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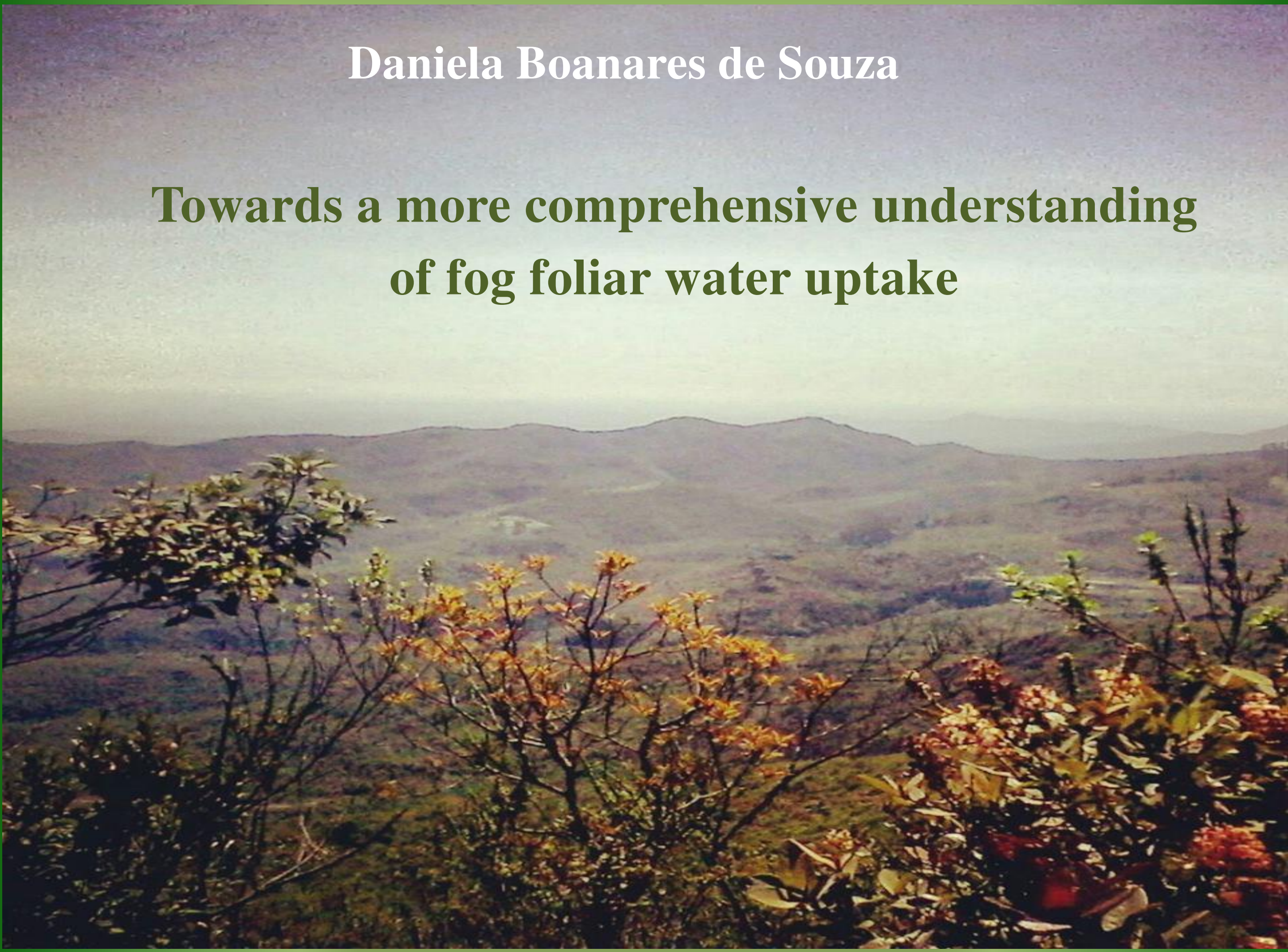
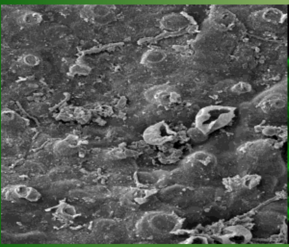
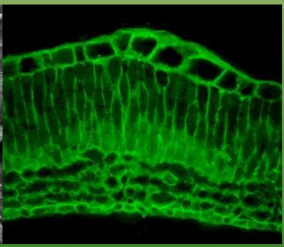
Instituto de Ciências Biológicas
Departamento de Botânica
Programa de Pós Graduação em Biologia Vegetal

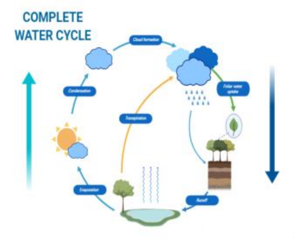


Daniela Boanares de Souza

Towards a more comprehensive understanding of fog foliar water uptake

Orientador: Prof. Dr. Marcel Giovanni Costa França
Coorientadora: Profª Drª Rosy Mary dos Santos Isaias

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UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
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DANIELA BOANARES DE SOUZA

TOWARDS A MORE COMPREHENSIVE UNDERSTANDING OF FOG FOLIAR WATER UPTAKE

Tese apresentada ao Programa de Pós Graduação em Biologia Vegetal do Departamento de Botânica do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito à obtenção do título de doutora em Biologia Vegetal

Área de concentração: Fisiologia e Ecologia Vegetal

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DEDICATÓRIA

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SUMÁRIO

RESUMO

A absorção foliar de água tem um papel importante na fisiologia de algumas espécies, principalmente aquelas que ocorrem em ambientes com presença de neblina. A neblina pode ser a única fonte de água para uma determinada vegetação, principalmente na estação seca, tornando essa condição climática uma fonte alternativa de água para a vegetação. Assim, o objetivo desta tese foi avaliar atributos ultraestruturais, fisiológicos, bioquímicos e fenológicos de espécies com diferentes capacidades de absorção foliar de água que ocorrem em ambientes sujeitos à neblina. Foi verificado que a composição química da cutícula e a proporção de componentes da parede celular interferem na absorção foliar de água. A assimilação de carbono é, momentaneamente, afetada pela absorção foliar de água, mas com diminuição na perda de água pela transpiração. O status hídrico, avaliado a partir do potencial hídrico, foi beneficiado pela absorção foliar de água, demonstrando valores similares de potencial hídrico na estação chuvosa e na estação seca com neblina. A absorção foliar de água aumentou a termotolerância, diminuiu a possibilidade de fotoinibição e melhorou o sistema antioxidante, principalmente nas espécies que absorvem grande quantidade de água. A presença de neblina, juntamente com a absorção de água pelas folhas interfere na fenologia de uma espécie decídua, retardando a queda de suas folhas. Assim, concluímos que a absorção foliar de água, principalmente de neblina tem um papel fundamental para a manutenção de várias plantas em seus habitats naturais; além disso, a estratégia de absorver maior quantidade de água, apesar de lentamente seria a melhor estratégia para as plantas lidarem, a longo prazo, com as mudanças climáticas previsíveis.

Palavras-chave: neblina, absorção foliar, fotossíntese, cutícula, fenologia

ABSTRACT

Foliar water uptake plays an important role in the physiology of some species, especially those that occur in foggy environments. Fog may be the only source of water for some particular vegetation, especially in the dry season, making the occurence o fog an alternative source of water for the vegetation. Thus, the objective of this thesis was to evaluate ultrastructural, physiological, biochemical and phenological attributes of plant species with different leaf absorption capacities that occur in environments subjected to fog. The chemical composition of the cuticle and the proportion of cell wall components interfere with leaf water uptake. Carbon assimilation is momentarily affected by leaf water absorption, but there has been a decrease in water loss through transpiration. The water status, evaluated through water potential, was benefited by leaf water uptake, demonstrating similar values of water potential in rainy season and in dry season with fog. Foliar water uptake increased thermotolerance, reduced the possibility of photoinhibition and improved antioxidant system, especially in species that absorb a large amount of water. The presence of fog, along with the absorption of water by the leaves, interferes with the phenology of a deciduous plant species, delaying the fall of its leaves. Thus, we conclude that leaf water uptake, especially fog, plays a key role in maintaining various plants in their habitats. The strategy of absorbing more water, although slowly, would be the best strategy for plants in the long term to cope with possible climate changes.

Keywords: fog, leaf absorption, photosynthesis, cuticle, phenology

INTRODUÇÃO GERAL

Diferentes estratégias moleculares, fisiológicas e bioquímicas são acionadas, rapidamente ou mais tardiamente, quando as plantas enfrentam estresse por falta de água. Algumas das estratégias podem ser facilmente observadas, quando, p.ex., plantas perdem água pelos estômatos na busca pelo equilíbrio do status hídrico foliar. Analogamente, vem sendo demonstrado que existem diferentes estratégias de captura de água pelas folhas, o que caracteriza o movimento contrário da água ao fluxo no sistema solo-planta-atmosfera. Esta absorção foliar acontece quando a água ultrapassa a superfície da folha em resposta ao gradiente de potencial hídrico difundindo-se para os tecidos internos da folha (Gouvra & Grammatikopoulos 2003). A absorção foliar de água pode ocorrer durante e logo após as diversas formas de precipitação, como a neblina.

Um exemplo de ecossistema tropical no qual a neblina é um evento atmosférico frequente é o campo rupestre ferruginoso, que ocupa nos topos de morros, em geral com altitudes acima de 1000 metros, no Quadrilátero Ferrífero. Devido à marcada sazonalidade de precipitação de chuvas e pela presença de fisionomias campestres ocorrendo sobre afloramentos rochosos ricos em ferro como itabiritos ou cangas, ou seja, predominantemente sobre solos rasos e descontínuos, acredita-se que a vegetação sobre campos rupestres ferruginosos experimente períodos de déficit hídrico durante a estação seca (Benites et al. 2003). Já foi visto que nos períodos sem neblina, ambientes tropicais de altitudes podem ser considerados áridos, caracterizados por uma alta demanda evaporativa (Leuschner, 2000).

A absorção foliar de água, a partir de agora referida como FWU (em inglês "foliar water uptake"), pode proporcionar uma vantagem ecológica para as plantas que ocorrem em locais que apresentam déficit hídrico, pelo menos sazonalmente, desempenhando papel importante na sua sobrevivência e crescimento (Limm et al. 2009; Eller et al. 2013). Das 1100 espécies estimadas para os campos rupestres ferruginosos (Jacobi e Carmo, 2012), foi verificado que pelo menos onze espécies têm capacidade de absorver água pelas folhas, mas com diferentes estratégias para esta entrada de água. Algumas espécies absorvem água rapidamente em quantidades menores enquanto outras espécies absorvem água lentamente em quantidades maiores (Boanares et al. 2018 a, b). Como existe um consenso de que FWU pode ser a norma e não a exceção em plantas (Berry et al. 2019), é de extrema importância o conhecimento mais aprofundado sobre como ocorre e o que influencia este mecanismo nas diferentes espécies de plantas.

Desta forma, o objetivo desta tese foi responder se diferenças nas características ultraestruturais da folha interferem na capacidade de FWU e se essas diferentes capacidades influenciam parâmetros ecofisiológico e ecossistêmicos das espécies estudadas. A tese é apresentada na forma de 7 capítulos conforme os objetivos propostos a seguir:

Como a cutícula é a primeira barreira física para a entrada de água na folha, o **Capítulo 1** aborda a relação da composição química da cera cuticular e das ornamentações da cera epicuticular com as diferentes capacidades de FWU. Considerando que a parede celular é a próxima barreira física à FWU, o **Capítulo 2** verifica a relação entre a composição química da parede celular, quanto aos seus componentes estruturais, pectinas e celulose, com as estratégias de FWU. Uma vez avaliada a interferência da química da cutícula e dos componentes da parede celular nas diferentes estratégias de FWU, o objetivo seguinte foi responder como estas estratégias podem interferir na fisiologia destas espécies. Assim, o **Capítulo 3** avalia se as diferentes estratégias de FWU podem interferir no balanço hídrico e de carbono das espécies ao longo de um ano, contemplando as estações climáticas seca e chuvosa. A premissa central deste capítulo é de que as mudanças climáticas globais podem interferir no regime hídrico e térmico nos diferentes ecossistemas. Por isso, o **Capítulo 4** aborda diferentes estratégias de FWU e sua relação com as respostas de termotolerância e de fotoinibição. No **Capítulo 5,** verifica-se se as mesmas estratégias de FWU podem minimizar o metabolismo oxidativo induzido pelos respectivos estresses ambientais, regime hídrico e amplitude térmica.

Uma vez entendido como parâmetros ultraestruturais e fisiológicos podem interferir na FWU de espécies de um ambiente tropical de altitude, partimos para verificar alguns desses parâmetros em plantas com diferentes respostas à presença de neblina em um ecótone Mediterrâneo - Temperado. Neste ambiente, já foi verificado uma redução dos eventos de neblina, impactando de forma distinta o crescimento destas espécies.

Desta forma, outro objetivo da tese foi verificar a importância da FWU e da neblina na ecofisiologia de espécies ocorrentes neste ecótone tendo como cenário questões sobre Mudanças Climáticas Globais. Assim, no **Capítulo 6** verificamos se a ocorrência de neblina e, consequentemente, sua absorção pelas folhas interfere na fisiologia e fenologia de duas espécies que coexistem no ecótone Mediterrâneo-Temperado.

Por fim, o **Capítulo 7** propõe uma abordagem multidisciplinar focando na genética, ultraestrutura, anatomia, fisioloiga e ecologia buscando o maior entendimento do fluxo reverso de água atmosfera-planta-solo, contrário ao que se observa na literatura clássica considerando o sistema solo-planta-atmosfera.

Assim, os objetivos específicos foram:

- Avaliar se a composição química e a ornamentação da cutícula interferem na FWU
- Avaliar se as diferentes proporções de celulose e pectina na parede celular interferem na FWU

- Verificar se as diferentes capacidades de FWU influenciam no balanço hídrico e de carbono e na termotolerância das espécies.
- Analisar se o metabolismo oxidativo tem relação com as distintas capacidades de FWU.
- Verificar o comportamento fisiológico e fenológico de espécies com distintas respostas à presença de neblina.

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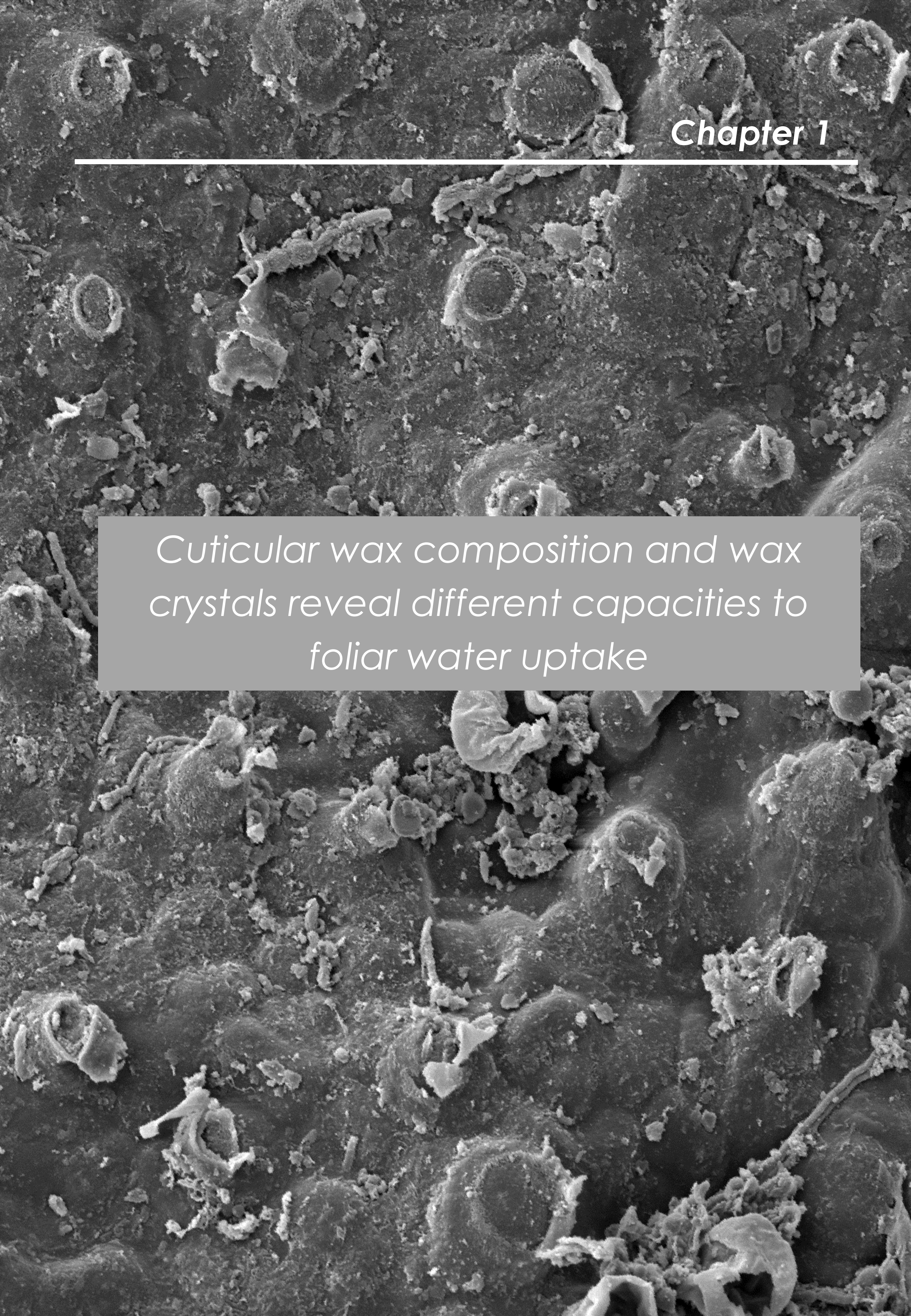
Lista de abreviaturas

FWU - foliar water uptake
LWU - leaf water uptake
CW - cuticular wax
EW - epicuticular wax
C_{max} - maximum leaf water absorption
k - foliar water absorption speed
C_{max}^{L} - total amount of water leaf lost
k^{L} - water leaf lost speed
GLM - General Linear model
LMM - linear mixed model
HGA - Homogalacturonans
RG - rhamnogalacturonans
LEM – leaf specific mass
LSA - leaf specific area
RWC - relative water content
WSD - water saturation deficit
A - net CO_2 assimilation
E - transpiration
g_s - stomatal conductance
Ci /Ca - internal carbon and ambient carbon ratio
WUE - water-use efficiency
iWUE – intrinsic water-use efficiency
Ψw – water potential
PD ds - Predawn dry season
PD ds fog - Predawn dry season with fog
MD ds - Midday dry season with fog
MD ds fog – Midday dry season with fog
PD rs - Predawn rainy season
MD rs - midday rainy season
SPAC - soil-plant-atmosphere continuum
Fv/Fm - potential quantum yield of photosystem II
T_{50} - temperature of Fv/Fm 50% decline
PSII - photosystem II
ROS - reactive oxygen species
CAT - catalase
SOD - superoxide dismutase
APX - ascorbate peroxidase
LQ - species that absorb less water more quickly
MS - species that absorb more water more slowly
PRI - photochemical reflectance index
NDVI - normalized difference vegetation index
APSC – atmosphere-plant-soil continuum



Chapter 1

Cuticular wax composition and wax crystals reveal different capacities to foliar water uptake

Cuticular wax composition and wax crystals reveal different capacities to foliar water uptake

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ABSTRACT

Leaf cuticle is the first physical barrier for foliar water uptake (FWU), since its main function is to prevent excessive water loss through transpiration. We assume that not only the cuticle itself but the chemical composition of the cuticular wax and the ornamentation of the epicuticular wax must influence the different capacities of FWU. To address this hypothesis, the chemical analysis of cuticular wax (CW) was performed by gas chromatography-mass spectrometry/flame ionization detection, and the epicuticular wax ornamentation was investigated by scanning microscopy on six species with different FWU capacities. The total chemical composition of CW related to the speed of foliar water absorption but did not relate to the amount of water absorbed. However, the classes of the chemical compounds analyzed separately had no relation to FWU. Current chemical data suggest that the permeability of CW does not regard to the amount of CW or to a specific class of chemical compound but to the presence and proportion of all classes together. The greater or lesser FWU capacity is related to a three-dimensional network of the chemical molecules. Also, the three-dimensional ornamentation of EW indicates the relation of the higher ornamentation complexity with the slowest water absorption capacity, due to the higher hydrophobicity of the leaf surface. Together, the composition of the CW and the crystals wax of the EW differentially affect the FWU of the studied species.

Key words: Foliar water uptake; ultraestrutural traits; epicuticular ornamentation; gas chromatography; *Campo rupestre*

List of abbreviation:

C_{max} - maximum water content in leaf lamina

k - water leaf uptake speed

CW - cuticular wax

EW – epicuticular wax

Author contributions

D.B., R.M.S.I. and M.G.C.F. designed the research. D.B., H.C.S, A.R.K., A.B. and A.X.S collected the data for the research, and D.B., L.P.S.P and D.R.M.N analyzed the data. D.B., M.R., R.M.S.I. and M.G.C.F wrote the manuscript.

HIGHLIGHTS

- Cuticular and crystals waxes was related to foliar water uptake (FWU)
- Chemical compounds analyzed separately were not related to FWU
- Complexity in the ornamentation gives greater hydrophobicity to the leaf surface

INTRODUCTION

Foliar water absorption (FWU) occurs when there is a difference of vapor pressure in atmosphere and inside the leaf, inducing a difference in water potential, which promote water o overtake the boundary layer and into the epidermal cells, causing hydration of leaf tissues (Todd and Dowson, 2004). The FWU can play an important role in the water relations of many terrestrial plants, especially in humid environments and in ecosystems where, in addition to rainfall, water is provided by mist, fog and dew causing frequent leaf wetting (Breshears et al., 2008; Limm et al., 2009; Limm and Dawson, 2010; Goldsmith et al., 2013; Mayr et al., 2014; Boanares et al., 2019).

Due to the emergence of new investigation methods, researches into the water pathways inside the leaf have increased in recent years (Eller et al, 2016; Goldsmith et al., 2017, Boanares et al, 2018; Brinks et al, 2019). The mechanism of leaf water entry is of great interest and several pathways have been proposed: water movement directly through the cuticle (Goldsmith et al., 2013; Ritpitakphong et al., 2016), through the pores (Schonherr, 2006), trichomes (Mencuccini and Ball, 2016; Boanares et al., 2018), hydathodes (Martin and von Willert, 2000), and also by stomata (Burkhardt et al., 2012; Berry et al., 2014; Fernandez et al., 2017).

Such observations about the pathway of water raises critical leaf surface issues associated to the FWU capacity, such as morphological (leaf angle and size), anatomical (stomatal location, leaf tissue thickness) and cuticle traits (Goldsmith et al., 2017; Boanares et al., 2018). The cuticle is the first physical barrier that water faces to enter leaf tissues. The plant cuticle is most typically associated to a fixed barrier to excessive water loss from transpiration. However, several secondary functions have evolved, such as the defense against pests and pathogens, the avoidance of dust and other debris due to many epicuticular crystals that promote a self-cleaning surface, and may also play the role of filtering excessive light radiation (Yeast and Rose, 2013).

Plant cuticles contain, beyond the cutin, wax as the second largest chemical component (Riederer and Schreiber, 2001). Wax composition may vary substantially with species, ontogeny, and environmental growth conditions (Jenks and Ashworth, 1999). This set of compounds is characterized by unbranched and fully saturated hydrocarbon backbones, which may have a primary oxygen-containing functionality, that is, a hydroxyl, carbonyl or carboxyl terminal group. Therefore, plant cuticle waxes are typically mixtures of primary n-alcohols, n-aldehydes and fatty acids, as well as n-alkanes and various lipophilic secondary metabolites such as pentacyclic triterpenoids and flavonoids, which may also be important components (Jetter et al., 2006; Yeast and Rose, 2013). Each of these classes of compounds comprises a homologous series of chemical compounds with chain lengths ranging from 20 to nearly 40 carbons, and a species-specific matrix that may comprise more than 50 chemical compounds (Riederer and Schreiber, 2001).

When considering cuticular permeability (Kerstiens, 1996; Riederer and Schreiber, 2001), interspecific and intraspecific differences in water entry capacity may partly result from differences in chemical composition, epicuticular wax morphology among species, and tissue age (Kerstiens, 2006). Some studies have evidenced that cuticular permeability to water is mainly determined by genetic control, and only slightly subject to environmental influence. Therefore, cuticular water permeability may be related to the plant life strategy to deal with different environmental constraints (Bueno et al., 2019).

As there is a consensus that FWU may be the norm rather than the exception in plants (Berry et al., 2019), in-depth knowledge of how it occurs and what influences FWU at the cuticular level is of utmost importance. Differences in the FWU capacity of species that occur in a Brazilian altitude ecosystem revealed species with low and fast water absorption by leaves (Boanares et al, 2018a,b) but the influence of cuticular morphology and cuticular wax chemical composition differences on leaf absorption speed has not been explored, yet. Taking for granted that the different water absorption speeds relate both to the chemical composition of cuticular wax (CW) and to the morphology of epicuticular wax, gas chromatography coupled to mass spectrometry or flame ionization detection and scanning electron micrography (SEM) were used to evaluate the composition of CW and the epicuticular wax (EW) ornamentation of six species of a Brazilian altitude ecosystem.

MATERIAL AND METHODS

Plant material

The studied species are present in the ferruginous *Campo Rupestre*. This ecosystem is a Brazilian OCBIL, characterized by herbaceous and shrub species growing on rock outcrops of itabirites or *cangas* of shallow soils (Hopper 2009; Valim *et al*., 2013; Silveira et al., 2016). These plant species were selected according to their different water absorption capacity from calculated by a differential equation, which provides data on maximum leaf water absorption (C_{max}) and leaf uptake velocity (k) (g g−1 dry mass) (Liang et al., 2009; Boanares et al., 2018b). The species *Leandra australis* (Melastomataceae), *Byrsonima variabilis* (Malpighiaceae) and *Ocotea pulchella* (Lauraceae) have a higher speed of leaf absorption and uptake a smaller amount of water (k=0.250±0.02; C_{max}=0.140±0.02). However, the species *Trembleya laniflora* (Melastomataceae), *Pleroma heteromallum* (Melastomataceae) and *Senna reniformis* (Fabaceae) are slow in absorption, but they uptake a greater amount of water through the leaves than the species of the first strategy (k=0.067±0.06; C_{max}=0.443±0.19).

Chemical analyses of cuticular waxes

Cuticular waxes were extracted by dipping the whole leaf (except the wounds of cut petioles) twice into chloroform (≥ 99.8%, Roth) at room temperature for 1.5 min. 10 µg of n-tetracosane (C24; ≥ 99.5%, Sigma-Aldrich) was added as an internal standard and the solutions were reduced to dryness under a gentle flow of nitrogen. Dry cuticular wax samples were derivatized with N,O bis(trimethylsilyl)trifluoroacetamide (BSTFA, Marchery-Nagel) in dry pyridine (≥ 99.5%, Roth) at 70°C for 30 min. Quantification of cuticular wax compounds was performed with a gas chromatograph equipped with a flame ionization detector and an on-column injector (7890A, Agilent Technologies). Separation of compounds was carried out on a fused-silica capillary column (DB1-ms, 30 m length × 0.32 mm inner diameter, 0.1 μm film thickness, Agilent Technologies) with hydrogen as a carrier gas. The temperature program consisted of injection at 50°C for 2 min, raised by 40°C min^{-1} to 200°C, held at 200°C for 2 min, and then raised by 3°C min^{-1} to 320°C, and held at 320°C for 30 min. Qualitative analysis was carried out using a gas chromatograph equipped with a mass spectrometric detector (5975 iMSD, Agilent Technologies) following the same gas chromatographic conditions but using helium as the carrier gas. Cuticular wax compounds were identified comparing a query mass spectrum with reference mass spectra in a library via spectrum matching and quantitated against the internal standards (Bueno et al., 2019).

Scanning electron microscopy

For scanning electron microscopy (SEM), leaves collected and fixed in Karnovsky (pH 7.2 in 0.1 M phosphate buffer; modified from Karnovsky 1965) were used to remove one fragment of 0.5 cm from the middle part of leaves of 4 individuals of each species. The material was dehydrated in increasing ethanol solutions (70%, 90% and 100%) and then the fragments were mounted in stubs and brought to dryness to the critical point with liquid CO_2 (Balzers CPD 020). Subsequently, the surfaces of the fragments were covered by a thin layer of metallic gold (Edwards Scancoat Six) and examined by scanning electron microscope (SEM) - DSM 950 Carl Zeiss.

Data analysis

We first explored the patterns of chemical differentiation between species by calculating their pairwise Euclidean Distance in chemical composition, and performing non-metric multidimensional scaling (NMDS) (McCune & Grace, 2002). We explored the results visually by plotting the species and their ecological strategies (fast or slow) in ordination space (NMDS) space, and then fitting the values of FWU parameters: leaf total water absorbed (C_{max}) and leaf water absorption speed (k), via generalized additive models (GAM). The NMDS and GAM analyses were conducted in the statistical package vegan (Oksanen *et al.*, 2016), in the R Statistical Environment (R Development Core Team, 2019). We further determined whether the chemic compound classes were related to FWU parameters (C_{max} and k) via generalised linear models (using Gaussian error distributions) with the compound class as the response variables and the species as explanatory variable.

RESULTS

Chemical composition of leaf cuticular waxes

The chemical composition of the cuticular waxes influences, qualitatively and quantitatively, the FWU of the six studied species. All species presented primary alkanols, *n*-alkanes, alkanoic acid, steroids and triterpenoids. Alkyl esters was present in all species except *P. heteromallum.* The simplest cuticular wax with only 4 aliphatic compound classes was found in *P. heteromallum* (Table 1). *Leandra australis* and *T. laniflora* had most complex cuticular wax with 7 compound classes (Table 1). Alfa-amyrin was detected in five of the six species studied (Supplementary data).

Among the species with fast FWU, the most abundant compound classes in *L. australis* and *O. pulchella* was the primary alkanols (0.418 ± 0.1 µg cm^{-2}, 29.6%, and 0.827 ± 0.111 µg cm^{-2}, 26.2%, respectively), and the steroids and triterpenoids in *B. variabilis* (9.094 ± 1.66 µg cm^{-2}, 35.9%). For the species with the lowest absorption speed strategy, *T. laniflora* (1.777 ± 0.186 µg cm^{-2}, 37.8%) and *P. heteromallum* (2.703 ± 0.668 µg cm^{-2} , 50.7%), the n-alkanes as the main class of compounds. Although the *n*-alkanes was the second largest class detected in *S. reniformis* (1.005 ± 0.165 µg cm^{-2}, 15.4%), the steroids and triterpenoids were the most abundant classes of compounds (3.144 ± 0.534 µg cm^{-2}, 47.8%), demonstrating a chemical composition similar to those of the fast water absorption species. The species with the highest amount of cuticular wax was *B. variabilis* (25.320 ± 7.50 µg cm^{-2}). The other species showed wax amounts between 1.41 to 6.51 µg cm^{-2} (Table 1).

The very long chain aliphatic compounds with around 20 to 34 carbons varied among species. *B. variabilis* and *O.pulchella* but the highest percentage were molecules with 29 C (6.207 ± 2.82 µg cm^{-2}, 50%) and 29 C (0.770 ± 0.14 µg cm^{-2}, 30%), respectively. *Leandra australis* was the exception, with molecules up to 36 carbons (Fig. 1) but the highest percentage were molecules with 28 C (0.125 ± 0.06 µg cm^{-2}, 16%). *P. heteromallum*, *S. renifomis* and *T. laniflora* presented chains around 20 to 37 carbons, in which the highest percentage was represented by molecules with 33 C (1.103 ± 0.395 µg cm^{-2}, 34%), 29 C (0.606 ± 0.15 µg cm^{-2}, 29%) and 26 C (0.541 ± 0.16 µg cm^{-2}, 14%), respectively (Fig. 2). The amount of CW and the amount of each compound of the CW analyzed separately were not related to the different FWU strategies (C_{max} and *k*). For all major classes of compounds no species separation occurred according to the FWU strategies (Fig. 3).

Table 1. Simplified chemical composition of leaf cuticular waxes of six species with different capacities of foliar water uptake. Each value represents the mean value ± SD (n = 3).

Compound class	Coverage (µg cm-2)					
	L.australis	*O. pulchella*	*B. variabilis*	*P. heteromallum*	*T. laniflora*	*S. reniformis*
primary alkanols	0.41 ± 0.10	0.82 ± 0.11	2.492 ± 0.765	0.321 ± 0.092	0.970 ± 0.605	0.798 ± 0.074
branched alkanols	0.04 ± 0.01					
n-alkanals	0.02 ± 0.00	0.17 ± 0.00			0.023 ± 0.014	0.130 ± 0.030
n-alkanes	0.24 ± 0.07	0.72 ± 0.20	8.584 ± 3.601	2.703 ± 0.668	1.777 ± 0.186	1.005 ± 0.165
branched alkanes					0.412 ± 0.130	
alkanol acetates						
alkyl esters	0.04 ± 0.01	0.110 ± 0.028	0.239 ± 0.064		0.243 ± 0.158	0.512 ± 0.008
alkanoic acids	0.02 ± 0.00	0.716 ± 0.075	1.093 ± 0.588	0.183 ± 0.052	0.368 ± 0.207	0.240 ± 0.036
flavonoids						
steroids and triterpenoids	0.38 ± 0.06	0.078 ± 0.038	9.094 ± 1.667	1.800 ± 0.865	0.436 ± 0.294	3.113 ± 0.534
not identified	0.25 ± 0.00	0.506 ± 0.023	3.816 ± 0.913	0.314 ± 0.091	0.462 ± 0.266	0.713 ± 0.160
Cuticular wax quantitiy total *	1.41 ± 0.21	3.14 ± 0.30	25.32 ± 7.50	5.32 ± 1.64	4.69 ± 1.75	6.51 ± 0.96

(*) means the total amount of cuticular wax also including the other non-aliphatic compounds described in this table.

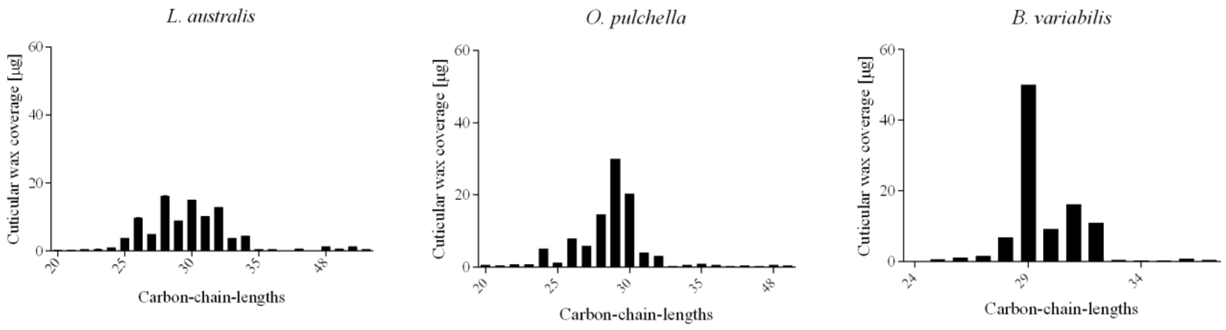

Fig. 1. Chain-length distributions of the very-long-chain aliphatic fraction of cuticular waxes of species with speed water absorption. Bars stand for the mole-based contribution of a single chain-length to the total very-long-chain aliphatic wax coverage.

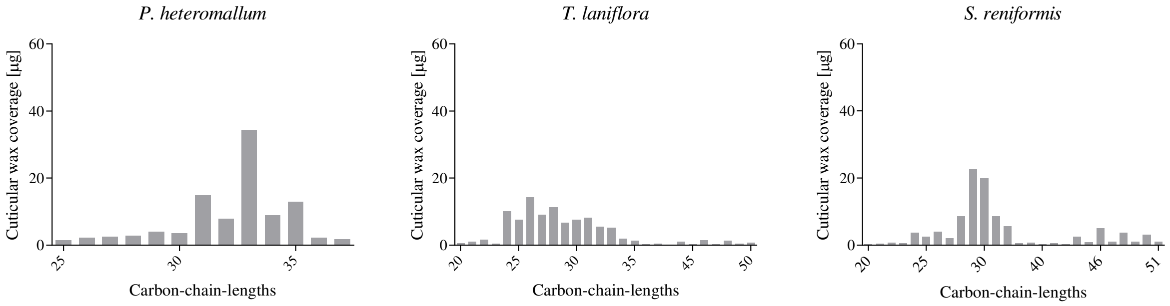

Fig. 2. Chain-length distributions of the very-long-chain aliphatic fraction of cuticular waxes of species with slow water absorption. Bars stand for the mole-based contribution of a single chain-length to the total very-long-chain aliphatic wax coverage.

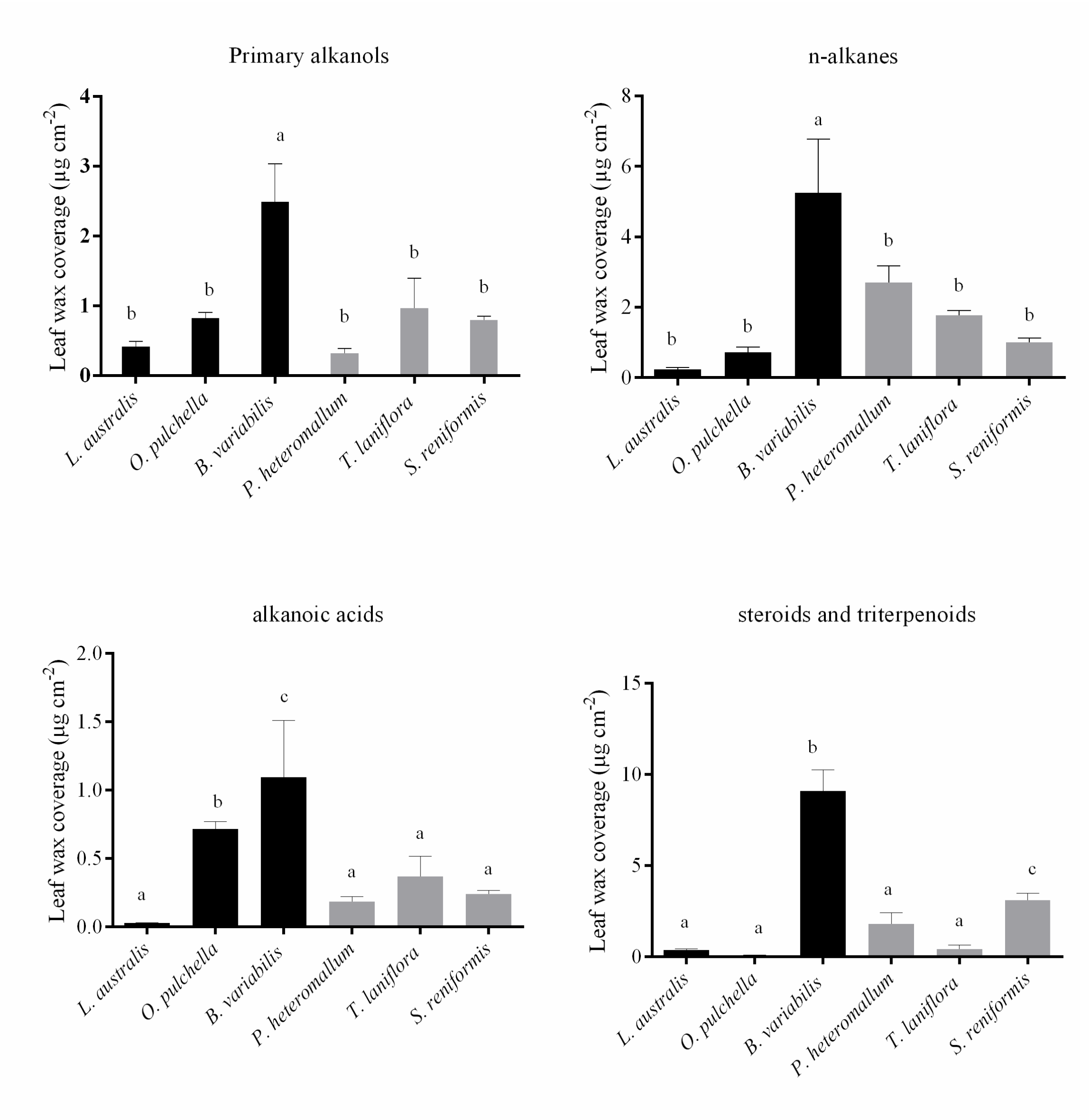

Fig. 3. Cuticular wax coverage of principal's compound class of six species leaves with different speed of FWU (*k*). Each value represents the mean value ± SD. Black and grey bars indicate species with higher *k* and lower *k*, respectively. Different letters indicate differences between species ($P \leq 0.05$). Error bars represent ± SE.

The first two NMDS axes (there was a negligible decrease in stress values by adding a third axis) were effective in segregating the two ecological strategies. Only species with a slow strategy (*T. laniflora, P. heteromallum* and *S. reniformis*) are placed in both negative values of the first NMDS axis and positive values of the second NMDS axis

(Fig. 4). We also found that the speed of water uptake (parameter *k*) represented a better fit to the relationship between species chemical composition and their water uptake parameter (AIC = -60.28) than the amount of water uptake (parameter C_{max}; AIC = -17.49) (Fig. 4).

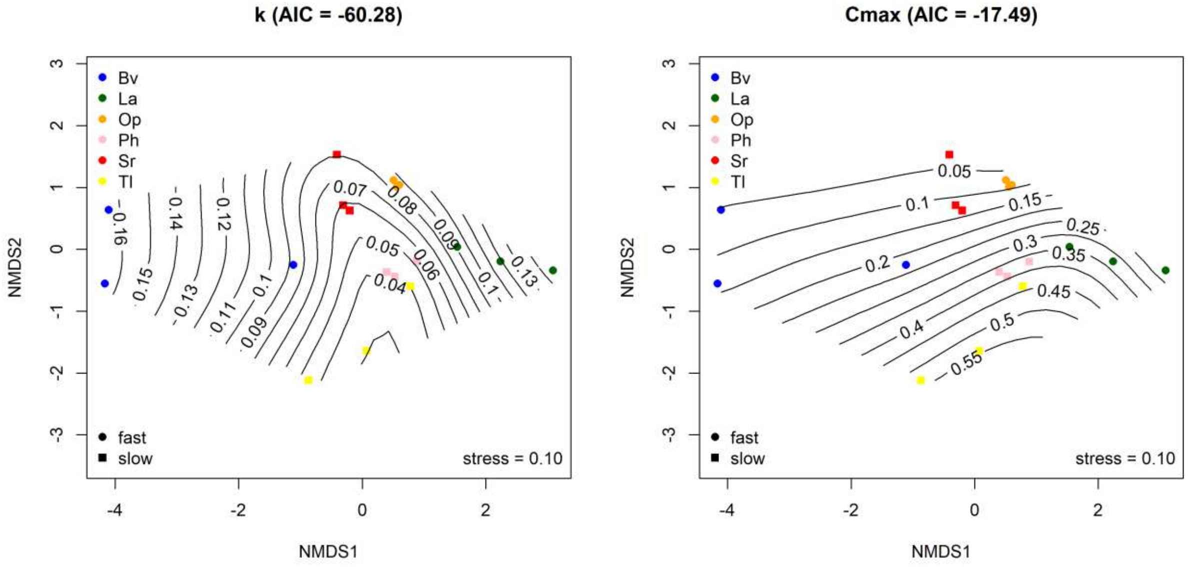

Fig. 4. NMDS plot of cuticular waxes composition of six species (a) maximum leaf water absorption (C_{max}) between species and (b) leaf water uptake speed (*k*) among species. BV: *B. variabilis*, La: *L. australis*, Op: *O. pulchella*, Ph: *P. heteromallum*, Sr: *S. reniformis* and Tl: *T. laniflora.*

Scanning electron microscopy analyses

The type of the epicuticular waxes (EW) of *B. variabilis* and *O. pulchella* is of crust with granules on their adaxial leaf surfaces (Fig. 5). *Ocotea pulchella* had the similar type but also presented sparse trichomes. On the abaxial face, *B. variabilis* presented the EW in crust with ribbons and *O. pulchella* presented the EW in film. In *L. australis*, both on the adaxial and abaxial leaf surfaces, the EW in crust with plateles was found. *Senna reniformis*, both on the adaxial and abaxial leaf surfaces, presented crust-like EW. In *T. laniflora* and *P. heteromallum*, the EW in crust with granules and threads was found on the adaxial leaf surface, and just EW in crust on the abaxial surface. The difference between *T. laniflora* was the presence of glandular trichomes and *P. heteromallum* was the presence of emergences on the adaxial surface (Fig. 5).

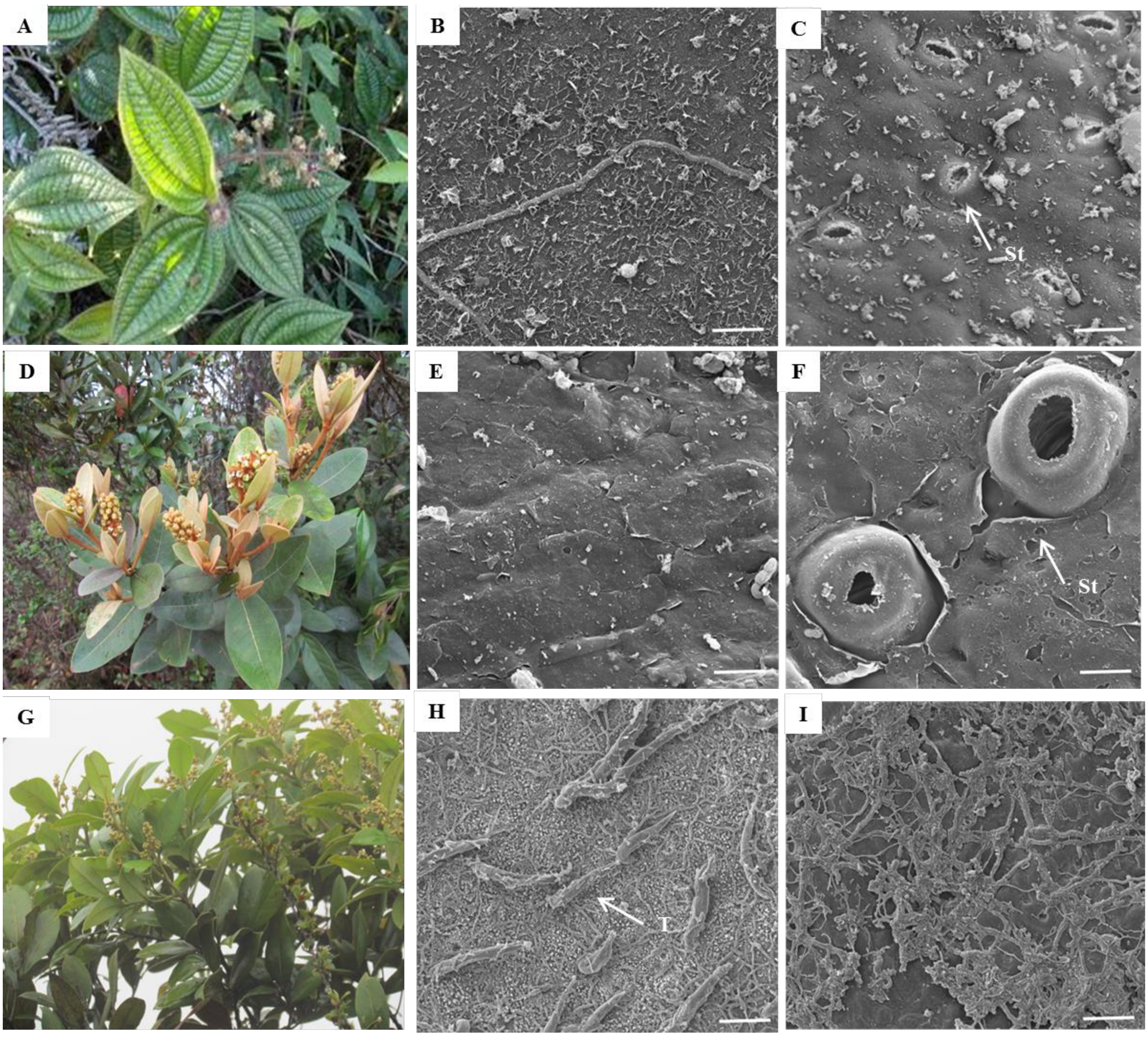

Fig. 5. Scanning electron micrographs of the adaxial and abaxial surfaces of species with speed water absorption. (A-B) *Leandra australis*, adaxial and abaxial surfaces respectively; (C-D) *Byrsonima variabilis*, adaxial and abaxial surfaces respectively; (E-F) *Ocotea pulchella*, adaxial and abaxial surfaces respectively. St: stomatal; T: Trichomes. Bars = 10 µm.

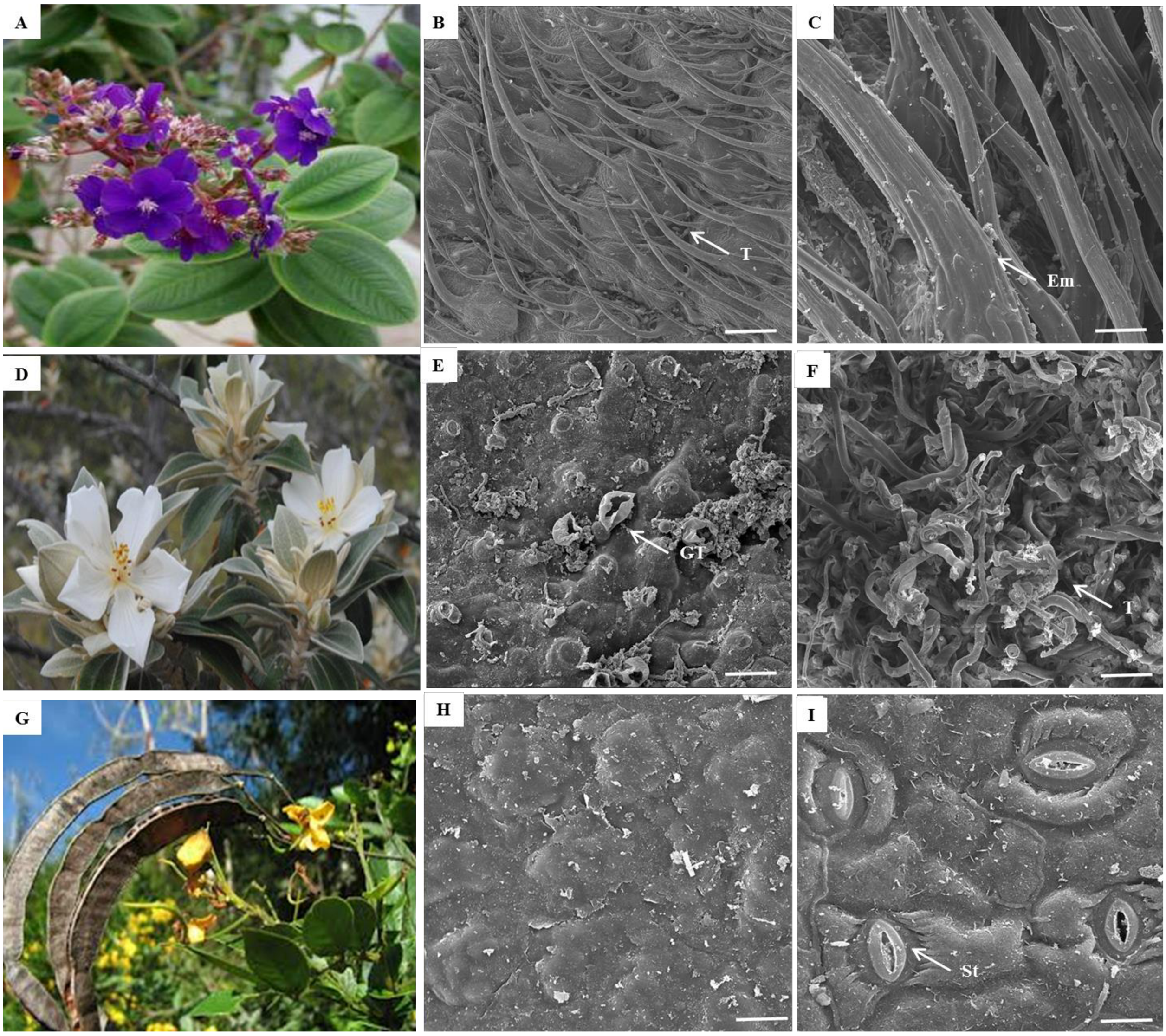

Fig. 6. Scanning electron micrographs of the adaxial and abaxial surfaces of species with slow water absorption. (A-B) *Pleroma heteromallum*, adaxial and abaxial surfaces respectively; (C-D) *Trembleya laniflora*, adaxial and abaxial surfaces respectively and (E-F) *Senna reniformis*, adaxial and abaxial surfaces respectively. St: stomatal; T: Trichomes; GT: glandular trichomes; St: stomatal. Bars = 10 µm

DISCUSSION

The ordinary role of the cuticle is to prevent excessive water loss from transpiration (Riederer and Schreiber, 2001; Burghardt and Riederer, 2006). Accordingly, several ecological and physiological roles have been attributed to the epicuticular wax, except leaf hydration from FWU. Contrary to the common sense, our hypothesis that different FWU capacities, especially regarding the absorption speed, are related to the cuticular wax composition of the six species of the rupestrian field was corroborated. The absorption speed (*k*) was much higher than the total amount of water absorbed (C_{max}), which is related to leaf surface features capable of controling the speed of water entry. The high C_{max} is better related to leaf anatomical traits, such as tissue thickness, and cell wall components such as pectin and cellulose (Boanares et al., 2018a, b).

The chemical features of some classes of compounds detected in cuticle wax are already known and may interfere in FWU. The *n*-alkanes, total nonpolar compounds, has no hydroxyl radical, and tend to be associated with a low permeability of the CW (Yeast and Rose, 2013). The *n*-alkanes confer compactness to the structure of CW, negatively interfering with FWU, consequently the large amount of *n*-alkanes found in some plant species relates to their low water absorption speed (*k*). Contrastingly, fast-absorbing species presented a higher proportion of classes of compounds, such as alkanoils, steroids, and triterpenoids, which have hydroxyl or oxygen radicals, and some degree of polarity, H-bond interaction, and a higher solubility parameter in relation to *n*-alkanes (Fernandez et al., 2016). Alkanois tend to decrease the nonpolar part, as hydroxyls can bind to water molecules, facilitating their entry into leaf tissues. Triterpenoids and steroides disrupt *n*-alkane compaction, disaggregate the crystalline cuticle layer, promoting polar spaces, aiding water movement (Reynhardt, 1997; Riederer and Schreiber, 2001), and thereby favoring FWU. This is consistent with a model in which CW are located in crystalline or amorphous domains of the cuticle, with aliphatic compounds forming the crystalline domain that is impermeable to water. The crystalline structure forces water to diffuse through a tortuous route through the amorphous domain that is formed by polar and cyclical compounds (Riederer and Schreiber, 1995), facilitating the FWU.

Triterpenoids such as *b*-amyrin also increase the volume of amorphous cuticle molecules, and decrease the size of the crystalline domain, blocking aggregation or increasing the distance between the nonpolar parts (Bushhaus and Jetter, 2012). Thus, the more polar compounds present in CW, the more routes through which water can diffuse may be opened or shortened, thereby decreasing the overall cuticle resistance. A common perception is that a thick cuticle is associated with lower water permeability and therefore low FWU capacity. However, the quantity/thickness of CW was unrelated to permeability or to FWU (Riederer and Schreiber 2001; Schuster, 2016; Bueno 2018). In fact, the proportion of *n*-alkanes is more significant to CW permeability than the total amount of waxes (Yeast and Rose, 2013), corroborating our data with plants and environmental condition of *Campo Rupestre*. The chemical complexity of CW was also unrelated to FWU possibly because compound classes may be equivalent in structure and function, and may not individually influence permeability. Commonly, the chain size of aliphatic compounds has been related to the water barrier (Vogg et al., 2004; Leide et al., 2007, 2011; Buschhaus and Jetter, 2012; Jetter and Riederer, 2016; Jetter and Riederer, 2016). Very long-chain aliphatic compounds build impermeable crystalline domains (Riederer and Schreiber, 1995) and constitute a barrier to water outflow and possibly to water entrance into plants, as well. Therefore, the fact that fast FWU species presented low percentages of very long-chain aliphatic compounds compared to water slow absorption species corroborates this premise.

Except for the *n*-alkanes, the other classes of compounds detected in the CW such as acids and alcohols, have some degree of polarity and H-bond interactivity. Thus, the polarity or potential H-binding properties of each compound found on plant surfaces are more important than their hydrophobic or lipophilic traits (Fernandez et al., 2016), because one molecule alone may be irrelevant but their association with other classes of compounds can produce more polar or nonpolar sites, which may, in fact, interfere with water entrance. After all, the predominant understanding of plant cuticle as independent and lipidic is largely based on Brongniart (1830) and von Mohl's (1847) hypotheses, which were already controversial at the time. Possibly the non-responsiveness of the elements separately regards to the importance of the association of cuticle compounds to the FWU. Contrastingly, it seems that CW permeability is mainly determined by the particular mixture of epicuticular and intracuticular waxes and their morphology and organization within the cuticle architecture, and not just by an isolated class of compounds (Yeast and Rose, 2013). The overall profile of the CW mixture plays important roles in cuticle structure models because the functional groups of the wax constituents engage in spontaneous reactions within the cuticle, and confer gradual modifications to the CW composition (Riederer and Schreiber, 2001). Thus, structure and compositional variability are of particular importance for cuticular permeability to water (Schreiber, 2005). Since also in our data we find that in addition to the proportion of each compound, a mixture of each class of compound interferes with CW permeability.

While intracuticular waxes function primarily as a barrier to excessive water loss through transpiration (Riederer and Schreiber, 1995), epicuticular waxes (EW) strongly influence surface wettability (Bargel et al., 2006). Scanning electron microscopy revealed several ornamentations of the EW. The most complex or more ornate EW was observed on the species with the lowest FWU speed (*P. heteromallum* and *T.laniflora*, except *S. reniformis*). These data are in accordance with the fact that three-dimensional EW crystals create microstructured surfaces with low wettability (Baker, 1982; Wagner et al., 2003). Therefore, a higher density of three-dimensional wax crystals provides a higher degree of leaf surface hydrophobicity (Koch, 2006). Although the presence of trichomes has a positive influence on FWU, it is important to emphasize that this influence is more related to the amount of water absorbed than to its speed (Boanares et al., 2018a). Contrastingly, the species with the highest water leaf absorption speed were the species with a simpler EW, and less ornamentation, which could have facilitated water entrance.

For some waxes, a distinct coherence between chemical composition and morphology in EW has been found (Koch and Ensikat, 2008). Platelets are a sub-type of plate wax observed on the adaxial face of *L. australis*, which has an intimate relationship with the EW chemical composition. It is well known that some plateles are formed by primary alcohols (Koch et al., 2006), which were the main components of the CW composition of *L. australis*. However, for some types of wax, the correlation between crystals, morphology, and chemistry is still unclear (Koch and Ensikat, 2008), which corresponds to the non-correlation between chemistry and cuticle ornamentation of the studied species.

For the six species of the Brazilian rupestrian fields, the different CW compositions and EW ornamentations are related to the different FWU capacities. The combination of all classes of compounds and not simply the isolated amount of one or another class of compound provides the three-dimensional structure that favors the different degrees water entrance into leaf tissues, interfering in the FWU.

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Table 1. Supplementary data. Total chemical composition of leaf cuticular waxes of six species with different FWU capacities. Each value represents the mean value ± SD (n = 3).

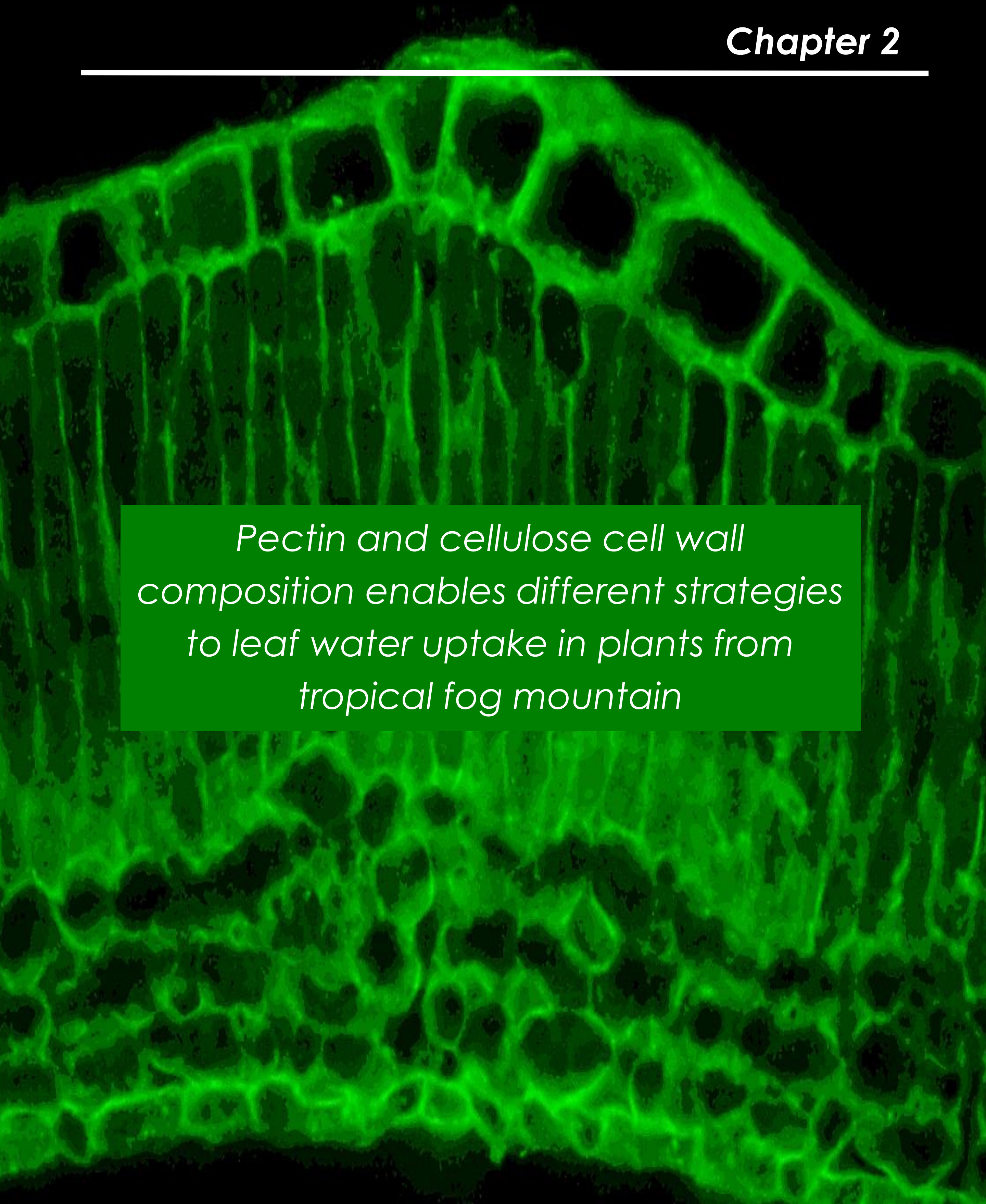
Compound class	Carbon-chain-length	Coverage (µg cm-2)					
		L.australis	*O. pulchella*	*B. variabilis*	*P. heteromallum*	*T. laniflora*	*S. reniformis*
n-alkanes	25	0.023 ± 0.008	0.016 ± 0.003	0.071 ± 0.049	0.051 ± 0.024	0.212 ± 0.04	0.018 ± 0.007
	26	0.024 ± 0.009	0.03 ± 0.013	0.077 ± 0.053	0.06 ± 0.028	0.229 ± 0.053	0.019 ± 0.009
	27	0.03 ± 0.01	0.112 ± 0.017	0.134 ± 0.028	0.058 ± 0.027	0.266 ± 0.032	0.027 ± 0.01
	28	0.035 ± 0.014	0.082 ± 0.057	0.227 ± 0.05	0.061 ± 0.017	0.181 0.048	0.064 ± 0.015
	29	0.047 ± 0.014	0.379 ±0.104	6.154 ± 2.81	0.112 ± 0.014	0.190 ± 0.019	0.606 ± 0.151
	30	0.02 ± 0.005	0.023 ± 0.007	0.157 ± 0.03	0.063 ± 0.013	0.116 ± 0.028	0.068 ± 0.006
	31	0.038 ± 0.011	0.083 ± 0.019	1.762 ± 0.88	0.428 ± 0.148	0.256 ± 0.103	0.201 ± 0.027
	32	0.012 ± 0.003			0.162 ± 0.052	0.083 ± 0.006	
	33	0.013 ± 0.001			1.054 ± 0.387	0.163 ± 0.086	
	34				0.165 ± 0.051	0.026 ± 0.002	
	35				0.397 ± 0.163	0.035 ± 0.011	
	36				0.03 ± 0.01	0.013 ± 0.002	
	37				0.056 ± 0.018		
Total n-alkanes		0.246 ± 0.078	0.728 ± 0.223	8.584 ± 3.90	2.703 ± 0.957	1.777 ± 0.436	1.005 ± 0.228
branched-alkanes	25					0.012 ± 0.005	
	26					0.029 ± 0.014	
	27					0.045 ± 0.020	
	28					0.017 ± 0.002	
	29					0.035 ± 0.004	
	30					0.036 ± 0.004	
	31					0.026 ± 0.006	
	32					0.031 ± 0.010	

	33					0.027 ± 0.016	
	34					0.026 ± 0.011	
	35					0.019 ± 0.007	
Total branched alkanes							
alcohol	21	0.001 ± 0.00	0.008 ± 0.001			0.014 ± 0.014	0.004 ± 0.002
	22	0.0009 ± 0.00	0.009 ± 0.00			0.043 ± 0.022	0.007 ± 0.003
	23	0.002 ± 0.00	0.01 ± 0.001			0.009 ± 0.004	0.008 ± 0.005
	24	0.004 ± 0.001	0.077 ± 0.017	0.018 ± 0.001		0.319 ± 0.208	0.072 ± 0.011
	25	0.004 ± 0.00	0.005 ± 0.00			0.025 ± 0.011	0.03 ± 0.013
	26	0.043 ± 0.034	0.118 ± 0.019	0.0248 ± 0.004		0.184 ± 0.115	0.049 ± 0.002
	27	0.008 ± 0.004	0.016 ± 0.002	0.029 ± 0.004	0.013 ± 0.003	0.012 ± 0.005	0.025 ± 0.002
	28	0.081 ± 0.045	0.104 ± 0.026	0.092 ± 0.028	0.01 ± 0.004	0.132 ± 0.081	0.167 ± 0.038
	29	0.012 ± 0.002	0.357 ± 0.044			0.017 ± 0.007	0.274 ± 0.098
	30	0.09 ± 0.015	0.093 ± 0.034	0.797 ± 0.268	0.032 ± 0.007	0.094 ± 0.058	0.123 ± 0.024
	31	0.0175 ± 0.002		0.171 ± 0.063	0.029 ± 0.01	0.016 ± 0.01	
	32	0.087 ± 0.015	0.018 ± 0.004	1.238 ± 0.379	0.069 ± 0.022	0.068 ± 0.045	0.016 ± 0.003
	33	0.016 ± 0.003	0.006 ± 0.00	0.076 ± 0.021	0.034 ± 0.01	0.009 ± 0.005	0.019 ± 0.004
	34	0.034 ± 0.003		0.043 ± 0.014	0.085 ± 0.026	0.022 ± 0.014	
	35	0.004 ± 0.00			0.021 ± 0.005		
	36	0.004 ± 0.00			0.025 ± 0.006		
Total primary alkanols		0.418 ± 0.131	0.827 ± 0.153	2.492 ± 0.786	0.321 ± 0.096	0.97 ± 0.605	0.798 ± 0.212
branched-alcohol	29	0.01 ± 0.002					
	30	0.007 ± 0.001					
	31	0.024 ± 0.13					
alkanals	24					0.013 ± 0.01	
	26	0.002 ± 0.001	0.016 ± 0.002				
	28		0.043 ± 0.002			0.01 ± 0.004	

	30		0.081 ± 0.011				0.13 ± 0.03
	32		0.028 ± 0.007				
Total alkanals	32		0.078 ± 0.012			0.023 ± 0.014	0.13 ± 0.03
Coumaric acid ester	24		0.013 ± 0.008				
	26		0.006 ± 0.003				
	28		0.007 ± 0.002				
	30		0.019 ± 0.006				
Alkanoic acid	20	0.002 ± 0.00	0.016 ± 0.00			0.022 ± 0.014	0.006 ± 0.003
	21	0.001 ± 0.00	0.005 ± 0.00			0.024 ± 0.015	0.006 ± 0.003
	22	0.002 ± 0.00	0.008 ± 0.00			0.022 ± 0.014	0.011 ± 0.002
	23	0.001 ± 0.00	0.01 ± 0.00			0.005 ± 0.003	0.01 ± 0.002
	24	0.004 ± 0.00	0.055 ± 0.002			0.054 ± 0.035	0.024± 0.007
	25	0.001 ± 0.00	0.01 ± 0.001	0.02 ± 0.006		0.007 ± 0.003	0.021 ± 0.006
	26	0.005 ± 0.00	0.04 ± 0.001	0.041 ± 0.022	0.01 ± 0.003	0.053 ± 0.031	0.037 ± 0.002
	27		0.023 ± 0.002	0.035 ± 0.015	0.008 ± 0.002	0.008 ± 0.003	
	28	0.007 ± 0.00	0.146 ± 0.017	0.537 ± 0.529	0.021 ± 0.008	0.077 ± 0.039	
	29		0.033 ± 0.005	0.052 ± 0.024	0.015 ± 0.004	0.013 ± 0.005	
	30		0.312 ± 0.039	0.191 ± 0.095	0.018 ± 0.004	0.042 ± 0.022	0.061± 0.017
	31		0.02 ± 0.005	0.087 ± 0.046	0.019 ± 0.006	0.009 ± 0.004	0.031 ± 0.006
	32		0.032 ± 0.007	0.126 ± 0.054	0.021 ± 0.006	0.026 ± 0.014	0.029 ± 0.008
	33				0.014 ± 0.004		
	34				0.036 ± 0.011		
	35						
	36				0.016 ± 0.004		
Total alkanoic acids		0.027 ± 0.00	0.717 ± 0.087	1.093 ± 0.793	0.183 ± 0.056	0.368 ± 0.208	0.24 ± 0.61
Alkanol acetate	26						
	28						

	30						
Total alkanol acetates							
alkyl esteres	40						0.006 ± 0.0
	41						
	42		0.015636365			0.015 ± 0.11	0.014 ± 0.003
	43					0.006 ± 0.004	0.006 ± 0.00
	44	0.001 ± 0.00	0.025856349	0.052 ± 0.025		0.037 ± 0.025	0.066 ± 0.009
	45					0.01 ± 0.006	0.021 ± 0.002
	46	0.005 ± 0.001	0.015 ± 0.002	0.108 ± 0.023		0.059 ± 0.40	0.137 ± 0.00
	47	0.002 ± 0.007	0.008 ± 0.001			0.014 ± 0.008	0.025 ± 0.001
	48	0.01 ± 0.003	0.012 ± 0.001	0.078 ± 0.027		0.053 ± 0.036	0.0977 ± 0.002
	49	0.005 ± 0.006	0.007 ± 0.007			0.015 ± 0.008	0.024 ± 0.001
	50	0.011 ± 0.003	0.014 ± 0.001			0.03 ± 0.018	0.084 ± 0.007
	51	0.003 ± 0.00	0.011 ± 0.00				
Total alkyl esteres		0.04 ± 0.011	0.11 ± 0.03	0.239 ± 0.076		0.243 ± 0.159	0.512 ± 0.031
alpha-amyrin		0.008 ± 0.002		1.136 ± 0.317	0.209 ± 0.090	0.269 ± 0.181	0.371 ± 0.025
amyrin derivative				0.363 ± 0.032	0.032 ± 0.014		0.17 ± 0.01
beta-amyrin				1.562 ± 0.345	0.274 ± 0.139	0.100 ± 0.068	0.248 ± 0.014
beta-amyrinon					0.014 ± 0.004		0.248 ± 0.045
beta-sitosterol		0.004 ± 0.00					
beta-tochopherol			0.012 ± 0.006	0.064 ± 0.045			
betulin				0.124 ± 0.036			0.092 ± 0.005
betulinic acid							0.051 ± 0.007
cholesterol		0.007 ± 0.00	0.017 ± 0.011	0.041 ± 0.014	0.01 ± 0.002		0.027 ± 0.003
delta-amyrin					0.023 ± 0.004		0.023 ± 0.001
delta-tocopherol			0.004 ± 0.001				
erythrodiol		0.005 ± 0.00			0.011 ± 0.004		0.102 ± 0.025

fridelin						0.268 ± 0.092
gama-tocopherol						0.017 ± 0.004
hederagenin	0.006 ± 0.00					
hederagenin derivative	0.005 ± 0.001					
lupenon			0.12 ± 0.055			0.705 ± 0.284
lupeol			4.927 ± 0.806	0.954 ± 0.699	0.051 ± 0.038	0.268 ± 0.059
lupeol derivative 1			0.395± 0.083	0.096 ± 0.085		0.439 ± 0.206
non-identified triterpenoid	0.043 ± 0.009		0.229 ± 0.09	0.086± 0.014		
oleanoic acid	0.074 ± 0.015		0.123 ± 0.014	0.019 ± 0.006		
ursolic acid	0.193 ± 0.041			0.017 ± 0.009	0.015 ± 0.008	
ursolic acid derivative	0.012 ± 0.002					
uvaol	0.018 ± 0.001			0.046 ± 0.016		0.076 ± 0.014
not identified	0.255 ± 0.006	0.506 ± 0.023	3.816 ± 0.913	0.314 ± 0.091	0.461 ± 0.266	0.714 ± 0.161
Cuticular wax total	1.41 ± 0.21	3.14 ± 0.30	25.32 ± 7.50	5.32 ± 1.64	4.69 ± 1.75	6.51 ± 0.96



Chapter 2

Pectin and cellulose cell wall composition enables different strategies to leaf water uptake in plants from tropical fog mountain

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Pectin and cellulose cell wall composition enables different strategies to leaf water uptake in plants from tropical fog mountain

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ABSTRACT

Leaf water uptake (LWU) has been observed in plants of different ecosystems and this process is distinct among different species. Four plant species from the Brazilian fog mountain fields were evaluated in order to detect if leaf water uptake capacity is related to the cell wall composition of leaf epidermis. LWU measurements and their relation to anatomical and biochemical traits were analyzed. Cell wall composition was verified through immunocytochemistry using monoclonal antibodies recognizing pectin compounds, and histochemistry with calcofluor white to track cellulose. Differences in LWU among the four species were clearly revealed. Two species presented higher maximum leaf water content and the lowest values of water absorption speed. The other two species presented opposite behavior, namely, low leaf water uptake and the highest values of water absorption speed. The anatomical traits associated with the cell wall composition corroborated the data on the different LWU strategies. The species with abundant detection of cellulose in their epidermal cell walls absorbed more water, but more slowly, while those with abundant detection of pectins absorbed water at a higher speed. These results indicate that cell wall composition regarding pectin and cellulose are significant for water uptake by the leaf epidermis. Pectin provides greater porosity and absorption speed, while cellulose provides greater hydrophilicity and greater water uptake capacity. Current data indicate that the composition of epidermal cell walls is a relevant trait for leaf water uptake.

Keywords:
Immunocytochemistry
Leaf traits
Rupestrian field
Water relations

Abbreviations: CW, cell walls; LWU, leaf water uptake; GalA, galacturonic acid; HGA, homogalacturonans; RG-I, rhamnogalacturonans I; RG-II, rhamnogalacturonans II; C_{max}, maximum water content; *k*, leaf uptake speed; LSM, leaf specific mass; LSA, leaf specific area; SU, succulence; RWC, relative water content; WSD, water saturation deficit; GLM, general linear model

1. Introduction

Leaf water uptake (LWU) is more easily observed when leaves are in the presence of a great amount of fog, and water steam can come from the atmosphere toward leaves through a difference in water potential (Burgess and Dawson, 2004; Oliveira et al., 2005; Goldsmith et al., 2013; Eller et al., 2015; Gotsch et al., 2015). Although LWU is responsible for a small portion of plant water content in relation to the amount that entries by roots, such absorption increases leaf hydration and water potential without a visible increase in soil moisture (Ewing et al., 2009; Simonin et al., 2009; Limm et al., 2009). Thus, LWU provides an ecological advantage for plants occurring at sites with water deficits, at least seasonally, playing an important role in plant survival and growth (Limm et al., 2009; Eller et al., 2013, 2016). Therefore, LWU capacity confers advantages to the vegetation by reducing the negative impacts of severe drought (Burgess and Dawson, 2004; Eller et al., 2013). Physiological studies have shown that LWU helps plants to better face drought events (Eller et al., 2016); and that it can be facilitated by leaf structures such as trichomes, stomata, emergencies, hydathodes, but can also occur through the ordinary epidermal cells (Martin and von Willert, 2000; Riederer and Schreiber, 2001; Burkhardt et al., 2012; Fernandez et al., 2014).

Leaf water uptake may occur in many plant species of different ecosystems (Martin and von Willert, 2000; Gouvra and Grammatikopoulos, 2003; Oliveira et al., 2005; Breshears et al., 2008; Limm et al., 2009; Eller et al., 2013; Goldsmith et al., 2013; Cassana et al., 2015; Gotsch et al., 2016; Eller et al., 2016) by several possible mechanisms. Nevertheless, no study has investigated whether the LWU capacity is related to the presence of some specific structural traits, such as special cell types or trichomes, or even the composition of epidermal cell walls.

The cell walls (CW) are one of the main barriers to the entrance of water into leaf cells. In plants, the CW are mainly composed of cellulose (Carpita and McCann, 2000), hemicelluloses and pectins (Wolf and Greiner, 2012). Cellulose is a linear homopolymer consisting of long chains of D-glucose units, linked through β-type glycosidic bonds (1 → 4). Each cellulose molecule is linked to the others by means of hydrogen bonds, forming sets identified as microfibrils (Zykwinska et al., 2005; Agoda-Tandjawa et al., 2012). The cellulose microfibrils are dispersed into water by shearing forces, when they may form gels, but when the microfibrils interact with each other, they may structure a strong network (Tatsumi et al., 2002; Pääkkö et al., 2007).

Pectins are a group of diversified polysaccharides rich in galacturonic acid (GalA) (Caffall and Mohnen, 2009). They are divided into distinct groups depending on the types of monosaccharides associated with GalA and the types of linkage between its monomers. Among the main groups of pectins, there are homogalacturonans (HGA), rhamnogalacturonans I (RG-I), and rhamnogalacturonans II (RG-II) (Ridley et al., 2001; Albersheim et al., 2011). The HGAs are homopolymers of GalA bound through α-(1 → 4) bonds (Willats et al., 2001; Derbyshire et al., 2007; Verhertbruggen et al., 2009; Oliveira et al., 2014), they are very abundant in the CW, generally comprising 60-65% of the pectin set (Bush et al., 2001). The RG-I, contrastingly, are branched polymers of GalA, generally representing 20-35% of the pectin matrix (Oliveira et al., 2014). They have many structurally different side chains attached through the C-4 of the central skeletal rhamnosil with arabinosyl and galactosyl residues as the predominant chain components. The RG-I can alter the degrees of the CW porosity, flexibility and cell-cell adhesion (Mohnen et al., 2008; Verhertbruggen et al., 2009; Oliveira et al., 2014).

Pectins can regulate cell-cell signalling, cell growth, morphogenesis and defence (Agoda-Tandjawa et al., 2012). Moreover, pectins may allow increased water storage and transit in the CW, and regulate the stiffness levels, according to the degrees of methyl esterification and chain branching (Willats et al., 2001; Alba and Kontogiorgos, 2016). The degree of methylation (DM) of the HGA molecules is the proportion of methyl esterified galacturonic acids in their chain. The HGAs are synthesized in a highly methyl esterified form, and the structure of the polymers may undergo varying de-methylesterification degrees throughout cell development

(Wolf et al., 2009). HGAs with low degree of methyl esterification, when hydrated, form gels in association with calcium ions at different levels of wall acidity (Axelos and Thibault, 1991).

It is speculated that the composition of the epidermal CW could influence the LWU of certain plant species, especially those naturally involved in a great amount of fog and water steam. As models of study, four native plant species from Brazilian tropical fog mountain fields were evaluated, using histochemical, immunocytochemical, and physiological approaches, to detect whether the leaf water uptake capacity was related to the leaf surface traits including the chemical composition of the epidermal CW.

2. Materials and methods

2.1. Plant material and leaf water uptake

Leaves of *Heteropterys campestris* (Malpighiaceae), *Tibouchina heteromalla* (Melastomataceae), *Trembleya laniflora* (Melastomataceae) and *Ocotea pulchella* (Lauraceae) were collected in ferruginous outcrops above 1,450 m of altitude at Serra da Brígida, Parque Cachoeira das Andorinhas, Minas Gerais state, Brazil, where fog is a very frequent meteorological event during the four seasons of the year.

To evaluate the water intake ability, we performed the following procedure: first the leaves (n = 4) were collected in the field and immediately weighed with a portable scale. Afterwards the leaves were taken to the laboratory and weighed once more. Subsequently, leaves were immersed in distilled water and weighed every 15 min for 2h, then every 30 min for 2 h. Finally, the leaves were immersed in water again for 1 h and weighed to obtain the maximum water absorption curve. Before each weighing, the leaves were first dried with ultra-absorbent microfiber synthetic towels to remove the surface water (Liang et al., 2009). Leaf water uptake was calculated by a differential equation, which provides data about the maximum water content in leaf lamina (C_{max}) and leaf uptake speed (k) (Liang et al., 2009).

2.2 Leaf functional traits

Leaves (n = 5) from 5 individuals of each species were collected in the field to obtain the leaf functional attributes. Ten leaf discs of 1 cm diameter were removed from each leaf to obtain the leaf specific mass (LSM), leaf specific area (LSA) (Rozendaal *et al.*, 2006), succulence (SU) (Mantovani, 1999), relative water content (RWC), and water saturation deficit (WSD) (Turner 1981).

2.3 Light microscopy

For anatomical analysis, expanded and undamaged mature leaves (n = 6) were collected from individuals (n = 3) of each species (Colleen, 2013). Leaf fragments (0.5 cm^2) were fixed in Karnovsky's solution (Karnovsky, 1965) for 48 h, dehydrated in n-butyl series (Johansen, 1940), embedded in Paraplast® (Kraus and Arduin, 1997), sectioned in a rotary microtome (12 µm) (Jung Biocut), and stained with 9:1 (v/v) solution of astra blue and safranin (Bukatsch, 1972, modified to 0.5 %).

Leaf traits that may affect the absorption capacity and water storage on leaves, such as the presence of trichomes, calcium oxalate crystals, secretory cavities, mucilage presence, and the degree of compactness of spongy parenchyma cells were evaluated.

2.4 Histochemical analyses

Cutin was detected by staining with Sudan red B (Brundett et al., 1991). This staining was also useful for the evaluation of cuticle thickness of the adaxial and abaxial leaf surfaces. Pectin was detected with ruthenium red (Johansen, 1940). Cellulose microfibrils were labelled with calcofluor white (Hughes and McCully, 1975), a specific dye that stains the β-glucan. All sections were mounted in water and examined under a light microscope (Leica DM500), or a fluorescence microscope (Leica DM 2500-LED). Photomicrographs were obtained with a Leica DFC 7000T camera coupled to the microscope.

2.5 Immunocytochemistry of cell wall epitopes

Part of the leaf samples fixed in Karnovsky's solution (Karnovsky, 1965), and prepared for anatomical analyses were incubated with the monoclonal antibodies JIM5, JIM7, LM5 and LM6 (Centre for Plant Sciences, University of Leeds, UK). These antibodies have specificity to binding epitopes of low methylesterified homogalacturonans (HGAs) (JIM5) and high methylesterified HGAs (JIM7), galactans (LM5) (Jones et al., 1997), and arabinans (LM6) (Willats et al., 1998). The sections were immersed in blocking solution with 3% (w

/ v) milk powder in phosphate-buffered saline (PBS) for 30 min, and incubated with primary antibodies in powder milk/PBS solution (1:10) for 2 h at room temperature. For control tests, the primary antibodies were not added. The sections were washed in PBS, incubated with secondary antibody for IgG FITC (Sigma) in powder milk/PBS solution (1:100) for 2 h in the dark. After washing in PBS, the sections were mounted in 50% glycerol and examined under the fluorescence microscope Leica DM 2500-LED with a Leica DFC 7000T coupled camera.

2.6 Data analyses

General linear models (GLMs) with Quasi Poisson errors were built to evaluate if the leaf specific mass (LEM), leaf specific area (LSA), succulence (SU), percentage of relative water content (RWC), and water saturation deficit (WSD) (independent variables) influenced the maximum water content in leaf (C_{max}), and the water uptake speed (k) (dependent variables) of the four species.

3. Results

3.1 Water uptake and functional leaf traits

Water uptake was fast in the first few minutes, but decreased in approximately 5 h, reaching zero value, due to leaf hydration, regarding the four plant species evaluated. According to the water absorption curves, *Trembleya laniflora* had the highest amount of water uptake, followed by *Tibouchina heteromalla* and *Heteropterys campestris*, with similar values. *Ocotea pulchella* had the lowest amount of water uptake (Fig. 1).

Trembleya laniflora had the highest rate of C_{max}, which was significantly different from the similar intermediary values of C_{max} in *T. heteromalla* and *H. campestris*. *Ocotea pulchella* differed significantly from the other species, with the lowest C_{max} values (Fig. 2A). The water uptake speed (k) was similar between *O. pulchella* and *H. campestris*, and it was higher than the values of the other two plant species. The lowest values of k, in *T. laniflora* and *T. heteromalla*, were statistically similar (Fig. 2B).

Among the analysed functional leaf traits, only the specific leaf area (SLA) had a positive and significant relationship with the C_{max} and k. The relative water content (RWC) affected the C_{max}, while the water saturation deficit (WSD) was more affected by the water uptake speed (k) (Fig. 5), and these results also presented a positive and significant relationship (Table 1). The other physiological leaf traits had no influence on the evaluated parameters.

3.2 Leaf anatomy and histochemistry

The leaves of three species are hypostomatic, and *T. heteromalla* is amphistomatic. The cuticle is thick on the adaxial leaf surface of two species, *T. laniflora* and *O. pulchella,* while the other two species, *T. heteromalla* and *H. campestris*, have thin cuticle. The cuticle is thin on the abaxial surface of all the four species (Fig. 3A-D). The four species have uniseriate epidermis with larger cells on the adaxial surface in relation to the abaxial leaf surface. Non-glandular trichomes occur on the abaxial leaf surface of *T. laniflora* and on the adaxial surface of *O. pulchella. Trembleya laniflora* also has short-pedunculated glandular trichomes, exclusively on the abaxial leaf surface (Fig. 3A). *Tibouchina heteromalla* has non-vascularized emergencies on both leaf surfaces, including on the venal region. These emergencies are lined by uniseriate epidermis, have some idioblasts containing druses on the cells of the ground system, and filiform sclereids interspersed (Fig. 3B). The mesophyll is dorsiventral in the four species, with 1-2 layered palisade parenchyma in three species and 3-layered in *T. laniflora*. The spongy parenchyma has 3-4-layers in *T. heteromalla*, *T. laniflora* and *H. campestris,* with variable area of intercellular spaces (Fig. 3C). *Ocotea pulchella* has the most distinct leaf lamina due to the occurrence of some round-shaped hypertrophied secretory idioblasts, containing oils, phenols and mucilage, interspersed to palisade parenchyma cells (Fig. 3D). The spongy parenchyma in this species has large intercellular spaces and has 5-7 cell layers. Two species, *T. laniflora* and *T. heteromalla,* have vascular bundles in bicollateral arrangement and two, *H. campestris* and *O. pulchella*, have vascular bundles in collateral arrangement (Fig. 3A-D). The bundle sheaths store phenolics and have interspersed sclereids in *T. laniflora*, and druses of calcium oxalate in *T. heteromalla* and *H. campestris* (Fig. 3 B-C). Sheath extensions with lignified fibres communicate the vascular bundles to both sides of the epidermis in *O. pulchella*. The abaxial epidermis has papillose cells and some glandular trichomes in *T. heteromalla*.

The leaves of *T. laniflora* and *T. heteromalla* have positive reaction for pectins detected by the ruthenium red in the CW of all tissues, including the vascular bundles, and the adaxial epidermis. In leaves of *H. campestris*, the positive reaction for ruthenium red occur in the CW of the adaxial epidermis, vascular bundle sheaths and collenchyma. There is also positive reaction for pectins in some cells of the abaxial epidermis, and in glandular trichomes. *Ocotea pulchella* has positive reaction on the CW on both epidermal surfaces and parenchyma cells (data not shown). All species have positive reaction for calcofluor white in epidermal cells, which has low level of recognition in *O. pulchella* (Fig. 3 E-H).

3.3 Immunocytochemistry of cell wall epitopes

In *T. laniflora,* the epitopes of low methyl-esterified HGAs were labelled by JIM5 overall the CW of the spongy parenchyma, idioblasts, and xylem and phloem (Fig. 3I). The epitopes of high methyl-esterified HGAs bound by JIM7 were restricted to some CW of the spongy parenchyma (Fig. 4A). No epitopes of galactans were labelled by LM5 (Fig. 4E). Epitopes of arabinans were abundantly labelled by LM6 in the CW of the palisade parenchyma (Fig. 4I).

In *T. heteromalla,* the low methyl-esterified HGAs had a low level of recognition by JIM5 in the CW of both epidermal surfaces, emergencies, spongy parenchyma, xylem and phloem (Fig. 3I). The high methyl-esterified HGAs had an overall but low level of recognition by JIM7 in the CW of all leaf tissues (Fig. 4B). The epitopes of galactans were labelled by LM5 throughout the CW of the palisade parenchyma and abaxial epidermis (Fig. 4F). The epitopes of arabinans labelled by LM6 were restricted to the stomata (Fig. 4J).

In *H. campestris,* the epitopes of low methyl-esterified HGAs were labelled by JIM5 in the CW of the adaxial epidermis (Fig. 3K). The high methyl-esterified HGAs had abundant detection by JIM7 in the CW of adaxial epidermis and palisade parenchyma (Fig. 4C). The epitopes of galactans labelled by LM5 were restricted to the CW of the idioblasts and the epitopes of arabinans were labelled by LM6 only in some cells of the vascular bundles (Fig. 4G, K).

In *O. pulchella,* the epitopes of low methyl-esterified HGAs were labelled by JIM5 in the CW of adaxial epidermis, in parenchyma cells, and in the vascular bundles (Fig. 3L). The high methyl-esterified HGAs labelled by JIM7 were overall abundant in the CW of all leaf tissues (Fig. 4D). The epitopes of galactans labelled by LM5 were restricted to a few cells of spongy parenchyma (Fig. 4H), while the epitopes of arabinans recognized by LM6 were restricted to some cells of the vascular bundles (Fig. 4L).

4. Discussion

4.1 LWU, morphology and leaf anatomy

Different strategies of LWU were clearly revealed in all the four species, with peculiarities among them. The amount and speed of water absorption are not only related to the presence of trichomes and thin cuticle, but also to more compactly arranged tissues and mucilaginous cells in the mesophyll. Water storage sites indicate greater capacity for water absorption, while the CW with high detection of pectin are related to an increase in water absorption speed in leaves.

As the RWC describes the plant water status at a given time (Kramer, 1969), it is interesting to note that there is a close relationship between the RWC and the LWU strategies. The fastest absorbing species are those with a higher initial RWC, when compared to the slower absorbing species. In other words, a higher RWC is a consequence of a higher absorption rate, since a smaller value of WSD positively interferes in the value of *k*. Thus, it may be highlighted that the species with faster absorption have higher RWC values, which may be a strategy to avoid drought effects. Moreover, different strategies were observed; *T. laniflora*, followed by *T. heteromalla* had higher rates of C_{max} and the smallest values of *k*. *O. pulchella* and *H. campestris* had the highest values of *k* and smallest values of C_{max} (Fig. 2).

Morphological and anatomical features may significantly interfere in the LWU. The cuticle is the main route through which water must pass during leaf absorption (Eller et al., 2013; 2016). However, the degree of permeability may be related to the cuticle chemical composition and to the arrangement of the epicuticular wax, and not necessarily to cuticle thickness (Schuster et al., 2016), as herein observed for the four species. The leaves of *T. laniflora* and *T. heteromalla*, despite the differences in cuticle thickness, absorbed similarly more water in the 5-hour period of the experiment, but more slowly. The trichomes positively influence the LWU (Lusa et al., 2014; Eller et al., 2016; Vitarelli et al., 2017), as observed in *H. campestris* and *O. pulchella*, the only species with non-glandular leaf trichomes. Accordingly, only *T. heteromalla* has emergencies on its leaves (Fig. 3B), which may also facilitate the LWU, while the mucilage in the leaf tissues of *H. campestris* and *O. pulchella* may enable water retention due to its hygroscopic trait, as indicated by Fahn and Cutler (1992).

The compactness of the spongy parenchyma in *T. laniflora* and *T. heteromalla* has a direct relationship with the strategy of these species to absorb a larger amount of water (Fig. 3A-B). Since water is mainly stored in the vacuoles, the higher the number of vacuolated large cells, the higher is the availability of cell compartments to store water. Thus, these anatomical attributes help water retention within leaf tissues, and with more compartments for water storage and therefore higher C_{max} (Tomlinson, 2003). Contrastingly, *O. pulchella*, which had the second smaller C_{max}, has a loose spongy parenchyma, with large intercellular spaces. Also, the presence of oil-containing idioblasts also decreases the number of vacuoles for water storage, corroborating the initial premises.

Current results indicate a trade-off between the LWU capacity and the water storage. Species with greater LWU capacity tend to have limited water storage capacity (Gotsch et al., 2015), compensating it with rapid leaf water uptake. However, they can avoid the loss of cell turgor, keeping high structural allocation and water storage, as seen in epiphytes of Costa Rica, which have a thicker hydrenquimal layer (Gotsch et al., 2015). Accordingly, similar strategies should occur with two of the four species currently studied, *T. laniflora* and *T. heteromalla,* which have a slower water uptake, but a higher degree of water storage.

4.2 Cell wall composition

There is direct relationship between the absorption rate, the amount of water absorbed, and the CW composition in the four species herein studied. Species that have abundant detection of cellulose in their epidermal CW, i. e., *T. laniflora and T. heteromalla*, can absorb more water, but more slowly, while those with abundant detection of pectins, especially HGAs, can absorb water at a higher rate.

Once water molecules have passed the cuticle, the next barrier is the external periclinal CW. As one of the main components of the CW, cellulose was histochemically detected in distinct levels of recognition in the CW of the adaxial epidermal cells in all the studied species by the calcofluor. For both *H. campestris* and *O. pulchella*, low fluorescence was considered indicative of less cellulose in their epidermal cells in relation to the other species. Cellulose microfibrils, when interacting with water, form gels that may increase the amount of water absorbed, but even though these gels have hygroscopic property and keep wall hydration, they slow the absorption rate due to their physical properties (Pääkkö et al., 2007; Agoda-Tandjawa et al., 2012). Current data indicate that the interlacing of cellulose microfibrils may reduce the CW porosity, and therefore slow the speed of water uptake.

Cell walls can compensate the low proportion of cellulose by the deposition of HGAs (His et al., 2001). The proportion of cellulose:pectin can differ between species, and once it is very dynamic, it can be modified in response to several external and internal factors (Cosgrove, 2005). Currently, pectins were detected by ruthenium red in the CW of the adaxial epidermal cells, in the four species studied. However, the MAbs detected slight differences in the pectin classes. The absence of labelling for all the tested MAbs in the CW of *T. laniflora* epidermis indicates low pectin proportion, as proposed as an overall assumption (Albersheim et al., 2011), making it difficult to be detected. The greater amount of water absorbed by *T. laniflora* can be explained by the overall and higher detection of cellulose microfibrils in the CW of the adaxial epidermis, which is reinforced by the CW traits of *T. heteromalla,* the second species with the higher LWU.

The HGAs of high and low methyl esterification are the most abundant pectin group in the CW (Verhertbruggen et al., 2009), as is true for two of the four species of the tropical fog mountain fields herein studied. The overall labelling of HGAs in *H. campestris* and *O. pulchella* corroborates the assumption that their cell walls are pectin-rich, mainly rich in HGAs, and indicates the role of HGAs as determinant in the increase of the water absorption speed in these two species. Thus, abundant pectins in the CW of the adaxial epidermis of this two species may explain the high speed of LWU.

The pectins form hydrated gels that displace the cellulose microfibrils, and as they fill in the microfibrillar spaces, they avoid the aggregation and collapse of the cellulose network (Wolf et al., 2009). A study carried out with *Phaseolus vulgaris* evidenced that reducing the amount of pectins at root apices under osmotic stress altered the structure of the CW, due to a rearrangement of wall polymers, affecting cell porosity (Cosgrove, 1997; Cosgrove, 2005). Therefore, pectins ensure the CW porosity, and consequently a higher water absorption rate, as observed for *H. campestris* and *O. pulchella*, which presented the fastest LWU.

The degree of methyl esterification of the HGAs has a direct influence on the CW structure and consequently on cell physiology (Wolf et al., 2009). The HGAs with low degree of methyl esterification form bonds with Ca^{2+}, forming an "egg-box" structure (Liners et al., 1989), and consequently, a greater interaction with the water. Therefore, the decrease in the degree of methyl esterification increases the interaction with water, and makes the CW more hydrophilic (Faravash and Ashtiani, 2007). Accordingly, the HGAs with a low degree of methyl esterification should be more related to LWU than those with a high degree of methyl esterification. The increasing in the detection of HGAs with low methyl esterification may indicate a compensation to the decreasing in the restricted detection of cellulose in the CW. In *H. campestris* and *O. pulchella*, cellulose microfibrils had a restricted detection, in the epidermal cells, by the calcofluor white, but the HGAs with low degree of methyl esterification were abundantly detected by JIM5. Consequently, it is interesting to note that an increase in the labelling of low methyl esterified HGAs can balance the apparently low synthesis and deposition of cellulose in the CW (His et al., 2001; Manfield et al., 2004).

The substitution of part of the HGAs by RG-I or RG-II is related to an increase in the CW porosity, and thus to the movement of proteins and hydrolytic enzymes within the wall matrix (Iraki et al., 1989). Peculiarly, *H. campestris* was the only species where galactans were restricted labelled in some abaxial epidermal cells, and as galactans are related to stability and rigidity in CW of the leaf epidermis (Xu et al., 2011), a greater amount of this lateral chains of RG-I may be associated with a higher rate of absorption. The CW composition of *H. campestris* seems to favour the porosity of the wall and the speed of water absorption in its leaves.

5. Conclusion

The plants that presented the fastest leaf water absorption have an overall labelling of pectins, mainly HGAs, in their epidermal CW. However, plants that absorb more water, even more slowly, have abundant detection of cellulose microfibrils in the CW of epidermal cells. These results indicate that cellulose and pectins are determinant in water absorption by leaf epidermis, conferring greater porosity and absorption speed, regarding pectin composition; or greater hydrophilicity and greater absorption capacity, regarding cellulose. In conclusion, it seems that the determining trait in leaf water absorption in the studied species is the composition of the epidermal CW, although other traits such as trichomes are known to influence the process. In addition, the presence of more compact tissues and mucilaginous cells in leaves may also influence leaf water absorption and water storage capacity.

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Figure captions

Fig. 1. Dynamic water content in the leaves by submerging time (min). Equations: *Tibouchina heteromalla* ($y=0.55751-e^{-0.0372t}$) $r^2=0.9341$; *Ocotea pulchella* $y=0.0789(1-e^{-0.0624t})$ $r^2= 0.971$; *Trembleya laniflora* $y=0.7985(1-e^{-0.026t})$ $r^2= 0.936$; *Heteropterys campestres* $y=0.4508(1-e^{-0.0621t})$ $r^2= 0.869$.

Fig. 2. Maximum water content (Cmax) in (A) and water uptake speed (k) in (B) of the evaluated species. The mean values (±SE) of the leaf uptake maximum amount standardized by projected leaf area. The standardized mean values (±SE). ($P \leq 0.05$ between treatments).

Fig. 3. Immunocytochemistry of plant cell wall pectins. (A, E, I) *Trembleya laniflora*. (B, F, J) *Tibouchina heteromalla*, (C, G, K) *Heteropterys campestris* (D, H, L) *Ocotea pulchela*. (A–D) Safranin and astra blue; (E-H) Calcofluor white; (I-L) JIM5. AE: adaxial epidermis; PP: palisade parenchyma; SP: spongy parenchyma; Em: emergencies; Ae: aerenchyma; SE: bundle sheath extension.

Fig. 4. Immunocytochemistry of plant cell wall pectins. (A, E, I) *Trembleya laniflora*. (B, F, J) *Tibouchina heteromalla*, (C, G, K) *Heteropterys campestris* (D, H, L) *Ocotea pulchela*. (A–D) JIM 7, (E-H) LM5, (I-L) LM6. AE: adaxial epidermis; PP: palisade parenchyma; SP: spongy parenchyma; ID: idioblast; ST: stomata; Xy: xylem; Ph: phloem.

Fig. 5. Relative water content (RWC) and water saturation deficit (WSD) (%) of *Heteropterys campestris*, *Ocotea pulchella*, *Tibouchina hetemalla* and *Tremblyea laniflora*.

Contributions

The study was conceived by MGCF and RMSI. DB, BGF, ARZ and HCS performed the experiments. DB computed and analyzed data and DB, MGCF and RMSI wrote the manuscript.

Table 1

Table 1

Water leaf content (C_{max}) and water uptake speed (k) related to leaf traits of the evaluated plants. Specific leaf weight (SLW), specific leaf area (SLA), succulence (SU) and relative water content (RWC) and water saturation deficit (WSD). In these analyses, dependent variables were maximum leaf water content (C_{max}) and water uptake speed (k).

Dependent variable	Independent variable	Deviance	F	DV	*P*
C_{max}	SLW	3.5368	0.1731	18	0.6837
	SLA	2.0768	15.1418	17	0.0016 **
	SU	2.0742	0.0268	16	0.8722
	RWC	1.6541	4.3563	15	0.05064 *
	WSD	1.5381	1.2034	14	0.2911
k	SLW	0.4944	0.0025	18	0.9607
	SLA	0.2512	23.2187	17	0.0002 ***
	SU	0.2421	0.8686	16	0.3671
	RWC	0.2289	1.2574	15	0.281
	WSD	0.1433	8.1740	14	0.0126**

The general linear models (GLMs), with Quasipoisson errors, for the biotic independent variables evaluated. In these analyses, dependent variables were maximum water leaf content (C_{max}) and water uptake speed (k). *** Indicates $P \leq 0.001$; **Indicates $P \leq 0.01$ and * Indicates $P \leq 0.05$.

Figure 1

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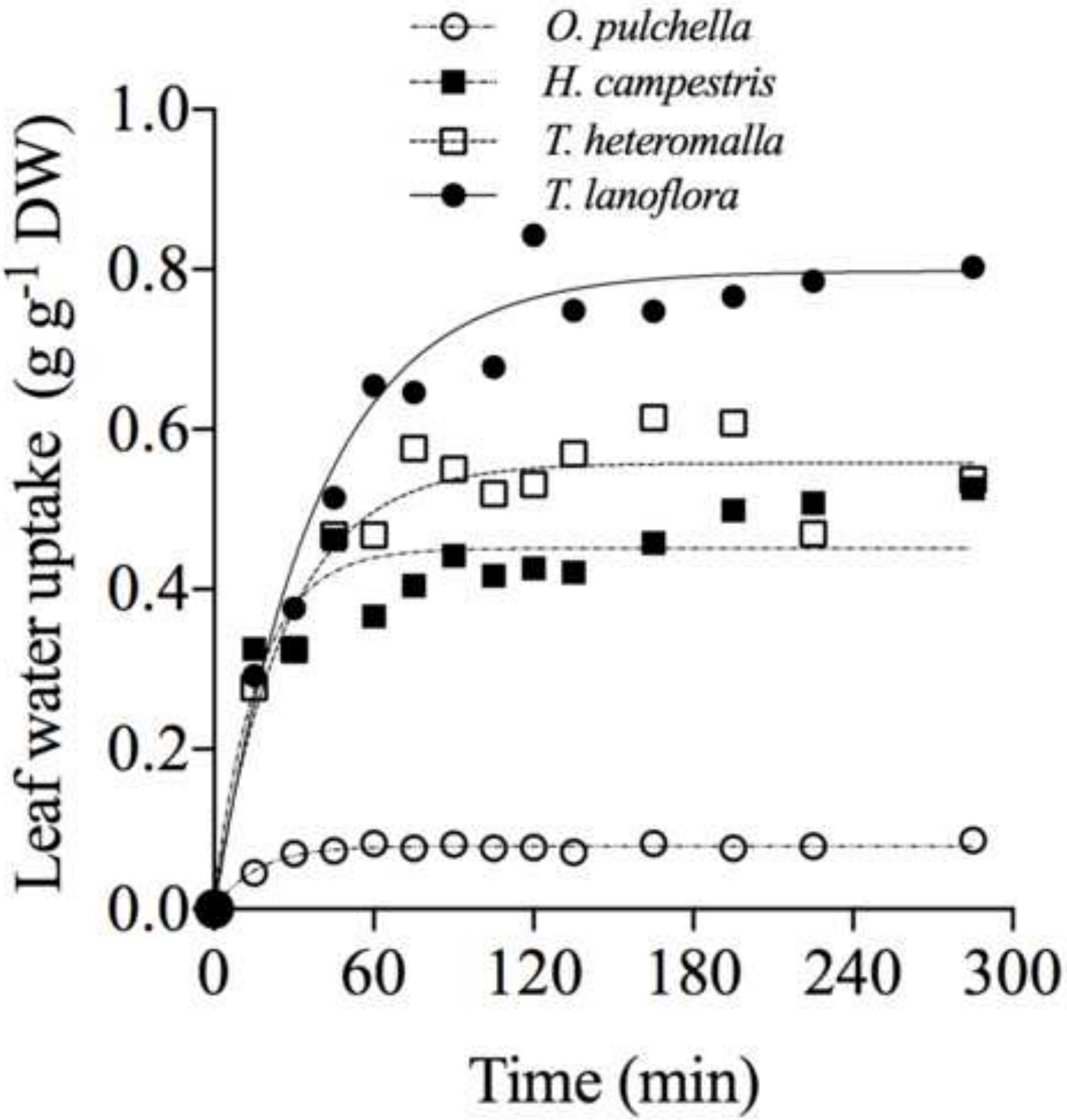

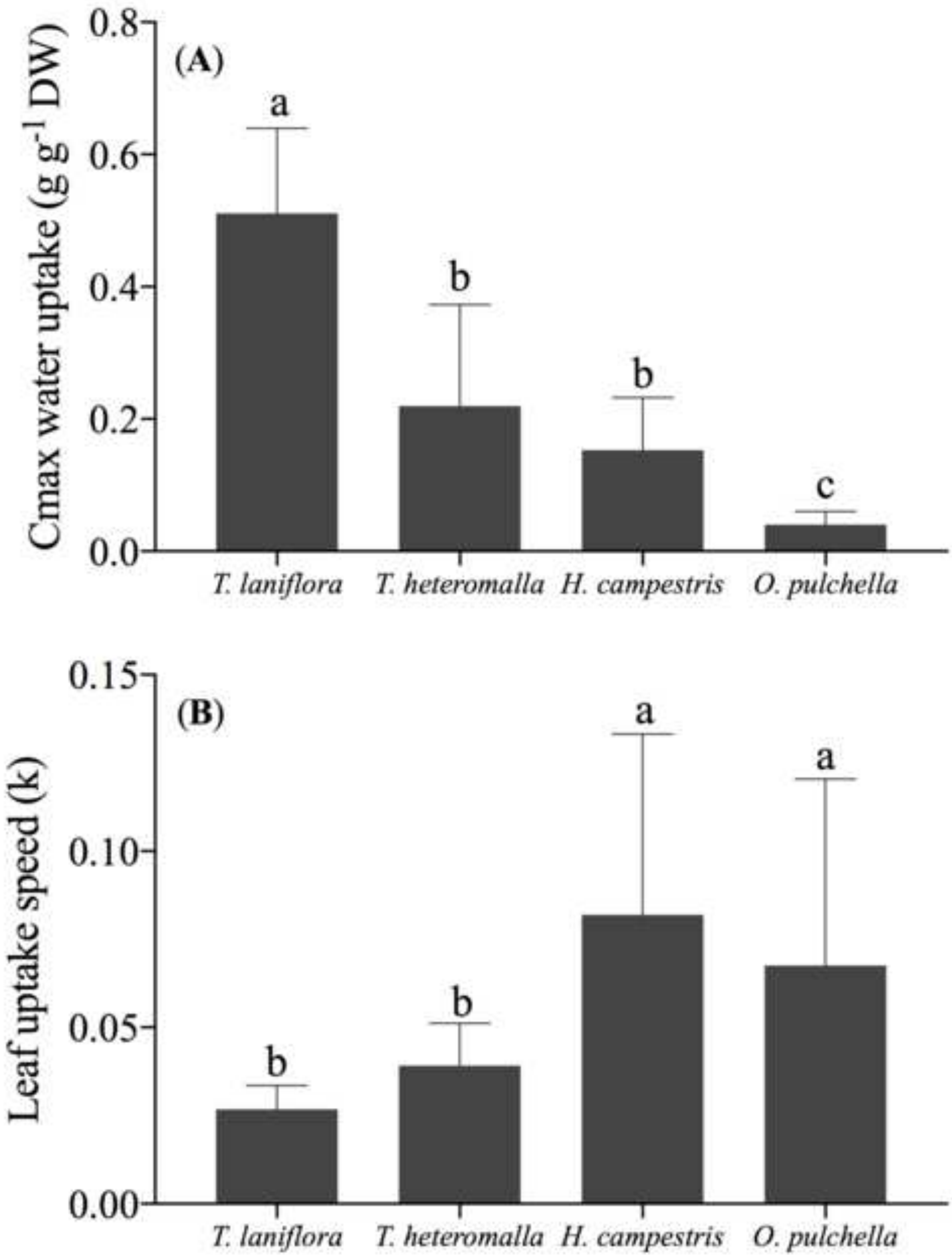
(A)
Cmax water uptake (g g^{-1} DW)
0.8
0.6
0.4
0.2
0.0
a
b
b
c
T. laniflora
T. heteromalla
H. campestris
O. pulchella
(B)
Leaf uptake speed (k)
0.15
0.10
0.05
0.00
b
b
a
a
T. laniflora
T. heteromalla
H. campestris
O. pulchella

Figure 3

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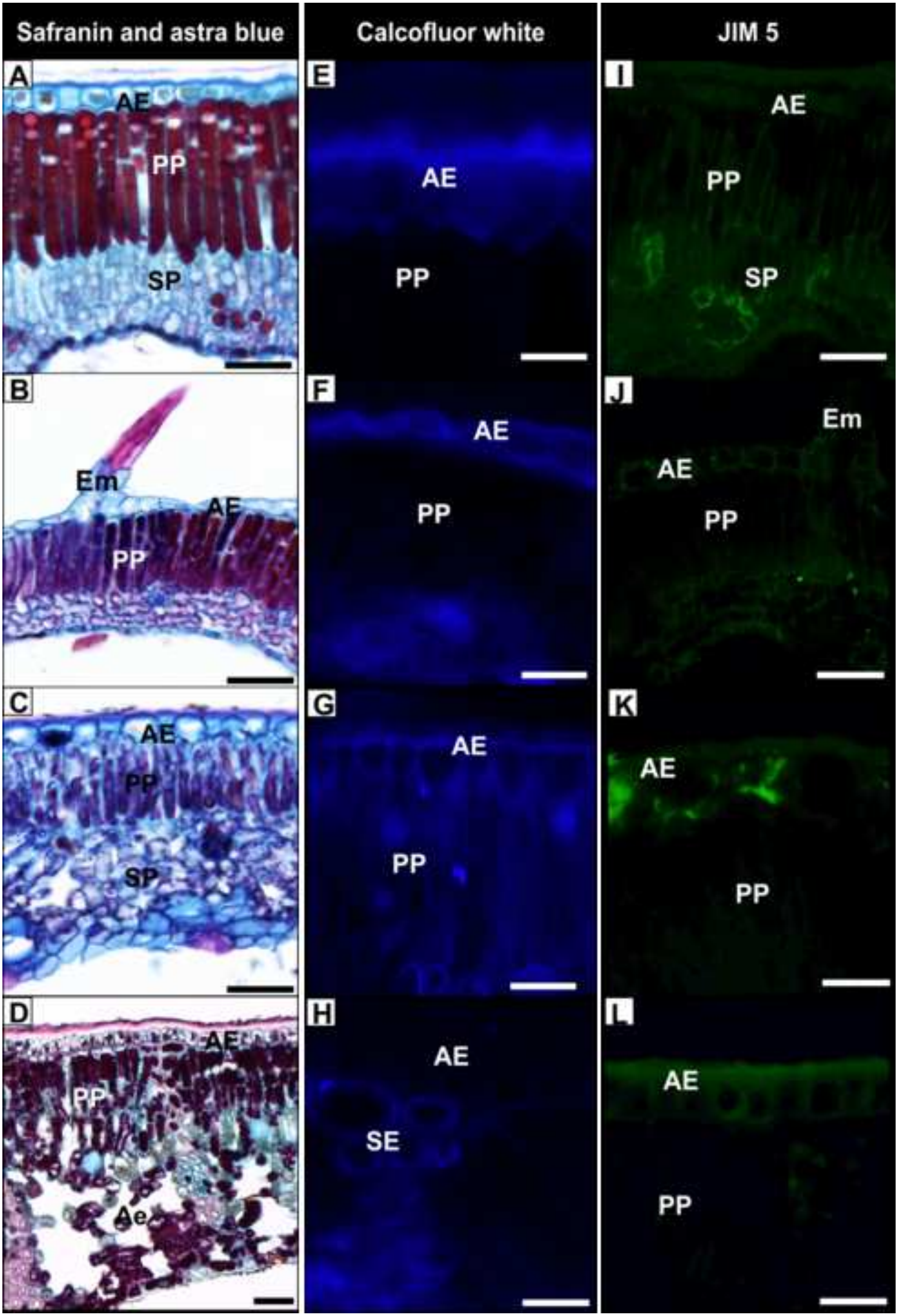

Figure 4
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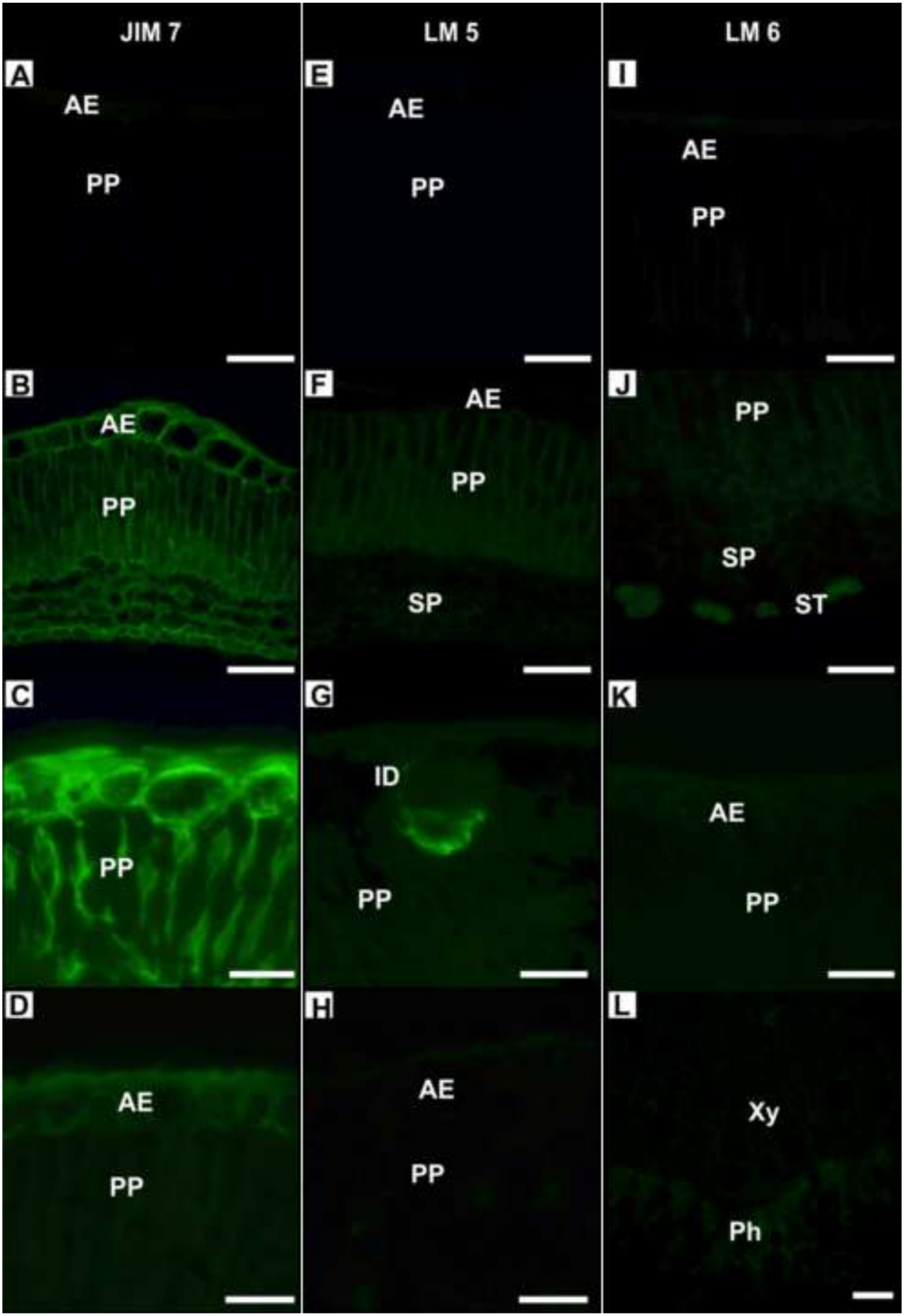

Figure 5

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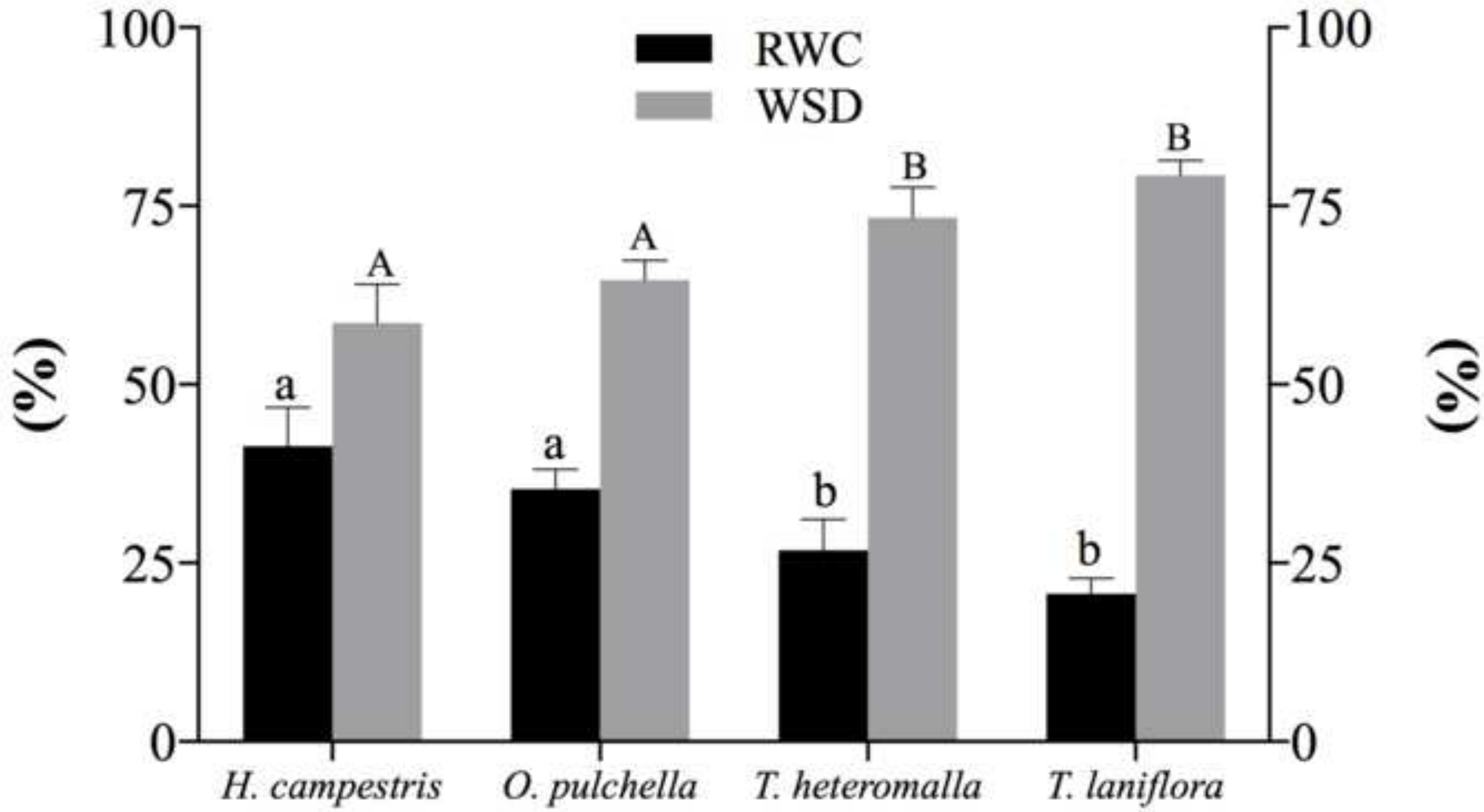

Chapter 3

Foliar water-uptake strategies are related to leaf water status and gas exchange in plants from a ferruginous rupestrian field

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Foliar water uptake strategies are related to both leaf water status and gas exchanges in plants from a ferruginous rupestrian field

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PREMISE OF THE STUDY: Fog plays an important role in the physiology of several plant species. It is known that foliar water uptake (FWU) of fog happens in different ways: a faster and a slower water fog uptake. However, water fog may play a negative role in CO_2 assimilation. Thus, the interference of different strategies of FWU in the water and carbon balance were evaluated to verify if fog may mitigate possible leaf water deficit.

METHODS: Four plant species with different FWU strategies were studied in a ferruginous rupestrian field, where fog is a frequent event. Measurements of gas exchanges and water potential in predawn and midday were performed during dry and rainy seasons, separating foggy from non-foggy days during the dry season.

KEY RESULTS: The FWU speed negatively influences CO_2 assimilation in the dry season, possibly because of its negative relationship with stomatal conductance, since reduced stomatal aperture impairs carbon entrance. Fog presence increased leaf water potential both in early morning and mid-day during the dry season. However, during the rainy season, the values of leaf water potential were higher at noon than at midday, which favors leaf gas exchanges.

CONCLUSIONS: FWU interferes negatively, but briefly with CO_2 assimilation. Nevertheless, FWU prevents water loss through transpiration and increases the water status of plants in the dry season. That is, FWU generates a compensation between CO_2 assimilation and foliar hydration, which, in fact, is beneficial to the plants of this ecosystem.

KEY WORDS; gas exchanges; photosynthesis; Rupestrian field, water relations.

The ferruginous rupestrian fields are considered an ancient climatically protected infertile landscape (OCBIL) such as Kwongkan in South-West Australia, the fynbos in South-West South Africa, and the tepuis in the Shield of Guyana in Northern South America (Hopper, 2009). This ecosystem has great diversity with high endemism, and rare species (Silveira et al., 2016). Ferruginous rupestrian fields are usually characterized by a mosaic of grassy-shrub and arboreal vegetation, prone to fire occurring on outcrops hematite rich sites, such as itabirites or *cangas*, that is, on shallow and discontinuous soils. A peculiar feature of this ecosystem is the presence of fog due to its elevated altitude. In addition to contributing to the hydrology of ecosystems, fog may play a relevant ecological role as an alternative source of water for vegetation (Burgess and Dawson, 2004), once many species depend on an alternative source of water in drought periods (Dawson, 1998; Williams et al., 2008). During periods without fog, tropical altitudinal environments can be considered arid due to the high evaporative demand (Leuschner, 2000).

When fog is present, foliar water uptake (FWU) is a strategy for leaf hydration. Foliar absorption occurs when water surpasses a leaf surface in response to the water potential gradient diffusing through the cuticle (Gouvra and Grammatikopoulos 2003), absorbent trichomes (Benzing et al., 1978) and through the hydatodes (Martin and von Willert, 2000). Water absorption and particles can occur soon after different forms of precipitation, such as fog and rain.

Although the interception and absorption of water by the leaves have been reported in publications since the 1960 (Stone 1963), studies on the mechanisms and pathways of foliar water uptake (FWU) have become more present in recent decades (Burgess and Dawson, 2004; Anderson, 2005; Lai et al., 2007; Liang et al., 2009; Qiu et al., 2010; Goldsmith, 2013; Eller et al., 2016; Berry et al., 2018). Several studies have already revealed the advantages of FWU in the physiology of plant species, such as increased water status and benefits for gas exchange (Grammatikopoulos and Mannetas, 1994; Martin and von Willert, 2008; Simonin et al., 2009). The importance of FWU for gas exchanges and leaf water status has not yet been described for any shrub and woody species of Brazilian ferruginous rupestrian fields. The water acquired by FWU is considered of ecological relevance for dry or seasonally dry environments, where fog events are frequent (Oliveira et al., 2014). This strategy may facilitate the physiological activity, growth and establishment of plant species, since the frequency of fog seems to be functionally linked to the patterns of distribution of certain species and types of ecosystems (Qiu et al., 2010).

Due to selective limitations imposed by water, species that occur in places with water deficit have different mechanisms of water capture or maintenance (Reich et al., 2003), and consequently different adaptive strategies. In some species, water can be absorbed quickly by leaves, but in smaller amounts. However, there are species whose leaves absorb more water but more slowly. Indeed, 11 species of this ecosystem have different water absorption capacities (Boanares et al., 2018a and b), and such knowledge may help clarify the coexistence of species adapted to the temporal and spatial water availability variations that the tropical environment is subject to. Such adaptations are key factors for the understanding of species diversity (Tyree et al., 2003), mainly in relation to climate changes. After all, pragmatically, metal-rich environments, such as ferruginous rupestrian fields, have a flora with restricted geographic distribution and subjected to several iron mining activities (Silveira et al., 2016). In this way, several species are also endangered due to this anthropic activity (Martinelli and Moraes, 2013).

The hypothesis of this study is that FWU strategies interferes positively in the water and carbon balance of the species studied. Thus, increased carbon assimilation and leaf hydration would attenuate possible water stress.

MATERIALS AND METHODS

Study area and plant species

This study was carried out in a region of ferruginous rupestrian field in the "Cachoeira das Andorinhas" Environmental Protection Area, located in the municipality of Ouro Preto, Brazil (20 ° 21'30 "S / 43 ° 30'11 "W). The local ecosystem is characterized by species of herbs, shrubs and treelets growing on shallow soils of rock outcrops of itabirites or *cangas* (Valim et al., 2013). The area has an average altitude of 1,492 m, where fog events are frequent (Fig.1). The study was conducted in February and July, 2017, the most evident months of rainy and dry seasons, respectively. The average annual temperature and precipitation were 16.9°C and

100.4 mm, respectively, with the total annual rainfall of 1,204.7 mm, 95.5% concentrated from October to March (Fig. 2). Fog accounts for approximately 13% of rainfall entries during the year. However, in the dry season, it contributed 10 times more in relation to rainfall (Baeta, 2012).

The studied Phanerophytes species were: *Leandra australis* (Cham.) Cogn. (shrub) and *Miconia corallina* Spring. (arboreal) (Melastomataceae), *Eremanthus erythropappus* (DC.) MacLeish, (Asteraceae) (arboreal), *Byrsonima variabilis* A. Juss., (Malpighiaceae) (shrub), all endemic of Brazil except *Leandra australis*.

These species were selected according to their different FWU capacities (Boanares et al., 2018b). *Leandra australis* and *Byrsonima variabilis* have a higher speed of leaf water absorption, but uptake a small amount of water. However, *Eremanthus erythropappus* and *Miconia corallina* have slow absorption, but uptake a large amount of water through the leaves (Table 1).

Measurements of foliar water uptake

To evaluate leaf water absorption capacity, leaves (n = 4) were collected in the field and immediately weighed with a portable digital scale. Leaves were then taken to the laboratory and weighed once more, and the petiole was sealed with hot paraffin. Subsequently, the leaves were immersed in distilled water and weighed every 15 min for 2 h, then weighed every 30 min for 2 h. Finally, the leaves were immersed in water again for 1 h and weighed to obtain the maximum water absorption curve. Before each weighing, the leaves were dried with synthetic towels of ultra-absorbent microfibers to remove surface water (Liang et al., 2009). Leaf water uptake was calculated by a differential equation, which provides data on maximum leaf water (C_{max}) and leaf uptake velocity (k) (g g^{-1} dry weight) (Liang et al., 2009).

Measurements of water potential and gas exchanges

The water potential (Ψ_w) was measured on three leaves of four individuals of each species at predawn (Ψpd) and at midday (Ψmd) with a Scholander type pressure chamber in the dry (July) and rainy seasons (February). Predawn measurements were taken between 5:00 and 6:00 am, and midday measurements were taken between 12:00 and 1 pm. In the dry season, Ψ_w was measured at midday with and without fog.

Gas exchanges were measured in leaves of four individuals of each species in the dry and rainy season. A portable infrared gas analyzer (IRGA) (LI6400, LI-COR, Lincoln, Nebraska) equipped with artificial light and an integrated fluorometer (Li-6400-40, Li-Cor, Inc) was used for light curves and one-off measurements. The light curve measurements were performed from 9:00 to 11:00 am, with the addition of photosynthetic active radiation (PAR) from 0 µmol m^{-2} s^{-1} to 2600 µmol m^{-2} s^{-1}. One-off measurements of gas exchanges were taken at 9:00 am with PAR saturation obtained by the light curve (1500 µmol m^{-2} s^{-1}). Measurements of net CO_2 assimilation (A), transpiration (E), stomatal conductance (g_s) and internal carbon and internal: ambient carbon ratio (Ci/Ca) were obtained. Water use efficiency (WUE = A/E) and intrinsic water use efficiency (iWUE = A/g_s) were also calculated.

Data analysis

Data normality was tested using the Kolmogorov-Smirnoff test with 5% significance. A variance analysis (ANOVA) followed by Tukey test at 5% was performed to verify if there was a difference in the FWU among plant species from a comparison between the mean values of C_{max} and k in the dry and rainy seasons. Linear Mixed Models (LMM) (Bolker et al.,

2009) were used to evaluate the influence of water absorption capacity on the physiological parameters related to gas exchange. The two parameters related to water absorption capacity, maximum water content (C_{max}) and leaf uptake speed (k) were used as fixed explanatory variables and the response variables were: A, E, g_s, Ci, Ci/Ca, WUE and iWUE. The identity of each measured plant was used as a random effect variable to control for the potential pseudoreplication biases in our models (*sensu* Hurlbert 1984). Highly correlated variables were removed from the models (Ci and iWUE).

To analyze the relationship between FWU and Ψ_w, a model selection was performed. Thus, an interaction between C_{max} and k was made as two categorical factors and, as explanatory variables, the value of the water potential at predawn (Ψpd) and at midday (Ψmd) in the dry and rainy seasons were selected. Individuals and species in both models were used as random factors (Appendix S1).

The full model was subjected to model selection using the Akaike Information Criteria of second order (AICc), and the best set of models was selected through multimodal inference analysis (Burnham et al., 2011). Any given model was considered superior to the other, whenever the deltaAICc between the top model and the others was higher than two (Burnham et al., 2011). For all adjusted models used in this study, residual analysis was performed to check for model suitability and adequacy of the distribution used (Crawley, 2012). All analyzes were performed on the R platform (R-Core-Team, 2015) and the computational packages used were: Ordinations and PERMANOVA - vegan, gdata, GLMM-lme4.

RESULTS

Strategies of FWU and gas exchanges

A significant relationship was observed among foliar water absorption rate (*k*) and *A*, g_s and *E*. There was no relationship between C_{max} and gas exchange parameters (Table 2), and *A*, g_s and *E* were inversely proportional to *k* (Fig. 3). *A* and g_s are significantly influenced by *k*, by species and by different seasons (Table 2). The differences between *A* and g_s in relation to *k* are more evident in the dry season (Fig. 3). The dry season influenced negatively these parameters, except for *E. erythropappus* that did not have differentiated values of *A* and g_s between the seasons. Only *M. corallina* and *E. erythropappus* differed significantly from each other in relation to *A*. For *M. corallina*, g_s is significantly different from *E. erythropappus* and *B. variabilis*. Unlike *A* and g_s, *E* was influenced only by *k* and by species and not by seasons. Only *M. corallina* and *E. erythropappus* differed significantly from each other.

The strategy of rapidly absorbing water i.e., high *k* favored *A*, but decreased the *E* with decreasing of g_s. However, with the decrease of g_s, there is also a negative influence on *A*. No distinct behavior was observed between species according to their strategy of foliar absorption and gas exchange.

Strategies of FWU and water potential

Only one best model remained after model selection, with a deltaAICc = 4 to the second-best model. In this model, leaf water potential varied significantly between predawn and midday and between dry and rainy seasons with and without fog (Fig. 4). The presence of fog increased the mean values of leaf water potential in all species during the dry season. The Ψ_w was positively influenced by *k* ($\beta = 5.68$) and dry season with fog ($\beta = 3.01$). However, the Ψ_w was negatively influenced by C_{max} ($\beta = -0.39$) and dry season without fog ($\beta = - 8.76$). These results corroborate with the antagonistic traits between C_{max} and *k* parameters. The presence of fog increased leaf Ψ_w in both predawn and midday, during the dry season. The fog water absorption generated higher values of leaf Ψ_w at midday during the dry season than at the rainy season.

DISCUSSION

Strategies of FWU and gas exchanges

The uptake of water by the leaves under different strategies has been proven in the species studied herein (Boanares et al., 2018b). These different strategies of FWU are a compensation because once the plant is slow to absorb water through its leaves, it compensates by absorbing a larger amount of water. In contrast, a plant with limitations in storing water in its tissues can adjust such disadvantage by absorbing faster, and hydrating its photosynthetic tissues (Boanares et al., 2018a and b). Currently, it is demonstrated that these strategies interfere in the water and carbon balance in *in situ* conditions, and the benefits water fog uptake in the dry season were verified. FWU increases the water potential of plant species, minimizing the water stress due to low rainfall of this season.

The k parameter, which confers speed to the FWU, negatively influences A in the dry season, because it also has negative relation with g_s. Thus, reduced stomatal conductance impairs gas exchange, and consequently carbon input due to the limited storage of water in plant tissues. Therefore, when leaves are less hydrated, stomatal closure reduces the g_s inducing a decrease in the transpiration rate, which is beneficial for the plant. For the plant species studied herein, the strategy of fast leaf water absorption through the leaves grants disadvantages in terms of carbon assimilation, but offers advantages against loss of water in the dry season. The stomatal closure restricts the damage caused by dehydration, since the potential for leaf transpiration is much greater than the water supply and / or storage capacity

(Medrano et al., 2015). Then, species with higher C_{max}, such as *E. erytrhopappus*, may be less able to control stomatal closure and opening, showing a greater dependence on the continuous supply of water for its tissues (Sperry et al., 2002).

Much wetting next to the leaves through fog or rainfall, provides a decrease in photon flux density, which can negatively interfere with light absorption due to its dispersion (Egri et al., 2010). Indeed, this wetting can also negatively interfere in CO_2 assimilation (Burgess and Dawson, 2004; Eugster et al., 2006). This process occurs because diffusing CO_2 through water is 10,000 times slower than through air, consequently photosynthetic carbon uptake is reduced (Brewer and Smith, 1995; Letts and Mulligan, 2005). There is also the possibility of water entering the stomata and consequently causing a decrease in leaf gas exchanges, since it has been seen that there is evidence supporting the role of stomata of FWU (Burgess and Dawson, 2004; Burkhardt, 2010). Stomatal clog up may also occur by water droplets without the actual absorption of this water, preventing CO_2 entrance (Gerlein-Safdi et al., 2018). However, the opposite behavior, i.e., leaf absorption varying positively in relation to gas exchange in different species of different ecosystems may occur (Grammatikopoulos and Mannetas, 1994; Martin and von Willert, 2008; Simonin et al., 2009; Burkhardt et al., 2012; Eller et al., 2013; Berry et al., 2014; Rosado et al., 2018). Leaf wetting changes leaf energy balance, decreasing temperature and consequently stomatal closure (Dawson and Goldsmith 2018; Gerlein-Safdi et a., 2018). Thus, in addition to leaf hydration, there is a decrease in transpiration. Due to the different availabilities of water and CO_2 resources, improvement of leaf wetting serves as a support to the limited water soil content (Boucher et al., 1995).

The wet soils during the rainy season does not represent a favorable condition for FWU (Simonin et al., 2009), which explain the non-significant relationship of the rainy season and FWU. Meteorological events of fog saturating the atmosphere, which reduce the incidence of photons, and consequently carbon assimilation, is a temporary situation. As soon as plant tissues hydrate and precipitation ceases, it will assimilate carbon again, and water stress will no longer impair photosynthesis, which means a compensation between CO_2 assimilation and leaf hydration.

The increase of gas exchange may be directly related to the increase of water potential in these species, which does not imply a decrease in CO_2 assimilation (Eller et al., 2013). During the rainy season, there is an increase in precipitation frequency, which can provide more favorable sunlight for photosynthesis, if compared to clear sky conditions. Thus, a reduction in the risk of photoinhibition from the penetration of diffuse and non-direct light may occur (Lüttge et al., 2007). The strategies of plant species to repel superficial water, as seen in many species of plants growing in areas with frequent leaf wetting, may favor the relation of FWU with gas exchanges (Brewer and Smith, 1995).

A decrease in gas exchange in the dry season may be related to the amount of water in leaf tissues, which is not enough for gas exchanges to occur in total capacity. Therefore, in the dry season, two of the four plant species studied here, *Byrsonima variabilis* and *Leandra australis* cannot store enough water to maintain leaf gas exchange. However, *E. erythropappus* presented similar *A* values between dry and rainy seasons, and possibly, this is the consequence of a large water storage in its tissues (the highest C_{max}), and therefore this species will not suffer constraints related to water loss, by keeping its stomata open. Also, the possible deep root and xylem hydraulic differences in *E. erythropappus* can be related to maintenance of this physiological process in dry season (Zimmermann, 1983).

Strategies of FWU and water potential

Several studies have demonstrated positive effects of fog on the water balance of some plant species (Johnson and Smith, 2008; Ritter et al., 2009; Eller et al., 2016). Currently, different strategies of foliar absorption (k and C_{max}) interfere in the Ψ_w of the species. Just as in FWU, there is an antagonistic relationship between k and C_{max}, as is also true for the Ψ_w. The Ψ_w is positively influenced by the k and negatively by the C_{max}. This relationship occurs because the foliar water rapid absorption (k) is directly related to the increase in plant water status. In contrast, a plant with slow absorption will not change its water status at the same speed as plants with fast absorption. However, considering the period of the day, the C_{max} positively influences the Ψ_w, and k negatively influences the the Ψ_w in predawn. Such influences may be explained by the strategy of plants with higher C_{max} to slowly absorbing larger amounts of water, losing less water than the plants that absorb fast, keeping their tissues more hydrated for a longer period of time. Thus, in the early hours of the day, their Ψ_w is larger than that of the fast-absorbing plants.

During the dry season when fog occurs, all the species increased their Ψ_w in relation to the dry season when fog was absent. *M. corallina* and *E. erythropappus* (C_{max}) increased their water status the most, when the midday of the dry season with no fog is compared to the midday of the dry season with fog. The positive relationship between FWU and gas exchange in the dry season is a consequence of the positive role of fog in plant physiology (Dawson, 1998; Limm et al., 2009; Eller et al., 2013; Cassana et al., 2015).

Fog can alter plant microclimate to a large extent by reducing the vapor pressure deficit, and consequently decreasing plant transpiration close to zero (Burgess and Dawson, 2004; Johnson and Smith, 2008; Vesala et al., 2017). Many studies have shown that despite the potentially negative effects, cloud forest species may benefit from cloud immersion as the transpiration can increase considerably in the dry season (Burgues and Dawson, 2004; Johnson and Smith, 2008). Also, the existence of some species in a given environment may be correlated to their FWU (Limm et al., 2009; Berry et al., 2014). The occurrence of fog in the ferruginous rupestrian fields confers a favorable water balance for all species studied, especially in the dry season. Thus, it can be observed that the studied species of the Brazilian ferruginous rupestrian fields do not experience water stress as much as expected formerly, since fog mitigate the damages caused by water deficit.

Current data corroborate the concept of decoupling of shoot physiology from soil water potential due to the foliar absorption capacity (Simonin et al., 2009). Transpiration and photosynthesis are independent of the amount of water in the soil, contrary to which was previously predicted by the unidirectional model of the continuous soil-plant-atmosphere (SPAC). Additionally, our results represent a complement to the concept of the SPAC model, with implications to the climatic models, once the strategy of leaf water absorption of these plants can be dissociated from the soil water potential (Goldsmith, 2013).

Although FWU interferes negatively in CO_2 assimilation, this interference is brief and prevents water loss through transpiration by increasing plant water status in the dry season. Accordingly, there is a compensation between CO_2 assimilation and foliar hydration, which is, in fact, beneficial to the plants of this ecosystem.

Studies on fog FWU in ferruginous rupestrian fields may provide information on the functional traits of species more or less adapted to carbon and water flux differences, considering changes in thermal and hydrological patterns, since global climate changes models predict a decrease in the fog formation (IPCC, 2013). Because of such changes, it is expected that the distribution of species and phytophysiognomies will be strongly changed in the landscape and that the same pattern will be found in other OCBILs. Such knowledge can provide subsidies which will help to understand the dynamics of the community, its operation and flows.

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Table 1. Mean values of C_{max} and *k* (g g^{-1} dry weight) in leaves of four species studied in dry and rainy seasons.

Species	Season	C_{max}		Species	Season	*k*	
E. erythropappus	Dry	0.9729	**a**	*L. australis*	dry	0.1822	**a**
M. corallina	Wet	0.8754	**ab**	*B. variabilis*	wet	0.1607	**a**
E. erythropappus	Wet	0.8363	**ab**	*B. variabilis*	dry	0.1387	**a**
M. corallina	Dry	0.6907	**b**	*L. australis*	wet	0.1196	**a**
L. australis	Wet	0.3521	**c**	*E. erythropappus*	wet	0.03356	**bc**
B. variabilis	Wet	0.2173	**c**	*M. corallina*	wet	0.03032	**bc**
B. variabilis	Dry	0.183	**c**	*E. erythropappus*	dry	0.02846	**bc**
L. australis	Dry	0.1805	**c**	*M. corallina*	dry	0.0194	**c**

Different letters indicate a significant difference ($P \leq 0.05$) between the means of the same column.

Table 2. Water leaf content (C_{max}) and water uptake speed (*k*) related to the gas exchange parameters and ratios of all four species analyzed that were related to the parameters of foliar water uptake. Net CO_2 assimilation (*A*), transpiration (*E*), stomatal conductance (g_s).

Independent variable	Dependent variable	F	DF	Deviance	p-value	
A	C_{max}	0.301	1	72.97	0.5845	
	k	6.717	1	110.95	0.0108	*
	Species	6.558	3	109.06	0.0004	***
	Season	64.923	1	109.92	0.0001	***
g_s	C_{max}	0.091	1	72.97	0.9244	
	k	13.126	1	110.95	0.0001	***
	Species	4.628	3	109.062	0.0043	**
	Season	11.135	1	109.92	0.0011	**
E	C_{max}	0.006	1	72.97	0.9341	
	k	6.276	1	110.97	0.0136	*
	Species	4.029	3	109.06	0.0092	**
	Season	2.94	1	109.92	0.0892	

The above data correspond to the general model. The data of the appropriate minimum model are in the supplementary data. *** Indicate $P \leq 0.001$; **Indicate $P \leq 0.01$ and * Indicates $P \leq 0.05$.

Appendix S1. Model selection table of the Global model call: lmer (formula = Potential ~ C_{max} + k + hour of day + season + C_{max}: season + k: season + (1 | Specie/Ind.)

Model	(Int)	C_{max}	Season	k	Hour of day	C_{max}: season	C_{max}: hour of day	Season:k	k: Hour of day	R^2	df	logLik	AICc	Delta	Weight
256	-8.75	-0.39	+	5.67	+	+	+	+	+	0.726	15	-230.531	497.1	0.00	0.742
240	-7.52	-1.84	+	1.23	+		+	+	+	0.724	13	-235.261	501.0	3.90	0.106
128	-8.05	-1.01	+	1.78	+	+	+	+		0.725	14	-234.269	501.7	4.66	0.072
224	-11.40	2.75	+	15.23	+	+		+	+	0.714	14	-234.409	502.0	4.94	0.063
112	-6.81	-2.46	+	-2.66	+		+	+	+	0.723	12	-238.992	505.7	8.68	0.010
208	-10.17	1.29	+	10.78	+			+	+	0.712	12	-239.162	506.1	9.02	0.008

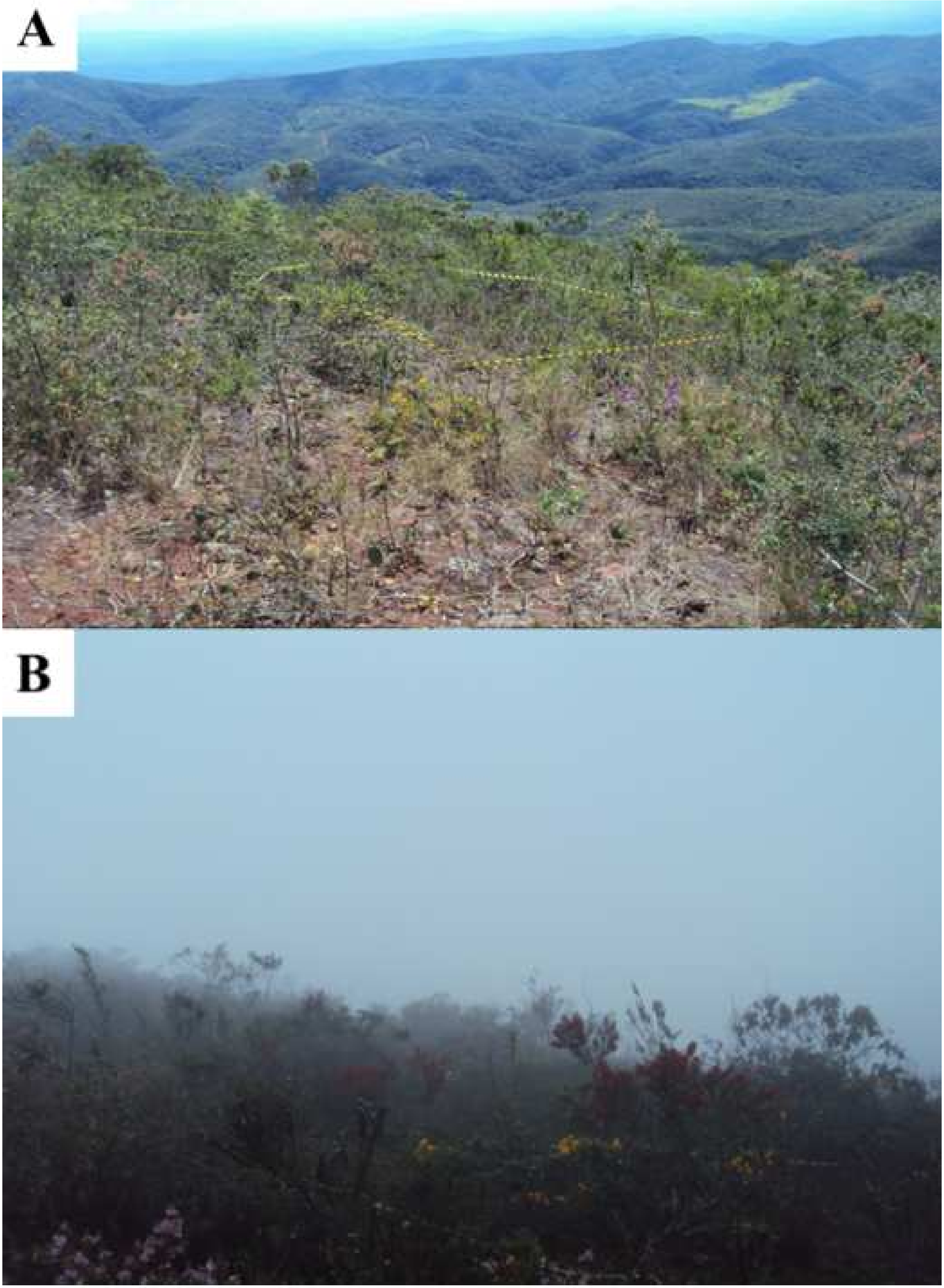
Figure legends

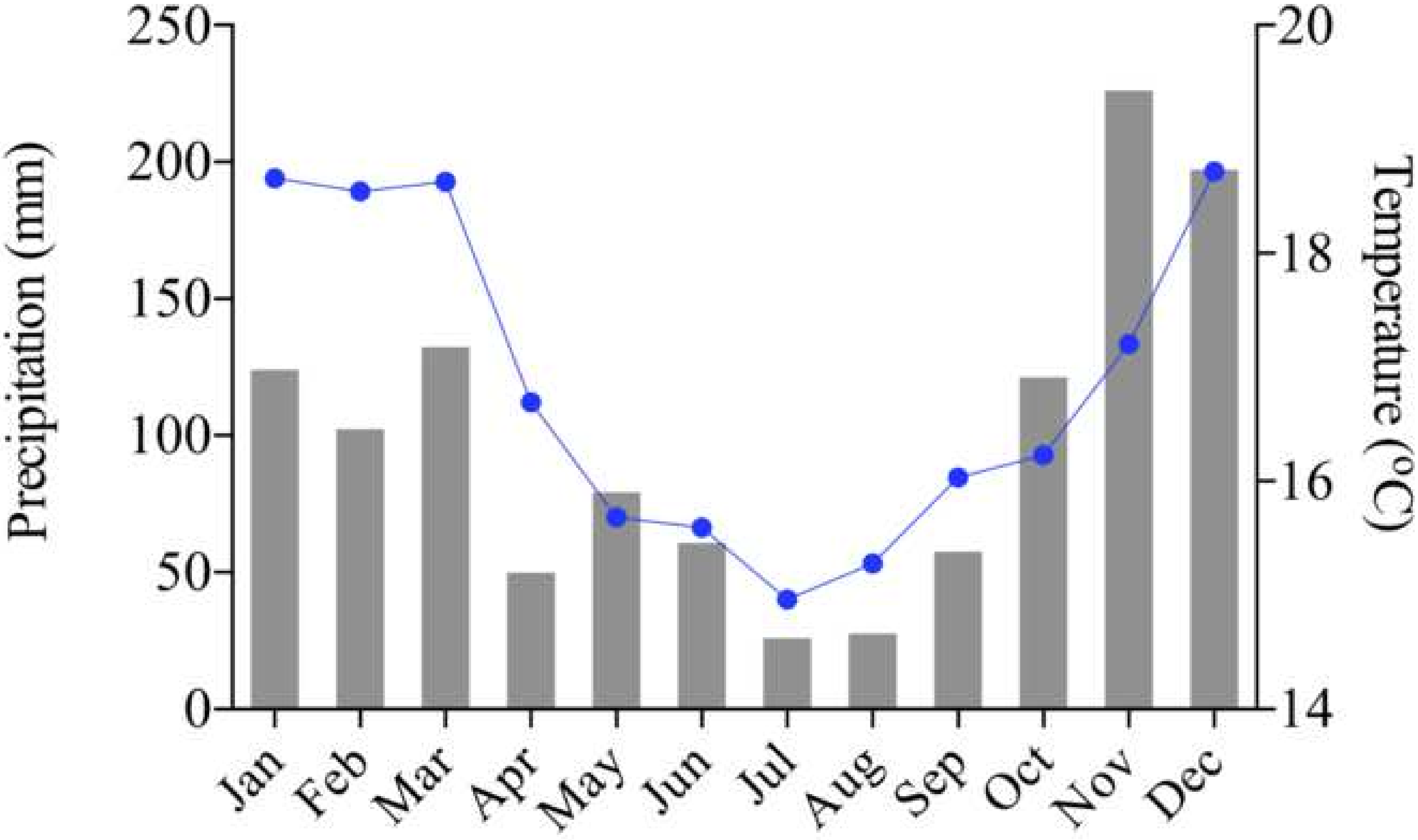
FIGURE 1. Ferruginous rupestrian field (A) without fog and (B) with fog.

FIGURE 2. The average annual rainfall and average annual temperature from "Serra da Brígida". Bars represent total precipitation volume (mm) and the line represents monthly average.

FIGURE 3. Gas exchanges in leaves of *Eremanthus erythropappus*, *Miconia corallina*, *Leandra australis* and *Byrsonima variabilis* in the rainy and dry seasons. Parameters that were significantly inflated by C_{max} and *k*. Net CO_2 assimilation (A), stomatal conductance (B) and transpiration (C).

FIGURE 4. Leaf water potential (Ψ_w in MPa) of the branches of the four species studied. Period: Predawn dry season without fog (PD d); Predawn dry season with fog (PD d + f), Predawn rainy season (PD r); Midday dry season without fog (MD d); Midday dry season with fog (MD d + f); Midday rainy season (MD r).


A
B


Precipitation (mm)
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Temperature (°C)
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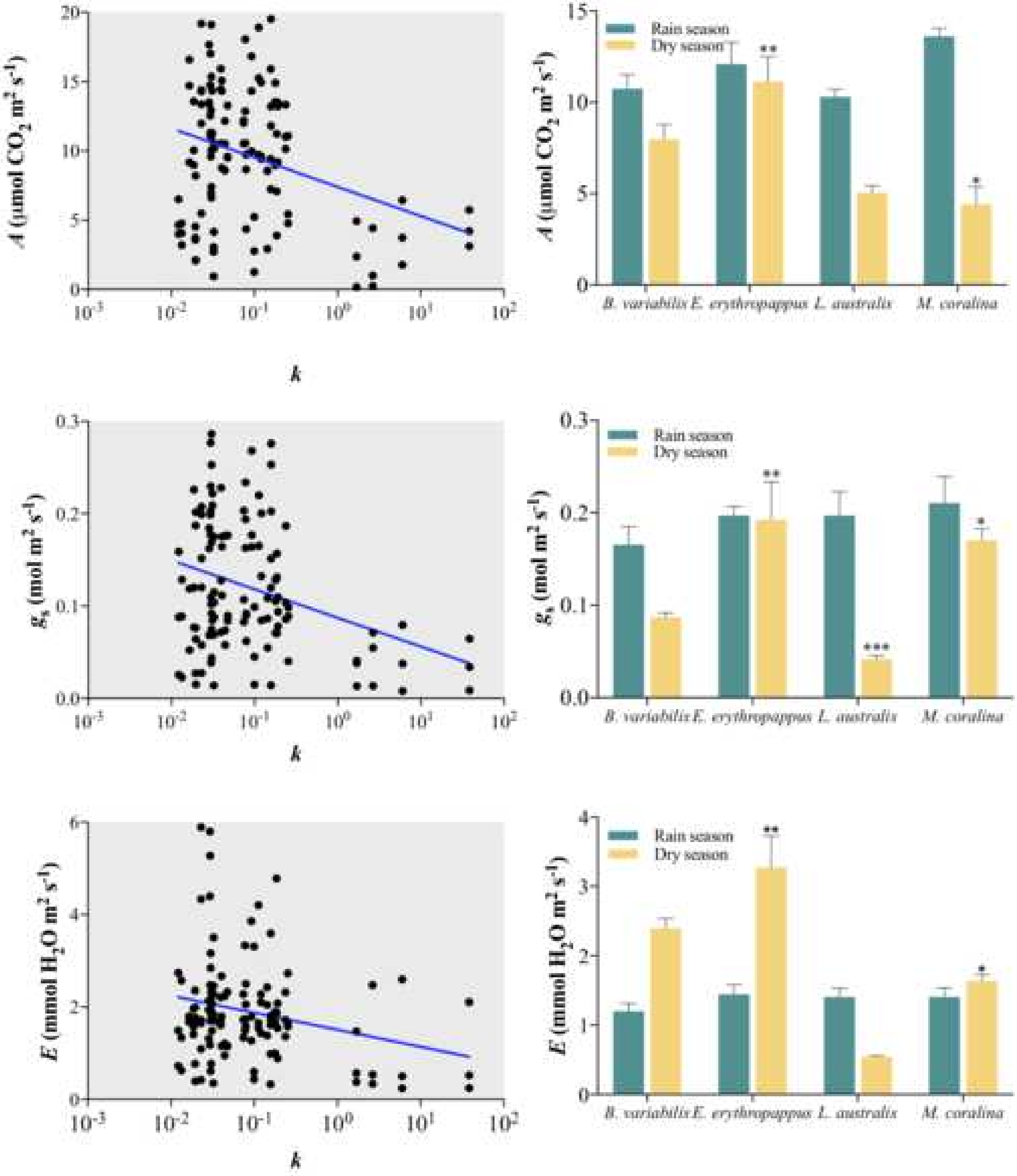
Figure 3


A (μmol CO$_2$ m^2 s^{-1})
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Rain season
Dry season
**
*
B. variabilis
E. erythropappus
L. australis
M. coralina
g_s (mol m^2 s^{-1})
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Rain season
Dry season
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***
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E (mmol H$_2$O m^2 s^{-1})
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4
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Rain season
Dry season
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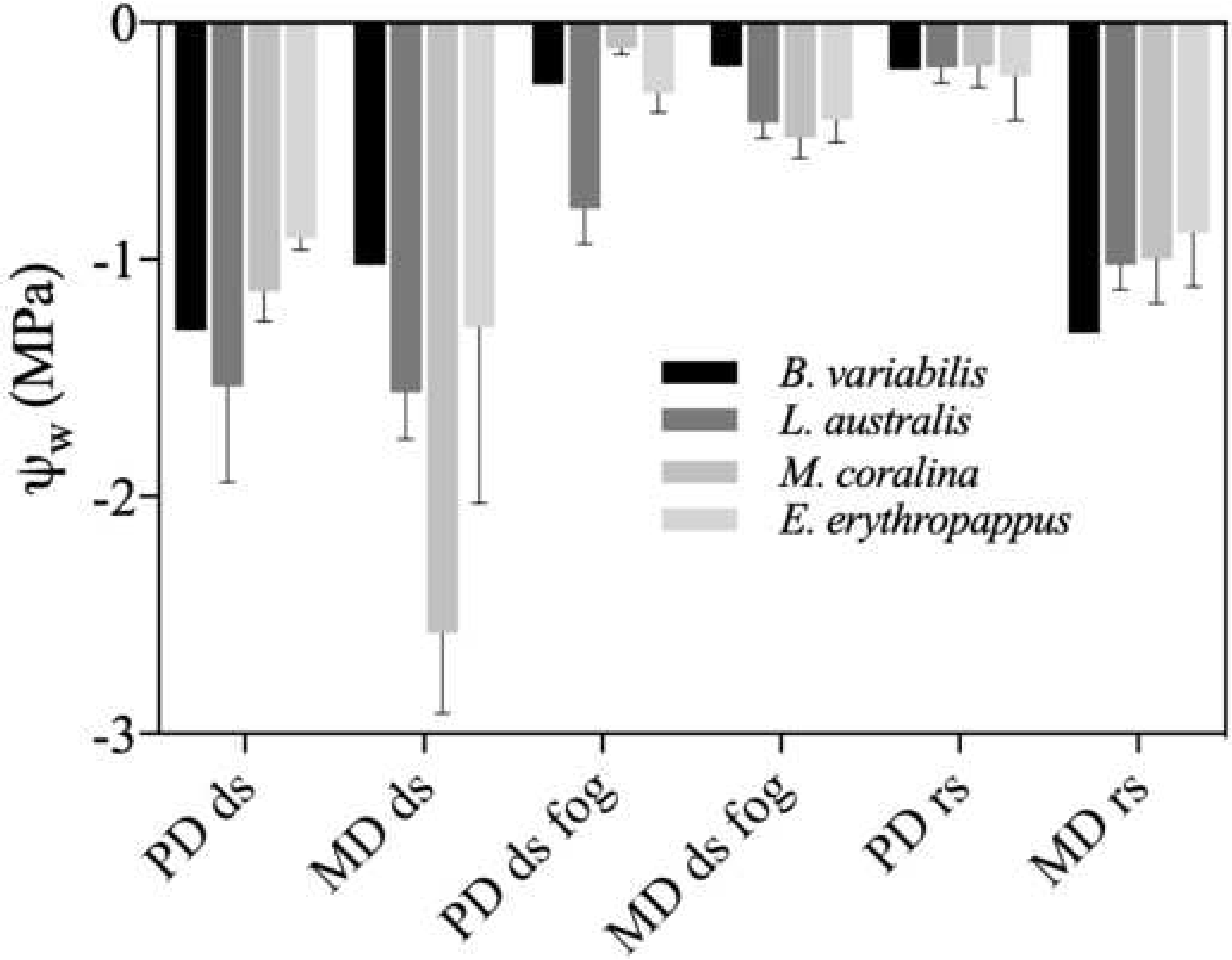
*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ψw (MPa)
B. variabilis
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M. coralina
E. erythropappus
PD ds
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PD rs
MD rs

Chapter 4

Foliar water uptake is an important trait related to thermotolerance responses in plants from ferruginous rupestrian fields

Chapter "under review" in Ecosystem

Photosynthetic heat tolerance in plants with different foliar water uptake strategies

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AUTHORS' CONTRIBUTIONS

D.B., J.P.L.F., R.M.S.I. and M.G.C.F. designed the research, D.B. collected the data for the research, and D.B. analyzed the data and D.B., J.P.L.F., R.M.S.I. and M.G.C.F wrote the manuscript. All the authors contributed critically to the drafts and gave final approval for publication.

Photosynthetic heat tolerance in plants with different foliar water uptake strategies

Abstract

1. The Campo rupestre is an old climatically buffered infertile landscape (OCBIL) and we have previously characterized in this protected ecosystem different strategies for foliar water uptake (FWU) as follows: quickly and lower water absorption (QL) or slower and higher water absorption (SH).
2. For the first time the relationship between FWU, photoinhibition and thermotolerance was investigated in seven species from this ecosystem.
3. The SH species are those that presented less photoinhibition. Contrastingly, the QL species presented greater thermotolerance when their leaves were totally hydrated. However, when there is greater leaf dehydration, the most thermotolerant species were those with SH strategy.
4. Results indicated that FWU is an important plant trait from this ecosystem to face excessive light intensity and higher temperatures. These plants may be differentially affected by future global warming and it was observed that the best strategy to deal with this expected climate change is the SH strategy.

KEYWORDS

Brazilian OCBIL, climate change, fog, foliar water uptake, heat tolerance, photosynthesis

1 | INTRODUCTION

The occurrence of fog is observed in the mountains of Minas Gerais, Brazil due to the high altitude characteristic of Tropical montane cloud Forests (Jarvis & Mulligan, 2011). This meteorological phenomenon plays an important role in the water balance of plants from Campo rupestre. In these tropical highland environments, the water balance is favored by fog episodes. The fog is intercepted by the canopy toward leaf water absorption, which is also favored by reduced evapotranspiration conditions (Aparecido et al., 2018). This is the case of the vegetation of the rupestrian fields, which also have shallow soils and rocky outcrops (Silveira et al., 2016), and are frequently subjected to high light incidence on sunny days. In addition, the high air vapor pressure deficit often results in restriction of transpiration due to the decrease of stomatal conductance. These overall conditions result in thermal stress by the increasing leaf temperature as described for species from the montane Atlantic forest of southeastern Brazil (Fauset et al., 2017).

Temperature is a strong driver of plant species distribution and even survival (Marias et al., 2016). Likewise, the increase of mean global temperature (IPCC, 2014) can cause thermal stress and induce a significant constraint for vegetation survival (Wahid et al., 2007). High temperatures can threaten the survival of sensitive species, resulting in local extinctions, large scale changes and altered vegetation composition (Taylor et al., 2019). Unfortunately, global warming may, by the end of this century, increase the temperature in the tropics resulting in a higher frequency and intensity of thermal stress in plants, and similarly compromise the hydric regime through the decrease of precipitations events, such as rain and fog (Corlett, 2011; IPCC, 2014). Therefore, this heating can change the structure, function, services and earth-atmosphere interactions in the ecosystems (Sastry & Barua, 2017). However, these high-temperature impacts on the physiological performance of plants are not fully understood (Marias et al., 2016).

Mountain species grown in the Campo rupestre (Jacobi et al., 2007) are the most susceptible to global climate change (Colwell et al., 2008). This tropical environment is considered an OCBIL for it is an old, climatically buffered, infertile landscape (Hopper, 2009). The progressive increase in mean atmospheric temperature will force the species to move to areas beyond their current distribution (Corlett & Westcott, 2013). However, such movement is improbable for several species have limited dispersion capacity in this environment (Colwell et al., 2008; Silveira et al., 2016). In this sense, global warming is a major threat for the survival of species that already suffer great anthropic pressure due to iron mining activity (Martinelli & Moraes, 2013).

Above a certain thermal limit, temperature increases result in damage to the photosynthetic apparatus, and a consequent decrease in photosynthetic rates (Berry & Bjorkman, 1980). The photosystem II (PSII) and the oxygen-evolving complexes are sensitive to stress factors, such as light excess and extreme temperatures (O'Sullivan et al., 2016). The temperature limits and the consequence of temperature increasing for tropical species are poorly known. Tropical species are subjected to interactions with several environmental constraints, and may present variations in the tolerance of the photosynthetic apparatus to progressive temperature increasing and light excess (Sastry & Barua, 2017). PSII tolerance to high temperatures can be enhanced by drought (Havaux, 1992; Godoy et al., 2011). Anyway, an increase in heat tolerance, even in a few degrees, can be extremely important for plant survival regarding the intensification of heat waves and drought episodes resulting from predicted climate change.

In addition, rupestrian field plants are exposed to high light intensity and under excess light conditions; non-photochemical thermal dissipation provides an energy imbalance between light absorption and utilization, minimizing the possibility of photooxidative damage (Demmig-Adams & Adams, 2006). The damage to the PSII under excessive light has been reported for some species in the rupestrian fields (Luttge et al., 2007; Morales et al., 2014; Castro et al., 2016).

Foliar water uptake (FWU) has been studied in the last decades (Berry et al., 2019) and this physiological process is characterized by the water entrance, whether in its liquid form or vapor through the leaf surface (Burgess & Dawson, 2004). The ecological role of this small but significant water intake has been studied (Berry et al., 2019). The FWU can improve the hydric status of the plant through increase of leaf water potential, decrease stomatal conductance and improvement of the photosynthesis capacity and water use efficiency (Burgess & Dawson, 2004; Eller et al., 2016; Vesala et al., 2017; Berry et al., 2019). It was observed that 11 species of the ferruginous rupestrian field have FWU capacity, but with different strategies of water influx. Some species absorb water rapidly in smaller amounts and other species absorb water slowly in greater amounts (Boanares et al., 2018 a, b).

Hypothetically, PSII heat tolerance can differ among the species with different FWU strategies, due to the increase in the water content in leaves, which allows a greater loss of water through transpiration providing the cooling of these leaves. According to the theory OCBIL, past climate stability in the Campo rupestre may have favored the existence of several areas of endemism and refuge (Collevatti et al., 2009; Bonatelli et al., 2014; Barbosa et al., 2015) and the temperature increase will represent a great challenge for the species of this environment. Therefore, understanding the relationship between FWU and thermotolerance and high light intensity is essential to assess the vulnerability of these species in the global climate change scenario (Wang et al., 2012; Sastry & Barua, 2017) and this is not yet investigated.

We investigate if species with differential FWU strategies also have differential PSII tolerance to heat, and if they respond differently to excessive light. Thus, we aim to verify if there is a better FWU strategy related to the increase of the mean temperatures expected due to climate change for studied species. We believe that species, which absorb a greater amount of water but slowly, present greater photosynthetic efficiency and thermotolerance than those species with rapid absorption, but with lesser amount of water.

2 | MATERIALS AND METHODS

2.1 | Plant material

This study was carried out in a Campo rupestre, a Brazilian altitudinal ecosystem (20° 21′30″S/43°30′11″W). The area has an average altitude of 1,492 m a.s.l., and fog is frequent throughout the year both during day night. Local vegetation is present on shallow soils on rock outcrops of quartzite, sandstone or ironstone with iron formation such as itabirites and *cangas* and is characterized by herbs, shrubs and treelets (Valim et al., 2013). Data were obtained in March 2019, duration the transition from rainy to dry season, and the average annual temperature was 16.9°C with a total annual rainfall of 1,204.7 mm.

The studied species were: *Leandra australis* (Cham.) Cogn. (Shrub), *Trembleya laniflora* (D. Don) Cogn. (Shrub), *Pleroma heteromallum* (D. Don) D. Don. (Shrub), *Miconia corallina* Spring. (tree) (Melastomataceae), *Eremanthus erythropappus* (DC.) MacLeish, (Asteraceae) (tree), *Byrsonima variabilis* A. Juss., (Malpighiaceae) (shrub) and *Ocotea pulchella* (Nees and Mart.) Mez, (Lauraceae) (tree). All species are native and endemic to Brazil, except *Leandra australis* and *Ocotea pulchella* that were not endemic to Brazil. *T. laniflora* is the species with the most restricted distribution, followed by *B. variabilis*. Both occur only in the Campo rupestre. Contrastingly, *Pleroma heteromallum, Eremanthus erythropappus* and *Ocotea pulchella* have the widest distribution among the species studied. These species were selected according to their different water absorption capacities (Boanares et al., 2018b). *Leandra australis*, *Ocotea pulchella* and *Byrsonima variabilis* have a higher speed of leaf water absorption, but uptake a small amount of water (Figure 1).

2.2 | Foliar water uptake measurement

To evaluate the different capacities of FWU, the following procedure was performed: first, mature and intact leaves (n = 4) of 5 individuals were collected in the field, and in laboratory conditions the leaves were immersed in distilled water and weighed during 4 h, every 15 min for 2 h. Then every 30 min for 2 h and 1 h to finish the experiment to obtain the maximum water absorption curve. Before each weighing, leaves were dried with ultra-absorbent microfiber synthetic towels to remove the surface water (Liang et al., 2009). Leaf water uptake was calculated by a differential equation, which provides data on the maximum water content in leaf lamina (C_{max}) and leaf uptake speed (k) (Liang et al., 2009). From a statistical analysis two opposite strategies were observed: species with higher C_{max} presented lower k and species with low C_{max} presented higher k (Boanares et al., 2018a, b).

2.3 | Leaf dehydration measurement

To assess the leaf dehydration capacity of these species, mature leaves (n = 4) of 5 individuals were totally submerged in distilled water, room temperature, for 4 h to full hydration after the petiole were sealed. These leaves were dried with ultra-absorbent microfiber synthetic towels to remove the surface water (Liang et al., 2009), weighed with an electronic scale, every 30 min in the first 6 h, and then they were weighed two more times after 12 h and 24 h. The leaves were kept in the light throughout the experiment. The total amount of water leaf lost (C_{max}^{L}) and water leaf lost speed (k^{L}) were defined from a differential equation according to Liang et al. (2009).

2.4 | Photosynthetic efficiency evaluation

In order to observe the occurrence of photoinhibition, measurements of the photosynthetic efficiency were performed three times a day, at 9 am, 3 pm and 6 pm, using a modulated fluorescence meter (MINI-PAM Waltz, Germany) in leaves of 5 individuals in the field. The average temperature ranged from 18º C to 32.5ºC, the humidity was 36% to 83%, and the maximum radiation was 3101 kJ/m^2. After adaptation of leaf lamina to the dark for 30 min the potential quantum yield (Fv/Fm) of photosystem II (PSII) was evaluated (Rascher et al., 2008). Subsequently, to calculate the *Fv/Fm* (Fv = Fm-F0), the Fv (variable fluorescence) was measured in leaves adapted to the dark, using leaf clips provided by the manufacturer, and Fm (maximum fluorescence) was measured in leaves adapted to the dark after a pulse of saturating light, and F0 is the basal fluorescence produced by leaves adapted to the dark.

2.5 | Photosystem II thermotolerance assessment

Leaf disks (2 cm^2) from 4 leaves from each species were collected at 9 am and also at 1 pm, totalling 10 discs per species for each sampling time, were used to evaluate heat tolerance immediately after the sampling. The thermotolerance assessment was conducted using a thermostatic bath. The leaf discs were placed on a thermally controlled plate, and covered with plastic film to avoid contact of the discs with water of thermostatic bath. To evaluate thermotolerance at different levels of leaf hydration, measurements were made on new obtained disks with 100%, 80% and 60% of the full foliar hydration. Leaf discs were kept on a workbench in order to obtain the hydration levels of 60% and 80%, weighted on analytical scale and considering the fully hydrated disc weights. Leaf discs were subjected to a progressive temperature increase from 25°C to 65°C, with gradual increases of 2°C every 3 min in the dark. The values of *Fv/Fm* were obtained in intervals of 2°C. The *Fv/Fm* decay temperatures at 50% (T_{50}) values were calculated from a sigmoidal equation adjustment between the *Fv/Fm* data at each temperature increase (Chaves et al., 2015).

2.6 | Data analysis

General linear models (GLMs) with gaussian errors were built to evaluate if the maximum water content in leaves, the water uptake speed and time of the day (9 am or 3 pm) (independent variables) influenced the potential quantum yield, and T50 (dependent variables) of the seven species. GLM were built to evaluate if there was a relation between the water parameter efflux (C_{max}^L and k^L) with thermotolerance data (T_{50}) and FWU parameters (C_{max} and k). To verify if different levels of hydration interfere in thermotolerance, a GLM was built, including levels of hydration (independent variable) and T_{50} (dependent variable). All analyses were performed using the R platform (R Core Team, 2018).

3 | RESULTS

3.1 | Leaf dehydration measurements

There was a negative relation between the amount of water lost by the leaves (C_{max}^L) and the foliar water absorption speed (k). The species with higher values of k are those that lose less water (C_{max}^L). Contrastingly, species that absorb a higher amount of water (C_{max}) lose water by the leaves more quickly (k^L) (Figure 2). The foliar water loss parameters influenced thermotolerance responses of all studied species. The most thermotolerant species were those that lost water more slowly and in smaller quantity, i.e., lower values of C_{max}^L and k^L. These species, *L. australis*, *O. pulchella* and *B. variabilis*, were represented by the lower values of k^L (Table 1). However, these species that absorb water faster (high k) and lose less water through their leaves (low C_{max}^L) are not the most resistant to dehydration. That is, with the decrease of their foliar hydration, these species reduced the thermotolerance responses.

3.2 | Photoinhibition evaluation

There was a significant relation between C_{max} and time of day with *Fv/Fm* values and species ($F_{1,82}$=22.7, $F_{2,79}$=14.6 and $F_{6,73}$=8,06, respectively; $P \leq 0.001$). The species with higher values of *Fv/Fm* were those that have the strategy of higher leaf water absorption and lower water absorption speed (high C_{max} and low k): *E. erythropappus*, *T. laniflora* and *P. heteromallum*. Contrastingly, the species with lower *Fv/Fm* values, *L. australis* and *B. variabilis*, presented opposite strategies, that is low C_{max} and high k. However, this second group of species includes *M. corallina,* which presents the FWU strategy similar to that of the first group of species (Figure 3). Importantly, *O. pulchella* that has the lowest value of C_{max}, also showed the lowest value of *Fv/Fm*. The species in which photoinhibition was detected (Fv/Fm values lower than 0.8) are those related to the strategy of higher speed (k) and a smaller amount of water absorbed (C_{max}) (Figure 3).

Regarding the different times of the day when *Fv/Fm* was measured, at 3 pm the lowest values of *Fv/Fm* were observed and, consequently, photoinhibition occurred, at least in RL species. On the other hand, the highest *Fv/Fm* values occurred at 6 pm, when there was solar radiation and lower temperature - environmental conditions which enable the occurrence of PSII recovery - in fact, *Fv/Fm* values at 9 am showed intermediate values.

3.3 | PSII thermotolerance assessment

The two parameters used to estimate FWU (C_{max} and *k*) and plant species were significantly related to T_{50} ($F_{1,33}$=21.04; $P \leq 0.001$, $F_{1,32}$= 4.13; $P \leq 0.05$ and $F_{6,26}$=5,03; $P \leq 0.01$, respectively). Species with higher values of T_{50}, that is, the most thermotolerant ones, were the species with higher foliar absorption speed (high *k*) and lower values of C_{max}, i.e, *B. variabilis*, *L. australis* and *O. pulchela* obtained at 46ºC. The parameters with opposite traits related to FWU distinguished the species according to the thermotolerance capacity. The less tolerant species to temperature increase were those that had the strategy to absorb water slowly by the leaves but in greater quantity: *E. erythropappus*, *T. laniflora*, *M. corallina* and *P. heteromallum* obtained at 44ºC (high C_{max}) (Figure 4).

Considering leaves hydration levels, there was a significant relation between the hydration levels ($F_{2,207}$=32.48; $P \leq 0.001$) and the different species ($F_{6,201}$=3.81; $P \leq 0.01$) in relation to T_{50} values (Figure 4). A contrary standard for thermotolerance results was observed, i.e., the most thermotolerant species to the decrease of dehydration were the species that have the strategy of absorbing a larger quantity of water but more slowly (high C_{max} and low *k*): *M. corallina, T. laniflora* and *P. heteromallum* obtained at 45ºC. The less thermotolerant species with the increase of dehydration were those of the opposite strategy with high *k* and low C_{max}: *L. australis*, *O. pulchella* and *B. variabilis* obtained at 43°C, except *E. erythropappus* that despite presenting high C_{max} remained in this group (Figure 5).

4 | DISCUSSION

4.1 | Water influx and efflux in leaves

The different FWU traits influenced the photosynthetic efficiency and thermotolerance of the species studied. Although the relation between leaf hydration levels with photosynthesis and thermotolerance was expected, the relation between leaf hydration from FWU with these parameters was not known. An observed pattern was that the species that absorb the most amount of water through the leaves (high C_{max}), but in a slower speed (low k) lose water more quickly (high k^L). Contrastingly, species that absorb less water (low C_{max}) more quickly (high k) lose less water (low C_{max}^L). These different water losses strategies are the cause of a lower value of relative water content (RWC) for the species with high C_{max} and high k^L, and higher values of RWC for species with high k and low C_{max}^L (Boanares et al., 2018b). However, the leaf ultrastructural and anatomical traits that grant these distinct FWU behaviors are not the same for water loss (Riederer & Schreiber, 2001). The cell wall composition with different proportions of cellulose and pectins that allow these different FWU strategies (Boanares et al., 2018b) does not influence the different water exit trait. Moreover, the total amount of water absorbed by the leaf (C_{max}) should also depend on a different set of traits, such as the leaf hydraulic capacitance (McCulloh et al., 2014; Berry et al., 2019).

Probably there is a compensation mechanism between FWU traits and water efflux. Species that absorb more water lose water faster, consequently, these leaf species need to absorb a greater amount of water. However, leaves that absorb water quickly cannot absorb a large amount of water due to their anatomical traits. Their anatomical structure is one of the causes of this restriction because their leaves have less space for water storage (Boanares et al., 2018a). On the other hand, they lose less water, compensating the limitation to absorb a greater amount of water (low C_{max}). However, even though an individual leaf can eventually stop absorbing water because its internal water storage capacity is saturated, in a whole-plant context this water would have been redistributed to the other tissues, and the leaf would be able to keep absorbing water (Burgess & Dawson, 2004; Eller et al., 2013).

4.2 | Photosynthetic efficiency

In turn, the species with the high C_{max} trait are more efficient photosynthetically than the species that absorb water faster (high k), due to higher values of water potential induced by fog (Boanares et al., 2019). Moreover, the late afternoon recovery of *Fv/Fm* values and the absence of photoinhibition are indicative of the effectiveness of mechanisms that dissipate the excess light of these plants (Castro et al., 2016). These species have anatomical traits, which offer more possibilities of water storage in their leaves, and this may protect the photosynthetic apparatus, since a higher amount of water plays a key role in this process. The species with rapid absorption (higher k) and low amount water absorption (low C_{max}) present anatomical structure that is one of the causes of this restriction, because their leaves have less space for water storage (Boanares et al., 2018a). On the other hand, they lose less water, compensating the limitation to absorb a greater amount of water (low C_{max}). Moreover, this opposite pattern interfered in the physiological parameters analyzed. The species that absorb rapidly and lose little water are not efficient enough photosynthetically, since photoinhibition was observed during a more intense irradiation period of the day. The lower values of CO_2 assimilation of the species with lower *k* corroborate these results (Boanares et al., 2019). This behavior occurs because the CO_2 suppression fixation may increase the extent of PSII photoinhibition, inhibiting the synthesis of repair proteins (Murata et al., 2007). The different strategies of water absorption are closely linked to the strategies of drought tolerance of these species. The slow absorption species can be closely related to the desiccation tolerance strategy, protecting the proteins linked to the photosynthesis (Challabathula et al., 2018). Contrastingly, species with higher speed absorption may be related to the strategy of drought avoidance, reducing the possibility of dehydration of their tissues, because they cannot deal with low leaf hydration.

4.3 | Heat tolerance

Water contributes to higher heat dissipation by its higher specific heat capacity, granting a greater leaf thermotolerance (Fauset et al., 2018), therefore attenuating the daily thermal gradient and the highest irradiance flux. A greater amount of water in leaves may favor greater transpiration and therefore heat dissipation. In addition, there is a variation in the thermotolerance degree because of the different gene expressions of the proteins related to thermotolerance (Liao et al., 2016). The difference in this protein expression may be directly related to the water amount in leaves (Bechtold et al., 2013). The species with high C_{max} are not as thermotolerant as the other species with high *k*, when their leaves are kept fully hydrated. This relation is plausible since species with faster FWU benefit from total hydration in a more efficient way to face high temperatures. In fact, the species with higher *k* lose less water to the environment and induce higher leaf hydration levels although they have a slower water uptake. There are several repair mechanisms to PSII, such as cyclic electron flux around PSI, xanthophyll cycle, photorespiratory pathway, ROS elimination systems and thermal dissipation of absorbed light energy (Szymanska et al., 2017). The species with high C_{max} are not as thermotolerant as the species with high *k* when their leaves are fully hydrated. So, it is probable that these species prevent or reduce the chances of photoinhibition through a more efficient system for thermal dissipation. Thus, it is possible that even large increases both in the internal repair temperature and in the external temperature can occur due to high light intensity, which is beyond the levels these species usually encounter.

Although the species with high *k* are more thermotolerant, this advantage is limited, once it decreases with the increasing leaf dehydration. Plants under water deficit have the stomatal opening reduced to maintain more internal water and, consequently, there may occur an increase in leaf temperature (Sharkey, 2005). It is possible that these species will not be able to metabolic rearrange to the extent of maintaining their thermotolerance with a low amount of water in leaf tissues. This rearrangement would help in tolerating the lower thermotolerance level of these species, when they are less hydrated.

Thermal stress causes loss of plant water, severely damaging membrane structure and function (Tiwari et al., 2018). However, species whose strategy is high C_{max} and high k^{L} present a higher tolerance to water deficit, since these species had the highest values of T_{50} in the less hydrated leaves. Is known that plant thermotolerance is influenced by exposure to higher temperatures, reduced water availability and high light intensities increasing heat tolerance (Havaux et al., 1992; Sastry & Barua, 2017). One of the explanations would be a rapid induction of proteins sensitive to dehydration (Sastry & Barua, 2017). The expression of these proteins as well as the chaperones are directly related to better tolerance against other abiotic stresses such as thermal stress (Liu et al., 1998; Sakuma et al., 2002) which prevents protein denaturation and protects organisms under thermal stress (Parsell & Lindquist, 1993). Furthermore, when plants suffer from heat stress there is the activation of several proteins related to dehydration generating defense mechanisms. These proteins play a crucial role in regulating gene expression in response to this constraint (Takahashi & Badger, 2011). In this way, these species manage to cope better with lower water content in their foliar tissues being better adapted because they lose water more quickly. Therefore, these species must protect themselves from the inherent water deficit in their physiological traits of water loss and, consequently, are more adapted to a lesser leaf hydration.

However, it was verified in this work that the different strategies of FWU have no relation with the degree of distribution (more or less restricted) of the species. An example are the species *T. laniflora* and *B. variabilis*, both of which are restricted to the rock field environment and present distinct ecophysiological traits. The same occurred with other species in this work, that is, species with different FWU strategies coexisting, corroborating the consensus that FWU is not an exception, but a rule for the species, differentiating only in its magnitude (Berry et al., 2019).

5 | CONCLUSIONS

This study indicates that native species may be vulnerable and affected differently by future global warming. Considering this climate scenario, among the different strategies of FWU and thermotolerance, it is noticed that the best strategy to deal with this expected climate change is the one that grants higher C_{max} to plants. These species with higher C_{max} will be able to better perform photosynthetically, when they experience water deficit due to rainfall decrease. These strategies will help finding out species more adapted to the future climate scenarios, and consequently indicated for revegetation of this ecosystem, which is already impacted, and it will also help to predict the possible changes in the landscape and which species will suffer the most.

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Figure captions

FIGURE 1 Relationship between water absorption speed (*k*) and maximum water content (C_{max}) $P \leq 0.05$ (a) and the C_{max} values among species (b). In black are the species that present slower and higher water absorption and in gray are the species that present quickly and lower water absorption. Data represent mean values (±SE). Different letters indicate differences between species ($P \leq 0.05$).

FIGURE 2 Maximum water loss (C_{max}^{L}) and Water speed loss (k^{L}). In black are the species that present quickly and lower water absorption and in gray are the species that present slower and higher water absorption. Different letters indicate differences between species ($P \leq 0.05$).

FIGURE 3 Potential quantum yield (*Fv/Fm*) measured after 30 min of dark adaptation from leaves of the different species observed at three different times of the day: 9 am, 3 pm and 6 pm. Different letters indicate differences between species at the same time of day at $P<0.05$. Bars represent ± SE.

FIGURE 4 Relationship between the temperature of *Fv/Fm* 50% decline (T_{50}) with (a) water loss leaf content (C_{max}^{L}) (g g^{-1} dry weight) and (b) loss water speed (k^{L}). Linear regressions at $P<0.05$.

FIGURE 5 Thermotolerance in different species and different leaf hydration levels. Error bars represent ± SE. Thermotolerance measured as decay temperatures at 50% (T_{50}) of PSII.

TABLE 1 Water loss leaf content (C_{max}^{L}) (g g^{-1} dry weight), loss water speed (k^{L}) and temperature (°C) drop 50% (T_{50}) related to the FWU parameters of all seven species analyzed. Water leaf content (C_{max}) and water uptake speed (k).

Independent variable	Dependent variable	F	DF	Deviance	*p*-value	
C_{max}^{L}	C_{max}	1.1983	1	0.178	0.28809	
	k	7.7568	1	1.153	0.01222	*
	k^{L}	0.872	1	5.8696	0.02618	*
	Species	39.493	6	109.92	0.0001	***
k^{L}	C_{max}^{L}	1.5374	1	0.0001	0.2309	
	C_{max}	26.852	1	0.002	0.0001	***
	k	0.5504	1	0.0004	0.4677	
	Species	13.369	6	0.0002	0.0001	***
T_{50}	C_{max}^{L}	34.873	1	23.481	0.0001	***
	k^{L}	33.373	1	22.471	0.0001	***
	Species	4.3844	6	17.713	0.006	**

Data correspond to the statistical analysis of linear general model (GLM). *** Indicate $P \leq 0.001$; ** Indicate $P \leq 0.01$ and * Indicates $P \leq 0.05$.

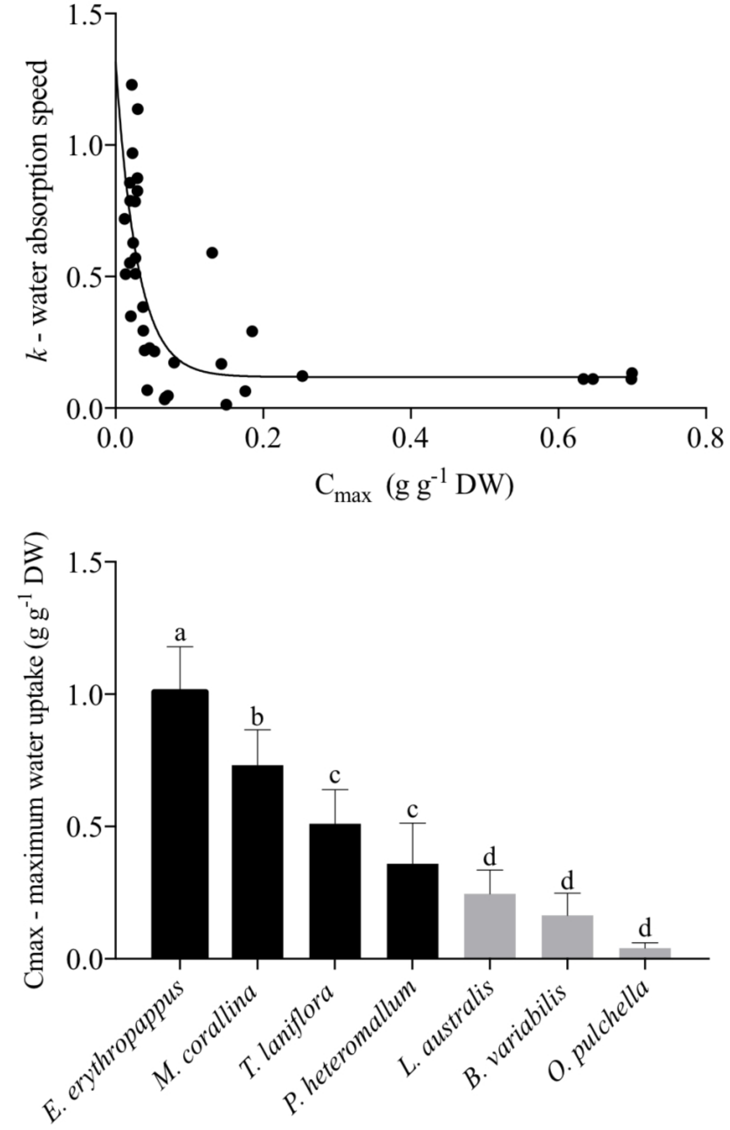

Figure 1

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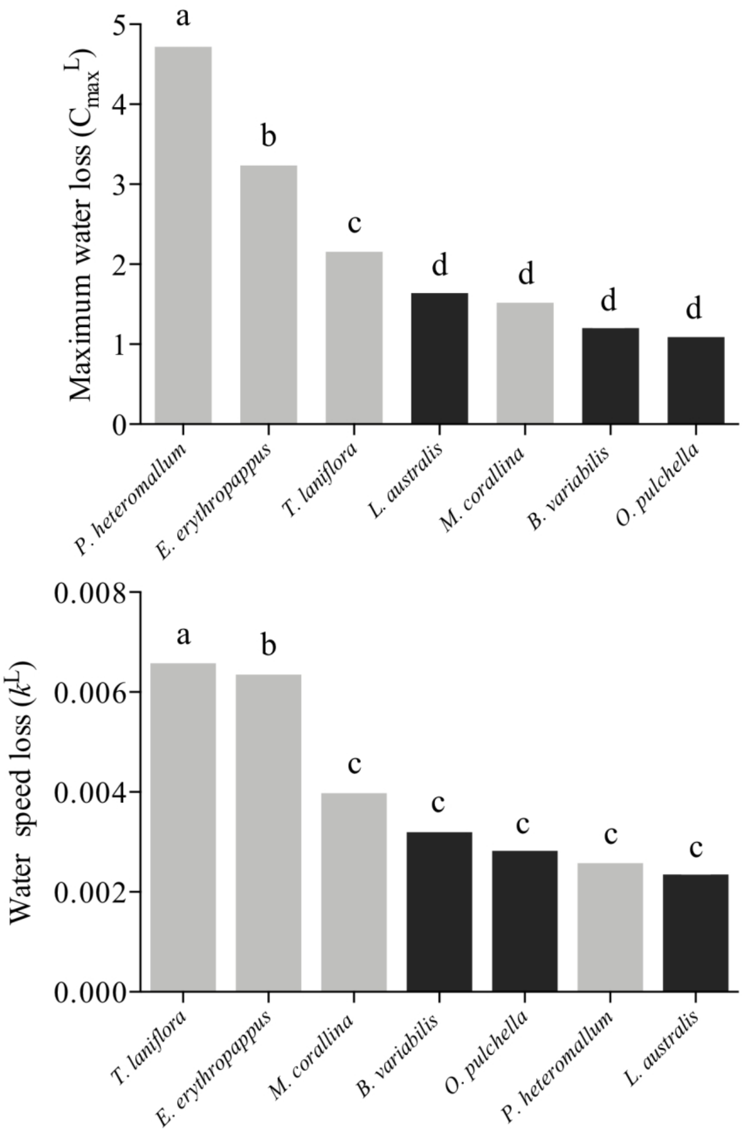

Figure 2

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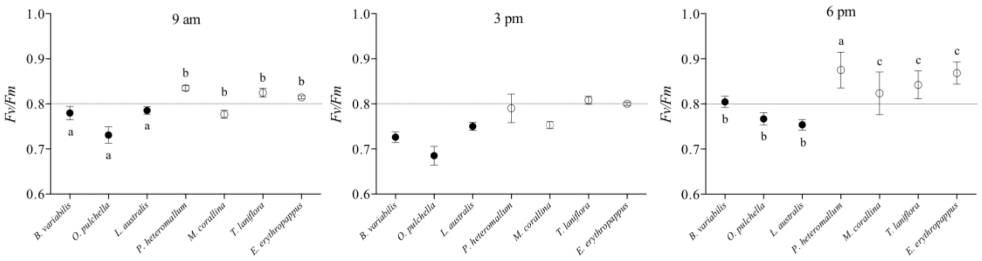

Figure 3

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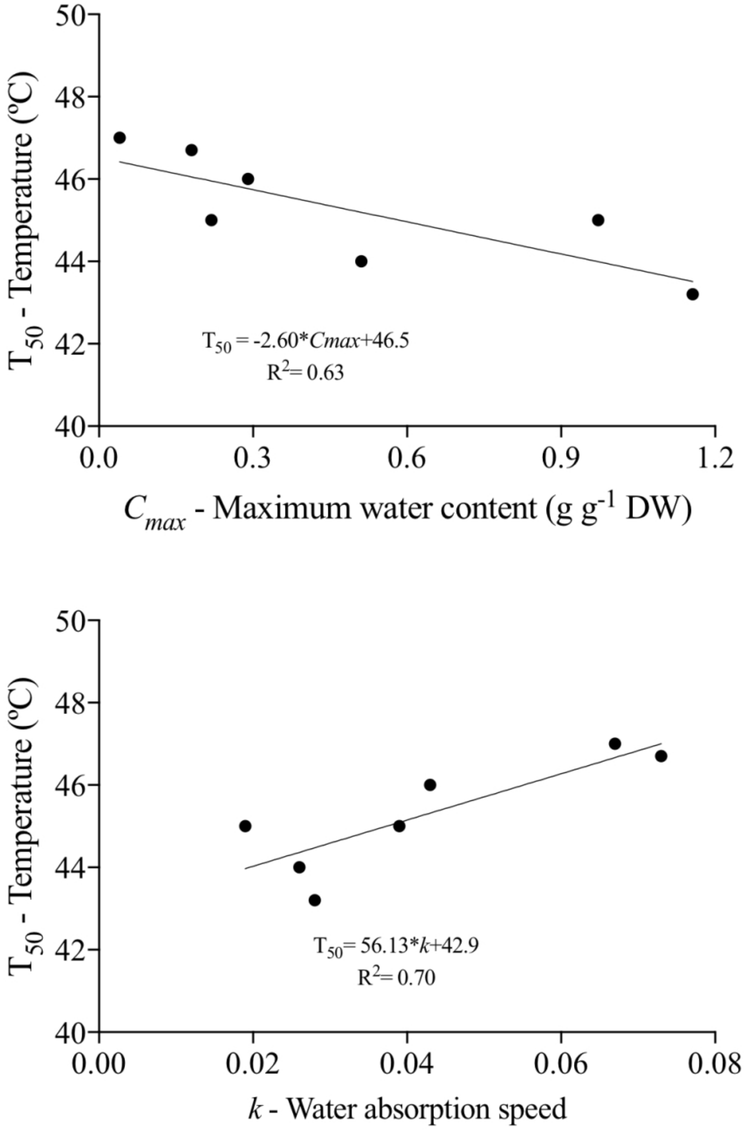

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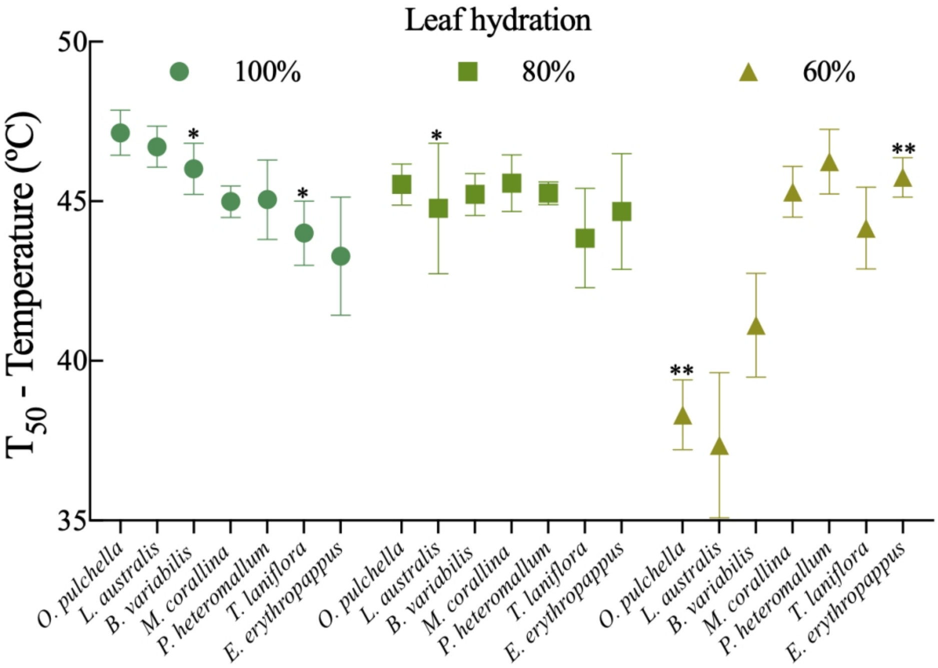

Figure 5

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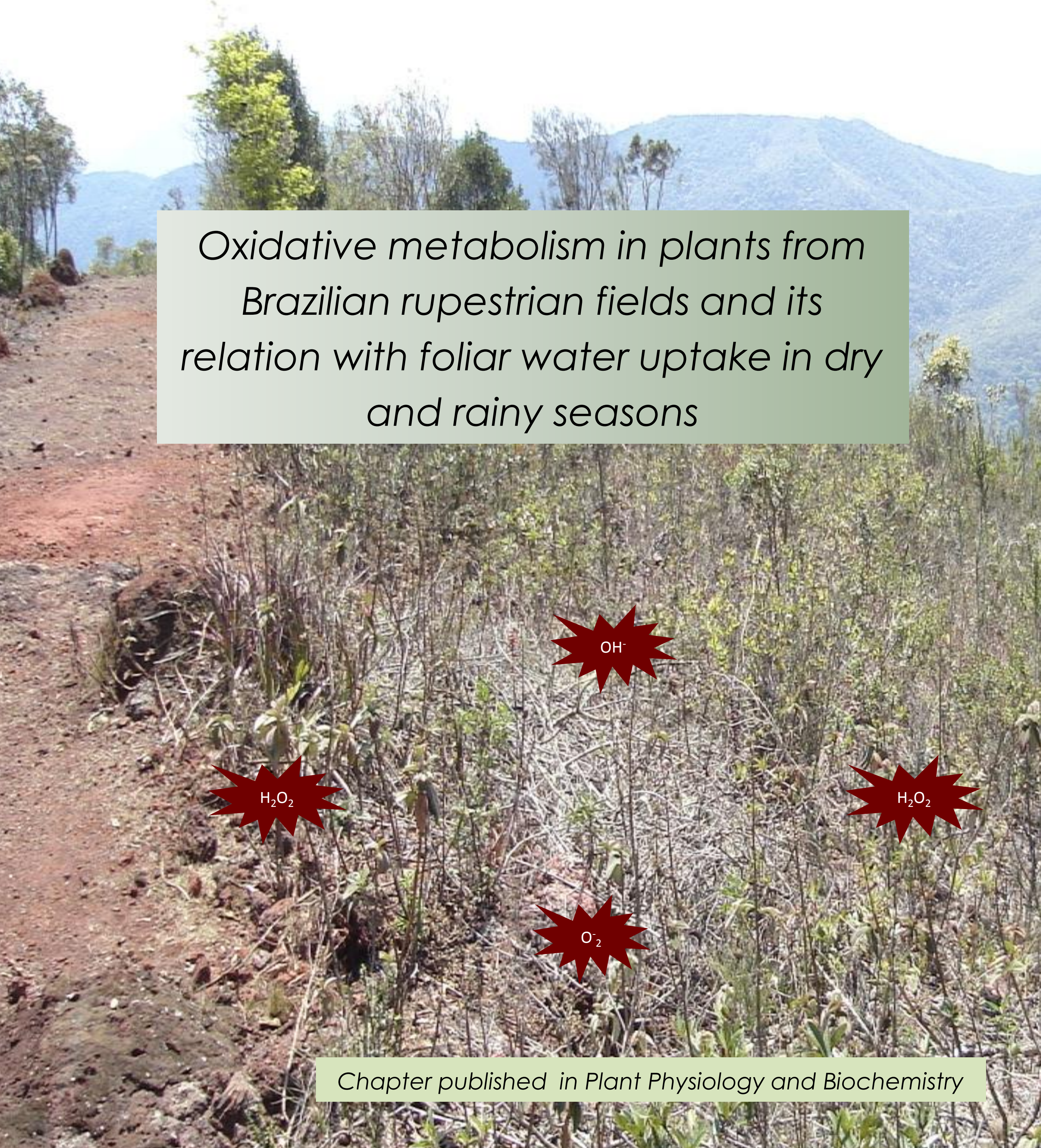
Chapter 5

Oxidative metabolism in plants from Brazilian rupestrian fields and its relation with foliar water uptake in dry and rainy seasons



Chapter published in Plant Physiology and Biochemistry

Oxidative metabolism in plants from Brazilian rupestrian fields and its relation with foliar water uptake in dry and rainy seasons

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ABSTRACT

In an altitudinal Brazilian ecosystem, fog is a frequent event in both the dry and rainy seasons. The drought stress is aggravated in the dry season due to elevated evaporative demand and this constraint can induce oxidative stress in plants. In this ecosystem, there are plants which present different foliar water uptake (FWU) capacities - species that absorb less water more quickly (LQ) and those that absorb more water more slowly (MS). In this study, the relationship between oxidative stress responses and the different FWU strategies was evaluated in dry and rainy seasons. The oxidative stress was assessed by H_2O_2 production and lipid peroxidation as well as by the antioxidant enzymes system as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX). During the dry season, plants had higher oxidative stress compared to rainy season plants which exhibited moderate oxidative damage. The FWU strategies were closely related to oxidative stress responses, since the LQ species presented the higher H_2O_2 content and oxidative defense system. Contrastingly, it was found that MS species have the lowest values of H_2O_2 and less SOD, CAT and APX activities. However, the lipid peroxidation did not present any relation with FWU strategies. Altogether results revealed that plants, which present MS strategy, are more adapted to cope with the higher H_2O_2 concentrations generated in the dry season and drought stress events than those that present LQ strategy.

Keywords:

Adaptive strategies

Drought stress

Reactive oxygen species

Cell signaling

1. Introduction

Because they are sessile, plants have developed several strategies to cope with the challenges (Mignolet-Spruyt et al., 2016) imposed by various biotic and abiotic constraints (Anjum et al., 2011). These different stresses are minimized by adaptive strategies that depend on the type, severity, and combination of environmental traits, and require specific and flexible combinations of signaling components to trigger adaptation and acclimatization responses (Suzuki et al., 2012, 2014). The reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion (O_2•-), the oxygen singlet ($1O_2$) and hydroxyl radical (HO•), which are formed mainly in chloroplast and mitochondria during the cellular oxidative process, are involved in the beginning and signaling regulation of the oxidative events (Baxter et al., 2014).

The excessive production of ROS can profoundly affect plant metabolism due to damage to cell membranes, proteins, and genetic material (Foyer and Noctor, 2013). To minimize oxidative stress, plants possess complex non-enzymatic and enzymatic antioxidant systems, that include superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) among others (Apel and Hirt, 2004). The cell signaling or oxidative damages occurrence is determined by balance between ROS and antioxidative enzyme activities (Moller et al., 2007). Previously, ROS were considered only negative byproducts for optimal cellular metabolism. Nevertheless, the role of these molecules has been better understood and has emerged as essential messengers involved in redox signaling (Mittler et al., 2011; Vaahtera et al., 2014).

Among the environmental constraints, drought is one of the most limiting factors to plant growth, establishment, and survival (Cruz de Carvalho, 2008). An ecosystem in which plants face very frequent water deficit mainly in the dry season is the Brazilian rupestrian fields. This ecosystem is characterized by a typically altitudinal tropical climate, with mainly two distinct seasons, a dry and a rainy one. However, because it is located in sites with an altitude above 1,000 m, fog is a frequent meteorological phenomenon (Bruijnzeel, 1990;

Baeta, 2012). Besides, this environment can be considered arid due to the high evaporative demand in the dry season without fog (Leuschner, 2000), emphasizing its importance to this ecosystem.

Moreover, many plants from this ecosystem can absorb vapor water or liquid water through the leaves (Boanares et al., 2018a). Foliar water uptake (FWU) occurs in response to the water gradient potential when water in the leaf surface diffuses into the internal tissues through the cuticle (Yates and Hutley, 1995; Gouvra and Grammatikopoulos, 2003), absorbent trichomes (Benzing et al., 1978) and through hydathodes (Martin and Willert, 2000). Thus, the FWU provides an ecological advantage for plants that occur in the environments that reveal water deficit, at least seasonally, playing an important role in their cell physiological process and plant growth (Limm et al., 2009; Eller et al., 2013).

The FWU capacity has already been verified in 11 species from this ecosystem and different FWU strategies have been revealed (Boanares et al., 2018a, b). There are plants which present different foliar water uptake FWU capacities - species that absorb less water more quickly (LQ) and those that absorb more water more slowly (MS) and coexist in this same ecosystem (Boanares at al., 2018a, b). Therefore, the hypotheses of this study are: 1) there is a relation between FWU capacity and oxidative stress/signaling response, mainly in the dry season when plants have to cope with water deficit; 2) that the different FWU strategies are related to this metabolic oxidative response.

2. Material and Methods

2.1. Site study

This study was carried out in a Brazilian ferruginous rupestrian field in the municipality of Ouro Preto (20° 21′30″S/43°30′11″W). The area has an average altitude of 1,492 meters above sea level, and fog is frequent and accounts for approximately 13% of the total precipitation during the year. However, in the dry season, it contributed 10-fold more than rainfall (Baeta, 2012). Local vegetation is present on shallow soils on rock outcrops of itabirites or *cangas* and is characterized by herbs, shrubs and treelets (Valim et al., 2013). Data were obtained in January and July 2018, characterized as the rainiest and the driest months of both seasons, respectively. The site, therefore, provides a possibility of drought stress for plants in the dry season. The average annual temperature was 16.9°C with the total annual rainfall of 1,204.7 mm (Fig. 1).

2.2. Plant material and FWU measurements

Leaves of *Pleroma heteromallum*, *Trembleya laniflora*, *Miconia australis* and *Miconia corallina* (Melastomataceae), *Ocotea pulchella* (Lauraceae) and *Byrsonima variabilis* (Malpighiaceae) were harvested to evaluate the FWU. Leaves (n = 4) were collected in the field and immediately weighed with a portable scale. After the leaves were taken to the laboratory, they were weighed once again. Then, leaves were immersed in distilled water and weighed every 15 min for 2 h, and every 30 min for 2 h. At last, leaves were immersed in water for 1 h and weighed to obtain the maximum water absorption curve. Before each weighing, leaves were then dried with ultra-absorbent microfiber synthetic towels to remove the surface water (Liang et al., 2009). FWU was calculated by a differential equation $\Delta C = \Delta C_{max}(1 - e^{-kt})$, where $\Delta C = (C - Ci)$ is the difference between the amount of water present in the leaves in an interval of 300 min, and $\Delta C_{max} = (Cs - Ci)$ the difference between the maximum amount of water after the end of the experiment, providing data on the maximum water content in leaf surface (C_{max}) and leaf uptake speed (*k*) (Liang et al., 2009).

2.3. H_2O_2 and lipid peroxidation evaluation

Frozen leaves of the six species studied (0.3 g) were ground into powder in mortar with liquid nitrogen and homogenized with 0.1 % (v/v) trichloroacetic acid (TCA). Then, plant samples were centrifuged at 13,000 × g at 4 ºC for 20 min and the supernatant was collected for further analysis of H_2O_2 and malondialdehyde (MDA). The amount of H_2O_2 was determined essentially as described by Velikova et al. (2000). In the reaction medium presenting 1 mM of phosphate buffer (pH 7.0) and 1 M potassium iodide, volumes of 300 mm^3 of supernatant were added. After, samples were incubated at 30 ºC for 30 min, absorbance was determined at 390 nm. The H_2O_2 concentrations were estimated based on a calibration curve.

The concentration of MDA was used as a parameter to evaluate lipid peroxidation. For this purpose, the supernatant was incubated in a reaction medium containing 0.5% thiobarbituric acid (w/v) and 10% TCA (w/v) at 90 ºC. The reaction was stopped with an ice bath for 10 min, 30 min later samples were centrifuged for 5 min at 13,000 × g. The absorbance was measured at 535 and 600 nm, and the MDA concentration was calculated as described by Cakmak and Horst (1991).

2.4. Activity of antioxidant enzymes evaluation

Frozen leaves of the six species studied (0.3 g) were ground in mortar with liquid nitrogen, and proteins were extracted with a 100 mM phosphate buffer (pH 7.8) containing 100 µM ethylenediaminetetraacetic acid, 20 mM ascorbic acid and 50% (w/w) polyvinylpolypyrrolidone. After centrifuging at 13,000 × g at 4 ^{o}C for 20 min, the supernatant was collected for further analysis of the superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) enzymes. The method of nitroblue tetrazolium (NBT) photoreduction (Giannopolitis and Ries, 1977) was used to evaluate SOD (EC 1.15.1.1) activity. One unit of SOD was defined as the amount of enzyme required to inhibit NBT reduction by 50%. To determine the CAT (EC 1.11.1.6) activity, plants extracts were incubated with 100 mM phosphate buffer (pH 7.0) and 12.5 mM H_2O_2 (Azevedo et al., 1998). For the enzyme activity determination, the molar extinction coefficient (ε) of = 39.4 M cm^{-1} from measurements of H_2O_2 degradation at 240 nm was used. To evaluate APX (EC 1.11.1.11) activity, 100 mM of phosphate buffer (pH 7.0), 0.5 mM ascorbic acid and 0.1 mM H_2O_2 was used (Nakano and Asada, 1981). The rate of ascorbic acid oxidation was monitored at 290 nm and the quantification was carried out using ε equal to 2.8 mM^{-1} cm^{-1}.

2.5. Data analyses

General linear mixed models (GLMMs) with gaussian errors were built to evaluate the influence of FWU capacity on oxidative response related to oxidative stress. The H_2O_2, MDA, SOD, APX, and CAT were used as fixed explanatory variables, and the response variables were maximum water content in leaf (C_{max}), the water uptake speed (*k*) and season. The identity of each measured plant was used as a random effect variable to control for potential pseudoreplication biases in our models (Hurlbert, 1984). For all models, distributions were tested through residual analysis. All analyses were performed using the R platform (R Core Team, 2015) using the function GLMER from package lme4 (Bates et al., 2015).

3. Results

There were significant differences among species analyzed according to the FWU strategies. Highest C_{max} and lowest *k* values were observed in *M. corallina*, *P. heteromallum* and *T. laniflora*. Contrastingly, *M. australis*, *B. variabilis* and *O. pulchella* presented a different pattern, that is, smaller values of C_{max} and higher values of k in dry season (Fig. 2) and rainy season (see Supplementary Fig. S1).

As expected, the highest values for all parameters of oxidative stress responses occurred in the dry season. The comparison between dry and rainy season data can be found in the Supplementary data (Fig. S2). However, H_2O_2, besides having a significant relationship among different plant species and seasons it is related to the two parameters of FWU - C_{max} and k (Table 1). This relation of FWU parameters is antagonistic since the increase of H_2O_2 was directly related to the amount of water absorbed (C_{max}) and inversely proportional to the rate of water absorption (k). In other words, the species with the highest values of C_{max} presented the highest H_2O_2 content and the species with higher values of k presented the lowest H_2O_2 content, mainly in the dry season (Fig. 3). These data corroborate the opposite pattern of the parameters C_{max} and k according to the statistical difference among species. Only *T. laniflora* that presents strategy with higher C_{max} value and lower k value, and *B. variabilis* that presents strategy with higher k value and lower C_{max} value did not fit the patterns of the different FWU strategies. However, the lipid peroxidation estimated by MDA values had no significant relation with any FWU parameters (C_{max} and k). This relation of lipid peroxidation was only found between species and seasons (Table 1). This non-relation with MDA and FWU was observed in the significant differences among species in which there was no clear separation between the FWU strategies, that is, species *M. corallina*, *P. heteromallum* and *T. laniflora* that have different FWU strategy than *B. variabilis* and *O. pulchella* showed no significant differences for lipid peroxidation (Fig. 3). However, the antioxidant enzymes SOD, APX and CAT analyzed were significantly related to C_{max}, plant species and season (Table 1). These enzymes had a positive relationship with C_{max} mainly in the dry season (Fig. 4). APX and CAT were the enzymes that best separated the species according to FWU strategies.

4. Discussion

It is widely known that oxidative metabolism, which comprises the production of ROS and the antioxidant response, is closely linked to the plant water status, as well as plant species and season. Also, we know that the ability of plants to absorb water through leaves is an adaptive advantage, because despite a small amount of water entry, this water is enough to enable rapid leaf hydration, which is the primary organ for photosynthesis and gas exchanges. Although the plant response to oxidative stress depends on the type of ROS molecule and its concentration, but also the site of production and interaction with other molecules (Moller et al., 2007), it was shown that the different strategies of FWU reflect in distinct H_2O_2 production. Among the different ROS molecules, H_2O_2 was analyzed for being relatively stable and present at higher levels of concentration concerning the other ROS molecules, being, therefore, the most representative molecule (Moller et al., 2007). In addition, H_2O_2 has the ability to cross membranes, possibly through aquaporins (Bienert et al., 2007), and is the main secondary messenger acting on a signal transduction pathway for stress response (Mittler, 2002; Vranova et al., 2002; Miller and Mittler, 2006). Thus, their concentrations can have different consequences for the plant, if maintained at relatively low levels, it is likely that the function is to act as a signaling pathway for stress, defense responses/stress acclimatization (Moller et al., 2007). However, upon reaching a certain level of concentration, ROS become extremely harmful, activating uncontrolled oxidative cascades that result in biochemistry stress and, eventually, cell death (Mignolet-Spruyt et al., 2016).

Therefore, the different concentrations of H_2O_2 in the studied species are related to characteristics of the species, but also the FWU strategies, culminating in differences in leaf hydration, especially in the dry season, when the highest concentration of this molecule occurred. In the rainy season, there was also the detection of H_2O_2, despite the lower concentration in relation to the dry season. Probably it is due to its role of signalization and not of its involvement in drought stress, since, in this condition, plants continue to produce ROS (Cruz de Carvalho, 2008; Zandalinasa et al., 2018). Plants can use a stable cellular ROS level to monitor their intracellular stress level (Mittler, 2002), but this stationary level must be strictly regulated to avoid an oxidative burst on the ROS accumulation, which would result in extensive cellular damage (Dinakar et al., 2010; Baxter et al., 2014; Choudhury et al., 2017).

Plants are extremely tolerant to H_2O_2 and antioxidant systems appear to function as rigid controllers of the cellular redox state rather than destroyers of all intracellular H_2O_2 (Mittler et al., 2004). In this sense, antioxidants are key components in the modulation of the ROS signal, since they determine lifetime and intensity of this signal (Foyer and Noctor, 2013). Regarding the antioxidant response and the FWU strategies, only C_{max} parameter had a significant influence on the SOD, CAT and APX enzymes production since the highest concentrations of these enzymes are proportional to H_2O_2 production. Thus, the species with the highest concentrations of antioxidant enzymes were those with the highest C_{max} values. SOD is the first enzyme in the line of defense against damages produced by ROS and the most general one, also having affinity with other ROS molecules (Boguszewska et al., 2010). One of its functions is the elimination of ROS during drought stress, possibly through the water-water cycle, thus protecting the photosynthetic apparatus from oxidative damage induced by drought (Cruz de Carvalho, 2008), since all species need a larger amount of water in their tissues for a more effective hydration, justifying a junction of the FWU strategies among the species and the concentrations of SOD.

Due to the high affinity of APX with H_2O_2, this activity increase in the dry season has shown that APX is responsible for the elimination of high intracellular levels of H_2O_2 produced under this constraint condition (Cruz de Carvalho, 2008). Thus, this result reveals the clear separation between different FWU strategies, that is, the highest APX concentrations was related to the highest H_2O_2 content present in species that have the highest C_{max} strategy. This combination of increased SOD and APX concentration in the dry season demonstrates a high efficiency in increasing tolerance to water deficit-induced oxidative damage due to simultaneous scavengers (Lee et al., 2007).

However, CAT has the characteristic of being a sequestering molecule less susceptible than APX in relation to the oxidative stress. This finding indicates that only under severe drought stress the activity of CAT is increased. However, under moderate drought stress, H_2O_2 removal is preferably done by ascorbic acid from the ascorbate / glutathione cycle (Moller et al., 2007). Because it has a lower affinity for H_2O_2 than APX, CAT only acts when there is excess of H_2O_2, because this may inhibit APX. Therefore, CAT activity is more probable to occur in maintaining APX activity under severe drought stress (Cruz de Carvalho, 2008).

Maintaining the normal level of intracellular ROS requires a delicate balance between ROS production and elimination. Considering the drought-induced water stress, this balance is shifted upwards, resulting in an increase in ROS production due to stomatal closure and the consequent limitation in CO_2 fixation, as seen in the results of oxidative stress/signaling and the antioxidant response in this study. However, rather than having an immediate detrimental effect, this increase in ROS production is likely to be beneficial for the plant to be kept under balanced control. Higher ROS production activates defense pathways and acclimatization responses, resulting in an adaptation of the plant to environmental changes (Shi et al., 2013; Golldack et al., 2014).

The majority of higher C_{max} strategy species had the highest values of H_2O_2 compared to k strategy species in the dry season. We believe that this higher production of H_2O_2 is related to its greater need for foliar hydration due to the lower speed of water in leaf tissue and slower absorption (Boanares et al., unpublished data). However, in the dry season, regardless of the FWU strategies and the H_2O_2 content observed, the lipid peroxidation occurred in all studied species, which was confirmed through the non-significant relation between C_{max} and k with the lipid peroxidation. So, the sensitivity to lipid peroxidation is related only to the plant species and the season. However, the production of H_2O_2 which can culminate in lipid peroxidation is related to both C_{max} and k. Although the antioxidant response was proportional to H_2O_2 content, the production of antioxidant enzymes was not sufficient to prevent lipid peroxidation. Therefore, the capacity of FWU, with the temporary and rapid hydration of the foliar tissues, should attenuate ROS production, reducing oxidative stress and decreasing the chances of chronic stress and consequent more cell damage. Thus, the plants of this environment depend on the alternative source of water from the fog and collected by the leaves present in the dry season to attenuate the water deficit imposed by this season.

Possibly, there is a fine adjustment between the foliar absorption capacity and the oxidative signaling. Perhaps due to water deficit pressure experienced by plants, especially in the dry season, a selection of different FWU strategies was induced. A possible relationship between FWU and ROS is in the role of H_2O_2 being involved in cell wall differentiation (Gapper and Dolan, 2006). Its role in the process of cell wall differentiation is not well known. However, it is known that H_2O_2 can act by raising the activity of cellulose synthases that are activated during the growth of cotton fiber. In this way, it can be inferred that H_2O_2 can increase the proportion of cellulose, which consequently increases the capacity to absorb a greater amount of water throughout the high C_{max} plants (Boanares et al., 2018b). This relationship may explain the higher content of H_2O_2 in these species, and may have a key role in the proportion of pectin/cellulose (Boanares et al., 2018b) in the cell wall to grant greater amount of absorbed water.

5. Conclusion

It was verified that species with the high *k* value strategy are more sensitive to lipid peroxidation than species with the high C_{max} value strategy, because they needed a smaller amount of ROS to induce the lipid peroxidation. Species that have a higher speed of leaf water absorption (higher *k*), but uptake a small amount of water (lower C_{max}) are more sensitive. Thus, these species should have faster foliar water absorption to mitigate the damage caused by ROS. The species with higher C_{max} value, because they tolerate a higher concentration of ROS, use H_2O_2 as a signal and thus possibly modify the proportion of the constituents of the leaf cell wall, generating a slower FWU, but in a larger quantity. In this way, it can be inferred that the oxidative stress response is associated with the FWU strategies (Fig. 5). Therefore, it is assumed that fog as an alternative source of water for vegetation has an important ecological role for the maintenance of these species, being the only source of water available to plants to deal with the oxidative stress/signaling induced by drought stress in this ecosystem.

Acknowledgments

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Figure captions

Fig. 1. Mean annual rainfall and mean annual temperature at Brazilian ferruginous rupestrian field at 2018. —— represent total precipitation volume (mm); - - - represents monthly temperature mean.

Fig. 2. Dynamic water content in leaves by submerging time (min) in dry season. Equations: *P. heteromallum*, $y = 0.5575(1-e^{-0.0372t})$, $r^2 = 0.93$; *T. laniflora*, $y = 0.7985(1-e^{-0.026t})$, $r^2 = 0.94$; *M. corallina*, $y = 0.0789(1-e^{-0.0624t})$, $r^2 = 0.97$; *B. variabilis*, $y = 0.4508(1-^{e-0.0621t})$, $r^2 = 0.87$; *L. australis*, $y = 0.4508(1-^{e-0.0621t})$, $r^2 = 0.87$; *O. pulchella*, $y = 0.0789(1-e^{-0.0624t})$, $r^2 = 0.97$.

Fig. 3. (A) Relationship between H_2O_2 and maximum leaf water absorption (C_{max}) and (B) leaf uptake speed (*k*) in the dry season. (C) Quantity of MDA that represent lipid peroxidation among species. Black and grey bars indicate species with higher C_{max} and species with lower C_{max}, respectively. Regression values are means of the four individuals for each species. Significant differences among species were tested by contrast analysis of R program with letters comparing among species ($P < 0.01$).

Fig. 4. Relationship between activities of antioxidant enzymes and maximum leaf water absorption (C_{max}) in dry season: (A) superoxide dismutase (SOD), (B) ascorbate peroxidase (APX) and (C) catalase (CAT). Black and grey bars indicate species with higher C_{max} and species with lower C_{max}, respectively. Regression values are means of the four individuals for each species. Significant differences among species were tested by contrast analysis of R program with letters comparing among species ($P < 0.01$).

Fig. 5. Schematic comparison of two contrasting foliar water uptake (FWU) strategies. (A) species that absorb less water more quickly (LQ) and (B) species that absorb more water more slowly (MS), indicating the differences in plant water regime and ROS (H_2O_2) and antioxidant enzyme (catalase, superoxide dismutase and ascorbate peroxidase) levels in the leaf.

Supplementary material

Fig. S1. Dynamic water content in leaves by submerging time (min) in rainy season. Equations: *P. heteromallum* $y = 0.0474(1\text{-}e^{-0.08t})$, $r^2 = 0.94$; *T. laniflora* $y = 0.0275(1\text{-}e^{-0.002t})$, $r^2 = 0.98$; *M. corallina* $y = 0.05117\ (1\text{-}e^{-0.004t})$, $r^2 = 0.96$; *B. variabilis* $y = 0.0333(1\text{-}^{e-0.113t})$, $r^2 = 0.84$; *L. australis* $y = 0.082(1\text{-}^{e-0.0145t})$, $r^2 = 0.83$; *O. pulchella* $y = 0.028(1\text{-}e^{-0.0055t})$ $r^2 = 0.98$.

Fig. S2. Relationship between H_2O_2 content and activities of antioxidant enzymes in different plant species from ferruginous rupestrian field (A) H_2O_2 content, (B) superoxide dismutase (SOD), (C) catalase (CAT) and (D) ascorbate peroxidase (APX). Black and grey bars indicate species with higher C_{max} and species with lower C_{max}, respectively. Significant differences among species were tested by contrast analysis of R program with letters comparing among species ($P < 0.01$).

Contribution

Daniela Boanares and Marcel Giovanni Costa França designed research, performed all analyses and wrote the first draft of the manuscript. Cristiane Jovelina Silva and Rosy Mary dos Santos Isaias performed the experiment and helped to collect data. All the authors contributed to the experimental design, and significantly improved later drafts.

Table 1. Oxidative stress parameters, such as H_2O_2 concentration, MDA concentration to evaluate lipid peroxidation and antioxidant enzymes (SOD), (APX) and (CAT) related to two FWU parameters: maximum leaf water content (C_{max}) and water uptake speed (k).

Dependent variable	Independent variable	Deviance	F	DV	*P* value
H_2O_2	C_{max}	390.04	6.3109	11	0.012*
	k	390.04	6.4441	11	0.0111*
	Species	390.04	41.843	11	0.0001***
	Season	390.04	87.873	11	0.0001***
MDA	C_{max}	479.3	0.763	11	0.3824
	k	479.3	3.0775	11	0.0793
	Species	479.3	21.424	11	0.0001***
	Season	479.3	84.928	11	0.0001***
SOD	C_{max}	94.826	8.7544	11	0.0030**
	k	94.826	2.1928	11	0.1387
	Species	94.826	36.791	11	0.0001***
	Season	94.826	56	11	0.0001***
APX	C_{max}	-7.1862	14.731	11	0.0001***
	k	-7.1862	0.662	11	0.4159
	Species	-7.1862	14.152	11	0.0146*
	Season	-7.1862	34.877	11	0.0001***
CAT	C_{max}	325.49	18.212	11	0.0001***
	k	325.49	1.4351	11	0.2309
	Species	325.49	29.63	11	0.0001***
	Season	325.49	35.365	11	0.0001***

The general linear mixed models (GLMMs) for the biotic independent variables evaluated. *** Indicates $P \leq 0.001$; **Indicates $P \leq 0.01$ and * Indicates $P \leq 0.05$.

Figure 1

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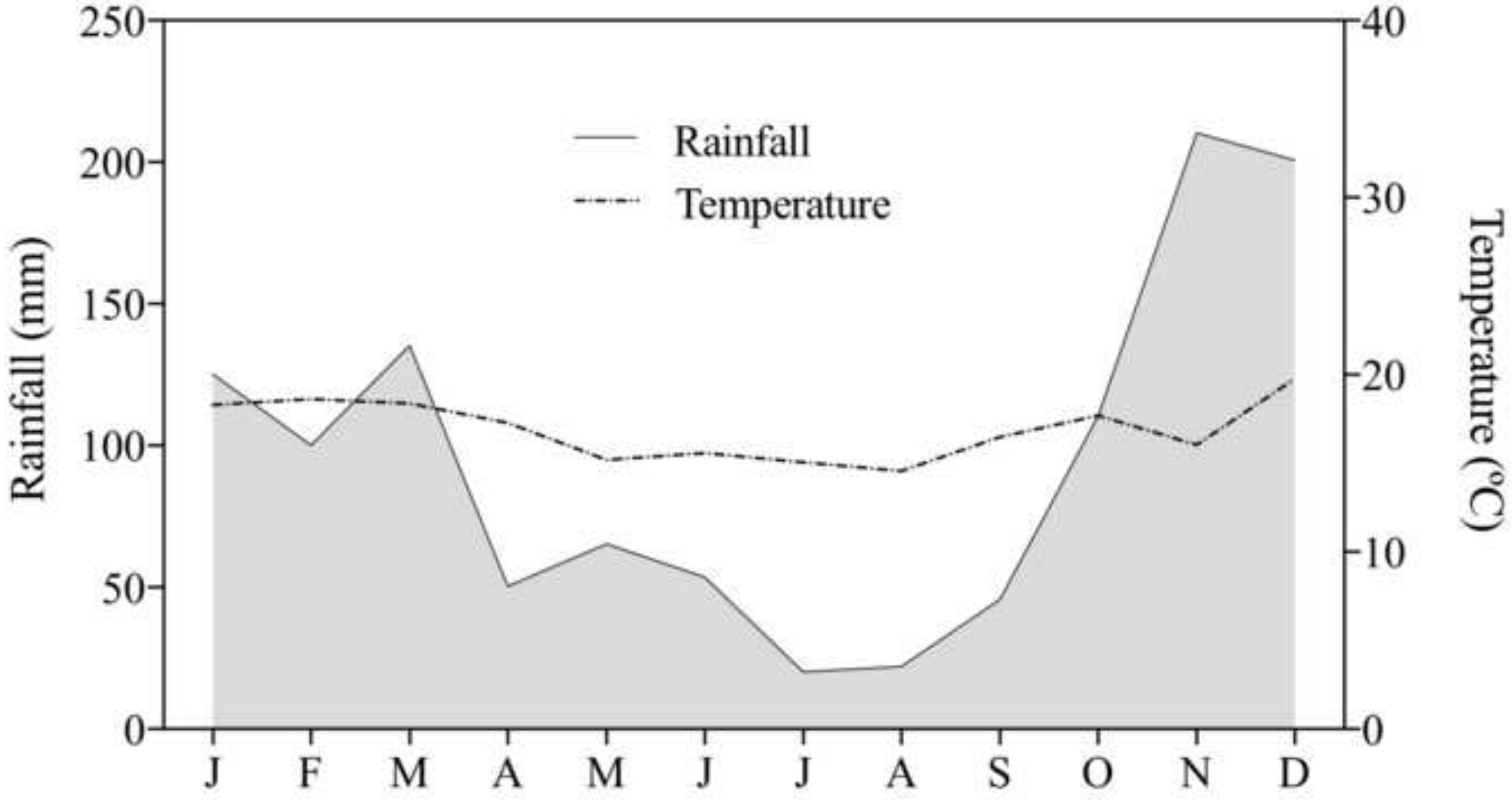

Figure 2

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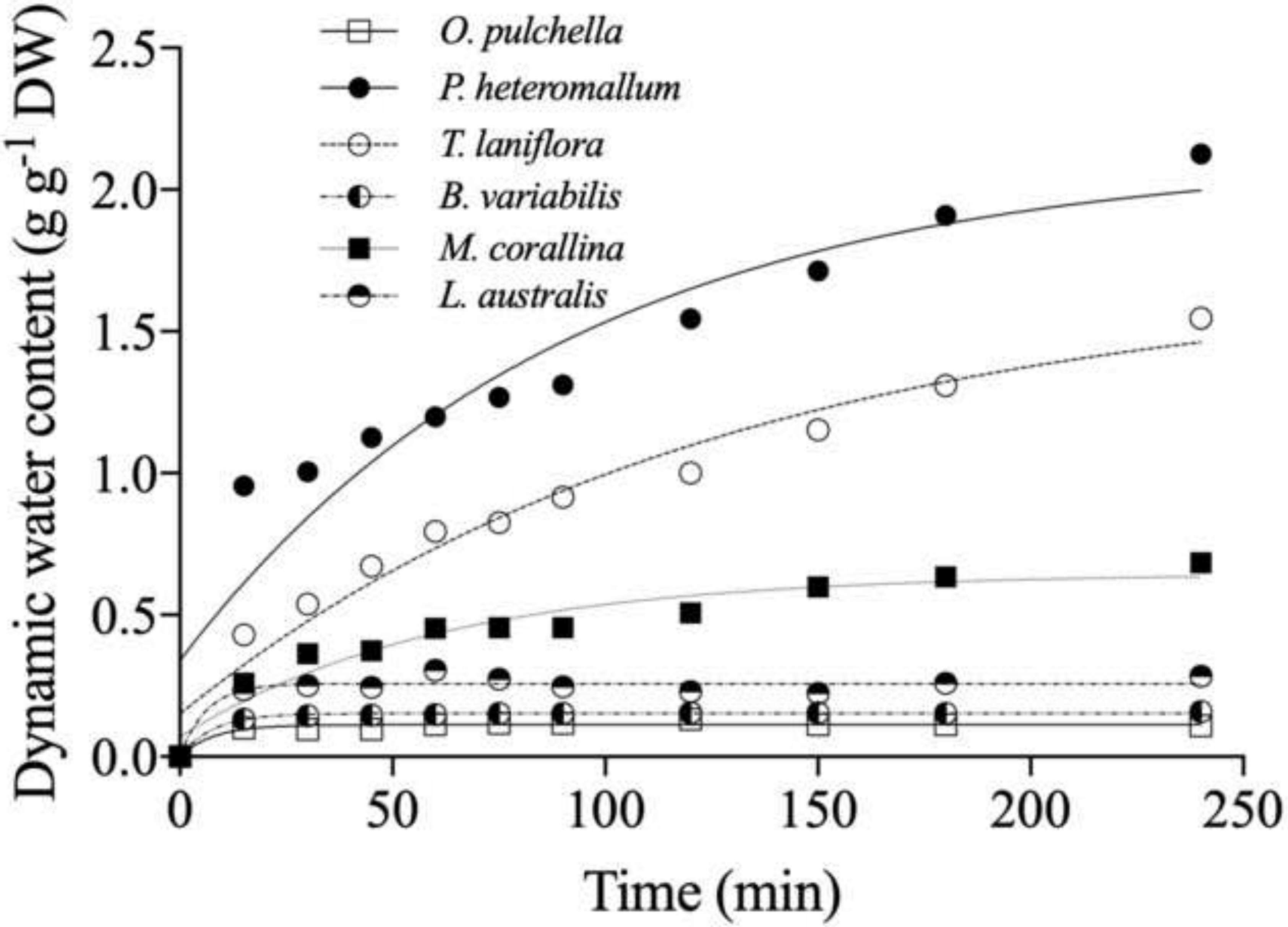


Figure 3

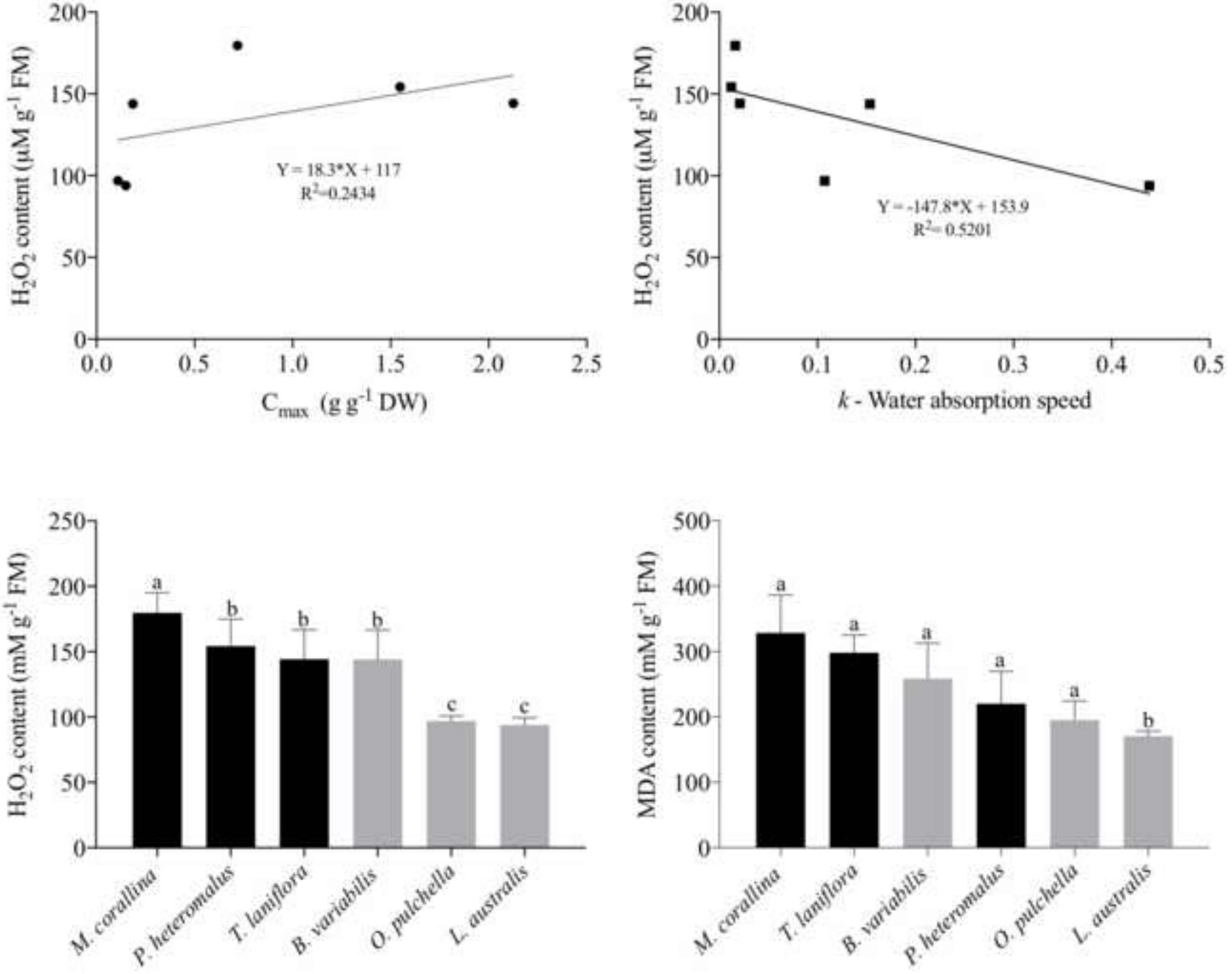
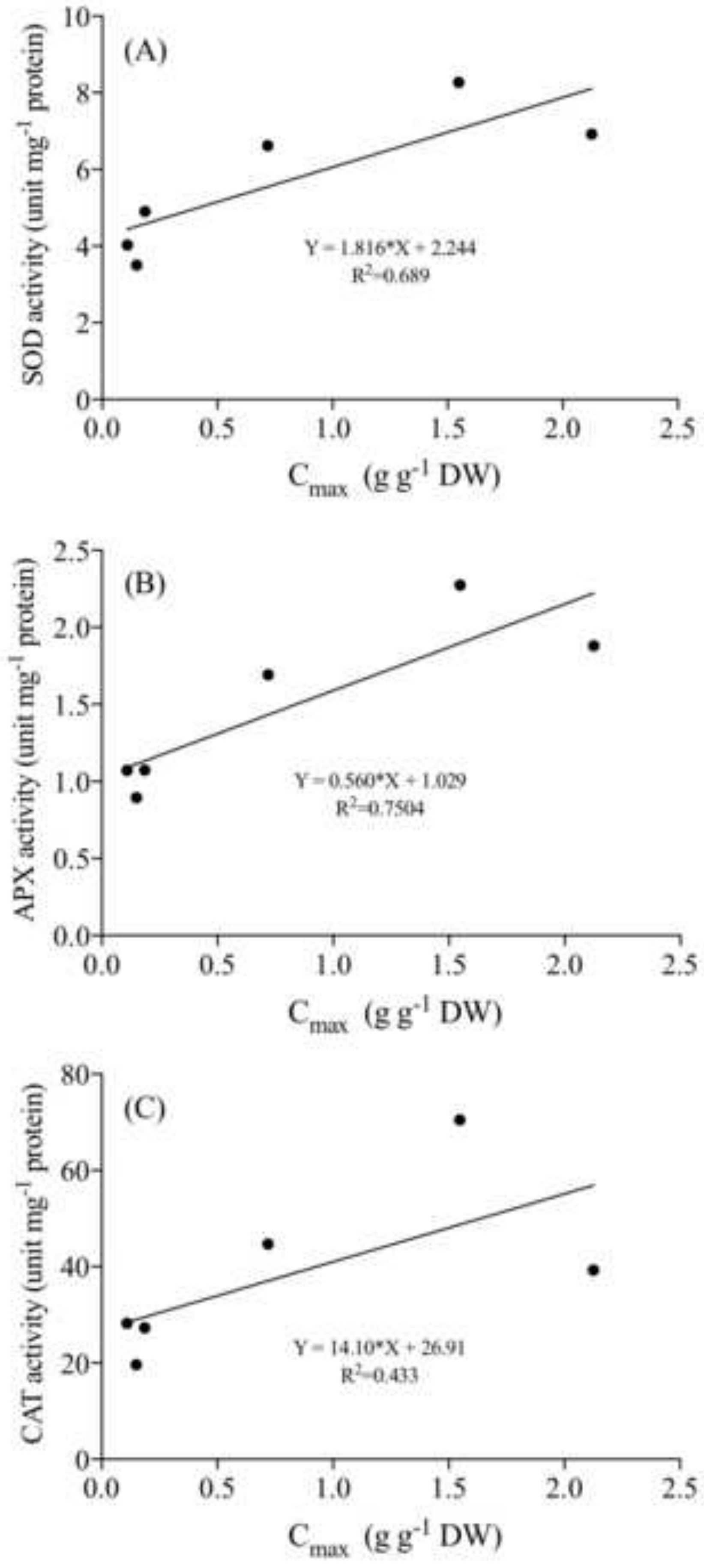
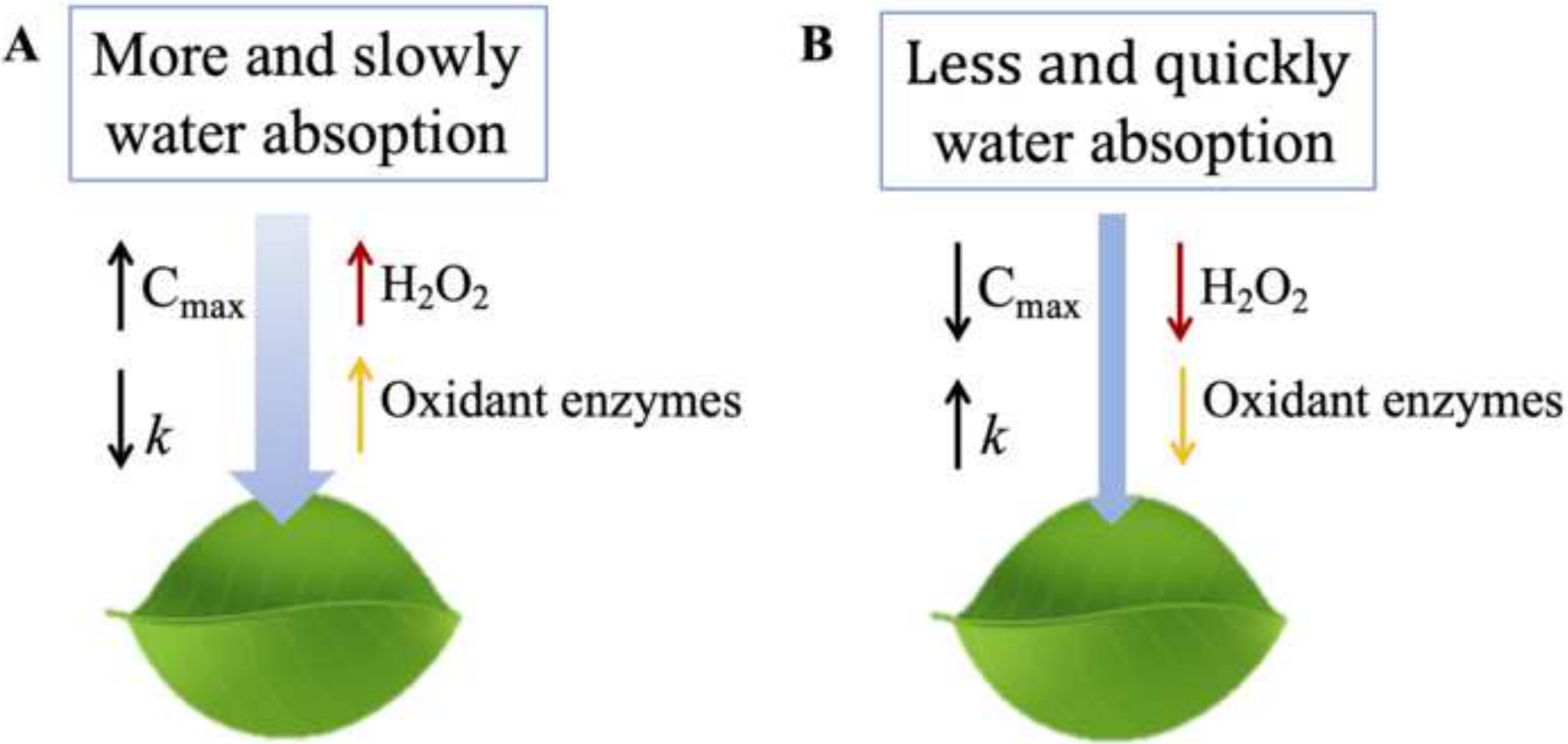
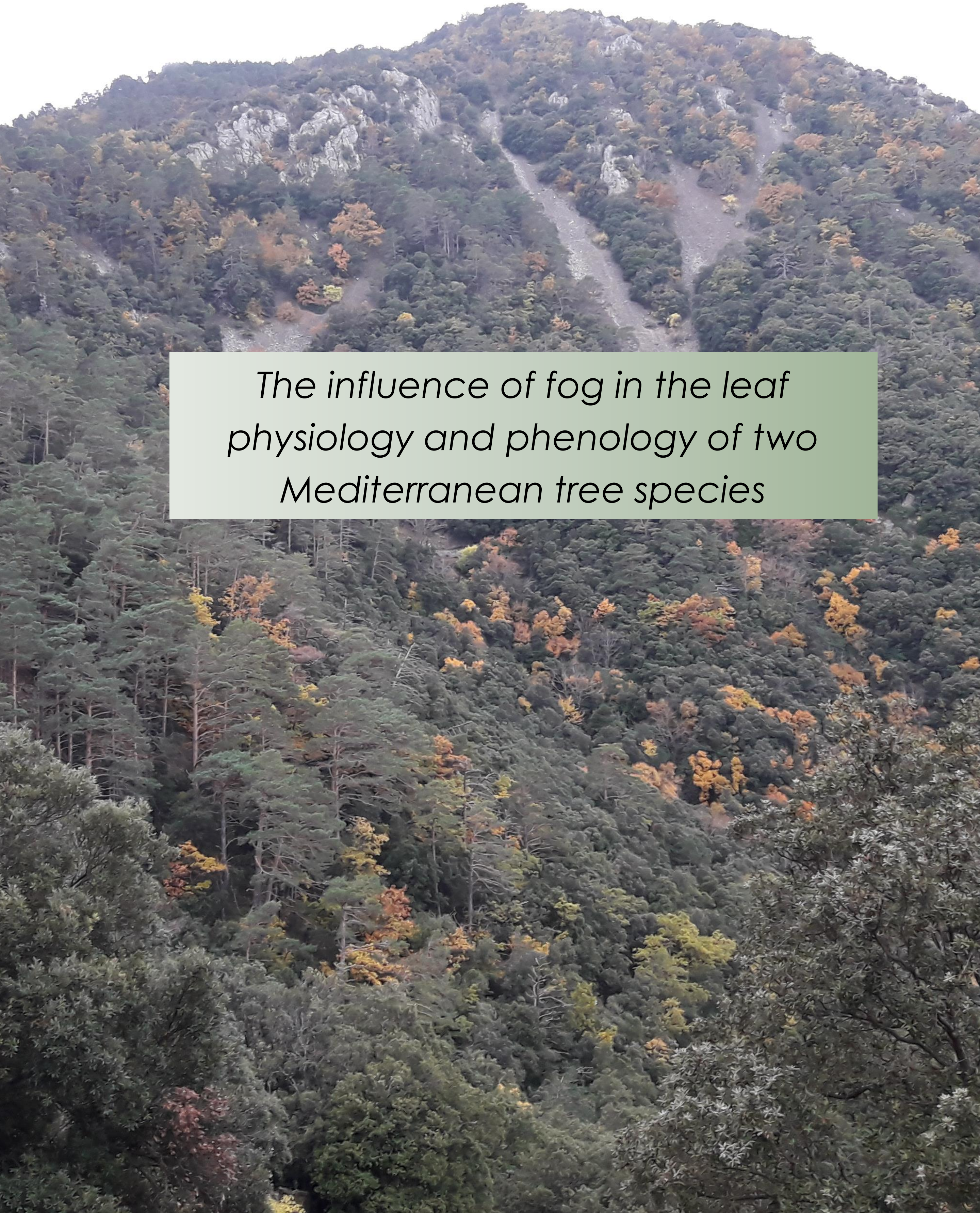

200
150
100
50
0
H2O2 content (µM g-1 FM)
0.0
0.5
1.0
1.5
2.0
2.5
Cmax (g g-1 DW)
Y = 18.3*X + 117
R2=0.2434
200
150
100
50
0
H2O2 content (µM g-1 FM)
0.0
0.1
0.2
0.3
0.4
0.5
k - Water absorption speed
Y = -147.8*X + 153.9
R2= 0.5201
250
200
150
100
50
0
H2O2 content (mM g-1 FM)
a
b
b
b
c
c
M. corallina
P. heteromalus
T. laniflora
B. variabilis
O. pulchella
L. australis
500
400
300
200
100
0
MDA content (mM g-1 FM)
a
a
a
a
a
b
M. corallina
T. laniflora
B. variabilis
P. heteromalus
O. pulchella
L. australis

Figure 4


(A)
SOD activity (unit mg^{-1} protein)
10
8
6
4
2
0
Y = 1.816*X + 2.244
R^2=0.689
0.0
0.5
1.0
1.5
2.0
2.5
C$_{max}$ (g g^{-1} DW)
(B)
APX activity (unit mg^{-1} protein)
2.5
2.0
1.5
1.0
0.5
0.0
Y = 0.560*X + 1.029
R^2=0.7504
0.0
0.5
1.0
1.5
2.0
2.5
C$_{max}$ (g g^{-1} DW)
(C)
CAT activity (unit mg^{-1} protein)
80
60
40
20
0
Y = 14.10*X + 26.91
R^2=0.433
0.0
0.5
1.0
1.5
2.0
2.5
C$_{max}$ (g g^{-1} DW)


A
More and slowly water absoption
↑ C_{max}
↓ k
↑ H_2O_2
↑ Oxidant enzymes
B
Less and quickly water absoption
↓ C_{max}
↑ k
↓ H_2O_2
↓ Oxidant enzymes

Chapter 6



The influence of fog in the leaf physiology and phenology of two Mediterranean tree species

The influence of fog in the leaf physiology and phenology of two Mediterranean tree species

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ABSTRACT

Fog may play a role as an alternative source of water for vegetation, and the leaf absorption capacity of fog is an important strategy for plants of different terrestrial ecosystems. Thus, our objective was to evaluate the importance of fog in the physiology of the most common native species of European forests, *Fagus sylvatica* and *Quercus ilex*, and leaf phenology of *F. sylvatica*, a deciduous species. We evaluated the physiological performance of plantlets subjected to 3 environmental conditions in a greenhouse experiment: artificial fog, irrigation, and water deficit. To assess whether the duration of *F. sylvatica* leaves was related to the presence of fog, the 11-year remote sensing data from four regions of a Mediterranean-temperate ecotone were analyzed. Fog significantly improve the physiology of both species, especially *F. sylvatica*, preventing or delaying the occurrence of photoinhibition, and increasing the water status of plants. Vegetative indices were responsive to fog only for *F. sylvatica*, whose phenology has a significant relationship with the presence of fog. Short-duration fog events accelerates leaf falling, therefore, fog not only improves the physiological parameters of the two studied species, but also plays an important role on *F. sylvatica* phenology.

Key words: *Fagus sylvatica; Quercus ilex;* Foliar water uptake; Photosynthesis

List of abbreviation:

C_{max} - maximum water content in leaf lamina

k - water leaf uptake speed

Fv/Fm - potential quantum yield of photosystem II

PRI - photochemical reflectance index

NDVI - Normalized Difference Vegetation Index

Author contributions

D.B., J.P., R.S.O. and M.G.C.F. designed the research. D.B., J.L., R.O. and I.F. collected the data for the research, and D.B. and analyzed the data. D.B., R.M.S.I. and M.G.C.F wrote the manuscript.

HIGHLIGHTS

- Leaf physiology was closely related to fog in *Fagus sylvatica* and *Quercus ilex*
- Fog prevented or delayed the occurrence of photoinhibition in plants
- Leaf phenology of *F. sylvatica* has a significant relationship with fog presence
- 11-years remote sensing data showed decrease in both, fog events and leaf duration

Introduction

Fog is an important meteorological phenomenon for the hydrological cycle in several terrestrial ecosystems (Holder 2004, 2006; Prada et al., 2009), especially at altitudes above 800 meters (Mc Jannet et al., 2007). Its formation can occur through the encounter of hot and cold air masses, when the cooling forms fog and low clouds (Burgess and Dawson 2004). The air mass stability, environment topography, and the flow and direction of humidity are conditions which may induce fog formation (Money 2000). In addition to contributing to the hydrology of ecosystems, fog may play a relevant ecological role as an alternative source of water for vegetation. First, it can decrease the vapor pressure deficit of the atmosphere, reducing evaporation and transpiration on plant surfaces (Juvik and Nullet 1995). If wind directs fog flow through the canopy, much of the fog water may be intercepted by the vegetation surfaces, where it would remain for some time, returning to the atmosphere through evaporation, or wetting the soil by the water runoff (Hutley et al., 1997). Also, there is the possibility of water absorption through plant leaves (Martin and von Willert 2000).

Foliar water uptake (FWU) occurs when water crosses leaf surface in response to the potential gradient diffusing into the internal plant tissues through the cuticle (Yates and Hutley 1995; Gouvra and Grammatikopoulos 2003), and/or absorbent trichomes (Benzing et al., 1978) and/or through hydathodes (Martin and von Willert 2000). FWU can occur during and after various forms of precipitation, such as rain and fog. Several studies have been carried out to evaluate the capacity of fog FWU, and its consequences for the ecology and ecophysiology of different plant species (Holder 2006; Simon et al., 2009). Although FWU represents a small portion of water entering the leaves in relation to the total entry of water by the roots, such absorption immediately increases leaf hydration and water potential (Ewing et al., 2009; Boanares et al., 2019). Nevertheless, it may take more than a month to be transported from roots into leaves in large species such as *Sequoia semprevirens* (Cassana et al., 2006; Eller et al., 2013).

Fog FWU may play an important role in the survival and growth of plant species occurring in places with water deficit (Limm et al., 2009). *Fagus sylvatica* L. (European beech) and *Quercus ilex* L. (Sessile oak), the most common native species of European forests (Corcuera et al., 2004; Petritan et al., 2014), have differences in growth response to air temperature and fog presence on a Mediterranean mountain (Barbeta et al., 2019). The growth of *Fagus sylvatica*, a deciduous species, is more responsive to fog presence than *Quercus ilex*, an evergreen species (Barbeta et al., 2019). Models predict the fog decrease and this will possibly have consequences to structure and functioning of ecosystems (Johnstone and Dawson, 2010; LaDochy and Witiw, 2011). A decrease in fog events in this region resulted in a decrease in growth of *F. sylvatica* along the years (Barbeta et al., 2019).

The influence of fog events is well known in tropical environments and little in temperate forests subject to the Mediterranean climate (Barbeta et al., 2019). The Mediterranean climate is characterized by wet winters, and hot and dry summers, during which low water availability severely limits carbon gain (Ackerly et al., 2002). Fog in high altitudes may interfere with the physiology of coexisting species by reducing water stress in periods when plants experience water deficit.

Our objective was to study physiological responses of *Fagus sylvatica* and *Quercus ilex* to fog events, and the interference of fog on the phenology of *F. sylvatica.* Measurements of the effect of fog on photosynthesis, water potential, and plant reflectance were conducted in greenhouse conditions, where the plants were subjected to three environmental conditions, artificial fog, irrigation, and water deficit. The reflectance indices can be used to estimates physiological status of plants that estimate of photosynthetic efficiency. Additionally, the duration of *F. sylvatica* leaves was evaluated by using remote sensing images from 11-years data in a Mediterranean-temperate ecotone.

Materials and methods

Study species

Natural vegetation of *Fagus sylvatica* and *Quercus ilex* coexists in Mediterranean-temperate ecotone in Montseny Natural Park (Catalonia, Spain, Northeastern Iberian Peninsula). *F. sylvatica* is a deciduous species and dominates the forests in the highest altitude range in the Montseny Mountains (750-1690 m a.s.l.), and *Q. ilex* is an evergreen species, and occurs in lower altitudes of the same mountains (300-1300 m a.s.l.) (Bolòs, 1983). Plantlets with approximately 1-1.20 meters used for this study were obtained from the Vivers Tortadès nursery in Saint Hilari Sacalm, Spain.

Greenhouse experiment

To evaluate the species responses to fog, plantlet of each species, 6 individuals for each treatment were subjected to different hydric conditions: artificial fog, soil irrigation and soil drought in the greenhouse of the Autonomous University of Barcelona (Barcelona, Spain).

Artificial fog was created by an air humidifier installed inside a chamber made with pallets and plastic (1.5 x 3.0 x 2.0 m), and was applied only overnight. The soil in pots had been sealed with parafilm and plastic bags to prevent fog from contacting the soil (Eller et al., 2004). Plants hydration status was guaranteed by everyday irrigation. Water deficit was induced by submitting the plants to a progressive drought stress, suppressing irrigation, all along the experiment for 30 days.

Physiological performance

Measurements of the potential quantum yield (Fv/Fm) were performed at 9:00 am, using a modulated fluorescence meter (MINI-PAM Waltz, Germany), to identify the occurrence of photoinhibition. Such measurements were taken at the first day of the experiment with artificial fog (day 1), after 4 days (day 4), 8 days (day 8), 18 days (day 18), and 24 days (day 24). After adaptation of leaf to the dark for about 30 min, the potential quantum yield (Fv/Fm) of photosystem II (PSII) (Rascher et al., 2000) were measured. In order to calculate the Fv/Fm (Fv = Fm-F0), the Fv (variable fluorescence) was measured in dark adapted leaves, Fm (maximum fluorescence) was measured in dark adapted leaves after a pulse of saturating light, and F0 is the basal fluorescence produced by dark adapted leaves.

Measurements of water potential

The water potential was measured on leaves of three individuals of each treatment at 10:00 am, the day before the beginning of the treatment with artificial fog (day 0), one day after the beginning of the treatment (day 1), and at day 4, day 8, day 18, and day 24, with a Scholander type pressure chamber (Scholander, 1964).

Reflectance Measurements

Spectral reflectance of leaves was measured in situ using a portable field spectroradiometer (GER1500, Geophysical & Environmental Research, Spectra Vista Corp., Poughkeepsie, NY, USA). Reflectance was calculated after standardization by canopy irradiance using a reference spectral panel (Spectralon, Labsphere, North Sutton, NH, USA) serving as a Lambertian reflector. All spectral measurements were from a nadir view angle approximately 0.1 m above the plant. Three scans were quickly recorded (around one second per record) in different positions of each plant as replicates after measuring the white standard spectrumThe photochemical reflectance index (PRI) and Normalized Difference Vegetation Index (NDVI) were calculated from the reflectance data (Rx implies reflectance in x nm) respectively as PRI = (R531 − R570)/(R531 + R570) (Gamon et al., 1992; Peñuelas et al., 1995) and NDVI= (R900 − R680)/(R900 + R680) (Tucker, 1979).

Remote sensing of Montseny Natural Park vegetation

The annual cycle of *F. sylvatica* vegetation phenology was characterized using remote sensing using data from enhanced vegetation index (EVI) (Huete et., 2002) in four regions of Montseny Natural Park (Catalonia, Spain, northeastern Iberian Peninsula; 41°49’28’’N, 2°24’20’’E). The regions were: Borderiol, l’Ase mort and Santa Fe with two altitudes (low and high altitudes) (Fig. 1). The data are from the period 2001 to 2014. Data based on MODIS - MCD12Q2 Land Cover Dynamics Product were obtained from the four moments that define the main phenological phases of vegetation dynamics at annual time scales (Zhang et al., 2003). The moments were: beginning of the photosynthetic activity; maturity, in which the plant has its maximum leaf area; senescence, where photosynthetic activity and green leaf area begin to decrease; and dormancy, which is when the physiological activity is close to zero (Zhang et al., 2003). To verify leaf duration, the ratio between the period of senescence (D) and the maximum of the photosynthetic activity (C) was calculated.

To assess the direct relationship between the presence of fog in the green canopy duration from 2001 to 2014, a linear regression was used. In order to make this regression, it was necessary to extrapolate the data of foggy days/year since the data obtained in the weather station captured data only in the period from 1950 to 2000. These data captured in these years were used to make a linear regression. From the generated equation, it was possible to estimate the data for the years 2001 to 2014. Thus, time with the obtained data, another linear regression between the foggy days/year and the green canopy duration was performed.

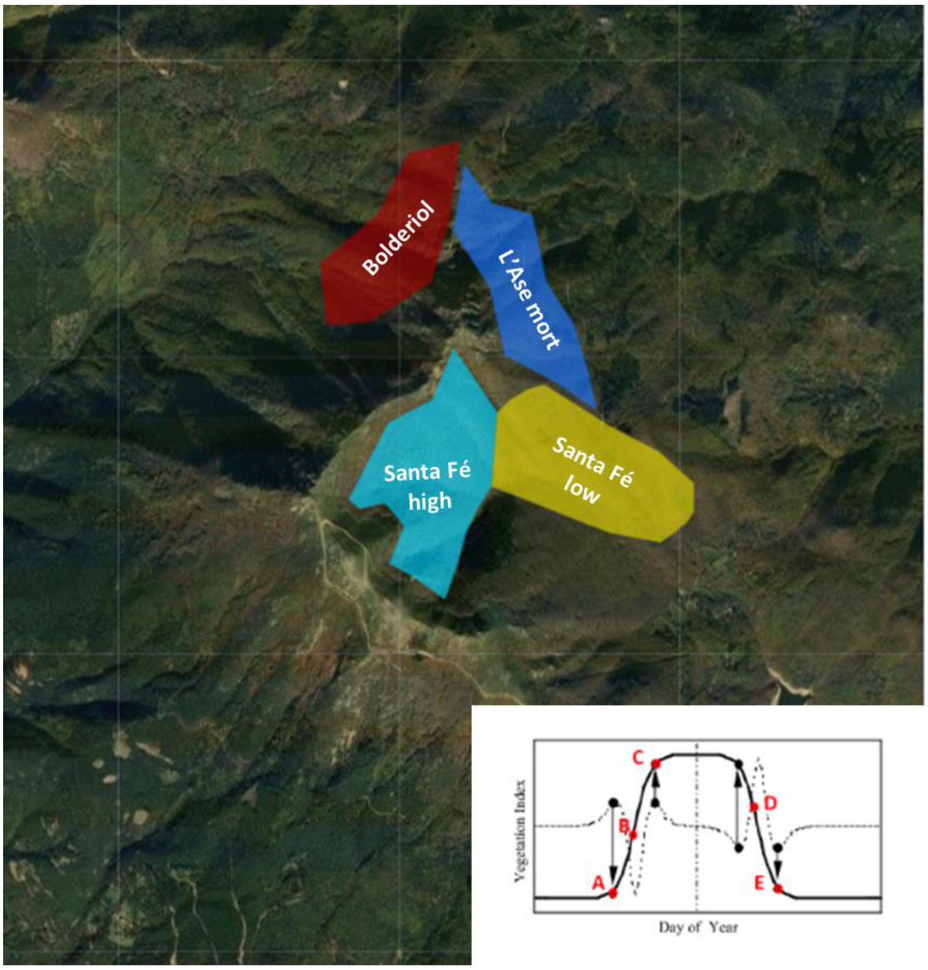

Fig. 1. Aerial photograph of the studied sites in a Mediterranean-temperate forest. The colors in the panel show the location of each site, which was used from remote sensing based on MODIS - MCD12Q2 Land Cover Dynamics Product data for *Fagus sylvatica*. The lower right panel shows the moments of the annual cycle vegetation phenology. Beginning of the photosynthetic activity (A and B); maturity, in which the plant is with the maximum leaf area (C); senescence (D), where photosynthetic activity and green leaf area begin to decrease; and dormancy, which is when the physiological activity is close to zero (E)

Data analyses

General linear models (GLMs) with Gaussian errors were built to evaluate if potential quantum yield, Photochemical Reflectance Index (PRI), Normalized Difference Vegetation Index (NDVI), and water potential (dependent variables) are influenced for the different treatments (with artificial fog; soil irrigation; soil drought) (independent variables) over the two species. The analyses were performed in the R platform (R Development Core Team 2015).

Results

Photoinhibition measurements

The different performances under the three water regimes treatments in greenhouse for F. *sylvatica* and *Q. ilex*, had verified. The potential quantum yield (Fv / Fm) of Q. ilex was not influenced by the fog, since the values were considered equal between the drought of the soil and the fog treatments and different from irrigation soil treatment ($F_{2.87}$=5.680; $P \leq 0.05$) (Fig. 2A). Contrastingly, *F. sylvatica* showed was more responsive in the presence of fog, after all, there was no difference in the values of Fv / Fm between the soil irrigation and fog treatment but were different from soil drought treatment ($F_{2.87}$=7.853; $P \leq 0.05$)(Fig. 2B). The plants submitted to treatments with artificial fog and soil irrigation had the highest values of potential quantum yield when compared to the plants submitted to soil drought. There was a potential of quantum yield decreasing during the experiment with water stress, mainly in *F. sylvatica*, denoting an evident photoinhibition effect. Under water stress, the Fv/Fm values of *F. sylvatica* were lower than the values of *Q. ilex,* evidencing *F. sylvatica* higher sensitiveness to water deficit.

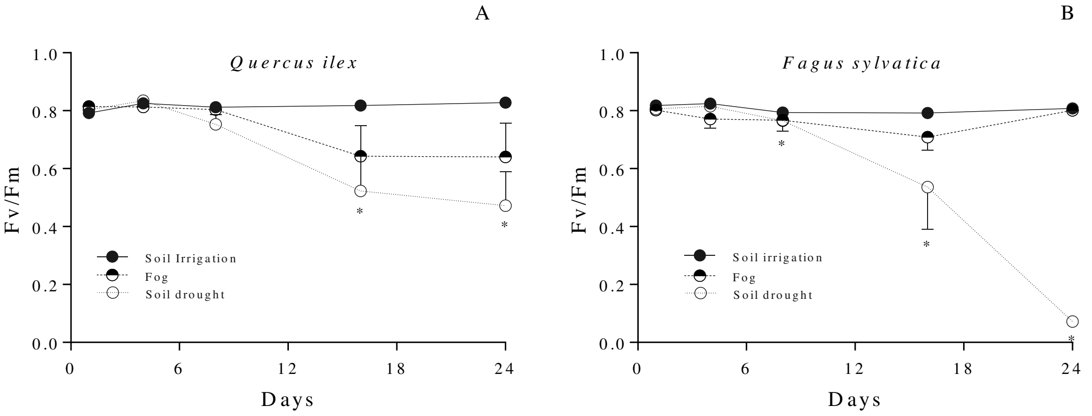

Fig 2. Measurements of PSII chlorophyll fluorescence parameters of seedlings submitted to soil irrigation, fog, and soil drought treatments. Potential quantum yield (Fv/Fm) of (A) *Q. ilex* and (B) *F. sylvatica*. Periods: before treatment with artificial fog (day 0), days 1, 4, 8, 16 and 24. Mean values, and standard deviation, n = 6 species per treatment. General linear models (GLMs) with Gaussian errors, *P*<0.05, followed by contrast analysis. Different letters denote significant difference among treatments.

Water potential

Leaf water potential did not vary significantly between plants subjected to artificial fog and soil irrigation treatments but it was significantly lower in plants subjected to soil drought in both species ($F_{2,105}$=24.845; P≤0.05) (Fig. 3). The artificial fog increased the mean values of leaf water potential both in *F. sylvatica* and *Q. ilex* generating water potential values similar to those of plants of soil irrigation treatment, mainly the first days. It is important to emphasize that at the beginning of the experiment, the water potential of the plants did not show significant difference between the different environmental conditions, demonstrating that all plants presented the same water status ($F_{5,100}$=28.357; P≤0.05). Water from artificial fog was enough to increase the water potential of these two species, improving their water status. This increase in water potential demonstrates the strategy of these species to absorb water by the leaves thus, the FWU capacity of both species is evident but possibly with different magnitudes.

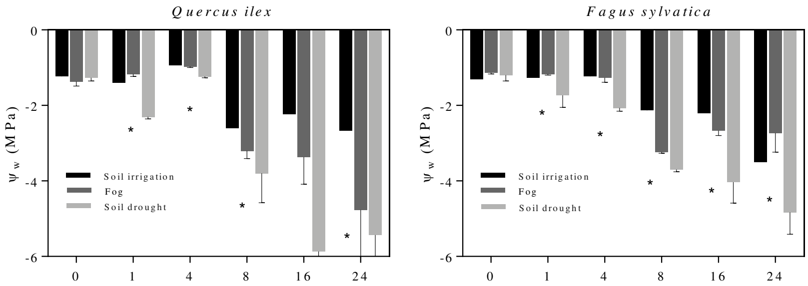

Fig 3. Leaf water potential (Ψw) of the branches of the two studied species along the three different treatments: soil irrigation, fog, and soil drought. Periods: before treatment with fog (day 0), and 1, 4, 8, 16 and 24 days after to beginning of the treatments.

Reflectance index

The performance of the two studied species regarding the reflectance index is different. The plants of *Q. ilex* showed no significant differences among the treatments for NDVI ($F_{2.87}$=1.204; *P*≥0.05) (Fig. 4). However, there were differences for PRI ($F_{2.87}$=5.863; *P*≤0.05) between plants submitted to the soil irrigation treatment and those submitted of soil drought and fog. For *F. sylvatica,* the NDVI values ($F_{2.87}$=7.498; *P*≤0.05) were different between plants submitted to the water deficit treatment and the plants submitted to the treatments of artificial fog and soil irrigation. Plants subjected to fog and the soil irrigation treatments had higher values of NVDI than plants submitted to the soil drought treatment, mainly in the initial days ($F_{4.83}$=6.744; *P*≤0.05). In contrast, the PRI values were similar between plants subjected to the artificial fog and to the soil drought treatments, and PRI values were higher in plants submitted to the soil irrigation treatment ($F_{2.87}=21.123$; $P\leq0.05$). *Q. ilex* showed overall higher values of NDVI and PRI than *F. sylvatica.*

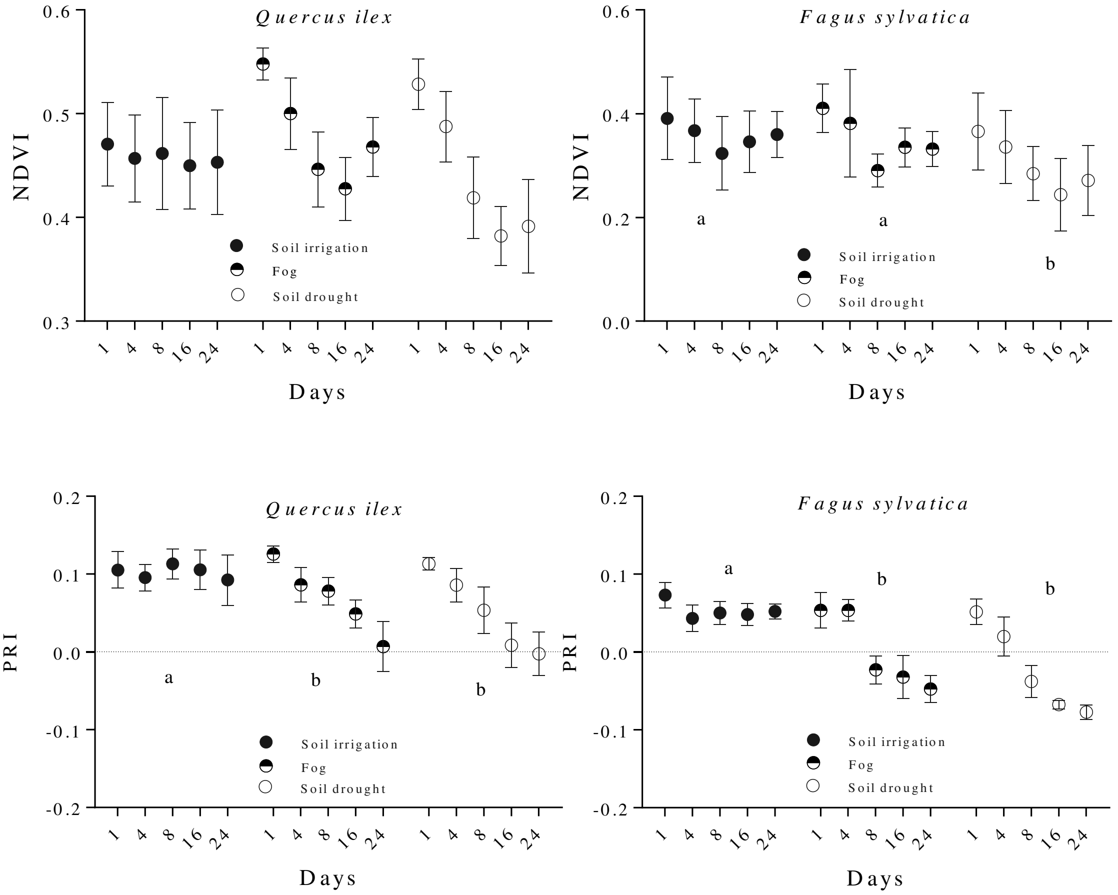

Fig. 4. Variation of the efficiency of the use of radiation in *Q. ilex* and *F. sylvatica*. The parameters used were NDVI (Normalized Difference Vegetation Index) and PRI (Photochemical Reflectance Index). Measurements occurred in first day to start the treatment with fog, 4, 8, 16 and 24 days. Error bars indicate the standard errors of the mean (n = 6 for each treatment). The significances of the GLM with *posthoc* test are represented using different letters to show differences between treatments ($P\leq0.05$).

Remote sensing - MODIS

There was a trend to extend the duration of *F. sylvatica* leaves with the presence of fog. Thus, the decrease in fog events negatively interferes in leaf duration from the EVI-MODIS data, mainly in the high altitude of Santa Fe (SE) (Fig. 5).

Using data from 14 years of fog occurrence in the region, there was a direct relationship between the fog events and the duration of the leaves of *F. sylvatica*. There is a trend in which, with the increase in the foggy days, there was an increase in the duration of the leaves from canopy greenness duration (Fig. 6).

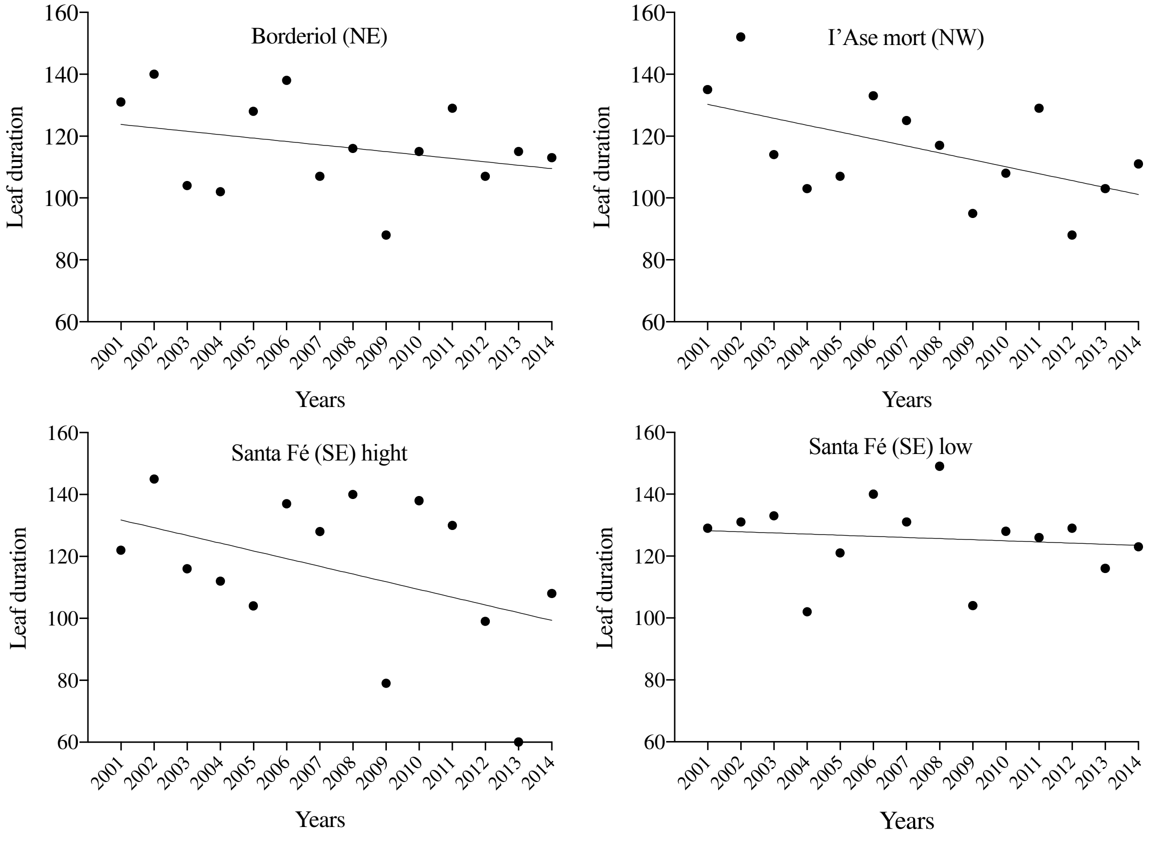

Fig. 5. Linear regression between the years and leaf duration from the product MCD12Q2 of *F. sylvatica* in the three regions of Montseny Park. Linear regression: Bolderiol (NE): EVI=-

1.097*years+2318; L'Ase mort (NW): EVI=-2.242*years+4616; Santa Fe (SE) high altitude: EVI=-2.497*years+5128 and Santa Fe (SE) low altitude: EVI=-0.3648*years+8583.

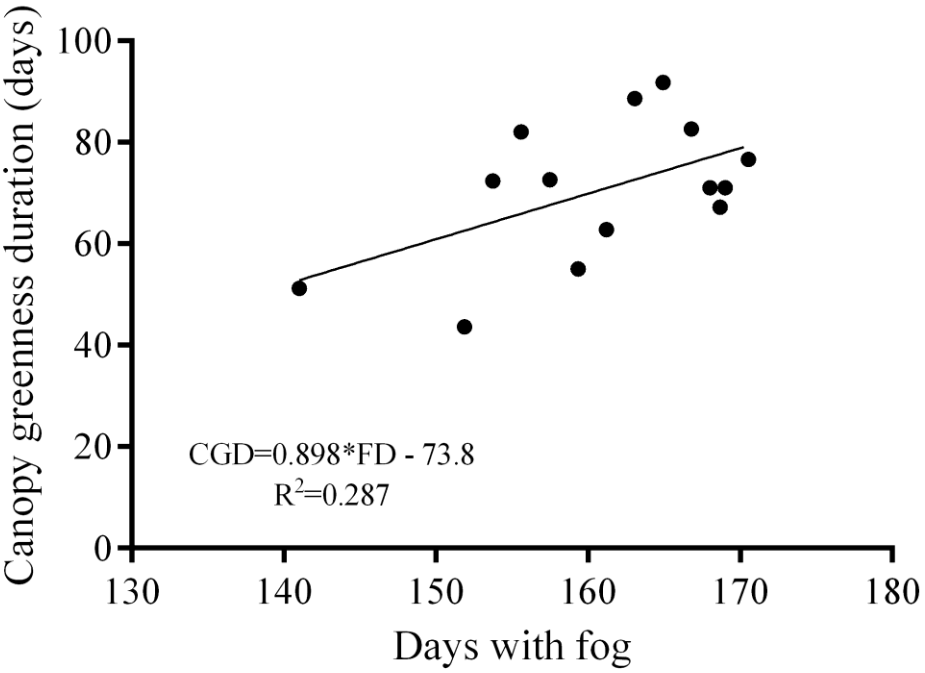

Fig. 6. Linear regression between the days with fog and canopy greenness duration from the product MCD12Q2 of *F. sylvatica* in the Montseny Natural Park. Data collected from 14 years (2001 -2014). Linear regression: CGD = canopy greenness duration and FD = fog days. *P*<0.05.

Discussion

Our physiological data complemented the understanding of the importance of fog in the Mediterranean environment for *F. sylvatica* and *Q. ilex*, corroborating that F. sylvatica is more responsive to fog than *Q. ilex* (Barbeta et al., 2019). Furthermore, we have verified, for the first time, the role of fog in the phenology of a deciduous species. Its decrease negatively affected the duration of *F. sylvatica* leaves.

The fog, and its consequent absorption by the leaf, increased the water status of the plants and then interfered in photosynthetic efficiency. The FWU generated by the artificial fog was enough to keep the photosynthetic apparatus stability by increasing the potential quantum yield (*Fv/Fm*) throughout the experiment and preventing possible photoinhibition effect for both species. Photoinhibition had already been demonstrated in *F. sylvatica* plants cultivated both under low and high light conditions (Tognetti et al., 1998), corroborating the importance of fog, since, besides providing an alternative water source for the plant, it also decreases radiation at the environment. Although *Q. ilex* showed no significant difference between treatments of drought and subjected to fog, artificial fog improved the photosynthetic efficiency not at the same intensity of the irrigated treatment, but efficient enough to avoid photoinhibition. Perhaps the non-significant difference between treatments is due to the higher tolerance of *Q. ilex* to photoinhibition (Méthy et al. 1996, Oliveira and Peñuelas 2000). Thus, the artificial fog was not enough to promote a statistical difference between the treatments of water deficit and fog.

The vegetation indices that also indicate the photosynthetic efficiency of the plants were influenced by the presence of artificial fog. The similarity between the vegetation status index for the NDVI in the irrigated treatment and in the artificial fog for *F. sylvatica* indicated the importance of fog for the maintenance of biomass-related biological variables such as leaf area index, chlorophyll by soil area, intercepted radiation fraction, leaf cover and green vegetation factor, which means the proportion of green vegetation in the canopy (Gamon et al., 1995; Rundquist 2002). The PRI index did not show a positive relationship in plants of *F. sylvatica* and *Q. ilex* subjected both to artificial fog and irrigated treatment. Accordingly, other leaf-scale studies have already seen reduced accuracy of PRI to track the photosynthetic activity of herbaceous and shrubby plants under water deficit (Winkel et al., 2002; Filella et al., 2004). Then, the reduced accuracy of PRI may also have prevented the detection of fog influence on the hydration of *F. sylvatica* and *Q. ilex* because theses plantas were seedlings, similar sizes to herbaceous plants.

A more comprehensive view of the importance of fog in determining the landscape of an ecosystem was the verification of *F. sylvatica* phenology. The shorter duration of *F. sylvatica* leaves may be influenced by a decrease in fog events, as it is already proposed (Barbeta et al., 2019). This phenological change in response to warming processes is a sensitive and easily observable indicator of climate global changes (Parmesan and Yohe 2003; Root et al., 2003), which also interfere with the structure and functioning of the ecosystems (Peñuelas and Filella 2001). It is well known that decreasing rainfall can change leaf phenology of drought-deciduous species, as rainfall may become insufficient to replenish soil water reserves regularly (Borchert 1998; Enquist and Enquist 2011). Although, the shortening of leaf duration as a consequence of low fog events is a slight trend for all the studied regions (Borderiol, l'Ase mort and Santa Fe with two altitudes), *F. sylvatica* behaves as an opportunistic species, because it maintains its leaves, and consequently, photosynthesis activity, while some type of water source for hydration is available. Thus, the presence of fog increase leaf duration, influence the phenology of *F. sylvatica*, by promoting instantaneous leaf hydration without the need for soil wetting.

Although both species have increased their water potential due to the presence of fog, there was a difference in behavior between the two species studied. This difference can be explained by the different phenological and physiological strategies of these species in relation to the availability of water. *F. sylvatica* is a deciduous species, that is, it has the strategy of avoiding the pressure imposed by water deficit by losing its leaves and limiting its physiological activity. On the other hand, *Q. ilex* is a perennial species, which has the strategy of withstanding periods of low water availability without stress, allowing plant survival during the dry season (Murphy and Lugo 1986; Bowman and Prior 2005; Lock 2006; Lehmann et al., 2009). Thus, the higher sensitivity of *F. sylvatica* to fog is related to its phenology, since the presence of fog relieves the stress that the plant would suffer, delaying leaf falling. Since lower water availability and environmental warming are the two most important environmental and selective pressures that restrict *F. sylvatica* along its geographical range (Leuzinger et al., 2005), fog plays a key role in alleviating these environmental constraints. In fact, the presence of *F. sylvatica* at high altitudes is positively influenced by cloudiness during the growing season and may even have an impact on the growth of this species (Rozas et al., 2015; Barbeta et al., 2019).

The distinct habitat of *F. sylvatica* and *Q. ilex* may also explain the different responses of these species to fog. As an evergreen sclerophyll, *Q. ilex* occurs in lower altitude areas, which consequently have less fog events (Corell et al., 2014), and are often subjected to stressful environmental conditions including water deficit (Fleck et al., 1998). However, the relationship between the occurrence of *F. sylvatica* and fog events is traditionally known (Bolòs 1983; Gutiérrez 1988; Barbeta et al., 2019), which is the reason for the dominance of this species in higher altitude locations (Peters 1997; Ellenberg 2009), corroborating the view that *F. sylvatica* is a drought-sensitive tree species. This statement can be reinforced by observing that the relationship between leaf duration and the presence of fog was in the highest altitude region, demonstrating the selective pressure of fog in this ecosystem.

Fagus sylvativa has the behavior of an isohydric species (Arando 2018), that is, during the dry season this plant species can control the stomatal opening, under ideal or moderate drought conditions, and consequently prevent a xylem hydraulic conductivity failure (Tardieu and Simonneau 1998; Peuke et al., 2002). However, under severe drought, this response may be disadvantageous, for hydraulic system cavitation may occur (Wortemann et al., 2011;

Choat et al., 2012; Gleason et al., 2016). The risk that hydraulic irreversibility dysfunction may occur demonstrates that *F. sylvatica* cannot withstand such a severe drought (Choat et al., 2012; Gleason et al., 2016). Current data corroborate *F. sylvatica* as drought-sensitive species (Backes and Leuschner 2000; Aranda et al., 2005; Bréda et al., 2006; Ellenberg, 2009; Cano et al., 2013; Bahamonde et al., 2018). However, *F. sylvatica* has some relative high resilience and ability to recover from moderate water stress (Sánchez-Gómez et al., 2013; Blessing et al., 2016; Bahamonde et al., 2018). Perhaps this resilience can be enhanced by the presence of fog as an alternative source of water.

We conclude *F. sylvatica* and *Q. ilex* had different physiological responses regarding photoinhibition prevention, water potential increasing, and improvement in vegetative indices in the presence of fog. Unheard information that the reduction of fog events provides decreased leaf duration of a deciduous species, *F. sylvatica*, in this case, is of great importance for understanding the environment functioning. *F. sylvatica* main response to the fog events is a delay in its leaf falling, which improves its growth (Barbeta et al., 2019), and physiology and phenology. This information can be useful in predicting the possible behavior of *F. sylvatica* and *Q. ilex* in a less foggy environment due to global climate change. *F. sylvatica* will be the most negatively affected species in all physiological and phenological parameters analyzed with decreasing fog events, since it was the most responsive to the presence of this type of precipitation. Therefore, fog is extremely important for the eco-hydrology of the Mediterranean mountain, where *F. sylvatica* and *Q. ilex* are the main species.

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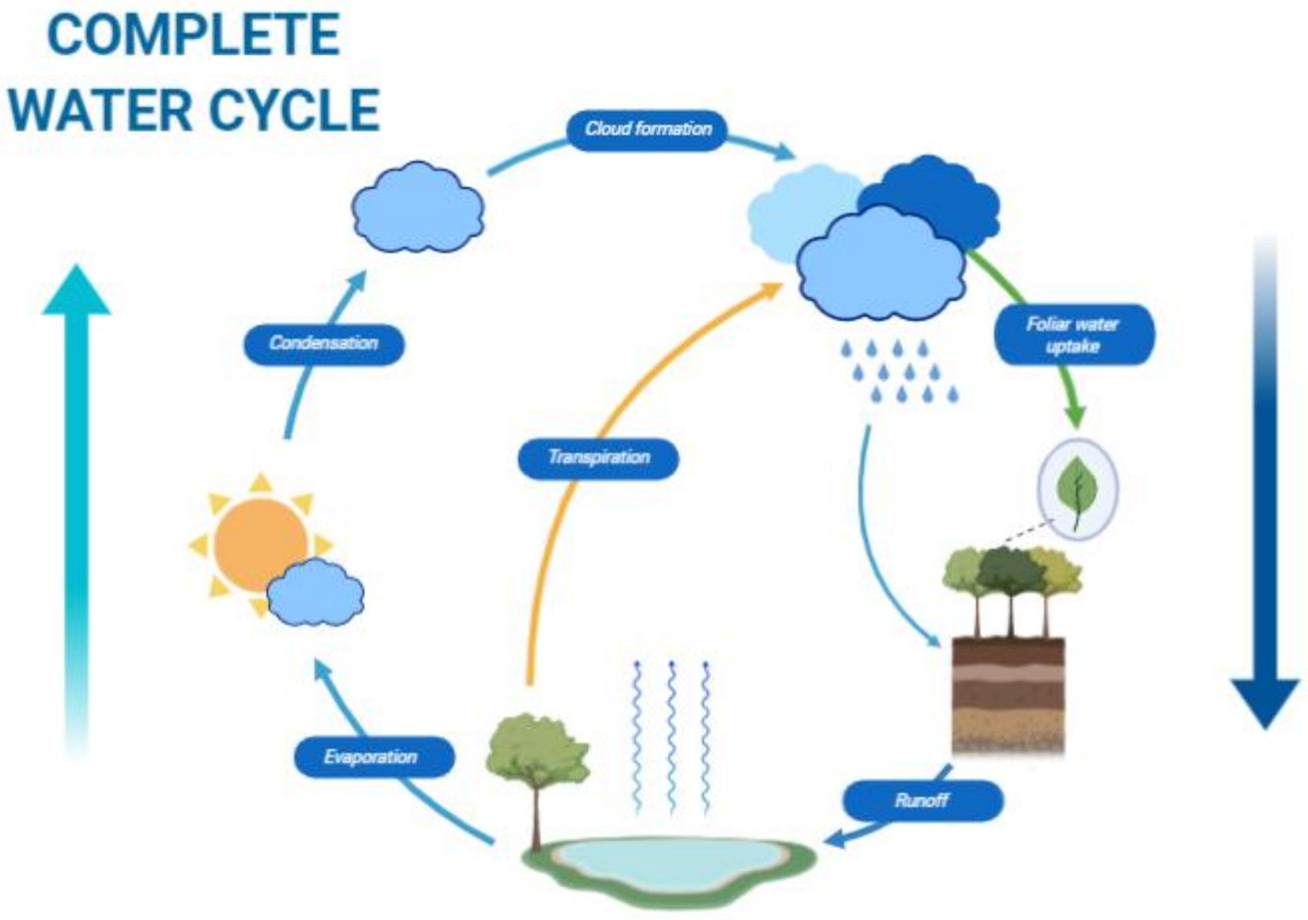
Chapter 7

Towards a more comprehensive understanding of water flux in plants



Chapter submitted to Trends in Plant Science

Towards a more comprehensive understanding of water flux in plants

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ABSTRACT

The reverse movement of water on the atmosphere - plant - soil continuum (APSC) driven by foliar water uptake has important consequences for plant performance and water flow in ecosystems. Here we discuss a novel perspective on the resistances that control the APSC in contrast to the soil-plant-atmosphere continuum (SPAC).

MAIN TEXT

Water movement through the soil-plant-atmosphere continuum (SPAC) has been traditionally seen as a unidirectional flux. Water flow starts from soil water absorption by plant roots through stems and leaves and diffuses to the atmosphere, in larger proportion, through transpiration, and to a lesser extent, by evaporation from leaf surfaces [1]. Water flow reversals induced by foliar water uptake (FWU) lead to an alternative water pathway [1] and here we propose to call this underappreciated pathway as atmosphere-plant-soil continuum (APSC). The bidirectional water movement is driven by differences in water potential among the atmosphere, leaves and soil as a consequence of the vapor pressure gradient generated among these compartments. This vapor gradient density between atmosphere and leaves is reduced, while relative air humidity between atmosphere and leaves is increased [1,2]. The objective of this commentary is to emphasize the differences between SPAC and APSC to highlight the importance of FWU as a major process driving water fluxes in ecosystems. Foliar water uptake starts when water overruns foliar surface due to vapor air saturation, resulting in full reversal in water potential. The reverse water flow from the atmosphere into the plant leaf tissues requires water to overcome different hydraulic resistances, which are not exactly the same as those in the SPAC pathway.

The first barrier that water encounters is the foliar boundary layer [3], where vapor density tends to be high and vapor-pressure deficit consequently tends to be low. This gradient of water vapor accordingly hinders the entrance of water through the foliar boundary layer. The saturation vapor pressure inside and outside the leaves equalizes and favors water vapor condensation. Liquid water, when reaching the foliar surface, adheres to superficial structures, such as emergences, trichomes or wrinkles in the cuticle [4], then takes this pathway of lower resistance and enters the foliar tissues.

The foliar surface is thus the second barrier to FWU [5,6], where trichomes, emergences, cuticular and stomatal structures and even endophytic fungi may contribute to the hydration of the foliar surface. These structures decrease water surface tension and can be a lower resistance pathway for water influx and to restore leaf hydraulic conductance [7]. The chemical nature of the cuticle is important to determine leaf surface properties and, constitutes a barrier to water entrance into foliar tissues [8]. The chemical properties of this layer vary with plant species climate conditions, and foliar phenology, leading to distinct FWU competences [8], since different proportions in the hydrophobic and hydrophilic structural compounds can occur [9].

After the cuticular barrier has been crossed, water will always flow towards sites of less resistance down the water potential gradient, either through the apoplastic or the symplastic pathway [3]. To cross the intercellular spaces, water must overcome gas pressure and the resistance in the middle lamella and cell walls, where pectins and cellulose [10] influence the permeability of the cell wall and influence FWU [10]. A high proportion of pectins confers porosity to cell walls and favors rapid water absorption, and a high proportion of cellulose confers hygroscopicity to cell walls, and favors high water absorption. Both cell-wall components strongly influence FWU through the cell walls [10]. Water can also enter foliar palisade cells or cells of the spongy parenchyma through plasmodesmata and aquaporins, integral membrane proteins, in the plasma membrane [2]. Water conductivity through the plasma membrane can be increased by a higher expression of genes encoding proteins of the aquaporin channels [2]. The regulation of aquaporin genes influences foliar wetting, which together with high atmospheric humidity increases FWU [2]. After reaching the symplast, water can be retained in the mucilaginous content of cells or can diffuse throughout the foliar mesophyll [2] as well as go through paraveinal mesophyll, an important site to water redistribution and storage [11].

Lignified vascular bundle sheaths represent the next barrier before water gets into xylem vessels [3], which reduces hydraulic conductance in the vascular bundles [11]. Phloem cells are an alternative pathway for water to reach the xylem vessels [2,12]. After water enters the vessels, hydraulic resistance may be influenced by the pits in the vessel walls parallel to the foliar surface, where occasional transpiration can induce an opposite water flow, increasing the resistance to water flow in APSS. Shoots and roots grow in opposite directions, so the water basipetal movement in the shoots, and its acropetal movement in the roots towards the root apices and soil, prevent transpiration. The soil-plant-atmosphere system flow can be completely reversed due to a favorable gradient of the soil-water potential attributed to the roots and transpiration suspension [1]. This reversed water flow has been observed in trunks and branches during precipitation and fog, in which the water absorbed by leaves reached the roots and occasionally the soil [1,13]. The resistance to water flow can increase if hydraulic conductivity varies due to the different calibers of the xylem vessels, which may imply an increase or decrease in water flow. In order for both the upward and downward movements start in roots and leaves, respectively, water flow will encounter resistance, which interferes with the entire water flow in bidirectional movements. Considering the downward movement, and all the resistances, especially that conferred by the cuticle, leaves impose the greatest resistance to FWU. However, water entrance through the leaves is not essentially the same as in the output movement. The cuticular pores and fissures, which are the main waterways for the APSC have much greater resistance compared to the ostiole and the boundary layer, when water vapor diffuses through the stomata in the SPAC. In contrast, for the upward flow, the greatest resistance to water flow from soil to the plant is found on the root endoderm, mainly due to the Casparian strips that force the water flow from the apoplastic to the simplastic pathway. Nevertheless, water absorption by the roots goes towards the driving forces of transpiration, driven by the established gradient of water potential, and propels the water into the atmosphere, causing the water upward flow to be greater than the downward movement.

About 9-70% of the water lost by transpiration can be recovered by FWU, depending on the species and environmental conditions [13-15]. FWU, although reduced for most species, plays a very important role in plant water balance, because it can rapidly increase water potential, reducing water deficit and supplying water to mesophyll cells or the vascular system [3,15]. The velocity of water absorbed, however, will depend on the traits of the foliar surface, mainly the composition and structure of the cuticle, which provide the major resistance to FWU. After this resistance is overcome, the ability of leaves to store water in their tissues can also interfere with the amount of water absorbed [4]. Most of the levels of hydraulic resistance observed during water flow from leaves to soil must overcome the biochemical composition of the leaves and the structural and anatomical traits of the entire plant body, i.e. leaves, stems and roots. These traits are genetically established but can also be strongly influenced by the environment, especially in plants with high phenotypic plasticity. Differences in environmental factors, such as solar radiation and precipitation (fog, rain and dew), modify the efficiency of FWU due to changes in molecular, anatomical and physiological plant traits. Plant plasticity enables distinct anatomical strategies, such as greater thickness of foliar tissues and presence of cells with mucilage, which affect FWU capacity [4]. The different FWU strategies: quickly and lower water absorption or slower and higher water absorption were characterized in some plant species [4]. That happens as a response to water deficits in individuals of a given vegetation type related to individuals of the same species from a site with less evaporative demand [4,15]. The flow of water in the APSC in recent years it has received a lot of attention with the aim of complementing the SPAC, since this bidirectional movement is more common than imagined [16].. We thus recommend considering the importance of resistances imposed to water flow in multiple directions and also an integrative morphological, structural, biochemical and physiological approach to obtain a better understanding of the entire water flow in plants.

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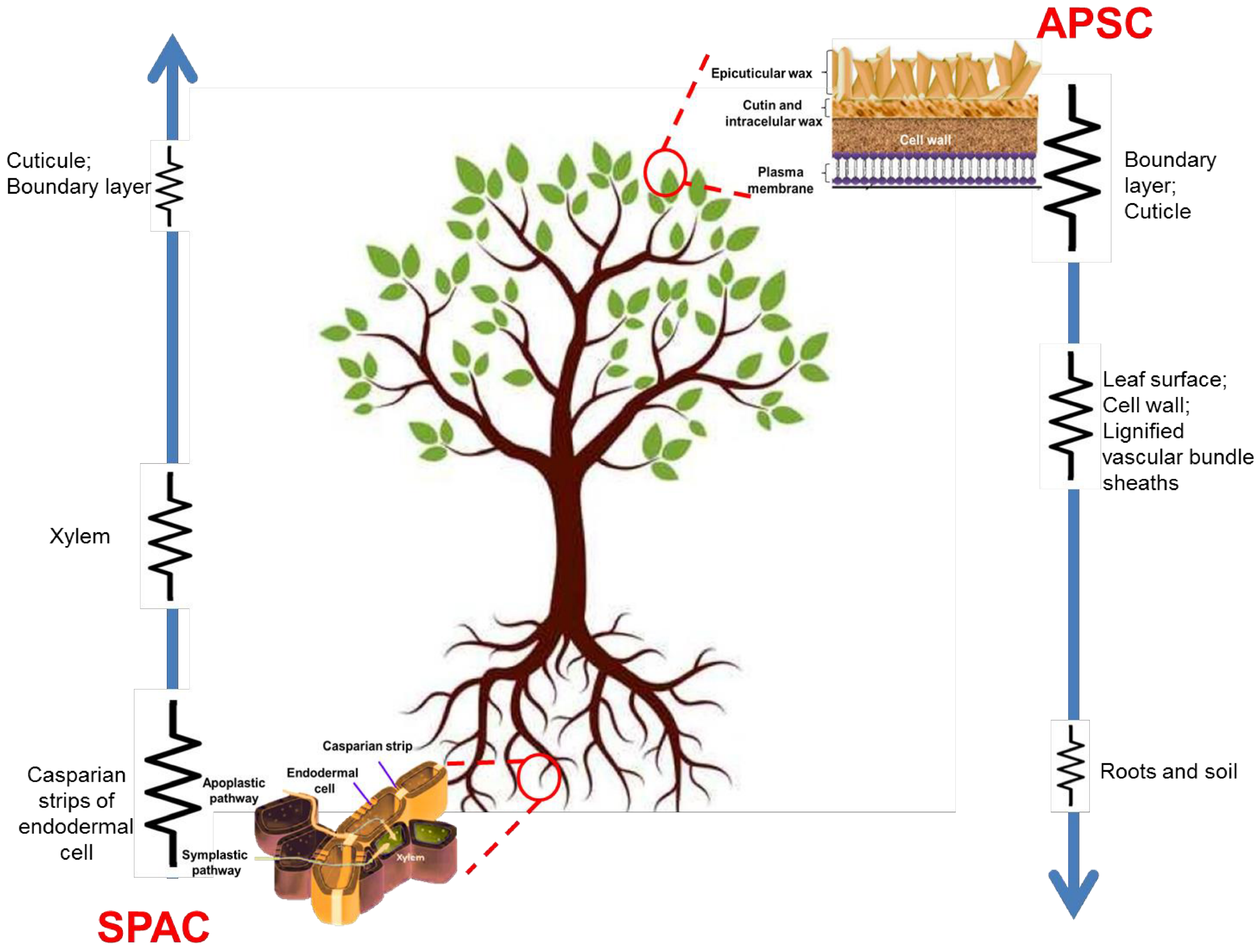
Figure caption



Scheme of resistances to ascending (SPAC) soil-plant-atmosphere continuum) and descending (APSC, atmosphere-plant-soil continuum) water flux. The size of resistance symbol represents the proportional resistances found respectively in SPAC and APSS water flux.

CONSIDERAÇÕES FINAIS

As plantas têm capacidades distintas de absorver água pelas folhas (foliar water uptake – FWU) e essa característica decorre de atributos particulares de cunho morfológico, anatômico e ultraestrutural. Esses atributos, consequentemente, contribuem para as diferentes respostas ecofisiológicas, de caráter bioquímico e fenológico. A possibilidade de absorver água pelas folhas pode influenciar na sobrevivência de espécies de um determinado ambiente, principalmente mediante as mudanças no regime de precipitação preditas nos modelos climáticos globais. Utilizando dois ecossistemas (Campo rupestre ferruginoso e ecótone-mediterraneo-temperado) e também sete espécies com diferentes capacidades de absorção foliar de água podemos verificar a influência da FWU em todos os atributos descritos acima e a importância da neblina para a ocorrência destas espécies nestes dois ambientes.

Quanto à ultraestrutura celular, foi mostrado que existe relação entre as ornamentações de cera epicuticular, composição química da cera cuticular e da parede celular com as diferentes capacidades de FWU. Verificou-se que a proporção dos componentes da cera e da parede celular interfere nas diferentes estratégias de absorver água rápido, porém em menor quantidade ou mais lentamente, mas em maior quantidade. Como perspectivas, o isolamento da cera cuticular para análises mais aprofundadas da permeabilidade cuticular permitirá o maior entendimento do fluxo reverso de água na epiderme das folhas.

Embora a FWU tenha interferido negativamente na assimilação de CO_2, essa interferência foi breve e evitou a perda de água através da transpiração, aumentando o status hídrico da planta na estação seca, onde a única forma de precipitação é a neblina. A neblina demonstrou ter um papel fundamental na melhoria do status hídrico das espécies na estação chuvosa e principalmente na estação seca na presença de neblina.

Verificou-se que entre as estratégias de FWU, as espécies que absorvem lentamente uma quantidade maior de água (*T. laniflora, P.heteromallum, M. corallina*, e *E. erytrhopappus*) tiveram melhor desempenho fotossintético com maior aumento da termotolerância e diminuição e/ou desaparecimento da fotoinibição comparado com as espécies que absorvem água mais rapidamente, mas em menor quantidade (*O. pulchella, B, variabilis* e *L. australis*). O metabolismo oxidativo também foi associado às estratégias de FWU. As espécies que apresentam a estratégia de maior velocidade de absorção foram também as mais sensíveis, já que houve aumento de espécies reativas ao oxigênio comparado com aquelas que possuem a estratégia de absorver mais lentamente maior quantidade de água.

Já foi demonstrado indiretamente em alguns destes parâmetros (trocas gasosas, eficiência fotossintética, termotolerância e metabolismo oxidativo) e diretamente através de medidas de potencial hídrico das plantas na estação seca com neblina, a importância da neblina no campo rupestre ferruginoso. Assim, visando avaliar a importância da neblina em outro ecossistema, foram utilizadas duas espécies do ecótone mediterrânico e temperado, visto que pesquisas da Universidade Autônoma de Barcelona, verificaram diferentes respostas no crescimento dessas espécies em relação à neblina. Como esperado, foram mostrados respostas fisiológicas diferentes em relação à prevenção de fotoinibição e melhora dos índices vegetativos na presença de neblina. Além disso, foi visto a interferência da neblina na fenologia de *Fagus sylvatica* até então nunca verificado, ou seja, a manutenção das folhas de uma espécie decídua em condições de estresse hídrico minimizado pela ocorrência de neblina. Com a diminuição nos eventos de neblina, ocorre uma aceleração da queda foliar. Para finalizar, compilamos todas as principais vias de entrada de água a partir das folhas para aprimorar o modelo o ciclo da água no sistema atmosfera-planta-solo.

Em conclusão, as plantas que apresentaram a estratégia de absorver lentamente maior quantidade de água tiveram maior sucesso nos parâmetros fotossintéticos, na termotolerância e no metabolismo oxidativo comparado com as espécies que absorvem água mais rapidamente mas em menor quantidade. Assim, o conhecimento de como cada estratégia interfere nestes atributos ajudará na escolha de espécies mais adaptadas aos futuros cenários climáticos para a revegetação destes ecossistemas, e também contribuirá na previsão do cenário das possíveis mudanças na paisagem e quais as espécies serão mais susceptíveis ou tolerantes a essas mudanças. Foi demonstrado que a neblina tem um papel importante para todas as espécies estudadas nos distintos ecossistemas (*T. laniflora, P.heteromallum, M. corallina*, *E. erytrhopappus, O. pulchella, B, variabilis*, *L. australis, F. sylvatica e Q. ilex*), sendo um fenômeno meteorológico importante e necessário para a sobrevivência destas plantas.

EPÍLOGO

Voar com asa ferida?
Abram alas quando eu falo.
Fiz, pequeno, quando o tempo estava todo do meu lado e o que se chama passado, passatempo, pesadelo só me existia nos livros.
Feliz, depois, dono de mim, quando tiver que escolher entre um abismo, o começo, e essa história sem fim.
Asa ferida, asa ferida,
Meu espaço, meu herói. A asa arde. Voar, isso não dói.

Paulo Leminski

A maior riqueza do homem é sua incompletude.
Nesse ponto sou abastado.
Palavras que me aceitam como sou
— eu não aceito.
Não aguento ser apenas um sujeito que abre
portas, que puxa válvulas, que olha o
relógio, que compra pão às 6 da tarde, que vai
lá fora, que aponta lápis, que vê a uva etc. etc.
Perdoai.
Mas eu preciso ser Outros.
Eu penso renovar o homem
usando borboletas.

Manoel de Barros

E valeu a pena!!!