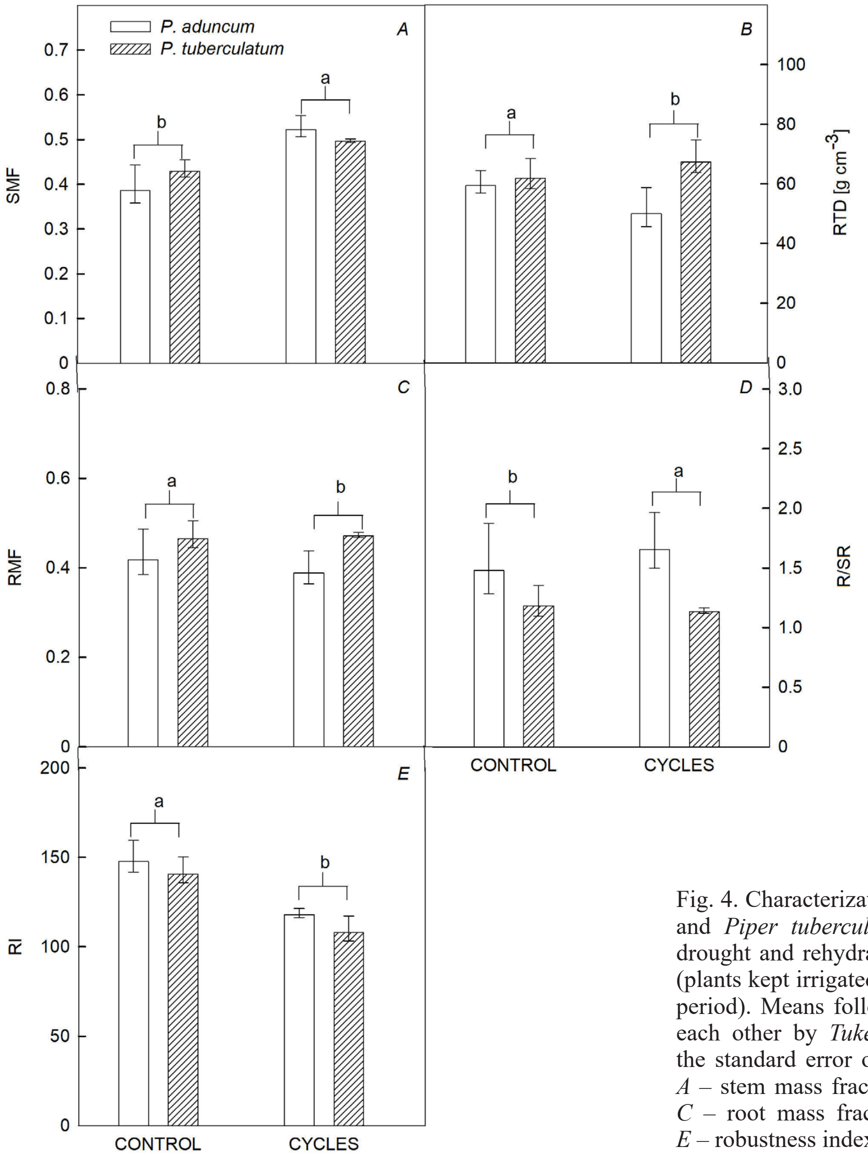

Fig. 4. Characterization of growth parameters in *Piper aduncum* and *Piper tuberculatum* plants submitted to three cycles of drought and rehydration (recovery), compared with the control (plants kept irrigated and evaluated throughout the experimental period). Means followed by *the same letter* do not differ from each other by *Tukey*'s test (p<0.05). *The bar* corresponds to the standard error of the average of four replicates of a plant. *A* – stem mass fraction (SMF), *B* – root tissue density (RTD), *C* – root mass fraction (RMF), *D* – root/shoot ratio (R/SR), *E* – robustness index (RI).

environments compared to *P. aduncum*. *P. tuberculatum* showed a higher density in plants that went through the dry cycles (Fig. 5*A*).

Palisade parenchyma thickness (PPT) (Fig. 5*D*), and vascular bundle length (LVBL) increased in *P. aduncum* control plants (Fig. 5*F*), followed by drought plants. *P. tuberculatum* showed no difference in cultivation environments. However, PPT and LVBL were higher in *P. tuberculatum* in the dry season compared to *P. aduncum*.

As for the anatomical structures of the stem, *P. tuberculatum* showed an increase in the length of the cortex vascular bundles (CSVBL) (Fig. 6*A*), length of the cortex xylem (XCSL), and the diameter of the xylem vessels (DCSV) in the control plants in comparison to *P. aduncum*. In *P. aduncum*, there was an increase in XCSL and DCSV in plants that were under drought (Fig. 6*A–C*). *P. aduncum* in the dry season showed a greater length in pith vascular bundles (LSMVB) and xylem length (LSMX) when compared to *P. tuberculatum* (Fig. 6*D*,*E*). However, *P. tuberculatum* showed an increase in LSMX in control plants compared to *P. aduncum*. The number of xylem vessels (NVMS) in *P. aduncum*, regardless of the culture environment, was higher than that in *P. tuberculatum*. Furthermore, *P. aduncum*, when subjected to drought cycles, showed an increase in the number of xylem vessels (NVMS) (Fig. 6*F*).

In the roots, both species presented a larger epidermis in the control plants (Fig. 7*C*). Xylem length (RXL), number of vessels (NRV), and vessel diameter increased in plants that underwent drought cycles in *P. aduncum* compared to *P. tuberculatum*. *P. tuberculatum*, regardless of the culture environment, showed a significant increase in the cortex when compared to *P. aduncum* (Fig. 6*A*,*B*,*D*).

Discussion

During vegetative growth, approximately 93% of the absorbed water is dissipated due to transpiration,

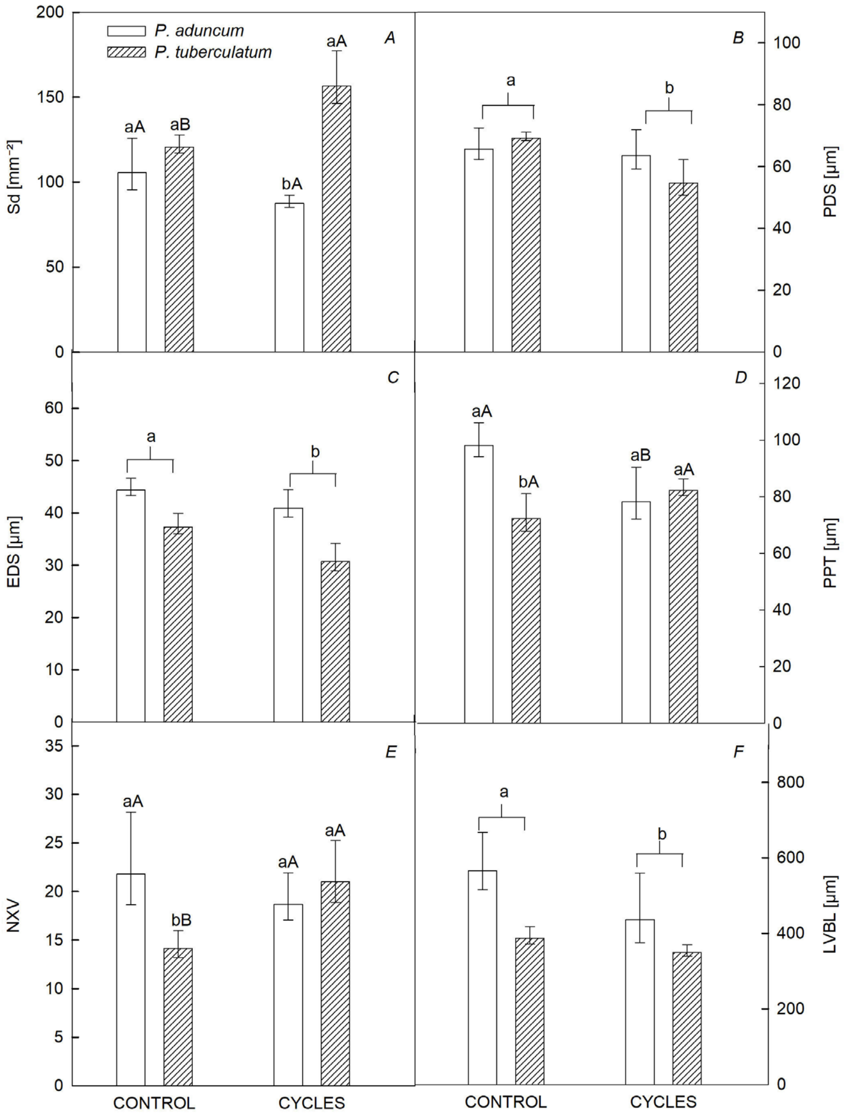

Fig. 5. Leaf anatomical variables in *Piper aduncum* and *Piper tuberculatum* plants submitted to three cycles of drought and rehydration (recovery), compared with the control (plants kept irrigated and evaluated throughout the experimental period). Means followed by *the same letter* do not differ from each other by *Tukey*'s test ($p<0.05$), where *capital letters* are used to compare species in each treatment (control and cycles) while *lowercase letters* compare treatments (control and cycles) in the function of each species. *A* – stomatal density (Sd), *B* – polar diameter of stomata (PDS), *C* – equatorial diameter of stomata (EDS), *D* – palisade parenchyma thickness (PPT), *E* – number of xylem vessels (NXV), *F* – vascular bundle length (LVBL).

the remainder is used in plant growth and maintenance, in the formation of carbon skeletons, in metabolic processes, and in photosynthesis (Krahmer *et al.* 2018). Plants need a large amount of water and have high evaporation, with a strong link between transpired water, carbon assimilation, and mass production (Krahmer *et al.* 2018).

Within the physiological processes, stomatal conductance (g_s) is one of the first and main ones to be sensitized when plants are under water deficit; typically, a parallel decrease occurs, meaning that as water availability decreases, there is also a corresponding reduction in g_s. Sweating reduces as a result of the drop in g_s (Brunetti *et al.* 2019). This was observed for the two species evaluated in both dry periods (Fig. 2*B*).

The plant body has its structures formed based on its genetic composition and, consequently, the physiological functioning as well, as it is directly linked to the organization chart of the anatomical structures. However, water availability has a great effect on plant growth, so anatomy and physiology can adjust to improve performance or just keep the plant alive (Krahmer *et al.* 2018).

The instantaneous efficiency of carboxylation (ICE) is directly linked with the assimilation of carbon (CO_2) where high values are associated with the increase in stomatal conductance (g_s); this is observed in the studied species, where there is a significant increase in ICE in both species kept irrigated (Qayyum *et al.* 2021). Thus, the increased values of ICE in the control plants are linked to the fact

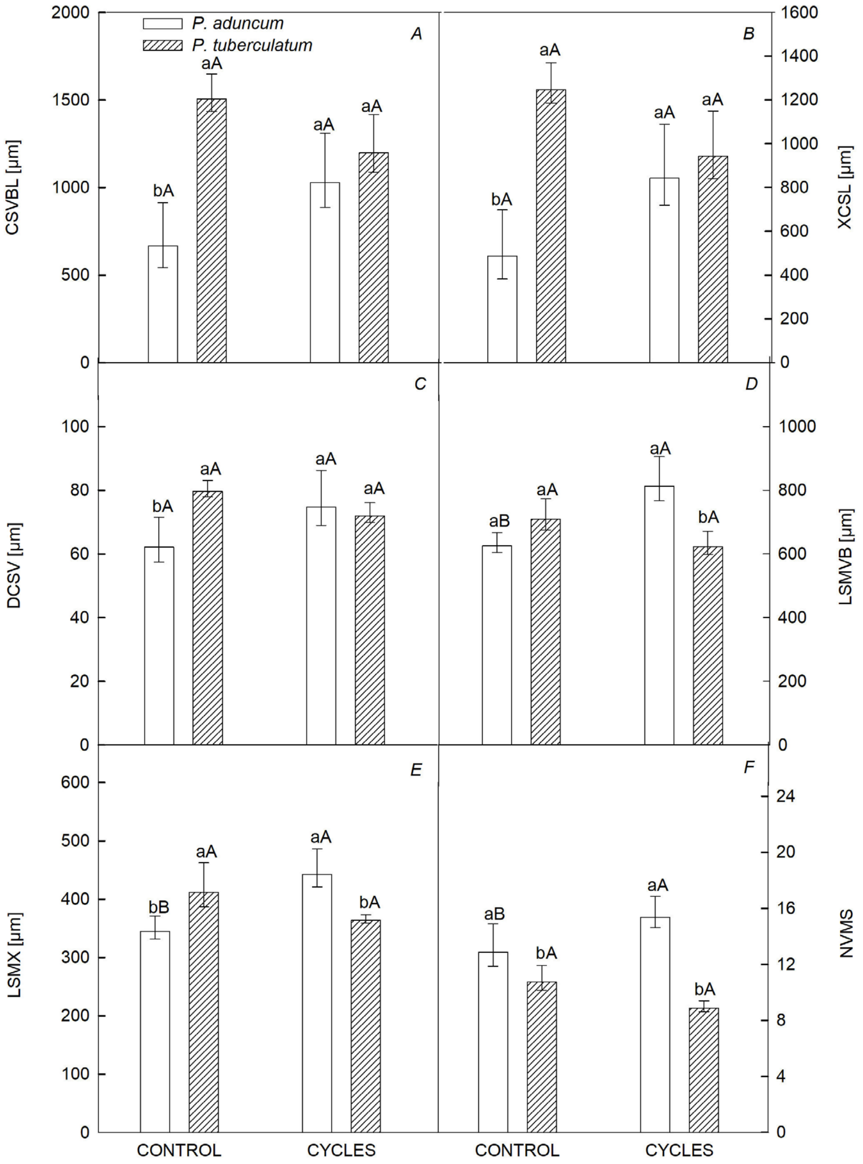

Fig. 6. Stem anatomical variables in *Piper aduncum* and *Piper tuberculatum* plants submitted to three cycles of drought and rehydration (recovery), compared with the control (plants kept irrigated and evaluated throughout the experimental period). Means followed by *the same letter* do not differ from each other by *Tukey*'s test ($p<0.05$), where *capital letters* are used to compare species in each treatment (control and cycles) while *lowercase letters* compare treatments (control and cycles) in the function of each species. *A* – cortex stem vascular bundle length (CSVBL), *B* – cortex xylem length (XCSL), *C* – xylem vessel diameter (DCSV), *D* – medulla vascular bundle length (LSMVB), *E* – medulla xylem length (LSMX), *F* – number of xylem vessels (NVMS).

that these plants are hydrated, consequently to an increase in the rate of net photosynthesis, where P_N was increased in both species in the control and after rehydration (Fig. 2*A*). In addition, the reduction in the water potential of the plants in all periods of drought (Fig. 1*A*,*B*) is also linked to the fact that these plants present a reduction in g_s because, in order to avoid water loss, the leaf water potential decreases, causing stomatal closure (Gao *et al.* 2018).

The reduction in water caused by water deficit affects physiological processes, especially the reduction in CO_2 caused by stomatal closure, which according to Zivcak *et al.* (2013) causes a reduction in electron transport. As well as the results presented by Zivcak *et al.* (2013), our study also supports that there is a limitation in photosynthesis caused by stomatal actions.

The maximum photochemical quantum yield (φP_0) reflects the photochemical efficiency of PSII (Chekanov *et al.* 2018). The reduction of φP_0 in *P. tuberculatum* in the environment under drought cycles occurred (Table 1) because the efficiency in electron transport was compromised (Jiang *et al.* 2008). The reduction in the quantum yield of electron transfer from Q_A^- to the electron transport chain beyond Q_A^- (φE_0) in *P. tuberculatum* in drought cycle environments (Table 1) is a result of failure in electron transport (Mathur *et al.* 2013). However, the

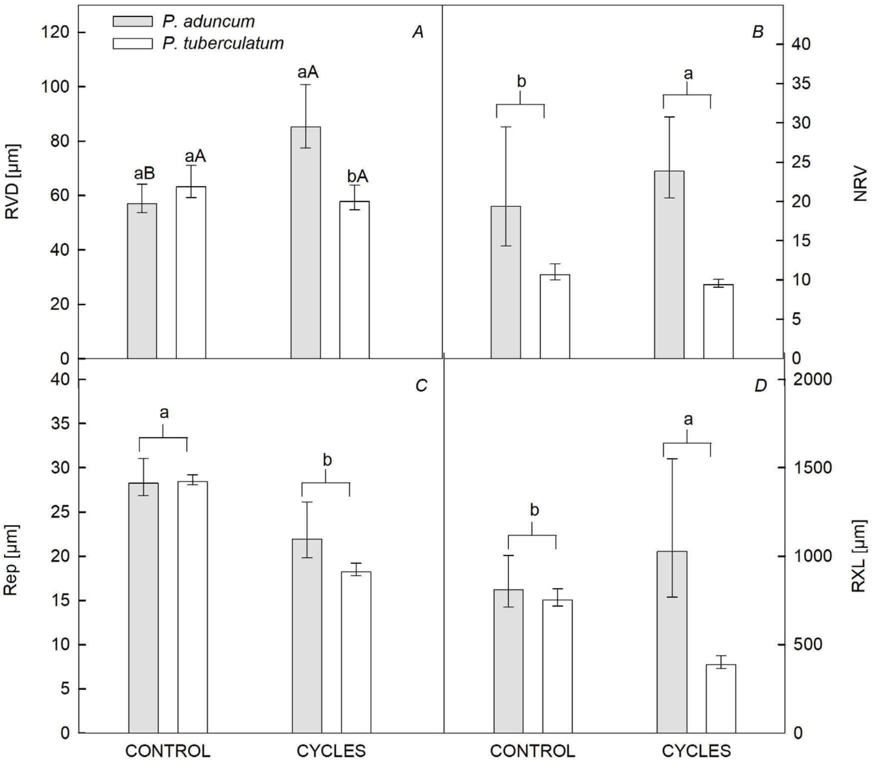

Fig. 7. Root anatomical variables in *Piper aduncum* and *Piper tuberculatum* plants submitted to three cycles of drought and rehydration (recovery), compared with the control (plants kept irrigated and evaluated throughout the experimental period). Means followed by *the same letter* do not differ from each other by *Tukey*'s test ($p<0.05$), where *capital letters* are used to compare species in each treatment (control and cycles) while *lowercase letters* compare treatments (control and cycles) in the function of each species. *A* – diameter of the xylem vessel (RVD), *B* – number of root vessels (NRV), *C* – root epidermis (Rep), *D* – length of the root xylem (RXL).

increase of φE_0 in *P. aduncum* plants in the water deficit indicates that the stress did not compromise the transport of electrons.

While *P. tuberculatum* showed an increase, *P. aduncum* showed a decline in the energy absorption flux per reaction center (ABS/RC). This increase is caused by the relative size of the antenna connected to each reaction center on change (Paunov *et al.* 2018). However, the decrease observed in *P. aduncum*, when cultivated under water deficit, indicates a stress defense mechanism, which causes changes in the size of the PSII antenna system and leads to the inactivation of a fraction of the reaction centers. The inactivation of reaction centers (reduction or heat dissipation centers) may be an indication of susceptibility to photoinhibition (Kalaji *et al.* 2018a).

The highest flux of energy dissipated per reaction center (DI_0/RC) in *P. tuberculatum* plants in the dry cycle occurs according to Kalaji *et al.* (2014) to protect the leaves of plants that are under stress from photooxidative damage (Table 1). However, *P. aduncum* showed a decrease in DI_0/RC because the energy required for the closure of all reaction centers and dissipation energy flux per reaction center (DI_0/RC) decreased with the imposition of stress (Lotfi *et al.* 2018). *P. aduncum* plants showed a greater increase in the $PI_{(abs)}$ compared to *P. tuberculatum* (Table 1). The reduction of $PI_{(abs)}$ suggests a decrease in overall photosynthetic performance associated with reduced electron transport capacity (Kalaji *et al.* 2018b).

Water deficit causes the degradation of pigments which causes a reduction in photosynthetic yield. This was observed in *P. aduncum* plants (Fig. 1S) (Uarrota *et al.* 2018) and caused by increased production of reactive oxygen species in thylakoids. The increase in carotenoids in *P. tuberculatum* in the dry cycle occurs because carotenoids can absorb energy that would be able to form reactive oxygen species, mainly zeaxanthin, and can also eliminate those that are formed (Ilić and Fallik 2017).

Both species showed a reduction in the aerial part in the dry cycles and *P. tuberculatum* showed an increase in the root system. This occurred (Figs. 3, 4, 2S) because plants that grow in environments with low water availability can invest in root system biomass and also reduce leaf area to reduce surface transpiration. With the decrease in transpiration there is an increase in the efficiency of water use, as the plant is losing less water to the environment (Ahemd *et al.* 2016).

The reduction in starch content in the roots of both species in drought cycles is due to the high energy demand that the plant needs to meet, in addition to being a strategy that helps in cellular osmoprotection, reducing the osmotic potential (Dong and Beckles 2019). However, it is clear that both species prioritized allocating starch in the roots, with the starch being transported from the leaves and stem to the root system (Fig. 3S*C*), which occurs due to the alarm of the plant, seeking an escape strategy (Maggio *et al.* 2018).

Biomass growth and allocation are controlled through investment in organs, seeking to minimize the effects of a limiting factor, seeking to escape stress (Maggio *et al.* 2018). Biomass allocation is a measure of plant performance, as it is a direct and final product of growth. Therefore, changes in biomass and morphology may suggest an alarm signal, so that it tolerates stress (Liu *et al.* 2016).

The increase in alarm reactions normally leads the plant to better survival and consequently, growth after the dissipation of the stress, as it happened in evolution. These genetic variations are good for preventing the plant from dying (Maggio *et al.* 2018).

The reduction of reducing sugars in *P. aduncum* (Fig. 3S*A*) is associated with low water availability in the soil, which makes it difficult to transport triose-phosphate from the chloroplasts of the mesophyll cells to the cytosol, where it is converted into sucrose (Dong and Beckles 2019). The accumulation of total soluble sugar (TSS) in the stem of both species in the dry cycles (Fig. 3S*B*) occurs to allow the plant to recover the carbon accumulation after the uptake of water in the soil. In addition, the greater storage of soluble sugar in the stem allows for rapid rehydration of the shoot biomass after the return of soil moisture (O'Brien *et al.* 2020).

The limitation in the photosynthetic process is influenced by changes in anatomical structures, mainly the thickness of the palisade parenchyma, and stomatal density, in addition to changes in the vascular system, particularly in the xylem and in the density and thickness of the vessels, which control the flow of CO_2 and the water entering the plant (Vieira *et al.* 2011). A species with the ability to modify its anatomical structures and its photosynthetic apparatus under different environmental conditions can escape, doing so to avoid serious damage due to stress (Maggio *et al.* 2018).

Inversely proportional to the polar (PDS) and equatorial (EDS) diameter of the stomata, the stomatal density (ED) of *P. tuberculatum* showed an increase in plants that went through dry cycles. This determines an irreversible reduction in the area and leaf thickening and an increase in stomatal density (Dardengo *et al.* 2017).

Therefore, the higher density in *P. tuberculatum*, under water deficit, can be explained as a water control mechanism. The cells expanded less due to the lower availability of water, hence the reduction in PDS and EDS, however, the stomata moved closer together resulting in greater humidity in the stomatal area, favoring water control (Ahemd *et al.* 2016).

The values of stomatal density, and the thickness of the palisade parenchyma were higher in *P. tuberculatum* in the dry cycles compared to the control (Fig. 5*A*). The stomatal density is linked to the increase in the conversion of light energy into chemical energy (Malone *et al.* 1993).

The greater thickness of the palisade parenchyma in plants under drought cycles is a mechanism to avoid photoinhibition (Dardengo *et al.* 2017). This allows the light to be transmitted more directly, preventing excess light from falling on the upper portion of the plant.

The increase in the xylem length (XCSL) and in the diameter of the xylem vessels (DCSV) and the reduction in the number of vessels in the stem and root of *P. aduncum* in the dry cycles (Figs. 6, 7) is associated with compensation in the water capture (Lenhard *et al.* 2013).

The reduction of root epidermis thickness in both species in dry cycles (Fig. 7*C*) is an adaptive strategy to facilitate water capture, as the plant is in an environment with low water availability and high transpiration demand. As a result, a lower Ψ_w was observed (Batista *et al.* 2010).

Conclusion: Both species studied showed a reduction in shoots in dry cycles, to reduce transpiration. They also showed an increase in the root system to optimize water capture. The rehydration cycles provided an increase in the photosynthetic rate of both species. In *P. aduncum* plants, there was a decrease in water potential in all periods of drought, indicating a mechanism of maintenance of turgor.

P. aduncum showed an increase in the quantum yield of electron transfer from Q_A^- to the electron transport chain beyond Q_A^-. The $PI_{(abs)}$ was also increased in *P. aduncum*. The damage to the photosynthetic apparatus was caused by the influence of electron flow attenuation in the transport chain. The stomatal density increased in *P. tuberculatum* in the dry cycles, and inversely related, it showed a decrease in the size of the stomata.

Thus, we concluded that the plants of the drought cycles showed alarm that consequently activated escape mechanisms to avoid or tolerate stress and *P. tuberculatum* showed more alarm signals and memory in the face of stress.

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