Universidad de Concepción

Facultad de Ciencias Naturales y Oceanográficas

Doctorado en Ciencias Biológicas, mención botánica

Photosynthetic and respiratory characterization in vascular and crop plant species: from an evolutionary perspective to a sustainable agriculture

Tesis presentada la Facultad de Ciencias Naturales y Oceanográficas de la Universidad de Concepción para optar al grado de Doctor en Ciencias Biológicas mención botánica.

POR: JOSÉ MIGUEL ORTIZ GUTIÉRREZ

PROFESOR GUÍA: Néstor Fernández del Saz

Marzo 2022

Concepción, Chile

Esta tesis fue desarrollada en el laboratorio de Fisiología Vegetal y el laboratorio de ECOBIOSIS, departamento de Botánica, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción.

Dr. Néstor Fernández del Saz (Profesor Guía) _____

Facultad de Ciencias Naturales y Oceanográficas

Universidad de Concepción

Dr. Lohengrin Cavieres G. (Profesor Co-guía) _____

Facultad de Ciencias Naturales y Oceanográficas

Universidad de Concepción

Dr. Pablo Guerrero M. _____

Director Programa de Doctorado en Cs. Biológicas, mención Botánica

Facultad de Ciencias Naturales y Oceanográficas

Universidad de Concepción

AGRADECIMIENTOS

Al Dr. Néstor Fernández del Saz por darme la oportunidad de aprender de su trabajo y pertenecer a su equipo.

A Carolina Sanhueza, Luisa Bascuñan y Carla Alvear por su buena disposición.

A las fuentes de financiamiento. Beca de Doctorado Nacional ANID N° 21180329 y al proyecto Fondecyt N° 1191118.

Contents

INDICE DE TABLAS

INDICE DE FIGURAS

Summary

The physical and chemical properties of the terrestrial environment were important for plant land colonization and later diversification, promoting physiological, morphological, and metabolic adaptations to deal with changes in energy input, gravity, and humidity. From a primitive thalloid-like shape, the photosynthetic organ evolved into the appearance of a large variety of leaf forms and stomata allowing increases of the efficiency of water transport and thermoregulation for the benefit of plant gas exchange. Besides, the replacement of an inefficient rudimentary rhizoid system in water and nutrient absorption, by an early symbiosis with soil microorganisms, allowed plant roots to adapt to poor nutrient soils. Along with these aboveground and belowground adaptations, the progressive evolution of an aerobic cell metabolism, involving oxygen consumption in mitochondria, increased both the efficiency of oxidative phosphorylation and energy homeostasis for the benefit of plant carbon metabolism. In the present thesis, I studied the relationship between energy homeostasis and carbon metabolism in plants along three chapters that explore different scenarios of plant adaptations to terrestrial

environments: (1) In chapter one, this relationship is explored through comparisons between terrestrial and palustrine plants. (2) In the second chapter, this relationship is studied during drought by exploring the importance of leaf shape for both photosynthetic capacity and energy dissipation as convective heat. (3) Finally, in the third chapter, this relationship is analyzed by reviewing the role of root respiration during symbiosis with soil microorganisms.

From photosynthetic and respiratory characterizations in vascular plants and crop species, the results of this thesis suggest that: (1) terrestrial plants display higher rates of photosynthesis and redox balance because they are exposed to higher reducing conditions in their environments; (2) leaf shape controls the energy input by a physical mechanism that benefit carbon assimilation and optimal temperature range; (3) root respiration may regulate the energy balance in plants under nutrient deficiency and during symbioses with soil microorganisms. Overall, these results contribute to understand the coordination between several biochemical and physiological mechanisms important for energy assimilation and later conversion into carbon compounds and plant growth, being of

interest for agricultural improvement and community development programs.

INTRODUCTION

Photosynthesis from carbon assimilation to the biochemical limitations

Carbon assimilation (A_N) in C_3 plants

Photosynthesis provides carbon skeletons required for growth, plant maintenance, and the metabolic precursors of other physiological processes like respiration and nutrient assimilation (Van Oijen et al. 2010; Sweetlove et al. 2010; Tcherkez et al. 2012; Krishnamurthy & Rathinasabapathi, 2013). From the appearance of photosynthesis in cyanobacterial algae about 3.5 billion years ago in an anoxygenic atmosphere (Olson & Blankenship, 2005), six pathways for carbon assimilation are recognized, being Calvin cycle pathway the most widespread in cyanobacteria and plant kingdom (Thauer, 2007; Buchanan et al. 2015). In plants, the Calvin cycle or photosynthesis is limited by several factors like CO_2, light, nutrients, optimal temperature, and water (Wright et al. 2004; Körner et al. 2015). These factors are expected to be altered due to global change, which predicts increases in drought events, temperature oscillation, and nutrient deprivation in some

areas (IPCC, 2017). To deal with this, several studies highlighted the importance of increasing carbon assimilation for the benefit of crop productivity through improvements of the photosynthetic limitations and the importance of the integration of these limitations into models for crop improvements (Evans et al. 2013; Flexas et al. 2016; Kubis & Van-Eben, 2019).

Light reactions

Photosynthesis is divided into two major stages: the first called "light reactions" involves the photon trap by chlorophyll antennas and subsequent liberation of O_2 by the photosystem II (Figure 1), the production of NADPH and creation of H^+ gradient in thylakoid lumen (Renger et al. 2010; Kramer et al. 2004; Lambers et al. 2019). From this H^+ gradient, the second stage involves the ATP synthesis by an ATPase located in chloroplast thylakoid membrane. ATP, NADPH and RuBP (ribulose biphosphate) are required for carbon assimilation by the action of RuBisCO (Tcherkez, 2013) in the so-called biosynthetic pathway or Calvin Cycle (Figure 2) (Kramer et al. 2004; Buchanan et al. 2015; Lambers et al. 2019). The RuBisCO is the most abundant protein on

Earth, and its biochemical properties, together with irradiance and CO_2 concentration, govern CO_2 fixation. RuBisCO possesses a dual activity, capable of fixing carbon for photosynthesis or fixing oxygen in photorespiration. Photorespiration takes place in mitochondria and peroxisomes (Bauwe et al. 2010; Erb & Zarzycki, 2018) and it was originally thought to be a wasted cycle. However, among other functions, photorespiration promotes energy balance during stress response, decreasing the accumulation of ROS (Peterhansen & Maurino, 2010; Ellsworth et al. 2015; Sunil et al. 2019).

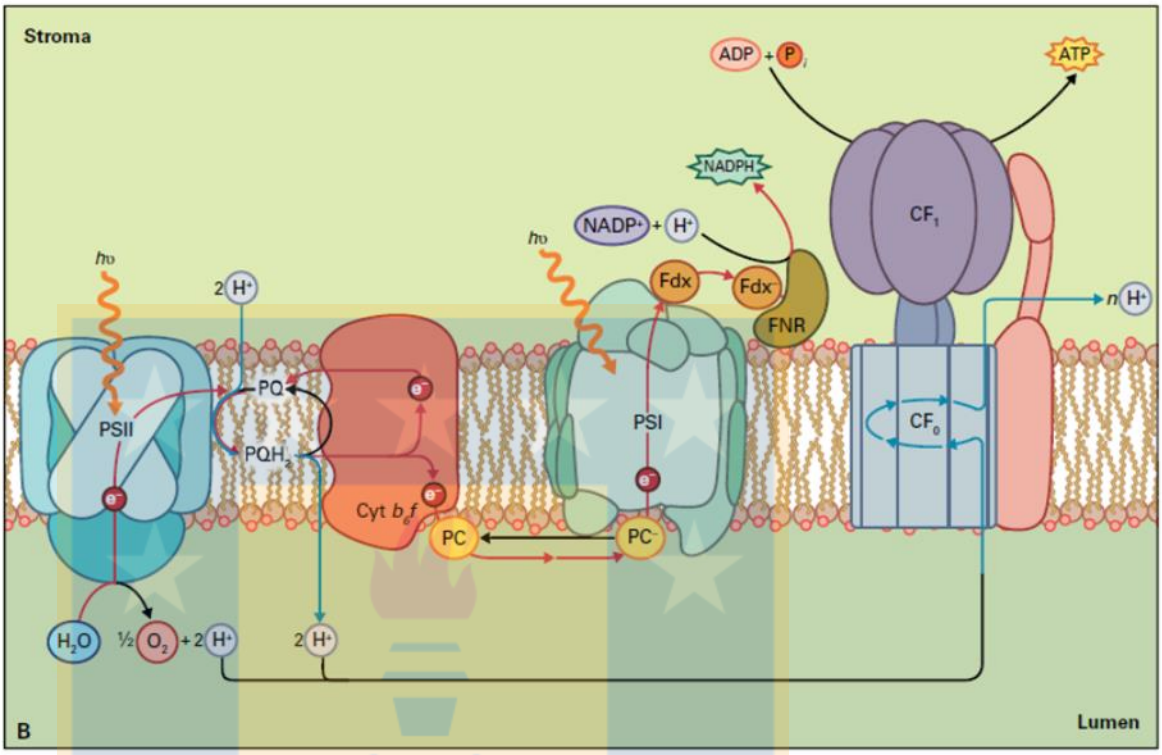


Figure 1. Reactions occurring in the light reactions inside chloroplast. Light is absorbed by the antenna complexes (not shown) and then passed through photosystem II in which the photolysis of water occurs. Electrons from PSII are delivered by plastoquinone, a transmembrane molecule which together with two H^+ from stroma space, deliver the electrons to cytochrome b_6f and translocate the H^+ to lumen space. From, Cytochrome b6f, electrons flow to photosystem I, where reduction of $NADP^+$ occurs thanks to ferredoxin reactions -Fdx, FNR-. With the H^+ accumulated in the photolysis of water and plastoquinone, a proton motive force is achieved and used by the ATPase for ATP synthesis. A side reaction occurs in PSI, in which one electron from PSI is delivered by plastocyanin to cyt b6f and plastoquinone is reduced, allowing the translocation of H^+ and the Q cycle. This cycle is preferred when plants need more ATP than NADPH by increasing the H^+ translocation. Figure obtained from Buchanan et al. 2015.

Dark reactions

The C_3 biochemical model of photosynthesis establishes that the diffusion of CO_2 from air to inside the leaves through stomata (g_s) and the biochemical limitations are the two main limiting factors (Farquhar 1980; Farquhar 1982). Biochemical limitations rely on the biosynthetic reactions or "dark reactions" involving several enzymatic steps for CO_2 assimilation (Figure 2). Briefly, the first stage named "carboxylation" involves the assimilation of CO_2 by a Ribulose 1,5 biphosphate (RuBP) by RuBisCO, forming a compound of six carbon. In the second stage, from NADHP and ATP obtained during the light reactions, the six-carbon molecule is reduced and broken into two three-carbon molecules named phosphoglycerate or PGA (Figure 2). This molecule is exported to cytosol in exchange of inorganic phosphate -Pi- for sucrose synthesis. The last reaction involves the regeneration of RuBP to maintain the continuity of the cycle (Buchannan et al. 2015; Lambers et al. 2019).

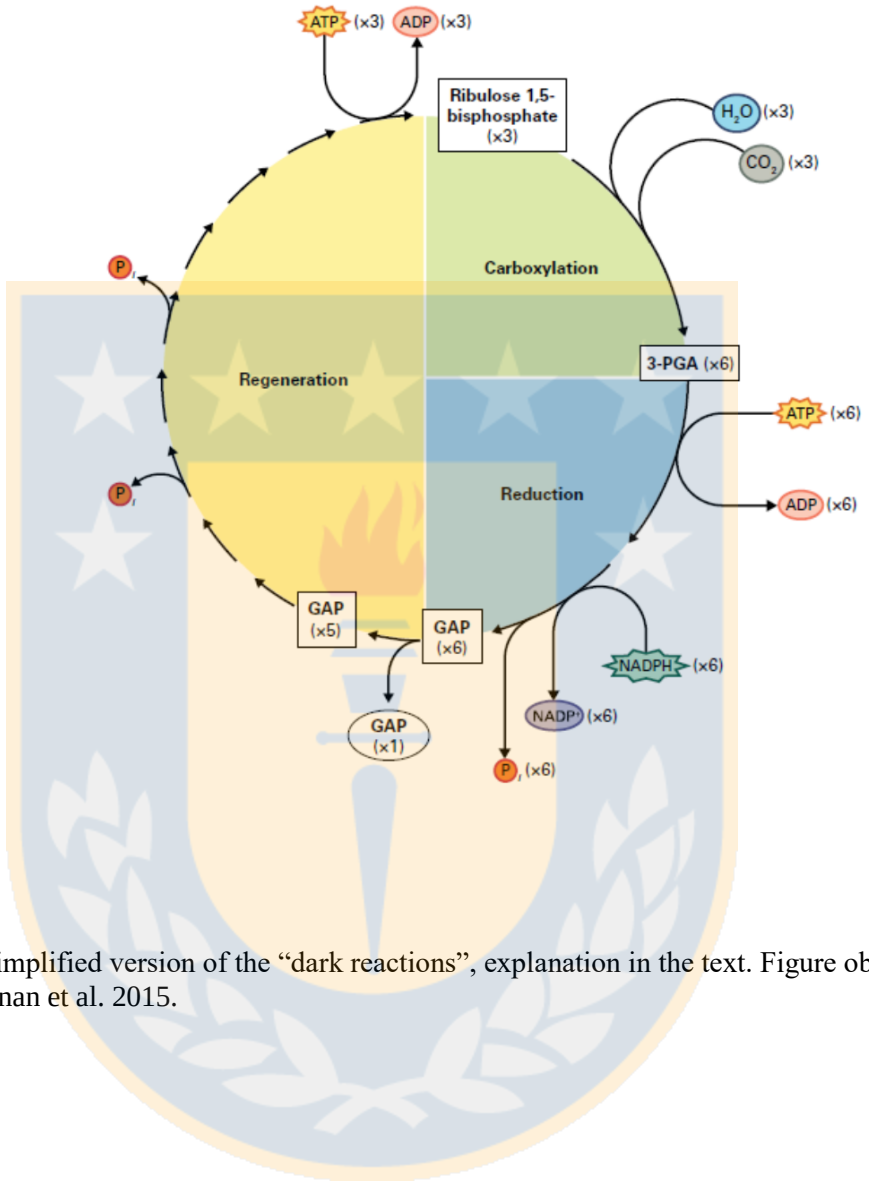


Figure 2. Simplified version of the "dark reactions", explanation in the text. Figure obtained from Buchanan et al. 2015.

Modelling *in vivo* C_3 carbon assimilation

Based on the above, the limitations of photosynthesis are related to the carboxylation velocity of RuBisCO (V_{cmax}) governed by CO_2 concentration and its specificity for this gas. The maximum electron

transport (J_{max}) limits the RuBP regeneration and is strongly related to irradiance. The limitation of triose phosphate (TPU) is related to Pi regeneration during sucrose synthesis (Farquhar et al. 1980; Farquhar and Sharkley, 1982; Ethier et al. 2004). Mesophyll conductance (g_m) is considered an additional important constraint to carbon assimilation in plants (Figure 3 & 4) (Ethier et al. 2004; Flexas et al. 2008; Niinemets et al. 2009a). The g_m has an anatomical component related to the shape, arrangement, and thickness of mesophyll cells being able to restrict photosynthesis by 70 % (Bernacchi et al. 2002; Flexas et al. 2008; Warren et al. 2008; Terashima, 2011; Tomas et al. 2013). Related to this, both the anatomy and the shape of the leaves can determine the maximum rates of carbon assimilation in plants by affecting the photosynthetic limitations (Tomas et al. 2013; Onoda et al. 2017).

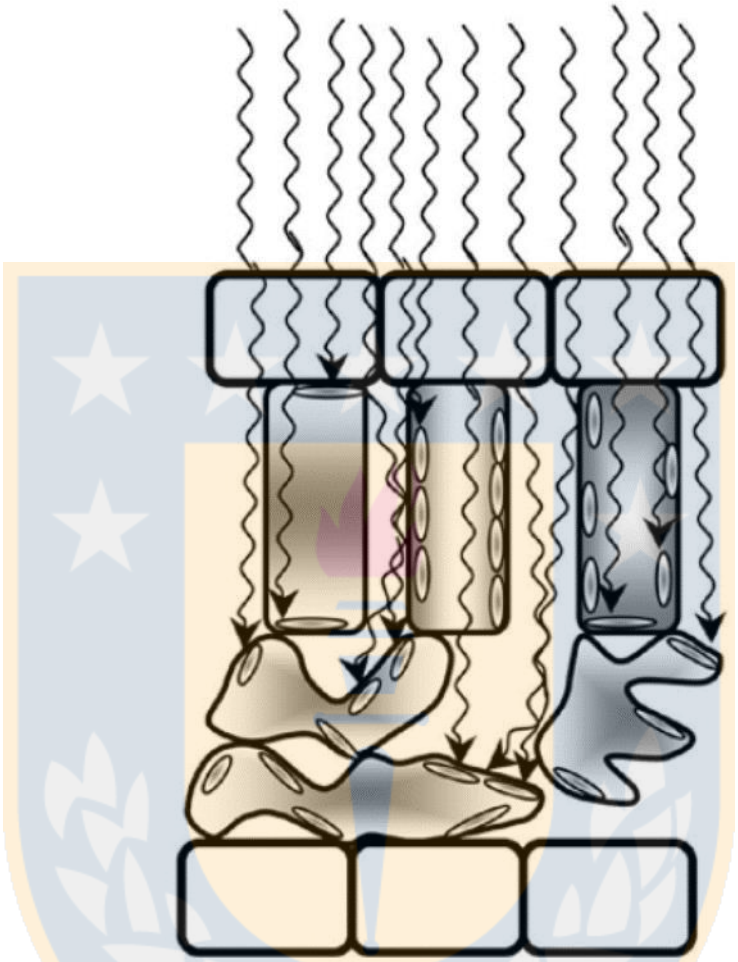


Figure 3. Representation of mesophyll structure. Mesophyll conductance is obtained by measuring the chlorophyll fluorescence in mesophyll cell during carbon assimilation with fluorescent values, and the electrons used for CO_2 assimilation are calculated. Then, chloroplastic CO_2 or Cc is obtained and a in vivo modeling of photosynthesis can be performed. Figure obtained from Flexas et al. 2012.

The vast knowledge about C_3 photosynthesis made possible to model accurately the rate of carbon assimilation. Gas exchange measurements are achieved based on the fluorescence of chlorophyll and the biochemical model of C_3 photosynthesis proposed by Farquhar et al. (1980). Fluorescence measurements are related to the light reaction of photosynthesis (Figure 1). During the transition of dark to light, chlorophylls of PSII absorb photons. Then, a protein located in the thylakoid membrane, with an electron carrier function named plastoquinone, delivers electrons of sunlight for: 1) light reactions or photochemistry, 2) heat dissipation by the non-photochemical quenching (NPQ); and 3) dissipation by emission of a low energy electron or fluorescence (Figure 4) (Maxwell, 2000; Piechulla, 2021; Buchanann 2015). Briefly, non-photochemical quenching or NPQ corresponds to a dissipation mechanism in which excited chlorophyll decays their energetic state as heat. This pathway is activated by the proton gradient occurring inside of chloroplast stroma when excessive input of energy occurs as under high light conditions (Figure 4) (Müller et al. 2001). As NPQ competes with fluorescence and photochemistry, by using

chlorophyll fluorescence, we can infer *in vivo* the electrons used in photochemistry (Maxwell, 2000; Flexas et al. 2012; Lambers et al. 2019).

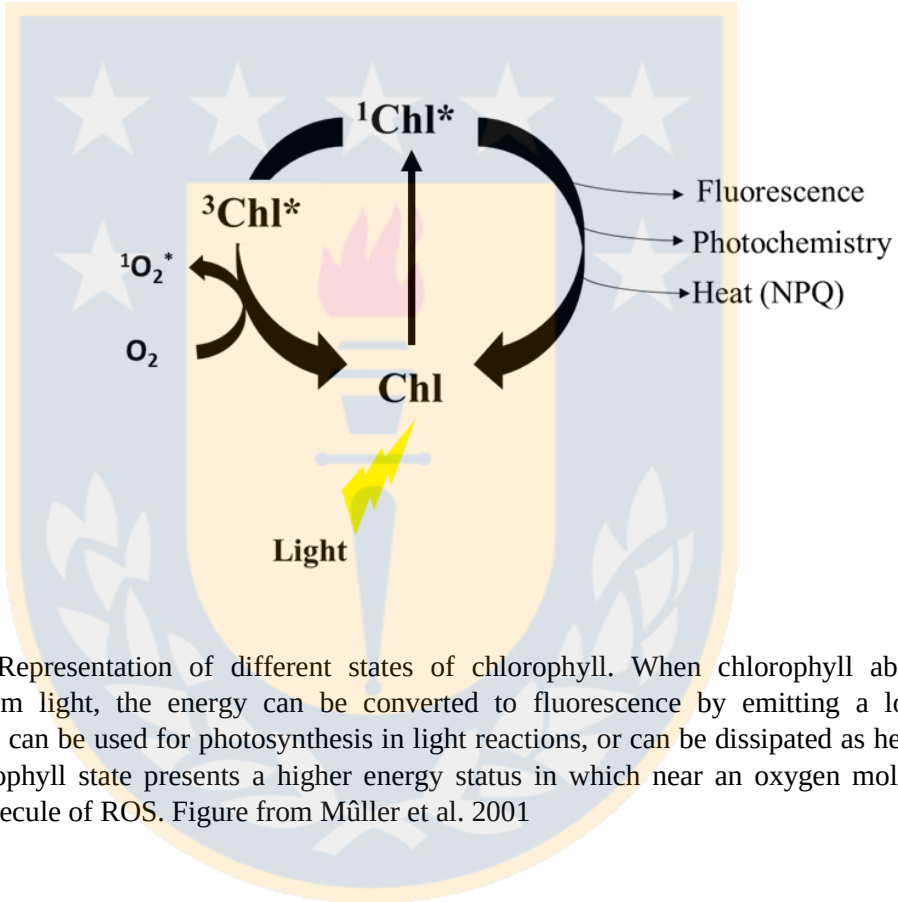


Figure 4. Representation of different states of chlorophyll. When chlorophyll absorbs photons from light, the energy can be converted to fluorescence by emitting a longer wavelength, can be used for photosynthesis in light reactions, or can be dissipated as heat. A triple chlorophyll state presents a higher energy status in which near an oxygen molecule forms a molecule of ROS. Figure from Mûller et al. 2001

Regard biochemical limitations, commercial IRGAs, are equipped with fluorescence cuvettes and regulation of CO_2 concentrations, light intensity, and temperature. By controlling these variables, we can obtain rates of photosynthesis versus increasing concentrations of CO_2 -A_N/C_i

curves-, allowing the quantification of the biochemical limitations (Figure 5). With this information and fluorescence values we can model the mesophyll conductance (Figure 3) obtaining the *in vivo* photosynthetic activity (Figure 5).

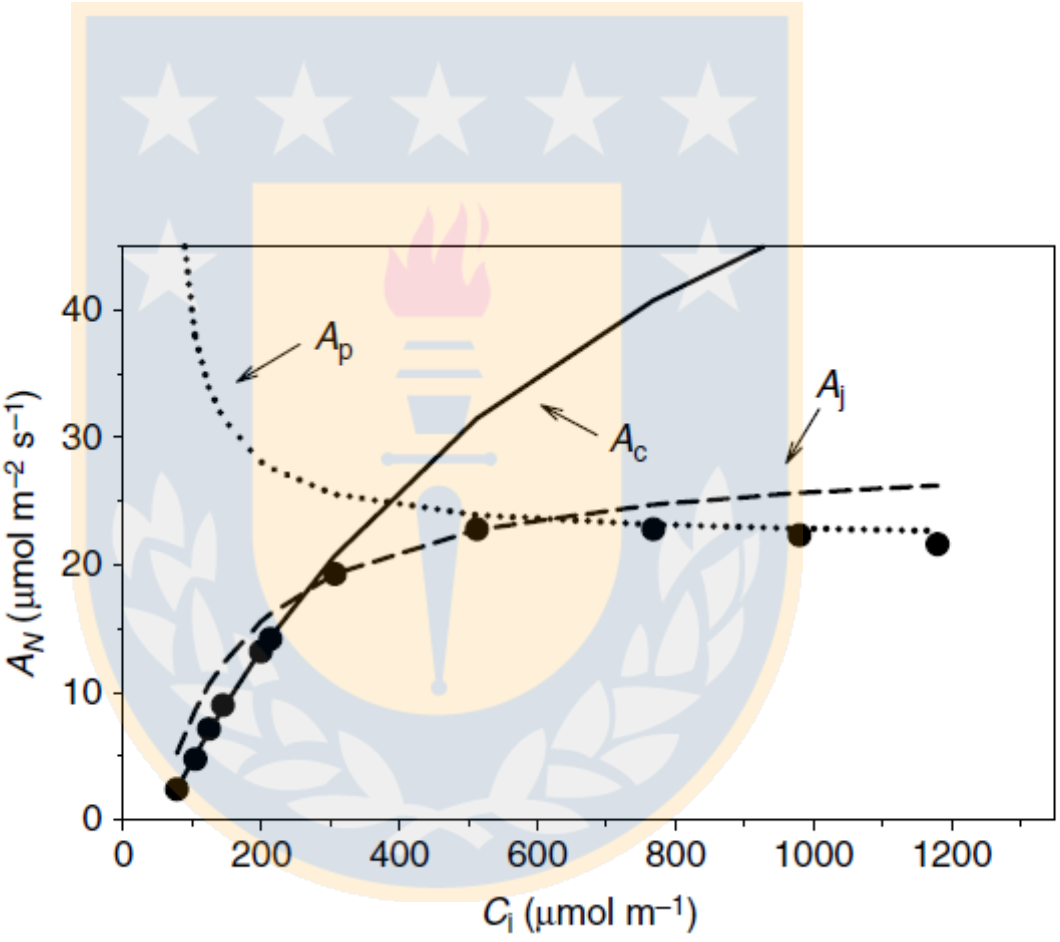


Figure 5. A/Ci or photosynthesis versus increments in CO_2 concentrations. Photosynthetic limitations are modelled based on data obtained in the curve. The A_c region corresponds to the limited region dominated by the RuBisCO carboxylation properties where CO_2 is the limiting source. The A_j region is related to the rate of ATP synthesis in the light reactions for reduction of PGA, in this region Pi and RuBP regeneration are the limiting factors. The A_p

region occurs commonly together with the A_j region due to the limited Pi release during regeneration phase of photosynthesis. Figure obtained from Flexas et al. 2012.

Ecophysiology of Photosynthesis: Drought and foliar traits

Physical components of thermoregulation in leaves

Leaves represent the organ involved in the transformation of sun energy into ATP through photochemical processes. Leaf energy balance is affected by temperature and drought causing perturbations in respiration and photosynthesis (Michaletz et al. 2015). As high foliar temperatures provoke the inactivation of several enzymes related to primary metabolism (Yamori et al. 2014), only an 8% of total solar input is used in biochemical reactions related to growth, while the remaining is lost as heat (Kume et a. 2017). Plants develop a physical mechanism to avoid thermal stress by evaporative cooling and evaporation through stomata, and convective cooling, a process directly associated with leaf size and shape (Gates, 1968; Nobel, 2020). Reductions in leaf size allows more surface exposed for convective cooling by mass heat exchange with the surrounding air. In dry areas, compound leaves were proposed as

dominant due to their tiny leaflets capable of more convective cooling and hence, less water loss through stomata (Givinish, 1989). In simple leaves, leaf size reductions are commonly related to lower g_m values and hence lower A_N (see later) (Flexas et al. 2008). In this sense, simple leaves could rely on more stomatal cooling than compound leaves. However, the effect of drought during the energy balance in both types of leaves is still unknown and may be conditioned to a trade-off existing between leaf area and leaf mass important for carbon assimilation and leaf temperature (Niinemets, 1998; Michaletz et al. 2015).

One of the threats of global change is the increase in water scarcity and extended drought events. Plants growing in dry areas develop several strategies to cope with seasonal droughts such as shrub or semi tree habit, sclerophyllous leaves, low leaf areas, increased efficiency in photosystem II, increases in the water use efficiency, RuBisCO specificity, and increases in leaf mass area -LMA- (Delfine et al. 2001; Galmés et al. 2005, 2007; Medrano et al. 2009; Galle et al. 2011; De Micco & Aronne, 2012; Flexas et al. 2014). Precisely, LMA represents the quantity of mass per area of foliar tissue, and the values may vary with growth forms and

environment (Niinemets, 1999; Wright et al. 2004; Poorter et al. 2009). Increases in LMA reflect a mechanism to tolerate drought, by packing the same photosynthetic tissue in a less projected area and decreasing transpiration losses and incoming radiation (Figure 6) (Nogués & Baker, 2000; Grassi & Magnani 2005; Pickup et al. 2005; Galmés et al. 2005; Tomás et al. 2013). Reductions in air spaces in the mesophyll decrease CO_2 conductance and increase the length for the CO_2 to reach the carboxylation sites (Figure 6) (Poorter et al. 2009; Hassiotou et al. 2010; Terashima 2011; Niinemets et al. 2011; Tholen et al. 2012; Tosens et al. 2012). For example, several functional groups like herbs, shrubs, and trees subjected to drought conditions show LMA increases together with decreases in g_m and A_N (Niinemets et al. 2009b; Tosens et al. 2012). In other studies, LMA increased with no change in A_N, as occurs in some *Banksia spp*. species (Hassioutou et al. 2010; Peguero-Pina et al. 2017). These changes reflect the dependence of A_N to leaf temperature during drought stress. Despite the above, relationships between photosynthesis, g_m and LMA in different leaf shapes such as simple, and compound leaves has not been studied so far. For example, Mediterranean simple

leaves species present the high LMA values with no photosynthetic decreases. Compared with compound species, which show three times less LMA, simple leaves present a 40% decrease in photosynthesis (Flexas et al. 2008; Niinemets et al. 2009b; Hassioutou et al. 2009, 2010). Simple leaves may achieve this compensation by exposing more mesophyll surface to CO_2 favoring access to the chloroplast (Hassiotou et al. 2010b). In contrast, compound leaves present low LMA, leaflets with lower size which promotes more photosynthetic area and heat convection during stomatal close. In this sense, LMA relates to leaf size, energy management and leaf temperature, and differences in the photosynthetic performance between compound and simple leaves can exist. Thus, research about leaf type adaptations and heat dissipation during stomatal close in dry areas may help to improve crop production and water use efficiency by selection of suitable leaf traits (Turner et al. 2005; Katerji et al. 2008; Michaletz et al. 2015; Flexas et al. 2016).

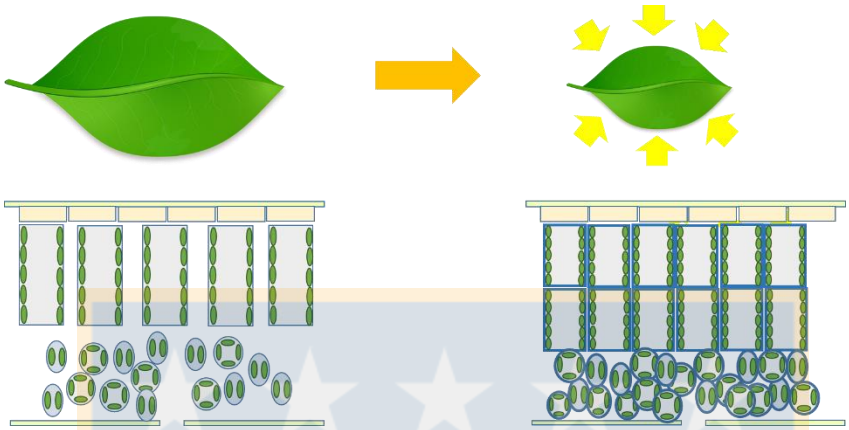


Figure 6. Representation of drought effects on leaf size and leaf mass area (LMA). Leaves with high specific leaf areas, or SLA, present a loose mesophyll structure, allowing faster CO_2 diffusion through carboxylation sites in chloroplast. In contrast, during drought, decreases in leaf size allow a more packing mesophyll structure, this adaptation compensates for a decrease in the photosynthetic area by increasing the mesophyll area. However, more mesophyll package decreases mesophyll conductance compared to species with lower LMA.

Respiration: from energy production to carbon balance

Respiration process

Both respiration in mitochondria, and photosynthesis in chloroplast, regulate plant growth and yield crop. In mitochondria, sugars that came from photosynthesis are respired and transformed into carbon skeletons used for biomass production and maintenance of plant metabolism.

Several works stated the importance of improving respiration, by increasing the ATP produced by sucrose consumed (Figure 7), allowing biomass increases in crops (Amthor, 2010). Before the oxygenation of earth, respiration was performed by chemolithotrophic microorganisms, which used the inorganic minerals as electron acceptors. (Broda 1975; Gomez & Amils, 2002). With increasing oxygen levels and the use of O_2, mitochondria achieved a highly exergonic reaction and greater efficiency in energy production. This makes oxygenic respiration a more energetic reaction compared to other electrons acceptors. However, due to the reactivity of oxygen with organic molecules, several mechanisms avoiding ROS were developed (Turrens et al. 2003; Landis & Tower, 2005; Krumova & Cosa, 2016). In this sense, mitochondria regulate the energy state of the cell, developing mechanisms including interaction with other organelles and metabolites levels derived from primary metabolism for redox homeostasis maintenance.

Glycolysis and TCA cycle: the producers of reducing power

In plants as in animals, cellular respiration occurs in mitochondria. The process begins with the oxidation of sucrose, and a three-carbon

molecule named pyruvate is produced with gain of two ATP and NADH molecules (Figure 7). In plants, malate is produced mostly instead of pyruvate, with no net gain of ATP or NADH (Lambers et al. 2019). These malate and pyruvate enter mitochondria in the TCA or tricarboxylic cycle (Figure 8). The function of TCA is to take advantage of all the energy contained in C3 carbon bonds, and to transfer this energy to the donor electrons molecules such as NADH and $FADH_2$ (Noguchi & Yoshida, 2007). During the TCA cycle, several decarboxylation reactions occur, releasing CO_2 that can be measured by IRGAs. The net ATP and NADPH generated during glycolysis and TCA depend on the substrate obtained from sucrose oxidation. When malate is the principal product, glycolysis and TCA produced 16 NADH, 4 $FADH_2$, and 4 ATP molecules (Sweetlove et al. 2010; Buchanan et al. 2015). Pyruvate produces 4 additional NADH and ATP molecules (Figure 7). The purpose of NADH and $FADH_2$ produced in these reactions is to generate the H^+ gradient required for ATP synthesis. At the same time, the mitochondrial electron transport chain, located in the

inner membrane of this organelle, uses the electrons of NADH and $FADH_2$ to reduce the O_2 to water (Figure 8).

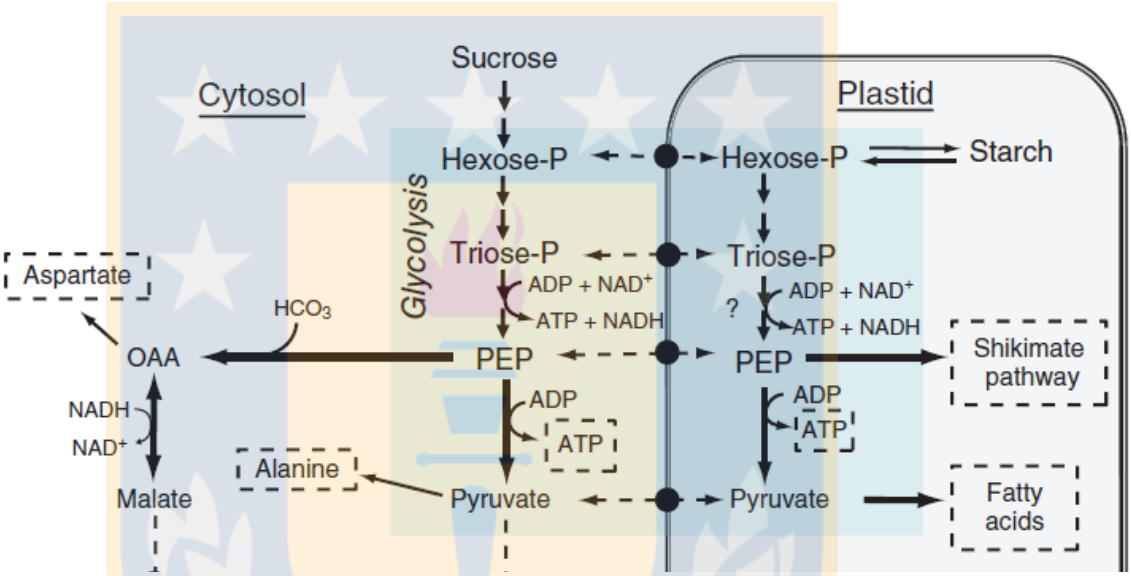


Figure 7. Reactions of glycolysis. In the cytosol, sucrose coming from another source or hexose phosphate coming from photosynthesis are subsequently transformed onto Pyruvate or Malate. When malate is synthetized no NADH is produced in glycolysis due to the use in the oxaloacetate reduction. Precursor of malate, the phosphoenol-pyruvate can enter to chloroplast and being part of shikimate pathway for secondary metabolism. Pyruvate also can enter to chloroplast for lipid synthesis or produce the aminoacid alanine. Figure from O`Leary & Plaxton, 2016

Mitochondrial electron transport chain, mETC

Once inside the mitochondrial inner membrane, NADH and $FADH_2$ deliver their electrons to several complexes to generate a proton motive force for ATP synthesis (Finnegan et al. 2004; Noguchi & Yoshida, 2007). The proton motive is created by complexes named from I to IV, using electron transport to move H^+ from the mitochondrial matrix to the intermembrane space. Ubiquinone -UQ- is a mobile protein that transports the electrons from complex I to complex III. During this electron transport, ubiquinone is reduced -UQH_2- accepting two H^+ from the mitochondrial matrix and releasing them in intermembrane space when electrons are passed to complex III. Finally, in the Complex IV or COX -for cytochrome oxidase-, the electrons from complex III are used for O_2 reduction to H_2O (Figure 8) (Del Saz & Ribas-Carbó, 2018; Lambers et al. 2019). Thus, ATP synthesis is dependent of the proton motive-force created by the flow of electrons through complexes I, II, III, and IV.

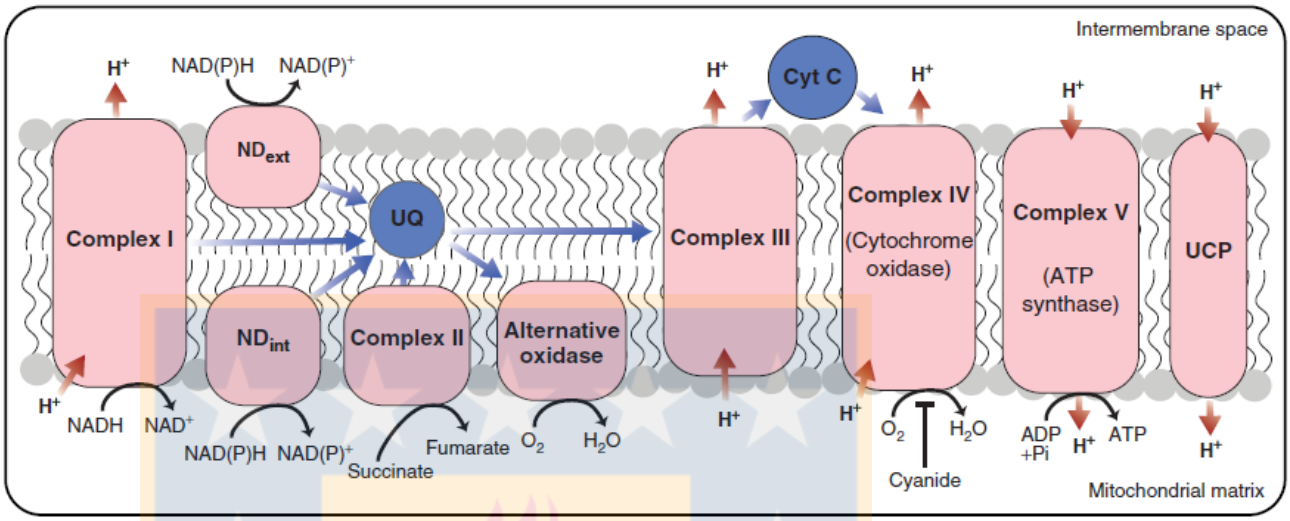


Figure 8. Mitochondrial electron chain reactions. Located in the intermembrane space the complexes received the electrons coming from NADH and $FADH_2$. The ubiquinone transports the electrons of complex I and II to the complex III. Complex I and II translocated H^+ from mitochondrial matrix to intermembrane space. As occurs in chloroplast, in mitochondria ubiquinol can operate a Q cycle in which electrons are transferred from UQ to complex III where proton extrusion into the intermembrane space takes place. The reduction of oxygen to water occurs in the cyanide sensitive complex IV or cytochrome oxidase. Complex V is an ATPase which create ATP from the H^+ concentration gradient. Note that due to its location, complex II does not translocate protons, but transfers electrons to ubiquinone. Figure from O`Leary & Plaxton, 2016

Besides COX, alternative oxidase (AOX) is activated in mitochondria when misbalances in cell energy status occur during biotic or abiotic stress. Alternative respiration delivers NADH electrons directly to the reduction of O_2 to water, lowering ATP production and hence, the energy efficiency of respiration (Gandin et al. 2014; Florez-Sarasa et al. 2016;

Del Saz et al. 2018; Vanlerberghe, 2020). AOX is a di-iron protein located in the inner membrane of mitochondria, and present in several phyla such as plants, animals, and proteobacteria (Finnegan et al. 2004; McDonald & Vanlerbergue, 2006). Due to its importance for energy status, AOX activity is regulated by four factors, 1) the status of UQ reduction; 2) the quantity of protein available; 3) the redox status of the cysteine residues, which activates or deactivates the enzyme; 4) concentrations of organic acid from TCA cycle such as pyruvate (Figure 8 and 9) (Finnegan et al. 2004; Vanlerbergue, 2013). The ancestral function of this pathway probably appeared in anaerobic bacteria and related to the avoidance of reactive oxygen species from O_2 metabolism during transition from anaerobic to oxygenic respiration (Gomes et al. 2001; Finnegan et al. 2004). This hypothesis is supported by the insensitivity of AOX -unlike COX- to sulfide, which was the primitive electron acceptor before oxygen. In this sense, AOX would allow the continuity of respiration during the transition of aerobic respiration in a still high sulfide atmosphere where COX was prone to be inhibited (Azcon-Bieto, 1986). With this in mind, AOX's presence in an

anoxygenic atmosphere probably helped with the transition of plants from water to land. In contrast to the aquatic environment, terrestrial plants are subject to higher energy input due to high light and conditions that promote stomatal close (Maberly et al. 2014). In this situation, the NAD(P)H/NAD(P) ratio increases while the respiratory demand decreases, leading to a cellular redox imbalance. It can be thought that during evolutive time AOX pathway could maintain the electron transport by dissipating excess of reducing equivalents and this could be especially relevant during land colonization (Yoshida et al. 2006; Vries & Achibald, 2017; Vanlerbergue et al. 2020). Thus, it seems that mitochondrial oxidases play a crucial role in cellular redox status regulation together with chloroplast signaling to promote higher CO_2 assimilation in a high reductant world (Gomes et al. 2001).

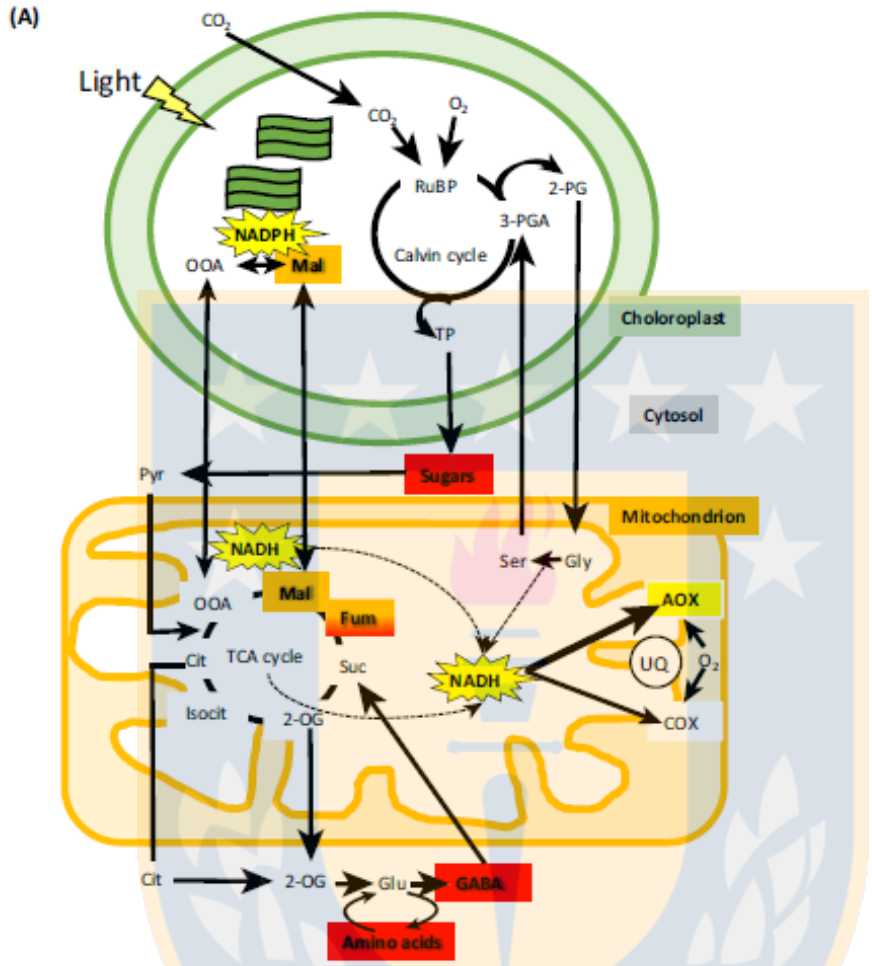


Figure 9. Synthesis of reactions which produced NADH and activates AOX. In conditions of high energy input such as drought, high light, or nutrient deprivation, the energy produced in the chloroplast in the NADPH form can enter mitochondria by malate valve, converted to NADH, and oxidized in AOX. Moreover, photorespiration, glycolysis, and TCA products are converted to NADH and oxidized via alternative respiration. Organic acid from TCA such as malate, fumarate, or citrate also can activate AOX and regulate the energy status. The GABA metabolism also relates to alternative respiration via amino acid metabolism. This picture shows the metabolic flexibility of respiration during stress and its coordination with chloroplast. Figure obtained from Del Saz et al. 2017.

As occurs with photosynthesis, and due to the importance of respiration on plant growth, several approaches are available for respiration measurements. One of the most accurate approaches is the IRMS, or the isotope ratio mass spectrophotometry (Ribas-Carbo et al. 1995, 2005; Muccio & Jackson, 2009; Kaklamanos et a. 2020). When operating as continuous flow (CF-IRMS) or dual inlet (DI-IRMS), the discrimination against heavier isotopes of several elements such as C, O, H, and N in different materials can be obtained (Tomaszek, 2005; Benson et al. 2006). These elements have a lighter version that is preferred in some biological reactions, such as the fixation of CO_2 by RuBisCO and the consumption of O_2 by the two terminal oxidases in mitochondria (O´Leary, 1981; Ribas-Carbo et al. 1995). The oxygen isotope fractionation technique is based in the differential discrimination of both oxidases against the heavy oxygen isotope ^{18}O. Due to the energy required to break the bond of a heavy ^{18}O isotope, being higher than for the light ^{16}O isotope, both oxidases prefer to react with the light isotope, but AOX discriminates more against the heavier oxygen isotope than COX (Ribas-Carbo et al. 1995; Henricksson et al. 2018). Because each

oxidase competes for electrons during respiration, the use of DI-IRMS allows estimations of the rate of electron flow to the alternative pathway in the absence of inhibitors, obtaining real contribution (or τ) of this oxidase to total respiration (Del Saz & Ribas-Carbó, 2018). Currently, metabolite profiling is another mass spectrophotometry technique, extensively used for elucidating the levels of primary metabolites during biotic or abiotic stress (Roessner et al. 2001; Trethewey et al. 2004; Broeckling et al 2005; Sweetlove et al. 2014). When observations obtained with DI-IRMS and metabolite profiling are combined, we can infer relationships between respiratory parameters and primary metabolites (Florez-Sarasa et al. 2016; Del Saz et al. 2016).

Ecophysiology of AOX respiration

Studies evaluating the impact of elevated atmospheric CO_2 on nutrient cycling report plant growth and root metabolism are the main source of carbon sequestration (de Graaff et al. 2006). In soil, root respiration accounts for 80% of the total soil respiration, with the microbial CO_2 release accounts for the resting 20% (Melillo et al. 2002; Davidson &

Janssens, 2006). Roots are a sink for carbohydrates due to energetics requirements for ion transport, nutrient assimilation, growth, and maintenance (Atkins et al. 2000; Nunes-Nesi et al. 2010; Foyer et al. 2011; Del Saz et al. 2017). Global warming can increase soil respiration and hence, acting as positive feedback for raising CO_2 concentrations (Amundson, 2001; Bergner et al. 2004; Chen et al. 2018). Increases in respiration can affect the productivity in some crops due to the linear relationship between respiration and temperature (Atkins et al. 2000; Atkin & Tjoelker, 2003; Del Saz et al. 2017). In this sense, agricultural production is challenged by an increase in demand for food by the growing human population (Vance et al. 2003). Climate change also affects the plant nutrient status by decreasing the nitrogen (N) and phosphorus (P) content in some biomes (Djikstra et al. 2012; Yuan & Chen, 2015). Global warming and the increase in CO_2 can influence the distribution of N: P in plant tissue, and this imbalance can affect the stoichiometry for correct growth and plant productivity (Yuan & Chen, 2015). Almost all nitrogen available for the plant is present in the reduced form of nitrate (NO_3^-), ammonia (NH_4^+), organic compounds,

and molecular nitrogen (N_2) in the air (Miller & Cramer, 2005, Lea & Miflin 2011). For Pi, the largest source of this element comes from the phosphate rock, monopolized by a few countries, and probably depleted by 2050, a date coinciding with an increase in food demand for humans (Cordell et al. 2009; Sattari et al. 2012). Several works showed the positive effect of AOX in abiotic stress such as nutrient deprivation, elevated CO_2 and drought (Gandin et al. 2009; Del Saz et al. 2017; Shane et al. 2014; Lambers et al. 2015). In this sense, the negative effects on ATP production are compensate by the role of AOX in carbon balance during nutrient stress allowing better plant performance and yield (Gandin et al. 2009; Dahal et al. 2015; Florez-Sarasa et al. 2020).

Another possible solution to deal with nutrient deprivation is the ecological fertilization process that occurs in nature by various microorganisms in soil (Vance & Lamb, 2001; Lambers et al. 2015). However, the roles of AOX activity during plant symbioses with mycorrhiza and rhizobia are unknown. With this goal in mind, understanding the physiological process that regulates metabolic

requirements and physiological aspects of N and P nutrition in plants can improve agricultural practices.

Role of AOX activity during N deficiency

N is linearly related to photosynthesis and respiration, due to its importance for enzyme synthesis (Evans, 1989). Assimilation of N requires the integration of photosynthesis, photorespiration, and respiration in different tissues organs, and cells (Atkin et al. 2000; Bykova et al. 2014; Igamberdiev & Kleckowski,2018). The preferred form N absorbed in roots is nitrate (Betti et al. 2012). To be integrated into amino acids, nitrate must be reduced by the nitrate and nitrite reductase (NR, NiR) in the ammonium -NH_4^+- form which uses NAD(P)H, which comes from photosynthesis or mitochondria when the assimilation occurs in leaves or roots, respectively (Foyer et al. 2011; Betti et al. 2021; Buchannan et al. 2015). Photorespiration is also another regulator of N metabolism by recycling the NH_4^+ that is released during the decarboxylation of glycine to serine, with lethal consequences when imbalances in these reactions occur (Oliver et al. 1990; Matt et al. 2001). In addition, respiration allows N assimilation by providing carbon

skeletons which added to NH_4^+, give rise to amino acids (Foyer et al. 2011). Thus, the availability of nitrogen determines the amount of carbon that must be assimilated by photosynthesis and oxidized in the TCA. With this in mind, components of respiratory chain are essential in regulate the redox status on cell, promoting flexibility for energy usage when N is scarce (Foyer et al. 2011).

In the case of low N supply, respiration decreases faster than photosynthesis promoting a high carbon environment (Richard-Molard et al. 2008; Körner, 2015). The latter occurs due to the reduction of electron requirements for nitrate reductase activity and lower requirements of carbon skeletons for amino acid synthesis, promoting carbon and NADH accumulation (Noctor & Foyer, 1999; Nunes-Nesi et al. 2010). This high energetic environment increases the carbon respired via AOX, and hence, this is not used for growth, but it is used to maintain the metabolism (Sieger et al. 2005). Regardless of the knowledge about AOX contribution in energy management during nutrient deprivation (Sieger et al. 2005; Gandin et al. 2014; Lambers & Plaxton, 2015), the *in vivo* activity of these pathways was not addressed so far, especially the

signaling which regulates AOX activity and its relationship with carbon metabolism. In this sense, during the lack of activity of nitrate reductase and hence, an increase of NADH not used for nitrogen assimilation, AOX is activated in order to avoid redox imbalance (Gandin et al. 2014). Thus, during nitrogen starvation, AOX probably reduces the NADH pool allowing the continuity of light reactions in the chloroplast.

Role of AOX during Pi deficiency

Phosphorous (P) or inorganic phosphate (Pi) is the limiting factor in the formation and accumulation of triose phosphate, sugars, and starch, as well as ATP synthesis during oxidative phosphorylation (Farquhar et al. 1980; Evans, 1989; Sieger et al. 2005; Vanlerbergue et al. 2019). As stated above, Pi has a marked effect in photosynthesis due to its regeneration from PGA for further carboxylation cycles. When Pi is scarce, an increase in sucrose contents occurs due to the low sink force exerted by plants organs due to the decrease in ATP produced in the mitochondria. Later, photosynthesis is downregulated by the higher sucrose contents and starch inside chloroplast (Huang et al. 2008; Hernandez et al. 2009; Obata et al. 2012). Photorespiration is also part of

the recycling process of Pi. During the phosphoglycerate export from chloroplast to mitochondria, a Pi is released in exchange for glycolate, and being decarboxylated and releases NH_4^+ inside mitochondria (Ellsworth & Lambers et al. 2014). In this case, the role of AOX is to regulate the carbon metabolism, by acting as a sink for energy, probably oxidating the NADH coming from TCA during sugar accumulation and avoiding its overaccumulation (Gandin et al. 2009; Vanlerbergue et al. 2019). This is observed when AOX is knocked out, and sugar phosphates, starch, and sucrose are accumulated (Vanlerbergue et al. 2019).

In roots, AOX provides a different role during Pi deficiency. A mechanism used by roots to explore and solubilize Pi involves lateral root growth, exudation of organic acids –carboxylates-, and Pi transporter synthesis, allowing exploration, solubilization, and Pi absorption, respectively (Florez-Sarasa et al. 2014; Lambers et al. 2015; Malhotra et al. 2019). The majority of P in soil is unable to be absorbed by plants because is immobilized by other metals, being part of cations or organic forms (Hao et al. 2002). Carboxylate exudation involves the release of

organic acids, such as malate or citrate in the soil adjacent to roots. Carboxylate possesses a negative charge, its exudation lowers the pH in soils, makes P accessible for remobilization and assimilation by plants (Otieno et al. 2015; Israr et al. 2016). During carboxylate exudation, respiration via AOX increases (Shane et al. 2004). This is due to both high level of organic acids and NADH in the TCA cycle (Florez-Sarasa et al. 2014). However, the role of AOX during plant symbioses with soil microorganisms that fix nitrogen or solubilize Pi in exchange for sugar coming from photosynthesis is today very blurry, but this role could be related to the synthesis of different primary metabolites important for the maintenance of the symbioses.

Respiration during symbiosis with nitrogen fixing bacteria and mycorrhiza: An in vivo role of AOX

Another way to cope with nutrient starvation includes the establishment of positive symbiotic associations with positive microorganisms living in soil (Lagunas et al. 2015). In this sense, two major symbioses exist, mycorrhizal (fungi) –ecto and endomycorrhizas- and *Rhizobium*-Legume, a plant-bacteria symbiosis (Paracer & Ahmadjian, 2000; Poole et al.

2018). The presence of rhizobium and mycorrhiza in roots imposes a carbon cost for the fertilizer effects in N and Pi (Kaschuk et al. 2009; 2012; Lagunas et al. 2015). Mycorrhizas were the first symbionts on Earth promoting the land transition of early vascular plants due to their positive effects on nutrient foraging (Selosse et al. 1998, 2004; Barman et al. 2016). One of the most recognized effects is the nutrient fertilizer effect, improved water content, protection by pathogen attack, and toxic metals (Selosse et al. 2004; Fritz et al. 2006; Pozo et al. 2007; Mitra et al. 2021). The fungus association with roots occurs as a controlled infection depending on the Pi concentration in soil medium (Smith et al. 2008; Barman et al. 2016). Roots release several metabolites such as strigolactones that are recognized by a specific fungus strain (Figure 9) (Smith et al. 2008). The fungi also release several chemical factors which interact with the root surface, allowing hyphae penetration within root cells. Once inside, mycorrhiza hyphae extent into the cytoplasm of root cells forming an arbuscular structure (Lagunas et al. 2015; Barman et al. 2016). Once established in roots, mycorrhiza acts as a new organ and hence, another sink for sugar (Figure 9) (Herold et al. 1980; Kaschuk et

al. 2010). The uptake and extension of hyphae beyond the deprivation zone of nutrients such as N and Pi require nearly 4-16% of the photoassimilates. These carbon requirements impact carbon metabolism in plants, by increasing A_N and respiration in leaves and roots, respectively (Hughes et al. 2008; Kaschuk et al. 2009; Romero-Munar et al. 2014). In return, thanks to the extension of hyphae, mycorrhiza can uptake Pi to sustain plant metabolism (Lagunas et al. 2015; Andrino et al. 2021).

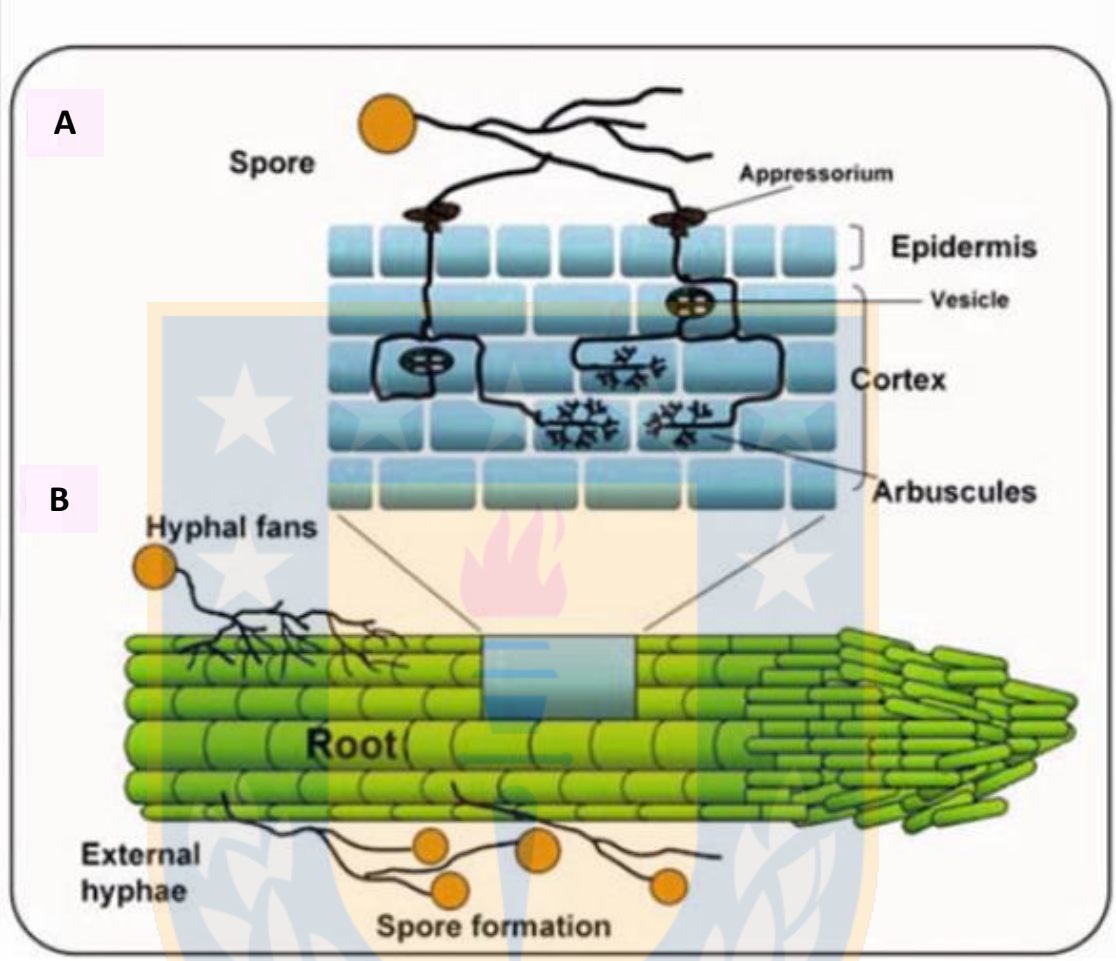


Figure 10. Steps in the controlled infection of arbuscular mycorrhiza. A) After the chemical communication and recognition, the fungus penetrates in the root cells. Hyphae penetrate inside the cortex of roots and stablish arbuscules inside cells, where the exchange of nutrients and carbon takes place. In B) the hyphae can extend beyond of the deprivation zone and explore soil for water and nutrients such as inorganic phosphate. Figure from Lambers et al. 2019.

The positive effects of mycorrhiza on plant growth depend on the fungus species and the carbon requirements, and they may include incremented energy efficiency of respiration (Lendenmann et al. 2011; Andrino et al. 2021). Mycorrhiza requires sucrose or TCA intermediates for energy production. The COX or AOX activity inside hyphae determines the rates of ATP used for Pi absorption and hence, the quantity of sugars needed for this task (Hughes et al. 2008). The positive effects in growth occur due to improvement in ATP synthesis in plants despite the carbon cost for the maintenance of mycorrhiza. For example, in *Nicotiana tobacco* roots, the presence of mycorrhizae decreases AOX activity and carbon exudation. By preventing carbon loss and enhancing COX, mycorrhiza promotes the accumulation of plant biomass (Del Saz et al. 2017). Moreover, both ATP concentration and COX expression increase probably due an increment in the efficiency in energy production. For example, the mycorrhizal roots of *Arundo donax* showed a decrease in total respiration due to a low COX activity. This suggested a lower ATP demand for nutrient uptake in mycorrhizal roots. With this in mind, changes in total respiration -contribution of COX and AOX- in

mycorrhizal roots are important for plant growth (Hodghes et al. 2008; Romero-Munar et al. 2014; Liu et al. 2014; Del Saz et al. 2017).

Insights into AOX activity and the Legume-Rhizobium symbiosis

The Fabaceae or Leguminosae is the third largest family of flowering plants and the second family most used for agricultural purposes (Graham & Vance, 2003). Legumes possess high amounts of vitamins, insoluble and soluble fiber contents, minerals, and secondary metabolites used for medicinal purposes (Gulewiez et al. 2014; Wanda et al. 2015). The high nitrogen content in leaves makes the leguminous plant a good forage for animals and sustainable agricultural purposes (Mckey, 1994; Crews, 1999; Vance, 2001; Dwivedi et al. 2015). Besides their nutritional properties, legumes establish symbiotic associations with nitrogen-fixing bacteria that enhance soil quality. Bacteria can fix atmospheric nitrogen and provide it to the plant in return for plant photoassimilates (Heath, 2010; Laranjo et al. 2014). Nitrogen-fixing bacteria belong to several genera and strains, generally called rhizobia, which form a mutualistic interaction with plant roots (Figure 10) (McKey, 1994; Masson-Boivin et al. 2009; Heath, 2010; Giraud & Fleichman, 2004 Laranjo et al. 2014).

This association requires 14% of plant photoassimilates to fuel nitrogenase reactions. By fixing molecular nitrogen (N_2) to organic form (NH_3), nodules exert a fertilizer effect in plants (Figure 10) (Minchin & Witt, 2005; Kaschuk et al. 2009; Piechulla & Heldt, 2011). Despite this cost, it has benefits such as tolerance to abiotic stress and enhanced plant growth (Barka et al. 2006; Heath & Lau, 2011; Laranjo et al. 2014). In this sense, understanding the importance of the links between nitrogenase activity, photosynthesis, and respiration is important to improve and understand nodule functioning in legumes and the physiological process regulating nitrogen fixation.

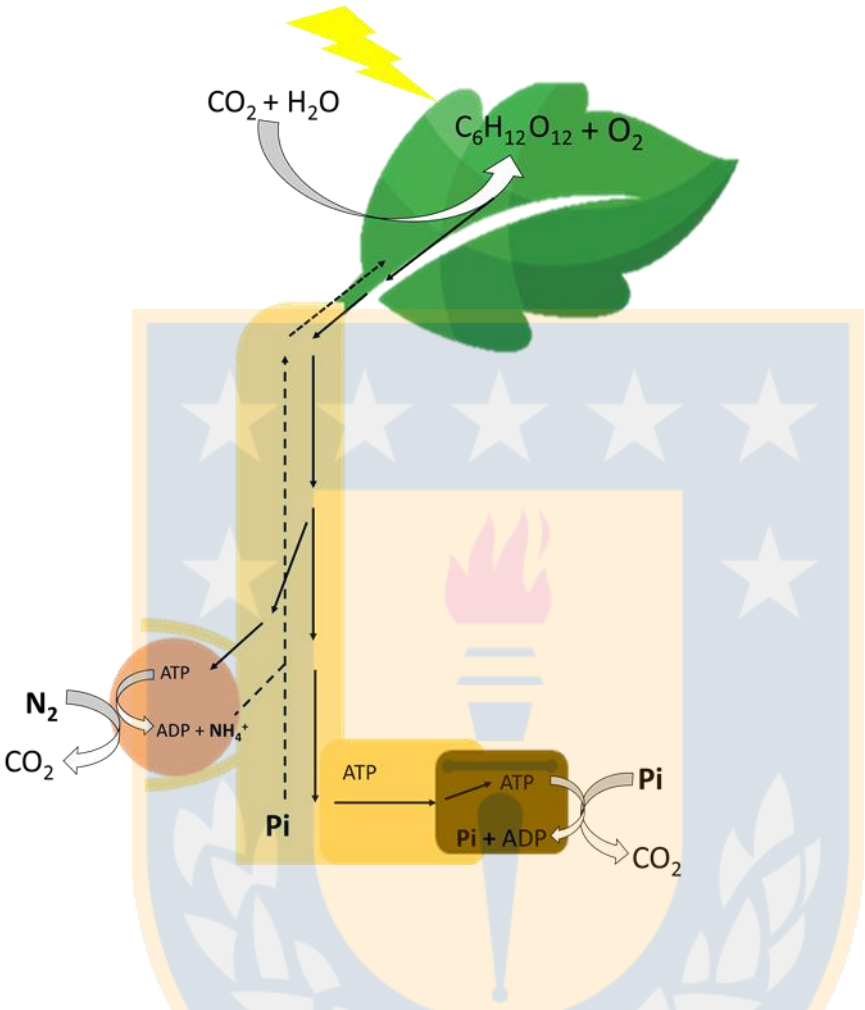


Figure 11. Representation of the presence of rhizobia and mycorrhiza in roots. The two types of symbiosis require carbon from photosynthesis to sustain their fertilizing effects. Rhizobia exchange organic forms of nitrogen for organic acids from TCA. On the other hand, mycorrhiza exchanges phosphate against a gradient for sucrose or organic acids. Note that the image is only representative because few species can maintain the two kinds of symbiosis.

The symbiosis of rhizobia enhanced photosynthesis and respiration. The above occurs by a decrease in limitations of photosynthesis to satisfy the carbon demand of the nodules (Figure 11) (Kaschuk et al. 2009). As

respiration and photosynthesis metabolism remain in equilibrium to sustain carbon balance (Noguchi & Yoshida, 2008), an increase in the rate of photosynthesis must increase respiration in growing organs such as stems, fruits, and roots due to sink strength of nodules (Pitelka, 1977; Herold, 1980; Dinakar et al. 2010; Ainsworth & Bush, 2011; Dietze et al. 2014). Thus, if increases in photosynthesis are not accompanied with the demand for the end products (ATP, carbon skeletons), the sugar can accumulate and alter carbon balance and growth in the plant (Sieger et al. 2005; Gandin et al. 2009). The strictly use of organic acid by nodules involves the activity of several enzymes of the TCA cycle inside root cells (Rosendahl et al. 1990; Poole et al. 2018). As nodules use malate for the nitrogenase activity, the NADH pool increases, and oxaloacetate cannot be regenerated. During this situation, activation of anaplerotic routes for de novo synthesis of oxalacetate allows the TCA continuity. With this in mind, increases in the respiratory components (COX and AOX) to balance this high input/demand of energy required by the nodule for nitrogen assimilation can be expected (Lambers et al. 1980; Green et al. 2000; Schulze et al. 2000; Sieger et al. 2005). In this line,

various reports showed that nodulated roots have more respiration rates than non-inoculated roots (Rao & Ito, 1988; Schulze et al. 2000; Mortimer et al. 2008). However, few studies have focused on the contribution of both oxidases in the total respiration of the inoculated plants.

Nodulated plants present higher biomass accumulation due to efficiency in bacteroid respiration per unit of fixed N_2, despite the carbon needs (Schulze et al. 2000; Esfahani et al. 2004). However, if rhizobia symbiosis imposes a similar effect on respiration as mycorrhizal symbiosis is unknown. In this sense, based on mycorrhizal effects on respiration, rhizobium probably promotes efficiency in ATP synthesis avoids excessive carbon respired, promoting biomass accumulation. The photosynthetic increase and the reduction of carbon loss by respiration are the cornerstones to improving yield programs (Amthor et al. 2019). With this in mind, the effect of the metabolite signaling and AOX activity during symbiosis in the two mitochondrial oxidases is worthy of evaluation in order to understand how maximize increases in plant yield during sustainable agriculture programs.

Problem statement

Photosynthesis was a major evolutionary event that allowed the oxygenation of the atmosphere and the diversification of diverse forms of life on earth (Van Oijen et al. 2010; Sweetlove et al. 2010; Tcherkez et al. 2012). However, plants being sessile organisms, cannot escape from excess radiation. Several studies show the origin of various protective molecules and morphological characteristics against light excess (Weng & Chapple, 2010; Bowman et al., 2017). However, few studies relate the respiratory capacity as a strategy that allows the management of solar energy, and therefore a greater capacity for energy utilization. As mentioned above, respiration is responsible for using the substrates from photosynthesis for growth and maintenance. The terrestrial habitat is characterized by high solar radiation and a dry atmosphere, factors that promote high reductive environment inside cells (Asada et al. 2006; Takagi et al., 2017; Zandalinas et al., 2021). However, the capacity and activity of mitochondrial oxidases, and thus, the partitioning of electrons in the two oxidases which determines the energy efficiency of respiration, has not been studied in detail. Based on the above, the first

part of this thesis attempted to determine how the transition to the terrestrial environment determined a greater capacity of electrons to the alternative oxidase, allowing the establishment and colonization of plants by higher metabolic management of electrons. In addition, the leaf was an evolutionary adaptation that allowed an improvement in photosynthetic capacity and the development of the hydraulic system in plants (Brodibb et al. 2010). However, by maximizing the photosynthetic area, a greater area of access to CO_2 is achieved, but in turn, more area is prompted to absorb solar radiation and hence prone to overheat (Givinish, 1989). This is of great importance in places where access to water decreases periods and added to stomatal closure, increases the probability of plants suffering from high temperatures (Michaletz et al. 2015). It has been established that a smaller leaf size would allow the correct regulation of energy due to its convective exchange with the environment (Gates, 1968). However, reductions in leaf size have consequences on the biochemical limitations of photosynthesis (Flexas et al. 2009; Tomas et al. 2013). Therefore, it is proposed that leaf shape

regulates the energy dissipation by physical mechanisms and controls photosynthesis by regulate leaf temperature.

Climate change predicts water and nutrient deficit (IPCC 2019). In this sense, both crop and native species face the same problem being capable of developing is an entire life cycle in a changing world. As the human population grows, all efforts are put into decreasing the high use of artificial fertilizers and their negative effects (Djikstra et al. 2012; Yuan & Chen, 2015). In this sense, understanding the metabolic reorganization occurring in plants during nutrient deficit is a new target for crop production programs. Nitrogen and Phosphorous deficiency also produce a high reductive environment in plants (Shane et al. 2004; Nunes-Nesi et al. 2010; Obata et al. 2012). As proposed in chapter one, changes in the activities of both mitochondrial oxidases could improve our understanding of the energy balance occurring during a nutrient deficit. Moreover, the role of the alternative oxidase during this stress probably helps plants cope with the high reductive environment by reprogramming the metabolism and metabolite signaling, which promotes plant viability.

Another role of respiration during nutrient deficit is the effects in plant metabolism the presence of symbiosis with rhizobia and mycorrhiza (Laranjo et al. 2014). Symbiosis exerts a different energy imbalance by promoting higher photosynthetic rates to cover the symbiont needs. In return, rhizobia and mycorrhiza give N and Pi, respectively (Hughes et al. 2008; Kaschuk et al. 2010). However, based on the results found during mycorrhiza symbiosis (Romero-Munar et al. 2018), the metabolic signaling leading to increases in the carbon needs and changes in AOX activity is not fully understood. Legumes is a perfect case of study to test this role of respiration during symbiosis due to high AOX transcripts and the recognition of symbiosis with rhizobia -a soil bacteria-. Several studies show the positive effects of rhizobia in growth and respiration rates (Barka et al. 2006; Heath & Lau, 2011; Hungria et al. 2014; Laranjo et al. 2014). However, the metabolite signaling and the role of the two mitochondrial oxidases in the energy balance during symbiosis are not studied so far.

Hypothesis

¿How does respiration influence photosynthetic and metabolic capacity in the terrestrial environment?

Due to terrestrial conditions generate a higher reducing environment for plants biochemistry reactions, the alternative oxidase would allow plants to increase their capacity to deal with incoming solar energy during the transition between land and water.

¿There are differences in the management of solar energy by simple and compound leaves?

Because the compound leaves present a smaller leaf area, they would present high values of mesophyll conductance and, therefore, higher photosynthetic capacity. In addition, under drought conditions, the compound leaves would have greater convective cooling.

¿Which is the *in vivo* role of alternative oxidase during nutrient deficit in plants?

Nutrient limitation causes energy misbalances, which promotes metabolic changes and differences in mitochondrial oxidases

contribution, in this sense, alternative oxidase regulates the redox homeostasis by respiring substrates allowing the TCA continuity and avoiding overreduction of respiratory chain.

¿How symbiosis with soil microorganism change the metabolic routes involved with respiration in plants?

The establishment of symbiosis with soil microorganism promotes higher photosynthetic capacity by sink force, hence, changes in both COX and AOX in plants are expected in order to maintain C/N status and to decrease ROS generation during symbiosis.

Thesis outline

This thesis consists of three articles. Each of the articles is represented in a chapter, being three chapters in total.

Chapter 1 corresponds to Article 1, where I tested the role of respiration in managing the high flow of energy under terrestrial conditions. This was tested by using palustrine species from semi-aquatic environments of different families and comparing them with terrestrial plant species belonging to the same families. Photosynthetic capacity and oxygen-isotope fractionation were quantified and its relationship with energy management was tested. In addition, a metabolite profiling was performed to determine which metabolic pathways were related to the homeostasis redox in both types of environments.

Chapter 2 is based on a trade-off between leaf type to photosynthetic limitations and energy management under control and water stress. In article 2, the role of leaf shape was established by comparing the photosynthetic limitations between compound leaves and simple leaves of the Chilean matorral. By exposing both types of leaf species to extreme drought, which is recurrent in the Chilean Mediterranean

climate, leaf temperature, the net rate of photosynthesis, stomatal conductance, and mesophyll were obtained. In addition, a logarithm response ratio (LnRR) was performed to determine if the drought affects negatively more in compound or simple leaves. Based on the photosynthetic data and leaf temperature, the role of leaf type in the leaf energy balance from dry areas such as the Mediterranean was assessed, with attention to the effects of climate change.

Finally, in chapter 3, the possible role of alternative oxidase in plant carbon metabolism during nutrient deficiency and in symbiosis with soil microorganisms is reviewed. First, article 3 shows several metabolic rearrangements occurring in plants subjected to N and Pi deficiency. The possible mechanism of metabolite signaling in both oxidases activity and its effects on carbon balance and energy status is discussed. In the second part, symbiosis's effects on plant carbon metabolism and changes in the mitochondrial metabolism adjustments are also discussed. Moreover, the role of metabolite signaling in the integration and flexibility of primary metabolism is addressed. The data obtained links the mitochondrial oxidases with carbon balance during plant-microorganism interaction.

Understanding the metabolic processes that increase crop yield has vital importance for sustainable agriculture programs.

General aim

The general aim of this thesis is to determine the changes occurring on the primary metabolism in leaves and roots of vascular species including crops, when are subjected to a misbalance in the energy status caused by the physical, chemical, and biotic conditions of the terrestrial life such as desiccation, nutrient scarcity, and symbiosis with soil microorganism.

The specific objectives that emerge from each chapter are the following:

-Chapter 1: Respiration: as an energy producer to a master regulator of nutrient and carbon metabolism in leaves.

Objective 1: Determine if land environment triggers respiratory differences between the aerial leaves of palustrine and terrestrial plants species.

Objective 2: Identify which metabolic routes are related to the AOX pathway in leaves of palustrine and land plants species.

The article covering these objectives is:

Article 1: **“Different Metabolic Roles for Alternative Oxidase in Leaves of Palustrine and Terrestrial Species”**

-**Chapter 2:** Photosynthesis: leaf shape as a key player in the energy management and biochemical limitations

Objective 1: Investigate if compound leaves and simple present differences in biochemical limitations based on A_N, g_s and g_m values.

Objective 2: To determine if heat dissipation is a mechanism that exists in both types of leaves to face the recurrent droughts that occur in the Central Chile matorral.

The article covering these objectives is:

Article 2: **“Chilean matorral compound and simple leaf woody species are equally affected by extreme drought”**

-**Chapter 3**: Ecophysiology of AOX respiration: role in nutrient deficit and during symbiosis

Objective 1: To assess the energy and carbon regulation during N and Pi deficiency in leaves and roots of plants

Objective 2: To identify metabolites involve in the *in vivo* AOX activity during N and Pi deficiency.

Objective 3: Study the metabolic regulation of AOX during symbiosis with mycorrhiza and rhizobium and its importance for crop programs.

The article covering these objectives is:

Article 3: **"In vivo Metabolic Regulation of Alternative Oxidase under Nutrient Deficiency. Interaction with Arbuscular Mycorrhizal Fungi and Rhizobium Bacteria"**

CHAPTER 1:

Respiration: energy producer and master regulator of nutrient and carbon metabolism in leaves.

Different Metabolic Roles for Alternative Oxidase in Leaves of Palustrine and Terrestrial Species

Authors and addresses:

Nestor Fernandez Del-Saz[1], Cyril Douthe[2], Marc Carriquí[2], Jose Ortíz[1], Carolina Sanhueza[1], Alicia Rivas-Medina[3], Allison McDonald[4], Alisdair R. Fernie[5], Miquel Ribas-Carbo[2], Jorge Gago[2], Igor Florez-Sarasa[6] and Jaume Flexas[2]

[1]Laboratorio de Fisiología Vegetal, Departamento de Botánica, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Concepción 4030000, Chile; [2]Research Group on Plant Biology under Mediterranean conditions, Departament de Biologia, Universitat de les Illes Balears / Institute of Agro-Environmental Research and Water Economy –INAGEA, Carretera de Valldemossa, 07122, Palma, Illes Balears, Spain; [3]Departamento de Ingeniería Civil, Facultad de Ingeniería, Universidad de Concepción, Concepción 4030000, Chile; [4]Department of Biology, Wilfrid Laurier University, Science Building,

75 University Avenue West, Waterloo, Ontario N2L 3C5, Canada; [5]Max-Planck-Institut für Molekulare Pflanzenphysiologie, Am Mühlenberg 1, 14476 Potsdam-Golm, Germany; [6]Centre for Research in Agricultural Genomics (CRAG) CSIC-IRTA-UAB-UB, Campus UAB Bellaterra, 08193 Barcelona, Spain.

Manuscript published in Frontiers in Plant Science:

Del-Saz, N. F., Douthe, C., Carriquí, M., Ortíz, J., Snahueza, C., Rivas-Medina, A., ... & Flexas, J. (2021). Different Metabolic Roles for Alternative Oxidase in Leaves of Palustrine and Terrestrial Species. *Frontiers in Plant Science*.

Summary

- The alternative oxidase pathway (AOP) is associated with excess energy dissipation in leaves of terrestrial plants. To address whether this association is less important in palustrine plants, we compared the role of AOP in balancing energy and carbon metabolism in palustrine and terrestrial environments by identifying metabolic relationships between primary carbon metabolites and AOP in each habitat.
- We measured oxygen isotope discrimination during respiration, gas exchange, and metabolite profiles in aerial leaves of ten fern and angiosperm species belonging to five families organized as pairs of palustrine and terrestrial species. We performed a partial least square model combined with variable importance for projection to reveal relationships between the electron partitioning to the AOP (τ_a) and metabolite levels.
- Terrestrial plants showed higher values of net photosynthesis (A_N) and τ_a, together with stronger metabolic relationships between τ_a and sugars, important for water conservation. Palustrine plants

showed relationships between τ_a and metabolites related to the shikimate pathway and the GABA shunt, to be important for heterophylly.

- Excess energy dissipation via AOX is less crucial in palustrine environments than on land. The basis of this difference resides in the contrasting photosynthetic performance observed in each environment, thus reinforcing the importance of AOP for photosynthesis.

Keywords: Alternative Oxidase Pathway (AOP); Cytochrome Oxidase Pathway (COP); electron partitioning to the AOP (τ_a); primary metabolism; terrestrial species; palustrine species; heterophylly

Introduction

Current life on Earth would not be possible without the evolution of biochemical processes that maintained energy entry in plants during land colonization (Delwiche & Cooper, 2015; de Vries *et al*., 2016; de Vries & Archibald, 2018; Gago *et al*., 2019). The earliest terrestrial plant ancestor, a charophycean alga, emerged from water approximately 500 million years ago (Bhattacharya & Medlin, 1998; Yoon *et al*., 2004; Harholt *et al*., 2016; Reski, 2018; Morris *et al*., 2018), undergoing physiological, structural, and biochemical changes to cope with the transition from an aqueous to a gaseous medium (Kenrick & Crane, 1997; Pires & Dolan, 2012; Vermeij, 2016). Among physiological and structural modifications from the first colonizing vascular land plants, specialized sexual organs, different kinds of leaves and roots, stomata, vascular and structural tissues allowed increases in plant size and water use efficiency (Kenrick *et al*., 2012; Assouline & Or, 2013; Proctor *et al*., 2014; Arteaga-Vazquez, 2016; Brodribb *et al*., 2020). At the biochemical level, changes in metabolic pathways favored the synthesis of phenolic compounds, lignin, plant hormones, isoprenes, heat shock proteins or

superoxide dismutase to favor photosynthetic performance and plant growth under a highly-stressful terrestrial environment (Lowry *et al*., 1980; Kenrick & Crane, 1997; Waters, 2003; Weng & Chapple, 2010; Bowman *et al*., 2017). As oxygenic photosynthesis requires water, survival in the dry atmosphere required that plants overcame desiccation forcing the first colonizing terrestrial plants to be close to sources of water, until new adaptations allowed their spread into the dry atmosphere of terrestrial habitats (Brodribb *et al*., 2020). In the meantime, a co-evolution of the antioxidant system and oxygenic photosynthesis allowed land plants to survive several deleterious types of environmental stressors worldwide that induce oxidative stress and damage to the photosynthetic apparatus (Asada *et al*., 2006; Thomas *et al*., 2008; Gill & Tuteja, 2010; Takagi *et al*., 2017; Zandalinas *et al*., 2021).

Currently, several metabolic pathways are identified as major energy-dissipating systems conferring metabolic adaptation in response to a large entry of sunlight energy in leaves (Niyogi, 1999; Raghavendra & Padmasree, 2003; Scheibe, 2004; Noguchi & Yoshida, 2008). Among these pathways, mitochondrial metabolism stands out for its interaction

with photosynthesis, photorespiration and nitrogen assimilation (Raghavendra & Padmasree, 2003; Florez-Sarasa *et al*., 2016; O'Leary *et al*., 2020). In the mitochondrial electron transport system, oxygen consumption takes place simultaneously through the activities of cytochrome oxidase (COX) and alternative oxidase (AOX). Several studies in genetically engineered AOX-modified terrestrial model plants have suggested a role of AOX activity in optimizing photosynthesis under stress (Dahal & Vanlerberghe, 2018; Del-Saz *et al*., 2018a) by favoring the dissipation of excess energy and thus balancing cellular redox metabolism (Raghavendra & Padmasree, 2003; Del-Saz *et al*., 2018a; Vanlerberghe, 2020). In fact, there is *in vivo* evidence of a fine tuning of respiratory metabolism via AOX activity in leaves of crops and model terrestrial plant species exposed to abiotic stress as a mechanism to dissipate excess energy (Florez-Sarasa *et al*., 2012, 2016; Del-Saz *et al*., 2018a,b). Indeed, across the divergence of the plant kingdom, AOX is widespread and conserved, and it is of vital importance for plants (McDonald *et al*., 2006; Del-Saz *et al*., 2018a; Selinski *et al*., 2018). Notably, AOX is hypothesized to have originated among anaerobic

bacteria in an anoxic atmosphere, being important for redox homeostasis during the transition to an oxygen-rich atmosphere 2.45 billion years ago during the Great Oxidation Event (Moore *et al*., 2002; Finnegan *et al*., 2003; Catling & Claire, 2005).

Several clades that appeared during the diversification of terrestrial plants, which include bryophytes, ferns and angiosperms, returned to aquatic environments, necessitating physiological, structural and biochemical modifications (Robe & Griffiths, 2000; Rascio, 2002; Maberly *et al*., 2014). This transition from terrestrial to aquatic habitats occurred gradually with dynamic environmental changes that provided habitats in the palustrine wetland system and emergent heterophyllous amphibious plants, which are characterized by submerged and aerial leaves, and are precursors of the fully submerged habit (Maberly & Spence, 1989; Maberly *et al*., 2014). The fully submerged habit led many aquatic leaves to display metabolic adaptations to enhance carbon gain (Bowes & Salvucci, 1989; Keeley & Santamaria, 1992; Maberly & Madsen, 2002; Huang *et al*. 2020) and the aeration status to allow oxidative phosphorylation (Gibbs & Greenway, 2003). It is unknown

whether the transition from land to the amphibious condition, involved respiratory and metabolic adjustments when oxygen was not a limiting factor. Such adjustments could have happened due to the different redox conditions that characterize both environments, with terrestrial habitats being less often inundated and more influenced by rain and ground water than palustrine habitats, resulting in vegetation adapted to different soil water conditions. Although palustrine habitats can be found within terrestrial environments in different biomes across the globe, a predominance of terrestrial plants in arid biomes may support the idea of water losses acting as the driving force for survival on land (Raven *et al*., 2002; Berry *et al*., 2010), where variation in vegetation type is more affected by climate than in palustrine habitats (Schlesinger & Bernhardt, 2020). Under this scenario marked by higher potential risks for both water conservation and redox balance in terrestrial environments, comparisons of respiratory metabolism in terrestrial vascular plants and their close amphibian relatives could provide clues to different metabolic routes important for the leaf biochemistry in each ecosystem under aerobic conditions. These comparisons could be performed in leaves of

amphibious plants because part of their foliage photosynthesizes and respires in the same gaseous medium as leaves of terrestrial plants. In this sense, the combination of "omics" technologies together with measurements of photosynthesis and respiration is optimal for further understanding of the metabolic regulation of plant physiological processes under different environmental conditions (Del-Saz *et al*., 2016; Florez-Sarasa *et al*., 2012, 2016, 2019; Flexas & Gago, 2018; Clemente-Moreno *et al*., 2019).

No previous study has evaluated the *in vivo* respiratory activities in ferns and palustrine angiosperms. In the present study, we compared ten species of ferns and angiosperms organized as pairs of palustrine and terrestrial species (from the same family). The *in vivo* respiratory activities, photosynthesis, and metabolite profiling of aerial leaves were determined using the oxygen isotope discrimination technique, leaf gas exchange and gas chromatography coupled to mass spectrometry (GC-MS), respectively. Further, to outline the climatic space occupied by these species, we overlapped values of mean annual temperature and annual precipitation with Whittaker's biomes classification (Whittaker,

1970; Wright *et al*., 2004). The main objective was to assess respiratory differences between terrestrial and palustrine plant species. In addition, relationships between metabolic routes and the AOX pathway were identified given their importance for leaf biochemistry in terrestrial and palustrine environments. We hypothesize that in terrestrial plants, these relationships could be important for the regulation of water conservation and redox state; whilst in palustrine plants, these relationships could be important for non-stress roles related to the adaptation to intermediate habitats between land and water (e.g. heterophylly).

Material and methods

Plant material and experimental design

We selected five families of vascular plants, which consisted of one terrestrial species and its palustrine counterpart: (1) *Acanthus mollis* L. and *Hygrophila stricta* (Vahl) L. in Acanthaceae (angiosperm); (2) *Arum italicum* Mill. and *Anubias heterophylla* Engl. in Araceae (angiosperm); (3) *Trachelium caeruleum* L. and *Lobelia cardinalis* L. in

Campanulaceae (angiosperm); (4) *Polypodium cambricum* L. and *Leptochilus pteropus* (Blume) Fraser-Jenk, in Polypodiaceae (fern); and (5) *Pteris vittata* L. and *Ceratopteris thalictroides* L. (Brongn) in Pteridaceae (fern) (Table 1). In the middle of autumn, terrestrial plant species were collected in the field with their underlying substrate (soil) at various coordinates in Mallorca (Spain; Table 1), and placed in plastic bags to be immediately transported to the University of Balearic Islands (Mallorca) where they were transplanted into plastic pots, using a sterile soil–peat mixture (3 : 1 v/v). Then, the pots were maintained in a growth chamber under controlled conditions of 25°C, moderate light intensity of 350 μmol m^{-2} s^{-1} of photosynthetic photon flux density (PPFD), relative humidity above 40%, 12 h photoperiod, and watered to full soil capacity every 3-4 days (fertilized once a week). At the same time, commercial amphibious plants were distributed inside the same growth chamber as the terrestrial plants in different 34 x 45 cm water-tanks containing 20 ± 5 cm water-level, rooted in gravel/ substrate for aquarium plants, and maintained under a moderate irradiance of 100 μmol m^{-2} s^{-1}, according to the low light demand required for growing aquarium species as described

in previous studies (Mommer *et al*., 2005; Koga *et al*., 2020). Four to six plants per terrestrial and palustrine species were maintained under different availability of light energy and water in each habitat. By doing this, we generated contrasting redox environments according to their different predominance in biomes with contrasting canopy openness and water availability as outlined in next subsection. All plants developed aerial leaves under growth chamber conditions until the beginning of experiments in the middle of winter. The upper-most fully expanded aerial leaves of all species were used for gas exchange, *in vivo* respiration, and metabolic profiling analyses.

Species spatial distribution

In order to assess the abundance of both terrestrial and palustrine plant species in locations and biomes with different environmental conditions, we studied the spatial distribution of these species considering data of MAT and mean annual precipitation (MAP) from the years 1980 to 2010. Different numbers of records among species were obtained from GBIF (Global Biodiversity Information Facility[1]): *A. italicum* (32875), *P. cambricum* (17980), *L. cardinalis* (5375), *P. vittata* (3906), *A.*

mollis (2628), *T. caeruleum* (2559), *C. thalictroides* (2037), *L. pteropus* (196), *A. heterophylla* (55), and *H. stricta* (7). For greater accuracy, we increased the number of records of palustrine plants in Araceae and Acanthaceae, by substituting *Higrophylla stricta* (7) for *Higrophylla ringens* (1264) and *Anubias heterophylla* (55) for *Anubias* spp. Schott. (617) because of their similar distribution records (Supplementary Figure 1). Then, a random selection of records equalized the number of samples in each family and habitat; 2000 in Campanulaceae; 1500 in Pteridaceae; 1000 in Acanthaceae; 600 in Araceae, and 150 in Polypodiaceae. Finally, the spatial distribution of records randomly selected was studied with QGIS, a GIS software that combines species occurrences from GBIF with climate layers from WorldClim[2]. QGIS rasterized species occurrences and extracted MAT and MAP data across all grid cells of the species occurrence region, at a spatial resolution of 30 arc-seconds (~1 km). Then, species classification into biomes was performed from a Whittaker diagram of MAT and MAP (Wright et al. 2004).

Leaf gas exchange measurements

Leaf gas exchange with Chl*a* fluorescence measurements were recorded every day from 10 am to 2 pm during the last 2 weeks of the experiment with an open infrared gas-exchange analyzer system (Li-6400; Li-Cor Inc., Lincoln, NE, United States) equipped with a leaf chamber fluorometer (Li-6400-40, Li-Cor Inc.) using aerial leaves of terrestrial and amphibious plants under light-saturating photosynthetic photon flux density (PPFD) of 1000 and 400 μmol m^{-2} s^{-1}, respectively (to avoid photodamage as a consequence of a high PPFD), with 10% blue light, a vapor pressure deficit (VPD) of 1.35 ± 0.32 kPa, a CO_2 concentration (C_a) of 400 μmol CO_2 mol^{-1}, and 25°C air temperature. Net photosynthesis (A_N) and stomatal conductance (g_s) were determined after a steady state was reached (after c. 20 min). Once the gas exchange stabilized, five readings were taken in four to six plants per species and averaged to be considered as the mean of the measured plant. Intrinsic WUEi was calculated as the ratio between A_N and g_s. After a minimum 30 min under dark conditions, leaf dark respiration (R_{dark}) was measured in three to five plants per species with at least five readings per plant, and

estimations of leaf carbon balance were obtained from the ratio of R_{dark} to A_N.

The quantum efficiency of the photosystem II (PSII)-driven electron transport was determined using the equation ΦPSII = $(F_m' - F_s)/F_m'$, where F_s is the steady-state fluorescence in the light (PPFD = 1000 and 400 μmol quanta m^{-2} s^{-1} for terrestrial and palustrine plants, respectively) and F_m' is the maximum fluorescence obtained with a light-saturating pulse (8000 μmol quanta m^{-2} s^{-1}). The electron transport rate (ETR) was calculated as ETR = ΦPSII × PPFD × αβ, where α is the leaf absorptance, assumed to be 0.84, and β is the distribution of absorbed energy between the two photosystems, assumed to be 0.5 (Gallé and Flexas, 2010). At least five readings in two to four plants per species were taken and averaged to be considered as ETR values of the measured plant. The average ETR value for each species was used for estimations of the ratio of ETR to A_N.

Respiration and oxygen-isotope fractionation measurements

For respiratory measurements, the aerial leaves of terrestrial and palustrine plants were harvested and cut into pieces after 30 min in

darkness to be placed in a 3 ml stainless-steel closed cuvette maintained at a constant temperature of 25°C. Air samples were sequentially removed from the cuvette and fed into the mass spectrometer (Delta XPlus; Thermo LCC, Bremen, Germany). Changes in the $^{18}O/^{16}O$ ratios and O_2 concentration were obtained to calculate the oxygen-isotope fractionation and the electron partitioning to the AOP (τ_a), allowing calculations of the *in vivo* activities of AOP and cytochrome oxidase pathway (COP) as described in Del-Saz et al. (2017a). Both end point fractionation values of the AOP (Δ_a) and the capacity of the alternative pathway (V_{alt}) were determined in leaves of terrestrial and palustrine plants treated with a solution of 10 mM potassium cyanide (KCN) for 30 min. For land plants, Δ_a values (n = 3) of 29.9 ± 0.2‰, 30.0 ± 0.2‰, 30.2 ± 0.5‰, 30.6 ± 0.2‰ and 30.3 ± 0.4‰ were obtained for *P. cambricum*, *P. vittata*, *A. italicum*, *A. mollis*, and *T. caeruleum*, respectively. For palustrine plants, Δ_a values of 32.5 ± 0.3‰, 30.8 ± 0.3‰, 31.2 ± 0.8‰, 31.4 ± 0.1‰, and 29.6 ± 0.2‰ were obtained for *A. heterophylla*, *C. thalictroides*, *H. stricta*, *L. cardinalis*, and *L. pteropus*, respectively. On the other hand, an assumed value of 20.0‰ for the end

point fractionation values of the COP (Δ_c) was used for the electron partitioning calculations as this has been shown to be fairly constant in most of the leaves and species examined (Ribas-Carbó et al., 2005). Total mitochondrial ATP production (ATP_{total}) together with ATP production *via* COP (ATP_{cop}) and AOP (ATP_{aop}) were modeled from the activities of the COP and AOP of each measurement, assuming that electron flow through the AOP drives the synthesis of 11 ATP for each 6 O_2 consumed whilst 29 ATP are formed for each 6 O_2 consumed *via* COP (Del-Saz et al., 2017b). Values presented are the mean of six to eight measurements performed in four to six plants per species that were performed from 9 am to 6 pm on the same days as gas exchange measurements were performed during the last 2 weeks of the experiment. In addition, the engagement of AOP (ρ) was calculated as a percentage of the ratio of the *in vivo* activity of AOP (v_{alt}) to V_{alt}.

Metabolite profiling

Terrestrial leaves of palustrine and terrestrial plants were simultaneously sampled after 30 min in darkness on the last day of the experimental period, immediately frozen in liquid nitrogen, and stored at –80°C until

further analysis. Metabolite extractions, derivatization and gas chromatography time of flight-mass spectrometry (GC-TOF-MS) analyses were carried out as previously described (Lisec et al., 2006). The GC-TOF-MS system was composed of a CTC CombiPAL autosampler, an Agilent 6890N gas chromatograph, and a LECO Pegasus III time-of-flight mass spectrometer running in EI + mode. Metabolites were identified by comparison with database entries of standards (Kopka et al., 2005; Schauer et al., 2005). The data of each terrestrial species were normalized to the mean of its respective palustrine counterpart (i.e., the value of all metabolites for each palustrine species was set to 1). The data represent averages of three to six measurements corresponding to material harvested from three to six individual plants per species.

Statistical analysis

Data of A_N, WUEi, total respiration (V_t), *in vivo* activity of COP (v_{cyt}), ATP_{cop}, and ATP_{total}, were log-transformed to meet homoscedasticity. A two-way analysis of variance ($p < 0.05$) was performed with habitat level (terrestrial, palustrine) and plant family (Acanthaceae, Araceae, Campanulaceae, Polypodiaceae, and Pteridaceae)

as fixed factors (Table 2), and Tukey's *post hoc* test ($p < 0.05$) was used to determine differences in each respiratory and photosynthetic parameter between species (Figures 2, 3, Tables 3, 4, and Supplementary Tables 2, 3). Student's *t*-tests were used for statistical analyses in Table 5 in order to compare data from terrestrial species with data from the respective palustrine counterpart in each family. To generate individual fold change data from the physiological parameters, we normalized each measurement of the terrestrial counterpart to the mean of the respective palustrine species, as for the GC-MS metabolite analyses, and Pearson coefficients were obtained with JMP®, Version 12.1.0 (SAS Institute Inc., Cary, NC, United States, 1989–2007; Table 6). Associations between the respiratory parameters and the metabolite profile were explored by applying the Partial Least Square (PLS) sparse regression as defined previously (Saccenti et al., 2014). Missing data in the metabolome dataset were imputed by employing a random forest imputation method before PLS analysis (Gromski et al., 2014). The "*pls*" package in R software was used to develop the PLS regression analysis. Also, this package includes a function to implement the variable importance for the

projection (VIP) for single-response orthogonal score *plsr* models (Wehrens and Mevik, 2007).

Results

Spatial patterns

A species classification into biomes was obtained from a Whittaker diagram of MAT and MAP (Figure 1 and Supplementary Table 1; Wright et al., 2004). We observed species records in all biomes, especially in shrubland, temperate forest, tropical seasonal forest, woodland, and desert (25.6, 24.0, 22.2, 12.6, and 9.89% total records). A low register was found in tropical rainforest, grassland, temperate rainforest, boreal forest, and tundra (4.06, 1.11, 0.48, 0.03, and 0.02% total records). In general, palustrine species were more abundant than terrestrial species in biomes with values of MAP ≥ 1000 mm, such as temperate forest (33.0% palustrine *vs.* 15.1% terrestrial), tropical seasonal forest (32.3% palustrine *vs.* 12.2% terrestrial), and tropical rainforest (6.40% palustrine *vs.* 1.72% terrestrial). In biomes with values of MAP ≤ 1000

mm, palustrine species were more abundant only in woodland (19.3% palustrine *vs.* 5.96% terrestrial), whilst terrestrial species were more abundant than palustrine species in arid biomes such as shrubland (46.4% terrestrial *vs.* 4.72% palustrine) and desert (17.6% terrestrial *vs.* 2.16% palustrine). Specific abundances in each type of biome can be found in Supplementary Table 1.

Leaf gas exchange

Regarding net photosynthesis (A_N), comparisons between groups showed no differences between angiosperms (Acanthaceae, Araceae, Campanulaceae) and ferns (Polypodiaceae, Pteridaceae) in terrestrial habitats; however among palustrine species, A_N was significantly lower in the two ferns species compared to the angiosperm *L. cardinalis* (Campanulaceae; Figure 2A). When comparing between counterparts in each family, A_N was significantly higher (by 2.5-fold) in terrestrial species of Acanthaceae, Araceae, Polypodiaceae, and Pteridaceae. Regarding g_s among terrestrial species, this parameter was significantly lower in the fern *P. cambricum* (Polypodiaceae) only when compared with the angiosperm *T. caeruleum* (Campanulaceae). Contrary

to what was observed for A_N, no differences were found in g_s when comparing between counterparts in each family (Figure 2B).

With regard to WUEi, no major differences were observed between ferns and angiosperms in terrestrial habitats; whilst among palustrine species, the two ferns species showed a significantly lower WUEi when compared to the angiosperm *A. heterophylla* (Araceae; Figure 2C). Very similar to the trends observed for A_N, WUEi was significantly higher (by 3.7-fold) in terrestrial counterparts of Acanthaceae, Polypodiaceae, and Pteridaceae, with the terrestrial fern *P. cambricum* (Polypodiaceae) showing the highest values of WUEi, and both the palustrine angiosperm *H. stricta* (Acanthaceae) and fern *L. pteropus* (Polypodiaceae) displaying the lowest values of WUEi (Figure 2C). On the other hand, palustrine plants showed higher averaged values of ETR/A_N (9.25) and R_{dark}/A_N (0.173) than terrestrial plants (ETR/A_N = 8.10, R_{dark}/A_N = 0.087) mainly because their small A_N, and secondary, because the lack of major variations in R_{dark} and ETR (Tables 2, 3 and Supplementary Table 2).

Respiration and electron partitioning to the AOX pathway

A high heterogeneity was found in V_t, v_{cyt}, and v_{alt} among all species. Considering that most of V_t takes place *via* COX activity, a similar heterogeneity was found in v_{cyt} and V_t, with both varying significantly by 3.3 and 2.7-fold, across species in the terrestrial and palustrine environments, respectively. Both v_{alt} and τ_a showed less variability than v_{cyt} and V_t across terrestrial species (2.0 and 1.6-fold, respectively). In palustrine environments, higher variability was found in v_{alt}, differing significantly 5.4-fold across species, whilst τ_a showed similar variability to v_{cyt} and V_t (2.6-fold). When comparing between counterparts in each family, V_t was significantly higher in terrestrial counterparts of Araceae (by 1.7-fold), and in palustrine counterparts from both fern families, Polypodiaceae and Pteridaceae (by 1.6-fold and 2.4-fold respectively; Table 2), differing slightly from v_{cyt}, which was no different in terrestrial counterparts of Araceae (Table 4). A different pattern was observed for v_{alt}, which was significantly higher in the terrestrial counterpart of Araceae (4.0-fold) and in the palustrine counterpart of Pteridaceae (2.0-fold). A similar behavior was observed for ATP production modeled from v_{cyt} and v_{alt} (Supplementary Table 3).

Regarding τ_a, the terrestrial counterparts of Acanthaceae, Araceae, and Polypodiaceae showed significantly higher values than their palustrine counterparts, 1.4, 2.3, and 1.4-fold, respectively. It is worth mentioning that in Polypodiaceae, the two ferns showed the highest values of τ_a in each habitat (Figure 3). On the other hand, leaves of *H. stricta* showed the highest engagement of AOP (ρ) (57%) mainly because the low V_{alt}, followed by leaves of plants in Polypodiaceae and Pteridaceae (25.5%) that showed variability in V_{alt} and v_{alt}, and by leaves of plants in Campanulaceae and of terrestrial plants in Araceae and Acanthaceae (14%) that displayed large V_{alt}. The palustrine *A. heterophylla* showed the lowest ρ (9%) because the low v_{alt} (Tables 3, 4 and Supplementary Table 2).

In order to better understand the changes in photosynthetic parameters driving the species-specific response of the respiratory parameters, fold changes of A_N, g_s and WUEi values were correlated with fold changes of V_t, τ_a, v_{cyt}, and v_{alt} as described in the statistical analyses section. The only significant correlation (r = 0.75) can be found between A_N and τ_a. Similarly, to study whether AOP contributes significantly to ATP

synthesis, fold changes of τ_a and ATP_{total} values were correlated with fold changes of τ_a, ATP_{cop} and ATP_{aop}. Significant correlations can be found between ATP_{total} and energy synthesis by each pathway (ATP_{cop} and ATP_{aop}; r = 0.98 and 0.87), and between τ_a and ATP_{aop} (r = 0.98; Table 6).

Relative metabolite levels

By using GC-MS-based metabolite profiling from the aerial leaves of palustrine and terrestrial plants, we annotated 40 metabolites (Supplementary Table 5), including sugars, amino acids, organic acids, antioxidants and secondary metabolite precursors, as well as sugar-alcohols (Table 5). Although the identification of 17 metabolites (glycine, asparagine, tryptophan, phosphoric acid, pyruvic acid, citric acid, malic acid, fumaric acid, 2-oxoglutaric acid, quinic acid caffeoyl, maltose, rhamnose, xylose, raffinose, melibiose, erythritol, and galactinol) were only partly detected (n = 2) or not detected at all (nd) in certain species, they were considered for a general interpretation of the results. Significant changes (Student's t test, p < 0.05) in metabolite levels were observed for each metabolite, in the comparison between

terrestrial and palustrine counterparts in each family, with the exception of threonine, pyruvic acid, fumaric acid, and caffeic acid.

Focusing on photosynthetic routes, we observed that Campanulaceae, the only family which showed no significant differences in A_N between palustrine and terrestrial counterparts, showed the largest number of metabolites (19), mainly sugars and organic acids, with reduced levels in the terrestrial species when compared to the palustrine counterpart (Table 5). In contrast, terrestrial species of Acanthaceae, Araceae, Polypodiaceae, and Pteridaceae, with higher values of A_N than their palustrine counterparts, showed higher levels of sugars such as sucrose, fructose or glucose (Table 5), suggesting a higher energy status. We also observed that Araceae, with significantly higher g_s in the terrestrial counterpart, was the only family also showing higher levels of metabolites such as malate and maltose, which are considered of interest due to their roles in determining stomatal movement (Fernie and Martinoia, 2009; Araújo et al., 2011; Gago et al., 2016).

Regarding respiratory routes, in Araceae, the only family showing higher V_t in the terrestrial counterpart, the lack of change and decrease in

citrate and 2-oxoglutarate levels, respectively, together with increases in downstream intermediates (succinate and malate) suggests a high TCA cycle activity (Table 5). This pattern was significantly different (increased citrate levels with no changes in 2-oxoglutarate and malate) in the two terrestrial fern species that displayed lower V_t and v_{cyt}, when compared to their palustrine counterparts, presumably due to lower TCA cycle decarboxylation activity. In this comparison, pronounced differences in γ-aminobutyric acid (GABA) levels – which are intimately connected to TCA cycle activity – between ferns and angiosperms suggest a different role for the GABA-shunt. In addition, the large accumulation of sugars such as sucrose, glucose, and fructose in ferns (Table 4) coincided with an accumulation of antioxidant and secondary metabolism precursors such as quinic acid and dehydroascorbic acid, likely indicative of a reduction in sugar oxidation by glycolysis and the TCA cycle while also promoting the accumulation of antioxidant and secondary metabolism precursors (Table 5). Notably, in Araceae, the only family showing higher values of v_{alt} in the terrestrial counterpart, we observed higher levels of metabolites such as valine, isoleucine, and

malate, which are considered of interest due to their positive correlation with v_{alt} in previous studies (Florez-Sarasa et al., 2012; Del-Saz et al., 2016).

Given the observed general tendency of several physiological parameters to correlate with several metabolites (Figures 2A, 3 and Table 5), we further investigated the observed respiratory patterns for each habitat group employing PLS statistical modeling combined with variable importance for projection (VIP) as a criterion to elucidate metabolite relevance from the generated models (Gago et al., 2016). This modeling helps to highlight putative metabolic networks that differentially drive the respiratory processes in the terrestrial as compared to the palustrine species studied. We used V_t, v_{cyt}, v_{alt}, and τ_a as response variables and, after cross-validation (CV) of the generated models by the PLS, only models for τ_a can be considered robust due to the display of a R^2 higher than 0.6, for both terrestrial ($R^2 = 0.62$) and palustrine ($R^2 = 0.7$) habitats. For palustrine species, significant associations with phosphoric acid, proline, glucose, malic acid, glyceric acid, quinic acid, quinic acid caffeoyl, fructose, GABA, and threonine were observed (Figure

4 and Supplementary Table 4). For terrestrial species, associations with τ_a were observed for trehalose, sucrose, glucose, threonic acid and glycerol (Supplementary Table 4). Interestingly, sugar metabolism was importantly related to τ_a for both lifestyle strategies, glucose being the only metabolite significantly associated in both; despite sugar metabolism in each family differing in the other metabolite associations. Terrestrial species associated mostly with levels of trehalose and sucrose, while palustrine species were mainly associated with phosphoric acid and proline.

Discussion

Habitats Are Associated With Different A_N, Water Use Efficiency and Electron Partitioning to Alternative Oxidase Pathway

In order to characterize terrestrial and palustrine species under the contrasting redox conditions that broadly differentiate both habitats, we decided to maintain plants under different light intensities to fall close to an optimum for each lifestyle. This is because palustrine plants are more often covered by dense canopy trees in humid forests than terrestrial plants in semi-arid Mediterranean forests, according to spatial

distribution of plant records and sample collection coordinates of terrestrial plants (Figure 1 and Table 1). Besides, in humid forest, ground layer plant species may display shade adaptations like low light saturation and light compensation points (Chazdon and Pearcy, 1991; Meng et al., 2014), which led us to photosynthetically characterize these species at different PPFD. We did not expose plants to changing light intensities because it is well known that changes in growth light intensity does not affect oxygen isotope discrimination or τ_a as observed in leaves of *Arabidopsis thaliana* (Florez-Sarasa et al., 2011) and of sun and shade species (Noguchi et al., 2001). However, we ensured that experimental conditions were non-stressful, and enough to allow ETR/A_N values typical of irrigated plants, positive leaf carbon balance and low AOP engagement (and enough overcapacity) in all species (Table 3).

As leaves of terrestrial plants have large energy input because in air the light level is high, the terrestrial species *A. mollis*, *A. italicum*, *P. cambricum*, and *P. vittata* showed higher A_N than their palustrine counterparts *H. stricta*, *A. heterophylla*, *L. pteropus*, and *C.*

thalictroides in Acanthaceae, Araceae, Polypodiaceae, and Pteridaceae, respectively (Figure 2A). This coincided with higher levels of sugars (e.g., sucrose, fructose, and glucose; Table 5), which were considered as markers of high photosynthetic activity (Gago et al., 2016). In contrast, no differences in A_N were found between *T. caeruleum* and *L. cardinalis* in Campanulaceae, which coincides with important reductions in sugars and organic acids in *T. caeruleum* with respect to *L. cardinalis* (Table 5). Because the higher A_N, WUEi, the ratio between A_N and g_s, was found to be larger in Acanthaceae, Polypodiaceae, and Pteridaceae (Figure 2C), which could be in line with previous studies describing a differential regulation of ecosystem (WUE) among biomes. In arid ecosystems, WUE is primarily controlled by evaporation; whilst in sub-humid regions, WUE is mostly regulated by assimilation (Yang R. et al., 2016), which could be partly due to a different predominance of palustrine and terrestrial records displaying contrasting values of WUEi (Figure 1 and Figure 2C) agreeing with the idea of water losses acting as a driving force for the evolution in land plants of gas exchange regulation system (Raven, 2002; Berry et al., 2010; Assouline and Or, 2013).

Contrary to A_N, total respiration (V_t) was not higher in the terrestrial species of Acanthaceae, Araceae, Polypodiaceae, and Pteridaceae than in their palustrine counterparts. Differences in V_t were found among families in each habitat and between ferns and angiosperms (Table 4), similar to previous studies (Choy-Sin and Suan, 1974; Boyce and Mohamed, 1987; Davey et al., 2004; Hilman and Angert, 2016; Zhu et al., 2021). Variability was also found regarding v_{alt} and v_{cyt} (Table 2). Respiration in leaves is highly variable among species as it depends on leaf characteristics such as leaf lifespan, nitrogen content, growth forms, and differential nutritional requirements, regardless of lifestyle or biome (Grime and Hunt, 1975; Reich et al., 1998; Lusk and Reich, 2000; Millenaar et al., 2001; Wright et al., 2004; Atkin et al., 2015). Moreover, the carbon cost for leaf growth and maintenance may differ among species (Lambers et al., 2008). This is why τ_a, which represents the contribution of AOX to V_t, represents a better proxy to evaluate the importance of AOX activity for plant respiration when comparing among different plant species. *In vivo* AOX activity accounted for 10-36% of V_t in both palustrine and terrestrial species considered here, which is

within the range of values observed under both stressful and non-stressful conditions in terrestrial species (10–50%; Del-Saz et al., 2018a), and here, it was strongly influenced by habitat (Table 2). The contribution of AOX to V_t was significantly higher in terrestrial species from Acanthaceae, Araceae, and Polypodiaceae (Figure 3). In model terrestrial plants, previous studies reported τ_a increases under abiotic stressors mainly due to reductions in v_{cyt} because the COX pathway is more sensitive to stressors than the AOX pathway (Del-Saz et al., 2018a), which helps to explain the different effect of habitat on both v_{cyt} and v_{alt} (Table 2). Considering the highest values of A_N and τ_a observed among terrestrial species (Figures 2A, 3) and the significant Pearson coefficient between these parameters (Table 6), the AOP is likely more important for the dissipation of excess energy in terrestrial plants than in palustrine plants, which is in line with previous studies describing higher oxygen isotope discrimination in sun leaves than in shade leaves (Noguchi et al., 2001). Moreover, this coincided with metabolic increases in the levels of several sugars and A_N (Figure 2A and Table 5). Interestingly, τ_a was variable among terrestrial and

palustrine species (Figure 3), suggesting that v_{alt} is coupled to fundamental metabolic processes under non-stress conditions that may differ among species (Florez-Sarasa et al., 2016). Regarding the differences observed between groups, previous studies suggested that the post-translational regulation of AOXs in ferns may differ from those of angiosperms because of the presence of a SerI residue instead of a CysI residue in the majority of the AOX protein sequences analyzed, which could presumably affect v_{alt} (Neimanis et al., 2013).

The electron partitioning to the AOP is linked to habitat-specific metabolic routes

A PLS approach through multivariate regression modeling identified significant relationships only between τ_a and several metabolites in each habitat (Figure 4 and Supplementary Table 4). In terrestrial plants, significant relationships were identified only between τ_a and metabolites related to sugar metabolism (sucrose, glucose, and trehalose). All of these carbohydrates are closely linked to glycolytic activity or sucrose synthesis that are highly dependent on leaf ATP synthesis or requirements (Lunn et al., 2006; Dimroth and von Ballmoos, 2008; Lim

et al., 2020). In addition, the accumulation of these sugars likely confers osmotolerance and redox homeostasis in both ecosystems (Robe and Griffiths, 2000). Sucrose is a metabolic precursor of trehalose, *via* trehalose-6-phosphate, which acts as a signal for high carbon availability in the form of sucrose (Schluepmann et al., 2004; Lunn et al., 2006; Paul et al., 2010; Fichtner and Lunn, 2021), which is in line with the high rates of A_N observed in terrestrial plants (Figure 2A). Trehalose is hydrolyzed by trehalase into glucose, and together with fructose (a product of the reactions catalyzed by both invertase and sucrose synthase) are metabolic precursors of ascorbic acid (AA), one of the most abundant antioxidants in plants (Smirnoff and Wheeler, 2000; Hossain et al., 2017). AA can be metabolized to compounds like threonate (Hancock and Viola, 2005; DeBolt et al., 2006; Smirnoff, 2018) which showed a significant relationship with τ_a in terrestrial plants. Notably, previous studies under salinity conditions highlighted a relationship between the AOP and erythronic acid (Del-Saz et al., 2016), a degradation product of AA (Green and Fry, 2005), reinforcing the role of the AOP in mitochondrial AA synthesis (Millar et

al., 2003; Bartoli et al., 2006; Del-Saz et al., 2016). In addition, threonate is also a precursor of osmoprotectants (Guerrier et al., 2000; Jouve et al., 2004; Muscolo et al., 2015). On the other hand, τ_a in terrestrial plants also showed a significant relationship with glycerol, which is a lipid precursor, that similar to trehalose, is thought to be produced as a consequence of an enhanced CO_2 assimilation in the Calvin-Benson cycle and/or from starch degradation (Liska et al., 2004), which corresponds to the highest values of photosynthesis, foliar carbon balance and oxygen isotope discrimination observed in terrestrial plants (Figures 1A, 3 and Table 3).

Palustrine plants displayed a higher energy efficiency of respiration bearing in mind their lower τ_a, the significant Pearson coefficient between ATP_{aop} and ATP_{total} (Table 6), and the highest VIP value obtained from the relationship between τ_a and phosphate (Supplementary Table 4), perhaps indicative of a tendency to save phosphorus during oxidative phosphorylation for the benefit of ATP synthesis *via* COX. Besides, we identified relationships between τ_a and primary metabolites related to sugar metabolism, photorespiration, secondary metabolism, the

TCA cycle and ammonium assimilation. Precisely, we found a significant relationship between τ_a and glycerate, corresponding to the described role of AOP in dissipating reducing equivalents from photorespiration (Watanabe et al., 2016; Timm and Hagemann, 2020), and suggesting a role of photorespiration in palustrine plants as previously described (Maberly and Spence, 1989). The relationships between τ_a and acyl-quinic acids (Qui, CQA; Figure 4) in palustrine plants suggest a participation of the AOP in modulating carbon supply for these chlorogenic acids, whose accumulation is associated with enhanced tolerance to oxidative stress (Tamagnone et al., 1998; Niggeweg et al., 2004), and competes with the accumulation of shikimate and derived metabolites (Marsh et al., 2009), such as phenylalanine and tryptophan. The reversible esterification of caffeoyl-CoA (whose metabolic precursor is CA) with Qui produces CQA. By the conversion of Qui to shikimate (Clifford et al., 2017), the shikimate pathway provides precursors for the synthesis of tryptophan that in turn is a metabolic precursor for the biosynthesis of auxins. In heterophyllous amphibious plants, auxin synthesis may be enhanced due to alterations in the perception of blue

light in submerged leaves. This is part of a mechanism to coordinate, together with other plant hormones, phenotypic plasticity in leaf form or heterophylly (Nakayama et al., 2012, 2014, 2017; Li et al., 2019, 2021). On the other hand, the significant relationships between τ_a and malate, GABA, and proline suggest that the AOP could also be related to the carbon supply for both the TCA cycle and ammonium assimilation. Through the mitochondrial 2-OG/malate transporter, malate can facilitate GABA transport (Ramesh et al., 2018; Bown and Shelp, 2020), whose synthesis mainly occurs from glutamate by the cytosolic glutamate decarboxylase, alternatively through polyamine degradation (Yang Y. et al., 2016), or by the oxidation of proline to glutamate in mitochondria (Fait et al., 2008; Shelp et al., 2012). Moreover, both GABA and proline may act as osmoprotectants and their catabolism in mitochondria can provide reducing equivalents as substrates for the AOP (Studart-Guimarães et al., 2007; Michaeli et al., 2011; Florez-Sarasa et al., 2021), which is in agreement with the relationships identified between τ_a and these metabolites in palustrine plants (Figure 4 and Supplementary Table 4). On top of this, GABA can act as a transducer of environmental stress

signals leading to the activation of genes for ethylene and abscisic acid biosynthesis (Kinnersley and Turano, 2000; Forde and Lea, 2007). Overall, the relationships between τ_a and metabolites related to hormone biosynthesis and signaling in palustrine environments could be especially relevant for heterophyllous amphibious plants. All these signaling metabolites, together with gibberellins, mediate perception and responses to fluctuations of water levels, and control the synthesis of new developing aerial leaves in the transition from a submerged to an aerial habit (Cox et al., 2004; Jackson, 2008; Chater et al., 2014; Kim et al., 2018). Whilst some evidence has suggested that plant hormones such as abscisic acid, ethylene, gibberellins, and auxins are part of signaling networks controlling AOX expression (Ivanova et al., 2014; Berkowitz et al., 2016), their control of *in vivo* AOX activity remains, even in model terrestrial plants, to be tested.

Concluding remarks

Here we performed a comparative study of photosynthesis, WUEi, and respiration in palustrine and terrestrial species of angiosperms and ferns widely distributed across biomes, and maintained at different availability

of energy and water in their habitats. Our experimental design does not allow the identification of the most important primary force (light or water) driving associations between the respiratory parameters and the metabolites. However, under different redox conditions that broadly characterize their habitats in nature, we found evidence of a large entry of energy into leaves of terrestrial plants considering their higher values of A_N, WUEi, and τ_a, as well as their significant relationships between τ_a and metabolites related to both sugar metabolism and osmotolerance. In palustrine plants, changes in τ_a could modulate the supply of carbon skeletons from sugars to metabolic routes involved in the production of hormones and signaling molecules important for heterophylly (e.g., the shikimate pathway and GABA shunt). Further experiments are needed in amphibious plants in order to study the precise regulation of the AOX pathway during the development of new aerial leaves during their emergence from water. In addition, the low τ_a observed together with the identification of τ_a relationships with phosphoric acid and other respiratory parameters suggests that mitochondrial electron partitioning

contributes to maximizing the ATP yield of respiration in palustrine plants.

Dedication

We would like to honor this manuscript to Prof. James N. Siedow. Jim taught me how to take science so seriously that only Duke basketball was at the same level. Jim could simultaneously smash you with the toughest question of the world, or plant biochemistry, and ensure that you could find the answer by yourself. The velocity of his brain was so high that by the time anyone could catch up with him, he was already smashing with the next joke. His jokes were always sharp, incisive, and funny. And, "so, What's your point?" – MR-C.

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author Contributions

JF, JG, MR-C, and ND-S conceived and designed the idea of this experiment. MC identified and recollected all plant species. CD carried

out the gas-exchange measurements. ND-S carried out the measurements of respiration. IF-S carried out the metabolic analysis. JG carried out the PLS approach. AR-M carried out the spatial distribution analysis. ND-S, JO, and CS wrote the first draft of the manuscript with subsequent inputs from all co-authors. All authors have read and agreed to the published version of the manuscript.

Funding

This work was mainly supported by Ministerio de Economía y Competitividad (MINECO, Spain) (project PGC2018-093824-B-C41) and the ERDF (FEDER). Others funding were provided by FONDECYT No. 1191118 from National Agency for Research and Development (ANID) and the Chilean Scholarship Program/Becas de doctorado nacional/2017–21180329, the European Union's Horizon 2020 Research and Innovation Program under the Marie Skłodowska-Curie grant agreement no. 753301, the 'Ramon y Cajal' contract RYC2019-027244-I/AEI/10.13039/501100011033 and the European Social Fund.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Acknowledgments

We are very grateful to Dr. Biel Martorell at the Serveis Cientifico-Tecnics of the Universitat de les Illes Balears for his help while running IRMS experiments, and to Chris Baldwin for his help with English corrections. Thanks to Bruce Osborne for fruitful discussions during the International Plant Ecophysiology Colloquium held at Katalapi's Park.

References

Araújo W.L., Tohge T., Ishizaki K., Leaver C.J. & Fernie A.R. (2011). Protein degradation–an alternative respiratory substrate for stressed plants. *Trends in plant science* **16:**489-498.

Arteaga-Vazquez M.A. (2016). Land plant evolution: listen to your elders. *Current Biology* **26:** 26-29.

Asada K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiology* **141:** 391–396.

Assouline S. & Or D. (2013). Plant water use efficiency over geological time–evolution of leaf stomata configurations affecting plant gas exchange. *PLoS One* **8:** e67757.

Atkin O.K., Bloomfield K.J., Reich P.B., Tjoelker M.G., Asner G.P., Bonal D., Bönisch G., Bradford M.G., Cernusak L.A., Cosio E.G., Creek D., Crous K.Y., Domingues T.F... Zaragoza-Castells J. (2015). Global variability in leaf respiration in relation to climate, plant functional types and leaf traits. *New Phytologist* **206:** 614-636.

Bartoli C.G., Yu J., Gomez F., Fernández L., McIntosh L. & Foyer C.H. (2006)**.** Inter-relationships between light and respiration in the control of ascorbic acid synthesis and accumulation in *Arabidopsis thaliana* leaves. *Journal of Experimental Botany* **57:**1621-1631.

Berkowitz O., De Clercq I., Van Breusegem F. & Whelan J. (2016). Interaction between hormonal and mitochondrial signaling during growth, development and in plant defense responses. *Plant, cell & environment* **39:**1127-1139.

Berry J.A., Beerling D.J. & Franks P.J. (2010). Stomata: key players in the earth system, past and present. *Current opinion in plant biology* **13:** 232-239.

Bhattacharya D. & Medlin L. (1998)**.** Algal phylogeny and the origin of land plants. *Plant physiology* **116:** 9-15.

Bowes G. & Salvucci M.E. (1989). Plasticity in the photosynthetic carbon metabolism of submersed aquatic macrophytes. *Aquatic Botany* **34:** 233-266.

Bowman J.L., Kohchi T., Yamato K.T., Jenkins J., Shu S., Ishizaki K., Yamaoka S., Nishihama R., Nakamura Y., Berger F., Adam C., Aki S.S... Schmutz J. (2017). Insights into land plant evolution garnered from the *Marchantia polymorpha* genome. *Cell* **171:** 287-304.

Bown A.W. & Shelp B.J. (2020). Does the GABA Shunt Regulate Cytosolic GABA?. *Trends in Plant Science* **25:** 422-424.

Boyce C.K., Brodribb T.J., Feild T.S. & Zwieniecki M.A. (2009). Angiosperm leaf vein evolution was physiologically and environmentally transformative. *Proceedings of the Royal Society B: Biological Sciences* **276:** 1771-1776.

Brodribb T.J. & Feild T.S. (2010). Leaf hydraulic evolution led a surge in leaf photosynthetic capacity during early angiosperm diversification. *Ecology Letters* **13:** 175-183.

Brodribb T.J., Carriquí M., Delzon S., McAdam, S.A.M. & Holbrook N.M. (2020)**.** Advanced vascular function discovered in a widespread moss. *Nature Plants* **6:** 273-279.

Carriquí M., Cabrera H.M., Conesa M.À., Coopman R.E., Douthe C., Gago J., Gallé A., Galmés J., Ribas-Carbó M., Tomàs M. & Flexas J. (2015). Diffusional limitations explain the lower photosynthetic capacity of ferns as compared with angiosperms in a common garden study. *Plant, Cell & Environment* **38:** 448-460.

Catling D.C. & Claire M.W. (2005). How Earth's atmosphere evolved to anoxic state: a status report. *Earth and Planetary Science Letters* **237:** 1–20.

Chater C.C., Oliver J., Casson S. & Gray J.E. (2014). Putting the brakes on: abscisic acid as a central environmental regulator of stomatal development. *New Phytologist* **202:** 376-391.

Choy-Sin H. & Suan W.Y. (1974). Photosynthesis and respiration of ferns in relation to their habitat. *American fern journal* **64:** 40-48.

Clemente-Moreno M.J., Omranian N., Sáez P., Figueroa C.M., Del-Saz N., Elso M., Poblete L., Orf I., Cuadros-Inostroza A... Gago J. (2019). Cytochrome respiration pathway and sulphur metabolism sustain stress tolerance to low temperature in the Antarctic species *Colobanthus quitensis. New Phytologist* **225:** 754-768.

Clifford M.N., Jaganath I.B., Ludwig I.A. & Crozier A. (2017). Chlorogenic acids and the acyl-quinic acids: discovery, biosynthesis, bioavailability and bioactivity. *Natural Products Report* **34:** 1391-1421.

Cox M.C., Benschop J.J., Vreeburg R.A., Wagemaker C.A., Moritz T., Peeters A.J. & Voesenek L.A. (2004)**.** The roles of ethylene, auxin, abscisic acid, and gibberellin in the hyponastic growth of submerged *Rumex palustris* petioles. *Plant Physiology* **136:** 2948-2960.

Dahal K. & Vanlerberghe G.C. (2018). Improved chloroplast energy balance during water deficit enhances plant growth: more crop per drop. *Journal of Experimental Botany* **69:** 1183–1197.

Davey P.A., Hunt S., Hymus G.J., DeLucia E.H., Drake B.G., Karnosky D.F. & Long S.P. (2004). Respiratory oxygen uptake is not decreased by an instantaneous elevation of [CO2], but is increased with long-term growth in the field at elevated [CO2]. *Plant physiology* **134:** 520-527.

De Boer H.J., Eppinga M.B., Wassen M.J. & Dekker S.C. (2012). A critical transition in leaf evolution facilitated the Cretaceous angiosperm revolution. *Nature Communications* **3:** 1-11.

De Vries J. & Archibald J.M. (2018). Plant evolution: landmarks on the path to terrestrial life. *New Phytologist* **217:** 1428-1434.

De Vries J., Stanton A., Archibald J.M. & Gould S.B. (2016). Streptophyte terrestrialization in light of plastid evolution. *Trends in Plant Science* **21:** 467-476.

DeBolt S., Cook D.R. & Ford C.M. (2006)**.** L-Tartaric acid synthesis from vitamin C in higher plants. *Proceedings of the National Academy of Sciences* **103:** 5608-5613.

Delaux P.M., Nanda A.K., Mathé C., Sejalon-Delmas N. & Dunand C. (2012). Molecular and biochemical aspects of plant terrestrialization. *Perspectives in plant ecology, evolution and systematics* **14:** 49-59.

Del-Saz N.F., Florez-Sarasa I., Clemente-Moreno M.J., Mahdhbi H., Flexas J., Fernie A.R. & Ribas-Carbó M. (2016). Salinity tolerance is related to cyanide-resistant alternative respiration in *Medicago truncatula* under sudden severe stress. *Plant, Cell & Environment* **39:** 2361-2369.

Del-Saz N.F., Romero-Munar A., Alonso D., Aroca R., Baraza E., Flexas J. & Ribas-Carbo M. (2017). Respiratory ATP cost and benefit of arbuscular mycorrhizal

symbiosis with *Nicotiana tabacum* at different growth stages and under salinity. *Journal of Plant Physiology* **218**: 243–248.

Del-Saz N.F., Ribas-Carbo M., McDonald A.E., Lambers H., Fernie A.R. & Florez-Sarasa I. (2018a). An *in vivo* perspective of the role(s) of the alternative oxidase pathway. *Trends in Plant Science* **23:** 206–219.

Del-Saz N.F., Romero-Munar A., Cawthray G.R., Palma F., Aroca R., Baraza E., Florez-Sarasa I., Lambers H. & Ribas-Carbó M. (2018b). Phosphorus concentration coordinates a respiratory bypass, synthesis and exudation of citrate, and the expression of high-affinity phosphorus transporters in *Solanum lycopersicum*. *Plant, Cell & Environment* **41:** 865–875.

Del-Saz N.F., Ribas-Carbo M., Martorell G., Fernie A.R. & Florez-Sarasa I. (2017). Measurements of electron partitioning between cytochrome and alternative oxidase pathways in plant tissues. In Plant Respiration and Internal Oxygen: Methods and Protocols; Gupta, J.K., Ed.; Humana Press: New York, NY, USA pp. 203–217.

Delwiche C.F. & Cooper E.D. (2015)**.** The evolutionary origin of a terrestrial flora. *Current Biology* **25:** 899-910.

Dimroth P. & von Ballmoos C. (2008)**.** ATP synthesis by decarboxylation phosphorylation. *Results and Problems in Cell Differentiation* **45:** 153-184.

Fait A., Fromm H., Walter D., Galili G. & Fernie A.R. (2008). Highway or byway: the metabolic role of the GABA shunt in plants. *Trends in Plant Science* **13:** 14–19.

Fernie A.R. & Martinoia E. (2009). Malate: jack of all trades or master of a few?. *Phytochemistry* **70:** 828–832

Fichtner F. & Lunn J.E. (2021). The role of trehalose 6-phosphate (Tre6P) in plant metabolism and development. *Annual Review of Plant Biology* **72:** 1-24.

Finnegan P.M., Umbach A.L. & Wilce J.A. (2003). Prokaryotic origins for the mitochondrial alternative oxidase and plastid terminal oxidase nuclear genes. *FEBS Letters* **555:** 425-430.

Flexas J. & Gago J. (2018). A role for ecophysiology in the 'omics' era. *The Plant Journal* **96:** 251–259.

Florez-Sarasa I., Flexas J., Rasmusson A.G., Umbach A.L., Siedow J.N. & Ribas-Carbo M. (2011). *In vivo* cytochrome and alternative pathway respiration in leaves of

Arabidopsis thaliana plants with altered alternative oxidase under different light conditions. *Plant, Cell & Environment* **34**: 1373–1383.

Florez-Sarasa I., Araújo W.L., Wallström S.V., Rasmusson A.G., Fernie A.R. & Ribas-Carbo M. (2012)**.** Light-responsive metabolite and transcript levels are maintained following a dark-adaptation period in leaves of *Arabidopsis thaliana*. *New Phytologist* **195:** 136-148.

Florez-Sarasa I., Ribas-Carbo M., Del-Saz N., Schwahn K., Nikoloski Z., Fernie A.R. & Flexas J. (2016). Unravelling the in vivo regulation and metabolic role of the alternative oxidase pathway in C3 species under photoinhibitory conditions. *New Phytologist* **212:** 66-79.

Florez-Sarasa I., Obata T., Del-Saz N.F., Reichheld J.P., Meyer E.H., Rodriguez-Concepcion M., Ribas-Carbo M. & Fernie A.R. (2019). The Lack of Mitochondrial Thioredoxin TRXo1 Affects In Vivo Alternative Oxidase Activity and Carbon Metabolism under Different Light Conditions. *Plant Cell Physiology* **60:** 2369-2381.

Florez-Sarasa I., Welchen E., Racca S., Gonzalez D.H., Vallarino J.G., Fernie A.R., Ribas-Carbo M. & Del-Saz N.F. (2021). Cytochrome c Deficiency Differentially Affects the In Vivo Mitochondrial Electron Partitioning and Primary Metabolism Depending on the Photoperiod. *Plants* **10:**444.

Forde B.G & Lea P.J. (2007)**.** Glutamate in plants: metabolism, regulation, and signalling. *Journal of experimental botany* **58:** 2339-2358.

Gago J., Carriquí M., Nadal M., Clemente-Moreno M.J., Coopman R.E., Fernie A.R. & Flexas J. (2019). Photosynthesis optimized across land plant phylogeny. *Trends in Plant Science* **24:** 948-957

Gago J., Daloso D.M., Figueroa C.M., Flexas J., Fernie A.R. & Nikoloski Z. (2016). Relationships of leaf net photosynthesis, stomatal conductance, and mesophyll conductance to primary metabolism: a multispecies meta-analysis approach. *Plant Physiology* **171:** 265–279.

Gibbs J. & Greenway H. (2003). Mechanisms of anoxia tolerance in plants. I. Growth, survival and anaerobic catabolism. *Functional Plant Biology* **30:** 1–47.

Gill S.S. & Tuteja N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant physiology and biochemistry* **48:** 909-930.

Green M.A & Fry S.C. (2005). Vitamin C degradation in plant cells via enzymatic hydrolysis of 4-O-oxalyl-L-threonate. *Nature* **433:** 83-87.

Grime J.P & Hunt R. (1975). Relative growth-rate: its range and adaptive significance in a local flora. *The Journal of Ecology* **63:** 393-422.

Gromski P.S., Xu Y., Kotze H.L., Correa E., Ellis D.I., Armitage E.G., Turner M.L. & Goodacre R. (2014). Influence of missing values substitutes on multivariate analysis of metabolomics data. *Metabolites* **4:** 433– 452.

Guerrier G., Brignolas F., Thierry C., Courtois M. & Kahlem G. (2000). Organic solutes protect drought-tolerant *Populus*× *euramericana* against reactive oxygen species. *Journal of plant physiology* **156:** 93-99.

Hancock R.D & Viola R. (2005). Biosynthesis and catabolism of L-ascorbic acid in plants. *Critical Reviews in Plant Sciences* **24:**167-188.

Harholt J., Moestrup Ø. & Ulvskov P. (2016). Why Plants Were Terrestrial from the Beginning. *Trends in Plant Science* **21:** 96-101.

Hilman B. & Angert A. (2016). Measuring the ratio of CO2 efflux to O2 influx in tree stem respiration. *Tree physiology* **36:** 1422-1431.

Hossain M.A., Munné-Bosch S., Burritt D.J., Diaz-Vivancos P., Fujita M. & Lorence A. (2017). *Ascorbic acid in plant growth, development and stress tolerance*. Eds.; Springer: Berlin/Heidelberg, Germany. 514 pp.

Huang W., Han S., Xing Z. & Li W. (2020). Responses of leaf anatomy and CO_2 concentrating mechanisms of the aquatic plant *Ottelia cordata* to variable CO_2. *Frontiers in Plant Science* **11:**1261.

Ivanova A., Law S.R., Narsai R., Duncan O., Lee J.H., Zhang B., van Aken O., Radomiljac J.D., van der Merwe M., Yi K. & Whelan J. (2014). A functional antagonistic relationship between auxin and mitochondrial retrograde signaling regulates alternative oxidase1a expression in Arabidopsis. *Plant physiology* **165:** 1233-1254.

Jackson M.B. (2008). Ethylene-promoted elongation: an adaptation to submergence stress. *Annals of Botany* **101:** 229-248.

Jouve L., Hoffmann L. & Hausman J.F. (2004). Polyamine, carbohydrate, and proline content changes during salt stress exposure of aspen (*Populus tremula* L.): involvement of oxidation and osmoregulation metabolism. *Plant Biology* **7:** 74-80.

Keeley J.E & Santamaría L. (1992). Carbon: freshwater plants. *Plant, Cell and Environment* **15:** 1021–1035.

Kenrick P. & Crane P.R. (1997). The origin and early evolution of plants on land. *Nature* **389:** 33.

Kenrick P., Wellman C.H., Schneider H. & Edgecombe G.D. (2012). A timeline for terrestrialization: consequences for the carbon cycle in the Palaeozoic. *Philosophical Transactions of the Royal Society B: Biological Sciences* **367:** 519-536.

Kim J., Joo Y., Kyung J., Jeon M., Park J.Y., Lee H.G., Chung D.S., Lee E. & Lee I. (2018**).** A molecular basis behind heterophylly in an amphibious plant, *Ranunculus trichophyllus*. *PLoS genetics* **14:** e1007208.

Kinnersley A.M. & Turano F.J. (2000). Gamma aminobutyric acid (GABA) and plant responses to stress. *Critical Reviews in Plant Sciences* **19:** 479-509.

Koga H., Doll Y., Hashimoto K., Toyooka K. & Tsukaya H. (2020). Dimorphic Leaf Development of the Aquatic Plant Callitriche palustris L. Through Differential Cell Division and Expansion**.** *Frontiers in plant science* **11:** 269.

Kopka J., Schauer N., Krueger S., Birkemeyer C., Usadel B., Bergmüller E., Dörmann P., Weckwerth W. & Willmitzer L. (2005). GMD@ CSB. DB: the Golm metabolome database. *Bioinformatics* **21:** 1635-1638.

Lambers H., Chapin F.S III. & Pons T.L. (2008). Plant Physiological Ecology. Eds. Springer-Verlag, New York, 10–95 pp.

Li G., Hu S., Hou H. & Kimura S. (2019)**.** Heterophylly: phenotypic plasticity of leaf shape in aquatic and amphibious plants. *Plants* **8:** 420.

Li G., Hu S., Zhao X., Kumar S., Li Y., Yang J. & Hou H. (2021). Mechanisms of the Morphological Plasticity Induced by Phytohormones and the Environment in Plants. *International Journal of Molecular Sciences* **22:** 765.

Lim S.L., Voon C.P., Guan X., Yang Y., Gardeström P. & Lim B.L. (2020). In planta study of photosynthesis and photorespiration using NADPH and NADH/NAD+ fluorescent protein sensors. *Nature communications* **11:** 1-12.

Lisec J., Schauer N., Kopka J., Willmitzer L. & Fernie A.R. (2006). Gas chromatography mass spectrometry-based metabolite profiling in plants. *Nature Protocols* **1:** 387–396.

Liska A.J., Shevchenko A., Pick U. & Katz A. (2004). Enhanced photosynthesis and redox energy production contribute to salinity tolerance in *Dunaliella* as revealed by homology-based proteomics. *Plant physiology* **136:** 2806-2817.

Lowry B., Hebant C. & Lee D. (1980). The origin of land plants – a new look at an old problem. *Taxon* **29:** 183–197.

Lunn J.E., Feil R., Hendriks J.H.M., Gibon Y., Morcuende R., Osuna D., Scheible W.R., Carillo P., Hajirezaei M.R. & Stitt M. (2006). Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADP glucose pyrophosphorylase and higher rates of starch synthesis in *Arabidopsis thaliana*. *Biochemical Journal* **397:** 139–148.

Lusk C.H. & Reich P.B. (2000). Relationships of leaf dark respiration with light environment and tissue nitrogen content in juveniles of 11 cold-temperate tree species. *Oecologia* **123:** 318-329.

Maberly S.C. (2014). The fitness of the environments of air and water for photosynthesis, growth, reproduction and dispersal of photoautotrophs: an evolutionary and biogeochemical perspective. *Aquatic Botany* **118:** 4-13.

Maberly S.C. & Spence D.H.N. (1989). Photosynthesis and photorespiration in freshwater organisms: amphibious plants. *Aquatic Botany* **34:** 267-286.

Maberly S.C. & Madsen T.V. (2002). Aquatic freshwater angiosperm carbon concentrating mechanisms: processes and patterns. *Functional Plant Biology* **29:** 393-405.

Marsh K.B., Boldingh H.L., Shilton R.S. & Laing W.A. (2009). Changes in quinic acid metabolism during fruit development in three kiwifruit species. *Functional Plant Biology* **36:** 463-470.

Martins S.C., McAdam S.A., Deans R.M., Da Matta F.M. & Brodribb T.J. (2016). Stomatal dynamics are limited by leaf hydraulics in ferns and conifers: results from simultaneous measurements of liquid and vapour fluxes in leaves. *Plant, cell & environment* **39:** 694-705.

McDonald A.E. & Vanlerberghe G.C. (2006)**.** Origins, evolutionary history, and taxonomic distribution of alternative oxidase and plastoquinol terminal oxidase. *Comparative Biochemistry and Physiolology Part D*: G*enomics Proteomics* **1:** 357–364.

Michaeli S., Fait A., Lagor K., Nunes-Nesi A., Grillich N., Yellin A., Bar D., Khan M., Fernie A.R., Turano F.J. & Fromm H. (2011). A mitochondrial GABA permease connects the GABA shunt and the TCA cycle, and is essential for normal carbon metabolism. *The Plant Journal* **67:** 485-498.

Millar A.H., Mittova V., Kiddle G., Heazlewood J.L., Bartoli C.G., Theodoulou F.L. & Foyer C.H. (2003)**.** Control of ascorbate synthesis by respiration and its implications for stress responses. *Plant Physiology* **133:** 443–447.

Millenaar F.F., Gonzalez-Meler M.A., Fiorani F., Welschen R., Ribas-Carbo M., Siedow J.N., Wagner A.M. & Lambers H. (2001). Regulation of alternative oxidase activity in six wild monocotyledonous species. An in vivo study at the whole root level. *Plant Physiology* **126:** 376-387.

Mommer L., de Kroon H., Pierik R., Bgöemann G.M. & Visser E.J.W. (2005). A functional comparison of acclimation to shade and submergence in two terrestrial plant species. *New Phytologist* **167:** 197–206.

Moore A.L., Albury M.S., Crichton P.G. & Affourtit C. (2002). Function of the alternative oxidase: is it still a scavenger?. *Trends in plant science* **7:** 478-481.

Morris J.L., Puttick M.N., Clark J.W., Edwards D., Kenrick P., Pressel S., Wellmar C.H., Yang Z., Schneider H. & Donoghue P.C. (2018). The timescale of early land plant evolution. *Proceedings of the National Academy of Sciences* **115**: 2274-2283.

Muscolo A., Junker A., Klukas C., Weigelt-Fischer K., Riewe D. & Altmann T. (2015). Phenotypic and metabolic responses to drought and salinity of four contrasting lentil accessions. *Journal of Experimental Botany* **66:** 5467-5480.

Nakayama H., Nakayama N., Nakamasu A., Sinha N. & Kimura S. (2012). Toward elucidating the mechanisms that regulate heterophylly. *Plant Morphology* **24:** 57-63.

Nakayama H., Nakayama N., Seiki S., Kojima M., Sakakibara H., Sinha N. & Kimura S. (2014). Regulation of the KNOX-GA gene module induces heterophyllic alteration in North American lake cress. *The Plant Cell* **26:** 4733-4748.

Nakayama H., Sinha N.R. & Kimura S. (2017). How do plants and phytohormones accomplish heterophylly, leaf phenotypic plasticity, in response to environmental cues. *Frontiers in plant science* **8:** 1717.

Nasrulhaq-Boyce A. & Mohamed M.H. (1987). Photosynthetic and respiratory characteristics of Malayan sun and shade ferns. *New phytologist* **105:** 81-88.

Neimanis K., Staples J.F., Hüner N.P. & McDonald, A.E. (2013). Identification, expression, and taxonomic distribution of alternative oxidases in non-angiosperm plants. *Gene* **526:** 275-286.

Niggeweg R., Michael A.J. & Martin C. (2004). Engineering plants with increased levels of the antioxidant chlorogenic acid. *Nature biotechnology* **22:** 746-754.

Niyogi K.K. (1999). Photoprotection revisited: genetic and molecular approaches. Annual Review of Plant *Physiology and Plant Molecular Biology* **50:** 333-359.

Noguchi K., Go C-S., Terashima I., Ueda S. & Yoshinari T. (2001). Activities of the cyanide-resistant respiratory pathway in leaves of sun and shade species. *Australian Journal of Plant Physiology* **28**: 27–35.

Noguchi K. & Yoshida K. (2008). Interaction between photosynthesis and respiration in illuminated leaves. *Mitochondrion* **8:** 87-99.

O'Leary B.M., Asao S., Millar A.H. & Atkin O. (2020). Core principles which explain variation in respiration across biological scales. *New Phytologist* **222:** 670-686.

Paul M.J., Jhurreea D., Zhang Y., Primavesi L.F., Delatte T., Schluepmann H. & Wingler A. (2010). Up-regulation of biosynthetic processes associated with growth by trehalose 6-phosphate. *Plant Signalling Behavior* **5:** 1–7.

Pires N.D & Dolan L. (2012). Morphological evolution in land plants: new designs with old genes. *Philosophical Transactions of the Royal Society B: Biological Sciences* **367:** 508-518.

Proctor M.C.F. (2014). The Diversification of Bryophytes and Vascular Plants in Evolving Terrestrial Environments. In Hanson D., Rice S. (eds) Photosynthesis in Bryophytes and Early Land Plants. Advances in Photosynthesis and Respiration (Including Bioenergy and Related Processes), vol 37. Springer, Dordrecht.

Raghavendra A.S. & Padmasree K. (2003). Beneficial interactions of mitochondrial metabolism with photosynthetic carbon assimilation. *Trends in Plant Science* **8:** 546-553.

Ramesh S.A., Kamran M., Sullivan W., Chirkova L., Okamoto M., Degryse F., Mclaughlin M., Giliham M. & Tyerman S.D. (2018). Aluminum-activated malate transporters can facilitate GABA transport. *The Plant Cell* **30:** 1147-1164.

Rascio N. (2002). The underwater life of secondarily aquatic plants: some problems and solutions. *Critical Reviews in Plant Sciences* **21:** 401-427.

Raven J.A. (2002). Selection pressures on stomatal evolution. *New Phytologist* **153:** 371-386.

Reich P.B., Walters M.B., Tjoelker M.G., Vanderklein D. & Buschena C. (1998). Photosynthesis and respiration rates depend on leaf and root morphology and nitrogen concentration in nine boreal tree species differing in relative growth rate. *Functional Ecology* **12:** 395-405.

Reski R. (2018). Enabling the water-to-land transition. *Nature plants* **4:** 67-68.

Ribas-Carbo M., Robinson S.A. & Giles L. (2005). The application of the oxygen isotope technique to assess respiratory pathway partitioning. Chapter 3 pp 31–42. In Lambers H and Ribas-Carbo (eds.). Plant Respiration: From Cell to Ecosystem, vol 18. Advances in Photosynthesis and Respiration Series. Springer, Dordrecht, The Netherlands.

Robe W.E & Griffiths H. (2000). Physiological and photosynthetic plasticity in the amphibious, freshwater plant, *Littorella uniflora*, during the transition from aquatic to dry terrestrial environments. *Plant, Cell & Environment* **23:** 1041-1054.

Saccenti E., Hoefsloot H.C., Smilde A.K., Westerhuis J.A. & Hendriks M.M. (2014). Reflections on univariate and multivariate analysis of metabolomics data. *Metabolomics* **10:** 361-374.

Schauer N., Steinhauser D., Strelkov S., Schomburg D., Allison G., Moritz T., Lundgen K., Roessner-Tunali U., Forbes M.G., Willmitzer L., Fernie A.R. & Kopka J. (2005). GC–MS libraries for the rapid identification of metabolites in complex biological samples. *FEBS letters* **579:** 1332-1337.

Scheibe R. (2004). Malate valves to balance cellular energy supply. *Physiologia Plantarum* **120:** 21-26.

Schlesinger W.H. & Bernhardt S.B. (2020). Wetland Ecosystems. Chapter 7 pp 249-291. In Schlesinger WH, Bernhardt SB (eds.). Biogeochemistry, Fourth Edition**,** Academic Press.

Schluepmann H., van Dijken A., Aghdasi M., Wobbes B., Paul M. & Smeekens S. (2004). Trehalose mediated growth inhibition of Arabidopsis seedlings is due to trehalose-6-phosphate accumulation. *Plant Physiology* **135:** 879–890.

Selinski J., Scheibe R., Day D.A & Whelan J. (2018). Alternative oxidase is positive for plant performance. *Trends in plant science* **23:** 588-597.

Shelp B.J., Mullen R.T. & Waller J.C. (2012)**.** Compartmentation of GABA metabolism raises intriguing questions. *Trends Plant Sciences* **17:** 57–59.

Smirnoff N. (2018). Ascorbic acid metabolism and functions: A comparison of plants and mammals. *Free Radical Biology and Medicine* **122:**116-129.

Smirnoff N & Wheeler G.L. (2000). Ascorbic acid in plants: biosynthesis and function. *Critical reviews in plant sciences* **19:** 267-290.

Studart-Guimarães C., Fait A., Nunes-Nesi A., Carrari F., Usadel B. & Fernie A.R. (2007)**.** Reduced expression of succinyl-coenzyme A ligase can be compensated for by up-regulation of the γ-aminobutyrate shunt in illuminated tomato leaves. *Plant Physiology* **145:** 626-639.

Takagi D., Ishizaki K., Hanawa H., Mabuchi T., Shimakawa G., Yamamoto H. & Miyake C. (2017). Diversity of strategies for escaping reactive oxygen species production within photosystem I among land plants: P700 oxidation system is prerequisite for alleviating photoinhibition in photosystem I. *Physiologia Plantarum* **161:** 56-74.

Tamagnone L., Merida A., Stacey N., Plaskitt K., Parr A., Chang C.F., Lynn D., Maxwell J., Roberts K. & Martin C. (1998). Inhibition of phenolic acid metabolism results in precocious cell death and altered cell morphology in leaves of transgenic tobacco plants. *The Plant Cell* **10:** 1801-1816.

Thomas D.J., Boling J., Crowell C.M., Eubanks L.M., McCarthy N., McSpadden T., Rector C., Schuchardt C.L., Spurlock C.J & Warrington J. (2008). A test of the oxygen paradox using antioxidant-deficient cyanobacteria. *Gravitation Space Biology* **21:** 27-28.

Timm S. & Hagemann M. (2020). Photorespiration—how is it regulated and how does it regulate overall plant metabolism? *Journal of experimental botany* **71:** 3955-3965.

Tosens T., Nishida K., Gago J., Coopman R.E., Cabrera H.M., Carriqui M., Laanisto L., Morales L., Nadal M., Rojas R., Talts E.... Flexas J. (2016)**.** The photosynthetic capacity in 35 ferns and fern allies: mesophyll CO2 diffusion as a key trait. *New Phytologist* **209:** 1576 – 1590 .

Valladares F, & Niinemets U. (2007). The architecture of plant crowns: from design rules to light capture and performance. In: Pugnaire FI, Valladares F, editors. Functional plant ecology. New York: Taylor and Francis; 2007. p. 101–50. https://doi.org/10.1201/9781420007626.ch4

Vanlerberghe G.C., Dahal K., Alber N.A. & Chadee A. (2020). Photosynthesis, respiration and growth: A carbon and energy balancing act for alternative oxidase. *Mitochondrion* **52:** 197-211.

Vermeij G.J. (2016). Plant defenses on land and in water: why are they so different?. *Annals of botany* **117:** 1099–1109.

Watanabe C.K., Yamori W., Takahashi S., Terashima I. & Noguchi K. (2016)**.** Mitochondrial Alternative Pathway-Associated Photoprotection of Photosystem II is Related to the Photorespiratory Pathway. *Plant Cell Physiology* **57:** 1426-1431.

Waters E.R. (2003). Molecular adaptation and the origin of land plants. *Molecular Phylogenetics and Evolution* **29:** 456–463.

Wehrens R. & Mevik B.H. (2007). The *pls* package: principal component and partial least squares regression in R. *Journal of Statistical Software* **18:** 1–23.

Weng J.K & Chapple C. (2010). The origin and evolution of lignin biosynthesis. *New Phytologist* **187:** 273-285.

Westbrook A.S. & McAdam S.A. (2020). Stomatal density and mechanics are critical for high productivity: insights from amphibious ferns. *New Phytologist* **229:** 877-889.

Whittaker R.H. (1970). Communities and Ecosystems. 2nd Revised Edition. Macmillan Publishing Co., New York.

Wright I.J., Reich P.B., Westoby M., Ackerly D.D., Baruch Z., Bongers F., Cavender-Bares J., Chapin T., Cornelissen J.H.C., Diemer M., Flexas J... Villar R. (2004). The Worldwide Leaf Economics Spectrum. *Nature* **428:** 821-827.

Yang R., Hui Q. & Gu Z. (2016b). Effects of ABA and CaCl2 on GABA accumulation in fava bean germinating under hypoxia-NaCl stress. *Bioscience, biotechnology, and biochemistry* **80:** 540-546.

Yang Y., Guan H., Batelaan O., McVicar T.R., Long D., Piao S., Liang W., Liu B., Jin Z. & Simmons C.T. (2016a). Contrasting responses of water use efficiency to drought across global terrestrial ecosystems. *Scientific Report* **6:** 23284.

Yoon H.S., Hackett J.D., Ciniglia C., Pinto G. & Bhattacharya D. (2004). A molecular timeline for the origin of photosynthetic eukaryotes. *Molecular Biology and Evolution* **21:** 809-818.

Zandalinas I., Fritschi B. & Mittler R. (2021). Global Warming, Climate Change, and Environmental Pollution: Recipe for a Multifactorial Stress Combination Disaster. *Trends in Plant Science*, https://doi.org/10.1016/j.tplants.2021.02.011.

Zhu L., Bloomfield K.J., Asao S., Tjoelker M.G., Egerton J.J., Hayes L., Lasantha K., Creek D., Grffin K.L., Hurry V., Liddell M., Meir P., Turnbull M.H. & Atkin O.K. (2021). Acclimation of leaf respiration temperature responses across thermally contrasting biomes. *New Phytologist* **229:** 1312-1325.

TABLES

Family	Habitat	Plant species	Life span	Description	GPS Coordinates
Acanthaceae	Palustrine	*Hygrophilla stricta*	Perennial	Angiosperm that reaches a height of 70 cm tall with lance-shaped shade leaves that can be up to 10-15 cm long and 2 cm wide	-----
	Terrestrial	*Acanthus mollis*	Perennial	Clump-forming angiosperm that reaches a maximum 180 cm in height with obovate leaves up to 40 cm long and 25 cm wide	39°45'34.2"N 2°42'39.5"E
Araceae	Palustrine	*Anubias heterophylla*	Perennial	Rhizomatous angiosperm that reaches 30 cm tall in height and develops oval shade leaves that can be up to 38 cm long and 13 cm wide	-----
	Terrestrial	*Arum italicum*	Perennial	Herbaceous angiosperm that reaches 30 cm tall in height with arrow-shaped 20-30 cm long leaves	39°45'34.2"N 2°42'39.5"E
Campanulaceae	Palustrine	*Lobellia cardinalis*	Perennial	Herbaceous angiosperm that grows up to 1.2 m tall in height with coarsely toothed shade leaves over 15 cm long and 4 cm wide	-----
	Terrestrial	*Trachelium caeruleum*	Perennial	Herbaceous angiosperm that grows 0.5-1 m tall with small lance-shaped leaves over 7.5-10 cm long	39°45'34.2"N 2°42'39.5"E
Polypodiaceae	Palustrine	*Leptochilus pteropus*	Perennial	Rhizomatous fern that reaches 15-30 cm tall in height with narrow and twisted shade leaves that can be up to 20 cm long	-----
	Terrestrial	*Polypodium cambricum*	Perennial	Rhizomatous fern that grows 60 cm tall with fronds over 5-30 cm in length	39°47'26.3"N 2°41'23.3"E
Pteridaceae	Palustrine	*Ceratopterys thalictroides*	Annual	Shade-adapted rhizomatous fern that grows 15-30 cm high and 10-20 cm wide with finely branched leaves	-----
	Terrestrial	*Pteris vittata*	Perennial	Rhizomatous fern that grows up to 1 m and with fronds that are from 30-80 cm long	39°45'51.3"N 2°42'33.6"E

Table 1 Classification, collection, and life histories of the different plant species used in this study. Note that amphibious species were obtained from commercial sources in Mallorca (Spain).

Table 2. Significance of sources of variation after two-way analysis of variance analyses for each parameter.

	Habitat	Family	Habitat x Family
ETR	***	*	ns
A_N	***	***	ns
g_s	ns	***	**
R_{dark}	ns	ns	*
WUEi	***	***	***
V_t	ns	***	***
τ_a	***	***	***
v_{cyt}	**	***	***
v_{alt}	ns	***	***
V_{alt}	*	*	***
ATP_{cop}	**	***	***
ATP_{aop}	ns	***	***
ATP_{total}	ns	***	***

Table 3. General characteristics of the studied terrestrial and palustrine plant species: the ratio of electron transport rate (ETR) to net photosynthesis (A_N), the ratio of dark respiration (R_{dark}) to A_N, and the ratio of v_{alt} to V_{alt} (ρ).

Family	Habitat	Plant species	ETR/A_N	R_{dark}/A_N	ρ (%)
Acanthaceae	Palustrine	*Hygrophilla stricta*	8.93	0.190	57
	Terrestrial	*Acanthus mollis*	6.83	0.086	11
Araceae	Palustrine	*Anubias heterophylla*	8.83	0.124	9
	Terrestrial	*Arum italicum*	5.90	0.110	12
Campanulaceae	Palustrine	*Lobelia cardinalis*	10.19	0.135	14
	Terrestrial	*Trachelium caeruleum*	8.25	0.057	19
Polypodiaceae	Palustrine	*Leptochilus pteropus*	8.46	0.158	23
	Terrestrial	*Polypodium cambricum*	9.96	0.094	33
Pteridaceae	Palustrine	*Ceratopterys thalictroides*	11.72	0.256	24
	Terrestrial	*Pteris vittata*	11.21	0.088	22

Table 4. Total respiration (V_t) and the *in vivo* activities of cytochrome oxidase (v_{cyt}) and alternative oxidase (v_{alt}) in aerial leaves of ten different terrestrial and palustrine plant species (see section "Materials and Methods").

Family	Habitat	Plant species	V_t (nmol O_2 g^{-1}DW)	v_{cyt} (nmol O_2 g^{-1}DW)	v_{alt} (nmol O_2 g^{-1}DW)
Acanthaceae	Palustrine	*Hygrophilla stricta*	12.84 ± 2.12 **ab**	10.74 ± 1.83 **abc**	2.10 ± 0.298 **bc**
	Terrestrial	*Acanthus mollis*	15.17 ± 1.45 **a**	11.74 ± 1.09 **ab**	3.43 ± 0.373 **ab**
Araceae	Palustrine	*Anubias heterophylla*	7.03 ± 0.483 **cd**	6.34 ± 0.484 **cd**	0.694 ± 0.109 **d**
	Terrestrial	*Arum italicum*	11.77 ± 0.975 **ab**	9.00 ± 0.724 **bcd**	2.78 ± 0.264 **abc**
Campanulaceae	Palustrine	*Lobelia cardinalis*	15.39 ± 1.51 **a**	12.35 ± 1.54 **ab**	3.05 ± 0.419 **abc**
	Terrestrial	*Trachelium caeruleum*	14.86 ± 0.896 **a**	11.24 ± 0.708 **ab**	3.63 ± 0.310 **a**
Polypodiaceae	Palustrine	*Leptochilus pteropus*	8.37 ± 0.820 **bc**	6.21 ± 0.619 **d**	2.16 ± 0.210 **bc**
	Terrestrial	*Polypodium cambricum*	5.19 ± 0.559 **d**	3.33 ± 0.350 **e**	1.86 ± 0.222 **cd**
Pteridaceae	Palustrine	*Ceratopterys thalictroides*	20.03 ± 2.67 **a**	16.31 ± 2.35 **a**	3.71 ± 0.331 **a**
	Terrestrial	*Pteris vittata*	8.29 ± 0.760 **bcd**	6.44 ± 0.579 **cd**	1.84 ± 0.198 **cd**

Table 5. Relative metabolite levels in leaves of 10 terrestrial and palustrine plant species belonging to five families of ferns and angiosperms as measured by GC-MS (see section "Materials and Methods").

	Acanthaceae		**Araceae**		**Campanulaceae**		**Polypodiaceae**		**Pteridaceae**	
	Hygrophylla stricta	*Acanthus mollis*	*Anubias heterophylla*	*Arum italicum*	*Lobelia cardinalis*	*Trachelium caeruleum*	*Leptochilus pteropus*	*Polypodium cambricum*	*Ceratopteris thalictroides*	*Pteris vittata*
Amino acids										
Alanine	1±0.40	1.62±0.30	1±0.16	0.98±0.47	1±0.29	0.80±0.22	**1±0.32**	**0.09±0.05**	1±0.45	2.15±0.65
Valine	1±0.56	1.64±0.32	**1±0.14**	**4.76±1.53**	1±0.19	0.79±0.29	1±0.36	0.79±0.28	1±0.32	2.78±1.07
Isoleucine	1±0.40	1.27±0.22	**1±0.13**	**2.57±0.41**	1±0.39	0.67±0.29	1±0.32	0.96±0.42	1±0.30	3.25±1.58
Glycine	1±0.75	1.12±0.57	**1±0.19**	**0.32±0.10**	nd		nd		1±0.75	0.69±0.23
Proline	**1±0.38**	**5.35±1.37**	**1±0.13**	**0.26±0.07**	**1±0.27**	**0.33±0.11**	1±0.48	0.20±0.13	**1±0.42**	**2.83±0.26**
Serine	1±0.42	1.98±0.25	1±0.10	0.80±0.30	1±0.32	1.03±0.39	**1±0.19**	**0.37±0.10**	1±0.30	2.49±0.80
Threonine	1±0.38	0.46±0.11	1±0.50	0.43±0.04	1±0.28	0.54±0.16	1±0.15	0.53±0.15	1±0.33	1.47±0.51
Phenylalanine	1±0.42	0.56±0.02	1±0.49	0.99±0.20	**1±0.16**	**0.47±0.13**	1±0.30	0.51±0.07	1±0.25	1.77±1.08
Asparagine	1±0.36	1.85±0.71	**1±0.16**	**2.31±0.05**	*1±0.38	0.47±0.01	**1±0.12**	**0.01±0.00**	1±0.26	1.93±0.80
Tryptophan	**1±0.38**	**0.13±0.02**	**1±0.48**	**0.47±0.11**	*1±0.20	0.36±0.00	1±0.35	2.58±0.80	1±0.26	2.02±1.14
Glutamic acid	**1±0.39**	**9.04±1.07**	**1±0.12**	**1.97±0.34**	1±0.23	1.14±0.34	1±0.35	0.52±0.12	**1±0.52**	**4.08±1.02**
Organic acids										
Glyceric acid	**1±0.31**	**7.39±1.90**	1±0.20	1.88±0.50	**1±0.19**	**0.20±0.04**	**1±0.17**	**0.33±0.13**	**1±0.19**	**0.18±0.04**
Pyruvic acid	1±0.19	1.67±0.39	nd		1±0.21	0.60±0.16	nd		*1±0.27	0.24±0.05
Citric acid	nd		1±0.26	1.17±0.45	nd		nd		**1±0.29**	**6.59±1.04**
Succinic acid	**1±0.37**	**6.58±0.79**	**1±0.14**	**2.83±0.55**	**1±0.23**	**0.24±0.02**	1±0.18	1.19±0.29	**1±0.41**	**3.67±0.19**
Fumaric acid	nd		1±0.30	0.51±0.08	1±0.24	1.01±0.49	1±0.68	0.09±0.03	1±0.34	0.40±0.03
Malic acid	nd		**1±0.25**	**14.9±3.56**	**1±0.22**	**0.18±0.05**	1±0.28	0.60±0.16	1±0.24	1.05±0.60
2-Oxoglutaric acid	**1±0.19**	**46.9±8.11**	**1±0.19**	**0.30±0.05**	*1±0.27	0.29±0.10	nd		1±0.35	0.25±0.05
Nicotinic acid	**1±0.12**	**6.50±1.95**	**1±0.10**	**0.40±0.08**	**1±0.13**	**0.65±0.07**	1±0.33	0.63±0.12	**1±0.20**	**0.26±0.03**
4-Aminobutyric acid	**1±0.21**	**0.48±0.05**	**1±0.19**	**0.21±0.06**	**1±0.17**	**0.13±0.04**	1±0.66	0.49±0.13	1±0.43	1.90±0.28
Threonic acid	1±0.24	1.66±0.32	**1±0.19**	**14.6±1.58**	**1±0.22**	**0.27±0.06**	1±0.37	1.32±0.41	**1±0.25**	**10.5±0.92**

Antioxidants and secondary metabolism precursor										
Quinic acid	**1±0.37**	**0.09±0.02**	**1±0.19**	**0.37±0.06**	1±0.15	1.97±0.17	1±0.38	2.07±0.28	**1±0.10**	**166±8.37**
Quinic acid caffeoyl	**1±0.27**	**0.01±0.00**	nd		**1±0.11**	**544±77.8**	1±0.16	1.37±0.11	*1±0.10	2.23±0.24
Dehydroascorbic acid	1±0.34	0.68±0.08	**1±0.14**	**1.70±0.20**	1±0.28	0.53±0.04	**1±0.18**	**20.2±3.79**	**1±0.40**	**45.6±8.01**
Caffeic acid	1±0.17	0.68±0.11	1±0.22	0.61±0.15	1±0.22	1.45±0.26	1±0.21	0.50±0.03	1±0.50	0.73±0.04
Sugars										
Maltose	nd		**1±0.33**	**8.55±1.47**	nd		**1±0.08**	**2.16±0.30**	nd	
Rhamnose	1±0.14	1.11±0.22	**1±0.22**	**6.33±0.51**	**1±0.13**	**2.47±0.33**	nd		nd	
1,6-Anhydroglucose	**1±0.20**	**0.28±0.03**	1±0.11	1.42±0.23	**1±0.16**	**13.5±2.91**	**1±0.28**	**4.70±0.96**	1±0.53	1.77±0.45
Fructose	**1±0.20**	**0.22±0.05**	1±0.07	1.05±0.06	**1±0.09**	**0.08±0.00**	**1±0.28**	**35.8±5.56**	1±0.23	1.00±0.05
Glucose	**1±0.29**	**8.35±1.93**	1±0.48	1.98±0.76	**1±0.29**	**0.04±0.01**	**1±0.38**	**183±24.6**	**1±0.64**	**26.5±1.92**
Xylose	*1±0.10	0.28±0.05	**1±0.31**	**2.05±0.23**	**1±0.03**	**0.53±0.13**	nd		nd	
Sucrose	1±0.26	1.92±0.26	1±0.16	1.14±0.36	1±0.23	0.91±0.12	1±0.37	1.17±0.10	**1±0.60**	**5.73±0.39**
Raffinose	1±0.23	1.69±0.77	**1±0.18**	**0.29±0.04**	**1±0.16**	**0.07±0.01**	**1±0.07**	**2.19±0.33**	nd	
Trehalose	**1±0.19**	**1.61±0.06**	**1±0.06**	**2.89±0.37**	1±0.14	2.24±0.68	1±0.33	1.66±0.36	1±0.78	0.33±0.04
Melibiose	1±0.19	1.53±0.44	**1±0.34**	**0.96±0.02**	nd		nd		1±0.53	2.10±0.11
Sugar-alcohols										
Erythritol	1±0.38	0.62±0.08	**1±0.19**	**8.21±2.04**	**1±0.13**	**1.64±0.10**	nd		nd	
Galactinol	**1±0.19**	**2.09±0.09**	1±0.23	1.50±0.56	**1±0.14**	**0.15±0.01**	*1±0.51	1.56±0.56	1±0.17	0.68±0.14
Glycerol	1±0.14	1.01±0.25	1±0.29	0.71±0.10	**1±0.16**	**2.53±0.19**	**1±0.21**	**0.33±0.11**	1±0.16	0.84±0.16
Myo-inositol	**1±0.21**	**2.18±0.22**	**1±0.22**	**19.6±6.25**	**1±0.05**	**1.35±0.11**	**1±0.13**	**0.20±0.04**	**1±0.42**	**25.8±10.5**
Other metabolites										
Phosphoric acid	*1±0.34	20.6±8.45	**1±0.17**	**0.18±0.11**	1±0.76	0.89±0.25	1±0.41	0.37±0.23	1±0.23	1.57±0.78

Table 6. Pearson correlation coefficients between fold changes in photosynthetic parameters levels (A_N, g_s, WUEi) and *in vivo* respiratory parameters levels (V_t, v_{cyt}, τ_a, v_{alt}), and between fold changes in respiratory parameters (τ_a and ATP_{total}) and ATP synthesis through each pathway (ATP_{cop} and ATP_{aop}), in leaves of ten species of palustrine and terrestrial vascular plants (Table 1).

	A_N	g_s	WUEi
V_t	-0.29	0.52	-0.45
τ_a	**0.75**	0.23	0.27
v_{cyt}	-0.44	0.55	-0.55
v_{alt}	0.19	0.48	-0.17
	ATP_{cop}	ATP_{aop}	τ_a
ATP_{total}	**0.98**	**0.87**	0.62*
τ_a	0.43	**0.92**	---

Supporting information Table 1. Specific abundances in biomes of the distribution of randomly selected and equalized plant records (≈10500) from different terrestrial and palustrine species belonging to the five families of ferns and angiosperms evaluated in this study (see Material and Methods). For each species, abundances of records were obtained from GBIF (http://www.gbif.org) and overlaid on the climate envelopes of Whittaker's biomes (Whittaker 1970; Wright *et al*., 2004). The number of selected records for each species is shown at the bottom of table. The percentage abundance of total, palustrine, and terrestrial records in each biome are shown on the right side of the table.

	Acanthaceae		**Araceae**		**Campanulaceae**		**Polypodiaceae**		**Pteridaceae**				
	Hygrophila ringens	*Acanthus mollis*	*Anubias spp.* Schott	*Arum italicum*	*Lobelia cardinalis*	*Trachelium caeruleum*	*Leptochilus pteropus*	*Polypodium cambricum*	*Ceratopteris thalictroides*	*Pteris vittata*	Total records	Palustrine records	Terrestrial records
Tropical rainforest	20	1	98	0	18	0	57	0	142	89	4.06%	6.40%	1.72%
Temperate rainforest	5	2	0	0	0	0	1	0	20	22	0.48%	0.50%	0.46%
Tropical seasonal forest	440	44	455	3	219	126	74	7	502	458	22.2%	32.3%	12.2%
Temperate forest	120	178	2	103	1241	183	2	44	361	281	24.0%	33.0%	15.1%
Boreal forest	0	0	0	0	1	1	0	1	0	0	0.03%	0.02%	0.04%
Tundra	0	0	0	0	2	0	0	0	0	0	0.02%	0.04%	0.00%
Woodland	386	29	40	8	150	67	12	0	419	208	12.6%	19.3%	5.96%
Shrubland	15	686	0	477	221	919	0	88	11	259	25.6%	4.72%	46.4%

Grassland	0	5	4	3	78	1	1	1	1	22	1.11%	1.61%	0.61%
Desert	8	54	0	6	69	703	1	9	35	151	9.89%	2.16%	17.6%
	994	999	599	600	1999	2000	148	150	1491	1490			

Supporting information Table 2. Values of photosynthetic electron transport rate (ETR); respiration (R_{dark}); and capacity of the alternative pathway (V_{alt}) in aerial leaves of ten different terrestrial and palustrine plant species. Different letters indicate significant differences with a p value < 0.05 determined by post hoc Tukey–Kramer's test. Values are means ± SE for 2-4, 3-5, and 3 biological replicates for ETR, R_{dark}, and V_{alt}, respectively. * denotes data obtained only in two plants per species.

Family	Habitat	Plant species	ETR (µmols electrons m^{-2} s^{-1})	R_{dark} (µmol CO_2 m^{-2} s^{-1})	V_{alt} (nmol O_2 g^{-1} $DW s^{-1}$)
Acanthaceae	Palustrine	*Hygrophilla stricta*	25.54 ± 2.67 **cd**	0.566 ± 0.120 **ab**	3.70 ± 0.131 **e**
	Terrestrial	*Acanthus mollis*	40.49 ± 7.25 **abcd**	0.529 ± 0.171 **ab**	30.90 ± 4.10 **a**
Araceae	Palustrine	*Anubias heterophylla*	30.11 ± 3.93 **bcd**	0.439 ± 0.126 **ab**	7.42 ± 0.866 **de**
	Terrestrial	*Arum italicum*	51.71± 8.95 **ab**	1.01 ± 0.191 **a**	23.54 ± 5.83 **ab**
Campanulaceae	Palustrine	*Lobelia cardinalis*	*46.11 ± 3.88 **abcd**	0.635 ± 0.098 **ab**	21.40 ± 1.82 **abc**
	Terrestrial	*Trachelium caeruleum*	65.21 ± 7.49 **a**	0.470 ± 0.054 **ab**	19.37 ± 3.19 **abcd**
Polypodiaceae	Palustrine	*Leptochilus pteropus*	15.78 ± 1.62 **d**	0.307 ± 0.058 **b**	9.37 ± 0.694 **cde**
	Terrestrial	*Polypodium cambricum*	49.16 ± 0.923 **abc**	0.484 ± 0.097 **ab**	5.72 ± 0.275 **de**
Pteridaceae	Palustrine	*Ceratopterys thalictroides*	25.90 ± 1.67 **cd**	0.634 ± 0.089 **ab**	15.40 ± 3.07 **bcde**
	Terrestrial	*Pteris vittata*	66.55 ± 4.83 **a**	0.542 ± 0.182 **ab**	8.47 ± 1.69 **cde**

Supporting information Table 3. Modeling of ATP production calculated from values of the *in vivo* activities of cytochrome oxidase (v_{cyt}) and alternative oxidase (v_{alt}) in aerial leaves of ten different terrestrial and palustrine plant species. It is considered that 11 ATP are formed for each 6 O_2 consumed by the AOP and 29 ATP are formed for each 6 O_2 consumed by the COP (see Materials and Methods). Values are the mean of six to eight measurements obtained from 4-6 plants per species. Different letters indicate significant differences with a p value < 0.05 determined by post hoc Tukey–Kramer's test.

Family	Habitat	Plant species	ATP_{cop} (nmol ATP g^{-1} DW s^{-1})	ATP_{aop} (nmol ATP g^{-1} DW s^{-1})	ATP_{total} (nmol ATP g^{-1} DW s^{-1})
Acanthaceae	Palustrine	*Hygrophilla stricta*	51.92 ± 8.82 **abc**	10.14 ± 1.44 **bc**	62.05 ± 10.23 **ab**
	Terrestrial	*Acanthus mollis*	56.73 ± 5.25 **ab**	16.58 ± 1.80 **ab**	73.31 ± 7.02 **a**
Araceae	Palustrine	*Anubias heterophylla*	30.63 ± 2.34 **cd**	3.36 ± 0.53 **d**	33.99 ± 2.34 **cd**
	Terrestrial	*Arum italicum*	43.48± 3.50 **bcd**	13.42 ± 1.28 **abc**	56.90 ± 4.71 **ab**
Campanulaceae	Palustrine	*Lobelia cardinalis*	59.69 ± 7.44 **ab**	14.73 ± 2.02 **abc**	74.42 ± 9.25 **a**
	Terrestrial	*Trachelium caeruleum*	54.32 ± 3.42 **ab**	17.52 ± 1.50 **a**	71.84 ± 4.33 **a**
Polypodiaceae	Palustrine	*Leptochilus pteropus*	30.01 ± 2.99 **d**	10.46 ± 1.02 **bc**	40.48 ± 3.96 **bc**
	Terrestrial	*Polypodium cambricum*	16.07 ± 1.69 **e**	9.00 ± 1.07 **cd**	25.07 ± 2.70 **d**
Pteridaceae	Palustrine	*Ceratopterys thalictroides*	78.85 ± 11.36 **a**	17.95 ± 1.60 **a**	96.81 ± 12.88 **a**

	Terrestrial	*Pteris vittata*	31.11 ± 2.80 **cd**	8.95 ± 0.96 **cd**	40.06 ± 3.67 **bcd**

Supporting information Table 4. Metabolites with higher VIP (variable importance for the projection) values obtained from partial least square sparse regression modeling outputs for each trait. The VIP ranking of metabolites is a representation of the most important metabolites for the models explaining each trait. High VIP values correspond to strong correlations.

	Palustrine τ_a				*Terrestrial* τ_a	
Metabolite	**Comp 1**	**Comp 2**	**Comp 3**	**Comp 4**	**Comp 1**	**Comp 2**
Phosphoric acid	2.6	2.4	2.4	2.3	---	---
Proline	1.9	1.8	1.8	1.8	---	---
Malic acid	1.8	1.7	1.7	1.7	---	---
Glyceric acid	1.8	1.7	1.7	1.6	---	---
Quinic acid	1.7	1.6	1.5	1.5	---	---
Caffeoylquinic acid	1.4	1.4	1.4	1.3	---	---
Fructose	1.4	1.3	1.3	1.3	---	---
GABA	1.2	1.2	1.2	1.1	---	---
Threonine	1.1	1.1	1.1	1.1	---	---
Glucose	1.9	1.8	1.7	1.7	2.4	2.2
Trehalose	---	---	---	---	4.2	3.8
Sucrose	---	---	---	---	2.8	2.6
Threonic acid	---	---	---	---	0	2.1
Glycerol	---	---	---	---	1.5	1.3

FIGURES

Fig. 1. The boundaries of global biome type in relation to the climate factors mean annual temperature (MAT) and mean annual precipitation (MAP; Whittaker, 1970; Wright et al., 2004). For each habitat (terrestrial and palustrine), 5250 plant records (randomly selected and equalized, see section "Materials and Methods") are overlaid on the climate envelopes of Whittaker's biomes. Terrestrial and palustrine records are represented as brown and blue dots, respectively. (1) Tropical rainforest; (2) temperate rainforest; (3) tropical seasonal forest; (4) temperate forest; (5) boreal forest; (6) tundra; (7) woodland, shrubland, and grassland; (8) desert. Biome boundaries are only approximate. Specific abundances in each type of biome can be found in Supplementary Table 1.

Fig. 2. (A) Net photosynthesis (A_N), **(B)** stomatal conductance (g_s), and **(C)** intrinsic water-use efficiency (WUEi) in all palustrine and terrestrial species tested in this study. In **(C)**, values were calculated from

mean values of A_N and g_s. Four to six plants were used to characterize each species. Different letters indicate significant differences with a *p*-value < 0.05 determined by a *post hoc* Tukey–Kramer's test.

Fig. 3. Electron partitioning to the alternative pathway (τ_a) in all palustrine and terrestrial species tested in this study. Values are the mean of six to eight measurements obtained from 4 to 6 plants per species. Different letters indicate significant differences with a *p*-value < 0.05 determined by a *post hoc* Tukey–Kramer's test.

Fig 1.

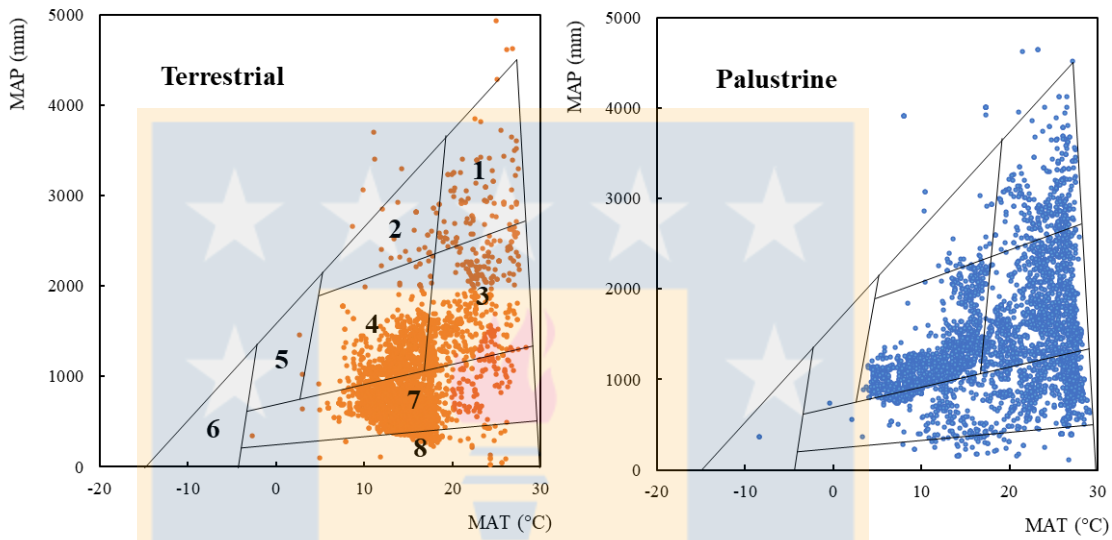

Terrestrial
Palustrine
MAP (mm)
MAT (°C)
5000
4000
3000
2000
1000
0
-20
-10
0
10
20
30
1
2
3
4
5
6
7
8

Fig 2.

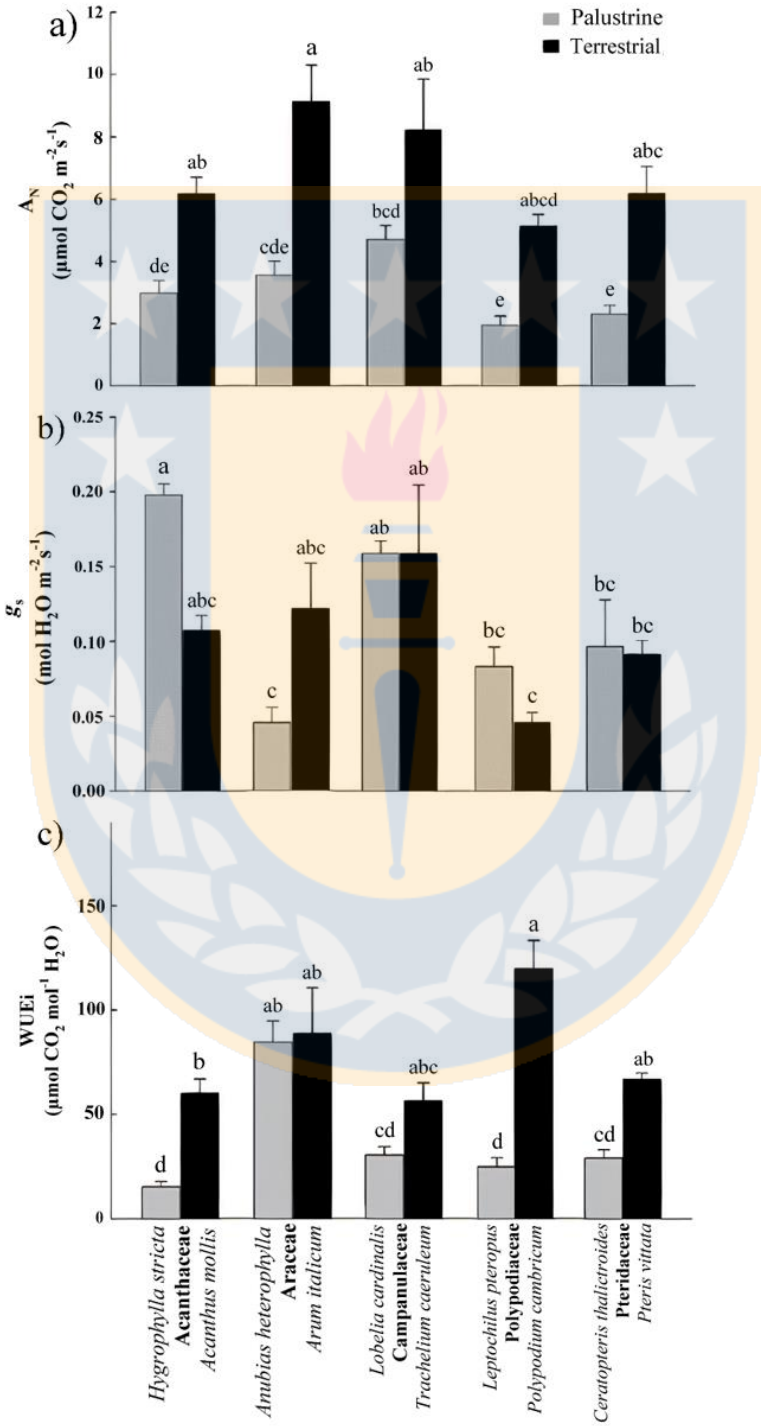
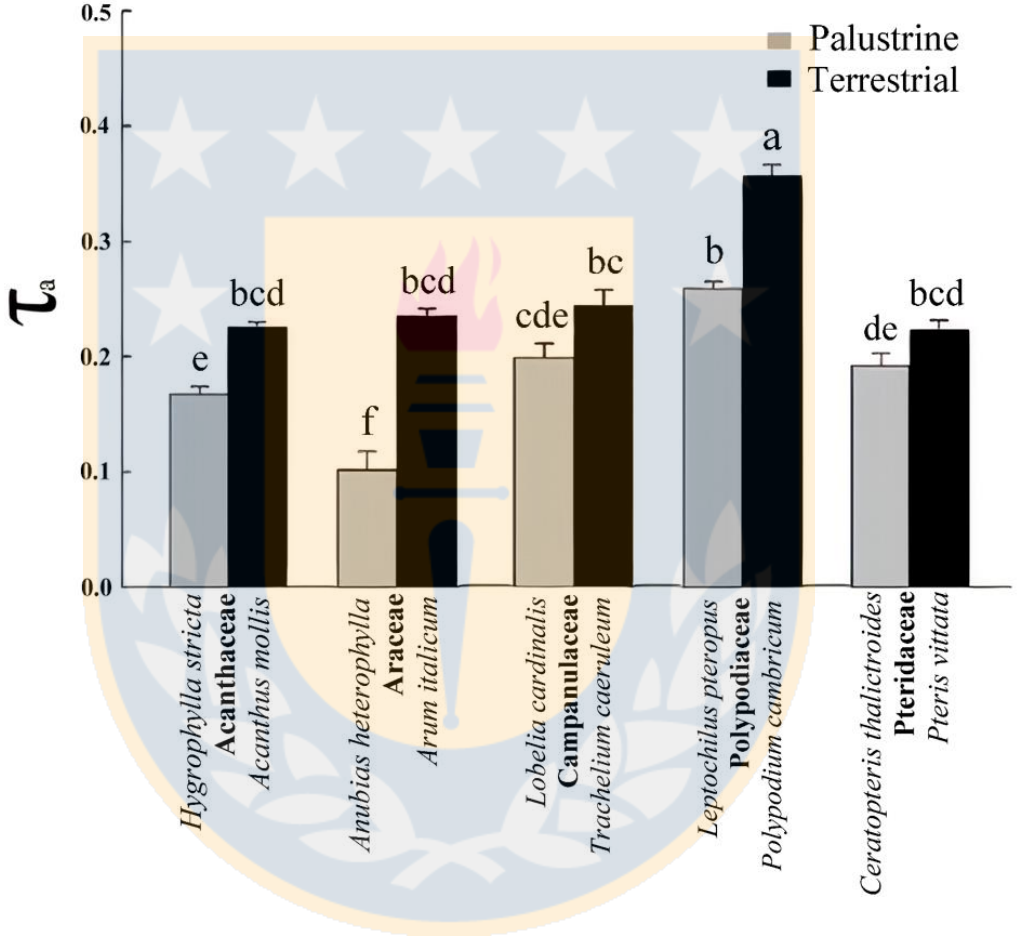
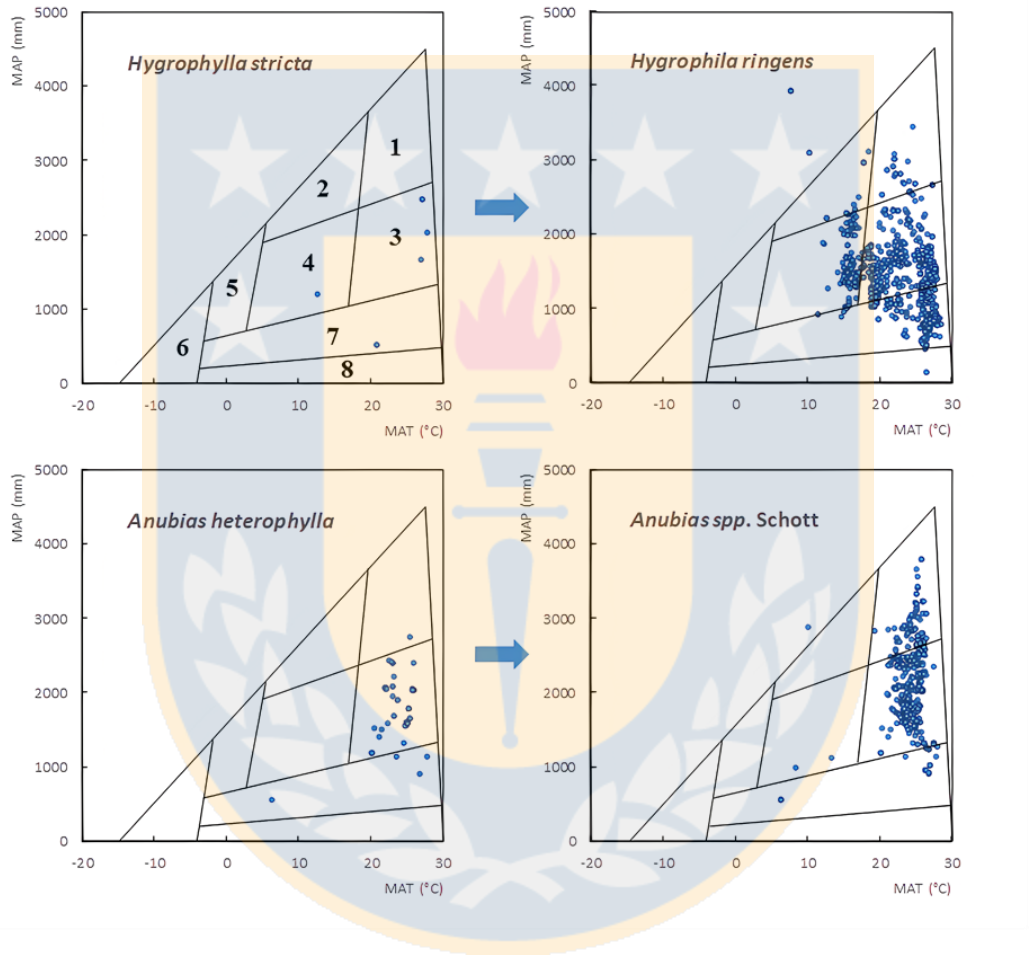

a)
b)
c)
Palustrine
Terrestrial
A_N (µmol CO_2 m^{-2} s^{-1})
g_s (mol H_2O m^{-2} s^{-1})
WUEi (µmol CO_2 mol^{-1} H_2O)
Hygrophylla stricta
Acanthaceae
Acanthus mollis
Anubias heterophylla
Araceae
Arum italicum
Lobelia cardinalis
Campanulaceae
Trachelium caeruleum
Leptochilus pteropus
Polypodiaceae
Polypodium cambricum
Ceratopteris thalictroides
Pteridaceae
Pteris vittata

Fig 3.


Palustrine
Terrestrial
τ_a
0.5
0.4
0.3
0.2
0.1
0.0
e
bcd
f
bcd
cde
bc
b
a
de
bcd
Hygrophylla stricta
Acanthaceae
Acanthus mollis
Amubias heterophylla
Araceae
Arum italicum
Lobelia cardinalis
Campanulaceae
Trachelium caeruleum
Leptochilus pteropus
Polypodiaceae
Polypodium cambricum
Ceratopteris thalictroides
Pteridaceae
Pteris vittata

Supporting information Figure 1. Species classification into major biome types of the world, performed from a Whittaker diagram of MAT and MAP, showing the number of records of species from Araceae and Acanthaceae, in which *Higrophylla stricta* (7 records) was substituted for *Higrophylla ringens* (1264 records), and *Anubias heterophylla* (55 records) for *Anubias spp.* Schott. (617 records) for a greater accuracy in the study of species distribution. 1, tropical rainforest; 2, temperate rainforest; 3, tropical seasonal forest; 4, temperate forest; 5, boreal forest; 6, tundra; 7, woodland, shrubland, and grassland; 8, desert.

Supporting Figure 1.



CHAPTER 2:

Photosynthesis: leaf shape as a key player for energy management and biochemical limitations

Chilean matorral compound and simple leaf woody species are equally affected by extreme drought

José M. Ortíz[1], Carolina Hernández-Fuentes[2], Patricia L. Sáez[3], Néstor Fernández Del-Saz[1] & Lohengrin A. Cavieres[2,3]

[1] Laboratorio de Fisiología Vegetal, Departamento de Botánica, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Concepción, Chile

[2] ECOBIOSIS, Departamento de Botánica, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Concepción, Chile.

[3] Laboratorio Cultivo de Tejidos Vegetales, Centro de Biotecnología, Departamento de Silvicultura, Facultad de Ciencias Forestales, Universidad de Concepción, Concepción, Chile

[4] Instituto de Ecología y Biodiversidad (IEB), Santiago, Chile.

***Manuscrito en proceso de Envío a la revista Plant Ecology**

Abstract

Vascular plants can have either compound or simple leaves. Compound leaves are believed to be photosynthetically more productive than simple ones, by having tiny leaflets increase in photosynthetic area is achieved. In addition, the leaflets allow greater cooling by convection, reducing transpiration costs especially in periods of drought. Simple leaves present other strategy, by invest more mass in less projected area during drought. As differences in the inversion of the foliar mass exist is expected that differences in mesophyll conductance (g_m) occurs. In Central Chile, species with simple and compound leaves coexist. Moreover, a mega drought event is occurring since 2015, causing browning and tree mortality. However, assessment in photosynthetic limitations on both types of leaves in drought conditions have not addressed so far. We measured photosynthetic limitations in well-watered and drought conditions, and a log response ratio were obtained. We found that g_m and CO_2 assimilation (A_N) were larger in compound leaves in well-watered conditions. Under drought conditions, both type of leaves respond negatively equal and foliar temperature in compound leaf species was 4°C lower. In this sense, megadrought events imposes an equal response in Chilean matorral species regardless leaf type.

Introduction

There are two basic leaf types among the vascular plant's realm: simple and compound leaves (Givnish, 1979). Whilst in simple leaves a single blade is inserted directly on the petiole, in compound leaves a blade has two or more subunits called leaflets that vary in number, form and connection to the petiole (e.g., palmately compound leaves *vs.* pinnately compound leaves). Compound leaves has been regarded as more productive than simple leaves due to their lower production cost (Givinish, 1979; Niinemets et al. 1999; Malhado et al. 2010). With the dissection of the photosynthetic area, compound leaves can maximize foliar area (diluting mass tissue in more projected area) for light capture and hence increase the growth rates (Givnish et al. 1979; Sack et al. 2003; Withfiel, 2006; Malhado et al. 2010). Further, the highly dissected venation usually found on compound leaves contributes with the mechanic support through the hydraulic force inside veins avoiding alterations in the leaf mass area (LMA) as occurs in simple leaves (Givnish et al. 1979; Whitfield et al. 2006; Li et al. 2008; Niinemets et al. 2010). For a similar area, compound leaves are more efficient in convective heat exchange, and thus less transpiration is required for cooling (Gates, 1968; Moya & Flexas, 2012; Michaletz et al. 2015). In fact, under

identical environmental conditions, the temperature of dissected leaves can be 4°C lower than that of simple leaves (Stokes et al. 2004). Convective heat exchange allows compound leaves to decrease water loss (Gurevitch et al. 1990; Xu et al. 2009), and to tolerate a wider range of temperatures for biochemical reactions to occur such as carbon assimilation (Michaletz et al. 2015). Thus, based on leaf thermoregulation trade-off related to leaf area and transpirational cooling, different responses to drought can be expected between compound and simple leaves (Givinish, 1979; Michaletz et al. 2015).

Compound leaves are on average thinner than simple leaves (Li et al. 2008; de la Riva et al. 2016). Thinner leaves tend to have higher mesophyll conductances (g_m), but lower tolerance to drought (Niinemets et al. 2011; Flexas et al. 2014). When exposed to drought simple leaf species increase LMA by packing mesophyll cells to avoid cellular lysis (Wright et al. 2004; Galmés et al 2007; Xu et al. 2009), whilst compound leaf species do not change LMA (Xu et al. 2009). In this sense, in well-watered conditions higher carbon assimilation can be achieved in compound leaves, however, under drought conditions, simple leaves can be more tolerant (Galmés et al. 2007; Alonso-Forn et al. 2020b).

It is well known that photosynthesis decreases with drought, but whether drought equally affects the two diffusive components of photosynthesis (i.e., stomatal and mesophyll conductances), remains somehow controversial (Grassi et al. 2005; Galle et al. 2009; Ferrio et al. 2012; Nadal et al. 2018a; Alonso-Forn et al. 2020a). While it seems almost universal that plants exposed to drought close stomata to avoid water losses in detriment of carbon assimilation (A_N) (Cornic et al. 2000; Nadal et al. 2018b; Alonso-Forn et al. 2020a), for the mesophyll conductance (g_m) some studies have shown that g_m decreases with drought (e.g. Galle et al. 2009; Cano et al. 2013; Ouyang et al. 2017) whilst others have found no changes (Galmés et al. 2007; Hommel et al. 2014; Ouyang et al. 2017). Further, to what extent leaf-shape related differences in diffusion photosynthetic traits affect carbon assimilation during drought remain elusive.

Mediterranean-type ecosystem are characterized by severe droughts during summer, and they occur only in five regions of the world: California, South Africa, southeast of Australia, the Mediterranean basin, and central Chile (Lawrence, 1987; Arroyo et al. 1995; Mooney et al. 2001; Armesto et al. 2007). Mediterranean plant species exhibit several morpho-physiological traits to deal with drought such as sclerophyllous leaves, low leaf areas,

increased efficiency in photosystem II, increases in the water use efficiency, and high RuBisCO specificity (Delfine et al. 2001; Galmés et al. 2005, 2007; Medrano et al. 2008; Galle et al. 2011; Flexas et al. 2014; Alonso-Forn et al. 2020ab). In the Mediterranean-type climate zone of central Chile species with simple and compound leaves coexist (Mooney & Dunn, 1970; Arroyo et al. 1995). Unlike other Mediterranean-type climate zones where some rainfall events usually occur during the growth season, in central Chile plants must cope with long droughts with no rain during months (Parson, 1976; Schultz, 2005). Moreover, central Chile has experienced an uninterrupted sequence of dry years since 2010 with mean rainfall deficits of 20–40% (Garreaud et al. 2020). The so-called Mega Drought (MD) is the longest event on record and with few analogues in the last millennia, with detrimental effects on water availability (Borzkut et al. 2018), vegetation and forest fires that have scaled into social and economic impacts (CR2 2017). Recently, Miranda et al. (2020) used temporal trends in the Normalized Difference Vegetation Index (NDVI) to show that the extreme drought of 2019 significantly reduced NDVI (browning) in near one-third of the region's forests and that the highest browning was observed in sclerophyllous forest dominated by species that have been catalogued as

tolerant to drought. Further, global climate models project that observed climate trends are likely to be preserved and the number of extreme drought events will be increasing during the rest of the 21st century, which may have a detrimental impact on these ecosystems (Matskovsky et al. 2021). Therefore, it is important to understand how different representative species of this ecosystem would be affected in their photosynthesis to future increased episodes of extreme drought as well as to assess the underlying mechanisms.

In the present study, we evaluated the photosynthetic response to an extreme experimental drought in compound and simple leaf species of the Central Chile matorral. We hypothesized that with no water limitation compound leaf species will show a higher A_N than simple leaf species associated with a higher CO_2 diffusion inside the leaves (g_m). Nevertheless, with an extreme drought simple leaf species will be less affected than compound leaf species because of their stress-tolerant physiology, showing fewer changes in their photosynthetic traits.

Materials and methods

Study species and growth conditions

We selected six tree species abundant in the central Chile Mediterranean-type zone; three of them have simple leaves whilst the other three compound leaves. The species with simple leaves were: *Peumus boldus* Mol. (Monimiaceae), *Lithraea caustica* Mol. (Hook et. Arn) (Anacardiaceae) and *Cryptocaria alba* (Mol.) Looser (Laureaceae), all of them characterized by ovoid to oval sclerophyllous coriaceous leaves (Fig. 1). Compound leaves species were: *Prosopis chilensis* (Mol.) Stuntz (Fabaceae), *Acacia caven* Mol. (Fabaceae) and *Sophora cassioides* (Phil) Sparre (Fabaceae). *P. chilensis* and *A. caven* have bipinnate leaves, while *S. cassioides* have compound paripinnate leaves (Fig. 1). All the study species are distributed between 30º and 41º south latitude (Fig. 1S; Rodriguez et al. 1983).

For each species, twenty individuals of similar age (1.5 or 2 years) and size were selected. Plants were acquired from a commercial garden ("Encanto Salvaje" http://www.encantosalvaje.cl) and transplanted into a 30 height x 15 cm diameter pots with soil taken from a natural matorral community near Farellones village (33ºS), located at 50 km east of Santiago, Chile. Plants were kept for 21 days in a greenhouse at 33/13 ºC (day/night mean temperature) and a PPFD of 1100 μmol photons m^{-2} s^{-1} with a 12/12 h

light/dark cycle, and 40-60% relative humidity. Pots were periodically irrigated (three times a week) at field capacity. After this period, plants of each species were randomly divided in two groups of 10 individuals, one group was maintained at field capacity (control treatment), whilst the other was exposed to a severe drought. For this, individuals under this treatment received no irrigation for 45 days. Then, to maintain this drought intensity comparable while we were conducting the gas exchange measures, pots were weighed every day and the water lost by evapotranspiration was refilled (usually < 50 ml). Plants were maintained 30 days under this water condition before the measurements.

Leaf gas exchange and chlorophyll a fluorescence measurement

Leaf gas exchange and chlorophyll fluorescence measurements were performed with a portable gas exchange system Li 6400XT (LI-COR Inc., Lincoln, NE, USA) equipped with a leaf chamber fluorometer (Li-6400-40; LI-COR Inc.).

The response of the net photosynthesis CO_2 uptake (A_N) to varying substomatal CO_2 concentration (Ci) was studied with A_N - Ci curves. For each species and growth condition, 10 replicates were performed. For this, a

fully expanded leaf was introduced in the IRGA's chamber and after stabilization (15 minutes) of A_N and g_s a measurement was recorded. Curves were performed by increasing CO_2 concentrations from 0 to 50, 100, 200, 300, 400, 600, 900, 1400 and 2000 µmol CO_2 mol-^{1}, at 1500 µmol photons m^{-2} s^{-1} and between 40 to 60% humidity. A_N -Ci curves were performed at both 21% and 2% of O_2, the latter to suppress photorespiration.

The actual photochemical efficiency of photosystem II (Φ_{PSII}) was determined, simultaneously to the A_N-C_i curves, by measuring steady-state fluorescence (F_s) and maximum fluorescence (F_m') during a light-saturating pulse of c.a. 8,000 photons m^{-2} s^{-1} following the procedures of Genty *et al*. (1989):

$$\Phi_{PSII} = (F_m' - F_s) / F_m'$$

Φ_{PSII} was used for the calculation of the linear rate of electron transport (ETR) according to Krall and Edwards (1992):

$$ETR = \Phi_{PSII} \cdot PPFD \cdot \alpha \cdot \beta$$

where α is the leaf absorbance and β is partitioning of absorbed quanta between photosystems I and II. The product $\alpha \cdot \beta$ was determined from PAR/A_N curves performed with 2% O_2 and at increasing PAR values of 30,

50, 75, 100, 150, 200, 500, 100, 1500, 2000 µmol photons m^{-2} s^{-1} (Yin et al. 2009; Bellasio et al. 2016). Corrections for the leakage of CO_2 into and out of the leaf chamber were applied to all gas-exchange data (Flexas et al., 2007).

Dark respiration (Rd) was measured by darkening the measuring leaf for 30 minutes with CO_2 concentration of 400 µmol mol m^{-1} at 25 ºC. Measured Rd value was used to correct the A_N - Ci curves.

Estimation of mesophyll conductance

From the combined A_N /Ci curves and chlorophyll *a* fluorescence the *in vivo* value of g_m was obtained following Harley et al. (1992):

$$g_m = A_N / (Ci-(\Gamma^*(ETR + 8 (A_N + R_L))/ (ETR – 4 (A_N + R_L))$$

where A_N and Ci were obtained from gas exchange measurements at saturating light. The rate of non-photorespiratory CO_2 evolution in the light (R_L) was determined as half of dark respiration. Chloroplast compensation point Γ* was obtained following Bernacchi et al. (2002), which used kinetic properties of RuBisCO and specificity factor ($S_{c/o}$) (von Caemmerer, 2000). ETR is the electrons that are managed by PSII, calculated with the αβ values obtained from the PAR/A_N curves (Yin et al. 2009).

Leaf temperature

To assess differences in the heat exchange between compound and simple leaves, infra-red thermal images were taken in six replicates per species at each growth condition. Leaf temperature was calculated by choosing 3 points in each leaf. Thermal images were obtained with a thermographic camera Testo 875 (Testo 875-2i, Germany) equipped with a display 3.5¨ LCD of with a resolution of 320 x 250 pixels and a field of view of 32°x 23°. All images were taken at midday when leaves reached their maximum temperature.

Statistical analyses

Linear mixed models were used to analyze the effect of drought on each parameter (photosynthetic rate (A_N), mesophyll conductance (g_m), stomatal conductance (g_s)) where leaf-type was the fixed factor and species a random factor. Log response ratios (LnRR) were calculated to assess the magnitude of the effect of drought on the different photosynthetic parameters between compound and simple leaves as:

$LRR = \log (X_d/X_c)$,

where X_d corresponded to the parameter X measured on plants exposed to drought and X_c to that parameter measured in control plants. Response ratios were calculated for photosynthetic rate (A_N), mesophyll conductance (g_m), stomatal conductance (g_s). Linear mixed models were used to analyze the response ratio of each parameter where leaf-type was the fixed factor and species a random factor. The analyses were done in R 3.0 using Ranova library.

Results

Under well-watered conditions (control conditions), on average, compound leaf species had greater photosynthetic capacity than simple leaf species, where the compound leaf tree species *Prosopis chilensis* and *Acacia caven* were the species showing the highest A_N value, corresponding with the highest g_s values for *A. caven* and the highest g_m value for *P. chilensis* (Fig. 2). Notwithstanding, the simple leaf species *Lithrea caustica* also showed higher A_N value corresponding with high g_s and g_m values (Fig. 2).

Drought negatively affected A_N, g_s and g_m in both leaf type species (Fig. 2, Table 1A), but leaf-type had no statistical effect on any of parameter evaluated (Table 1A). Size effect estimations with the LRR showed that

drought affected the photosynthetic parameters in similar magnitudes (Fig. 3), with no statistical effects of leaf-type (Table 1B).

Regarding species-specific responses, the compound leaf species *Sophora cassioides* and the simple leaf species *Peumus boldus* were the relatively less affected in terms of A_N, g_s and g_m (Fig. 3). In contrast, the compound leaf species *Prosopis chilensis* and the simple leaf *Cryptocaria alba* were the more negatively affected on these parameters (Fig. 3).

Under well-watered conditions, foliar temperature of compound-leaf species did not differ significantly from that of compound leaf species (30.4 *vs* 29.8 °C in compound and simple leaf species, respectively). Nonetheless, the foliar temperature of control plants in compound leaf species was significantly 4°C lower than that of plants under drought, whilst no differences between treatments were observed in simple leaf species (Table 2, Figure S2).

Discussion

Plants possess a great diversity of leaf shapes and sizes (Nicotra et al. 2011; Shi et al. 2020), and several studies remark that leaf shape plays crucial role

in process such as photosynthesis, thermoregulation, hydraulic conductivity, nitrogen content and growth (Wright et al. 2004; Michaletz et al. 2015; Oguchi et al. 2018; Alonso-Forn et al. 2020b). Nevertheless, few studies have characterized the photosynthetic responses to drought between compound and simple leaf species, which is particularly important for Mediterranean species that are increasingly exposed to severe droughts due to climate change (Miranda et al. 2020). We found that a severe drought negatively affect in a similar magnitude compound and simple leaf species from the Chilean matorral.

We observed that with no soil moisture limitations (control conditions), on average, compound leaf species had greater photosynthetic capacity than simple leaf species, where the compound leaf tree species *Prosopis chilensis* and *Acacia caven* were the species showing the highest A_N value. In *P. chilensis*, this could be due to the high values of g_m observed (0.29 mol CO_2 $m^{-2}s^{-1}$) that are on the range of values typically found on herbs (Tomas et al. 2013; Nadal et al. 2018b), being greater than the g_m values reported for other Mediterranean species that typically ranged between 0.18 and 0.08 CO_2 $m^{-2}s^{-1}$ (Niinemets et al. 2005; Galmes et al. 2007; Niinnemets et al. 2009b; Peguero-Pina et al. 2012; Tomas et al. 2013; Flexas et al.

2014; Peguero-Pina et al. 2017; Alonso-Fern et al. 2020b). In *A. caven*, the high A_N value seems to be related to a greater stomatal conductance that would allow higher CO_2 diffusion inside leaves consequently increasing the amount of carbon in carboxylation sites (Cc). Therefore, it seems that compound leaf species tend to show higher A_N and g_m than simple leaf species, as expected due to their lower LMA values (Table S1). As an exception, the compound leaf species *Sophora cassioides* showed the lowest A_N, analogous to the values observed in simple leaf species, probably due to their low g_m.

The lower A_N values were observed on the simple leaf species *P. boldus* and *C. alba*, and these species have high LMA values (Table S1), suggesting a strong constraints for CO_2 diffusion inside their leaves, and thus for A_N (Niinemets et al. 2009b; Tosens et al. 2012; Niinemets et al. 2015; Veroman-Jüergenson et al. 2019, 2020). Indeed, the g_m values obtained for simple leaf species in well-watered conditions are near to the limit of the foliar spectrum for g_m, and in the range of values found in other Mediterranean sclerophyllous species (<0.1 mol CO2 m^2s^{-1} Niinnemets et al. 2009a; Peguero-Pina et al. 2012; Flexas et al. 2014; Peguero-Pina et al. 2018; Alonso-Forn et al. 2020a). On the other hand, the simple leaf species

L. caustica showed the highest values of A_N, with values similar to those reported in previous studies (e.g. Dunn, 1975; Lawrence, 1987, Brito et al. 2014). The g_m value of 0.1 mol CO_2 $m^{-2}s^{-1}$ *L. caustica* was that expected for a LMA of 160 g m^{-2} as proposed by Flexas et al. (2009), and to the value modelled for sclerophyllous species for a similar LMA value (Hassioutou et al. 2010). However, despite the high value of LMA in *L. caustica*, higher A_N value found on this species are due to low diffusional limitation of photosynthesis according to the high g_s and g_m value respect the other simple leaves species.

In contrast to our expectations, leaf-type did not affect photosynthetic plant responses to drought. Although compound leaves species were 4ºC cooler under drought compared to simple leaves species, supporting that a dissected leaf anatomy offer a great advantage in drought conditions by convective heat exchange (Givinish, 1979), all compound leaf species were strongly affected by drought in An, g_s and g_m. In addition, *A. caven* is a winter-deciduous species (Specht, 1988, Aronson, 1992), thus a high control of evaporative demands is critical for maintaining the carbon assimilation during short leaf life span (Mooney & Dunn, 1970).

In this study, we focused on the gas exchange response of Chilean matorral plants to severe drought conditions where no differences were observed between compound and simple-leaf species. This suggests that the "browning" of vegetation (Miranda et al. 2020) caused by the intense and extended mega-drought (Gerraud et al. 2019) is a general response of vegetation where all species, regardless their leaf-type, are seriously affected. However, to forecast how these species will respond to further increases in drought require additional analyses. For example, anatomy and mesophyll arrangement data are required if exists some type of adaptation during drought or changes in enzymatic RuBisCO properties/concentration of this species is related to leaf type (Onoda et al. 2017; Galmes et al. 2019; Alonso-Fom et al. 2020). Further experiments accounting for the recovery phase are needed to know whether compound and simple leaves show differences.

Conflict of interest statement

Authors declare no conflict of interest

Acknowledgements

This study was funded by project Conicyt-PIA CCTE AFB170008 and Chilean Scholarship Program/Becas de Doctorado nacional/2017–21180329 (ANID). We thank Carolina Sanhueza for her comments and Cindy Cerna for her friendship.

References

Alonso-Forn, D., Peguero-Pina, J. J., Ferrio, J. P., Mencuccini, M., Mendoza-Herrer, Ó., Sancho-Knapik, D., & Gil-Pelegrín, E. (2020a). Contrasting functional strategies following severe drought in two Mediterranean oaks with different leaf habit: Quercus faginea and Quercus ilex subsp. Rotundifolia. Tree Physiology.

Alonso-Forn, D., Sancho-Knapik, D., Ferrio, J. P., Peguero-Pina, J. J., Bueno, A., Onoda, Y., ... & Gil-Pelegrín, E. (2020b). Revisiting the functional basis of sclerophylly within the leaf economics spectrum of oaks: Different roads to Rome. Current Forestry Reports, 1-22.

Armesto, J.J., Arroyo, M.K. & L.F., Hinojosa. 2007. The Mediterranean environment of Central Chile. In: Veblen, T.T., Young, K.R. & A.R. Orme (Eds). The Physical Geography of South America. P 184-199.Oxford University Press.

Aronson, J. (1992). Evolutionary biology of Acacia caven (Leguminosae, Mimosoideae): infraspecific variation in fruit and seed characters. Annals of the Missouri Botanical Garden, 958-968.

Arroyo, M. T. K., Zedler, P. H., & Fox, M. D. (Eds.). 1995. Ecology and Biogeography of Mediterranean Ecosystems in Chile, California, and Australia (Vol. 108). Springer Science & Business Media.

Bellasio, C., Beerling, D. J., & Griffiths, H. (2016). An excel tool for deriving key photosynthetic parameters from combined gas exchange and chlorophyll fluorescence: theory and practice. Plant, Cell & Environment, 39(6), 1180-1197.

Bernacchi, C. J., Portis, A. R., Nakano, H., Von Caemmerer, S., & Long, S. P. (2002). Temperature response of mesophyll conductance. Implications for the determination of Rubisco enzyme kinetics and for limitations to photosynthesis in vivo. Plant Physiology, 130(4), 1992-1998.

Brito, C. E., Bown, H. E., Fuentes, J. P., Franck, N., & Perez-Quezada, J. F. 2014. Mesophyll conductance constrains photosynthesis in three common sclerophyllous species in Central Chile. Revista Chilena de Historia Natural, 87(1), 8.

Cornic, G. (2000). Drought stress inhibits photosynthesis by decreasing stomatal aperture–not by affecting ATP synthesis. Trends in Plant Science, 5(5), 187-188.

Delfine, S., Loreto, F., & Aalvino, A. 2001. Drought-Stress effects on physiology, growth and biomass production of rainfed and irrigated bell pepper plants in the

Mediterranean region. Journal of The American Society For Horticultural Science, 126(3), 297-304.

Evans, J. R., & Clarke, V. C. (2019). The nitrogen cost of photosynthesis. Journal of Experimental Botany, 70(1), 7-15.

Ferrio, J. P., Pou, A., Florez-Saraza, I., Gessler, A., Kodama, N., Flexas, J., & Ribas-Carbó, M. (2012). The Péclet effect on leaf water enrichment correlates with leaf hydraulic conductance and mesophyll conductance for CO2. Plant, Cell & Environment, 35(3), 611-625.

Flexas, J., Barbour, M. M., Brendel, O., Cabrera, H. M., Carriquí, M., Diaz-Espejo, A., ... & Gallé, A. (2012). Mesophyll diffusion conductance to CO2: an unappreciated central player in photosynthesis. Plant Science, 193, 70-84.

Flexas, J., Díaz-Espejo, A., Berry, J. A., Cifre, J., Galmés, J., Kaldenhoff, R., ... & Ribas-Carbó, M. (2007). Analysis of leakage in IRGA's leaf chambers of open gas exchange systems: quantification and its effects in photosynthesis parameterization. Journal of Experimental Botany, 58(6), 1533-1543.

Flexas, J., Diaz-Espejo, A., Gago, J., Gallé, A., Galmés, J., Gulías, J., & Medrano, H. (2014). Photosynthetic limitations in Mediterranean plants: a review. Environmental and Experimental Botany, 103, 12-23.

Flexas, J., Ribas-Carbo, M. I. Q. U. E. L., DIAZ-ESPEJO, A. N. T. O. N. I. O., Galmes, J., & Medrano, H. (2009). Mesophyll conductance to CO2: current knowledge and future prospects. Plant, Cell & Environment, 31(5), 602-621.

Galle, A., Florez-Sarasa, I., Aououad, H. E., & Flexas, J. (2011). The Mediterranean evergreen Quercus ilex and the semi-deciduous Cistus albidus differ in their leaf gas exchange regulation and acclimation to repeated drought and re-watering cycles. *Journal of Experimental Botany*, *62*(14), 5207-5216.

Galle, A., Florez-Sarasa, I., Tomas, M., Pou, A., Medrano, H., Ribas-Carbo, M., & Flexas, J. (2009). The role of mesophyll conductance during water stress and recovery in tobacco (Nicotiana sylvestris): acclimation or limitation?. *Journal of Experimental Botany*, *60*(8), 2379-2390.

Galmés, J., Capó-Bauçà, S., Niinemets, Ü., & Iñiguez, C. (2019). Potential improvement of photosynthetic CO2 assimilation in crops by exploiting the natural variation in the temperature response of Rubisco catalytic traits. Current opinion in plant biology, 49, 60-67.

Galmés, J., Flexas, J., Keys, A. J., Cifre, J., Mitchell, R. A., Madgwick, P. J., Haslam, R.P., Medrano, H. & Parry, M. A. 2005. Rubisco specificity factor tends to be larger in plant species from drier habitats and in species with persistent leaves. Plant, Cell & Environment, 28(5), 571-579.

Galmés, J., Medrano, H., & Flexas, J. (2007). Photosynthetic limitations in response to water stress and recovery in Mediterranean plants with different growth forms. New Phytologist, 175(1), 81-93.

Galmés, J., Molins, A., Flexas, J., & Conesa, M. À. (2017). Coordination between leaf CO2 diffusion and Rubisco properties allows maximizing photosynthetic efficiency in Limonium species. Plant, Cell & Environment, 40(10), 2081-2094.

Garreaud, R. D., Boisier, J. P., Rondanelli, R., Montecinos, A., Sepúlveda, H. H., & Veloso-Aguila, D. (2020). The central Chile mega drought (2010–2018): a climate dynamics perspective. *International Journal of Climatology*, *40*(1), 421-439.

Gates, D. M. (1968). Transpiration and leaf temperature. Annual Review of Plant Physiology, 19(1), 211-238.

Genty, B., Briantais, J. M., & Baker, N. R. (1989). The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochimica et Biophysica Acta (BBA)-General Subjects, 990(1), 87-92.

Givnish T. 1979. On the adaptive significance of leaf form. In: Solbrig O.T., Jain S., Johnson G.B., Raven P.H. (Eds) Topics in plant population biology. Palgrave, London. Https://Doi.Org/10.1007/978-1-349-04627-0_17

Grassi, G., & Magnani, F. (2005). Stomatal, mesophyll conductance and biochemical limitations to photosynthesis as affected by drought and leaf ontogeny in Ash and Oak trees. Plant, Cell & Environment, 28(7), 834-849.

Gurevitch, J., & Schuepp, P. H. (1990). Boundary layer properties of highly dissected leaves: an investigation using an electrochemical fluid tunnel. *Plant, Cell & Environment*, *13*(8), 783-792.

Harley, P. C., Loreto, F., Di Marco, G., & Sharkey, T. D. 1992. Theoretical considerations when estimating the mesophyll conductance to CO2 flux by analysis of the response of photosynthesis to CO2. Plant Physiology, 98(4): 1429-1436.

Hassiotou, F., Renton, M., Ludwig, M., Evans, J. R., & Veneklaas, E. J. (2010). Photosynthesis at an extreme end of the leaf trait spectrum: how does it relate to high

leaf dry mass per area and associated structural parameters?. Journal of Experimental Botany, 61(11), 3015-3028.

Kattge, J., & Knorr, W. (2007). Temperature acclimation in a biochemical model of photosynthesis: a reanalysis of data from 36 species. Plant, Cell & Environment, 30(9), 1176-1190.

Krall, J. P., & Edwards, G. E. (1992). Relationship between photosystem II activity and CO2 fixation in leaves. Physiologia Plantarum, 86(1), 180-187.

Lawlor, D. W., & Cornic, G. (2002). Photosynthetic carbon assimilation and associated metabolism in relation to water deficits in higher plants. Plant, cell & environment, 25(2), 275-294.

Lawrence, W.T. 1987. Gas exchange characteristics of representative species form the scrub vegetation of Central Chile. In: Tenhunen, J. D., Catarino, F. M., Lange, O. L., & Oechel, W. C. (Eds.). 1987. Plant Response to Stress: functional analysis in Mediterranean Ecosystems (Vol. 15), P 279-304. Springer Science & Business Media.

Li, G., Yang, D., & Sun, S. (2008). Allometric relationships between lamina area, lamina mass and petiole mass of 93 temperate woody species vary with leaf habit, leaf form and altitude. Functional Ecology, 22(4), 557-564.

Malhado, A. C., Whittaker, R. J., Malhi, Y., Ladle, R. J., Ter Steege, H., Phillips, O., ... & Higuchi, N. (2010). Are compound leaves an adaptation to seasonal drought or to rapid growth? Evidence from the Amazon Rain Forest. Global Ecology and Biogeography, 19(6), 852-862.

Michaletz, S. T., Weiser, M. D., Zhou, J., Kaspari, M., Helliker, B. R., & Enquist, B. J. (2015). Plant thermoregulation: energetics, trait–environment interactions, and carbon Economics. Trends in Ecology & Evolution, 30(12), 714-724.

Miller, P.E. (Ed.) 1981. Resource use by Chaparral and Matorral. Springer-Verlag, Berlin.

Miranda, A., Lara, A., Altamirano, A., Di Bella, C., González, M. E., & Camarero, J. J. (2020). Forest browning trends in response to drought in a highly threatened Mediterranean landscape of South America. Ecological Indicators, 115, 106401

Mooney, H. A., & Dunn, E. L. 1970. Photosynthetic systems of Mediterranean-Climate shrubs and trees of California and Chile. The American Naturalist, 104(939), 447-453

Mooney, H. A., Arroyo, M. T. K., Bond, W. J., Canadell, J., Hobbs, R. J., Lavorel, S., & Neilson, R. P. 2001. Mediterranean-Climate Ecosystems. In global biodiversity in a changing environment. Pp. 157-199. Springer, New York, Ny.

Moya, I., & Flexas, J. (2012). Remote sensing of photosynthesis. Terrestrial photosynthesis in a changing environment: a molecular, physiological, and ecological approach, 219-236.

Nadal, M., & Flexas, J. (2018a). Mesophyll conductance to CO2 diffusion: effects of drought and opportunities for improvement. In *Water scarcity and sustainable agriculture in semiarid environment* (pp. 403-438). Academic Press.

Nadal, M., Flexas, J., & Gulías, J. (2018b). Possible link between photosynthesis and leaf modulus of elasticity among vascular plants: a new player in leaf traits relationships?. Ecology letters, 21(9), 1372-1379.

Nicotra, A. B., Leigh, A., Boyce, C. K., Jones, C. S., Niklas, K. J., Royer, D. L., & Tsukaya, H. (2011). The evolution and functional significance of leaf shape in the angiosperms. Functional Plant Biology, 38(7), 535-552.

Niinemets, U. (1999). Components of leaf dry mass per area—thickness and density—alter photosynthetic capacity in reverse directions in woody plants. New Phytol, 144, 35-47.

Niinemets, Ü. (2001). Global-Scale climatic controls of leaf dry mass per area, density, and thickness in trees and shrubs. Ecology, 82(2), 453-469.

Niinemets, Ü. L. O., Cescatti, A., Rodeghiero, M., & Tosens, T. 2005. Leaf internal diffusion conductance limits photosynthesis more strongly in older leaves of Mediterranean evergreen broad-leaved species. Plant, Cell & Environment, 28(12), 1552-1566.

Niinemets, Ü., & Keenan, T. (2014). Photosynthetic responses to stress in Mediterranean evergreens: mechanisms and models. Environmental and Experimental Botany, 103, 24-41.

Niinemets, Ü., Díaz-Espejo, A., Flexas, J., Galmés, J., & Warren, C. R. (2009). Role of mesophyll diffusion conductance in constraining potential photosynthetic productivity in the field. Journal of Experimental Botany, 60(8), 2249-2270.

Niinemets, Ü., Flexas, J., & Peñuelas, J. (2011). Evergreens favored by higher responsiveness to increased CO2. Trends in Ecology & Evolution, 26(3), 136-142.

Niinemets, Ü., Keenan, T. F., & Hallik, L. (2015). A worldwide analysis of within-canopy variations in leaf structural, chemical and physiological traits across plant functional types. New Phytologist, 205(3), 973-993.

Niinemets, Ü., Wright, I. J., & Evans, J. R. (2009). Leaf mesophyll diffusion conductance in 35 Australian sclerophylls covering a broad range of foliage structural and physiological variation. Journal of Experimental Botany, 60(8), 2433-2449.

Nobel, P. S. (1977). Internal leaf area and cellular CO2 resistance: photosynthetic

Oguchi, R., Onoda, Y., Terashima, I., & Tholen, D. (2018). Leaf anatomy and function. In The leaf: a platform for performing photosynthesis (pp. 97-139). Springer, Cham.

Olsovska, K., Kovar, M., Brestic, M., Zivcak, M., Slamka, P., & Shao, H. B. (2016). Genotypically identifying wheat mesophyll conductance regulation under progressive drought stress. Frontiers in Plant Science, 7, 1111.

Onoda, Y., Wright, I. J., Evans, J. R., Hikosaka, K., Kitajima, K., Niinemets, Ü., ... & Westoby, M. (2017). Physiological and structural tradeoffs underlying the leaf economics spectrum. New Phytologist, 214(4), 1447-1463.

Peguero-Pina, J. J., Flexas, J., Galmes, J., Niinemets, U., Sancho-Knapik, D. O. M. I. N. G. O., Barredo, G., ... & Gil-Pelegrín, E. U. S. T. A. Q. U. I. O. (2012). Leaf anatomical properties in relation to differences in mesophyll conductance to co2 and photosynthesis in two related Mediterranean Abies species. Plant, Cell & Environment, 35(12), 2121-2129.

Peguero-Pina, J. J., Mendoza-Herrer, Ó., Gil-Pelegrín, E., & Sancho-Knapik, D. (2018). Cavitation limits the recovery of gas exchange after severe drought stress in holm oak (Quercus ilex L.). Forests, 9(8), 443.7

Peguero-Pina, J. J., Sisó, S., Flexas, J., Galmés, J., García-Nogales, A., Niinemets, Ü., & Gil-Pelegrín, E. (2017). Cell-Level anatomical characteristics explain high mesophyll conductance and photosynthetic capacity in sclerophyllous Mediterranean oaks. New Phytologist, 214(2), 585-596.

Poorter, H., Niinemets, Ü., Poorter, L., Wright, I. J., & Villar, R. (2009). Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. New Phytologist, 182(3), 565-588.

Rodriguez, R. R., Matthei, S. O., & Quezada, M. M. 1983. Flora arbórea de chile. Chile, Editorial de la Universidad de Concepción.

Sack, L., Cowan, P. D., Jaikumar, N., & Holbrook, N. M. (2003). The 'Hydrology' of leaves: co-ordination of structure and function in temperate woody species. Plant, Cell & Environment, 26(8), 1343-1356.

Shi, P., Niinemets, Ü., Hui, C., Niklas, K. J., Yu, X., & Hölscher, D. (2020). Leaf bilateral symmetry and the scaling of the perimeter vs. the surface area in 15 vine species. Forests, 11(2), 246.

Shiklomanov, A. N., Cowdery, E. M., Bahn, M., Byun, C., Jansen, S., Kramer, K., ... & Dietze, M. C. (2020). Does the leaf economic spectrum hold within plant functional types? A Bayesian multivariate trait meta-analysis. Ecological Applications, 30(3), e02064.

Stokes, V. J., Morecroft, M. D., & Morison, J. I. (2006). Boundary layer conductance for contrasting leaf shapes in a deciduous broadleaved forest canopy. Agricultural and Forest Meteorology, 139(1-2), 40-54.

Tomás, M., Flexas, J., Copolovici, L., Galmés, J., Hallik, L., Medrano, H., ... & Niinemets, Ü. (2013). Importance of leaf anatomy in determining mesophyll diffusion conductance to CO2 across species: quantitative limitations and scaling up by models. Journal of Experimental Botany, 64(8), 2269-2281.

Tosens, T., Niinemets, U., Vislap, V., Eichelmann, H., & Castro Diez, P. (2012). Developmental changes in mesophyll diffusion conductance and photosynthetic capacity under different light and water availabilities in Populus tremula: How structure constrains function. Plant, Cell & Environment, 35(5), 839-856.

Wright, I. J., Reich, P. B., Westoby, M., Ackerly, D. D., Baruch, Z., Bongers, F., ... & Flexas, J. (2004). The Worldwide Leaf Economics Spectrum. Nature, 428(6985), 821-827.

Xu, F., Guo, W., Xu, W., Wei, Y., & Wang, R. (2009). Leaf morphology correlates with water and light availability: what consequences for simple and compound leaves?. Progress In Natural Science, 19(12), 1789-1798.

Yin, X., Struik, P. C., Romero, P., Harbinson, J., Evers, J. B., Van Der Putten, P. E., & Vos, J. A. N. (2009). Using combined measurements of gas exchange and chlorophyll fluorescence to estimate parameters of a biochemical c3 photosynthesis model: a critical appraisal and a new integrated approach applied to leaves in a Wheat (Triticum Aestivum) canopy. Plant, Cell & Environment, 32(5), 448-464.

TABLES

Table 1.

A. Mixed linear model for photosynthesis, A_N, mesophyll conductance, g_m, and stomatal conductance, g_s. Asterisks indicates statistical effects Ranova Test ($p<0.05$).

	Source:	**Df**	**t value**	**Pr(>|t|)**	
A_N					
	Type of Leaf	5	-0.061	0.954	
	Treatment	64	14.08	0.000	*
	Type of leaf x Treatment	64	-4.937	0.000	*
g_m					
	Type of Leaf	5	-0.242	0.816	
	Treatment	64	7.538	0.000	
	Type of leaf x Treatment	64	-4.151	0.000	*
g_s					
	Type of Leaf	5	-0.033	0.975	
	Treatment	64	12.350	0.000	*
	Type of leaf x Treatment	64	-2.200	0.031	*

B. Mixed Lineal model for LnRR of carbon assimilation, A_N. Mesophyll conductance, g_m. Stomatal conductance, g_s.

Source		Df	t value	Pr(>|t|)
A_N	Type of Leaf	4	-0.396	0.712
g_m	Type of Leaf	4	0.165	0.876
g_s	Type of Leaf	4	0.483	0.654

Table 2. Leaf temperature of compound and simple leaf species from the central Chile matorral growing under control and drought conditions. Values are mean ± S.D. (n = 6).

Leaf type	Species	Condition	Leaf Temperature (C°)
Compound	*A. caven*	Control	27.4 ± 0.21
		Drought	27.2 ± 0.31
	P. chilensis	Control	32 ± 0.44
		Drought	29.8 ± 0.07
	S. cassioides	Control	31.8 ± 0.54
		Drought	28.5 ± 0.18
Simple	*P. boldus*	Control	28.4 ± 0.62
		Drought	32.7 ± 0.55
	C. alba	Control	28.8 ± 0.6
		Drought	33.3 ± 0.56
	L. caustica	Control	32.1 ± 055
		Drought	33.9 ± 0.47

FIGURES

Figure 1. Compound (A-C) and simple leaves (D-E) used in this study. A, Prosopis chilensis. B, Sophora cassioides. C, Acacia caven. D, Cryptocarya alba. E, Lithraea caustica. F. Peumus boldus. Straight line indicates scale in cm.

Figure 2. Photosynthesis (A_N), stomatal conductance (g_s) and Mesophyll conductance (g_m) of simple and compound leaf species of the central Chile matorral. Values are mean ± two standard errors.

Figure 3. Log response ratio (LnRR) to drought on photosynthesis (A_N), stomatal conductance (g_s), mesophyll conductance (g_m) of two leaf-type species. Values are mean ± two standard errors. Zero line indicates no change.

Fig 1.

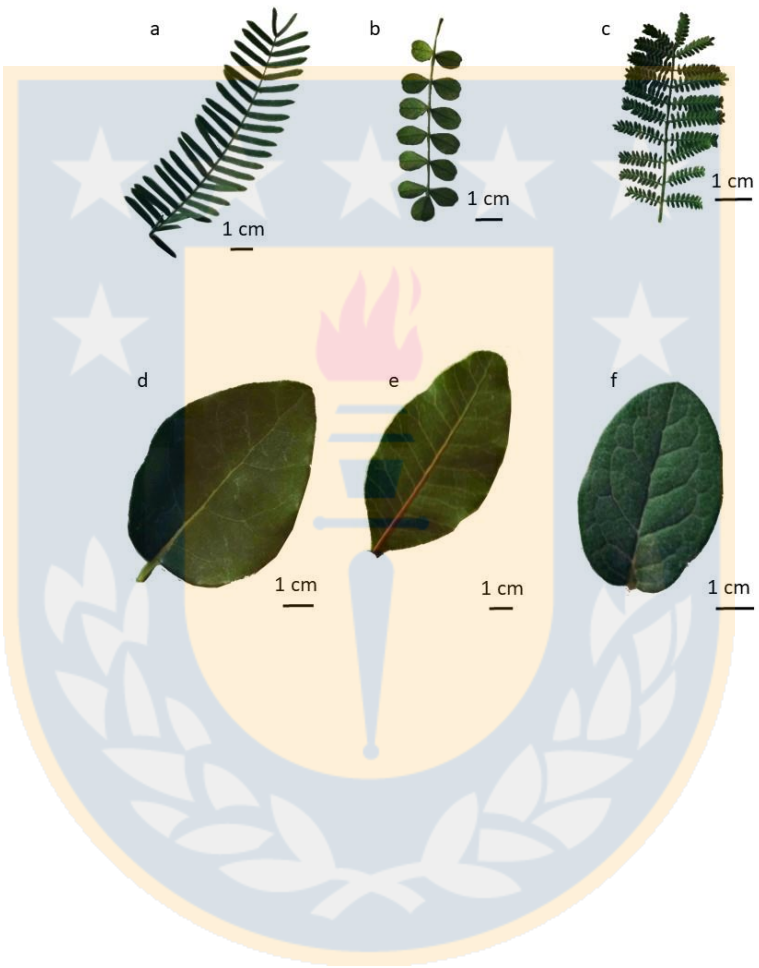


Fig 2.

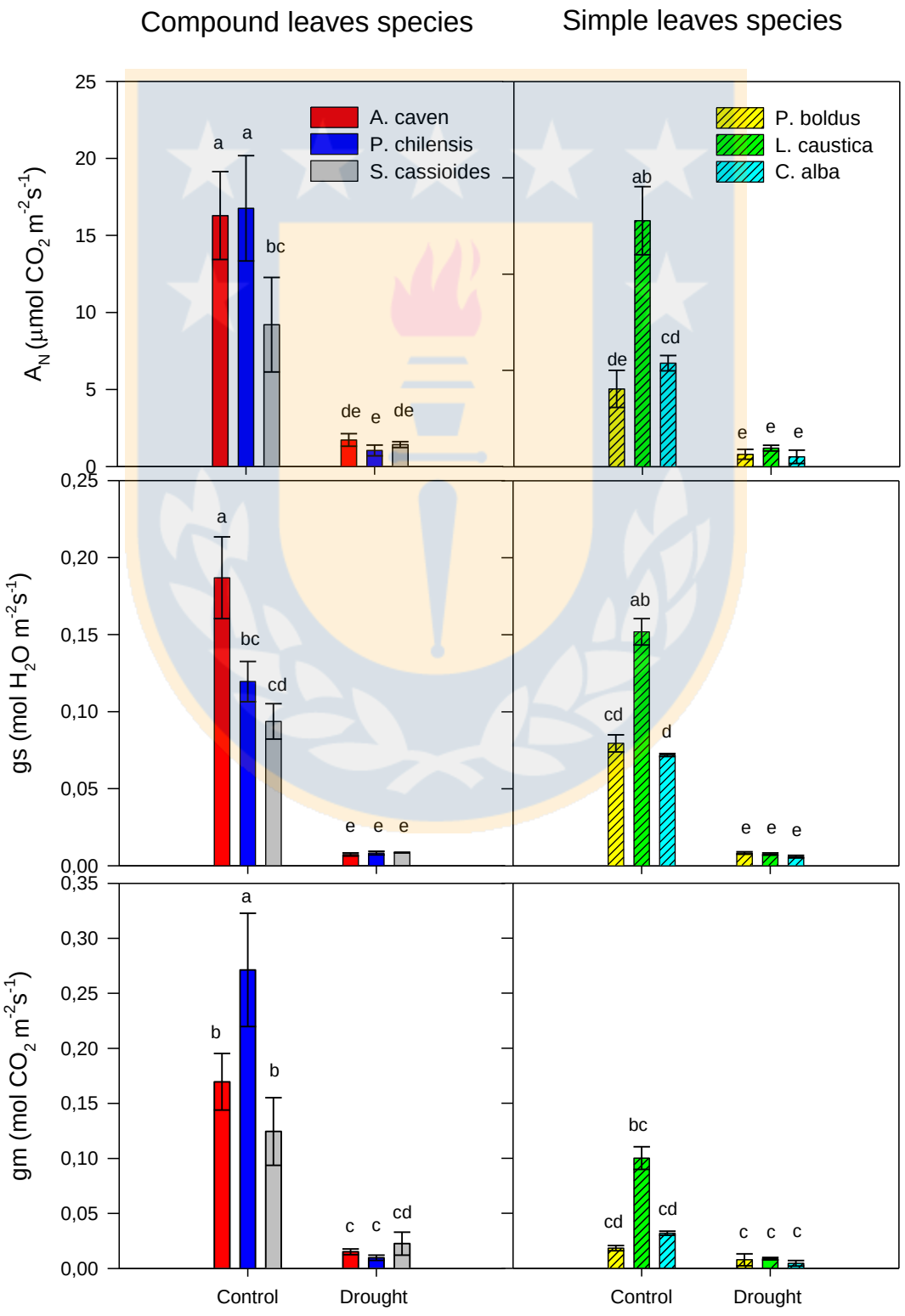
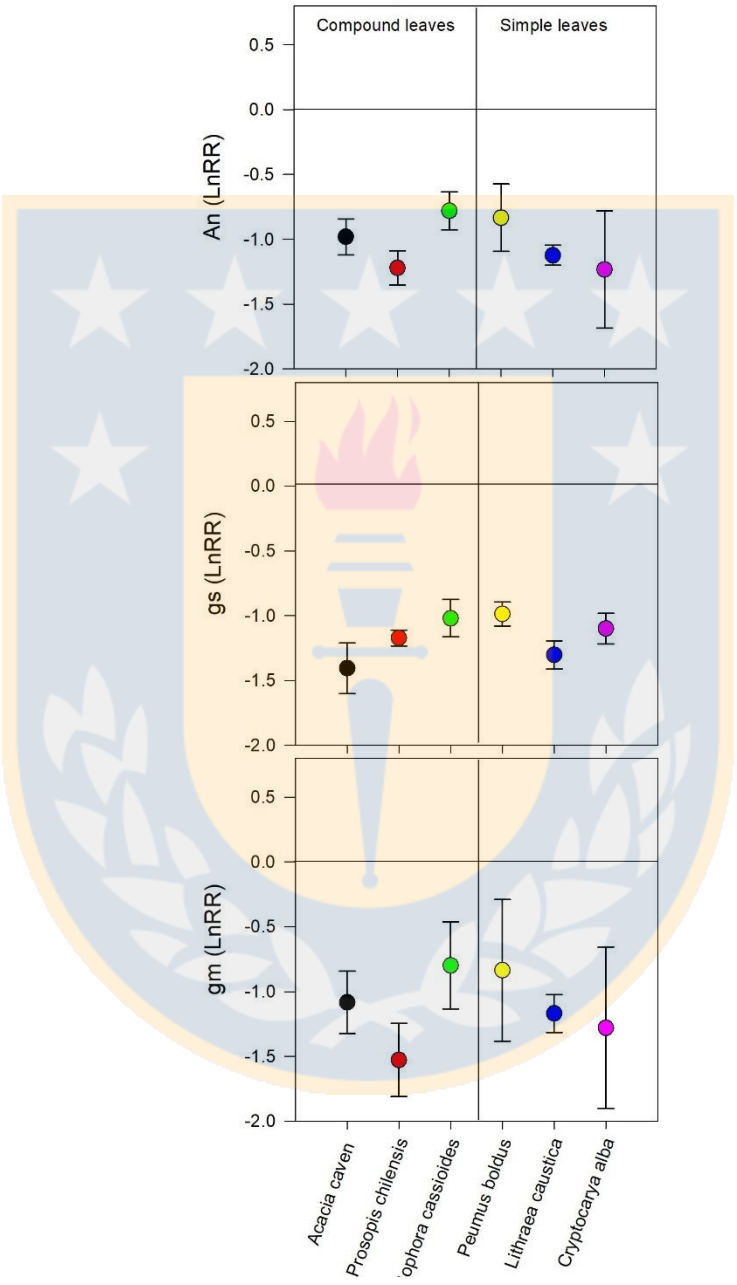

Compound leaves species
Simple leaves species
A. caven
P. chilensis
S. cassioides
P. boldus
L. caustica
C. alba
A_N (µmol CO_2 $m^{-2}s^{-1}$)
gs (mol H_2O $m^{-2}s^{-1}$)
gm (mol CO_2 $m^{-2}s^{-1}$)
Control
Drought
Control
Drought

Fig 3.


Compound leaves
Simple leaves
An (LnRR)
gs (LnRR)
gm (LnRR)
0.5
0.0
-0.5
-1.0
-1.5
-2.0
Acacia caven
Prosopis chilensis
ophora cassioides
Peumus boldus
Lithraea caustica
Cryptocarya alba

CHAPTER 3:

Ecophysiology of the alternative respiration in roots: A role for AOX during nutrient deficiency and symbiosis with soils microorganisms

In vivo Metabolic Regulation of Alternative Oxidase under Nutrient Deficiency. Interaction with Arbuscular Mycorrhizal Fungi and Rhizobium Bacteria

José Ortíz[1], Carolina Sanhueza[1], Antònia Romero-Munar[2], Javier Hidalgo-Castellanos[3], Catalina Castro[1], Luisa Bascuñán-Godoy[1], Teodoro Coba de la Peña[4], Miguel López-Gómez[3], Igor Florez-Sarasa[5], Néstor Fernández Del-Saz[1*]

[1] Laboratorio de Fisiología Vegetal, Departamento de Botánica, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Concepción, Chile

[2] Centro de Estudios Avanzados en Fruticultura (CEAF), Camino Las Parcelas 882, km 105 Ruta Sur, Sector los Choapinos, Rengo, 2940000, Chile

[3] Department of Plant Physiology, Faculty of sciences, University of Granada, Granada 18071, Spain

[4] Centro de Estudios Avanzados en Zonas Áridas (CEAZA), La Serena, Chile

[5] Centre for Research in Agricultural Genomics (CRAG) CSIC-IRTA-UAB-UB, Campus UAB Bellaterra, Barcelona, Spain

* Correspondence: nesfernandez@udec.cl; Fax number: +56-41-2221569

Manuscript published in the International Journal of Molecular Sciences:

Ortiz, J., Sanhueza, C., Romero-Munar, A., Hidagol-Castellanos, J. et al. In vivo Metabolic Regulation of Alternative Oxidase under Nutrient Deficiency. Interaction with Arbuscular Mycorrhizal Fungi and Rhizobium Bacteria. International journal of molecular sciences, 21(12), 4201 (2021).

https://doi.org/10.3390/ijms21124201

Abstract: The interaction of the alternative oxidase (AOX) pathway with nutrient metabolism is important for understanding how respiration modulates ATP synthesis and carbon economy in plants under nutrient deficiency. Although AOX activity reduces the energy yield of respiration, this enzymatic activity is upregulated under stress conditions to maintain the functioning of primary metabolism. The *in vivo* metabolic regulation of AOX activity by phosphorus (P) and nitrogen (N) and during plant symbioses with Arbuscular mycorrhizal fungi (AMF) and *Rhizobium* bacteria is still not fully understood. We highlight several findings and open questions concerning the *in vivo* regulation of AOX activity and its impact on plant metabolism during P deficiency and symbiosis with AMF. We also highlight the need for the identification of which metabolic regulatory factors of AOX activity are related to N availability and nitrogen-fixing legume-rhizobia symbiosis in order to improve our understanding of N assimilation and biological nitrogen fixation.

Keywords: Alternative oxidase, Arbuscular mycorrhizal fungi, Nitrogen and Phosphorus nutrition, *Rhizobium*, Plant primary metabolism

Introduction

Nitrogen (N) and phosphorus (P) are the two essential macronutrients for plants. Short supply of these nutrients may lead to the appearance of stress symptoms affecting photosynthesis, respiration and thus plant growth [1-6]. Under abiotic stress conditions, oxygen consumption in mitochondria may be less constrained than carbon fixation in chloroplasts due to the nature of the non-phosphorylating alternative pathway of respiration. This can help to maintain the functioning of primary metabolism and carbon balance even when photosynthesis is severely restricted [7, 8]. The singularity of this respiratory behavior can be especially notorious in roots, because respiration may increase when plants are nutrient limited, because the need for nutrient uptake requires the majority of carbon assimilated [8-10]. Roots are a sink for carbohydrates due to the energy requirements for ion transport, nutrient assimilation, growth and maintenance [3, 11-13]. Although alternative respiration is linked to carbon respiratory losses detrimental for plant growth, roots subjected to nutrient deficiency can reduce the energy efficiency of respiration through a respiratory bypass via

alternative oxidase (AOX) as part of a coordinated response directed to maximize the efficiency of nutrient acquisition. Mycorrhizas and N_2-fixing legume root nodules are recognized as the two major plant root symbioses for enhancing nutrient uptake and plant nutrient status [14, 15]. The regulation of AOX activity in plants during symbiosis is of vital importance for the determination of both the energy efficiency of respiration and the costs of carbon and energy (ATP) associated with plant symbioses. This knowledge can be of great interest for breeding programs to improve crop production through plant symbiosis with soil microorganisms. Although this line of research is still in its infancy, recent studies have evaluated the metabolic regulation of the *in vivo* AOX activity in leaves and roots of plants in symbiosis with soil microorganisms [13]. Their observations have provided first evidences of how alternative respiration of both plant organs can be affected in the presence of microbial symbionts.

Plant respiration is the combination of redox reactions, mostly involving both mitochondrial tricarboxylic acid (TCA) cycle and electron transport chain (mETC), which produce carbon skeletons, carbon dioxide (CO_2), and ATP coupled to the consumption of oxygen (O_2) and reducing equivalents [NAD(P)H and $FADH_2$] through the activities of two respiratory pathways

which compete for electrons from the ubiquinone (UQ) pool [16]. The cytochrome oxidase pathway (COP) is the primary respiratory pathway, while the alternative oxidase pathway (AOP) decreases energy efficiency of respiration. AOX contributes to dissipate the excess of reducing equivalents from chloroplasts and mitochondria and provides metabolic flexibility when COX is impaired under several abiotic stressors [8]. The *in vivo* electron partitioning between the two pathways and the activities of cytochrome oxidase (COX) and AOX can be measured by using the oxygen-isotope fractionation technique that allows measurements of O_2 consumption combined with its isotopic modification during plant respiration [17]. Over the last couple of decades, the regulation of AOX activity under a large range of abiotic and biotic stresses has been extensively studied in plants as recently reviewed by Del-Saz et al. [8]. In the last few years, several studies have focused on the *in vivo* AOX regulation at the post-translational level, reporting simultaneous changes in both AOX activity and levels of metabolites belonging to different metabolic pathways that produce and/or dissipate reducing equivalents [8]. These observations suggest that AOX activity can confer the metabolic flexibility needed for the continuity of primary metabolism, protein turnover and plant growth under stress [8, 18].

Plant symbioses with soil microorganisms may increase plant growth and affect levels of primary metabolites through the exchange of carbon and nutrients between plant roots and microsymbionts [19, 20]. The enhancement of plant growth is due to a double effect. On one hand, the microsymbiont induces an increase of nutrient content in plant organs, increasing carbon assimilation during photosynthesis [21-23]. On the other hand, increased rates of photosynthesis allow plants to satisfy the microsymbiont's demand for carbon compounds. In other words, by the decrease of photosynthetic limitations, microsymbionts´s lead plants to produce large amounts of carbon compounds required for maintain its own metabolism [19]. This phenomenon, called positive-feedback of photosynthesis [24], could be accompanied by adjustments in the energy efficiency of respiration, considering the tight relationship between photosynthesis and AOX activity [25-28]. Thus, adjustments in the energy efficiency of respiration can be conditioned by the metabolic costs of microsymbiont maintenance or by the metabolic benefits of improved nutrition, which in turn may depend on the microbial symbiont's energy efficiency of respiration.

2. Regulation of AOX activity by P availability

The main P resource for plants in soils is inorganic phosphate (Pi), which mostly can be retained or complexed by cations (*e.g.*, Ca^{2+} and Mg^{2+}) [29]. The other P pool in soil comprises organic P compounds derived from the degradation of plant litter, microbial detritus and organic matter [30]. Pi is involved in cellular bioenergetics and metabolic regulation, and it is also important as a structural component of essential biomolecules such as DNA, RNA, phospholipids, ATP and sugar-phosphates [2, 31]. A decrease in cytosolic Pi may restrict oxidative phosphorylation leading to an increased proton gradient and membrane potential. In turn, this prompts an over-reduction of the components of the electron transport chain, inhibiting oxygen consumption through the COX pathway, which is coupled with ATP synthesis. This creates a decrease in the re-oxidation of NADH produced in the TCA cycle [4, 16]. Furthermore, the accumulation of NADH in the mitochondrial matrix also inhibits the TCA cycle dehydrogenases, decreasing the activity of the TCA cycle and limiting the production of important metabolic intermediates [32].

A plant trait that enhances the capacity to acquire P in the poorest P soils is the production of cluster roots in members of the Proteaceae family, most of which do not form mycorrhizal associations [33-36]. Cluster roots are very

effective at acquiring P that is largely absorbed into soil particles, because of their pronounced capacity to exude carboxylates [9]. Cluster roots of *Lupinus albus* release much more citric and malic acid than lupin roots of plants grown under P sufficiency. Florez-Sarasa et al. [37] observed that growth under P limitation increased the activity of AOX in cluster roots of *L. albus* together with the synthesis of citrate and malate, as in *Hakea prostata* plants under the same conditions [38]. It is thought that the production of vast amounts of citrate in cluster rootlets is inexorably associated with the production of NADH [38, 39, 40]. This led Florez-Sarasa et al. [37] to state that AOX allows the continuity of TCA cycle activity by re-oxidizing the high levels of NADH produced during citrate synthesis when COX activity is restricted due to the P-deficiency-induced adenylate restriction.

The capacity to synthesize acidifying and/or chelating compounds is not restricted to species with morphological structures such as cluster roots and dauciform roots, several species can produces organic acid in P deficiency [9]. In roots without these adaptations, and in the absence of mycorrhiza, the levels of enzymes involved in organic acid biosynthesis, such as PEP carboxylase, often increase in response to P starvation in pea, tomato and

Brassica nigra [41]. This increase in enzyme levels was related to a higher amount of organic acids being produced for root exudation. This capacity is not only present in roots; leaves of plants grown under P limitation may accumulate carboxylates such as citrate, malate, and fumarate [41, 42]. Carboxylates in leaves can be transported via the phloem and directed to roots for exudation [41, 42]. Pioneering studies reported an adaptive response of respiratory metabolism and the mitochondrial electron transport chain to P limitation in NM roots [43-44], including increased AOX capacity [44, 45-46]. This in the line with previous studies reporting imbalances of C/N ratio and ROS levels in AOX-deficient cells under P deficiency [46, 48] although the situation at tissue level has been recognized to be more complex [18]. Recent studies have observed increases of AOX activity in roots of non-cluster roots for species grown under P limitation, such as *Nicotiana tabacum* and *Solanum lycopersicum* in the absence of mycorrhiza [13, 50]. In these species, increments of AOX activity were observed, coinciding with a higher synthesis of carboxylates citrate and malate. In leaves, there were reports of pioneer studies reported increases of AOX activity in *Phaseolus vulgaris* and *Gliricidia sepium* plants grown under P limitation, but a decrease of foliar AOX activity was observed in

Nicotiana tabacum, although this disparity was not related to any respiratory metabolite [51]. A recent study in *Solanum lycopersicum* plants grown at P-sufficient and limiting conditions, and exposed to sudden short-term (24 h) P-sufficient pulse, observed foliar respiratory bypasses via AOX and an increased accumulation of citrate, together with an enhanced expression of high-affinity P transporters *LePT1* and *LePT2* in conditions of limited P concentration [50]. These observations suggest that P concentration in plant organs regulates AOX activity in coordination with biochemical and molecular adjustments, functioning as a mechanism directed to maximize P acquisition [50]. Despite these findings, there is still a lack of understanding about the entire metabolic puzzle leading to the synthesis of citrate and increases in AOX activity. Studies combining metabolite profiling and measurements of electron partitioning between COX and AOX in P deficient plants could certainly shed light on the metabolic role of AOX in plant species adapted to P deficiency, which increase carbon use efficiency by decreasing Pi consumption in leaves as represented in Figure 1, below. The rate of photosynthesis and the export of its products from the chloroplast are determined by the availability of Pi in both chloroplast and cytosol [9, 51]. Low chloroplast Pi availability induces

a decrease in the rate of photosynthesis by decreasing both ATP synthesis and Calvin-Benson cycle activity, which results in a reduced availability of intermediates, *e.g.*, ribulose 1,5-biphosphate (RuBP), and decreased carboxylation activity of Rubisco [52]. Low cytosolic Pi availability decreases the export rate of the products of the Calvin-Benson cycle, leading to increasing amounts of triose-phosphate and starch in the chloroplast [51, 52]. Consequently, sucrose formation and glycolysis can be reduced, which may limit carbon supply into mitochondria, thus decreasing both tricarboxylic acid (TCA) cycle activity and respiration [5, 30], and therefore, plant growth and yield. In order to save Pi, leaves reduce Pi consumption in phosphorylation of sugar metabolites by converting phosphorylated metabolites (glucose-6-P, fructose-6-P, inositol-1-P and glycerol-3-P) to non-P-containing di- and tri-saccharides, as observed in *Hordeum vulgare* and *Eucalyptus globulus* P-deficient plants [53, 54]. In these studies, such changes coincided with reduced levels of organic acid intermediates of the TCA cycle, suggesting a short entry of carbon into mitochondria. Bearing in mind that the conversion of di- and tri-saccharides to organic acids requires Pi, it is unlikely that they can be further respired [53]. Under this circumstance, the use of alternative carbon resources would

allow the continuity of TCA cycle reactions to produce organic acids, *e.g.*, citrate for secretion and to sustain the mitochondrial electron transport chain. In this sense, changes in levels of amino acids glutamine, arginine and asparagine was observed in P-deficient plants [53, 54]. A similar response was recently observed in *Hordeum vulgare* [42]. These amino acids were suggested to provide carbon skeletons to mitochondria when plants reduce the consumption of Pi [42]. It is known that plants can metabolize proteins and lipids as alternative respiratory substrates when carbohydrates are scarce in plant cells [55, 56]. Carbon consumption of these alternative respiratory substrates could be associated with the generation of NADH in the TCA cycle, whose re-oxidation would be favored by AOX activity when COX is restricted under P deficiency (Figure 1).

2.1. Regulation of AOX activity by arbuscular mycorrhizal symbiosis

More than 90% of terrestrial plants are associated with root-colonizing fungi, establishing a durable and close mutualistic symbiosis, called mycorrhiza [58]. The endotrophic arbuscular mycorrhiza is the most common type, occurring in about 80% of plant species [59]. The

establishment of the association between AMF and plants implies the generation of roots with representative structures typical of this symbiosis such as (1) intraradical mycelium, which is a fungal structure that inhabits the plant intracellular space; (2) arbuscule, which is the space where the carbon and nutrient exchange between fungus and plant takes place; (3) the vesicles, storage structures and (4) the extraradical mycelium, which is a structure that extends from the root surface to the soil, beyond the root P-depletion zone and has access to a greater volume of soil compared to roots and root hairs alone [60]. Mycorrhizal associations act as 'scavengers' for Pi uptake in the soil solution. Compared to non-mycorrhizal (NM) plants, the advantages of increased P acquisition and photosynthesis increase with decreasing soil P availability [61]. The increase in photosynthesis in plants with mycorrhiza is related to an increased demand for carbohydrates supplied to the fungus [19, 62]. Some carbohydrates produced in leaves during photosynthesis are transported to roots, where they are broken down in respiration to produce ATP and carbon skeletons required for protein synthesis. Around 20% of the carbon fixed by photosynthesis is destined to form soluble sugars and organic acids in order to supply energy metabolism in fungal cells [63]. These metabolic carbon requirements of AM symbiosis

may affect plant respiration [64-67] as well as the levels of primary metabolites in plant organs [68-70]. In fact, AM symbiosis decreases the carboxylate-releasing strategy as observed in ten *Kennedia* species and five species of legumes [71, 72]. The mechanism for the reduction in rhizosphere carboxylates with AM symbiosis could be a consequence of the reduction of carbon availability in roots due to the demand of AMF for carbon compounds, or it could be a consequence of higher plant P concentration due to improved nutrition. Measurements of *in vivo* AOX activity and the accumulation of carboxylates in roots of *Nicotiana tabacum* and *Arundo donax*, showed that AM symbiosis decreased root respiration via COX and AOX in *N. tabacum*, decreased respiration via COX in *A. donax*, and decreased synthesis and exudation of citrate and malate in *A. donax* and *N. tabacum*, respectively [13, 73]. On top of this, both species showed symptoms of ameliorated physiological status and increased biomass accumulation in shoots. These results probably denote that the synthesis of rhizosphere exudates in non-AM plants imposes an important carbon cost detrimental for plant growth as compared with AM plants, which do not invest as much carbon in the synthesis of carboxylates, thus respiring less and allowing carbon to accumulate. Bearing all this in mind, it

would be logical to assume that the mechanism for the reduction in rhizosphere carboxylates is related to improved plant P status rather than less carbon availability. In fact, previous studies described that increasing P availability tends to reduce the amount of carboxylate in rhizosphere soil [74, 75], and the carboxylate-releasing strategy requires more carbon when P availability is in the range at which AM plants are functional [76]. Nevertheless, it is important to highlight that the effect of AM symbiosis on plant growth is variable because it depends on the host plant and the fungal species [77]. In this sense, *in vivo* AOX measurements have been made only in positive symbiotic interactions (beneficial for plant growth), and there is still a lack of studies that test the role of alternative respiration in defective symbiotic interactions (detrimental for plant growth). Besides, it has been reported that the effect of AMF on plant growth depends on the stage of colonization [60]. In this sense, a recent study in *N. tabacum* showed that symbiosis with *Rhizophagus irregularis* differently affects both respiration and ATP synthesis in leaves at different growth stages, when plants grow in P deficient soils. AM symbiosis represented an ATP cost (via decreased COX activity) for tobacco leaves that was detrimental for shoot growth at early stages, presumably because fungal structures were still under

construction. At the mature stage, this cost turned into an ATP benefit (via incremented COX activity), which allowed for faster growth presumably because symbiosis was functional, bearing in mind the observed increase in both foliar P status and shoot growth [78].

AM symbiosis can improve nutrient acquisition because AM provide an additional means of nutrient uptake, the mycorrhizal nutrient uptake pathway [79-80], which can bypass the pathway of direct nutrient uptake in a P availability-dependent manner [81-86]. Studies relating the functioning of the mycorrhizal nutrient uptake pathway to the *in vivo* electron partitioning to AOX are required, keeping in mind that AOX is also present in various fungi including *Rhizophagus intraradices* [87], and that P acquisition by AMF requires energy, which is obtained during oxidative phosphorylation in fungal mitochondria. Precisely, ATP is needed for P uptake by the external hyphae, P transport and export to the internal hyphae and P uptake by the plant at the arbuscule (Figure 2). It would be logical to assume that positive AMF-plant interactions display high rates of COX activity in extra radical mycelium to ensure ATP availability and to energize the mycorrhiza pathway uptake. Measurements of the *in vivo* COX and AOX activities together with techniques like multicompartment plant

growth systems [88] and ^{13}C and ^{33}P isotopic labeling [89] may help to identify AMF-plant associations with efficient energy rates of extra radical mycelium respiration when the mycorrhizal nutrient uptake pathway is active. This could contribute to expand our view on the interplay between nutrient uptake pathways in plants with mycorrhiza.

3. Regulation of AOX activity by N availability

Nitrogen is required by plants in greater quantities than any other mineral element. Almost all the N available for plants is present in the reduced form of nitrate (NO_3^-), ammonium (NH_4^+), organic compounds and molecular nitrogen (N_2) in the air [90, 91]. The major source of N in soils resides in the atmosphere, through both biological N_2 fixation and the deposition of NO_3^- and NH_4^+ in precipitation. In soils, NH_4^+ and NO_3^- move towards roots through transpiration-driven mass flow because they are water soluble [92]. Both NH_4^+ and NO_3^- enter the plant cells via specific transporters [93, 94]. In order to be incorporated, NO_3^- is reduced to NH_4^+ by nitrate reductase (NR) and nitrite reductase [995]. Then, NH_4^+ is further converted into glutamine and glutamate, in a reaction catalyzed by glutamine synthetase/glutamine 2-oxoglutarate amino-transferase (GS/GOGAT) cycle

[94]. At the cellular level, nitrogen assimilation is finely regulated according to its supply and demand. Nitrogen controls the regulation of nitrate transporters, activities of nitrate and nitrite reductase, the functioning of primary metabolic pathways associated with the production of reducing equivalents, and the production of organic acids required for N assimilation into amino acids [2].

There is a correlation between leaf N content and rates of respiration [96-99]. Short supply of N leads to decreasing rates of both photosynthesis and respiration by reducing protein turnover and increasing breakdown of nucleic acids and enzymes [9, 100]. Under these circumstances, a minimum supply of TCA metabolites (*e.g.*, 2-oxoglutarate, isocitrate, and citrate) may maintain optimum N assimilation and amino acid biosynthesis [101-103]. Indeed, TCA cycle enzymes fumarase, NAD-dependent isocitrate dehydrogenase, and NAD-dependent malic enzyme are up-regulated under low N [95, 104], and protein degradation acts as an alternative respiratory substrate [55]. In this situation, AOX activity could play a role in maintaining the functioning of primary metabolism by allowing adjustments in energy efficiency of respiration. However, there are no studies that test the regulation of AOX activity *in vivo* under N limitation.

Such a hypothetical role should be evaluated in plant species with constitutively high levels of the AOX protein, or AOX activity. Plants possess a higher threshold for AOX capacity [8, 26, 29, 105, 106], which is variable depending on the plant species and environmental conditions [29, 105]. It is thought that this high threshold eliminates the need for de novo AOX protein synthesis, granting alternative respiration the ability to respond to sudden changes in levels of reducing equivalents [8, 26, 107]. In this sense, a short supply of N in plant species with high levels of AOX protein such as legumes, could induce a decrease of both AOX protein and capacity to some extent without compromising the accuracy of AOX activity measurements, allowing us to evaluate the *in vivo* role of AOX under stress. Preliminary results in *Lotus japonicus* have shown that total respiration decreases (via COX and AOX) in leaves and roots when plants grow under short supply of KNO_3, in comparison with plants grown at sufficient KNO_3 supply. Interestingly, a short supply of KNO_3 induces an increase of the energy efficiency of respiration (via decreased contribution of AOX activity to total respiration) only in leaves (Ortíz et al. unpublished), which suggests the existence of a differential regulation

between organs directed to maximize ATP synthesis in leaves, most likely for maintenance purposes.

On the other hand, the source of N could be another regulatory factor of AOX activity in leaves. Classic studies have observed that the expression and activity of several glycolytic and TCA cycle enzymes were differentially affected following NH_4^+ or NO_3^- uptake [101-103, 108], which could be accompanied with respiratory adjustments. It is known that nitrate uptake and its conversion to ammonium require large amounts of ATP and reducing equivalents [109-110]. However, respiration increases when ammonium is present as the main N source [112-114]. This has been explained as a consequence for the lack of the important reductant sink exerted by nitrate reductase, thus leading to an increase of reducing equivalents in cytosol, that are dissipated by mitochondrial electron transport chain. Under this scenario, AOX could play a significant role during this dissipation of reductants, considering its roles in maintaining the cell redox balance [8, 115, 116]. In this way, the accumulation of NH_4^+ and its associated toxicity is prevented by the action of the GS/GOGAT cycle activity [117] in parallel to the mitochondrial dissipation of reductants. In fact, previous studies have shown an increase in AOX capacity and

enhanced of several AOX isoforms in plants grown under NH_4^+ supply [114, 115, 118-120]. Moreover, negative correlations between AOX capacity and nitrate concentrations were observed [114], although the electron flow through AOX under aerobic conditions can be important for the reduction of NO generation associated to nitrate reduction [121]. Interestingly, the growth of AOX-overexpressing plants is less restricted as compared to wild type (WT) *Arabidopsis* plants grown under NH_4^+ nutrition, although the metabolic causes of this phenotype remain uncertain [118]. The hypothetical role of AOX in conferring metabolic flexibility during NH_4^+ nutrition still needs to be tested by *in vivo* activity measurements (Figure 3).}

3.1. Regulation of AOX activity in the Rhizobium-legume symbiosis

Legumes are good candidates to study the regulation of AOX activity by N availability because these plants have been suggested to display faster rates of foliar AOX activity under stress as they constitutively express high levels of AOX protein under normal growth conditions [122-124]. In fact, measurements of *in vivo* AOX activity have been performed in leaves of six legumes species: common bean (*Phaseolus vulgaris*), garden pea (*Pisum*

sativum), barrel medic (*Medicago truncatula*), soybean (*Glycine max*), mung bean (*Vigna radiata*) and faba bean (*Vicia faba*). These experiments have been important to evaluate the regulation of the AOX activity under P limitation, high light, salinity, pathogen infection and variable temperatures [25-27, 29, 106, 125, 126]. Furthermore, legumes are suitable for the study of the regulation of AOX activity by N availability in roots because of their ability to establish symbiosis with a group of soil bacteria collectively designated as rhizobia. Rhizobia is a group of diazotrophs, most of them belonging to the α-proteobacteria, that include the genera *Rhizobium*, *Mesorhizobium*, *Ensifer* (formerly *Sinorhizobium*), *Bradyrhizobium* and *Azorhizobium*, among others) [127]. The rhizobia-legume symbiosis provides a suitable biological system to evaluate variations in both nutrient status and metabolite levels in plant organs due to the exchange of carbon and nutrients between host plants and bacteroids (that is, the differentiated endosymbiotic form of the bacteria able to fix nitrogen).

Legumes can fix atmospheric nitrogen (N_2) through the nitrogenase activity that reduces N_2 to NH_3^- and is located in the root nodule bacteria [128-130]. Biological N_2 fixation in leguminous plants requires the development of a specific symbiotic relationship between rhizobia soil bacteria and the plant

root in conditions of limited nitrogen availability in soil [131]. In bacteroids, the nitrogenase reaction requires a great deal of energy, consuming at least 16 ATP and 4 pairs of electrons for every molecule of N_2 reduced to ammonia [83]. This energy is obtained from plant carbon compounds in the form of TCA cycle intermediates (fumarate, succinate or malate) via a dicarboxylic-acid transport system [132-134]. Similar to mycorrhizal symbiosis, the nodule imposes a carbon cost in roots that cannot exceed their nutritional benefit. However, it is unknown whether respiratory adjustments in nodulated roots contribute to the regulation of the carbon economy in legumes. In this sense, preliminary results obtained in roots of *L. japonicus* nodulated by *Mesorhizobium loti*, revealed higher rates of total respiration via COX (and diminished AOX activity) when compared to non-nodulated roots of plants grown at low KNO_3 (Ortíz et al. unpublished). These results are in agreement with the previous studies describing high rates of respiration in nodulated roots [135-137]. On the other hand, we observed similar rates of respiration in nodulated roots when compared to non-nodulated roots of plants grown at sufficient KNO_3 (Ortíz et al. unpublished). Based on these results, it seems that the effect of rhizobia on root respiration could be related to an improved N status rather

than to carbon costs of nodule maintenance and nitrogenase activity. Biological nitrogen fixation leads to the production of ammonium in bacteroids, which is transferred to the host plant through the symbiosome membrane and initially assimilated to glutamine, and then to either ureides or amides to ameliorate N status in leaves [130, 134, 138-140]. Similar to roots, rhizobia inoculation did not significantly change the activities of the two terminal oxidases in leaves of *L. japonicus* plants grown under KNO_3 sufficiency, thus suggesting a similar N status between these plants. Furthermore, leaves of non-nodulated plants grown at low KNO_3 displayed the lowest rates of ATP synthesis via decreased COX and AOX (Ortíz et al. unpublished). Based on this preliminary results, it seems that the activities of both COX and AOX in plant organs depend on N availability. Another regulatory factor of AOX activity in leaves could be determined by the type of nodule. The determinate legume root nodules, characteristic of some tropical legumes as soybean and common bean, primarily exports ureides (allantoin and allantoate) as fixed-N compounds to be metabolized in leaves. These compounds are converted to glycine, which in turn will be converted to serine as part of the photorespiration pathway that is associated to the mitochondrial release of ammonia in and its re-assimilation into

nitrogenated compounds [133, 141]. On the other hand, indeterminate nodules, characteristic of certain temperate legumes as barrel medic and pea, assimilate amides in the form of asparagine (Asn) and glutamine (Gln) [141, 142], which are exported to the aerial part to be directly incorporated into leaf metabolism. This bypasses the production of reducing equivalents related to the decarboxylation of glycine to serine in leaf mitochondria [12] that is observed in determinate nodules, which could be associated with changes in AOX activity (Figure 4).

Although nitrogenase enzyme requires O_2 for ATP synthesis, this enzyme is extremely O_2-labile, being inhibited above a certain O_2 concentration. This was called “the oxygen paradox” [143]. In order to maintain respiration and ATP synthesis in the infected cells, the nodule displays several mechanisms for delivering a regulated flux of O_2, whilst maintaining free O_2 concentration at low levels in infected cells. One of these mechanisms is the occurrence of an O_2 diffusion barrier to the nodule central zone, where nitrogen-fixation takes place [144]. Besides, it is thought that rates of bacteroid respiration are high enough to ensure a quick consumption of O_2, as soon as the gas diffuses into the central zone to avoid its accumulation [145]. This behavior is achieved by the presence of terminal oxidases with

different Km for oxygen that prevents nitrogenase inhibition and allows rapids changes in oxygen concentration and modulates the consumption [146, 147, 148]. In soybean nodules, terminal oxidase presents a high affinity for oxygen, showing higher activities at 0,1 µmol and little activity between 1-3 µmol O2 dissolved respectively [146, 148]. However, fast rates of nitrogenase activity would increase the demand for O_2 concentration in the infected cells. To increase O_2 diffusion to bacteroids, the infected cells contain leghemoglobin that acts as an O_2 carrier. This is an iron protein with high affinity for O_2, which regulates O_2 diffusion from the cytosol to the bacteroid in adequate concentrations to fuel its respiration, preventing inhibition of nitrogenase [149, 150]. Thus, the ability of the nodule to respond to sudden increases of O_2 in infected cells is very important because of their repercussions on biological nitrogen fixation. AOX has been found in nodules infected by several species of rhizobia such as *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* [151, 152], although with lower abundance and capacity than any other tissue of the same plant as was described in soybean root nodules [123]. Thus, it is unlikely that AOX activity may play a significant role in nodule respiration as it does in plant cells [153, 154]. However, the observed upregulation of

AOX mRNA levels in senescent bean nodules was proposed to contribute to the redox balance in mitochondria [151]. Until this date, there are no results of *in vivo* activities of COX and AOX in legumes nodules. Accurate estimates of the activities of COX and AOX in plant nodules would require *in vivo* measurements in bacteroid and mitochondria by using on-line liquid-phase systems [152, 154, 155]. They are worthy for the corroboration of the high energy efficiency of nodule respiration, which can be assumed to be tightly coordinated with the nitrogenase activity, considering its dependence on O_2 consumption for ATP synthesis [156, 157]. In fact, abiotic stressors result in the inhibition of carbon metabolism in host legumes as well as in the increase of nodule resistance to O_2 diffusion in order to constrain respiration and nitrogenase activity and save carbon that will eventually become scarce [158]. It is worth mentioning the existence of different metabolic responses to Pi deficiency observed among legumes when biological nitrogen fixation is suppressed under Pi deficiency [133]. One of these responses is the enhancement of Pi uptake and recycling in nodules [139, 159, 160] that may lead to the use of alternative respiratory substrates of carbon compounds such as amino acids, as observed in the metabolic profiles performed during symbiotic nitrogen fixation in

phosphorus-stressed common bean [161]. Although methodological improvements are still needed for the study of nodule respiration, it seems that measurements of both apparent nitrogenase activity and total nitrogenase activity, in combination with O_2 isotope fractionation and metabolite profiling in plant organs, is a good starting point to understand how biological nitrogen fixation and plant respiratory metabolism are connected in legumes.

Concluding remarks

The study of the metabolic regulation of AOX activity in plant species that establish symbioses with soil microorganisms under nutrient deficiency is important for further understanding of plant growth responses to abiotic stress and global climate change. Previous and preliminary studies analyzing the *in vivo* response of AOX in roots and leaves of plants in symbioses with AMF and rhizobia under conditions of P and N limitation suggest that the absence of the symbiont imposes nutritional restrictions for ATP synthesis. Hence, plant symbioses with soil microorganisms confer energetic benefits due to improved plant nutrition. Moreover, the supply of N and P from the microbial symbiont to the plant depends on the ATP

availability in the microbial symbiont, which is regulated by its demand for carbon compounds. This regulation could involve high energy efficient rates of respiration for the benefit of ATP synthesis in hyphae and legume roots nodules under sudden changes in the demand for carbon compounds. Thus, regulation of respiration in plants by N and P, and its interaction with AMF and nitrogen-fixing bacteria, merits further attention in order to expand the view of the molecular mechanisms related to microsymbiont respiration. The identification of new regulatory factors (*e.g.*, of AOX activity) can be taken into account in breeding programs for improvement of crop production.

Author Contributions: N.F.D-S. conceived and designed the idea of this review. J.O., C.S., A.R-M. and N.F.D-S. wrote the first draft of the paper with subsequent inputs from all co-authors. C.C., and J.O. developed the figures and bibliographic compilation. J.H-C., M.L-G, L.B.-G, T.C.P and I.F-S contributed to writing the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the project FONDECYT-1191118 from the Fondo Nacional de Desarrollo Científico y Tecnológico (Chile)

and by the National Agency for Research and Development (ANID) / Scholarship Program / DOCTORADO BECAS CHILE/2017 – 21180329. IFS has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement no. 753301.

Acknowledgments: The authors would like to Prof. Chris Baldwin for his help with English corrections, and special thanks to Prof. Miquel Ribas-Carbo for his supervision during the doctoral thesis of N.F.D-S.

Conflict of Interest: The authors declare no conflict of interest.

Abbreviations

AMF	Arbuscular mycorrhizal fungi
AOP	Alternative oxidase pathway
AOX	Alternative oxidase
ATP	Adenosine tri-phosphate
COP	Cytochrome oxidase pathway
GOGAT	Glutamine oxoglutarate aminotransferase

GS/GOGAT	Glutamine Synthetase-Glutamate Synthase
NM	Non-mycorrhizal
NR	Nitrate reductase
mETC	Mitochondrial electron transport chain
LePT	*Lycopersicon esculentum* phosphate transporter
RuBP	Ribulose 1,5-biphosphate
TCA	Tricarboxylic acid
UQ pool	Ubiquinine pool

References

1. Lynch, J.P.; Brown, K.M. Topsoil foraging–an architectural adaptation of plants to low phosphorus availability. *Plant Soil*. **2001**, *237*, 225-237.
2. Vidal, E.; Rodrigo, A.; Gutiérrez. A. Systems view of nitrogen nutrient and metabolite responses in Arabidopsis. *Curr. Opin. Plant. Biol*. **2008**, *11*, 521-529.
3. Foyer, C.H.; Noctor, G.; Hodges, M. Respiration and nitrogen assimilation: targeting mitochondria-associated metabolism as a means to enhance nitrogen use efficiency. *J. Exp. Bot.* **2011**, *62*, 1467-1482.
4. Plaxton, W.C.; Tran, H.T. Metabolic adaptations of phosphate-starved plants. *Plant Physiol.* **2011**, *156*, 1006-1015.
5. Nunes-Nesi, A.; Araújo, W.L.; Obata, T.; Fernie, A.R. Regulation of the mitochondrial tricarboxylic acid cycle. Curr. Opin. Plant Biol. 2013, 16, 335-343.
6. Sulieman, S.; Tran, L.S.P. Phosphorus homeostasis in legume nodules as an adaptive strategy to phosphorus deficiency. Plant Sci. 2015, 239, 36-43.
7. Flexas, J.; Bota, J.; Galmes, J.; Medrano, H.; Ribas-Carbó, M. Keeping a positive carbon balance under adverse conditions: responses of photosynthesis and respiration to water stress. Physiol. Plant. 2006, 127, 343-352.
8. Del-Saz, N.F.; Ribas-Carbo, M.; McDonald, A.E.; Lambers, H.; Fernie, A.R.; Florez-Sarasa, I. An in vivo perspective of the role (s) of the alternative oxidase pathway. Trends Plant Sci. 2018a, 23, 206-219.
9. Lambers, H.; Chapin F.S III, Pons T.L. Plant Physiological Ecology. Springer-Verlag, New York, 2008, pp. 10-95.
10. Nielsen, K.L.; Eshel, A.; Lynch, J.P. The effect of phosphorus availability on the carbon economy of contrasting common bean (Phaseolus vulgaris L.) genotypes. J. Exp. Bot. 2001, 52, 329-339.
11. Atkin, O.K.; Edwards, E.J.; Loveys, B. R. Response of root respiration to changes in temperature and its relevance to global warming. New Phytol. 2000, 147, 141-154.
12. Nunes-Nesi, A.; Fernie, A. R.; Stitt, M. Metabolic and signaling aspects underpinning the regulation of plant carbon nitrogen interactions. Mol Plant. 2010, 3, 973-996.
13. Del-Saz, N.F.; Romero-Munar, A.; Cawthray, G.R.; Aroca, R.; Baraza, E.; Flexas, J.; Lambers, H.; Ribas-Carbó, M. Arbuscular mycorrhizal fungus colonization in Nicotiana tabacum decreases the rate of both carboxylate exudation and root respiration and increases plant growth under phosphorus limitation. Plant soil. 2017a, 416, 97-106.
14. Lambers, H.; Mougel, C.; Jaillard, B.; Hinsinger, P. Plant-microbe-soil interactions in the rhizosphere: an evolutionary perspective. Plant soil. 2009, 321, 83-115.

15. Lamont, B.B.; Pérez-Fernández, M. Total growth and root-cluster production by legumes and proteas depends on rhizobacterial strain, host species and nitrogen level. Ann. Bot. 2016, 118, 725-732.
16. Lambers, H.; Robinson, S.A.; Ribas-Carbo M. Regulation of respiration in vivo. In Plant Respiration: From Cell to Ecosystem, Vol 18. Advances in Photosynthesis and Respiration Series (eds H. Lambers & M. Ribas-Carbo). Springer, Dordrecht, The Netherlands. 2005, pp. 1-15.
17. Del-Saz, N.F.; Ribas-Carbo, M.; Martorell, G.; Fernie, A.R.; Florez-Sarasa, I. Measurements of electron partitioning between cytochrome and alternative oxidase pathways in plant tissues. In Plant Respiration and Internal Oxygen. Methods in Molecular Biology;Gupta, J.K., Ed.; Humana Press, New York, NY. 2017b; 1670, pp. 203-17.
18. Vanlerberghe, G.C. Alternative oxidase: A mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. Int. J Mol. Sci. 2013, 14, 6805-6847.
19. Kaschuk, G.; Kuyper, T.W.; Leffelaar, P.A.; Hungria, M.; Giller, K.E. Are the rates of photosynthesis stimulated by the carbon sink strength of rhizobial and arbuscular mycorrhizal symbioses? Soil Biol Bioch. 2009, 41, 1233-1244.
20. Kaschuk, G.; Leffelaar, P.A.; Giller, K.E.; Alberton, O.; Hungria, M.; Kuyper, T. W. Responses of legumes to rhizobia and arbuscular mycorrhizal fungi: a meta-analysis of potential photosynthate limitation of symbioses. Soil Biol Bioch. 2010, 42, 125-127.
21. Evans, J.R. Photosynthesis and nitrogen relationships in leaves of C3 plants. Oecol. 1989, 78, 9-19.
22. Wright, I.J., Reich, P.B., Westoby, M., Ackerly, D.D., Baruch, Z., Bongers, F.; Cavender-Bares, J.; Chapin, T.; Cornelissen, J.H.C.; Diemer, M.; Garnier, E.; Groom, P. K.; Gulias, J.; Hikosaka, K.; Lamont, B.B.; Lee, T.; Lee, W.; Lusk, C.; Midgey, J.J.; Navas, M.; Niinemets, C.; Thomas, S.C.; Tjoelker, M.G.; Veneklass, E. J; Villar, R. The worldwide leaf economics spectrum. Nature. 2004, 428, 821-827.
23. Kaschuk, G.; Yin, X.; Hungria, M.; Leffelaar, P.A.; Giller, K.E.; Kuyper, T.W. Photosynthetic adaptation of soybean due to varying effectiveness of N2 fixation by two distinct Bradyrhizobium japonicum strains. Environ. Exp. Bot. 2012, 76, 1-6.
24. Herold, A. Regulation of photosynthesis by sink activity–the missing link. New Phytol. 1980, 86, 131-144.
25. Del-Saz, N.F.; Florez-Sarasa, I.; Clemente-Moreno, M.J.; Mhadhbi, H.; Flexas, J.; Fernie, A.R.; Ribas-Carbo, M. Salinity tolerance is related to cyanide-resistant alternative respiration in Medicago truncatula under sudden severe stress. Plant Cell Environ. 2016, 39, 2361-2369.

26. Florez-Sarasa, I.; Ribas-Carbo, M.; Del-Saz, N.F.; Schwahn, K.; Nikoloski, Z.; Fernie, A. R.; Flexas, J. Unravelling the in vivo regulation and metabolic role of the alternative oxidase pathway in C3 species under photoinhibitory conditions. New Phytol. 2016, 212, 66-79.
27. Batnini, M.; Fernández Del-Saz, N.; Fullana-Pericàs, M.; Palma, F.; Haddoudi, I.; Mrabet, M.; Ribas-Carbo, M.; Mhadhbi, H. The alternative oxidase pathway is involved in optimizing photosynthesis in Medicago truncatula infected by Fusarium oxysporum and Rhizoctonia solani. Physiol. Plant. 2020, doi:10.1111/ppl.13080
28. Vanlerberghe, G.C.; Dahal, K.; Alber, N.A.; Chadee, A. Photosynthesis, respiration and growth: a carbon and energy balancing act for alternative oxidase. Mitochondrion. 2020, 197-211.
29. Gonzàlez-Meler, M.A.; Giles, L.; Thomas, R.B.; Siedow, J. N. Metabolic regulation of leaf respiration and alternative pathway activity in response to phosphate supply. Plant Cell Environ. 2001, 24, 205-215.
30. Shen, J.; Yuan, L.; Zhang, J.; Li, H.; Bai, Z.; Chen, X.; Zhan, W.; Zhang, F. Phosphorus dynamics: from soil to plant. Plant Physiol. 2011, 156, 997-1005.
31. Hawkesford, M.; Horst, W.; Kichey, T.; Lambers, H.; Schjoerring, J.; Møller, I.S.; White, P. Functions of macronutrients. In Marschner's mineral nutrition of higher plants, 3rd ed; Marchner, P.; Ed. Academic Press. 2012, pp. 135-189.
32. Araújo, W.L.; Nunes-Nesi, A.; Nikoloski, Z.; Sweetlove, L.J.; Fernie, A, R. Metabolic control and regulation of the tricarboxylic acid cycle in photosynthetic and heterotrophic plant tissues. Plant Cell Environ. 2012, 35, 1-21.
33. Shane, M.W.; Lambers, H. Cluster roots: a curiosity in context. In Root physiology: from gene to function, 1st ed.; Lambers, H.; Colmer, T. D.; Eds.; Springer, Dordrecht. 2005, pp. 101-125.
34. Lambers, H.; Shane, M.W.; Cramer, M.D.; Pearse, S. J.; Veneklaas, E. J. Root structure and functioning for efficient acquisition of phosphorus: matching morphological and physiological traits. Ann. Bot. 2006, 98, 693-713.
35. Lambers, H.; Brundrett, M.; Raven J.; Hopper, S. Plant mineral nutrition in ancient landscapes: high plant species diversity on infertile soils is linked to functional diversity for nutritional strategies. Plant Soil 2010, 334: 11-31.
36. Zemunik, G.; Lambers, H.; Turner, B.L.; Laliberté, E.; Oliveira, R. S. High abundance of non-mycorrhizal plant species in severely phosphorus-impoverished Brazilian campos rupestres. Plant Soil. 2018, 424, 255-271.
37. Florez-Sarasa, I; Lambers, H.; Wang, X.; Finnegan, P.M.; Ribas-Carbo, M. The alternative respiratory pathway mediates carboxylate synthesis in white lupin cluster roots under phosphorus deprivation. Plant Cell Environ. 2014, 37, 922-928.

38. Shane, M. W.; Cramer, M. D.; Funayama-Noguchi, S.; Cawthray, G. R.; Millar, A. H.; Day, D. A.; Lambers, H. Developmental physiology of cluster-root carboxylate synthesis and exudation in harsh hakea. Expression of phosphoenolpyruvate carboxylase and the alternative oxidase. Plant Physiol. 2004, 135(1), 549-560.
39. Lambers, H.; Plaxton, W.C. Phosphorus: Back to the Roots, in Annual Plant Reviews Phosphorus Metabolism in Plants. Plaxton, W. C; Lambers, H. John Wiley & Sons, Inc., Hoboken, NJ, USA. 2015, volume 48, pp. 1-22.
40. Vanlerberghe, G. C.; Mclntosh, L. Signals regulating the expression of the nuclear gene encoding alternative oxidase of plant mitochondria. Plant Physiol. 1996, 111(2), 589-595.
41. López-Bucio, J.; Nieto-Jacobo, M.F.; Ramırez-Rodrıguez, V.; Herrera-Estrella, L. Organic acid metabolism in plants: from adaptive physiology to transgenic varieties for cultivation in extreme soils. Plant Sci. 2000, 160, 1-13.
42. Alexova, R.; Nelson, C.J.; Millar, A.H. Temporal development of the barley leaf metabolic response to Pi limitation. Plant Cell Environ. 2017, 40, 645-657.
43. Theodorou, M.E.; Elrifi, I.R.; Turpin, D.H.; Plaxton, W. C. Effects of phosphorus limitation on respiratory metabolism in the green alga Selenastrum minutum. Plant Physiol. 1991, 95, 1089-1095.
44. Parsons, H.L.; Yip, J.Y.; Vanlerberghe, G.C. Increased respiratory restriction during phosphate-limited growth in transgenic tobacco cells lacking alternative oxidase. Plant Physiol. 1999, 121, 1309-1320.
45. Rychter, A.M.; Mikulska, M. The relationship between phosphate status and cyanide-resistant respiration in bean roots. Physiol. Plant, 1991, 79, 663-667.
46. Funayama-Noguchi, S.; Noguchi, K.O.; Terashima, I. Comparison of the response to phosphorus deficiency in two lupin species, Lupinus albus and L. angustifolius, with contrasting root morphology. Plant Cell Environ. 2015, 38, 399-410.
47. Hoefnagel, M.H.; Van Iren, F.; Libbenga, K.R.; Van der Plas, L.H. Possible role of adenylates in the engagement of the cyanideresistant pathway in nutrient-starved Catharanthus roseus Cells. Physiol. Plant, 1994, 90, 269-278.
48. Juszczuk, I.; Malusà, E.; Rychter, A.M. Oxidative stress during phosphate deficiency in roots of bean plants (Phaseolus vulgaris L.). J. Plant Physiol. 2001, 158,1299-1305.
49. Sieger, S.M.; Kristensen, B.K.; Robson, C.A.; Amirsadeghi, S.; Eng, E.W.; Abdel-Mesih, A.; Møller, I.M.; Vanlerberghe, G.C. The role of alternative oxidase in modulating carbon use efficiency and growth during macronutrient stress in tobacco cells. J. Exp. Bot. 2005, 56, 1499-1515.
50. Del-Saz, N.F.; Romero-Munar, A.; Cawthray, G.R.; Palma, F.; Aroca, R.; Baraza, E.; Florez-Sarasa, I.; Lambers, H.; Ribas-Carbó, M. Phosphorus concentration coordinates a respiratory bypass, synthesis and exudation of citrate, and the expression of high-affinity

phosphorus transporters in Solanum lycopersicum. Plant Cell Environ. 2018b, 41, 865-875.
51. Campbell, C.D.; Sage, R.F. Interactions between the effects of atmospheric CO2 content and P nutrition on photosynthesis in white lupin (Lupinus albus L.). Plant Cell Environ. 2006, 29, 844-853.
52. Hermans, C.; Hammond, J. P.; White, P.J.; Verbruggen, N. How do plants respond to nutrient shortage by biomass allocation? Trends Plant Sci. 2006, 11, 610-617.
53. Huang, C.Y.; Roessner, U.; Eickmeier, I.; Genc, Y.; Callahan, D. L.; Shirley, N.; Langridge, P.; Bacic, A. Metabolite profiling reveals distinct changes in carbon and nitrogen metabolism in phosphate-deficient barley plants (Hordeum vulgare L.). Plant Cell Physiol. 2008, 49, 691-703.
54. Warren, C.R. How does P affect photosynthesis and metabolite profiles of Eucalyptus globulus? Tree physiol. 2011, 31, 727-739.
55. Araújo, W.L.; Tohge, T.; Ishizaki, K.; Leaver, C.J.; Fernie, A. R. Protein degradation–an alternative respiratory substrate for stressed plants. Trends Plant Sci. 2011, 16, 489-498.
56. Hildebrandt, T.M.; Nesi, A.N.; Araújo, W.L.; Braun, H. P. Amino acid catabolism in plants. Mol. Plant. 2015, 8, 1563-1579.
57. Szal, B.; Podgorska, A. The role of mitochondria in leaf nitrogen metabolism. Plant Cell Environ. 2012, 35, 1756-1768.
58. Brundrett, M.C.; Tedersoo, L. Evolutionary history of mycorrhizal symbioses and global host plant diversity. New Phytol. 2018, 220, 1108-1115.
59. Strack, D.; Fester, T.; Hause, B.; Schliemann, W.; Walter, M. H. Arbuscular mycorrhiza: biological, chemical, and molecular aspects. J. Chem. Ecol. 2003, 29, 1955-1979.
60. Smith, S. E.; Read, D. J. Mycorrhizal symbiosis, 3th edn. Academic Press, London. 2008, pp. 1-769.
61. Nagy, R.; Drissner, D.; Amrhein, N.; Jakobsen, I.; Bucher, M. Mycorrhizal phosphate uptake pathway in tomato is phosphorus-repressible and transcriptionally regulated. New Phytol. 2009, 181, 950-959.
62. Hughes, J.K.; Hodge, A.; Fitter, A.H.; Atkin, O.K. Mycorrhizal respiration: implications for global scaling relationships. Trends Plant Sci. 2008, 13, 583-588.
63. Bago, B.; Pfeffer, P.E.; Shachar-Hill, Y. Carbon metabolism and transport in arbuscular mycorrhizas. Plant Physiol. 2000, 124, 949-958.
64. Baas, R.; Kuiper, D. Effects of vesicular-arbuscular mycorrhizal infection and phosphate on Plantago major ssp. pleiosperma in relation to internal cytokinin concentrations. Physiol. Plant. 1989, 76, 211-215.
65. Hodge, A. Impact of elevated CO2 on mycorrhizal associations and implications for plant growth. Biol. Fertil. Soils. 1996, 23, 388-398.

66. Johnson, D.; Leake, J.R.; Read, D.J. Transfer of recent photosynthate into mycorrhizal mycelium of an upland grassland: short-term respiratory losses and accumulation of 14C. Soil Biol. Biochem. 2002, 34, 1521-1524.
67. Atkin, O.K.; Sherlock, D.; Fitter, A.H.; Jarvis, S.; Hughes, J.K.; Campbell, C.; Hurry, V.; Hodge, A. Temperature dependence of respiration in roots colonized by arbuscular mycorrhizal fungi. New Phytol. 2009, 182, 188-199.
68. Hohnjec, N.; Vieweg, M.F.; Pühler, A.; Becker, A.; Küster, H. Overlaps in the transcriptional profiles of Medicago truncatula roots inoculated with two different Glomus fungi provide insights into the genetic program activated during arbuscular mycorrhiza. Plant Physiol. 2005, 137, 1283-1301.
69. Schliemann, W.; Ammer, C.; Strack, D. Metabolite profiling of mycorrhizal roots of Medicago truncatula. Phytochemistry. 2008, 69, 112-146.
70. Laparre, J.; Malbreil, M.; Letisse, F.; Portais, J.C.; Roux, C.; Bécard, G.; Puech-Pagès, V. Combining metabolomics and gene expression analysis reveals that propionyl-and butyryl-carnitines are involved in late stages of arbuscular mycorrhizal symbiosis. Mol. plant. 2014, 7, 554-566.
71. Ryan, M.H.; Tibbett, M.; Edmonds, T.; Suriyagoda, L.D.; Lambers, H.; Cawthray, G. R.; Pang J. Carbon trading for phosphorus gain: The balance between rhizosphere carboxylates and arbuscular mycorrhizal symbiosis in plant phosphorus acquisition. Plant Cell Environ. 2012, 35, 2170–2180.
72. Nazeri, N.K.; Lambers, H.; Tibbett, M.; Ryan, M.H. Moderating mycorrhizas: arbuscular mycorrhizas modify rhizosphere chemistry and maintain plant phosphorus status within narrow boundaries. Plant Cell Environ. 2014, 37, 911-921.
73. Romero-Munar, A.; Del-Saz, N.F.; Ribas-Carbó, M.; Flexas, J.; Baraza, E.; Florez-Sarasa, I.; Fernie, A.R.; and Gulías, J. Arbuscular mycorrhizal symbiosis with Arundo donax decreases root respiration and increases both photosynthesis and plant biomass accumulation. Plant Cell Environ. 2017, 40, 1115– 1126.
74. Pearse, S.J.; Veneklaas, E.J.; Cawthray, G.; Bolland, M.D.; Lambers, H. Carboxylate composition of root exudates does not relate consistently to a crop species' ability to use phosphorus from aluminium, iron or calcium phosphate sources. New Phytol. 2007, 173, 181-190.
75. Pang, J.; Ryan, M.H., Tibbett, M.; Cawthray, G.R.; Siddique, K.H.M.; Bolland, M.D. A.; Denton, M.D.; Lambers, H. Variation in morphological and physiological parameters in herbaceous perennial legumes in response to phosphorus supply. Plant Soil. 2010, 331, 241–255.
76. Raven, J.A.; Lambers, H.; Smith, S. E.; Westoby, M. Costs of acquiring phosphorus by vascular land plants: patterns and implications for plant coexistence. New Phytol. 2018, 217, 1420-1427.

77. Stribley, D.P.; Tinker, P.B.; Rayner, J.H. Relation of internal phosphorus concentration and plant weight in plants infected by vesicular-arbuscular mycorrhizas. New Phytol. 1980, 86, 261-266.
78. Del-Saz, N.F.; Romero-Munar, A.; Alonso, D.; Aroca, R.; Baraza, E.; Flexas, J.; Ribas-Carbo, M. Respiratory ATP cost and benefit of arbuscular mycorrhizal symbiosis with Nicotiana tabacum at different growth stages and under salinity. J. Plant Physiol. 2017c, 218, 243-248.
79. Smith, S.E.; Smith, F.A.; Jakobsen, I. Functional diversity in arbuscular mycorrhizal (AM) symbioses: the contribution of the mycorrhizal P uptake pathway is not correlated with mycorrhizal responses in growth or total P uptake. New phytol. 2004, 162, 511-524.
80. Bucher, M.; Hause, B.; Krajinski, F.; Küster, H. Through the doors of perception to function in arbuscular mycorrhizal symbioses. New Phytol. 2014, 204, 833-840.
81. Watts-Williams, S.J.; Jakobsen, I.; Cavagnaro, T.R.; Grønlund, M. Local and distal effects of arbuscular mycorrhizal colonization on direct pathway Pi uptake and root growth in Medicago truncatula. J. Exp. Bot. 2015, 66, 4061-4073.
82. Bucher, M. Functional biology of plant phosphate uptake at root and mycorrhiza interfaces. New Phytol. 2007, 173, 11-26.
83. Karandashov, V.; Bucher, M. Symbiotic phosphate transport in arbuscular mycorrhizas. Trends plant Sci. 2005, 10, 22-29.
84. Sawers, R.J.; Svane, S.F.; Quan, C.; Grønlund, M.; Wozniak, B.; Gebreselassie, M.N.; Gonzalez-Muñoz, E.; Chavez, R.A.; Baxter, I.; Goudet, J.; Jakobsen, I.; Paszkowski, U. Phosphorus acquisition efficiency in arbuscular mycorrhizal maize is correlated with the abundance of root-external hyphae and the accumulation of transcripts encoding PHT1 phosphate transporters. New Phytol. 2017, 214, 632-643.
85. Smith, S.E.; Jakobsen, I.; Grønlund, M.; Smith, F.A. Roles of arbuscular mycorrhizas in plant phosphorus nutrition: interactions between pathways of phosphorus uptake in arbuscular mycorrhizal roots have important implications for understanding and manipulating plant phosphorus acquisition. Plant Physiol. 2011, 156, 1050-1057.
86. Facelli, E.; Duan, T.; Smith, S.E.; Christophersen, H.M.; Facelli, J.M.; Smith, F.A. Opening the black box: Outcomes of interactions between arbuscular mycorrhizal (AM) and non-host genotypes of Medicago depend on fungal identity, interplay between P uptake pathways and external P supply. Plant Cell Environ. 2014, 37, 1382–1392.
87. Campos, C.; Cardoso, H.; Nogales, A.; Svensson, J.; Lopez-Ráez, J.A.; Pozo, M.J.; Nobre, T.; Schneider, C.; Arnholdt-Schmitt, B. Intra and inter-spore variability in Rhizophagus irregularis AOX gene. PLoS One. 2015, 10, 1-24.

88. Fellbaum, C.R.; Mensah, J.A.; Cloos, A.J.; Strahan, G.E.; Pfeffer, P.E.; Kiers, E.T.; Bücking, H. Fungal nutrient allocation in common mycorrhizal networks is regulated by the carbon source strength of individual host plants. New Phytol. 2014, 203, 646-656.
89. Williams, A.; Manoharan, L.; Rosenstock, N.P.; Olsson, P.A.; Hedlund, K. Long-term agricultural fertilization alters arbuscular mycorrhizal fungal community composition and barley (Hordeum vulgare) mycorrhizal carbon and phosphorus exchange. New Phytol. 2017, 213, 874-885.
90. Miller, A.J; Cramer, M.D. Root nitrogen acquisition and assimilation. Plant Soil. 2005, 274, 1–36.
91. Lea, P.J.; Azevedo, R.A. Nitrogen use efficiency. 2. Amino acid metabolism. Ann. Appl. Biol. 2007, 151, 269-275.
92. Barber, S. Soil nutrient bioavailability: A mechanistic approach, 2nd ed. Wiley, New York. 1995. 384 pp.
93. Rennenberg, H.; Wildhagen, H.; Ehlting, B. Nitrogen nutrition of poplar trees. Plant Biol. 2010, 12, 275–291.
94. Gan, H.; Jiao, Y.; Jia, J.; Wang, X.; Li, H.; Shi, W.; Peng, Ch.; Polle, A.; Zhi-Bin, L. Phosphorus and nitrogen physiology of two contrasting poplar genotypes when exposed to phosphorus and/or nitrogen starvation. Tree Physiol. 2016, 36, 22–38.
95. Xu, G.; Fan, X.; T, Miller. Plant nitrogen assimilation and use efficiency. Annu. Rev. Plant Biol. 2012, 63, 153-182.
96. Terashima, I.; Evans, J.R. Effects of light and nitrogen nutrition on the organization of the photosynthetic apparatus in spinach. Plant Cell Physiol. 1988, 29, 143–155.
97. Makino, A.; Osmond, B. Effects of nitrogen nutrition on nitrogen partitioning between chloroplasts and mitochondria in pea and wheat. Plant Physiol. 1991, 96, 355–362.
98. Byrd, G.T.; Sage, R.F.; Brown, R.H.A comparison of dark respiration between C3 and C4 plants. Plant Physiol. 1992, 100, 191–198.
99. Lusk, C.; Reich, P. Relationships of leaf dark respiration with light environment and tissue nitrogen content in juveniles of 11 cold-temperate tree species. Oecologia. 2000, 123, 318–329.
100. Richard-Molard, C.; Krapp, A.; Brun, F.; Ney, B.; Daniel-Vedele, F.; Chaillou, S. Plant response to nitrate starvation is determined by N storage capacity matched by nitrate uptake capacity in two Arabidopsis genotypes. J. Exp. Bot. 2008, 59, 779-791.
101. Larsen, D.P.; Schults, D.W.; Malueg, K.W. Summer internal phosphorus supplies in Shagawa Lake, Minnesota. Limnol Oceanogr. 1981. 26, 740-753.
102. Scheible, W.R; Gonzalez-Fontes, A.; Lauerer, M.; Müller-Röber, B.; Caboche, M.; Stitt M. Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. Plant Cell. 1997, 9, 783–798.

103. Lancien, M.; Ferrario-Mery, S.; Roux, Y.; Bismuth, E.; Masclaux, C.; Hirel, B; Gadal, P.; Hodges, M. Simultaneous expression of NAD-dependent isocitrate dehydrogenase and other Krebs cycle genes after nitrate re-supply to short-term starved Nicotiana tabacum. Plant Physiol. 1999, 120, 717–726.
104. Noguchi, K.; Terashima, I. Responses of spinach leaf mitochondria to low N availablity. Plant Cell Environ. 2006, 29, 710–719.
105. Millenaar, F.F.; Gonzàlez-Meler, M.A.; Fiorani, F.; Welschen, R.; Ribas-Carbo, M.; Siedow, J.N.; Wagner, A.M.; Lambers, H. Regulation of alternative oxidase activity in six wild monocotyledonous species. An in vivo study at the whole root level. Plant Physiol. 2001. 126, 376-387.
106. MacFarlane, C; Hansen, L.D.; Florez-Sarasa, I.; Ribas-Carbo, M. Plant mitochondria electron partitioning is independent of short-term temperature changes. Plant Cell Environ. 2009, 32, 585-591.
107. Rasmusson, A.G.; Fernie, A.R.; Van Dongen, J.T. Alternative oxidase: a defence against metabolic fluctuations? Physiol Plant. 2009, 137, 371-382.
108. Stitt, M. Nitrate regulation of metabolism and growth. Curr Opin Plant Biol. 1999, 2, 178–186.
109. Hachiya, T.; Terashima, I.; Noguchi, K. Increase in respiratory cost at high growth temperature is attributed to high protein turnover cost in Petunia x hybrida petals. Plant Cell Environ. 2007, 30, 1269–1283.
110. Noctor, G.; Foyer, C.H. A re-evaluation of the ATP:NADPH budget during C3 photosynthesis: a contribution from nitrate assimilation and its associated respiratory activity? J. Exp. Bot. 1998, 49, 1895–1908.
111. Scheurwater, I.; Clarkson, D.T.; Purves, J.V.; Van Rijt, G.; Saker, L.R.; Welschen, R.; Lambers, H. Relatively large nitrate efflux can account for the high specific respiratory costs for nitrate transport in slow-growing grass species. Plant Soil. 1999, 215, 123–134
112. Britto, D.T.; Kronzucker, H.J. NH4+ toxicity in higher plants: a critical review. J. Plant Physiol. 2002, 159, 567–584.
113. Escobar, M.A.; Geisler, D.A.; Rasmusson, A.G. Reorganization of the alternative pathways of the Arabidopsis respiratory chain by nitrogen supply: opposing effects of ammonium and nitrate. Plant J. 2006, 45, 775–788.
114. Hachiya, T.; Watanabe, C.K; Boom, C.; Tholen, D.; Takahara, K.; Kawai-Yamada, M., Uchimiya, H.; Uesono, Y.; Terashima, I.; Noguchi, K. Ammonium-dependent respiratory increase is dependent on the cytochrome pathway in Arabidopsis thaliana shoots. Plant Cell Environ. 2010, 33, 1888-97.
115. Hachiya, T.; Noguchi, K. Integrative response of plant mitochondrial electron transport chain to nitrogen source. Plant Cell Rep. 2011, 30, 195–204.

116. Podgórska, A.; Mazur, R.; Ostaszewska-Bugajska, M.; Kryzheuskaya, K.; Dziewit, K.; Borysiuk, K.; Wdowiak, A.; Burian, M.; Rasmusson, A.G.; Szal, B. Efficient Photosynthetic Functioning of Arabidopsis thaliana Through Electron Dissipation in Chloroplasts and Electron Export to Mitochondria Under Ammonium Nutrition. Front. Plant. Sci. 2020, 103, 1-18.
117. Esteban, R.; Ariz, I.; Cruz, C.; Moran, J.F. Mechanisms of ammonium toxicity and the quest for tolerance. Plant Sci. 2016, 248, 92-101
118. Frechilla, S.; Lasa, B; Aleu, M.; Juanarena, N., Lamsfus, C., Aparicio-Tejo, P.M. Short-term ammonium supply stimulates glutamate dehydrogenase activity and alternative pathway respiration in roots of pea plants. J. Plant Physiol. 2002. 159, 811–818
119. Lasa, B.; Frechilla, S.; Aparicio-Tejo, P.M.; Lamsfus, C. Alternative pathway respiration is associated with ammonium ion sensitivity in spinach and pea plants. Plant Growth Regul. 2002. 37, 49–55.
120. Patterson. K.; Cakmak, T; Cooper, A.; Lager, I.; Rasmusson, A.G.; Escobar, M.A. Distinct signalling athways and transcriptome response signatures differentiate ammonium- and nitrate-supplied plants. Plant Cell Environ. 2010, 33, 1486-501.
121. Gupta, K.J.; Kumari, A.; Florez-Sarasa, I.; Fernie, A.R.; Igamberdiev, A. U. Interaction of nitric oxide with the components of the plant mitochondrial electron transport chain. J. Exp. Bot. 2018, 69, 3413-3424.
122. Kearns, A.; Whelan, J.; Young, S.; Elthon, T.E.; Day D.A. Tissue specific expression of the alternative oxidase in soybean and siratro. Plant Physiol. 1992, 99, 712-717.
123. Finnegan, P.M.; Whelan, J.; Millar, A.H.; Zhang, Q.S.; Smith, M.K.; Wiskich, J.T.; Day D.A. Differential expression of the multigene family encoding the soybean mitochondrial alternative oxidase. Plant Physiol. 1997, 114, 455-466.
124. Sweetman, C.; Soole, K.L.; Jenkins C.L.D.; Day, D.A. Genomic structure and expression of alternative oxidase genes in legumes. Plant Cell Environ. 2019, 42, 71-84.
125. Gonzàlez-Meler, M.A.; Siedow J.N. Inhibition of respiratory enzymes by elevated CO2: does it matter at the intact tissue and whole plant levels? Tree Physiol. 1999, 19, 253– 259.
126. Martí, M.C.; Florez-Sarasa, I.; Camejo, D.; Ribas-Carbó, M.; Lázaro, J.J.; Sevilla, F.; Jiménez, A. Response of mitochondrial thioredoxin PsTrxo1, antioxidant enzymes, and respiration to salinity in pea (Pisum sativum L.) leaves. J. Exp. Bot. 2011, 62, 3863–3874.
127. Coba de la Peña, T.; Pueyo, J.J. Legumes in the reclamation of marginal soils, from cultivar and inoculant selection to transgenic approaches. Agron sustain dev. 2012, 32, 65-91.

128. Okazaki, S.; Kaneko, T.; Sato, S.; Saeki, K. Hijacking of leguminous nodulation signaling by the rhizobial type III secretion system. Proc. Nat. Acad. Sci. 2013. 110, 17131–17136.
129. Gourion, B.; Berrabah, F.; Ratet, P.; Stacey, G. Rhizobium-legume symbioses: the crucial role of plant immunity. Trends Plant Sci. 2015, 20, 186–194.
130. Masson-Boivin, C.; Sachs, J.L. Symbiotic nitrogen fixation by rhizobia — the roots of a success story. Curr. Opin. Plant Biol. 2018. 44, 7–15.
131. Coba de la Peña, T.; Fedorova, E.; Pueyo, J.J.; Lucas, M.M. The Symbiosome: Legume and Rhizobia Co-evolution toward a Nitrogen-Fixing Organelle?. Front. Plant Sci. 2018, 8, 2229.
132. Day, D.A. Carbon metabolism and compartmentation in nitrogen fixing legume nodules. Plant Physiol. Biochem. 1991, 29, 185–201.
133. Liu, J.; Contador, C.A.; Fan, K.; Hon-Ming, L. Interaction and Regulation of Carbon, Nitrogen, and Phosphorus Metabolisms in Root Nodules of Legumes. Front Plant Sci. 2018, 9, 1860.
134. Lodwig, E.M.; Hosie, A.H.; Bourdes, A.; Findlay, K.; Allaway, D.; Karunakaran, R.; Dowie, J.A.; Poole, P.S. Amino-acid cycling drives nitrogen fixation in the legume–Rhizobium symbiosis. Nature. 2003, 422, 722-726.
135. Rao, T.P.; Ito, O. Differences in root system morphology and root respiration in relation to nitrogen uptake among six crop species. Jpn. Agr. Res. Q. 1998, 32, 97-104.
136. Schulze, J.; Beschow, H.; Adgo, E.; Merbach, W. Efficiency of N2 fixation in Vicia faba L. in combination with different Rhizobium leguminosarum strains. J. Plant. Nutr. Soil Sci. 2000, 163, 367-373.
137. Mortimer, P.E.; Pérez-Fernández, M.A.; Valentine, A.J. The role of arbuscular mycorrhizal colonization in the carbon and nutrient economy of the tripartite symbiosis with nodulated Phaseolus vulgaris. S. Biol. Bioch. 2008, 40, 1019-1027.
138. Wang, Y.Y.; Hsu, P.K.; Tsay, Y.F. Uptake, allocation and signaling of nitrate. Trends Plant Sci. 2012. 17:458–467.
139. Udvardi, M.; Poole, P.S. Transport and metabolism in legume-rhizobia symbioses. Annu. Rev. Plant Biol. 2013. 64, 781–805.
140. Downie, J.A. Legume nodulation. Curr Biol. 2014. 24, 184–190.
141. Winkler, R.D.; Blevins, D.G.; Polacco, J.C.; Randall, D.D. Ureide catabolism in soybeans. II. Pathway of catabolism in intact leaf tissue. Plant Physiol. 1987, 83, 585–591
142. Sprent, J.I. Legume nodulation: a global perspective. Wiley-Blackwell, Oxford, UK, 2009, pp. 1-183
143. Marchal, K.; Vanderleyden, J. The" oxygen paradox" of dinitrogen-fixing bacteria. Biol. Fert. Soils. 2000, 30, 363-373.

144. Dakora, F.D.; Atkins C.A. Adaptation of nodulated soybean (Glycine max L. Merr.) to growth in rhizospheres containing non-ambient pO2. Plant Physiol. 1991, 96, 728– 736.
145. Brown, S.M.; Walsh, K.B. Anatomy of the legume nodule cortex with respect to nodule permeability. Aust. J. Plant Physiol. 1994, 21, 49-68.
146. Bergersen, F. J.; Turner, G. L. Leghaemoglobin and the supply of O2 to nitrogen-fixing root nodule bacteroids: presence of two oxidase systems and ATP production at low free O2 concentration. Microbiol. 1975, 91(2), 345-354.
147. Bergersen, F. J.; Turner, G. L. Properties of terminal oxidase systems of bacteroids from root nodules of soybean and cowpea and of N2-fixing bacteria grown in continuous culture. Microbiol. 1980, 118(1), 235-252.
148. Millar, A.H.; Day, D.A.; Bergersen, F.J. Microaerobic respiration and oxidative phosphorylation by soybean nodule mitochondria: implications for nitrogen fixation. Plant Cell Environ. 1995, 18, 715-726
149. Gordon, A. J.; Minchin, F. R.; Skot, L.; James, C. L. Stress-induced declines in soybean N2 fixation are related to nodule sucrose synthase activity. Plant Physiol. 1997, 114, 937-946
150. Becana, M.; Navascués, J.; Pérez-Rontomé, C.; Walker, F. A.; Desbois, A.; Abian, J.; Leghemoglobins with Nitrated Hemes in Legume Root Nodules. In: Biological Nitrogen Fixation.;Bruijn, F.J., Ed.;John Wiley & Sons, Inc, Hoboken, NJ, USA. 2015, pp. 705-713
151. Matamoros, M.A.; Fernández-García, N.; Wienkoop, S.; Loscos, J.; Saiz, A.; Becana, M. Mitochondria are an early target of oxidative modifications in senescing legume nodules. New Phytol. 2013, 197, 873-885.
152. Millar, A.H.; Finnegan, P.M.; Whelan, J.; Drevon, J. J.; Day, D.A. Expression and kinetics of the mitochondrial alternative oxidase in nitrogen-fixing nodules of soybean roots. Plant Cell Environ. 1997, 20, 1273-1282.
153. Bryce, J.H.; Day, D.A. Tricarboxylic acid cycle activity in mitochondria from soybean nodules and cotyledons. J.Exp. Bot. 1990, 41, 961-967.
154. Day, D.A.; Price, G.D.; Gresshoff, P.M. Isolation and oxidative properties of mitochondria and bacteroids from soybean root nodules. Protoplasma, 1986, 134, 121-129.
155. Cheah, W.Y.; Show, P.L.; Chang, J.S.; Ling, T.C.; Juan, J.C. (2015). Biosequestration of atmospheric CO2 and flue gas-containing CO2 by microalgae. Bioresour. Technol. 2015, 184, 190-201.
156. de Visser, R.; Lambers, H. Growth and the efficiency of root respiration of Pisum sativum as dependent on the source of nitrogen. Physiol. Plant. 1983, 58, 533-543.

157. Preisig, O.; Zufferey, R.; Thöny-Meyer, L.; Appleby, C.A.; Hennecke, H. A high-affinity cbb3-type cytochrome oxidase terminates the symbiosis-specific respiratory chain of Bradyrhizobium japonicum. Journal of Bacteriol. 1996, 178, 1532-1538.
158. López, M.; Herrera-Cervera, J.A.; Iribarne, C.; Tejera, N.A.; Lluch, C. Growth and nitrogen fixation in Lotus japonicus and Medicago truncatula under NaCl stress: nodule carbon metabolism. J. Plant Physiol. 2008, 165, 641-650.
159. Wang, X.; Shen, J.; Liao, H. Acquisition or utilization, which is more critical for enhancing phosphorus efficiency in modern crops? Plant Sci. 2010. 179, 302–306.
160. Qin, L.; Zhao, J.; Tian, J.; Chen, L.; Sun, Z.; Guo, Y.; Lu, X.; Gu, M.; Xu, G.; Liao, H. The High affinity phosphate transporter GmPT5 regulates phosphate transport to nodules and nodulation in soybean. Plant Physiol. 2012, 159, 1634–1643
161. Hernandez, G.; Valdes-Lopez, O.; Ramirez, M.; Goffard, N.; Weiller, G.; Aparicio-Fabre, R.; Fuentes, S.I.; Erban, A.; Kopka, J.; Udvardi, M.; Vance, C. Global changes in the transcript and metabolic profiles during symbiotic nitrogen fixation in phosphorus-stressed common bean plants. Plant Physiol. 2009. 151, 1221–1238.

FIGURES

Figure 1. Schematic representation of the TCA cycle and its connection with Pi consumption in leaves of plant species adapted to P deficiency. Low Pi availability limits both photosynthesis and respiration. In chloroplasts, the export rate of the Calvin-Benson cycle products, which are needed for the synthesis of sucrose, decreases under P limitation. This leads to increasing amounts of triose-phosphate and starch in chloroplasts. In cytosol, an accumulation of non-P-containing saccharides allows the cell to save Pi, but it aggravates the short supply of respiratory substrates into mitochondria. In contrast, protein degradation provides carbon skeletons to mitochondria via hydroxyglutarate synthesis that can be used for the synthesis and exudation of rhizosphere carboxylates citrate and malate, and feeds electrons to the mETC through to the ubiquinol pool via an electron-transfer flavoprotein:ubiquinone oxidoreductase (ETFQO) [56]. Similarly, the γ-aminobutyrate (GABA) shunt allows the entry of carbon skeletons in the form of acetyl-CoA, pyruvate, succinate, oxalacetate and α-ketoglutarate into the TCA cycle from amino acids alanine, glutamate and asparagine [58]. The re-oxidation of NADH generated in the TCA cycle may be

favored by AOX activity when COX is restricted by low Pi availability. TCA, tricarboxylic acid cycle.

Figure 2. Simplified overview of the interaction between respiratory metabolism of plant organs and mycorrhiza, conditioned by the demand for ATP synthesis and P uptake. Photosynthetic soluble sugars are used in respiration in leaves or transported to the root in order to fuel respiration and produce carbon skeletons for the fungal symbiont. Soluble sugars and organic acids can be exported to the fungal symbiont to fuel respiration in both intra and extraradical mycelium. ATP is required for P uptake and transport across organisms. TCA, tricarboxylic acid cycle. Modified from Hughes et al. [60].

Figure 3. A simplified schematic overview of the compartmentation of some of the interactions between primary metabolism pathways during ammonium and nitrate assimilation. Nitrate is mainly transported from roots to leaves via xylem where is converted into nitrite with the consumption of reducing equivalents in cytosol. In the chloroplast, the reducing power of

light-activated electrons drives the conversion of nitrite to ammonium from cytosolic nitrate reductase (NR)-derived nitrite by a nitrite reductase (NiR) activity, and its assimilation by the GS/GOGAT cycle. 2-oxoglutarate which is required for ammonium assimilation, is exported to the chloroplast by a 2-oxoglutarate /malate translocator. Ammonium uptake bypasses the nitrate reductase reaction in cytosol thus increasing the reducing equivalents available that can be dissipated during respiration. During photorespiration, the retrieval of CO_2 and NH_4^+ during the glycine cleavage reaction in mitochondria leads to an increased NADH/NAD^+ ratio in the mitochondrial matrix that has been suggested to be related to changes in AOX activity. TCA, tricarboxylic acid cycle.

Figure 4. Simplified overview of the nitrogen-fixing pathways in nodulated legumes, conditioned by the demand for ATP synthesis for nitrogenase activity in determinate and indeterminate nodules. Soluble sugars are catabolized via glycolysis to respiratory substrates for the synthesis of TCA metabolites, which are transported across the peribacteroid and bacteroid membranes to fuel the TCA cycle and respiration in the bacteroid. The

ammonia produced during nitrogenase activity is exported to the plant and assimilated by GS and GOGAT enzymes. In determinate nodules, glutamine is converted to ureides (allantoin), that are decarboxylated in metabolic pathways of photorespiration, contributing to the accumulation of NADH in mitochondria. In indeterminate nodules, glutamine and glutamate are further converted to asparagine and aspartate to be incorporated into the nitrogen metabolism of leaves. ASN, asparagine; ASP, aspartic acid; TCA, tricarboxylic acid cycle. Modified from Liu et al. [131].

Fig 1.

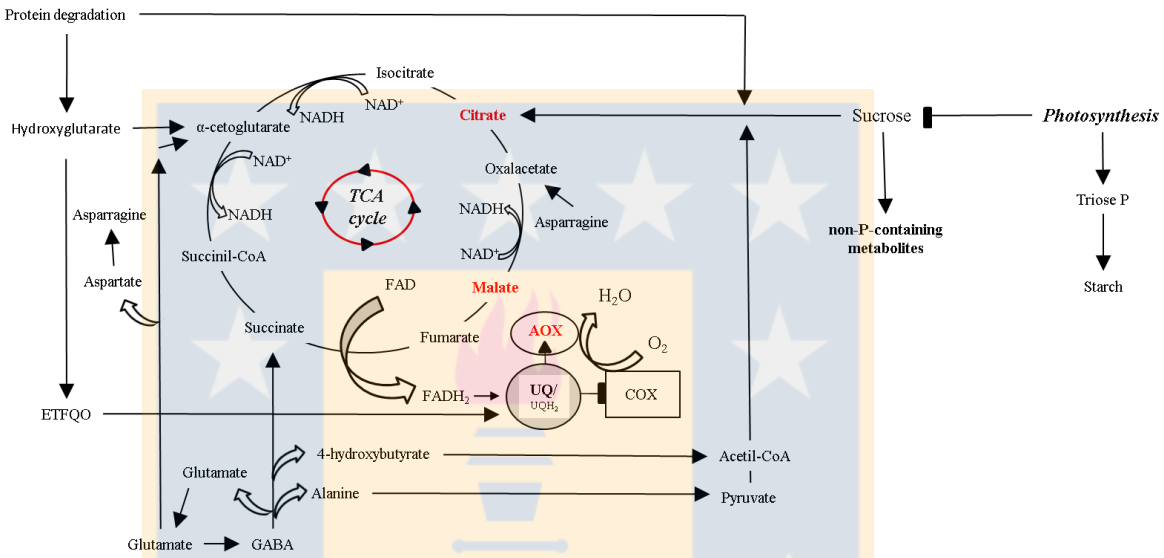

Protein degradation
Isocitrate
NADH
NAD+
Citrate
Hydroxyglutarate
α-cetoglutarate
Sucrose
Photosynthesis
NAD+
Oxalacetate
Triose P
TCA cycle
Asparragine
NADH
Asparragine
non-P-containing metabolites
Succinil-CoA
NADH
NAD+
Aspartate
FAD
Malate
H2O
Succinate
Fumarate
AOX
O2
FADH2
UQ/UQH2
COX
Starch
ETFQO
4-hydroxybutyrate
Acetil-CoA
Glutamate
Alanine
Pyruvate
Glutamate
GABA

Fig 2.

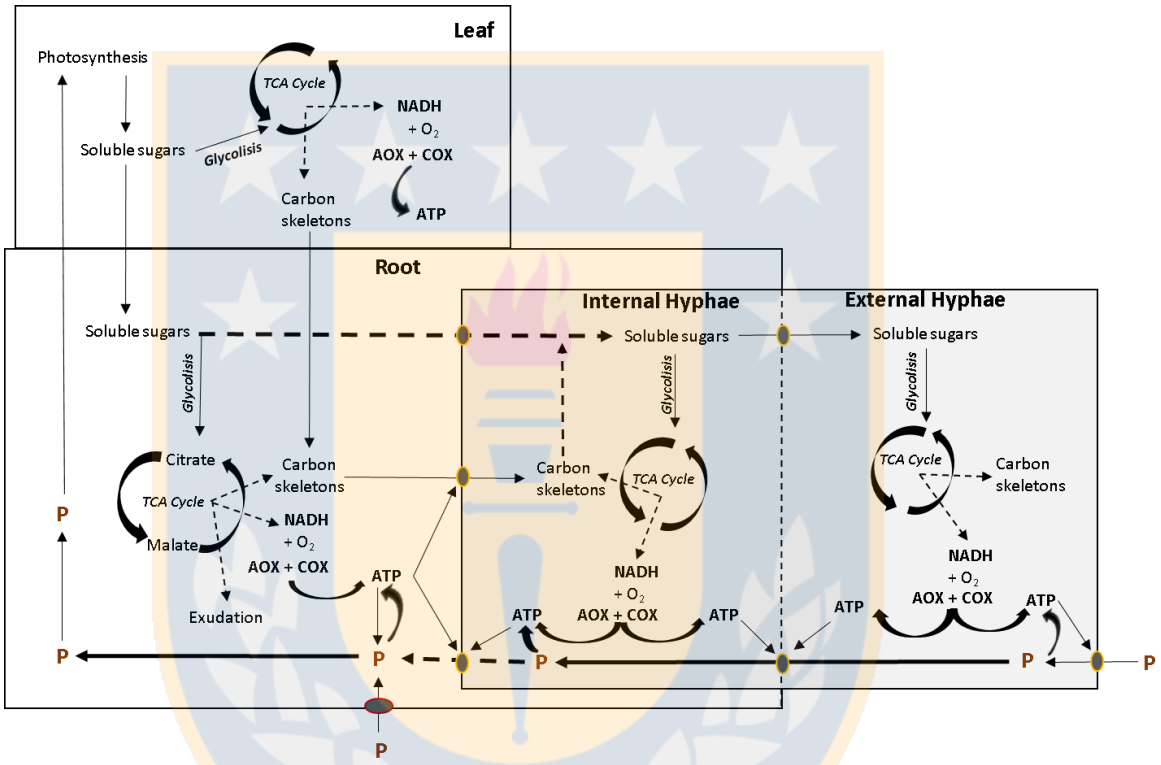

Leaf
Photosynthesis
TCA Cycle
NADH
+ O2
AOX + COX
Soluble sugars
Glycolisis
Carbon
skeletons
ATP
Root
Internal Hyphae
External Hyphae
Soluble sugars
Soluble sugars
Soluble sugars
Glycolisis
Glycolisis
Glycolisis
Citrate
TCA Cycle
Malate
Carbon
skeletons
NADH
+ O2
AOX + COX
ATP
Exudation
Carbon
skeletons
TCA Cycle
NADH
+ O2
AOX + COX
ATP
ATP
ATP
TCA Cycle
Carbon
skeletons
NADH
+ O2
AOX + COX
ATP
ATP
P
P
P
P
P
P
P
P

Fig 3.

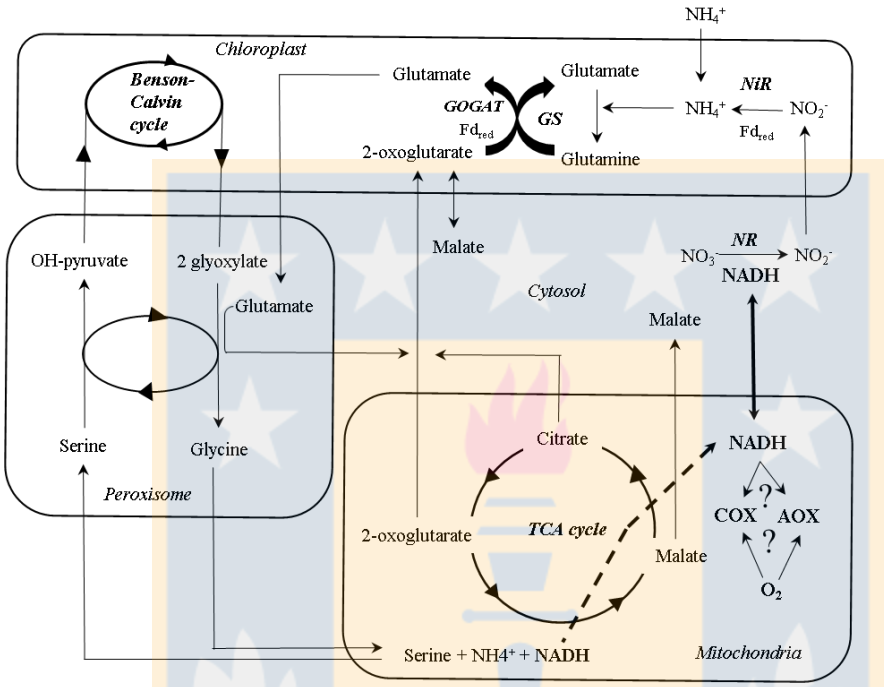
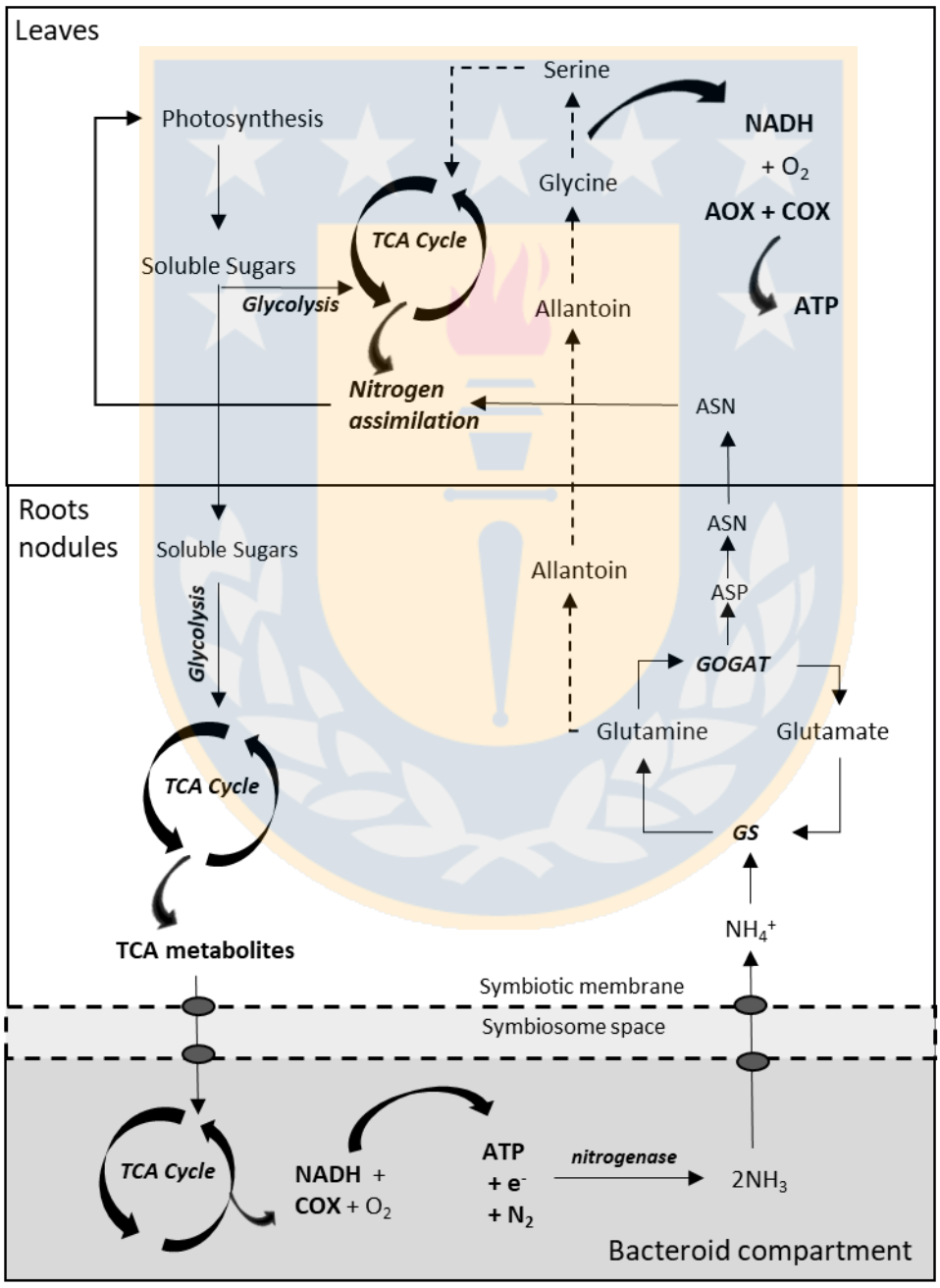

NH4+
Chloroplast
Benson-Calvin cycle
Glutamate
GOGAT
Fdred
GS
Glutamate
Glutamine
2-oxoglutarate
NiR
NH4+
NO2-
Fdred
Malate
NR
NO3-
NO2-
NADH
Cytosol
OH-pyruvate
2 glyoxylate
Glutamate
Malate
Serine
Glycine
Peroxisome
Citrate
NADH
COX
AOX
?
?
2-oxoglutarate
TCA cycle
Malate
O2
Serine + NH4+ + NADH
Mitochondria

Fig 4.


Leaves
Serine
Photosynthesis
NADH
+ O_2
Glycine
AOX + COX
TCA Cycle
Soluble Sugars
Glycolysis
Allantoin
ATP
Nitrogen assimilation
ASN
Roots nodules
Soluble Sugars
ASN
Allantoin
ASP
Glycolysis
GOGAT
Glutamine
Glutamate
TCA Cycle
GS
NH_4^+
TCA metabolites
Symbiotic membrane
Symbiosome space
TCA Cycle
NADH +
COX + O_2
ATP
+ e^-
+ N_2
nitrogenase
$2NH_3$
Bacteroid compartment

GENERAL DISCUSSION

The data contained in this thesis support the idea that plants possess several biochemical and physical ways for the management and regulation of energy input (Articles 1 and 2). As proposed in hypothesis 1, the high solar incidence and dry atmosphere of the terrestrial environment impose a great flow of electrons to the alternative oxidase in terrestrial plants promoting a higher energy balance (Article 1). In line to the above, leaf is the organ in charge of receiving solar energy and converting it into chemical energy through photosynthesis while at the same time avoiding excessive water loss and heating. The results obtained suggest that leaf type is essential in plant energy regulation. Thanks to changes in shape and size, plants can physically and biochemically regulate the energy received by the sun (Article 2, Figure 11). As occur in leaves during land conditions, nutrient deficiency causes energy misbalances in plant metabolism. Our results based on Article 3 demonstrate that mitochondrial respiration allows to plant great metabolic flexibility during conditions of N and Pi deprivation by regulating the substrate respired. In this sense, hypothesis 3 is accepted, due to the role of respiration and alternative oxidase in this metabolic

flexibility. Also, during symbiosis with soil microorganisms such as mycorrhiza and rhizobia, the role of respiration includes regulation of sugar metabolism by changes in the photosynthetic rates and hence, nitrogen metabolism. Based on this observation, hypothesis 4 is accepted due to the different changes occurring in the activities of two oxidases during symbiosis with soil microorganisms, which maintain the energetic balance in plants.

Environmental mechanisms for both dissipation and management of energy excess in plants: how leaf form and habitat can determine photosynthetic limitations and respiratory adjustments

Several reports highlighted the role of mitochondrial oxidases in energy regulation status on plants subjected to stress (Vanlerbergue et al. 2013; Florez-Sarasa et al. 2014; Del Saz et al. 2017; Florez-Sarasa et al. 2019). In addition, leaf type also regulates energy incoming by changes in leaf area for more light capture and gas exchange or reducing it to avoid energy excess and water loss during drought (Sack et al. 2002; Givinish et al. 1979; Vogel et al. 2009). In this sense, terrestrial lifestyle promotes simple leaves form to increase light capture, and hence photosynthesis and growth (Li et al. 2019; Van Veen & Sasidharan, 2019). In article 1,

terrestrial species seems to show higher activity of both glycolysis and TCA reactions. These changes are related to high conversion fluxes for these metabolites and thence "faster" metabolism (Fell, 2005). Also, the higher τ value in terrestrial species indicates a higher partitioning of electrons to alternative respiration (Figure 12). Regard palustrine species, the return to the aquatic ecosystem from land may have decreased the AOX contribution to total respiration, a consequence of diminished exposure to high-reducing environments (Figure 12). For example, during leaf shape alternation or heterophylly, submerged leaves exposed to terrestrial conditions, such as high light and dry atmosphere produce aerial leaves (Iida et al. 2016). The morphological changes are mediated by ABA signaling. These phytohormone promote stomatal density, thick cuticle, and oblong shape such a simple leaf form (Kim et al. 2018; Li et al. 2019; Van Veen & Sasidharan, 2019). The ABA effects also regulate genes related to zeaxanthin reactions, used in the NPQ for dissipating the excess of energy during stressful conditions (He et al. 2008). In this sense, the terrestrial environment promotes the apparition of a simple leaf form orchestrated by ABA signaling, which may help to increase photosynthesis while avoiding water loss (Brodibb et al. 2010; Komatsu et al. 2020). In addition, the

partitioning of electrons to alternative respiration probably allows plant mitochondria to move the energy threshold to a higher level, promoting a greater flow of metabolites and hence, increasing metabolism and growth rates.

The existence of another type of leaf: the compound one, presents another mechanism for regulating the management of the energy incoming by plants related to carbon assimilation capacity (Article 2, Figure 12). Contrary to aquatic conditions, the terrestrial atmosphere does not present stable temperatures (Maberly et al. 2014). During water-limited conditions, increases in leaf temperature caused by lower stomata aperture and incoming light reduced the photosynthetic integrity (Asada, 2006). The presence of leaflets, short in size and thin, allowed in compound leaves higher A_N, g_s, and g_m values than simple leaves in well water conditions. This data gives a physiological explanation of the hypothesis that establishes that compound leaves present higher A_N due to their tiny leaflets (Givinish, 1979). However, the negative effects of drought are observed in both types of leaves, but with a few differences. For example, simple leaves species presented a low A_N reduction during drought compared to compound leaves. One explanation is due to the high LMA in simple leaves

(Figure 12). Increases in LMA are a natural response of plants in low water conditions by increasing the thickness of mesophyll cells and cells walls, the cellular integrity is achieved (Flexas et al. 2014). The compound leaves, by having low LMA, probably are prone to cellular lysis during an extended drought period and therefore present a higher loss in photosynthetic capacity (Article 2). Another difference between both types of leaves was the convective heat exchange. During drought conditions, compound leaves presented 4ºC lower than simple leaves. This data reflects the physical mechanism that allows plants to manage the energy input during stress such as drought. In this sense, the strategy used in simple leaves is to develop higher LMA and sclerophylly with lower net carbon assimilation, ensuring the photosynthetic integrity during growth season. In contrast, compound leaves promote a better physiological convection temperature, which guarantees a higher A_N until the fragile leaflets are damaged.

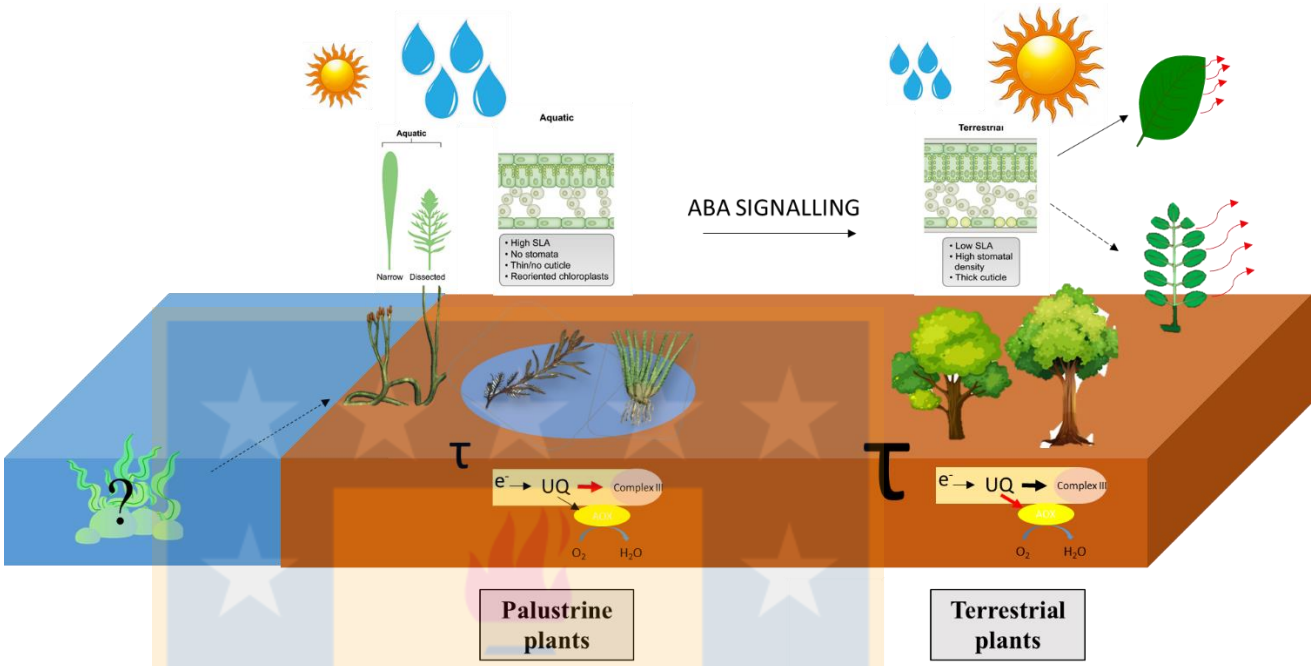


Figure 12. Graphic representation of chapter 1 and 2. As land conditions represent a high energy input in leaves, changes in the partitioning of electrons to alternative oxidase and a leaf shape that promotes convective cooling represent the mechanism for land colonization. Details in the discussion.

Respiratory mechanism for the dissipation of energy excess in roots: role of AOX activity during nutrient starvation and symbiosis with soil microorganisms

Chapters 1 and 2 show the role of respiration and leaf shape on energy balance, promoting higher carbon assimilation and tolerance to drought

conditions. Roots are organs that continuously require ATP from respiration to maintain the vital functions of plants, such as the absorption of nutrients and water. Several works stated root respiration as a sensor of energy status on plants during nutrient deficiency (Sieger et al. 2005; Florez-Sarasa et al. 2014; Gandin et al. 2014; Del Saz et al. 2017). Chapter 3 shows the role of root respiration in rearranging and balancing the source-sink relationship during N and Pi deficiency. In these conditions, low requirements for growth during nutrient limitation decrease ATP needs and COX activity. The low ATP demand for biosynthetic processes causes an accumulation of photoassimilates (Körner, 2015). The root respiration maintains an energy balance by an oxidizing surplus of NADH and increases the carbon respired via AOX during Pi and N deprivation (Figure 13). Thus, AOX maintains carbon homeostasis during nutrient limitation by metabolite signaling from TCA cycle, and carbon assimilation in the leaves, is regulated according to the requirements of metabolism. In this sense, roots respiration acts as a sensing mechanism that continually communicates the nutrient status and balances it to carbon assimilation in leaves.

In the presence of rhizobia and mycorrhiza symbionts, complex respiration adjustments maintain plant energy balance (Article 3, Figure 13).

As the rhizosphere possesses several microorganisms, roots can establish positive associations with them since ancient times (Selosse et al. 1998, 2004). Mycorrhizal and rhizobia act as a new sink for photoassimilates. By exerting a positive effect in photosynthesis to maintain their carbon needs, both symbioses promote plant growth by the fertilizer effect (Kaschuck et al. 2009, 2010). Based on the differential contribution of COX and AOX, and the positive growth effect during mycorrhizal symbiosis, I decided to evaluate if rhizobium symbioses could exert a similar response on the respiratory metabolism. I found that COX and AOX activities in leaves of the legume *L. japonicus* are more related to nutrient status and have no effect on the carbon symbiont requirements. In contrast, roots present a significant increase in COX contribution to total respiration whit reductions of AOX respiration. Thus, symbiosis promoted biomass accumulation by decreasing carboxylate exudation and nutrient uptake in the case of mycorrhiza. In the case of legume-rhizobia symbiosis, the biomass accumulation is probably by increasing the efficiency in the ATP produced by O_2 consumed and hence a COX improvement.

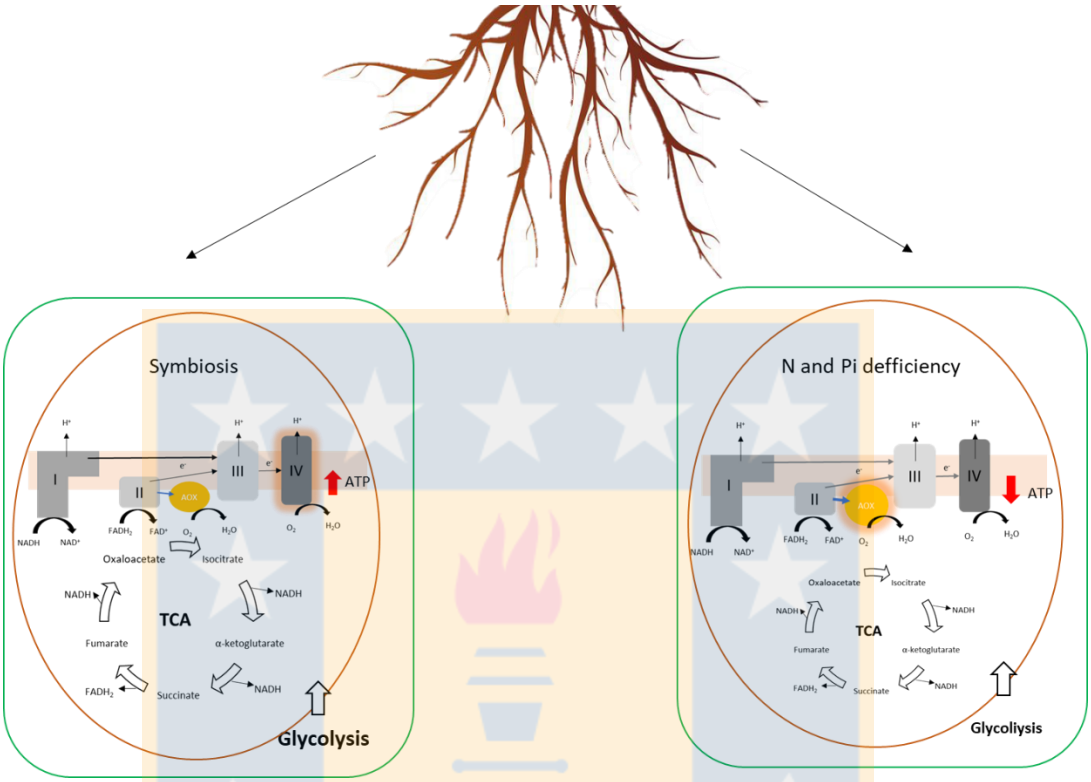


Figure 13. The root respiration during symbiosis and nutrient deficiency. The changes in energy status during N and Pi deficiency lowering the carbon assimilation and glycolysis. The low ATP demand increases AOX contribution in order to increase the sink strength for carbon and avoid ROS generation during nutrient stress. In contrast, during symbiosis, the effect of carbon assimilation increases the glycolysis pathway and COX efficiency for ATP production. In this way, despite de cost of symbiosis growth is maintained. In chapter 3, more detail of metabolic changes during nutrient deficiency and symbiosis can be found.

In summary, in this thesis, I found that plants cope and manage imbalances of energy input under both, biotic and abiotic stresses, being able of reprogramming its primary metabolism either in leaves or roots (Chapter I-III). In addition, I found that the contribution of AOX in leaves may promote higher photosynthetic capacity by increases of metabolite fluxes during land colonization (Chapter I). Regarding differences in the energy management and photosynthetic capacity based on biochemical limitations, I found that compound leaves present higher carbon assimilation and dissipation of heat during drought (Chapter II), demonstrating that the foliar form in terrestrial conditions appeared as an adaptation to regulate the amount of energy received (Chapter I and II). Regarding root respiration, the energy requirements for nutrient uptake process is only provided by oxidized sucrose coming from leaves. In this sense, role of roots in the maintenance of the stoichiometry required for correct biomass investment allows it to balance the energy from photosynthesis with the nutrient availability. Based on this, I found that AOX's contribution to root respiration depends on the limiting nutrient (Chapter III). In addition, symbioses impose carbon costs in plant photosynthesis which must be balanced by respiratory adjustments to improve plant yield. Thus, I stated

that AOX contribution during symbiosis decreases, and in parallel with increases in COX activity, promotes biomass accumulation in plants (Chapter III). Thus, photosynthetic and respiratory characterizations are important to understand the changes that occur during limitations in plant growth and yield, taking account the predicted climate change scenario.

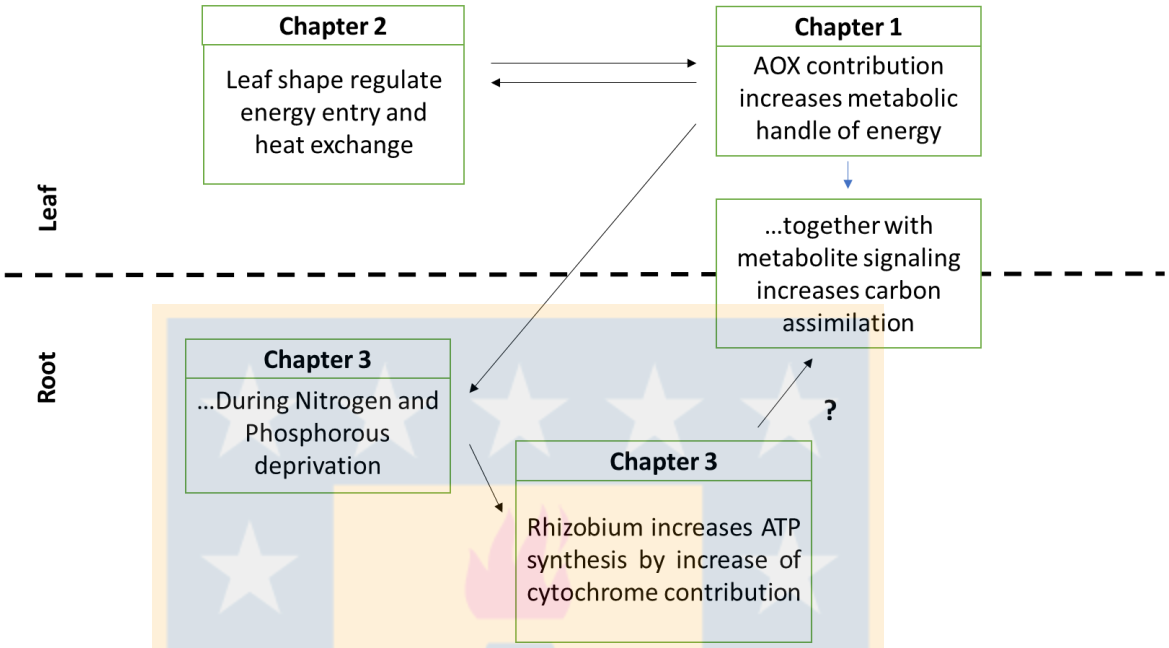


Figure 14. Summary of the conclusions obtained in the three chapters of the thesis.

CONCLUSIONS

1) In vascular plants, AOX could have contributed to cope with high reducing environments during aquatic-to-land transition, and progressively could have increased photosynthesis rates allowing a large management of energy in leaves.

2) Leaf type and shape regulates the energy used by photochemical process, allowing more photosynthesis by decreases in biochemical limitation and heat exchange drought conditions. However, trade-off between foliar mass and drought tolerance dominates photosynthetic rates and response to stress.

3) Symbiosis under nutrient deficiency alters primary metabolism and *in vivo* activities of both mitochondrial oxidases in plants. The presence of mycorrhiza and rhizobium promotes growth, producing a fertilizing effect, which contribute to maintain carbon balance in plants.

4) Legume-rhizobia symbiosis increases the contribution of the cytochrome oxidase over alternative oxidase for the benefit of ATP synthesis. However, nitrogen status in plants is more important for respiration than the presence of the symbiont.

FUTURE PERSPECTIVES

In this thesis, we used an integrative approach for searching responses of respiration and photosynthesis during abiotic and biotic stress. I integrated the role of mitochondrial oxidases in an evolutionary context using palustrine amphibious plants as a proxy for land transition to evaluate if AOX may exert a different function in respiration and carbon metabolism (Neimanis et al. 2013). These results show the energy management occurring in plants, its regulation by the mitochondrial metabolism, and its coordination with chloroplast.

In addition, our results showed that the physical properties of the leaf regulate the energy budget that the plant received from the sun. In this sense, compound, and simple leaves present different carbon assimilation rates, and this is due to leaf size and shape differences, which in turn affect the photosynthetic limitations. In this line, investigations into how the size of leaves affects the biochemical reactions related to energy management that occur in leaves are needed. In addition, Research in this field is required in different biomes and crops species (e.g legumes) for agriculture improvement programs.

Regard root physiology, nitrogen and phosphorous are significant for plant respiration and photosynthesis. The symbioses with mycorrhiza and rhizobium present an opportunity for solving fertilizer cost and availability by improving nutrient status in plants. Several works show the importance of the two oxidases in photosynthesis and mitochondria (Del-Saz et al. 2017; Selinski et al. 2018; Vanlerbergue et al. 2020). However, this is the first attempt to integrate plant response during symbiosis with metabolites that improved ATP synthesis and growth. In this sense, metabolite profiling arises as a tool for understanding changes in the primary metabolism of roots and leaves, which occurs during abiotic stress such as drought and nutrient deprivation. Works in this line are urgent based on extreme drought events and fertilizer exhaustion predictions, both dangers for sustain and feeding the human population.

References

Amthor, J. S. (2010). From sunlight to phytomass: on the potential efficiency of converting solar radiation to phyto-energy. New Phytologist, 188(4), 939-959.

Atkin, O. K., & Tjoelker, M. G. (2003). Thermal acclimation and the dynamic response of plant respiration to temperature. Trends in plant science, 8(7), 343-351.

Atkin, O. K., Edwards, E. J., & Loveys, B. R. (2000). Response of root respiration to changes in temperature and its relevance to global warming. The New Phytologist, 147(1), 141-154.

Barka, E. A., Nowak, J., & Clément, C. 2006. Enhancement Of Chilling Resistance Of Inoculated Grapevine Plantlets With A Plant Growth-Promoting Rhizobacterium, Burkholderia Phytofirmans Strain Psjn. Appl. Environ. Microbiol., 72(11), 7246-7252.

Bauwe, H., Hagemann, M., & Fernie, A. R. (2010). Photorespiration: players, partners and origin. Trends in plant science, 15(6), 330-336.

Benson, S., Lennard, C., Maynard, P., & Roux, C. (2006). Forensic applications of isotope ratio mass spectrometry—a review. Forensic science international, 157(1), 1-22.

Betti, M., García-Calderón, M., Pérez-Delgado, C. M., Credali, A., Estivill, G., Galván, F., ... & Márquez, A. J. (2012). Glutamine synthetase in legumes: recent advances in enzyme structure and functional genomics. International Journal of Molecular Sciences, 13(7), 7994-8024.

Broda, E. (1975). The history of inorganic nitrogen in the biosphere. Journal of molecular evolution, 7(1), 87-100.

Broeckling, C. D., Huhman, D. V., Farag, M. A., Smith, J. T., May, G. D., Mendes, P., ... & Sumner, L. W. (2005). Metabolic profiling of Medicago truncatula cell cultures reveals the effects of biotic and abiotic elicitors on metabolism. Journal of experimental botany, 56(410), 323-336.

Buchanan, B. B., Gruissem, W., & Jones, R. L. (Eds.). (2015). Biochemistry and molecular biology of plants. John wiley & sons.

Bykova, N. V., Møller, I. M., Gardeström, P., & Igamberdiev, A. U. (2014). The function of glycine decarboxylase complex is optimized to maintain high photorespiratory flux via buffering of its reaction products. Mitochondrion, 19, 357-364.

Cordell, D., Drangert, J. O., & White, S. (2009). The story of phosphorus: global food security and food for thought. Global environmental change, 19(2), 292-305.

Dahal, K., Martyn, G. D., & Vanlerberghe, G. C. (2015). Improved photosynthetic performance during severe drought in Nicotiana tabacum overexpressing a nonenergy conserving respiratory electron sink. New Phytologist, 208(2), 382-395.

Davidson, E. A., & Janssens, I. A. (2006). Temperature sensitivity of soil carbon decomposition and feedbacks to climate change. Nature, 440(7081), 165-173.

De Graaff, M. A., Van Groenigen, K. J., Six, J., Hungate, B., & van Kessel, C. (2006). Interactions between plant growth and soil nutrient cycling under elevated CO2: a meta-analysis. Global Change Biology, 12(11), 2077-2091.

de la Riva, E. G., Olmo, M., Poorter, H., Ubera, J. L., & Villar, R. (2016). Leaf mass per area (LMA) and its relationship with leaf structure and anatomy in 34 Mediterranean woody species along a water availability gradient. PloS one, 11(2), e0148788.

De Micco, V., & Aronne, G. (2012). Morpho-anatomical traits for plant adaptation to drought. In Plant responses to drought stress (pp. 37-61). Springer, Berlin, Heidelberg.

de Vries, J., Stanton, A., Archibald, J. M., & Gould, S. B. (2016). Streptophyte terrestrialization in light of plastid evolution. Trends in Plant Science, 21(6), 467-476.

Delfine, S., Loreto, F., & Alvino, A. (2001). Drought-stress effects on physiology, growth and biomass production of rainfed and irrigated bell pepper plants in the Mediterranean region. Journal of the American Society for Horticultural Science, 126(3), 297-304.

Del-Saz, N. F., Florez-Sarasa, I., Clemente-Moreno, M. J., Mhadhbi, H., Flexas, J., Fernie, A. R., & Ribas-Carbó, M. (2016). Salinity tolerance is related to cyanide-resistant alternative respiration in Medicago truncatula under sudden severe stress. Plant, Cell & Environment, 39(11), 2361-2369.

Del-Saz, N. F., Ribas-Carbo, M., McDonald, A. E., Lambers, H., Fernie, A. R., & Florez-Sarasa, I. (2018). An in vivo perspective of the role (s) of the alternative oxidase pathway. Trends in Plant Science, 23(3), 206-219.

Dijkstra, F. A., Pendall, E., Morgan, J. A., Blumenthal, D. M., Carrillo, Y., LeCain, D. R., ... & Williams, D. G. (2012). Climate change alters stoichiometry of phosphorus and nitrogen in a semiarid grassland. New Phytologist, 196(3), 807-815.

Ellsworth, D. S., Crous, K. Y., Lambers, H., & Cooke, J. (2015). Phosphorus recycling in photorespiration maintains high photosynthetic capacity in woody species. Plant, Cell & Environment, 38(6), 1142-1156.

Erb, T. J., & Zarzycki, J. (2018). A short history of RubisCO: the rise and fall (?) of Nature's predominant CO2 fixing enzyme. Current opinion in biotechnology, 49, 100-107.

Ethier, G. J., & Livingston, N. J. (2004). On the need to incorporate sensitivity to CO2 transfer conductance into the Farquhar–von Caemmerer–Berry leaf photosynthesis model. Plant, Cell & Environment, 27(2), 137-153.

Evans, J. R. (1989). Photosynthesis and nitrogen relationships in leaves of C 3 plants. Oecologia, 78(1), 9-19.

Evans, J. R. (2013). Improving photosynthesis. Plant physiology, 162(4), 1780-1793.

Farquhar, G. D., & Sharkey, T. D. (1982). Stomatal conductance and photosynthesis. Annual review of plant physiology, 33(1), 317-345.

Farquhar, G. D., von Caemmerer, S. V., & Berry, J. A. (1980). A biochemical model of photosynthetic CO 2 assimilation in leaves of C 3 species. Planta, 149(1), 78-90.

Finnegan, P. M., Soole, K. L., & Umbach, A. L. (2004). Alternative mitochondrial electron transport proteins in higher plants. In Plant mitochondria: from genome to function (pp. 163-230). Springer, Dordrecht.

Flexas, J., Díaz-Espejo, A., Conesa, M. A., Coopman, R. E., Douthe, C., Gago, J., ... & Niinemets, Ü. (2016). Mesophyll conductance to CO2 and Rubisco as targets for improving intrinsic water use efficiency in C3 plants. Plant, Cell & Environment, 39(5), 965-982.

Flexas, J., Diaz-Espejo, A., Gago, J., Gallé, A., Galmés, J., Gulías, J., & Medrano, H. (2014). Photosynthetic limitations in Mediterranean plants: a review. Environmental and Experimental Botany, 103, 12-23.

Flexas, J., Loreto, F., & Medrano, H. (Eds.). (2012). Terrestrial photosynthesis in a changing environment: a molecular, physiological, and ecological approach. Cambridge University Press.

Flexas, J., Ribas-Carbo, M., Diaz-Espejo, A, Galmes, J., & Medrano, H. (2008). Mesophyll conductance to CO2: current knowledge and future prospects. Plant, cell & environment, 31(5), 602-621.

Florez-Sarasa, I, Lambers, H., Wang, X., Finnegan, P. M., & Ribas-Carbó, M. (2014). The alternative respiratory pathway mediates carboxylate synthesis in white lupin cluster roots under phosphorus deprivation. Plant, Cell & Environment, 37(4), 922-928.

Florez-Sarasa, I., Fernie, A. R., & Gupta, K. J. (2020). Does the alternative respiratory pathway offer protection against the adverse effects resulting from climate change?. Journal of experimental botany, 71(2), 465-469.

Florez-Sarasa, I., Ribas-Carbo, M., Del-Saz, N. F., Schwahn, K., Nikoloski, Z., Fernie, A. R., & Flexas, J. (2016). Unravelling the in vivo regulation and metabolic role of the alternative oxidase pathway in C3 species under photoinhibitory conditions. New Phytologist, 212(1), 66-79.

Foyer, C. H., Noctor, G., & Hodges, M. (2011). Respiration and nitrogen assimilation: targeting mitochondria-associated metabolism as a means to enhance nitrogen use efficiency. Journal of Experimental Botany, 62(4), 1467-1482.

Galle, A., Florez-Sarasa, I., Aououad, H. E., & Flexas, J. (2011). The Mediterranean evergreen Quercus ilex and the semi-deciduous Cistus albidus differ in their leaf gas exchange regulation and acclimation to repeated drought and re-watering cycles. Journal of Experimental Botany, 62(14), 5207-5216.

Galmes, J., Flexas, J., Keys, A. J., Cifre, J., Mitchell, R. A., Madgwick, P. J., ... & Parry, M. A. (2005). Rubisco specificity factor tends to be larger in plant species from drier habitats and in species with persistent leaves. Plant, Cell & Environment, 28(5), 571-579.

Galmés, J., Medrano, H., & Flexas, J. (2007). Photosynthetic limitations in response to water stress and recovery in Mediterranean plants with different growth forms. New phytologist, 175(1), 81-93.

Galmés, J., Molins, A., Flexas, J., & Conesa, M. À. (2017). Coordination between leaf CO 2 diffusion and Rubisco properties allows maximizing photosynthetic efficiency in Limonium species. Plant, Cell & Environment, 40(10), 2081-2094.

Gandin, A., Denysyuk, M., & Cousins, A. B. (2014). Disruption of the mitochondrial alternative oxidase (AOX) and uncoupling protein (UCP) alters rates of foliar nitrate and carbon assimilation in Arabidopsis thaliana. Journal of experimental botany, 65(12), 3133-3142.

Gandin, A., Lapointe, L., & Dizengremel, P. (2009). The alternative respiratory pathway allows sink to cope with changes in carbon availability in the sink-limited plant Erythronium americanum. Journal of experimental botany, 60(15), 4235-4248.

Gomes, C. M., Le Gall, J., Xavier, A. V., & Teixeira, M. (2001). Could a diiron-containing four-helix-bundle protein have been a primitive oxygen reductase?. Chembiochem, 2(7-8), 583-587.

Gomez, F., & Amils, R. (2002). Evolution of microbial energy conservation: from chemolithotrophy to photosynthesis. In The Evolving Sun and its Influence on Planetary Environments (Vol. 269, p. 217).

Grassi, G., & Magnani, F. (2005). Stomatal, mesophyll conductance and biochemical limitations to photosynthesis as affected by drought and leaf ontogeny in ash and oak trees. Plant, Cell & Environment, 28(7), 834-849.

Hao, X., Cho, C. M., Racz, G. J., & Chang, C. (2002). Chemical retardation of phosphate diffusion in an acid soil as affected by liming. Nutrient Cycling in Agroecosystems, 64(3), 213-224.

Hassiotou, F., Ludwig, M., Renton, M., Veneklaas, E. J., & Evans, J. R. (2009). Influence of leaf dry mass per area, CO2, and irradiance on mesophyll conductance in sclerophylls. Journal of Experimental Botany, 60(8), 2303-2314.

Hassiotou, F., Renton, M., Ludwig, M., Evans, J. R., & Veneklaas, E. J. (2010). Photosynthesis at an extreme end of the leaf trait spectrum: how does it relate to high leaf dry mass per area and associated structural parameters?. Journal of Experimental Botany, 61(11), 3015-3028.

Heath, K. D., & Lau, J. A. 2011. Herbivores Alter The Fitness Benefits Of A Plant–Rhizobium Mutualism. Acta Oecologica, 37(2), 87-92.

Heldt, H. W., & Piechulla, B. (2021). Plant biochemistry. Academic Press.

Henriksson, N., Marshall, J., Lundholm, J., Boily, Å., Boily, J. F., & Näsholm, T. (2019). Improved in vivo measurement of alternative oxidase respiration in field-collected pine roots. Physiologia plantarum, 167(1), 34-47.

Hernández, G., Valdés-López, O., Ramírez, M., Goffard, N., Weiller, G., Aparicio-Fabre, R., ... & Vance, C. P. (2009). Global changes in the transcript and metabolic profiles during symbiotic nitrogen fixation in phosphorus-stressed common bean plants. Plant Physiology, 151(3), 1221-1238.

Huang, C. Y., Roessner, U., Eickmeier, I., Genc, Y., Callahan, D. L., Shirley, N., ... & Bacic, A. (2008). Metabolite profiling reveals distinct changes in carbon and nitrogen metabolism in phosphate-deficient barley plants (Hordeum vulgare L.). Plant and Cell Physiology, 49(5), 691-703.

Hungria, M., & Kaschuk, G. 2014. Regulation Of N2 Fixation And NO3−/NH4+ Assimilation In Nodulated And N-Fertilized Phaseolus Vulgaris L. Exposed To High Temperature Stress. Environmental And Experimental Botany, 98, 32-39.

Igamberdiev, A. U., & Kleczkowski, L. A. (2018). The glycerate and phosphorylated pathways of serine synthesis in plants: the branches of plant glycolysis linking carbon and nitrogen metabolism. Frontiers in Plant Science, 9, 318.

Israr, D., Mustafa, G., Khan, K. S., Shahzad, M., Ahmad, N., & Masood, S. (2016). Interactive effects of phosphorus and Pseudomonas putida on chickpea (Cicer arietinum L.) growth, nutrient uptake, antioxidant enzymes and organic acids exudation. Plant physiology and biochemistry, 108, 304-312.

Kaklamanos, G., Aprea, E., & Theodoridis, G. (2020). Mass spectrometry: Principles and instrumentation. In Chemical Analysis of Food (pp. 525-552). Academic Press.

Katerji, N., Mastrorilli, M., & Rana, G. (2008). Water use efficiency of crops cultivated in the Mediterranean region: review and analysis. European Journal of Agronomy, 28(4), 493-507.

Körner, C. (2015). Paradigm shift in plant growth control. Current opinion in plant biology, 25, 107-114.

Kramer, D. M., Avenson, T. J., & Edwards, G. E. (2004). Dynamic flexibility in the light reactions of photosynthesis governed by both electron and proton transfer reactions. Trends in plant science, 9(7), 349-357.

Krishnamurthy, A., & Rathinasabapathi, B. (2013). Oxidative stress tolerance in plants: novel interplay between auxin and reactive oxygen species signaling. Plant signaling & behavior, 8(10), e25761.

Krumova, K., & Cosa, G. (2016). Overview of reactive oxygen species.

Kubis, A., & Bar-Even, A. (2019). Synthetic biology approaches for improving photosynthesis. Journal of Experimental Botany, 70(5), 1425-1433.

Lagunas, B., Schäfer, P., & Gifford, M. (2015). Housing helpful invaders: the evolutionary and molecular architecture underlying plant root-mutualist microbe interactions. Journal of Experimental Botany, 66(8), 2177-2186.

Lambers, H & Oliveira, R.S. Plant Physiological Ecology. 2019. SpringerNature. Switzerland.

Lambers, H., & Plaxton, W. C. (2015). Phosphorus: back to the roots. Annual plant reviews, 48, 3-22.

Lambers, H., Martinoia, E., & Renton, M. (2015). Plant adaptations to severely phosphorus-impoverished soils. Current opinion in plant biology, 25, 23-31.

Landis, G. N., & Tower, J. (2005). Superoxide dismutase evolution and life span regulation. Mechanisms of ageing and development, 126(3), 365-379.

Lea, P. J., & Miflin, B. J. (2011). Nitrogen assimilation and its relevance to crop improvement. Annu. Plant Rev, 42, 1-40.

Li, G., Yang, D., & Sun, S. (2008). Allometric relationships between lamina area, lamina mass and petiole mass of 93 temperate woody species vary with leaf habit, leaf form and altitude. Functional Ecology, 22(4), 557-564

Maberly, S. C. (2014). The fitness of the environments of air and water for photosynthesis, growth, reproduction and dispersal of photoautotrophs: an evolutionary and biogeochemical perspective. Aquatic Botany, 118, 4-13.

Malhotra, H., Sharma, S., & Pandey, R. (2018). Phosphorus nutrition: plant growth in response to deficiency and excess. In Plant nutrients and abiotic stress tolerance (pp. 171-190). Springer, Singapore.

Matt, P., Geiger, M., Walch-Liu, P., Engels, C., Krapp, A., & Stitt, M. (2001). The immediate cause of the diurnal changes of nitrogen metabolism in leaves of nitrate-replete tobacco: a major imbalance between the rate of nitrate reduction and the rates of nitrate uptake and ammonium metabolism during the first part of the light period. Plant, Cell & Environment, 24(2), 177-190.

Maxwell, K., & Johnson, G. N. (2000). Chlorophyll fluorescence—a practical guide. Journal of experimental botany, 51(345), 659-668.

McDonald, A. E., & Vanlerberghe, G. C. (2006). Origins, evolutionary history, and taxonomic distribution of alternative oxidase and plastoquinol terminal

oxidase. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics, 1(3), 357-364.

Medrano, H., Flexas, J., & Galmés, J. (2009). Variability in water use efficiency at the leaf level among Mediterranean plants with different growth forms. Plant and Soil, 317(1), 17-29.

Medrano, H., Flexas, J., & Galmés, J. (2009). Variability in water use efficiency at the leaf level among Mediterranean plants with different growth forms. Plant and Soil, 317(1), 17-29.

Melillo, J. M., Steudler, P. A., Aber, J. D., Newkirk, K., Lux, H., Bowles, F. P., ... & Morrisseau, S. (2002). Soil warming and carbon-cycle feedbacks to the climate system. Science, 298(5601), 2173-2176.

Michaletz, S. T., Weiser, M. D., Zhou, J., Kaspari, M., Helliker, B. R., & Enquist, B. J. (2015). Plant thermoregulation: energetics, trait–environment interactions, and carbon economics. Trends in ecology & evolution, 30(12), 714-724.

Miller, A. J., & Cramer, M. D. (2005). Root nitrogen acquisition and assimilation. Plant and soil, 274(1), 1-36.

Muccio, Z., & Jackson, G. P. (2009). Isotope ratio mass spectrometry. Analyst, 134(2), 213-222.

Müller, P., Li, X. P., & Niyogi, K. K. (2001). Non-photochemical quenching. A response to excess light energy. Plant physiology, 125(4), 1558-1566.

Niinemets, Ü. (1999). Research review. Components of leaf dry mass per area–thickness and density–alter leaf photosynthetic capacity in reverse directions in woody plants. The New Phytologist, 144(1), 35-47.

Niinemets, Ü., Díaz-Espejo, A., Flexas, J., Galmés, J., & Warren, C. R. (2009). Role of mesophyll diffusion conductance in constraining potential photosynthetic productivity in the field. Journal of experimental botany, 60(8), 2249-2270.

Niinemets, Ü., Flexas, J., & Peñuelas, J. (2011). Evergreens favored by higher responsiveness to increased CO2. Trends in Ecology & Evolution, 26(3), 136-142.

Niinemets, Ü., Wright, I. J., & Evans, J. R. (2009b). Leaf mesophyll diffusion conductance in 35 Australian sclerophylls covering a broad range of foliage structural and physiological variation. Journal of experimental botany, 60(8), 2433-2449.

Noctor, G., & Foyer, C. H. (1998). A re-evaluation of the ATP: NADPH budget during C3 photosynthesis: a contribution from nitrate assimilation and its associated respiratory activity?. Journal of Experimental Botany, 49(329), 1895-1908.

Noguchi, K., & Yoshida, K. (2008). Interaction between photosynthesis and respiration in illuminated leaves. Mitochondrion, 8(1), 87-99.

Nogués, S., & Baker, N. R. (2000). Effects of drought on photosynthesis in Mediterranean plants grown under enhanced UV-B radiation. Journal of experimental botany, 51(348), 1309-1317.

Nunes-Nesi, A., Fernie, A. R., & Stitt, M. (2010). Metabolic and signaling aspects underpinning the regulation of plant carbon nitrogen interactions. Molecular plant, 3(6), 973-996.

Obata, T., & Fernie, A. R. (2012). The use of metabolomics to dissect plant responses to abiotic stresses. Cellular and Molecular Life Sciences, 69(19), 3225-3243.

Oliver, D. J., Neuburger, M., Bourguignon, J., & Douce, R. (1990). Glycine metabolism by plant mitochondria. Physiologia Plantarum, 80(3), 487-491.

Olson, J. M., & Blankenship, R. E. (2005). Thinking about the evolution of photosynthesis. Discoveries in Photosynthesis, 1073-1086.

Onoda, Y., Wright, I. J., Evans, J. R., Hikosaka, K., Kitajima, K., Niinemets, Ü., Westoby, M. (2017). Physiological and structural tradeoffs underlying the leaf economics spectrum. New Phytologist, 214(4), 1447-1463.

Otieno, N., Lally, R. D., Kiwanuka, S., Lloyd, A., Ryan, D., Germaine, K. J., & Dowling, D. N. (2015). Plant growth promotion induced by phosphate solubilizing endophytic Pseudomonas isolates. Frontiers in microbiology, 6, 745.

Peguero-Pina, J. J., Sisó, S., Flexas, J., Galmés, J., García-Nogales, A., Niinemets, Ü., ... & Gil-Pelegrín, E. (2017). Cell-level anatomical characteristics explain high mesophyll conductance and photosynthetic capacity in sclerophyllous Mediterranean oaks. New Phytologist, 214(2), 585-596.

Peterhansel, C., & Maurino, V. G. (2011). Photorespiration redesigned. Plant physiology, 155(1), 49-55.

Pickup, M., Westoby, M., & Basden, A. (2005). Dry mass costs of deploying leaf area in relation to leaf size. Functional Ecology, 19(1), 88-97.

Poorter, H., Niinemets, Ü., Poorter, L., Wright, I. J., & Villar, R. (2009). Causes and consequences of variation in leaf mass per area (LMA): a meta-analysis. New phytologist, 182(3), 565-588.

Renger, G. (2010). The light reactions of photosynthesis. Current Science, 1305-1319.

Ribas-Carbo, M., Berry, J. A., Yakir, D., Giles, L., Robinson, S. A., Lennon, A. M., & Siedow, J. N. (1995). Electron partitioning between the cytochrome and alternative pathways in plant mitochondria. Plant Physiology, 109(3), 829-837.

Ribas-Carbo, M., Robinson, S. A., & Giles, L. (2005). The application of the oxygen-isotope technique to assess respiratory pathway partitioning. In Plant Respiration (pp. 31-42). Springer, Dordrecht.

Richard-Molard, C.; Krapp, A.; Brun, F.; Ney, B.; Daniel-Vedele, F.; Chaillou, S. Plant response to nitrate starvation is determined by N storage capacity matched by nitrate uptake capacity in two Arabidopsis genotypes. J. Exp. Bot. 2008, 59, 779-791.

Roessner, U., Luedemann, A., Brust, D., Fiehn, O., Linke, T., Willmitzer, L., & Fernie, A. R. (2001). Metabolic profiling allows comprehensive phenotyping of genetically or environmentally modified plant systems. The Plant Cell, 13(1), 11-29.

Shane, M. W., Cramer, M. D., Funayama-Noguchi, S., Cawthray, G. R., Millar, A. H., Day, D. A., & Lambers, H. (2004). Developmental physiology of cluster-root carboxylate synthesis and exudation in harsh hakea. Expression of phospho enol pyruvate carboxylase and the alternative oxidase. Plant Physiology, 135(1), 549-560.

Sieger, S. M., Kristensen, B. K., Robson, C. A., Amirsadeghi, S., Eng, E. W., Abdel-Mesih, A., ... & Vanlerberghe, G. C. (2005). The role of alternative oxidase in modulating carbon use efficiency and growth during macronutrient stress in tobacco cells. Journal of Experimental Botany, 56(416), 1499-1515.

Sunil, B., Saini, D., Bapatla, R. B., Aswani, V., & Raghavendra, A. S. (2019). Photorespiration is complemented by cyclic electron flow and the alternative oxidase pathway to optimize photosynthesis and protect against abiotic stress. Photosynthesis research, 139(1), 67-79.

Sweetlove, L. J., Beard, K. F., Nunes-Nesi, A., Fernie, A. R., & Ratcliffe, R. G. (2010). Not just a circle: flux modes in the plant TCA cycle. Trends in plant science, 15(8), 462-470.

Tcherkez, G. (2013). Modelling the reaction mechanism of ribulose-1, 5-bisphosphate carboxylase/oxygenase and consequences for kinetic parameters. Plant, cell & environment, 36(9), 1586-1596.

Tcherkez, G., Boex-Fontvieille, E., Mahé, A., & Hodges, M. (2012). Respiratory carbon fluxes in leaves. Current opinion in plant biology, 15(3), 308-314.

Terashima, I., Hanba, Y. T., Tholen, D., & Niinemets, Ü. (2011). Leaf functional anatomy in relation to photosynthesis. Plant physiology, 155(1), 108-116.

Thauer, R. K. (2007). A fifth pathway of carbon fixation. Science, 318(5857), 1732-1733.

Tholen, D., Ethier, G., Genty, B., Pepin, S., & ZHU, X. G. (2012). Variable mesophyll conductance revisited: theoretical background and experimental implications. Plant, Cell & Environment, 35(12), 2087-2103.

Tomás, M., Flexas, J., Copolovici, L., Galmés, J., Hallik, L., Medrano, H., ... & Niinemets, Ü. (2013). Importance of leaf anatomy in determining mesophyll diffusion conductance to CO2 across species: quantitative limitations and scaling up by models. Journal of experimental botany, 64(8), 2269-2281.

Tomaszek, J. A. (2005). Automated Analysis of Stable Isotopes of H, C, N, O and S by Isotope Ratio Mass Spectrometry. Chemistry for the Protection of the Environment 4, 151-168.

Trethewey, R. N. (2004). Metabolite profiling as an aid to metabolic engineering in plants. Current opinion in plant biology, 7(2), 196-201.

Turner, N. C., & Asseng, S. (2005). Productivity, sustainability, and rainfall-use efficiency in Australian rainfed Mediterranean agricultural systems. Australian Journal of Agricultural Research, 56(11), 1123-1136.

Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. The Journal of physiology, 552(2), 335-344.

Van Oijen, M., Schapendonk, A., & Höglind, M. (2010). On the relative magnitudes of photosynthesis, respiration, growth and carbon storage in vegetation. Annals of Botany, 105(5), 793-797.

Vance, C. P., & Lamb, J. F. S. (2001). Application of biochemical studies to improving nitrogen fixation. Australian journal of experimental agriculture, 41(3), 403-416.

Vance, C. P., Uhde-Stone, C., & Allan, D. L. (2003). Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. New phytologist, 157(3), 423-447.

Vanlerberghe, G. C. (2013). Alternative oxidase: a mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. International journal of molecular sciences, 14(4), 6805-6847.

Vanlerberghe, G. C., Dahal, K., & Chadee, A. (2019). Does the stromal concentration of Pi control chloroplast ATP synthase protein amount in contrasting growth environments?. Plant signaling & behavior, 14(12), 1675473.

Vanlerberghe, G. C., Dahal, K., Alber, N. A., & Chadee, A. (2020). Photosynthesis, respiration and growth: A carbon and energy balancing act for alternative oxidase. Mitochondrion, 52, 197-211.

Wright, I. J., Reich, P. B., Westoby, M., Ackerly, D. D., Baruch, Z., Bongers, F., ... & Villar, R. (2004). The worldwide leaf economics spectrum. Nature, 428(6985), 821-827.

Yoshida, K., Terashima, I., & Noguchi, K. (2006). Distinct roles of the cytochrome pathway and alternative oxidase in leaf photosynthesis. Plant and Cell Physiology, 47(1), 22-31.

Yuan, Z. Y., & Chen, H. Y. (2015). Decoupling of nitrogen and phosphorus in terrestrial plants associated with global changes. Nature Climate Change, 5(5), 465-469.