UNIVERSIDADE FEDERAL DO CEARÁ

CENTRO DE CIÊNCIAS

DEPARTAMENTO DE BIOQUÍMICA E BIOLOGIA MOLECULAR

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

SILVIO ALENCAR CÂNDIDO SOBRINHO

ROLE OF GUARD CELL METABOLISM AND MESOPHYLL-DERIVED METABOLITES ON STOMATAL MOVEMENT REGULATION IN ANGIOSPERM AND FERN MODELS

FORTALEZA

2020

SILVIO ALENCAR CÂNDIDO SOBRINHO

ROLE OF GUARD CELL METABOLISM AND MESOPHYLL-DERIVED
METABOLITES ON STOMATAL MOVEMENT REGULATION IN ANGIOSPERM AND
FERN MODELS

FORTALEZA

2020

SILVIO ALENCAR CÂNDIDO SOBRINHO

ROLE OF GUARD CELL METABOLISM AND MESOPHYLL-DERIVED
METABOLITES ON STOMATAL MOVEMENT REGULATION IN ANGIOSPERM AND
FERN MODELS

Dissertação apresentada ao Curso de Mestrado Em Bioquímica do Departamento de Bioquímica e Biologia Molecular da Universidade Federal do Ceará, como requisito parcial à obtenção do título de Mestre em Bioquímica. Área de Concentração: Bioquímica Vegetal.

Orientador: Prof. Dr. Danilo de Menezes Daloso

Aprovada em: _____/_____/_____

BANCA EXAMINADORA

_____

Prof. Dr. Danilo de Menezes Daloso (Orientador)
Universidade Federal do Ceará (UFC)

_____

Prof. Dr. Humberto Henrique Carvalho
Universidade Federal do Ceará (UFC)

_____

Dr. Fabrício Eulálio Leite Carvalho
Universidade Federal do Ceará (UFC)

Aos meus pais, Eunice e Dário.

AGRADECIMENTOS

"Não seria a bondade, a recompensa da bondade?" (Alcorão, 55:60)

RESUMO

Plantas usam água em muitos processos fisiológicos como reações metabólicas, condução de seiva pelos vasos condutores e dissipação de calor via transpiração. Estômatos, estruturas encontradas principalmente na epiderme foliar, são compostos por duas células guarda e um poro, são a principal via de entrada de CO_2 para fotossíntese e de saída de água através da transpiração, sendo assim, reguladores chave da eficiência do uso da água da planta. Estímulos ambientais e endógenos combinadamente regulam os movimentos estomáticos através de mudanças no metabolismo das células guarda. No entanto, ainda não é claro como os mecanismos da regulação estomática desenvolveram-se como caracteres evolutivos. Esta pesquisa, disposta em dois capítulos, consiste em uma revisão sobre o metabolismo de células guarda (Capítulo I), e uma investigação de quais metabólitos e vias metabólicas estão envolvidas na regulação dos movimentos estomáticos em angiospermas e samambaias (Capítulo II). Neste último, iniciou-se a pesquisa a partir de uma meta-análise com dados de trocas gasosas e metabolômica encontrados em literatura. Posteriormente, elencamos os metabólitos glutamato, malato e sacarose para investigar a dinâmica de resposta estomática em feijão cáupi, e em duas espécies de samambaias sob aplicação exógena destes metabólitos. Utilizamos, também, um tratamento com ácido abscísico (ABA) e outro com manitol, este último como controle osmótico. Os resultados demonstraram que as espécies de samambaias utilizadas neste estudo não apresentaram mudanças na condutância estomática (g_s) sob ABA, enquanto uma drástica redução foi observada em angiosperma sob este fitormônio. A aplicação exógena de malato e sacarose induziu o fechamento estomático em todas espécies, porém com maior velocidade na angiosperma. Estes resultados sugerem que a menor velocidade de resposta e níveis de g_s amplamente observada nas samambaias, provavelmente envolve, também, alteração na resposta estomática à metabólitos oriundos das células mesofílicas. Assim, nossos resultados sugerem que as angiospermas, além de possuírem mecanismos sensíveis ao ABA, demonstram a contribuição do metabolismo das células do mesófilo para a velocidade da resposta estomática em angiospermas.

Palavras-chave: Angiospermas. Samambaias. Controle estomático. Evolução estomática. Metabolômica.

ABSTRACT

Plants use water in many physiological processes such as in metabolic reactions, sap conduction through vessels and heat quenching through transpiration. Stomata, structures mainly found at the leaf epidermis, are the main inlet of CO_2 for photosynthesis and outlet of water, being thus key regulators of plant water use efficiency. Combined environmental and endogenous stimuli regulate stomatal movements by changes in the metabolismo of guard cells. However, it remains nuclear how such mechanisms of stomatal regulation have developed as evolutive traits. This research, divided in two chapters, consists in a literature review of guard cell metabolism (Chapter I), and the investigation of which metabolites and metabolic pathways are involved on stomatal movement regulation in angiosperms and ferns (Chapter II). This later, started the research from a meta-analysis of gas enchange and metabolomics data available in literature. Further, the metabolites glutamate, malate sucrose were chosen to investigate the response ons tomatal dynamics in cowpea bean, and two fern species under exogenous application of these metabolites. Abcisic acid (ABA) and mannitol, this later used as na osmotic control were also used. Results demonstrate fern species used in this study did not show significant changes on stomatal conductance (g_s) using ABA, whilst a drastic reduction has been observed in angiosperms using this phytohormone. The application of exogenous malate and sucrose induced the stomatal closure in all species, however at higher intensity and velocity in angiosperm. This results suggest lesser g_s intensity and velocity observed in ferns, may be due changes on stomatal responses to metabolites driven from mesophyll cells. Thus, the results suggest angiosperms possess differential mechanisms which increase sensitivity to ABA and of the increased contribution of mesophyll-driven metabolites to velocity of stomatal responses in angioperms.

Keywords: Angiosperms. Ferns. Stomatal control. Stomatal evolution. Metabolomics.

CONTENTS

1 GENERAL INTRODUCTION

Plants acquired different traits during their evolutionary course that granted them to adapt to the terrestrial environment. The development of stomata, leaf epidermal structures composed by a pore surrounded by a pair of guard cells, was essential for land plant colonization. (LAWSON, 2009). Changes in guard cell metabolism are responsible for adjusting stomatal aperture thus controlling gas exchange between leaf and the external environment (CHATER et al., 2017). Thus, beyond being ecologically important for the maintenance of both CO_2 and H_2O biogeochemistry cycles, stomatal movements are the master regulators of plant water use efficiency (*WUEi*) by directly influencing both transpiration and photosynthesis.

Photosynthesis dynamically fluctuates throughout the day according to the availability of several environmental cues such as light, CO_2, water and nutrient supply (MCAUSLAND et al., 2016). Beyond that, several endogenous and environmental signals such as CO_2 concentration, light exposure, phytohormones and ions directly affect stomatal conductance (g_s), which, in turn, modulates the daily course of photosynthesis. Thus, photosynthesis and stomatal movements are tightly regulated. Evidence supporting this idea comes from the fact that photosynthetic and mesophyll-derived metabolites exert great influence on stomatal regulation (LAWSON et al., 2014; STADLER, 2003). However, it has been shown that the net photosynthetic rate quickly adjusts to environment variations whilst g_s adjust at a slower rate, leading to an undesirable reduction in *WUEi* under certain circumstances. (LAWSON; VIALET-CHABRAND, 2019). Notably, it is clear that angiosperms have acquired a higher velocity of stomatal responses when compared to basal lineage of plants (BRODRIBB et al., 2009; BRODRIBB; MCADAM, 2011; LIMA et al., 2019). However, it is unclear which mechanisms enable angiosperms to respond faster to changes in CO_2, light/dark and ABA.

Here, we provided an update on guard cell metabolism in the first chapter and investigated the role of leaf metabolism on stomatal movement regulation in ferns and angiosperms in the second chapter. Considering that there are conserved mechanisms governing g_s along with plant evolution, we revisited gas exchange and metabolomics data from angiosperms and ferns available in the literature and explored them by using different statistical approaches aiming to investigate which metabolites (or metabolic pathways) modulate g_s responses in these plant groups. We further extended the metabolic characterization of ferns and angiosperms by performing a LC-MS-based metabolite profiling

of leaves harvested throughout the day. Lastly, we carried out an experiment to verify the importance of mesophyll-derived metabolites on g_s dynamics in cowpea bean (*Vigna unguiculata* (L.) Walp) and two ferns, the evergreen monark fern (*Microsorum scolopendria*, Burm f.) and the golden polypody (*Phlebodium aureum* (L.) J.Sm.).

2 HYPOTHESIS

Key metabolites derived from mesophyll impact on stomatal movement regulation.

3 OBJECTIVES

The aim of this study was to verify the impact of mesophyll-derived metabolites which can regulate stomatal conductance.

3.1 Specific objectives

- Assess differences on primary and secondary metabolism along the groups;
- Assess key metabolites on stomatal conductance modulation (g_s);
- Point key metabolites and pathways as candidates for further strategies involving improving water use efficiency.

4 CHAPTER I - GUARD CELL METABOLISM

(Published book chapter in Encyclopedia of Life Sciences)

Cândido-Sobrinho, S. A.[1], Lima, V. F.[1], & Daloso, D. M[1]*.

[1] Departamento de Bioquímica e Biologia Molecular da Universidade Federal do Ceará, Fortaleza (Ceará). Ac. Público 907, CEP: 60.020-181, Brasil.
* Corresponding author

Tel: + 55 (85) 3366-9821
E-mail address: daloso@ufc.br

Abstract

Guard cells are found in leaf epidermis as a pair of cells surrounding the stomatal pore. Stomatal aperture is regulated by the metabolism of guard cells according to the prevailing environmental condition. Guard cell metabolism is thus adapted to support stomatal movement, which demands a high control of osmolytes content within the cell as well as the need to balance the exchange of ions and metabolites between the cytoplasm and the apoplastic space. Guard cells have several characteristics of sink cells. This is probably an alternative given the low photosynthetic rate and the few number of chloroplasts found in these cells. The glycolysis, the tricarboxylic acid cycle and the degradation of starch and lipids seem to be activated in the light to support stomatal opening. This indicates that guard cell metabolism is differentially regulated compared to mesophyll cells. The particularities of guard cell metabolism are reviewed here.

Keywords: glycolysis; malate; photosynthesis; respiration; stomatal movement; sucrose; tricarboxylic acid cycle.

Introduction

Life on earth depends on photosynthetic organisms such as cyanobacteria, algae and plants. The photosynthetic process of C3 plants is basically separated in two inter-connected phases, the photochemical reactions that absorb light and generated O_2 and chemical energy for the synthesis of NADPH and ATP, and the biochemical reactions of the Calvin-Benson cycle in which the CO_2 is fixed by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). The flux of CO_2 from the atmosphere to the site of carboxylation of RuBisCO has two main resistances, firstly the aperture of the stomatal pore and afterwards the resistance of mesophyll cells. Stomata is a complex composed by two guard cells that surround a pore, by which its aperture enable the exchange of CO_2, H_2O and other gases between plant and atmosphere. Thus, stomatal aperture not only regulates the amount of CO_2 for photosynthesis, but also the rate of water loss by the transpiration process. Given the importance of these processes for plant growth and also to maintain the dynamic of the CO_2 and H_2O biogeochemistry cycles, the regulation of stomatal movement has been extensively studied. Furthermore, understanding the functioning and the regulation of guard cell metabolism is of pivotal importance to comprehend how plants growth and tolerate different (a)biotic stresses (GAGO et al., 2014). See also: DOI: 10.1002/9780470015902.a0025268, DOI: 10.1002/9780470015902.a0026526

Plant cells are divided in sink or source cells, which depends on the capacity of the cell to produce and export photoassimilates. Guard cells seem likely to be a sink-like cell. This idea is based in the fact that guard cells have few and small chloroplasts, low level of chlorophyll (WILLMER; FRICKER, 1996) and low content and activity of RuBisCO (RECKMANN; SCHEIBE; RASCHKE, 1990), which leads to a limited photosynthetic capacity (GOTOW; TAYLOR; ZEIGER, 1988). Guard cells have also high activity of sink marker enzymes (DALOSO et al., 2015) and high expression of sugar transporters (BATES et al., 2012), which may be related to the import of sugars to the cell (RITTE et al., 1999). Given these characteristics, guard cell metabolism has tremendous differences compared to the neighbour mesophyll cells. For instance, given the low photosynthetic rate found in guard cells, it seems likely that the main function of the chloroplast metabolism is to meet the demand of reducing power rather than producing photoassimilates via the Calvin-Benson cycle (Figure 1a).

Beyond their lower photosynthetic capacity, evidence suggests that the distribution of the C photosynthesized is also different in guard cells. The C photosynthesized

by the mesophyll cells is mainly used to sustain starch and sucrose synthesis (Figure 1a). Several plants present a linear increase in starch content along the diel course, as a mechanism to store C for the subsequent dark period. By contrast, starch metabolism is differentially regulated in guard cells, in which it is degraded in the initial light period rather than accumulated (Figure 1b) (HORRER et al., 2016). Furthermore, the use of sucrose and the regulation of glycolysis and the mitochondrial metabolism are also differentially regulated in guard cells and seem to work to meet the demand of osmolytes needed for stomatal aperture regulation. It seems likely therefore that guard cells modulate their metabolism according to the environmental condition that leads to stomatal opening or closure. Here we describe the particularities and the differences in the regulation of guard cell metabolism compared to mesophyll cells and how guard cell metabolic pathways work to sustain stomatal movements.

Guard Cell Photosynthetic Metabolism

Plant metabolism depends fundamentally on the CO_2 assimilated by the photosynthetic process of photoautotrophic tissues. The CO_2 is mainly fixed by reactions catalysed by either RuBisCO or phospho*enol*pyruvate carboxylase (PEPc). The assimilated CO_2 is incorporated in a metabolite of 3 carbons (C3 cells) or 4 carbons (C4 and CAM cells) and used either in the source photosynthesizing tissue or exported to sink tissues such as young leaves and non-green organs. Leaves thus represent the most important photosynthesizing organ. By contrast, guard cells have fewer and smaller chloroplasts, lower chlorophyll and RuBisCO content and their thylakoid structures appear less developed, presenting less grana stacks, compared to mesophyll cells (RECKMANN; SCHEIBE; RASCHKE, 1990; WILLMER; FRICKER, 1996). These characteristics suggest that guard cell photosynthetic capacity is limited. Indeed, guard cell CO_2 fixation rate is generally lower than mesophyll cells (GOTOW; TAYLOR; ZEIGER, 1988; OUTLAW; TARCZYNSKI; ANDERSON, 1982), enquiring whether guard cell photosynthesis is sufficient to sustain their own energetic and metabolic demands. See also: DOI: 10.1038/npg.els.0001385

Guard cell CO_2 metabolism has been subject of long debate. For instance, RuBisCO-mediated CO_2 fixation was initially proposed to be absent in guard cells (OUTLAW et al., 1979). However, this idea has changed after the confirmation of the presence of RuBisCO in guard cells by mass spectrometry approaches (ZHAO et al., 2008) and by the fact that the ^{13}C photosynthetic flux distribution indicates that guard cells are able to fix CO_2 by both RuBisCO and PEPc (DALOSO et al., 2015). Given the low level of

RuBisCO found in guard cells and the fact that the guard cell $^{14}CO_2$ fixation is mainly incorporated into metabolites of the C4 cycle, PEPc-mediated CO_2 fixation has long been proposed as the main photosynthetic pathway (OUTLAW, 2003). Initial study using *Vicia faba* guard cell protoplasts supplied with $^{14}CO_2$ indicate that 95% of the dark-incorporated CO_2 has been assimilated in stable products derived from PEPc such as aspartate and malate (BROWN; OUTLAW, 1982). This evidence suggests that guard cells may have a C4-like metabolism having malate synthesis as primary photosynthetic pathway. In fact, several lines of evidence indicate that malate metabolism is key for stomatal movement regulation (discussed below in the section Respiratory metabolism), which supports the idea of a C4-like metabolism in guard cells. However, despite these metabolic evidences, a recent RNAseq-based transcriptomic study suggests that genes of the C4 metabolism are not highly expressed in guard cells compared to mesophyll cells (AUBRY et al., 2016), as preconized by previous microarray-based transcriptomic studies (BATES et al., 2012). It remains unclear therefore whether guard cells present the C4-cycle mode of CO_2 fixation or, alternatively, have a particular non-yet described mode of CO_2 fixation.

Beyond their capacity to fix CO_2, the O_2 evolution and the linear electron transport rate (ETR) has also been reported in guard cells. Initial studies sought to elucidate if guard cell chloroplasts have functional photosystems, demonstrating that in fact guard cells have both photosystems I and II, slightly lower ETR but three times higher O_2 evolution (on chlorophyll basis) compared to mesophyll cells (LAWSON et al., 2002; SHIMAZAKI, 1989; TOMINAGA; KINOSHITA; SHIMAZAKI, 2001). Recent evidence suggests that guard cell chloroplasts are essential to generate the ATP and NADPH required for guard cell turgor control, given that stomata of chlorophyll-less guard cells are not able to open (AZOULAY-SHEMER et al., 2015). This is probably due the fact that chloroplastic-derived ATP may be important to maintain plasma membrane H^+-ATPase activity, which is pivotal to generate a H^+ gradient across guard cell plasma membrane and thus enable K^+ influx and consequently stomatal opening (Figure 1a). It seems likely therefore that stomatal opening depends on cytosolic levels of ATP and NADPH produced by photophosphorylation. See also: DOI: 10.1002/9780470015902.a0021630

Given the apparent lower photosynthetic capacity exhibited by guard cells, it is reasonable to hypothesize that these cells have lower capacity to produce both starch and sucrose. However, guard cell sucrose and starch metabolism have been linked to the regulation of stomatal movements. In the next section, we highlight the particularities and the

importance of both guard cell sucrose and starch metabolism for the regulation of stomatal movement.

Sucrose and Starch Metabolism in Guard Cells

Sucrose is the major photoassimilate transported throughout vascular plants. As stated previously, guard cells have low photosynthetic rate and thus low capacity to produce sucrose. Therefore, the transport of sucrose from mesophyll to guard cells is important to maintain the C feedstock of these cells (Figure 1a). The leaf photosynthetic-derived sucrose is exported to sink tissues via the phloem and to guard cells via the transpiration stream (LU et al., 1997). Since most of mature guard cells lack plasmodesmata connection (WILLMER; FRICKER, 1996), sucrose uptake into guard cells is mediated by sucrose transporters or, alternatively, by hexose transporters, when sucrose is degraded into hexoses by cell wall invertase (RITTE et al., 1999). Beyond that, sucrose can also be produced via starch breakdown and gluconeogenesis within guard cells, after the activity of PEPc, pyruvate carboxylase, PEP carboxykinase and several other enzymes (Figure 1a). However, the extent by which sucrose can be produced by either of these pathways remain unclear. See also: DOI: 10.1002/9780470015902.a0021259.pub2

Sucrose has multifaceted roles in guard cells. Evidence suggests that guard cell sucrose metabolism is involved in the regulation of both stomatal opening and closure and also in the trade-off between photosynthesis and stomatal conductance (GAGO et al., 2016; KELLY et al., 2013a; MEDEIROS et al., 2018). During stomatal opening, sucrose was initially proposed to act as an osmolyte. However, recent results support the idea that the role of sucrose in this process is primarily energetic, by providing C skeletons to sustain glycolysis, a differential non-cycle mode activity of the tricarboxylic (TCA) cycle and glutamine biosynthesis (Figure 1a) (DALOSO et al., 2015, 2016; MEDEIROS et al., 2018). On the other hand, in periods of high photosynthetic rate, it has been suggested that the sucrose produced in the mesophyll cells would induce stomatal closure by its overaccumulation in the apoplastic space of guard cells (LU et al., 1997) or, alternatively, by its dissimilation within guard cells in a mechanism mediated by abscisic acid (ABA) and dependent of hexokinase activity (KELLY et al., 2013a).

In plants, not all CO_2 assimilated is used for sucrose synthesis and then transported to sink tissues; part of them is stored as starch in leaf chloroplasts. The rate of starch synthesis and degradation ranges according to the light condition. Substantial

differences are found in the mechanisms by which mesophyll and guard cells regulate starch turnover. For instance, mesophyll cells gradually accumulate starch during the day and degrade during the night to avoid C starvation before the end of the night (DOS ANJOS et al., 2018). By contrast, a different circadian rhythm is observed in guard cells. It has been shown that the dark-to-light transition trigger starch degradation (ANTUNES et al., 2017), in a mechanism mediated by the blue-light photoreceptors, phototropins (Figure 1b) (HORRER et al., 2016). Light-induced starch degradation would be a mechanism to induce the accumulation of malate, an important counter-ion of K^+ in guard cells (Figure 1a). Although still not clear, this contrasting circadian rhythm between mesophyll and guard cells may be due to a differential regulation of key enzymes of starch metabolism. For instance, whilst leaf enzymes related to the synthesis and degradation of starch are activated and inhibited in the light, respectively, the opposite may be observed in guard cells. In fact, guard cells preferentially use isoforms for starch degradation that are not required by leaf starch metabolism under non-stressed condition, such as β-amylase 1 (BAM1) and α-amylase 3 (AMY3) (Figure 1a) (ZANELLA et al., 2016). See also: DOI: 10.1002/9780470015902.a0020124.pub2plant

Starch metabolism has also been shown to be involved in stomatal movement regulation. Reverse genetic study demonstrated that starch synthesis in guard cells, but not in mesophyll cells, is crucial for CO_2 stomatal responses (AZOULAY-SHEMER et al., 2016), although this response may vary according to the day length in which the plants are grown (AZOULAY-SHEMER et al., 2018). It has been proposed that starch synthesis in guard cells chloroplasts would act as a sink for C skeletons during stomatal closure. As a non-osmolytic metabolite, the synthesis of starch would be important to remove the excess of osmolytes within guard cells. Hence, disrupted starch synthesis implicates in an impairment of guard cells capability to close the stomatal pore.

According to the difference in starch turnover among mesophyll and guard cells, it is likely that specific mechanisms differentially regulate starch metabolism in each cell type. Both the diel leaf starch dynamics and the stomatal movements are under control of the circadian clock. Additionally, recent evidence suggests that trehalose-6-phosphate (T6P), a sucrose-signaling metabolite, is also involved in the regulation of starch metabolism and stomatal movements (FIGUEROA; LUNN, 2016). However, although the role of T6P in modulating the rate and the velocity of leaf starch degradation has been recently demonstrated (DOS ANJOS et al., 2018), few studies have investigated the function of the trehalose metabolism in guard cells. Interestingly, guard cells display high expression of trehalose-

related genes (BATES et al., 2012). Furthermore, Arabidopsis *tre1* mutant, which lacks the trehalase 1 enzyme, shows impairment in ABA or drought-induced stomatal closure (VAN HOUTTE et al., 2013). This result suggests that trehalose metabolism is also involved in the regulation of stomatal movement. However, it remains unclear whether this response is related to the T6P-mediated mechanisms of starch metabolism regulation.

Respiratory metabolism

Glycolysis and the subsequent biochemical reactions in the mitochondria are part of the respiratory metabolism. Respiration is the main source of ATP in animal cells. Plants, notwithstanding, also produce ATP in the chloroplasts and have additional components in the mitochondrial electron transport chain such as the alternative oxidase (AOX) enzyme. The glycolytic pathway has several bi-directional reactions. When these reactions occur in the direction of glucose synthesis, it receives the name of gluconeogenesis. The function of gluconeogenesis in guard cells is obscure. It is known that the knockout of *PHOSPHOENOLPYRUVATE CARBOXYKINASE 1*, a gluconeogenic enzyme responsible for the conversion of oxaloacetate (OAA) to PEP, alters the stomatal responses to light (PENFIELD et al., 2012). This suggests that this enzyme is important for the stomatal aperture regulation. However, the idea is that this effect is mainly due an altered malate metabolism rather than a disruption in the gluconeogenic pathway. In fact, guard cell malate metabolism has long been pointed out as an important regulator of stomatal aperture. Several plants with altered malate metabolism shown variation in stomatal movements (ARAÚJO et al., 2011; DONG et al., 2018; MEDEIROS et al., 2016, 2017). The function of malate seems to be different according to the subcellular location. It has been shown that malate acts as a counter ion of potassium (K^+) in the vacuole, as an activator of vacuolar chloride channels or plasma membrane S-type anion channels or may serve as respiratory substrate in the numerous mitochondria found in guard cells (Figure 1a) (LAWSON et al., 2014). By contrast to the observed during light-induced stomatal opening condition, malate is either degraded to produce starch (Figure 1a) or released from guard cells through anion channels during stomatal closure conditions (HEDRICH; MARTEN, 1993; MEDEIROS et al., 2016). Guard cell malate metabolism is thus key for stomatal movement regulation. Given this, several characteristics of guard cell metabolism such as CO_2 fixation *via* PEPc (DALOSO et al., 2015) and blue light-induced starch degradation (HORRER et al., 2016) seems to occur to sustain a high rate of malate synthesis (Figure 1a). Furthermore, several enzymes of malate

metabolism such as malate dehydrogenase dependent of NAD and NADP, NADP-malic enzyme, succinate dehydrogenase and fumarase have higher activity in guard cells compared to mesophyll cells (WILLMER; FRICKER, 1996), indicating the importance of malate metabolism for guard cells. See also: DOI: 10.1002/9780470015902.a0001301.pub3

Given the few number of chloroplasts found in guard cells, the mitochondrial metabolism assumes a pivotal importance for the maintenance of guard cell homeostasis, especially under light conditions. Indeed, guard cells are known to have numerous mitochondria and higher respiration rate than mesophyll cells (ARAÚJO et al., 2011; WILLMER; FRICKER, 1996). It has been shown that several proteins of the chloroplast and mitochondrial metabolism are redox regulated, which makes them to be sensitive to the redox state of the cell and thus to be differentially regulated by light and dark conditions. In this vein, evidence indicates that leaf respiration is inhibited in the light, although this does not seem to be the case of guard cells. In leaves, key mitochondrial enzymes such as pyruvate dehydrogenase, fumarase and succinate dehydrogenase are deactivated in the light (GEIGENBERGER et al., 2017), which inhibit the flux of C to the tricarboxylic acid (TCA) cycle (TCHERKEZ et al., 2012). By contrast, the C coming from PEPc fixation or from sucrose breakdown seems to be used by the TCA cycle either to sustain malate or glutamine biosynthesis (DALOSO et al., 2015; MEDEIROS et al., 2018). Furthermore, recent evidence suggests that lipid degradation by β-oxidation pathway and the peroxissomal malate-acetate shunt are also important for malate synthesis and stomatal opening (DONG et al., 2018; MCLACHLAN et al., 2016). Taken together, these results suggest that the C fluxes through the TCA cycle are differentially regulated between mesophyll and guard cells. What remains unclear is which mechanisms lead to such differential regulation in guard cells. Given the importance of post-translational modifications for the regulation of plant metabolic networks, it is reasonable to assume that guard cell enzymes may be differentially regulated according to the light/dark and consequently redox conditions. This idea is supported by the fact that pyrophosphate-dependent phosphofructokinase (PFP), a glycolytic enzyme, is activated during dark-to-light transition in guard cells (HEDRICH; RASCHKE; STITT, 1985). It seems likely therefore that both glycolysis and the TCA cycle are differentially regulated in guard cells. Further studies aiming to identify how enzymes from glycolysis and the TCA cycle are regulated in guard cells will certainly provide important information to improve our understanding on the regulation of guard cell metabolism.

Lipids

Lipids comprise a group of diverse, biologically active non-polar metabolites. Unlike the other major constituents of plants (proteins, carbohydrates, and nucleic acids), lipids are defined on the basis of their physical properties rather than their common chemical structure. Fatty acids and acylglycerols are important energy and carbon sources, whilst phospholipids, glycolipids and ether lipids are important constituents of plant cell membranes. By contrast of animal cells, the synthesis of plant lipids involves an interconnection between plastids and endoplasmic reticulum. Notably, the plastidial-generated fatty acids can be converted into glycerolipids within the plastids (namely the prokaryotic pathway) or transported out to the cytosol and endoplasmic reticulum via the eukaryotic pathway (NEGI et al., 2018). Plant lipid metabolism has received great attention in the last years given their potential for biofuel production. The regulation of both leaf and seed lipid metabolism are relatively well-understood, in which key regulators have already been described (ZHAI et al., 2017). However, lipid metabolism is relatively less investigated in guard cells. Initial studies have reported that the content of both storage and structural lipids differs compared to mesophyll cells. Remarkably, guard cells have higher content of phosphatidylcholine, phosphatidyilinositol and C18 fatty acids, whilst the content of C16, C20 and C22 fatty acids are comparable to those found in mesophyll cells (SAKAKI et al., 1995; SATO, 1985). Recent evidence suggests that guard cell lipid synthesis rely on the eukaryotic rather than the prokaryotic pathway (NEGI et al., 2018), which is in agreement with the fact that guard cell plastids are usually smaller and less developed.

Lipids are known to be a source of storage carbon in plant cells, which is important for hormone metabolism and stress responses. In animal cells, the degradation of phosphatidyilinositol produce diacylglycerols (DAG) that acts as a second-messenger activating Ca^{2+} channels (EXTON, 1988). In this context, it has been proposed that sphingolipids, another class of structural lipids, modulates plant response to (a)biotic stress conditions via ABA and Ca^{+2} signalling pathway (NG et al., 2001). For instance, it was shown that sphingosine-1-phosphate induces stomatal closure by increasing the cytosolic content of Ca^{2+} (NG; HETHERINGTON, 2001). It seems reasonable to assume therefore that guard cell lipid metabolism may be important under ABA-enriched conditions such as drought stress, in which ABA and Ca^{+2} are key for stomatal closure and consequently for the maintenance of water use efficiency.

Lipids and proteins form single-layered globules in most plant cells, known as lipid droplets, which has been shown to be abundant in epidermal and guard cells (PAUTOV

et al., 2016). Lipid droplets have also been implicated with the regulation of stomatal movements. As discussed above, it is well-known that the activity of H^+-ATPase is of pivotal importance for stomatal movement regulation. It has been shown that blue-light triggers starch and triacylglycerol (TAG) breakdown with concomitant H^+-ATPase activation in a mechanism dependent of phototropins (Figure 1a) (HORRER et al., 2016; MCLACHLAN et al., 2016). Interestingly, the blue light-induced stomatal opening is delayed in TAG catabolism mutant. This suggests that TAG breakdown would be a mechanism to feed β-oxidation and mitochondrial ATP production, which would be then used to sustain H^+-ATPase activity under blue light-induced stomatal opening condition (Figure 1a). Thus, guard cell lipid metabolism is also adapted to meet the energetic demands of stomatal movements. Whilst TAG breakdown is important during stomatal opening, sphingosine-1-phosphate promotes stomatal closure through Ca^{+2} signalling, highlighting the importance of guard cell lipid metabolism for stomatal movement regulation. See also: DOI: 10.1002/9780470015902.a0027976plant

Conclusion

Guard cells have been extensively used as model to understand phytohormone signalling pathways. However, the study of guard cell metabolism has been relatively neglected in the last decades. The use of genetic reverse and mass spectrometry approaches in guard cells has improved our understanding of guard cell metabolism regulation. Recent evidence suggests that glycolysis, starch degradation, lipid degradation and the malate-acetate shunt are important mechanisms to feed the TCA cycle, which may work to sustain malate and/or glutamine biosynthesis in the light. Interestingly, the opposite is observed in mesophyll cells, in which the photosynthetic C is used to sustain sucrose and starch synthesis in the light. Given that trehalose-6 phosphate and the circadian clock control the trade-off between leaf starch and sucrose metabolism, it remains unclear whether these mechanisms of regulation are also observed in guard cells. It is clear therefore that further metabolomic and proteomic studies aiming to identify how guard cell enzymes and their pathways associated are regulated assumes a paramount importance to improve our understanding on how guard cell metabolism is regulated.

Acknowledgments

Glossary

Stomata – Structure located on the surface of leaf tissues. Stomata complex consists of microscopic pores surrounded by a pair of guard cells and, in certain cases, by subsidiary cells. Stomatal pore is thus an aperture in the leaf that enables the exchange of gases between the plant and the surrounding atmosphere.

Phototropins – Blue-light photoreceptors located at the plasma membrane of guard cells. Phototropins are thus the proteins responsible for the recognition of blue light. As consequence, blue light perception induces several metabolic changes within guard cells leading to stomatal opening.

Source and sink cells – Plant cells are identified as sink or source cells. These terms are designated to cells, tissues and organs related to their capacity to produce and export photoassimilates. The cell that is able to produce their own photoassimilate, sustain their own metabolism and still be able to export to the phloem is called source cells. By contrast, cells that import photoassimilates are called sink cells. Fully developed leaves are the most common source organ whilst young leaves, stems, roots, flower, fruits and seeds are typical sink organs.

C3, C4 and CAM plants – Plant cells can also be identified by their mode of CO_2 fixation. The CO_2 can be assimilated by two main enzymes namely Ribulose,1-5,bisphosphate carboxylase/oxygenase (RuBisCO) and phospho*enol*pyruvate carboxylase (PEPc). The CO_2 fixation mediated by RuBisCO and PEPc produce a molecule of 3 (C3) or 4 (C4) carbons, respectively. Thus, the mode of CO2 fixation of a cell is determined by which enzyme RuBisCO or PEPc is the main carboxylase of tre cell, in which in C3 cells the CO_2 is mainly fixed by the RuBisCO while in C4 and CAM cells the CO_2 is mainly fixed by the PEPc. RuBisCO and PEPc have several biochemical differences and are located in different subcellular compartments, which the first is chloroplastic and the second a cytosolic enzyme.

Although C4 and CAM plants have PEPc as the main carboxylase, the latter fix CO_2 in the dark, which is due, in part, by the unique pattern of stomatal opening in the night.

Feeding experiments – Consist on experiments that feed cells, tissues, organs or the entire organism with substrates enriched with radioactive or stable labelled isotopes (e.g,: ^{13}C, ^{14}C, ^{15}N). This type of experiment has long been used to understand the distribution of different C sources in guard cells. The incorporation of the supplied labelled isotope and its distribution throughout guard cell metabolism is determined either by radiolabeled techniques or mass spectrometry approaches. Nuclear resonance magnetic can also be used to perform such analysis, but its use has not yet been applied to the study of guard cell metabolism.

Further reading list

Daloso D.M., dos Anjos L. & Fernie A.R. (2016) Roles of sucrose in guard cell regulation. *New Phytologist* **211**, 809–818.

Daloso D.M., Medeiros D.B., dos Anjos L., Yoshida T., Araújo W.L. & Fernie A.R. (2017) Metabolism within the specialized guard cells of plants. *New Phytologist* **216**, 1018–1033.

Engineer C., Hashimoto-sugimoto M., Negi J., Israelsson- M., Azoulay-shemer T., Rappel W., … Jolla L. (2016) CO2 sensing and CO2 regulation of stomatal conductance: advances and open questions. **21**, 16–30.

Lawson T. (2009) Guard cell photosynthesis and stomatal function. *New Phytologist* **181**, 13–34.

Lima V.F., Medeiros D.B., Dos Anjos L., Gago J., Fernie A.R. & Daloso D.M. (2018) Toward multifaceted roles of sucrose in the regulation of stomatal movement. *Plant Signaling & Behavior* **00**, 1–8.

Misra B.B., Acharya B.R., Granot D., Assmann S.M. & Chen S. (2015) The guard cell metabolome: functions in stomatal movement and global food security. *Frontiers in Plant Science* **6**, 1–13.

Santelia D. & Lawson T. (2016) Rethinking Guard Cell Metabolism. *Plant Physiology* **172**, 1371–1392.

Santelia D. & Lunn J.E. (2017) Transitory Starch Metabolism in Guard Cells: Unique Features for a Unique Function. *Plant Physiology* **174**, 539–549.

Vavasseur A. & Raghavendra A.S. (2005) Guard cell metabolism and CO_2 sensing. *New Phytologist* **165**, 665–682.

Zeiger E., Talbott L.D., Frechilla S., Srivastava A. & Zhu J. (2002) The guard cell chloroplast: A perspective for the twenty-first century. *New Phytologist* **153**, 415–424.
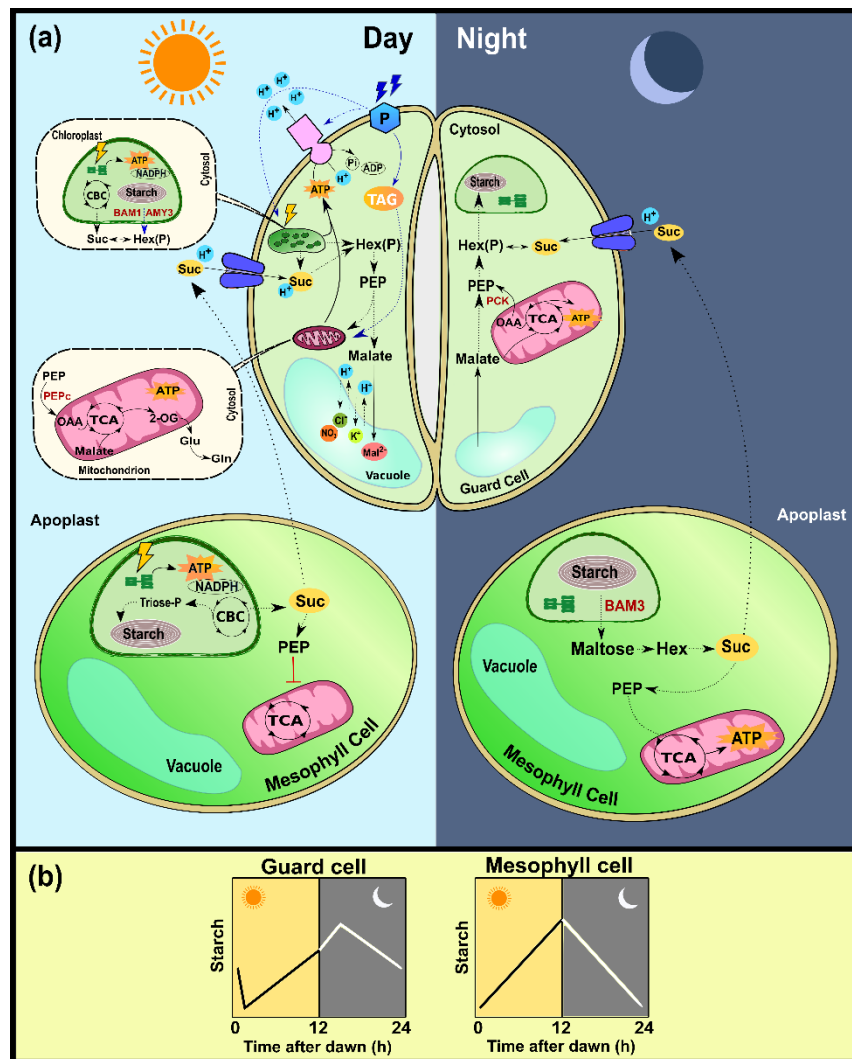
Figure 1. (a) Guard cell metabolism during day and night conditions. During the day, guard cell photosynthesis and mitochondria provide the ATP needed to maintain the activity of plasma membrane H^+-ATPases, which is important to create a H^+ gradient across guard cell plasma membrane. This H^+ gradient is dissipated by the transport of ions between the apoplastic space and the symplast of guard cells during stomatal opening. Phototropins, blue light receptors located in plasma membrane of guard cells, stimulates H^+-ATPase pump. Phototropin signalling is also a mechanism to stimulate starch and lipid (TAG) degradation. By contrast to mesophyll cells, in which respiration is light inhibited, guard cells seem to degrade starch, lipids and sucrose to sustain mitochondrial metabolism and glutamine biosynthesis. In this vein, guard cells have higher anaplerotic CO_2 fixation mediated by PEPc, when compared to mesophyll cells. Given the low RubisCO-mediated photosynthetic capacity of guard cells, sucrose may also be imported from neighbouring mesophyll cells. Therefore, sucrose is a putative signal from mesophyll cells that connects mesophyll photosynthetic process with stomatal movement. Another important metabolite for guard cell metabolism is malate, which can be imported from mesophyll cells and synthesized within guard cells from starch breakdown and PEPc fixation. Malate accumulation in the vacuole acts as a counter-ion of K^+. During the night, the content of guard cell osmolyte molecules such as malate and

sugars seems to be converted into starch. Furthermore, sucrose accumulation at the apoplastic space and its degradation within guard cells has been reported as mechanisms to induce stomatal closure. Thus, the transport of sucrose mesophyll cells may also contribute to stomatal closure during stomatal closure conditions. However, these hypotheses have yet to be tested. (b) Diel course of starch synthesis and degradation in both mesophyll and guard cells. Whilst mesophyll cells have a linear pattern of starch synthesis in the light and starch degradation in the night, guard cells linearly degrade starch at the beginning of the diel course and accumulate starch in the rest of the day and during the beginning of the night. For this, different isoforms of AMY and BAM enzymes seems to be involved in starch degradation in mesophyll and guard cells (see Figure (a)).

5 CHAPTER II - ROLE OF LEAF METABOLISM AND MESOPHYLL-DERIVED METABOLITES ON STOMATAL MOVEMENT REGULATION IN FERNS AND ANGIOSPERMS

(Unpublished manuscript)

Silvio A. Cândido-Sobrinho[1], Valéria F. Lima[1], and Danilo M. Daloso[1]

[1]Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Fortaleza-CE 60451-970, Brasil;

Author for Correspondence:
Danilo M. Daloso
Tel: +55 85 33669821
Email: daloso@ufc.br

Summary

Stomatal responses to changes in light and CO_2 concentration are much slower in ferns than angiosperms. We hypothesize that leaf metabolism and mesophyll-derived metabolites are crucial to regulate this response. Here, we have investigated which leaf metabolites correlate with stomatal conductance (g_s) throughout the day, performed an unprecedented LC-MS metabolite profiling of leaves harvested throughout the day and analyzed the effect of application of five exogenous metabolites over the kinetics of g_s responses in angiosperms and ferns. The results highlight ferns and angiosperms differ substantially in both primary and secondary metabolism. The angiosperm presented a higher accumulation of primary metabolites, whilst ferns have shown a higher accumulation of secondary metabolites. Furthermore, ferns showed no significant g_s response to exogenous ABA and a slower g_s response to sucrose and malate, while the angiosperm g_s decrease very fast in response to these compounds. These results strengthen our idea that metabolism-mediated mechanisms are key to regulate the faster stomatal velocity found in angiosperms. We discuss how the differential carbon allocation towards primary or secondary metabolism affect both photosynthesis-stomatal movement and growth-defence trade-offs in angiosperms and ferns.

Introduction

During the evolution, plants have acquired important traits such as stomata which enabled them to adapt to the terrestrial environment. Fossil records suggest that this trait exists since at least 400 million years ago (EDWARDS; KERP; HASS, 1998), being thus found from Bryophyta to Tracheophyta (LIGRONE; DUCKETT; RENZAGLIA, 2012). Stomata complex is composed of a pore surrounded by a pair of guard cells and, in certain cases, by subsidiary cells (LAU, 2017). Stomatal movements control the gas exchange between the leaf and the environment (CHATER et al., 2017), which thus regulate the intrinsic water use efficiency (*WUEi*) (GAGO et al., 2014), defined as the ratio between the rate of net CO_2 assimilation (A_N) and stomatal conductance to water vapour (g_s) (MCAUSLAND et al., 2016). Thus, beyond being essential for land plant establishment, stomata are key to climate changes by regulating carbon and water availability worldwide (FRANKS et al., 2017). Understanding the mechanisms that modulate g_s responses are thus pivotal to comprehend the dynamic of natural ecosystems and also to breed plants towards WUE enhancement (EISENACH et al., 2017; JALAKAS et al., 2018).

Changes in atmospheric and intracellular CO_2 concentration, light exposure and quality, phytohormones and ions modulate stomatal movements (CAI et al., 2017; DOI et al., 2004; PANDEY; ZHANG; ASSMANN, 2007; RUSZALA et al., 2011; SCHROEDER, 2003). Additionally, it has been shown that exogenous application of metabolites and genetic alteration of key metabolic enzymes substantially alter stomatal aperture/conductance (ARAÚJO et al., 2011; KELLY et al., 2013b; LI et al., 2016; MEDEIROS et al., 2018; YOSHIDA et al., 2016), indicating that mesophyll-derived metabolites have great influence on stomatal movement regulation (LAWSON et al., 2014; STADLER, 2003). This idea is based in the fact that guard cells have low photosynthetic activity, depending thus on energetic compounds derived from mesophyll and/or subsidiary cells (LIMA et al., 2018; WANG et al., 2019). These imported metabolites are crucial not only to supply the energetic demand of guard cells (GAGO et al., 2016; LAWSON et al., 2014) but also for the A_N-g_s trade-off regulation. However, despite the recent advances in our understanding regarding g_s regulation in model angiosperms species, it remains unclear whether these mechanisms are also present in basal lineage plant species.

Tremendous efforts have been recently placed in an attempt to understand the evolutive origin of the mechanisms that regulates stomatal movements (MCADAM; BRODRIBB, 2012). It is important to highlight that beyond being important to comprehend the evolution of land plants, understanding such mechanisms across different plant evolutionary groups also offer the possibilities to unravel important targets for WUE improvement. In this context, it was initially proposed that stomatal movements have passed from passive to active control through the evolution from ferns to angiosperms (BRODRIBB; MCADAM, 2011). However, recent studies have demonstrated that ferns are also able to respond to environmental signals, but in a much lower velocity (FRANKS; BRITTON-HARPER, 2016; HÕRAK; KOLLIST; MERILO, 2017; LIMA et al., 2019). It is thus currently accepted that angiosperms, gymnosperms and ferns share a common machinery that controls stomatal movements in response to light and CO_2 (SUSSMILCH et al., 2019). However, despite numerous evidence showing stomata from angiosperms are faster than ferns, the mechanisms that regulate such stomatal velocity remain to be elucidated.

It has been proposed that the differential stomatal velocity among ferns and angiosperms is rooted in the genetic differences between these plant groups (SUSSMILCH et al., 2019). Furthermore, we have recently proposed that mesophyll-derived metabolic signals, especially those associated with sucrose and malate, may be also important to modulate the differential stomatal velocity between ferns and angiosperms (LIMA et al., 2019). Here, we

have further investigated which leaf metabolites correlate with g_s throughout the day, performed an unprecedented LC-MS metabolite profiling of leaves of angiosperms and ferns harvested throughout the day and analyzed the effect of exogenous application of metabolites on the dynamic of g_s responses. The results are discussed in the context of evolution of stomatal movement regulation.

Material and Methods

Gas exchange and metabolomics meta-analysis

The gas exchange data were collected from previous published dataset consisting of maximum photosynthetic rate (A_{max}) and g_s in 58 angiosperms and 32 ferns species (GAGO et al., 2019) along with data retrieved from a previous study (LIMA et al., 2019). The parameters were evaluated at their absolute values and subjected to a maximum-minimum transformation according to the equation below:

$$f(x) = \frac{xi - \min(x)}{\max(x) - \min(x)}$$ Eqn 1

where xi stands for an observation of the dataset. This transforms parameters to a 0-1 scale along the time length (05:00 h, 08:00 h, 14:00 h and 17:00 h) where smallest value is set to zero (0) and the highest average value to one (1), then proportionally transforming the values beween the maximum and minimum observed (DEANS et al., 2019). Given angiosperms present absolute gas exchange data up to three times higher than ferns, this transformation allow a better comparison between A_N and g_s kinetics and A_N *vs* g_s relationship between the groups.

Plant material and growth conditions

This study has used two ferns *Microsorum scolopendria* (Burman) Copel. and *Phlebodium aureum* (L.) J. Sm., both obtained at the sporophyte stage, and two angiosperms *Vigna unguiculata* (L.) Walp. and *Nicotiana tabacum* L. Angiosperms were germinated in laboratory and the seedlings were transferred either to 2.5 L pots filled with soil for *N. tabacum* or in a hydroponic system for *V. unguiculata* using Hoagland solution (HOAGLAND; ARNON, 1950), as previously described (LIMA et al., 2019). The plants were kept at greenhouse under 12 hours natural photoperiod maximum photosynthetic photon

flux density (PPFD) of 500 µmol m^{-2} s^{-1}, ambient temperature of 30 ± 4 °C and relative humidity 62 ± 2 °C.

Stomatal kinetic assays

The assay of stomatal closure kinetics was carried out as described by Ceciliato et al. (2019). Briefly, leaves were detached and the petiole tip immediately submerged in deionized water in a petri dish. After that, a second oblique cut was made on petiole and transferred to a 2 mL microcentrifuge tube with deionized water and submitted to gas exchange analysis by an infrared gas exchange analyzer equipped with a 6 cm² leaf chamber (Li-6400XT, LI-COR Biosciences, Inc. Licoln, NE, USA), setting the flow rate to 400 rpm and 1500 µmol m^{-2} s^{-1} PPFD. The parameters net photosynthetic rate (A_N) (µmol CO$_2$ m^{-2} s^{-1}) and stomatal conductance to water vapor (g_s) (mmol H$_2$O m^{-2} s^{-1}) were recorded every 10 seconds for 40 minutes. Stock solution of metabolites were prepared then applied in order to reach the desired final concentration as follow: ABA (5 µM), glutamate (1 mM), malate (25 mM) and sucrose (25 mM) and mannitol (25 mM); this last was used as osmotic control. After the addition of these compounds to the tube containing the detached leaf, A_N and g_s were recorded for more 40 minutes.

Metabolomic analyses with GC-MS and LC-MS

We carried out a meta-analysis from previous published works that retrieves gas exchange and/or metabolomics data. We have deeply analyzed data from our previous published work which consists of gas exchange and GC-MS-based primary metabolite profiling of two angiosperms and two ferns harvested throughout the diel course (05:00 h, 08:00 h, 14:00 h and 17:00 h) (LIMA et al., 2019).

Leaf samples from angiosperms and ferns were harvested throughout the day (05:00 h, 08:00 h, 14:00 h and 17:00 h), frozen and the metabolites were extracted and analyzed by a liquid chromatography coupled to mass spectrometry (LC-MS) platform. We first carried out a metabolic fingerprinting followed by the identification of leaf secondary metabolites. The mass spectral data was initially pre-processed by MS-DIAL software, followed by XCMS and metaboanalystR packages on R.

Statistical analyses

In order to obtain possible metabolite candidates that coordinate g_s throughout the diel course, metabolite profiling data was retrieved from LIMA et al., (2019) and *one-way* ANOVA was carried out using multicomp package in R followed by Dunnett's test using 5:00 h as control. Pearson correlation ($p > 0.7$) was performed between g_s and metabolite levels. The data was further analysed by principal component analysis (PCA) by using the Statistical Analyses module in metaboanalystR package. We have further subjected the data into the Pathway Analysis module in metaboanalystR package in order to investigate which pathways could modulate g_s responses (CHONG; YAMAMOTO; XIA, 2019). After that, both metabolites and gas exchange parameters (A_N and g_s) were transfomed using Eqn 1 and the values were clustered by Euclidean distance using the dtwclust package in R to verify similar trends throughout the day. The data from LC-MS analysis were used to compared ferns with angiosperms through Student's *t*-test ($p < 0.05$).

Stomatal kinetics data were analyzed as average in raw and normalized data by curve fitting the data using a non-linear regression by SigmaPlot 12 (Systat Software Inc., San Jose, CA, USA). Regressions were performed using models which best fit the observed data considering the highest R^2. After, derivatives of the regression equations were calculated in order to assess: absolute and relative fold change, rate of change and maximum velocity (V_{max}) of g_s response. Rates of change were plotted *versus* time and undergone clustering classification for time-series comparison analysis using dtwclust package in R calculating distances with Euclidean method. Whenever V_{max} behaved exponential, dg_s/dt undergone linear plateau model fitting (y ~ a + b × (x - c) × (x<c).

Results

Meta-analysis of gas exchange and water use efficiency in ferns and angiosperms species

To obtain better insights into the relationship between photosynthesis (A_N) and stomatal conductance (g_s) in different evolutionary groups, previous gas exchange data was retrieved (LIMA et al., 2019) and transformed using Eqn 1. Both A_N and g_s increased considerably with the daylight and had maximum peaks around 10 am in both clades. Although angiosperms showed at least three times higher absolute A_N and g_s values than those observed in ferns (Figures 1a,c), no differences in relative values throughout the diel course were observed (Figures 1b,d). Similarly, the A_N-g_s relationship demonstrates higher absolute values for angiosperms but with similar trend in the relative values (Figures 2 a,b).

We next transformed A_N and g_s values (using Eqn 1) and fitted the data into an exponential rise to maximum model according to the equation below which presented *a* (0.7375) and *b* (4.4237) as global parameters (R^2 = 0.7339, A_{N0} = 0.0387 fitted in Angiosperms and A_{N0} = -0.0705 in Ferns). By performing a global curve fit with such significant R^2 and shared coefficients we can assume this mathematical model describes the phenomena observed in both plant groups, which is in accordance with other evidence that A_N and g_s increase following a similar trend.

$$A_N = A_{N0} + a \ (1 - e^{-b \ gs}) \hspace{4cm} \text{Eqn 2}$$

Both data presented in Figure 2a and Figure 2b (transformed with Eqn 1) undergone regression and their curves were obtained in Figure 2c and 2d. The figure 2c present the regression curves using exponential model rise to maximum of A_N *vs* g_s relationship. The A_N - g_s relationship observed in ferns best fitted to exponential rise to maximum both in absolute (R^2 = 0.777) and transformed (R^2 = 0.864) despite they seem to be linear (R^2 = 0.711 and R^2 = 0.863). The Figure 2d clearly demonstrates g_s impose a higher limitation to A_N in ferns when compared to angiosperms. We next evaluated the A_N-g_s ratio (*WUEi*) by using gas exchange data collected at the 8:00 h and 14:00 h timepoints, which held the A_{max} observed during the light period of the day (Figure 1a,c), and compared with those previously reported in GAGO et al., (2019). Interestingly, ferns have demonstrated higher *WUEi* than angiosperms by Student's *t*-test ($P < 0.001$) (Figures 3a,b).

Discriminating ferns and angiosperms at metabolic level

Ferns and angiosperms have extremely different evolutionary trajectories, which can result in different metabolite profiling. In order to discriminate the plant groups and to find out metabolites that most contribute to this differentiation, we carried out Principal Component Analyses merged with Loadings Plot (PCA Biplot). The results showed a clear distinction between the groups at each time point observed (Figures 4a-d). Although all observations were found into 95% confidence interval in all scatters, the two Angiosperms have been set more distant to each other in comparison with the two ferns, which were clustered together in most of the time points (Figures 4a-d). It is noteworthy that Loadings Plot reveals the features that most influenced the separation of the groups. Interestingly, in all time analyzed, the few secondary metabolites present in GC-MS data such as nicotinic acid,

cinnamic acid and quinic acid had a great contribution to the separation of the species. Additionally, the two main amino acids that are used as a substrate to the synthesis of secondary compounds namely tryptophan and phenylalanine also contributed to separate the plant groups (Figures 4a-d). Indeed, ferns have a higher accumulation of these compounds than angiosperms (LIMA et al., 2019).

Given that primary and secondary metabolisms are mostly related to growth and defense, respectively, the results described above suggest that ferns and angiosperms have a different resource allocation to primary and secondary metabolisms. In order to obtain better insights into this observation, we next performed a Pathway analysis to investigate which pathways most contribute to separate these plant groups and carried out an unprecedented metabolite profiling of leaf secondary metabolites by LC-MS analysis. The pathway analysis showed the pathways involving glycine-serine-threonine, alanine-aspartate-glutamate and phenylalanine, isoquinone alkaloids and tyrosine metabolism have high pathway impact in the GC-MS data set (Figures S1a-d). These results point ferns and angiosperms differ substantially at the photorespiratory, amino acid and secondary metabolisms. In fact, Partial Least Square Discriminant Analysis (PLS-DA) of the LC-MS metabolic fingerprinting indicates that ferns and angiosperms differ considerably in the secondary metabolism (Figures 5a-d), in which ferns present a higher number of features with higher accumulation when compared to the angiosperms (Figures S2-S5).

We next identified the features obtained from LC-MS analysis that are significantly different between ferns and angiosperms and compared the relative changes of these features and those obtained from GC-MS analysis during the dark to light transition (5:00 h to 8:00 h) between the plant groups. The results revealed that angiosperms and ferns differ substantially during the dark to light transition (Figure 6a), in which the feature vectors towards angiosperms are from primary metabolism whilst metabolites assigned to secondary metabolites are more significant to discriminate ferns (Figure 6b). Furthermore, ferns have a higher accumulation of secondary metabolites than angiosperms (Figure 7).

Seeking metabolite candidates that coordinate stomatal responses in ferns and angiosperms

Gas exchange and 57 metabolites GC-MS data previously reported by Lima and collaborators (LIMA et al., 2019) were analysed using uni/multi-variate statistical approaches. The changes in metabolite level along the diel course were assessed by performing an *one-way* analysis of variance (ANOVA) and Dunnett's test, having the timepoint 5:00 h as

control. Beyond A_N and g_s, 12 metabolites (mainly amino acids and organic acids) showed significant difference along the diel course (Table S1). We next assessed the Pearson's correlation (P ≥ 0.6, P ≤ -0.6) between metabolites and A_N and g_s. (Figure 8), which exhibited the data of those metabolites that presented at least two significant correlations with A_N or g_s. Valine, tyrosine, tryptophan and proline were negatively correlated with gas exchange parameters whilst alanine, serine, glycine, glycerate, Succinate and Sucrose were positively correlated in all species observed.

Given that stomatal movements and plant metabolism undergoes circadian rhythm, we next decided to investigate which metabolites have the same dynamic of g_s throughout the diel course. For this, metabolite and gas exchange data were normalized (Eqn 1) and their relative levels subjected to time-series comparison using Dynamic Time Warping (DTW) analysis, which clustered the data with similar trends over the diel course. DTW analysis generated 15 clusters (Figure 9; Table S2). The cluster 1 held all gas exchange parameters (A_N and g_s) from angiosperms and ferns as well as several amino acids (e.g. alanine, aspartate, serine, glutamate, and glutamine) sugars and sugar alcohols (e.g. sucrose, mannitol, idose, lyxose) and certain secondary metabolites (caffeic acid and quinic acid).

Effect of exogenous application of metabolites on the kinetic of stomatal movements

After obtaining the correlation and the trends between leaf metabolites and gas exchange parameters, along with metabolites known to regulate stomatal movements, we next tested the effect of exogenous application of ABA, glutamate, malate, mannitol and sucrose on the kinetic of stomatal movements. We aim to investigate whether mesophyll-derived metabolites have a differential impact on the dynamic of stomatal movements in ferns and angiosperms. ABA is a well-known phytohormone that induces stomatal closure (YOSHIDA et al., 2019). Here, the ferns used did not show any ABA response in the time period evaluated, although a slight tendency to decrease g_s in the last time points was observed (Figure 10). By contrast, the angiosperm species used for this analysis, *V. unguiculata*, have a substantial decrease in g_s (87 %) in response to ABA (Table 1). Glutamate (1 mM) slightly increased g_s on angiosperm and both ferns species tested (16% and 36%, respectively). Malate, sucrose and mannitol also decreased g_s in all plants analyzed, although angiosperms quickly reached steady-state and ferns kept decreasing g_s over time (Figure 10). This result indicates that the time to reach steady state was much lower in angiosperms than ferns, suggesting that angiosperms present a faster stomatal response to sucrose and malate. In order

to verify this, we next investigated how g_s changed in response to all metabolites tested by curve fitting the data by non-linear regression. The equations derived from the regression analysis were obtained and the changes in the velocity throughout the time were assessed. The results showed that ABA response on angiosperm have been fit to exponential decay and shown the highest velocity, in agreement on what is described in the literature (Table 2, Eqn 3). Ferns g_s kinetics under ABA treatment exhibited linear asymptotic curve and zero rate of change (Table 2, Eqn 4). Glutamate treatment slightly diminishes g_s on the beginning followed by an increase on angiosperms (Table 2, Eqn 5), which was best fitted to an exponential growth curve. By contrast, an increase was also observed in ferns but best fitting to exponential rise to maximum curve fitting (Table 2, Eqn 6).

Assuming that mannitol does not trigger other response rather than osmotic, we have used this treatment as osmotic control of the malate and sucrose treatments. In both plant groups, mannitol, malate and sucrose decreased g_s, but a higher velocity was observed in angiosperms (Table 2, Eqn's 7-9), especially under sucrose and malate treatments when compared to ferns (Table 2, Eqn 10-12). The regression curves were subsequently transformed (Eqn 1), to allow time-series comparison (Figure S6), and then clustered using dtwclust package and euclidean distance as the calculation method. Five clustered were generated. The cluster 1 contains both mannitol treatments from angiosperms and ferns, the ABA treatment from angiosperms and sucrose and malate treatments from ferns (Figure S6). To detect if these treatments reached a maximum velocity (V_{max}), all rate of changes (dg_s/dt) were analysed using easyreg package to check if the exponential rise or decay reached a plateau. We observed that both malate and sucrose treatments in angiosperms reached V_{max} at 421 s and at 238 s, respectively, whilst glutamate treatment reached V_{max} at 1062 s in Ferns (Figure S7). The slopes were calculated using the primitives equations obtained from regression using non-normalized data (not shown) considering all time frame except when reached a plateau, which in this case the slopes were calculated considering the time needed to reach the plateau (Table 3).

Discussion

Ferns have a similar circadian rhythm of A_N and g_s but higher WUEi when compared to angiosperms

It has been shown that the magnitude of both A_{max} and g_s are lower in ferns than angiosperms (BRODRIBB et al., 2009; GAGO et al., 2019), even when plants are acclimated under greenhouse conditions or under high CO_2 condition (LIMA et al., 2019). However, it is unclear whether these plant groups also differ in the dynamic of A_N and g_s diel course in relative terms. In order to investigate this, we have applied a normalization approach (Eqn 1) (DEANS et al., 2019) to previous published gas exchange data . Our results revealed that, in relative terms, the diel course of these parameters show identical diel circadian rhythm between ferns and angiosperms, with a maximum g_s observed in the initial period of the day (Figures 1a-d), as typically observed under tropical conditions (ANTUNES et al., 2017). These results indicate that ferns stomata are able to respond to the natural circadian rhythm, which is controlled by a complex regulatory network associated to changes in environmental cues such as temperature, air humidity, CO_2 concentration and light quality and quantity (GRAF et al., 2010; SHALIT-KANEH et al., 2018). This strengthens the idea that ferns stomata are responsive to environmental signals, although the velocity of these responses are much lower than those observed in angiosperms (discussed later in the section *The impact of the metabolic differences between ferns and angiosperms on g_s regulation*).

Despite the fact that ferns stomata have a similar circadian rhythm, it is still unclear why the magnitude of A_N and g_s is substantially lower in ferns, when compared to angiosperms, it has been proposed that mesophyll conductance (g_m) is the major limitation of A_{max} in the basal lineage of plants (GAGO et al., 2019). In close agreement with this, the results of the relative A_N-g_s relationship showed similar trends between ferns and angiosperms ($R^2 = 0.7841$ for angiosperms and $R^2 = 0.7339$ for ferns) (Figure 2b), suggesting that A_N is similarly limited by g_s among ferns and angiosperms in the diel course. The higher mesophyll limitation of basal lineage of plants have been associated to mesophyll traits such as cell wall thickness and the chloroplast surface area exposed to the intercellular air spaces per leaf area, which in one side leads to a reduced A_N per leaf mass area and/or N content in ferns compared to angiosperms (GAGO et al., 2019), but in another side may be a strategy for desiccation resistance (DEANS; BRODRIBB; MCADAM, 2017). In fact, although ferns have lower A_N and g_s when compared to angiosperms, they have higher *WUEi* (Figures 3a,b), similar to the observed in 35 ferns species compared to 60 angiosperms species (GAGO et al., 2019). This capacity of plants to dynamically adjust stomatal opening according to the surrounding environment dictates *WUEi*, i.e. the balance between carbon uptake and water loss via transpiration (FRANKS et al., 2017).

In the growth-defense trade-off, ferns seems to prioritize defense

The reduced absolute values of g_s limits A_N in ferns, but leads to a higher *WUEi* (Figures 2a,c; 3a,b). It seems likely therefore that angiosperms evolved toward a higher assimilation capacity strategy despite losing efficiency on water use. On the other hand, ferns are more efficient in the use of water but present less yield and slower g_s responses. It remains unclear however whether the slower stomatal responses of ferns are due to a miscommunication between mesophyll and guard cells or if this is strictly related to the metabolism of guard cells (LAWSON; VIALET-CHABRAND, 2019). Furthermore, our personal observations[1], together with previous findings, suggest that ferns have a higher tolerance to abiotic (PITTERMANN; BRODERSEN; WATKINS, 2013) and biotic stress (GRACELIN et al., 2012).

Ferns and other early evolutionary plants have been shown to be important models to investigate and understand the mechanisms that regulate the outstanding plant decision: to grow or to defend? Unraveling the mechanisms that regulate the growth-defense trade-off not only contribute to understand the dynamic of complex natural ecosystems but also provide great possibilities for plant breeding programs toward plant stress tolerance (BRODRIBB; SUSSMILCH; MCADAM, 2019; GAGO et al., 2014). In order to obtain better insights into this topic and given that metabolism is key to determine growth capacity and/or stress tolerance in plants (AMTHOR et al., 2019; TOHGE et al., 2016) we have performed a LC-MS-based secondary metabolite profiling, which, combined with the previous published GC-MS-based primary metabolite profiling (LIMA et al., 2019), provide a clear picture among the metabolic differences between ferns and angiosperms. Results also suggest ferns prioritize carbon allocation toward secondary metabolism, instead of the primary metabolism. This idea is supported by the higher accumulation of the majority of sugars and amino acids in angiosperms (LIMA et al., 2019) and the higher accumulation of secondary metabolites in ferns (Figures S2-5). Thus, in the growth-defense trade-off, it seems likely that ferns have higher stress tolerance due to a differential resource investment toward stress tolerance mechanisms, which is usually associated to the accumulation of secondary metabolites (MAZID; KHAN; MOHAMMAD, 2011; TOHGE; DE SOUZA; FERNIE, 2017). These results contribute to answer an elusive question on why the growth rate of ferns are much lower than angiosperms and shed lights on the challenge that plant breeding programs face to produce stress tolerant genotypes with minor impacts on plant productivity.

The impact of the metabolic differences between ferns and angiosperms on g_s regulation

Ferns and angiosperms present distinct metabolite profiles. PCA analysis of the GC-MS data showed that angiosperms were grouped by similarity on primary metabolites such as amino acids and oligosaccharides, whilst ferns were clustered according to the accumulation

---

[1]This idea is supported by empirical observations in which the ferns used in this work did not have problems with the attack of pathogens, whilst several angiosperm species such as rice, tobacco, beans and cotton have been demonstrated to be susceptible to pathogens present in our plant growth conditions).

of certain organic acids and secondary metabolites (Figures 4a-d). Given the importance of amino acids and oligosaccharides for plant growth and productivity, these results reinforce the of angiosperms dominance worldwide playing a crucial ecological and commercial role as primary producers. Furthermore, it is interesting to highlight that A_N and g_s were highly positively correlated to amino acids and photorespiratory-related metabolites such as alanine, serine, glycine, glycerate, glycolate, glutamate and glutamine, implying that these metabolites may be important for the A_N-g_s trade-off regulation. Taking this into account, we next tested whether the g_s response to exogenous application of metabolites differ between ferns and angiosperms. For this, we have used two ferns (*M. scolopendria* and *P. aureum*) and a representative angiosperm species (*V. unguiculata*). The results showed that the angiosperm species is high sensitivity to almost all compounds tested, except glutamate in which the response was minor. Notably, exogenous application of ABA clearly reduced g_s in the angiosperm whilst ferns stomata did not respond to this phytohormone in the time period analyzed here (Figure 10). Despite the fact that ferns possess the genes related to ABA response (CAI et al., 2017), it seems that the sensitivity to this phytohormone and changes in CO_2 concentration may differ among ferns species and growth conditions (BRODRIBB; MCADAM, 2011; HÕRAK; KOLLIST; MERILO, 2017).

Glutamate and glutamine have been associated with stomatal movement regulation (LIMA et al., 2019; MEDEIROS et al., 2018; YOSHIDA et al., 2016). Here, these metabolites were positively correlated to g_s in both ferns and angiosperms (Figure 8) the exogenous application of glutamate have positive effect on g_s (Figure 10). By contrast, the addition of malate, sucrose and mannitol reduced g_s in both groups. However, it is interesting to highlight that ferns and the angiosperm differ substantially in the velocity of the g_s response to malate and sucrose (Figure 10). These metabolites have reached a maximum velocity (V_{max}) in *V. unguiculata* (Figure 11), pointing out that angiosperms have a greater capacity to

rapidly respond to the accumulation of these metabolites. This demonstrates the importance of mesophyll-derived signals to coordinate photosynthesis status with stomatal movements. These results confirm our previous hypothesis that mesophyll-derived signals are key to modulate the faster stomatal responses in angiosperms (LIMA et al., 2019). We thus postulate that mesophyll-derived malate and sucrose are key to regulate stomatal movements by rapidly inducing stomatal closure. Given that mannitol also induced stomatal closure in ferns and angiosperms species (KOTTAPALLI et al., 2018), although in a slower velocity when compared to sucrose (Figure S6), we propose that sucrose and malate induce stomatal closure by two different mechanisms: (i) − by an osmotic mechanism, probably associated to the accumulation of these metabolites in the apoplastic space of guard cells (KANG et al., 2007); and (ii) by a signalling mechanism, in which these compounds would be perceived by guard cells and the stomatal closure triggered by signalling transduction pathways, most probably associated to ABA and hexokinase (KELLY et al., 2013a; KOTTAPALLI et al., 2018). However, experimental evidence supporting this hypothesis is still lacking.

Concluding remarks

In agreement with previous reports, both ferns used in this study have lower A_N and g_s but higher *WUEi* when compared to angiosperms. However, in relative terms, ferns and angiosperms respond similarly to the circadian rhythm. These plant groups not only diverge in *WUEi* but also in how they allocate the resources between the primary and secondary metabolisms. Ferns seem to prioritize the synthesis of secondary metabolites, which has several implications to improve stress tolerance at the cost of a reduced growth. Our data further suggest that angiosperms have developed mechanisms that enable them to respond to compounds such as ABA, sucrose and malate faster than ferns. Thus, our study provides important evidence on the role of metabolism-mediated mechanisms on the evolution of stomatal movement regulation.

Table 1. Maximum g_s difference (Δg_{smax}) observed in ferns and angiosperms after exogenous application of different metabolites. The first line holds the raw values whilst the relative decrease or increase (in the case of glutamate) are presented in the second line.

Group	ABA	Glutamate	Malate	Mannitol	Sucrose
Angiosperms	-0.3465	0.1081	0.1654	0.0556	0.0356
	89.8%	16.7%	30.7%	42.0%	21.2%
Ferns	0.0006	0.005	0.01	0.0132	0.016
	-	35%	39%	43%	48%

Fonte: dados da pesquisa.

Table 2. List of derivatives calculated from non-linear regression obtained with curve fitting on SigmaPlot 12.

Treatment	Plant Group	Derivative function	
ABA (5 µM)	Angiosperms	$dg_s/dt = -0.0006\,(\,0.6879\,e^{-0.0006\,t}\,)$	(Eqn 3)
ABA (5 µM)	Ferns	$dg_s/dt = 0$	(Eqn 4)
Glutamate (1 mM)	Angiosperms	$dg_s/dt = 0.0053\,(\,1.34\;10^{-6}\,e^{0.0053\,t}\,)$	(Eqn 5)
Glutamate (1 mM)	Ferns	$dg_s/dt = -0.0013\,(\,0.004\,e^{-0.0013\,t}\,)$	(Eqn 6)
Mannitol (25 mM)	Angiosperms	$dg_s/dt = -0.0009\,(\,0.0813\,e^{-0.0009\,t}\,)$	(Eqn 7)
Malate (25 mM)	Angiosperms	$dg_s/dt = -0.0048\,(\,0.0141\,e^{-0.0048\,t}\,)$	(Eqn 8)
Sucrose (25 mM)	Angiosperms	$dg_s/dt = -0.0084\,(\,0.0365\,e^{-0.0084\,t}\,)$	(Eqn 9)
Mannitol (25 mM)	Ferns	$dg_s/dt = -0.0011\,(\,0.0144\,e^{-0.0011\,t}\,)$	(Eqn 10)
Malate (25 mM)	Ferns	$dg_s/dt = -0.0008\,(\,0.0138\,e^{-0.0008\,t}\,)$	(Eqn 11)
Sucrose (25 mM)	Ferns	$dg_s/dt = -0.001\,(\,0.0188\,e^{-0.001\,t}\,)$	(Eqn 12)

Fonte: dados da pesquisa.

Table 3. Slopes calculated from dgs/dt linear phases, in mol $H_2O \cdot m^{-2} \cdot s^{-2}$.

Group	ABA	Glutamine	Malate	Mannitol	Sucrose
Angiosperms	$-2.6 \cdot 10^{-3}$	$4.2 \cdot 10^{-6}$	$-2.91 \cdot 10^{-5}$	$-3.86 \cdot 10^{-5}$	$-1.3 \cdot 10^{-3}$
Ferns	$-7.5 \cdot 10^{-7}$	$2.82 \cdot 10^{-6}$	$-7.4 \cdot 10^{-6}$	$-9.4 \cdot 10^{-6}$	$-1.2 \cdot 10^{-5}$

Fonte: dados da pesquisa.
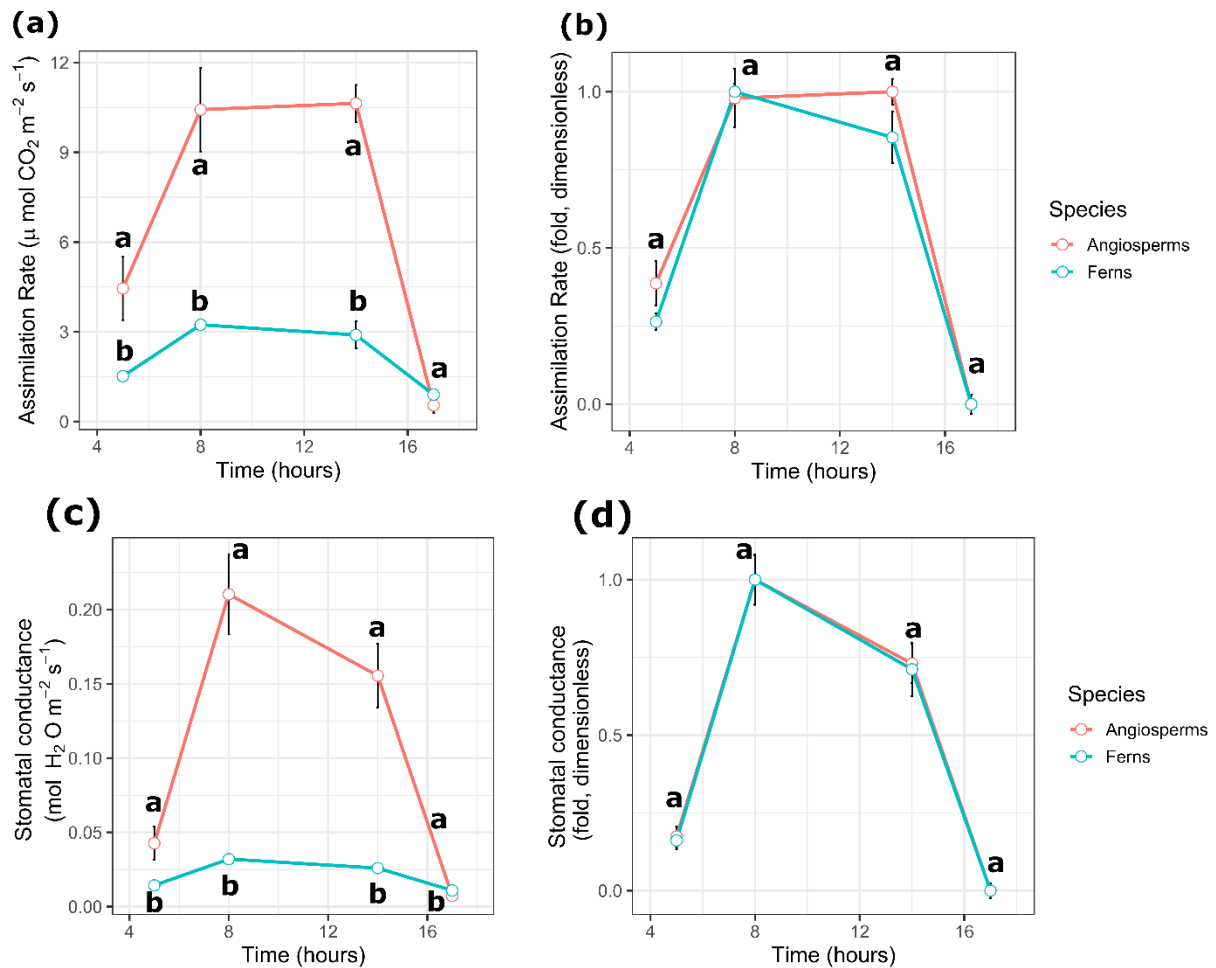
Figures 1. Diel course of gas exchange in ferns and angiosperms. (a-b) Absolute (µmol CO_2 m^{-2} s^{-1}) and relative (dimensionless) photosynthetic assimilation rate. (c-d) Absolute (mol H_2O m^{-2} s^{-1}) and relative (dimensionless) stomatal conductance. Data was retrieved from Lima et al. (2019). The data represents average ± SE (n = 4). Different letters indicate statistical differences among ferns and angiosperms at each time point by Student's *t*-test ($P < 0.05$).

Fonte: Lima et al, 2019. (a, c), dados da pesquisa (b, d).
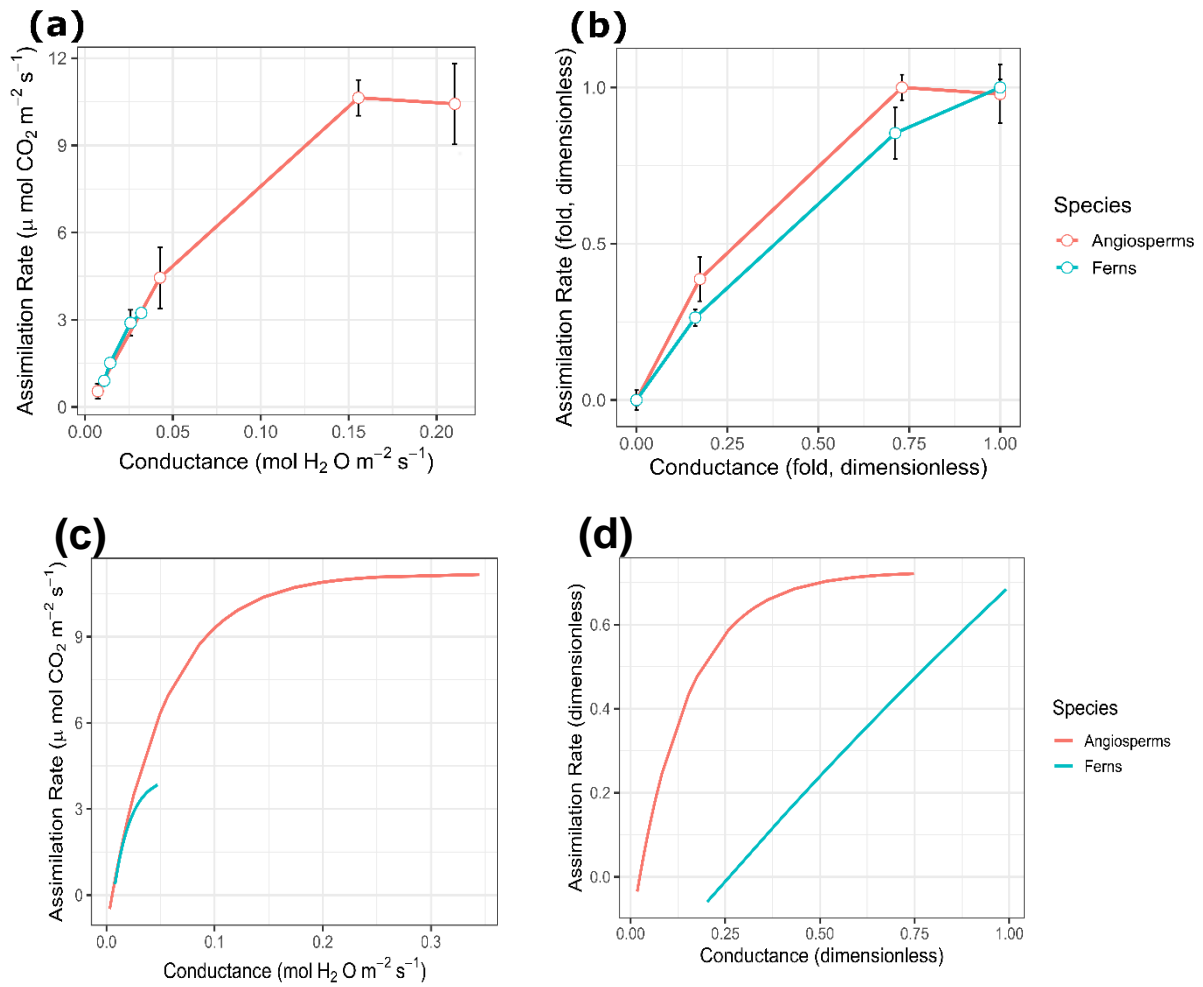
Figures 2. Absolute (a) and transformed (b) A_N *vs* g_s curves retrieved from (LIMA et al., 2019) dataset. A relative approach on dataset reveals A_N and g_s fold-change is linear in both groups. A global curve fit using SigmaPlot 12 software was performed setting as shared variables the *a* and *b* coefficients in Eqn 2. Regression curves obtained from gas exchange parameters were: (c) Angiosperms: $A_N(g_s) = -1.1678 + 12.3558(1 - e^{-18.7956\,gs})$, Ferns: $A_N(g_s) = -2.1014 + 6.2533(1 - e^{-63.6509\,gs})$. (d) Angiosperms: $A_N(g_s) = -0.1362 + 0.8619(1 - e^{-7.0696\,gs})$, Ferns: $A_N(g_s) = -0.2762 + 4.1705(1 - e^{-0.268\,gs})$

Fonte: Lima et al, 2019. (a, c), dados da pesquisa (b, d).
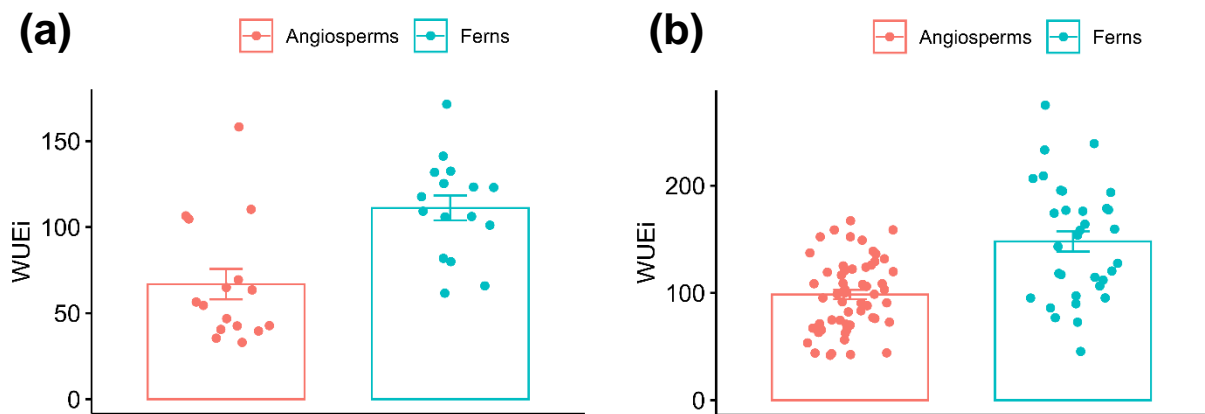
Figures 3. Intrinsic water use efficiency calculated from A_{max} with their respective g_S . The data was collected from (LIMA et al., 2019) (Angiosperms n = 16, Ferns n = 16) (a) and Gago et al. (2019) (Angiosperms n = 58, Ferns = 33) (b). *WUEi* was statistically different among ferns and angiosperms by Student's *t*-test (P < 0,001).

Fonte: Lima et al, 2019 (a), Gago et al 2019 (b).
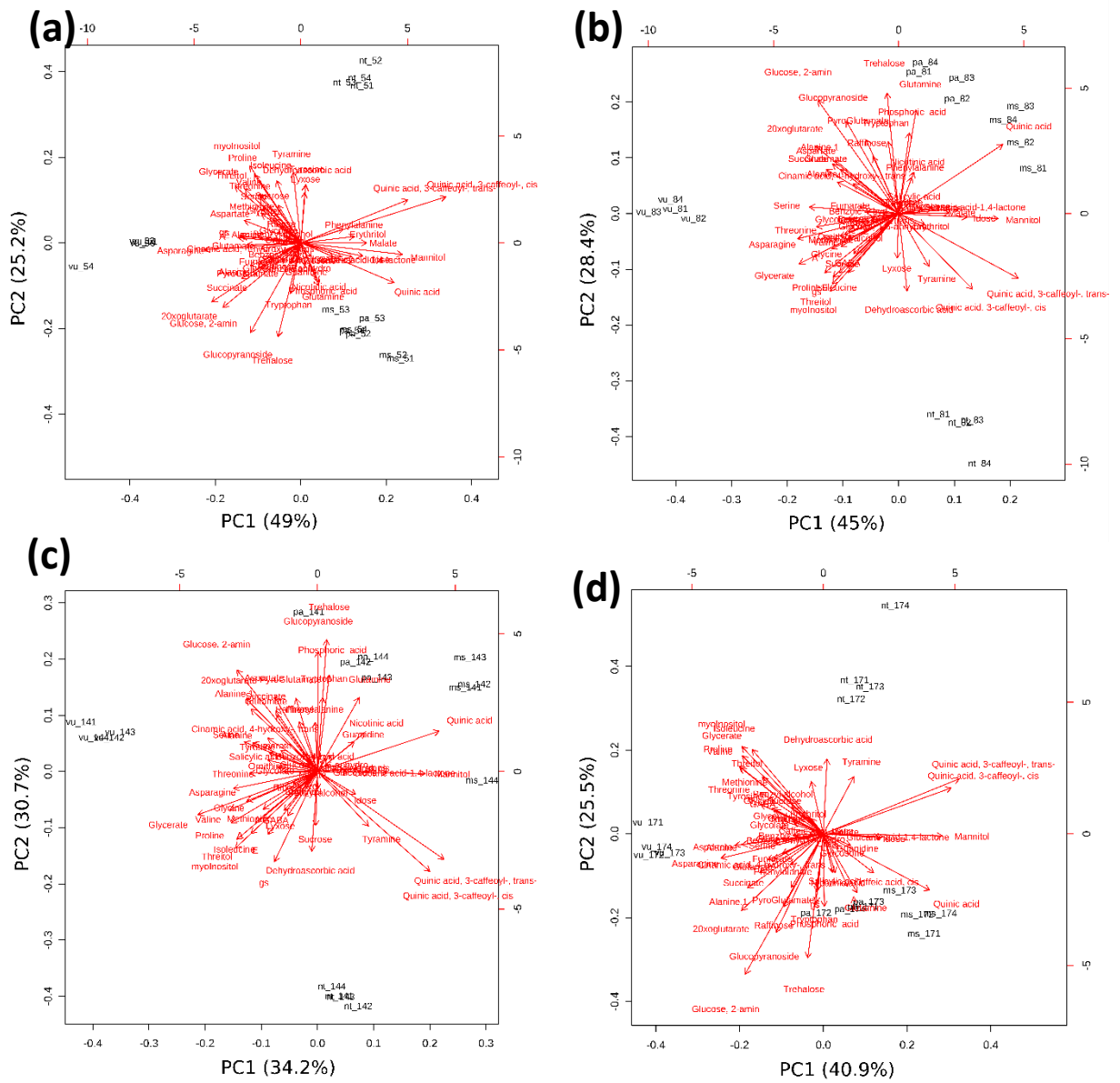
Figures 4. PCA Biplot of leaf metabolites harvested at 5:00 h (a), 8:00 h (b), 14:00 h (c) and 17:00 h (d). The orthogonal vectors demonstrate which metabolites are responsible for distinction among ferns and angiosperms.

Fonte: dados da pesquisa.
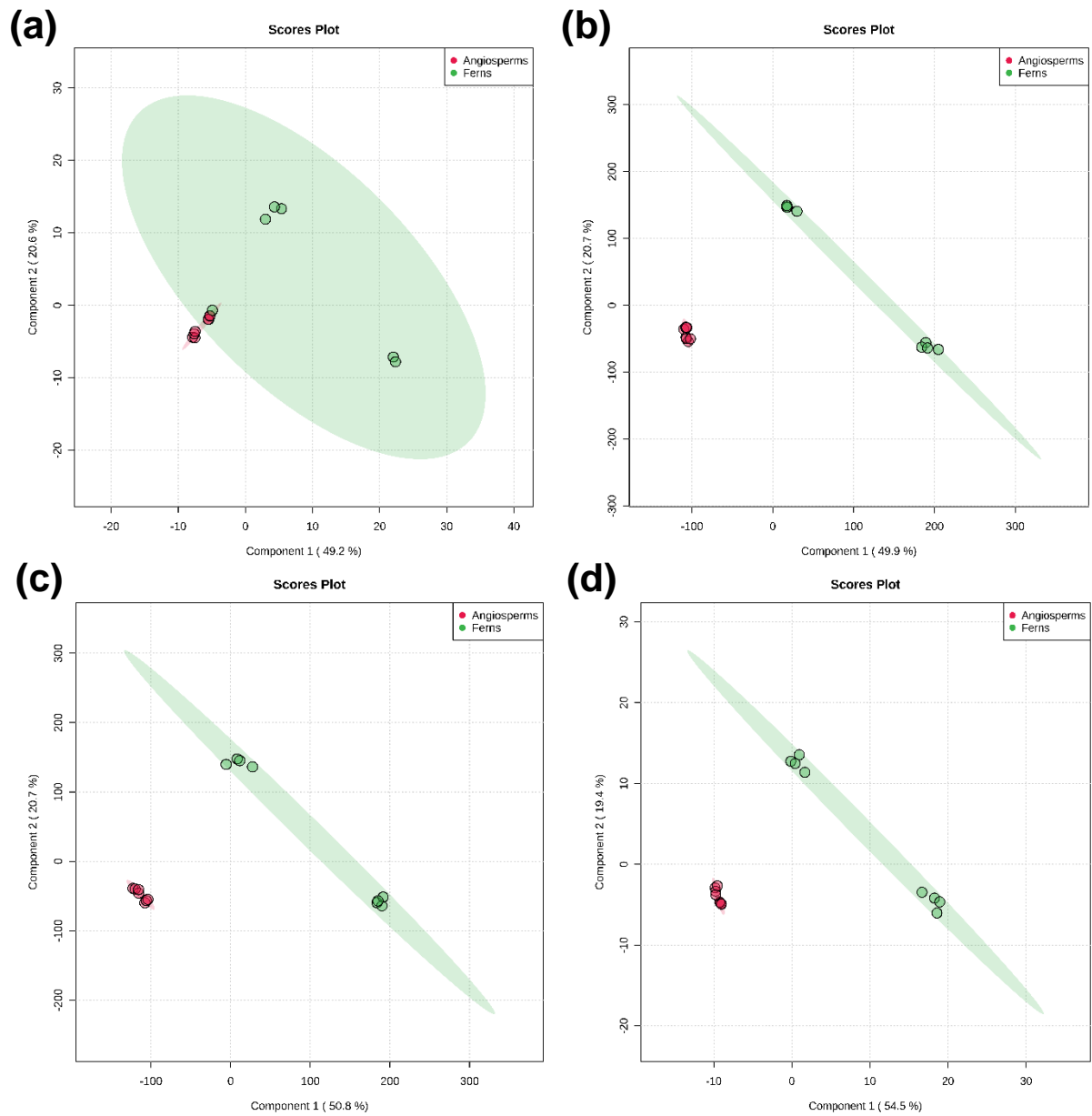
Figures 5. Partial least square discriminant analysis (PLS-DA) generated using LC-MS metabolic fingerprinting from samples harvested at 5:00 h (a), 8:00 h (b), 14:00 h (c) and 17:00 h (d).

Fonte: dados da pesquisa.
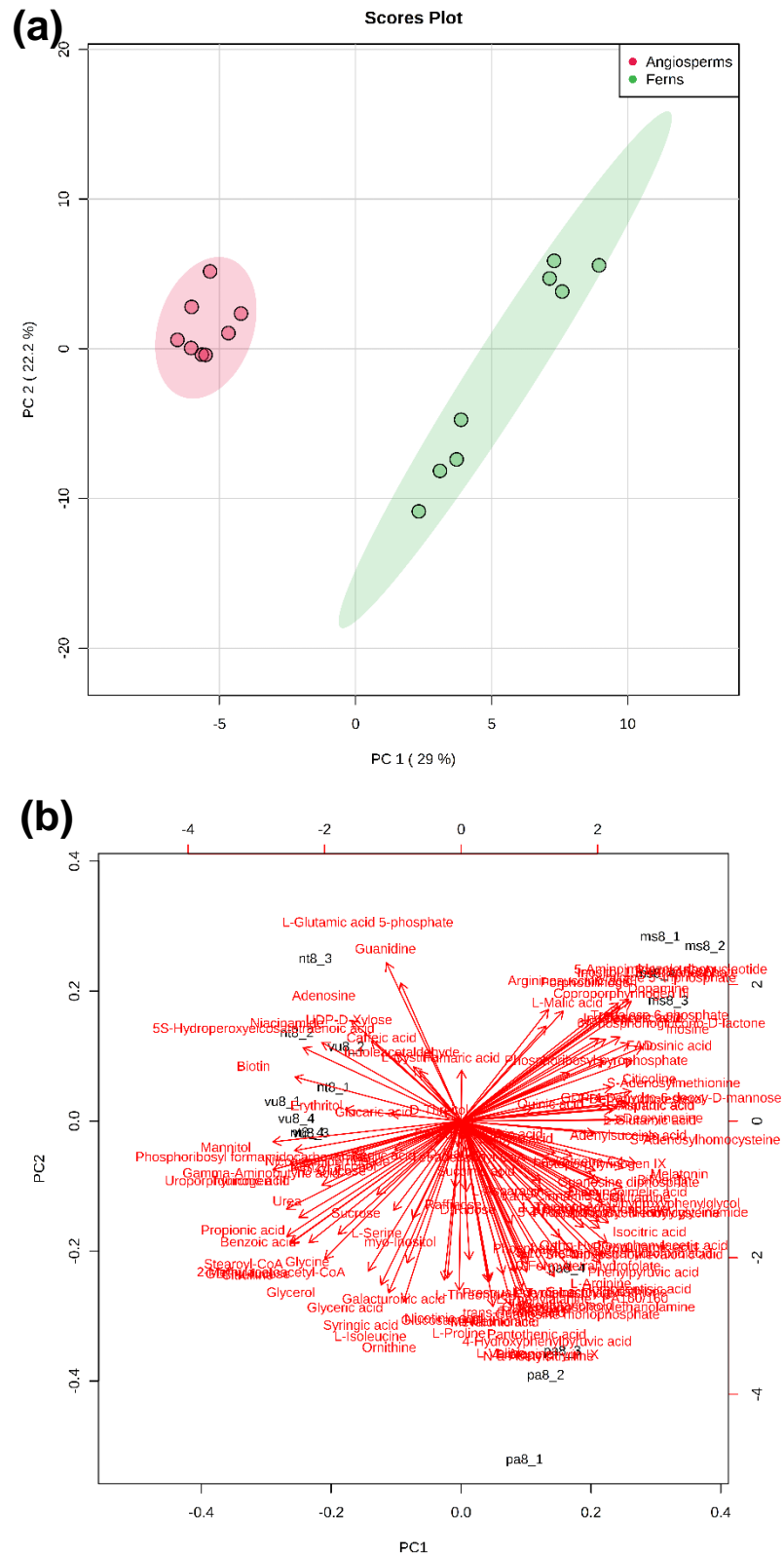
Figure 6. Principal Component Analysis (a) and the overlapped PCA Biplot (b) from detected in leaf by targeted GC-MS and LC-MS analyses.

Fonte: dados da pesquisa.

Figure 7. Heatmap showing 51 significant features (Student's *t* test, $P < 0.05$, n = 4) detected in leaf by targeted GC-MS and LC-MS analyses during the dark to light transitions. Note ferns present higher levels of identified features when compared to angiosperms.

Fonte: dados da pesquisa.

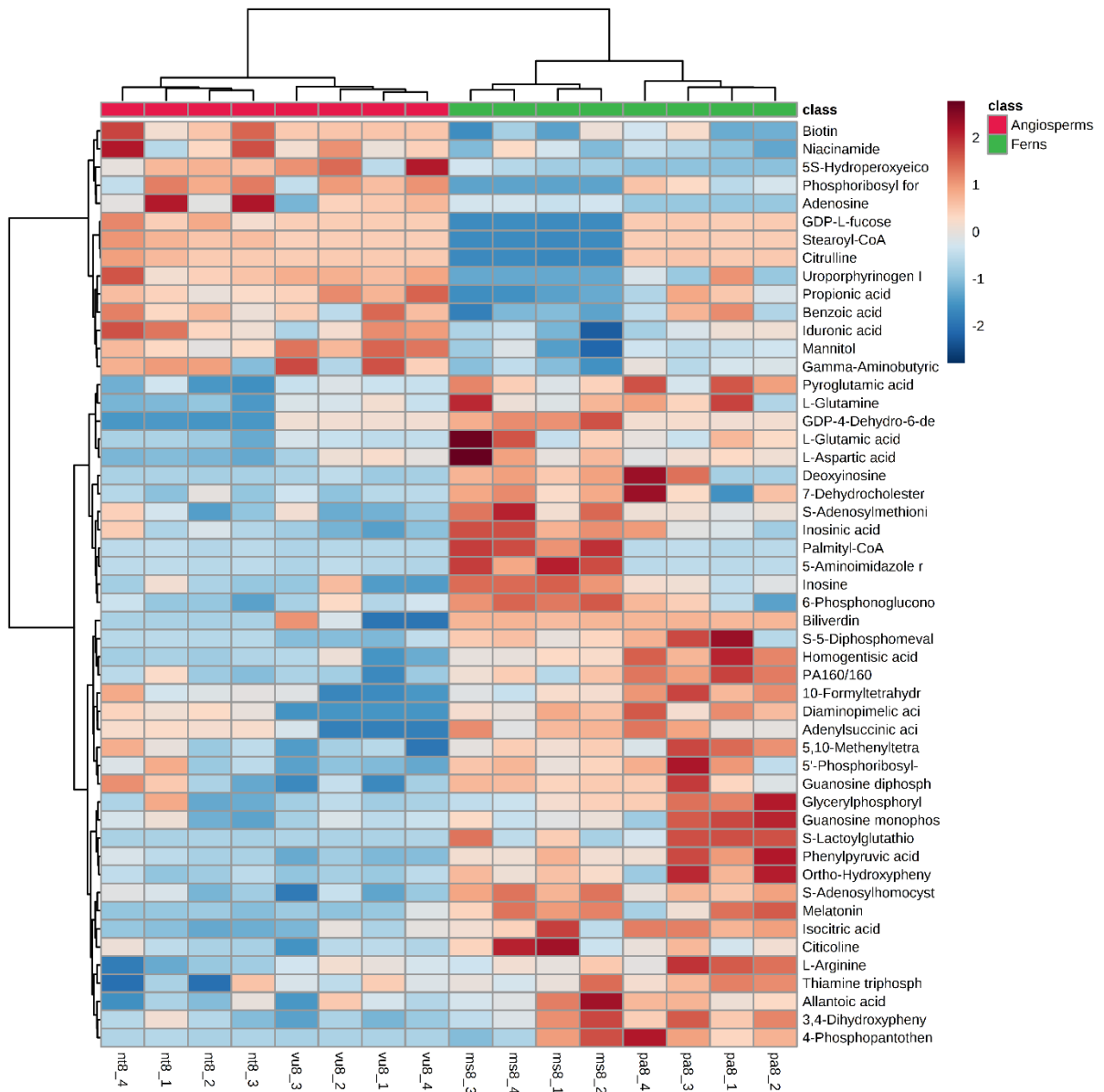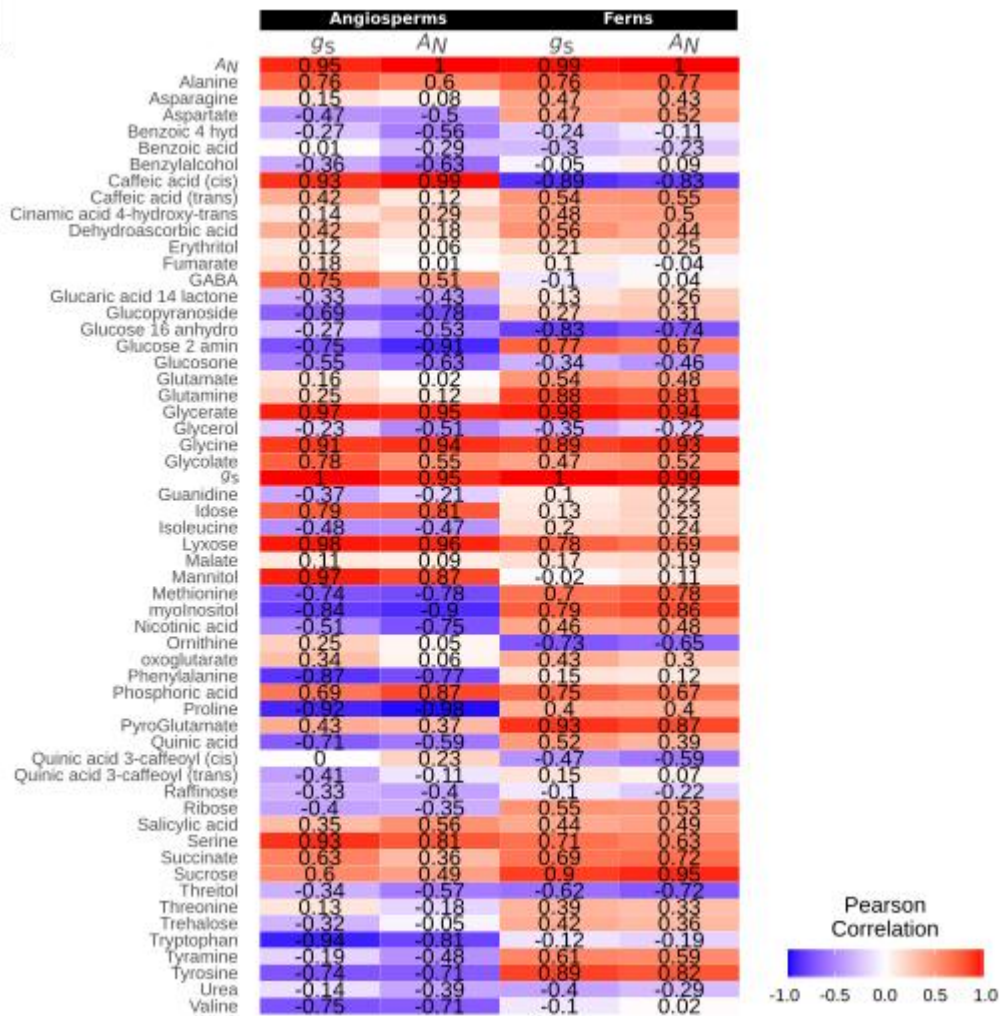|	Angiosperms		Ferns	
	g_s	A_N	g_s	A_N
A_N	0.95	1	0.99	1
Alanine	0.76	0.6	0.76	0.77
Asparagine	0.15	0.08	0.47	0.43
Aspartate	-0.47	-0.5	0.47	0.52
Benzoic 4 hyd	-0.27	-0.56	-0.24	-0.11
Benzoic acid	0.01	-0.29	-0.3	-0.23
Benzylalcohol	-0.36	-0.63	-0.05	0.09
Caffeic acid (cis)	0.93	0.99	-0.89	-0.83
Caffeic acid (trans)	0.42	0.12	0.54	0.55
Cinamic acid 4-hydroxy-trans	0.14	0.29	0.48	0.5
Dehydroascorbic acid	0.42	0.18	0.56	0.44
Erythritol	0.12	0.06	0.21	0.25
Fumarate	0.18	0.01	0.1	-0.04
GABA	0.75	0.51	-0.1	0.04
Glucaric acid 14 lactone	-0.33	-0.43	0.13	0.26
Glucopyranoside	-0.69	-0.78	0.27	0.31
Glucose 16 anhydro	-0.27	-0.53	-0.83	-0.74
Glucose 2 amin	-0.75	-0.91	0.77	0.67
Glucosone	-0.55	-0.63	-0.34	-0.46
Glutamate	0.16	0.02	0.54	0.48
Glutamine	0.25	0.12	0.88	0.81
Glycerate	0.97	0.95	0.98	0.94
Glycerol	-0.23	-0.51	-0.35	-0.22
Glycine	0.91	0.94	0.89	0.93
Glycolate	0.78	0.55	0.47	0.52
g_s	1	0.95	1	0.99
Guanidine	-0.37	-0.21	0.1	0.22
Idose	0.79	0.81	0.13	0.23
Isoleucine	-0.48	-0.47	0.2	0.24
Lyxose	0.98	0.96	0.78	0.69
Malate	0.11	0.09	0.17	0.19
Mannitol	0.97	0.87	-0.02	0.11
Methionine	-0.74	-0.78	0.7	0.78
myoInositol	-0.84	-0.9	0.79	0.86
Nicotinic acid	-0.51	-0.75	0.46	0.48
Ornithine	0.25	0.05	-0.73	-0.65
oxoglutarate	0.34	0.06	0.43	0.3
Phenylalanine	-0.87	-0.77	0.15	0.12
Phosphoric acid	0.69	0.87	0.75	0.67
Proline	-0.92	-0.98	0.4	0.4
PyroGlutamate	0.43	0.37	0.93	0.87
Quinic acid	-0.71	-0.59	0.52	0.39
Quinic acid 3-caffeoyl (cis)	0	0.23	-0.47	-0.59
Quinic acid 3-caffeoyl (trans)	-0.41	-0.11	0.15	0.07
Raffinose	-0.33	-0.4	-0.1	-0.22
Ribose	-0.4	-0.35	0.55	0.53
Salicylic acid	0.35	0.56	0.44	0.49
Serine	0.93	0.81	0.71	0.63
Succinate	0.63	0.36	0.69	0.72
Sucrose	0.6	0.49	0.9	0.95
Threitol	-0.34	-0.57	-0.62	-0.72
Threonine	0.13	-0.18	0.39	0.33
Trehalose	-0.32	-0.05	0.42	0.36
Tryptophan	-0.94	-0.81	-0.12	-0.19
Tyramine	-0.19	-0.48	0.61	0.59
Tyrosine	-0.74	-0.71	0.89	0.82
Urea	-0.14	-0.39	-0.4	-0.29
Valine	-0.75	-0.71	-0.1	0.02

Pearson Correlation

-1.0 -0.5 0.0 0.5 1.0

Figure 8. Heatmap showing Pearson's correlation between the accumulation of metabolites with A_N (net photosynthetic rate - µmol CO_2 m^{-2} s^{-1}) and g_s (stomatal conductance to water vapor - mol H_2O m^{-2} s^{-1}).
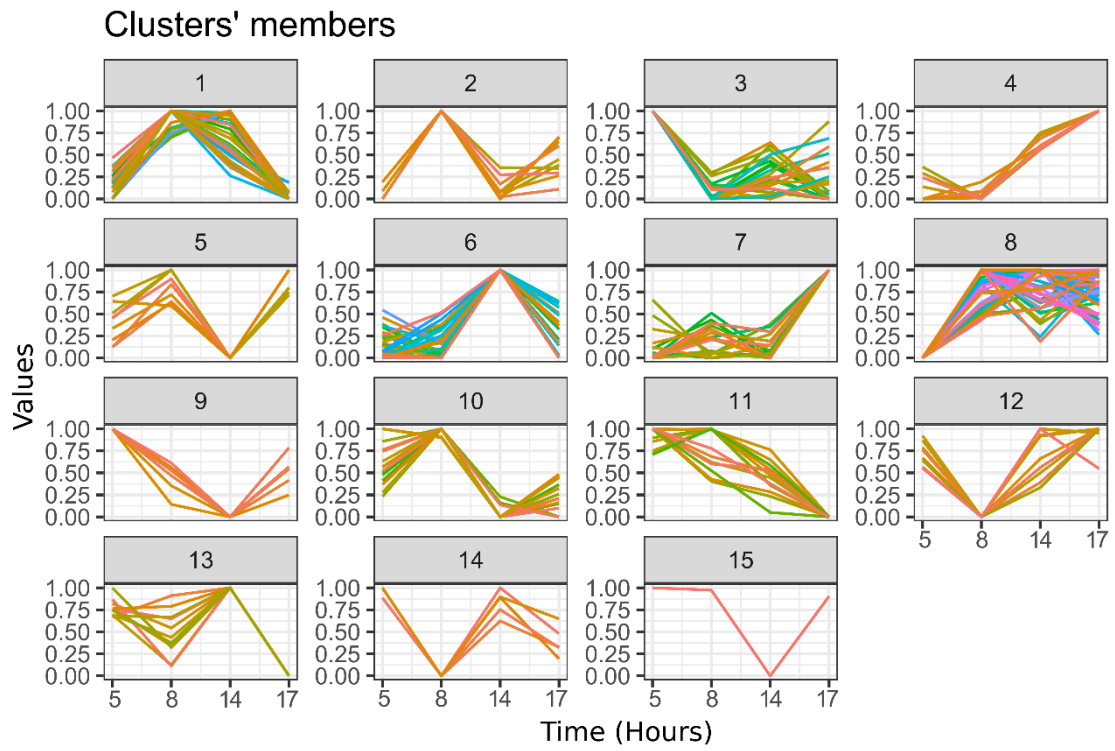
Fonte: dados da pesquisa.

Figure 9. Clusters generated by dtwclust package using euclidean distances for calculations. Cluster 1 holds similarity between gas exchange parameters and metabolites observed in four plants. Cluster 1 members are listed in Table 3.
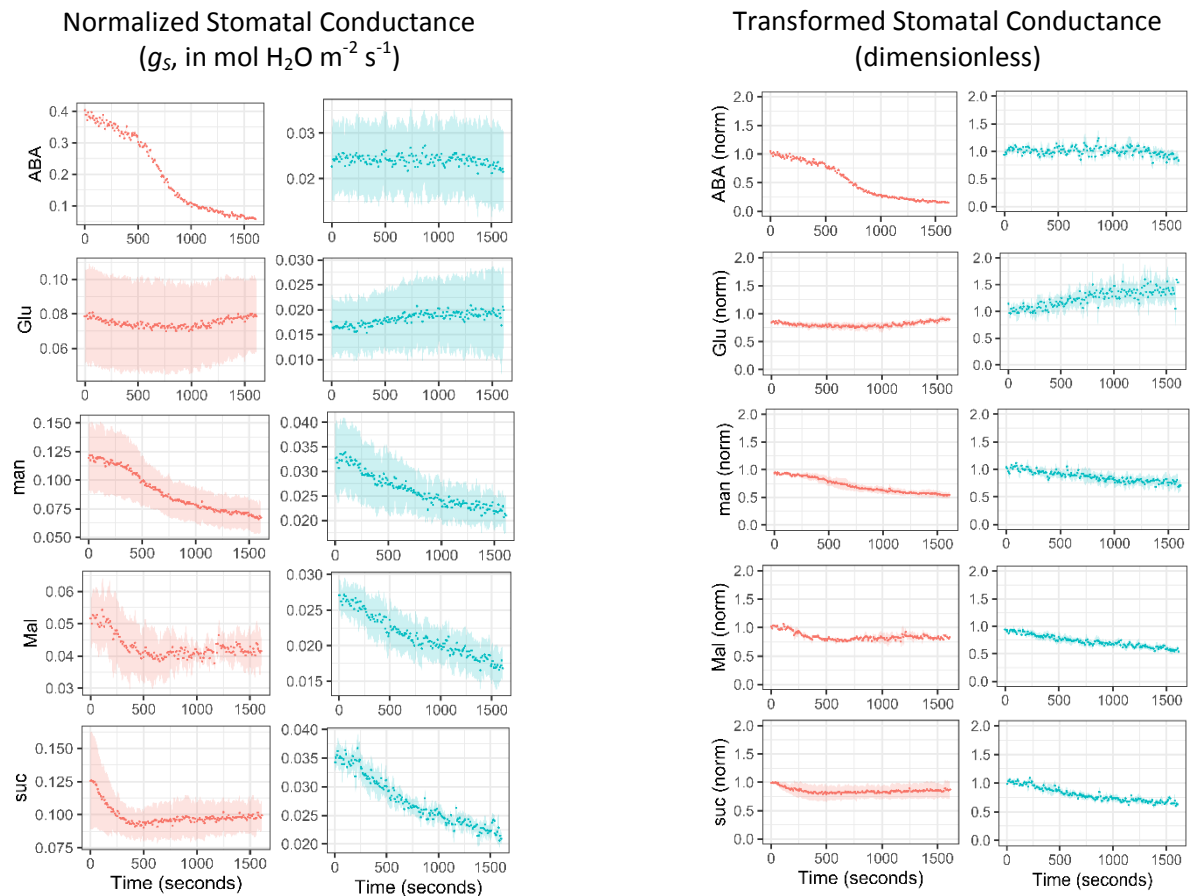
Fonte: dados da pesquisa.

Figure 10. Kinetic of stomatal movements in response to metabolites. Averages of g_s kinetics (left panel) observed under exogenous application of different metabolites in angiosperms (red) and ferns (blue). The right panel demonstrates data transformed using Eqn 1. Legend: Abscisic Acid (ABA), Glutamate (Glu), Mannitol (Man), Malate (Mal) and Sucrose (Suc). All data is represented as average ± SE (n = 3).
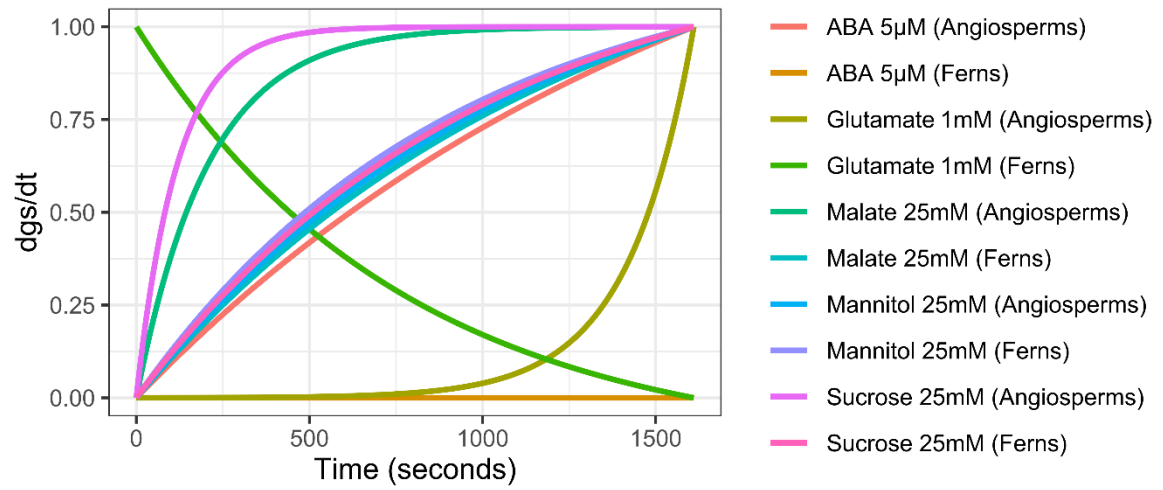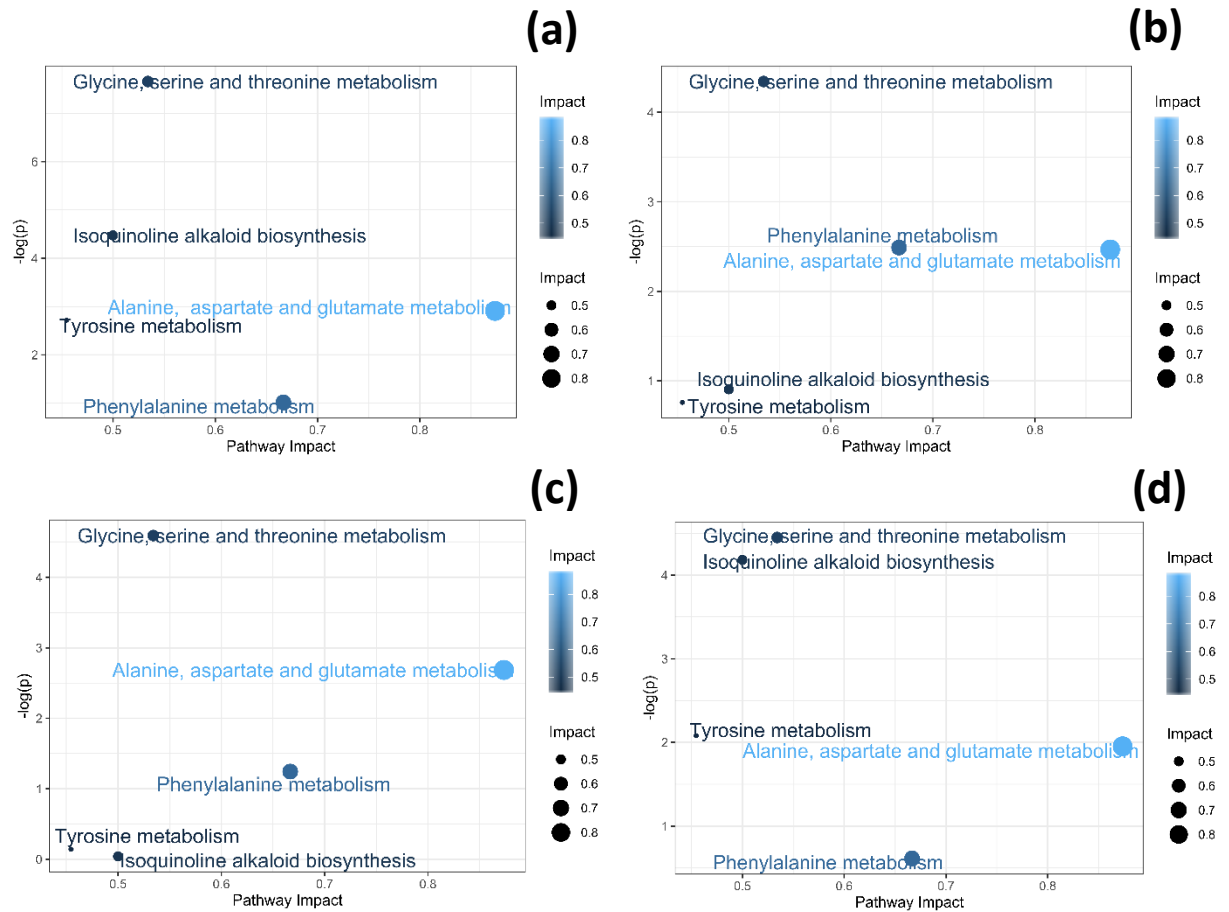
Fonte: dados da pesquisa.

Figure 11. Time-resolved derivatives showing the development of rate of change of g_s along time.

Fonte: dados da pesquisa.

Figures S1. Pathway analyses using data of 57 metabolites annotated. The method used was Global Analysis of Covariance and shows us the impact of pathways while comparing the plant groups on diel course. The pathways shown here are those who reached score above 0.45 on Pathway Impact. Legend: 5:00 h (a), 8:00 h (b), 14:00 h (c) and 17:00 h (d).
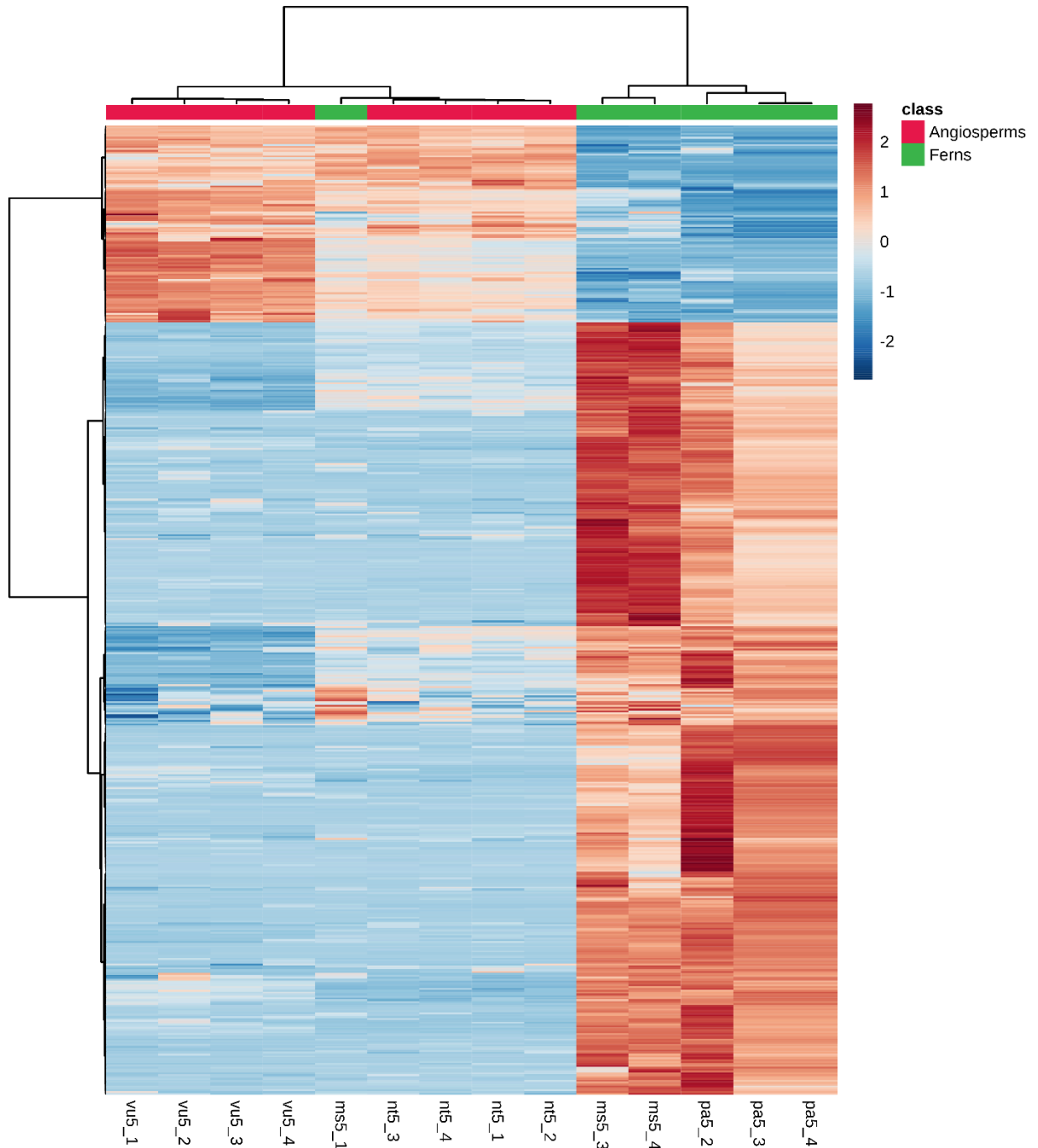
Fonte: dados da pesquisa.

Figure S2. Heatmap showing 517 significant features (Student's *t* test, P < 0.05) detected by LC-MS metabolic fingerprinting analysis in leaf samples harvested at 05:00 h. Note that despite having lower significant difference on variability of features found on both clades, ferns (n = 3) presented the higher levels than angiosperms (n = 4).
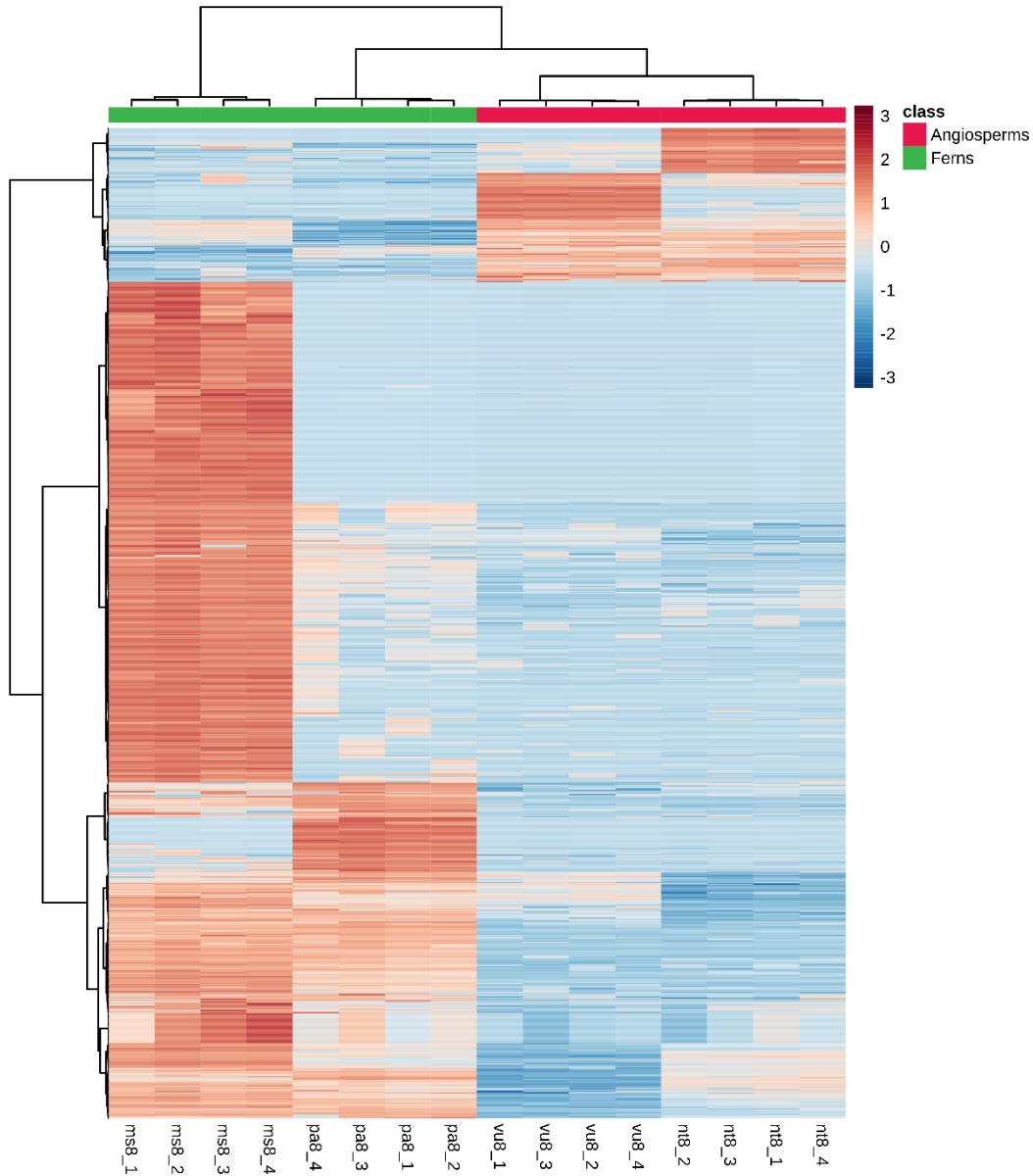
Fonte: dados da pesquisa.

Figure S3. Heatmap showing 4550 significant features (Student's t-test P < 0.05, n = 4) detected by LC-MS metabolic fingerprinting observed in samples collected at 8:00 h. Note there is an increase on amount of significant features noticed in both angiosperms and ferns species but keeping the same trend of higher levels found in ferns.
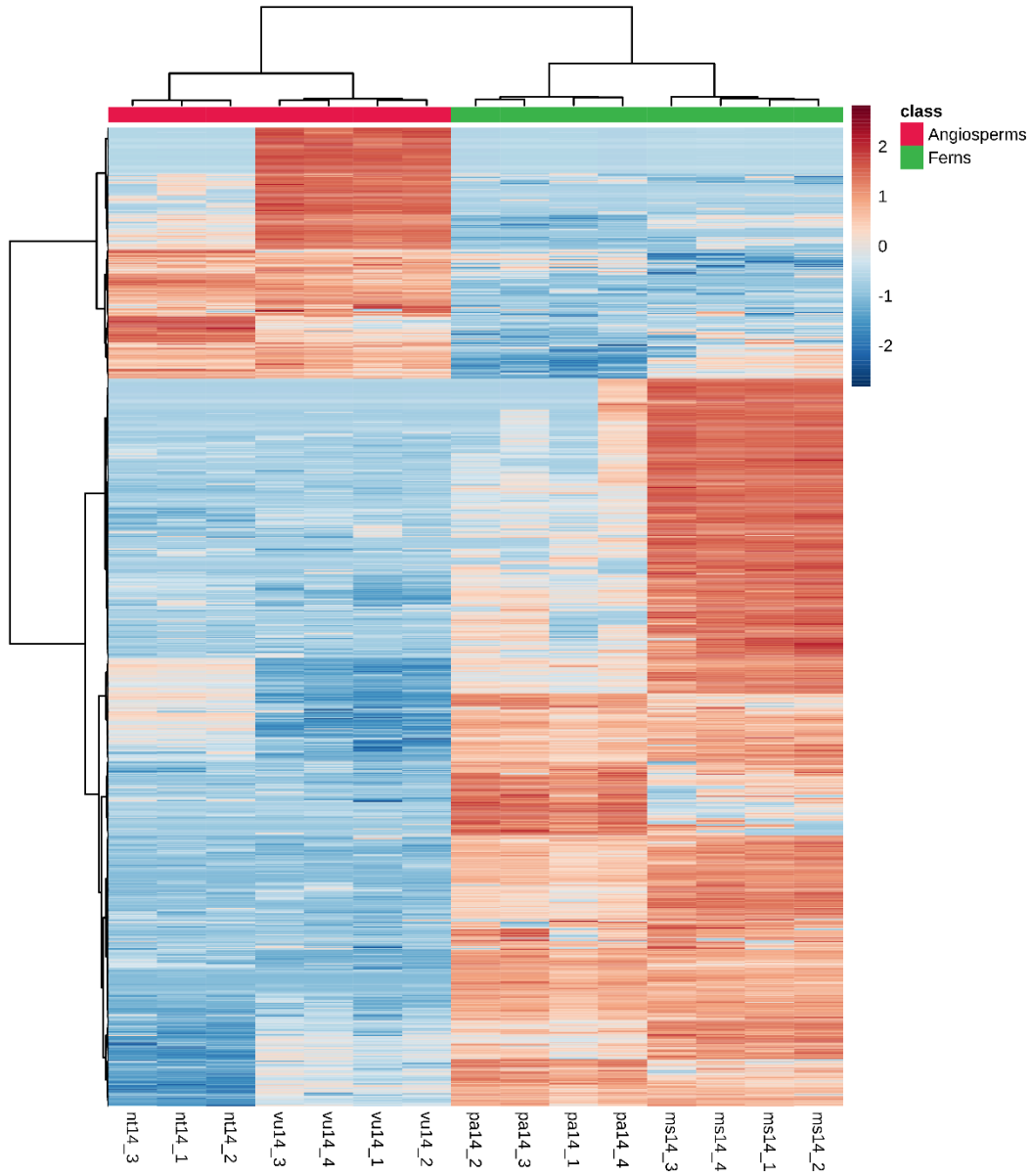
Fonte: dados da pesquisa.

Figure S4. Heatmap showing 2251 significant features (Student's t-test P < 0.05, n = 4) detected by LC-MS metabolic fingerprinting observed in samples collected at 14:00 h.
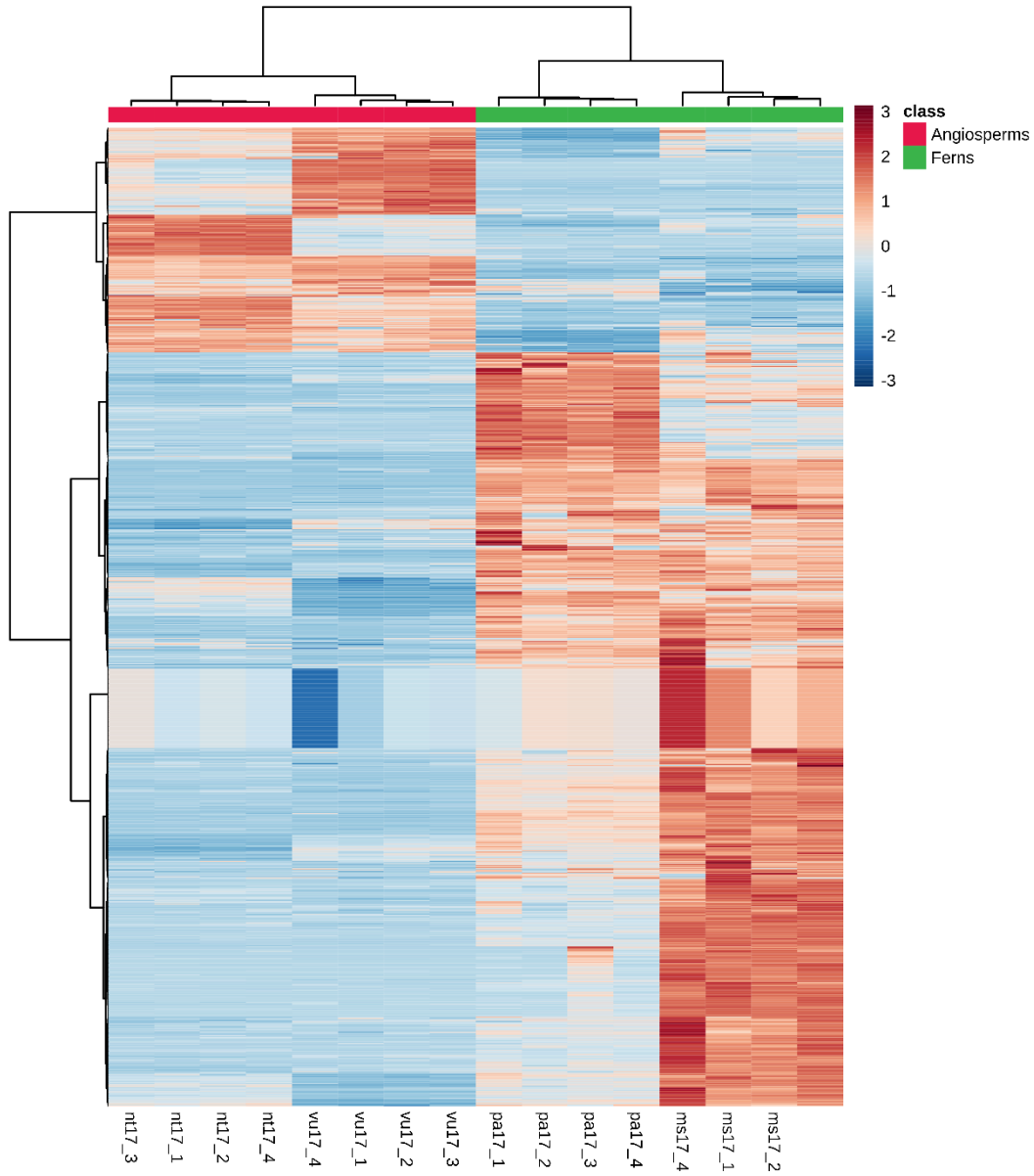
Fonte: dados da pesquisa.

Figure S5. Heatmap showing 1855 significant features (Student's t-test P < 0.05) detected by LC-MS metabolic fingerprinting found in samples collected at 17:00 h.

Fonte: dados da pesquisa.

Table S1. Results of the Dunnett's test of the gas exchange and GC-MS data harvested throughout the diel course. All parameters were tested considering 5 h measurements as control (n = 4). Legend: $P < 0.001$ (***), $P < 0.01$ (**), $P < 0.05$ (*), $P < 0.1$ (•).

Time / Parameters	Angiosperms			Ferns		
	8:00	14:00	17:00	8:00	14:00	17:00
A	***	***	*	***	**	
g_s	***	***		***	**	
E	***	***	•	***	***	
Valine	•		**			
Isoleucine		***	***			
Glycine	*	**				
Glycerate	*	•				
Benzoic acid		•				
Serine	•					
Erythritol		*	*			
GABA	•					
Aspartate		**				
Glutamine		•				
Tyrosine			**			
Malate		•	•			

Fonte: dados da pesquisa.

Table S2. Features held in Cluster 1 from dtwclust result with gas exchange parameters and metabolic levels which had the same trend along the diel course.

| Species	Feature
V. unguiculata	A
V. unguiculata	gs
V. unguiculata	E
V. unguiculata	Glycine
V. unguiculata	Glycerate
V. unguiculata	Serine
V. unguiculata	Mannitol
V. unguiculata	Caffeic acid
V. unguiculata	Quinic acid
N. tabacum	A
N. tabacum	gs
N. tabacum	E
N. tabacum	Glycine
N. tabacum	GABA
N. tabacum	Lyxose
N. tabacum	Mannitol
N. tabacum	Idose
M. scolopendria	A
M. scolopendria	gs
M. scolopendria	E
M. scolopendria	Aspartate
P. aureum	A
P. aureum	gs
P. aureum	E
P. aureum	Alanine
P. aureum	Glycerate
P. aureum	Glutamine
P. aureum	Pyroglutamate
P. aureum	Idose
P. aureum	Caffeic acid
P. aureum	Sucrose

Fonte: dados da pesquisa.
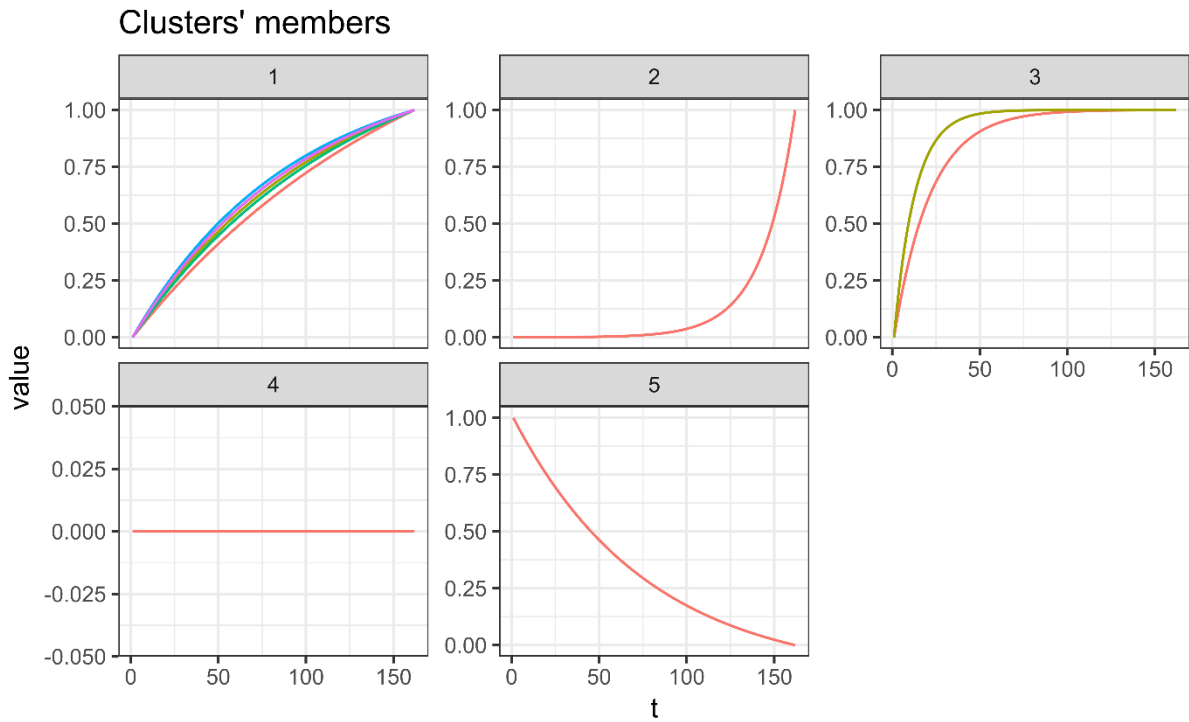
Figure S6. Observed dg_s/dt rates of change observed in the stomatal kinetic experiments. Cluster 1 members: ABA (Angiosperms), Mannitol (Angiosperms), Malate (Ferns), Mannitol (Ferns) and Sucrose (Ferns). Cluster 2 members: Glutamine (Angiosperms). Cluster 3 members: Malate (Angiosperms), Sucrose (Angiosperms). Cluster 4 member: ABA (Ferns). Cluster 5 members: Glutamine (Ferns).

Fonte: dados da pesquisa.

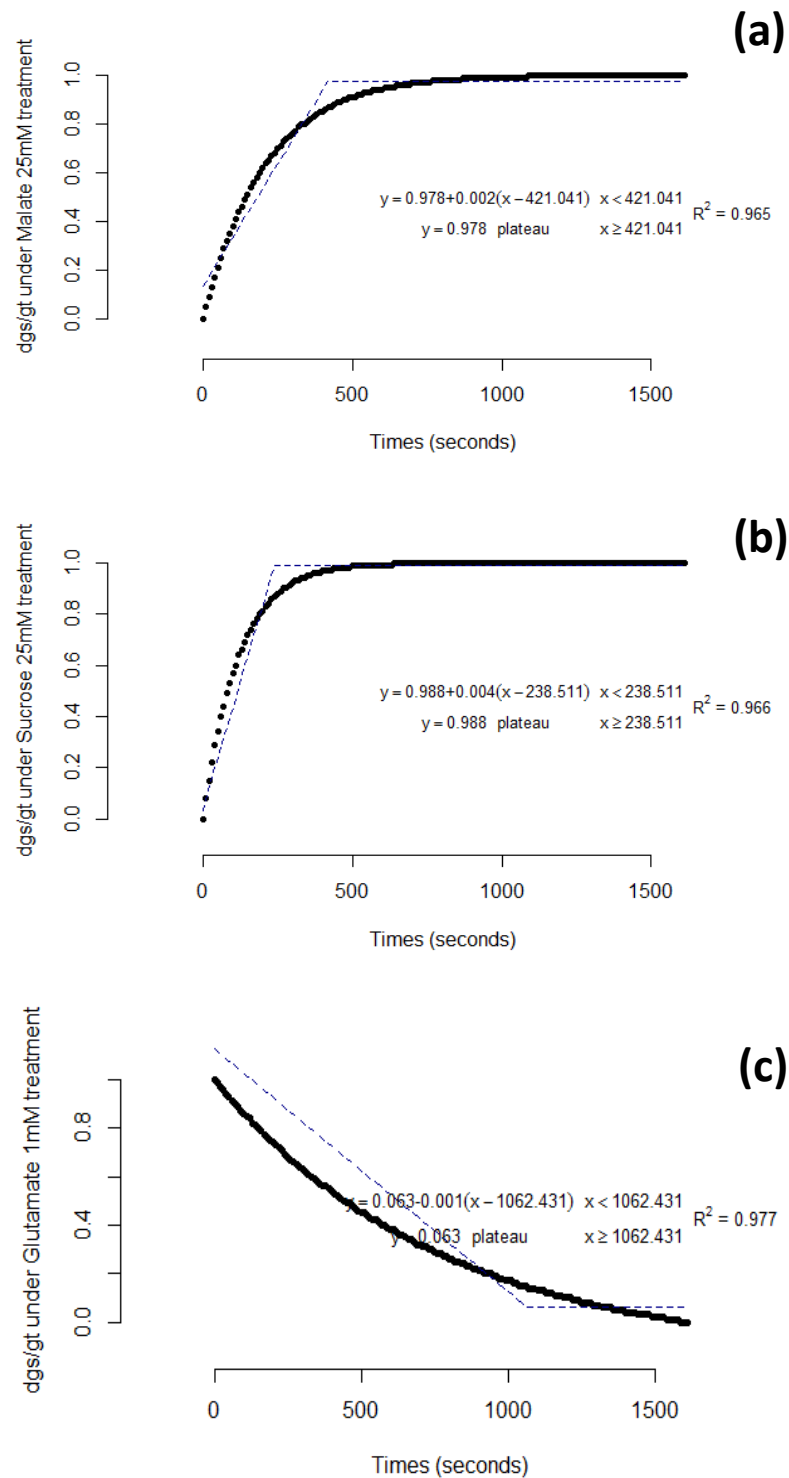**Figure S7.** dg_s/dt over malate 25mM (a), sucrose 25 mM (b) treatments in angiosperms and glutamate 1mM treament in ferns. Sucrose treatment was the fastest treatment presented to reach Vmax (238 s) followed by malate treatment, which reached Vmax at 421 s. Glutamate 1mM treatment induced an increase in gs where this velocity decreased reaching stability after 1062 s.

Fonte: dados da pesquisa.

6 CONCLUSIONS

Guard cells possess a specialized metabolism when compared to their mesophyll counterparts, which seem to have co-evolved a sensitivity for certain key metabolites the coordinate photosynthesis with stomatal movements regulation. Angiosperms evolved traits that leads to a higher carbon fixation at cost on lower efficiency on water use. By contrast, ferns seem to have a strategy that maximize carbon fixation with less consumption of water; i.e. higher water use efficiency; and this seems to be also associated with higher production of secondary metabolites which can also confer other benefits to ferns such as stress tolerance.

REFERENCES

AMTHOR, J. S. et al. Engineering strategies to boost crop productivity by cutting respiratory carbon loss. **The Plant Cell**. Reston, EUA, v. 31, n. 2, p. 297–314, 2019.

ANTUNES, W. C. et al. Guard cell-specific down-regulation of the sucrose transporter SUT1 leads to improved water use efficiency and reveals the interplay between carbohydrate metabolism and K^+ accumulation in the regulation of stomatal opening. **Environmental and Experimental Botany**. [*s. l.*], v. 135, p. 73–85, 2017.

ARAÚJO, W. L. et al. Antisense Inhibition of the Iron-Sulphur Subunit of Succinate Dehydrogenase Enhances Photosynthesis and Growth in Tomato via an Organic Acid–Mediated Effect on Stomatal Aperture. **The Plant Cell**. Reston, EUA, v. 23, n. 2, p. 600–627, fev. 2011.

AUBRY, S. et al. A Specific Transcriptome Signature for Guard Cells from the C4 Plant Gynandropsis gynandra. **Plant Physiology**. Herndon, EUA, v. 170, n. 3, p. 1345–1357, 2016.

AZOULAY-SHEMER, T. et al. Guard cell photosynthesis is critical for stomatal turgor production, yet does not directly mediate CO_2- and ABA-induced stomatal closing. **Plant Journal**. Oxford, Reino Unido, v. 83, n. 4, p. 567–581, 2015.

AZOULAY-SHEMER, T. et al. Starch biosynthesis in guard cells but not in mesophyll cells is involved in CO2-induced stomatal closing. **Plant physiology**. Herndon, EUA, v. 171, n. 2, p. 788–98, 2016.

AZOULAY-SHEMER, T. et al. Starch biosynthesis by AGPase, but not starch degradation by BAM1/3 and SEX1, is rate-limiting for CO2-regulated stomatal movements under short-day conditions. **FEBS Letters**. Oxford, Reino Unido, v. 592, n. 16, p. 2739–2759, 2018.

BATES, G. W. et al. A Comparative Study of the Arabidopsis thaliana Guard-Cell Transcriptome and Its Modulation by Sucrose. **PLoS ONE**. San Francisco, EUA, v. 7, n. 11, 2012.

BRODRIBB, T. J. et al. Evolution of stomatal responsiveness to CO2and optimization of water-use efficiency among land plants. **New Phytologist**. Lancaster, Reino Unido, v. 183, n. 3, p. 839–847, 2009.

BRODRIBB, T. J.; MCADAM, S. A. M. Passive origins of stomatal control in vascular plants. **Science**. Washington D.C., EUA, v. 331, n. 6017, p. 582–585, 2011.

BRODRIBB, T. J.; SUSSMILCH, F.; MCADAM, S. A. M. From reproduction to production, stomata are the master regulators. **Plant Journal**. Oxford, Reino Unido, p. 0–2, 2019.

BROWN, P. H.; OUTLAW, W. H. Effect of Fusicoccin on Dark CO(2) Fixation by Vicia faba Guard Cell Protoplasts. **Plant Physiology**. Herndon, EUA, v. 70, n. 6, p. 1700–1703, 1982.

CAI, S. et al. Evolutionary Conservation of ABA Signaling for Stomatal Closure. **Plant Physiology**. Herndon, EUA, v. 174, n. 2, p. 732–747, 2017.

CECILIATO, P. H. O. et al. Intact leaf gas exchange provides a robust method for measuring the kinetics of stomatal conductance responses to abscisic acid and other small molecules in Arabidopsis and grasses. **Plant Methods**. [*s. l.*], v. 15, n. 1, p. 38, 17 dez. 2019.

CHATER, C. C. C. et al. Origins and Evolution of Stomatal Development. **Plant Physiology**. Herndon, EUA, v. 174, n. 2, p. 624–638, 2017.

CHONG, J.; YAMAMOTO, M.; XIA, J. MetaboAnalystR 2.0: From raw spectra to biological insights. **Metabolites**. [*s. l.*], v. 9, n. 3, 2019.

DALOSO, D. M. et al. Tobacco guard cells fix CO_2 by both Rubisco and PEPcase while sucrose acts as a substrate during light-induced stomatal opening. **Plant Cell and Environment**. [*s. l.*], v. 38, n. 11, p. 2353–2371, 2015.

DALOSO, D. M. et al. Guard cell-specific upregulation of sucrose synthase 3 reveals that the role of sucrose in stomatal function is primarily energetic. **New Phytologist**. Lancaster, Reino Unido, v. 209, n. 4, p. 1470–1483, 2016.

DEANS, R. M. et al. Plant water-use strategy mediates stomatal effects on the light induction of photosynthesis. **New Phytologist**. Lancaster, Reino Unido, v. 222, n. 1, p. 382–395, abr. 2019.

DEANS, R. M.; BRODRIBB, T. J.; MCADAM, S. A. M. An Integrated Hydraulic-Hormonal Model of Conifer Stomata Predicts Water Stress Dynamics. **Plant Physiology**. Herndon, EUA, v. 174, n. 2, p. 478–486, 2017.

DOI, M. et al. A transgene encoding a blue-light receptor, phot1, restores blue-light responses in the Arabidopsis phot1 phot2 double mutant. **Journal of Experimental Botany**. [*s. l.*], v. 55, n. 396, p. 517–523, 1 fev. 2004.

DONG, H. et al. Modulation of Guard Cell Turgor and Drought Tolerance by a Peroxisomal Acetate–Malate Shunt. **Molecular Plant**. [*s. l.*], v. 11, n. 10, p. 1278–1291, 2018.

DOS ANJOS, L. et al. Feedback regulation by trehalose 6-phosphate slows down starch mobilization below the rate that would exhaust starch reserves at dawn in Arabidopsis leaves. **Plant Direct**. [*s. l.*], v. 2, n. 8, p. e00078, 2018.

EDWARDS, D.; KERP, H.; HASS, H. Stomata in early land plants: an anatomical and ecophysiological approach. **Journal of Experimental Botany**. Lancaster, Reino Unido, v. 49, n. Special, p. 255–278, 1 mar. 1998.

EISENACH, C. et al. ABA-Induced Stomatal Closure Involves ALMT4, a Phosphorylation-Dependent Vacuolar Anion Channel of Arabidopsis. **The Plant Cell**. Reston, EUA, p. tpc.00452.2017, 2017.

EXTON, J. H. Mechanisms of action of calcium-mobilizing agonists: some variations on a young theme. **The FASEB Journal**. Bethesda, EUA, v. 2, n. 11, p. 2670–2676, 1988.

FIGUEROA, C. M.; LUNN, J. E. A Tale of Two Sugars: Trehalose 6-Phosphate and Sucrose.

Plant Physiology. Herndon, EUA, v. 172, n. 1, p. 7–27, 2016.

FRANKS, P. J. et al. Stomatal Function across Temporal and Spatial Scales: Deep-Time Trends, Land-Atmosphere Coupling and Global Models. **Plant Physiology**. Herndon, EUA, v. 174, n. 2, p. 583–602, 2017.

FRANKS, P. J.; BRITTON-HARPER, Z. J. No evidence of general CO2 insensitivity in ferns: one stomatal control mechanism for all land plants? **The New phytologist**. Lancaster, Reino Unido, v. 211, n. 3, p. 819–827, 2016.

GAGO, J. et al. Opportunities for improving leaf water use efficiency under climate change conditions. **Plant Science**. [*s. l.*], v. 226, n. 0, p. 108–119, 2014.

GAGO, J. et al. Relationships of Leaf Net Photosynthesis, Stomatal Conductance, and Mesophyll Conductance to Primary Metabolism: A Multispecies Meta-Analysis Approach. **Plant Physiology**. Herndon, EUA, v. 171, n. 1, p. 265–279, 2016.

GAGO, J. et al. Photosynthesis Optimized across Land Plant Phylogeny. **Trends in Plant Science**. [*s. l.*], v. 24, n. 10, p. 947–958, 2019.

GEIGENBERGER, P. et al. The Unprecedented Versatility of the Plants Thioredoxin System. **Trends in Plant Science**. [*s. l.*], v. 22, n. 3, p. 249–262, 2017.

GOTOW, K.; TAYLOR, S.; ZEIGER, E. Photosynthetic carbon fixation in guard cell protoplasts of Vicia faba L. **Plant physiology**. Herndon, EUA, v. 86, p. 700–705, 1988.

GRACELIN, D. H. S. et al. ANTIBACTERIAL SCREENING OF A FEW MEDICINAL FERNS AGAINST ANTIBIOTIC RESISTANT PHYTO PATHOGEN. **International Journal Of Pharmaceutical Sciences And Research**. [*s. l.*], v. 3, n. 03, p. 868–873, 2012.

GRAF, A. et al. Circadian control of carbohydrate availability for growth in Arabidopsis plants at night. **Proceedings of the National Academy of Sciences of the United States of America**. [*s. l.*] v. 107, n. 20, p. 9458–9463, 2010.

HEDRICH, R.; MARTEN, I. Malate-induced feedback regulation of plasma membrane anion channels could provide a C02 sensor to guard cells. **EMBO Journal**, Heidelberg, Alemanha v. 12, n. 3, p. 897–901, 1993.

HEDRICH, R.; RASCHKE, K.; STITT, M. A role for fructose 2,6-bisphosphate in regulating carbohydrate metabolism in guard cells. **Plant physiology**. Herndon, EUA, v. 79, n. 4, p. 977–82, 1985.

HOAGLAND, D. R.; ARNON, D. I. **The Water-Culture Method for Growing Plants without Soil. THE COLLEGE OF AGRICULTURE**. California, EUA, 2 ed., 1950.

HÕRAK, H.; KOLLIST, H.; MERILO, E. Fern Stomatal Responses to ABA and CO$_2$ Depend on Species and Growth Conditions. **Plant Physiology**. Herndon, EUA, v. 174, n. 2, p. 672–679, 2017.

HORRER, D. et al. Blue light induces a distinct starch degradation pathway in guard cells for

stomatal opening. **Current Biology**. [*s. l.*], v. 26, n. 3, p. 362–370, 2016.

JALAKAS, P. et al. ABA-mediated regulation of stomatal density is OST1-independent. **Plant Direct**. [*s. l.*], v. 2, n. 9, p. e00082, 2018.

KANG, Y. et al. Guard-cell apoplastic sucrose concentration - A link between leaf photosynthesis and stomatal aperture size in the apoplastic phloem loader Vicia faba L. **Plant, Cell and Environment**. [*s. l.*], v. 30, n. 5, p. 551–558, 2007.

KELLY, G. et al. Hexokinase mediates stomatal closure. **Plant Journal**. Oxford, Reino Unido, v. 75, n. 6, p. 977–988, 2013a.

KELLY, G. et al. Hexokinase mediates stomatal closure. **The Plant Journal**. Oxford, Reino Unido, v. 75, n. 6, p. 977–988, set. 2013b.

KOTTAPALLI, J. et al. Sucrose-induced stomatal closure is conserved across evolution. **PLoS ONE**. San Francisco, EUA, v. 13, n. 10, p. 1–17, 2018.

LAU, O. S. Stomata. In: **eLS**. Chichester, UK: John Wiley & Sons, Ltd, 2017. v. 1p. 1–9. LAWSON, T. et al. Responses of Photosynthetic Electron Transport in Stomatal Guard Cells and Mesophyll Cells in Intact Leaves to Light , CO 2 , and Humidity 1. **Encyclopedia of Life Sciences**. [*s. l.*] v. 128, n. January, p. 52–62, 2002.

LAWSON, T. Guard cell photosynthesis and stomatal function. **New Phytologist**. Lancaster, Reino Unido, v. 181, n. 1, p. 13–34, 2009.

LAWSON, T. et al. Mesophyll photosynthesis and guard cell metabolism impacts on stomatal behaviour. **New Phytologist**. Lancaster, Reino Unido, v. 203, n. 4, p. 1064–1081, 2014.

LAWSON, T.; VIALET-CHABRAND, S. Speedy stomata, photosynthesis and plant water use efficiency. **New Phytologist**. Lancaster, Reino Unido, v. 221, n. 1, p. 93–98, jan. 2019.

LI, Y. et al. Glucose- and mannose-induced stomatal closure is mediated by ROS production, Ca 2+ and water channel in Vicia faba. **Physiologia Plantarum**. Lund, Suécia, v. 156, n. 3, p. 252–261, mar. 2016.

LIGRONE, R.; DUCKETT, J. G.; RENZAGLIA, K. S. Major transitions in the evolution of early land plants: a bryological perspective. **Annals of Botany**. Oxford, Reino Unido, v. 109, n. 5, p. 851–871, abr. 2012.

LIMA, V. F. et al. Toward multifaceted roles of sucrose in the regulation of stomatal movement. **Plant Signaling & Behavior**. [*s. l.*], v. 13, n. 8, p. 1–8, ago. 2018.

LIMA, V. F. et al. The sucrose-to-malate ratio correlates with the faster CO_2 and light stomatal responses of angiosperms compared to ferns. **New Phytologist**. Lancaster, Reino Unido, v. 223, n. 4, p. 1873–1887, 26 set. 2019.

LU, P. et al. A new mechanism for the regulation of stomatal aperture size in intact leaves (accumulation of mesophyll-derived sucrose in the guard-cell wall of Vicia faba). **Plant physiology**. Herndon, EUA, v. 114, n. 1, p. 109–118, 1997.

MAZID, M.; KHAN, T. A.; MOHAMMAD, F. Role of secondary metabolites in defense mechanisms of plants. **Biology and Medicine**. Barcelona, Spain, v. 3, n. 2 SPECIALISSUE, p. 232–249, 2011.

MCADAM, S. A. M.; BRODRIBB, T. J. Stomatal innovation and the rise of seed plants. **Ecology Letters**. Montpellier, França, v. 15, n. 1, p. 1–8, 2012.

MCAUSLAND, L. et al. Effects of kinetics of light-induced stomatal responses on photosynthesis and water-use efficiency. **The New phytologist**. Lancaster, Reino Unido, v. 211, n. 4, p. 1209–1220, 2016.

MCLACHLAN, D. H. et al. The Breakdown of Stored Triacylglycerols is Required during Light-Induced Stomatal Opening. **Current Biology**. [*s. l.*], v. 26, n. 5, p. 707–712, 2016.

MEDEIROS, D. B. et al. Enhanced Photosynthesis and Growth in *atquac1* Knockout Mutants Are Due to Altered Organic Acid Accumulation and an Increase in Both Stomatal and Mesophyll Conductance. **Plant Physiology**. Herndon, EUA, v. 170, n. 1, p. 86–101, 2016.

MEDEIROS, D. B. et al. Impaired malate and fumarate accumulation due the mutation of tonoplast dicarboxylate transporter. **Plant Physiology**. Herndon, EUA, v. 175, n. November, p. pp.00971.2017, 2017.

MEDEIROS, D. B. et al. Sucrose breakdown within guard cells provides substrates for glycolysis and glutamine biosynthesis during light-induced stomatal opening. **Plant Journal**. Oxford, Reino Unido, v. 94, n. 4, p. 583–594, 2018.

NEGI, J. et al. Eukaryotic lipid metabolic pathway is essential for functional chloroplasts and CO_2 and light responses in *Arabidopsis* guard cells. **Proceedings of the National Academy of Sciences.** Washington D.C., EUA, p. 201810458, 2018.

NG, C. K. Y. et al. Calcium-based signalling systems in guard cells. **New Phytologist**. Lancaster, Reino Unido, v. 151, n. 1, p. 109–120, 2001.

NG, C. K. Y.; HETHERINGTON, A. M. Sphingolipid-mediated signalling in plants. **Annals of Botany**.Oxford, Reino Unido, v. 88, n. 6, p. 957–965, 2001.

OUTLAW, W. H. et al. Photosynthetic carbon reduction pathway is absent in chloroplasts of *Vicia faba* guard cells. **Proceedings of the National Academy of Sciences of the United States of America**. Washington D.C., EUA, v. 76, n. 12, p. 6371–5, 1979.

OUTLAW, W. H. J. Integration of Cellular and Physiological Functions of Guard Cells. **Critical Reviews in Plant Sciences**. [*s. l.*], v. 22, n. 6, p. 503–5229, 2003.

OUTLAW, W. H.; TARCZYNSKI, M. C.; ANDERSON, L. C. Taxonomic survey for the presence of ribulose-1,5-bisphosphate carboxylase activity in guard cells. **Plant physiology**. Herndon, EUA, v. 70, p. 1218–1220, 1982.

PANDEY, S.; ZHANG, W.; ASSMANN, S. M. Roles of ion channels and transporters in guard cell signal transduction. **FEBS Letters**. Oxford, Reino Unido, v. 581, n. 12, p. 2325–

2336, 2007.

PAUTOV, A. et al. Large lipid droplets in leaf epidermis of angiosperms. **Flora: Morphology, Distribution, Functional Ecology of Plants**. [*s. l.*], v. 219, p. 62–67, 2016.

PENFIELD, S. et al. Expression and manipulation of PHOSPHOENOLPYRUVATE CARBOXYKINASE 1 identifies a role for malate metabolism in stomatal closure. **Plant Journal.** Oxford, Reino Unido, v. 69, n. 4, p. 679–688, 2012.

PITTERMANN, J.; BRODERSEN, C.; WATKINS, J. E. The physiological resilience of fern sporophytes and gametophytes: Advances in water relations offer new insights into an old lineage. **Frontiers in Plant Science**. Lausane, Suíça, v. 4, n. AUG, 2013.

RECKMANN, U.; SCHEIBE, R.; RASCHKE, K. Rubisco activity in guard cells compared with the solute requirement for stomatal opening. **Plant physiology**. Herndon, EUA, v. 92, n. 1, p. 246–53, 1990.

RITTE, G. et al. Rates of sugar uptake by guard cell protoplasts of Pisum sativum L. related to the solute requirement for stomatal opening. **Plant physiology**. Herndon, EUA, v. 121, n. 2, p. 647–656, 1999.

RUSZALA, E. M. et al. Land plants acquired active stomatal control early in their evolutionary history. **Current Biology**. [*s. l.*] v. 21, n. 12, p. 1030–1035, 2011.

SAKAKI, T. et al. Lipids and fatty acids in guard-cell protoplasts from Vicia faba leaves. **Phytochemistry**. [*s. l.*], v. 40, n. 4, p. 1065–1070, 1995.

SATO, N. Lipid Biosynthesis in Epidermal, Guard and Mesophyll Cell Protoplasts from Leaves of Vicia faba L. **Plant and Cell Physiology.** [*s. l.*], v. 26, n. 5, p. 805–811, jul. 1985.

SCHROEDER, J. I. Knockout of the guard cell K^+ out channel and stomatal movements. **Proceedings of the National Academy of Sciences**. Washington, D.C., EUA, v. 100, n. 9, p. 4976–4977, 2003.

SHALIT-KANEH, A. et al. Multiple feedback loops of the Arabidopsis circadian clock provide rhythmic robustness across environmental conditions. **Proceedings of the National Academy of Sciences of the United States of America**. Washington, D.C., EUA], v. 115, n. 27, p. 7147–7152, 2018.

SHIMAZAKI, K. Ribulosebisphosphate carboxylase activity and photosynthetic 02 evolution rate in Vicia guard-cell protoplasts. **Plant Physiology**. Herndon, EUA, v. 91, p. 459–463, 1989.

STADLER, R. Diurnal and Light-Regulated Expression of AtSTP1 in Guard Cells of Arabidopsis. **Plant Physiology**. Herndon, EUA, v. 133, n. 2, p. 528–537, 2003.

SUSSMILCH, F. C. et al. Acquiring Control: The Evolution of Stomatal Signalling Pathways. **Trends in Plant Science**. [*s. l.*], v. 24, n. 4, p. 342–351, 2019.

TCHERKEZ, G. et al. Respiratory carbon fluxes in leaves. **Current Opinion in Plant**

Biology. [*s. l.*], v. 15, n. 3, p. 308–314, 2012.

TOHGE, T. et al. Characterization of a recently evolved flavonol-phenylacyltransferase gene provides signatures of natural light selection in Brassicaceae. **Nature Communications**. Berlin, Alemanha, v. 7, n. August, p. 1–11, 2016.

TOHGE, T.; DE SOUZA, L. P.; FERNIE, A. R. Current understanding of the pathways of flavonoid biosynthesis in model and crop plants. **Journal of Experimental Botany**. Lancaster, Reino Unido, v. 68, n. 15, p. 4013–4028, 2017.

TOMINAGA, M.; KINOSHITA, T.; SHIMAZAKI, K. I. Guard-cell chloroplasts provide ATP required for H+pumping in the plasma membrane and stomatal opening. **Plant and Cell Physiology**. [*s. l.*] v. 42, n. 8, p. 795–802, 2001.

UNIVERSIDADE FEDERAL DO CEARÁ. **Guia de normatização de trabalhos acadêmicos da Universidade Federal do Ceará**. Fortaleza, Brasil. Biblioteca Universitária/Comissão de Normaalização, , 2017. Disponível em: <http://www.biblioteca.ufc.br/wp-content/uploads/2015/08/guia-normalizacao-trabalhos-ufc-2013.pdf>

VAN HOUTTE, H. et al. Overexpression of the Trehalase Gene AtTRE1 Leads to Increased Drought Stress Tolerance in Arabidopsis and Is Involved in Abscisic Acid-Induced Stomatal Closure. **Plant Physiology**. Herndon, EUA, v. 161, n. 3, p. 1158–1171, 2013.

WANG, H. et al. A Subsidiary Cell-localized Glucose Transporter Promotes Stomatal Conductance and Photosynthesis. **The Plant Cell**. Reston, EUA, p. tpc.00736.2018, 2019.

WILLMER, C.; FRICKER, M. **Stomata.** London, Reino Unido, Chapman & Hall, 1996.

YOSHIDA, R. et al. Glutamate functions in stomatal closure in Arabidopsis and fava bean. **Journal of Plant Research**. [*s. l.*] v. 129, n. 1, p. 39–49, 2016.

YOSHIDA, T. et al. Revisiting the Basal Role of ABA – Roles Outside of Stress. **Trends in Plant Science**. [*s. l.*] v. 24, n. 7, p. 625–635, 2019.

ZANELLA, M. et al. β-amylase 1 (BAM1) degrades transitory starch to sustain proline biosynthesis during drought stress. **Journal of Experimental Botany.** [Lancaster, Reino Unido], v. 67, n. 6, p. 1819–1826, 2016.

ZHAI, Z. et al. Sugar Potentiation of Fatty Acid and Triacylglycerol Accumulation. **Plant Physiology**, London, Reino Unido, p. pp.00828.2017, 2017.

ZHAO, Z. et al. Functional Proteomics of Arabidopsis thaliana Guard Cells Uncovers New Stomatal Signaling Pathways. **The Plant Cell Online.** [*s. l.*], v. 20, n. 12, p. 3210–3226, 2008.