Universidade de São Paulo
Instituto de Biociências

Priscila Pires Bittencourt

# Influência da disponibilidade hídrica sobre o comportamento fotossintético de *Portulaca oleracea* L.

# Influence of water availability on the photosynthetic behavior of *Portulaca oleracea* L.

São Paulo
2018

Priscila Pires Bittencourt

# Influência da disponibilidade hídrica sobre o comportamento fotossintético de *Portulaca oleracea* L.

# Influence of water availability on the photosynthetic behavior of *Portulaca oleracea* L.

Dissertação apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de título de Mestre em Ciências, na área de Botânica.
Orientador: Dr. Luciano Freschi

São Paulo
2018

# Ficha Catalográfica

Bittencourt, Priscila Pires
Influência da disponibilidade hídrica sobre o comportamento fotossintético de *Portulaca oleracea* L.

Número de páginas: 53
Dissertação (Mestrado em Ciências) – Programa de Pós-Graduação em Botânica, Departamento de Botânica, Instituto de Biociências, Universidade de São Paulo, São Paulo, 2018.

1.Eficiência no uso da água. 2.Estresse hídrico. 3.Fotossíntese. 4.Metabolismo ácido das Crassuláceas. 5.*Portulaca*. Universidade de São Paulo. Instituto de Biociências. Departamento de Botânica.

# Comissão Julgadora

_____ _____

Prof(a). Dr(a). Prof(a). Dr(a).

_____

Prof. Dr. Luciano Freschi
Orientador

# RESUMO

BITTENCOURT, Priscila Pires. Influência da disponibilidade hídrica sobre o comportamento fotossintético de *Portulaca oleracea* L. 2018. 53f. Dissertação (Mestrado em Ciências – Área Botânica) – Instituto de Biociências, Universidade de São Paulo, São Paulo, 2018.

O metabolismo ácido das crassuláceas (CAM) e a via $C_4$ são adaptações fotossintéticas que melhoram significativamente a eficiência no uso da água (WUE). Esses dois mecanismos concentradores de $CO_2$ (CCMs) compartilham semelhanças, incluindo a pré-fixação de $CO_2$ na forma de ácidos orgânicos através da atividade da enzima fosfo*enol*piruvato carboxilase (PEPC) e a subsequente descarboxilação desses ácidos próximos ao sítio ativo da Rubisco. A ocorrência simultânea de ambos os CCMs no mesmo tecido é vista como bioquimicamente conflitante; no entanto, a existência de espécies de *Portulaca* capazes de alternar entre as vias $C_4$ e CAM numa mesma folha desafia essa potencial incompatibilidade. Ao monitorar as características anatômicas, os parâmetros fotossintéticos, o acúmulo noturno de ácidos e a abundância de transcritos de genes relacionados aos comportamentos $C_4$ e CAM, o presente estudo buscou caracterizar as mudanças induzidas pela seca na expressão do CAM em tecidos foliares e caulinares de *Portulaca oleracea*. Enquanto a ocorrência do CAM nas folhas parece ocorrer de modo totalmente facultativo, tanto fatores ontogenéticos quanto ambientais parecem controlar a expressão desse comportamento fotossintético nos caules. Os dados revelaram que, dependendo das condições ambientais e da idade das plantas, os caules de *P. oleracea* podem realizar $C_3$ ou CAM, mas não a fotossíntese $C_4$. As análises de trocas gasosas e de fluorescência da clorofila *a* nas folhas das plantas submetidas à seca sugerem que a descarboxilação diurna dos ácidos orgânicos acumulados durante a noite forneceria $CO_2$ à Rubisco em níveis suficientes para manter a integridade e funcionamento do aparato fotossintetizante mesmo após exposição prolongada à seca. Em geral, nenhuma alteração anatômica marcante foi observada nas folhas ou caules durante a indução do CAM, sugerindo que as reprogramações da expressão gênica e do metabolismo respondem pela maior parte das mudanças associadas à transição de $C_4$ para CAM e de $C_3$ para CAM nas folhas e caules, respectivamente. Dados de expressão gênica também indicaram que a transição entre as vias $C_3$ e CAM nos caules requer alterações no perfil transcricional de um conjunto mais numeroso de genes relacionados aos CCMs do que a transição de $C_4$ para CAM nos tecidos foliares. Em conjunto, as dados obtidos revelam uma notável plasticidade fotossintética em *P. oleracea* e fornecem pistas importantes sobre os mecanismos responsáveis pela compatibilidade entre as vias $C_4$ e CAM nesta espécie vegetal.

**Palavras-Chave:** Eficiência no uso da água. Estresse hídrico. Fotossíntese. Metabolismo ácido das Crassuláceas. *Portulaca*.

# ABSTRACT

BITTENCOURT, Priscila Pires. Influence of water availability on the photosynthetic behavior of *Portulaca oleracea* L. 2018. 53p. Thesis (Master in Science – Botany) – Bioscience Institute, University of Sao Paulo, Sao Paulo, 2018.

Crassulacean acid metabolism (CAM) and the $C_4$ pathway are photosynthetic adaptations that significantly improve plant water use efficiency (WUE). These two $CO_2$-concentrating mechanisms (CCMs) share many similarities, including the pre-fixation of $CO_2$ as organic acids via phospho*enol*pyruvate carboxylase (PEPC) and the subsequent decarboxylation of these acids near the active site of Rubisco. The simultaneous occurrence of both CCMs in the same tissues has long been regarded as biochemically conflicting; however, the existence of *Portulaca* species that can switch between $C_4$ and CAM pathways within a single leaf challenges this assumption. By monitoring anatomical traits, photosynthetic parameters, nocturnal acid accumulation and transcript abundance of $C_4$- and CAM-related genes, this study aimed to characterize the drought-induced changes in CAM expression in both leaf and stem tissues of *Portulaca oleracea*. CAM was shown to be expressed in an entirely facultative fashion in leaves whereas both ontogenetic and environmental cues seem to control CAM induction in the stem tissues. Data revealed that depending on the environmental conditions and plant age, *P. oleracea* stems can perform either $C_3$ or CAM, but not $C_4$ photosynthesis. Gas exchange and chlorophyll *a* fluorescence analysis suggested that the daytime decarboxylation of the organic acids accumulated overnight in CAM-performing leaves supplied $CO_2$ to Rubisco behind closed stomata at sufficient levels to maintain the photosynthetic apparatus integrity and functioning even after prolonged drought exposure. Overall, no marked anatomical changes were observed in *P. oleracea* leaves or stems during the CAM induction, suggesting that gene expression and metabolism reprogramming may account for most of the $C_4$-to-CAM and $C_3$-to-CAM transition in leaves and stems, respectively. Gene expression data also indicated that the switch between $C_3$ and CAM pathways in the stems requires the transcriptional regulation of a more extensive set of CCM-related genes than the $C_4$-to-CAM transition in the leaves. Altogether, our findings reveal a remarkable photosynthetic plasticity in *P. oleracea* and provide important clues about the mechanisms behind the compatibility between the $C_4$ and CAM pathways in this plant species.

**Key-words:** Water use efficiency. Drought stress. Photosynthesis. Crassulacean acid metabolism. *Portulaca*.

# 1. Introduction

**Photosynthesis in plants**

Photosynthesis is arguably one the most important biochemical processes on the planet. It initiates in the thylakoid membrane of the chloroplasts with the conversion of light energy into chemical energy in the form of two energy-transporting molecules, ATP and NADPH. The energy stored in ATP and NADPH molecules is subsequently used in a series of reactions in the stroma known as the $C_3$ cycle, the Calvin-Benson cycle or the photosynthetic carbon reduction cycle (PCR cycle), which is responsible for the fixation and reduction of $CO_2$ into sugars. The cycle begins when $CO_2$ is combined with a five-carbon acceptor molecule, 1,5-ribulose bisphosphate (RuBP), generating an unstable six-carbon molecule that splits into two molecules of a three-carbon compound, 3-phosphoglycerate (3-PGA). This reaction is catalyzed by RuBP carboxylase/oxygenase (Rubisco), a key enzyme abundantly found in all photosynthetic organisms. Subsequent reactions in the Calvin cycle lead to the conversion of 3-PGA into trioses phosphate as well as the regeneration of RuBP (Whitney *et al.*, 2011; Raven and Beardall, 2016).

Importantly, Rubisco catalyzes either the carboxylation or oxygenation of RuBP depending upon the relative molecular concentration of carbon dioxide or oxygen near the enzyme active site. Under low $CO_2$ availability, Rubisco oxygenase activity is intensified resulting in the production of PGA and phosphoglycolate (PG), the latter being a toxic molecule capable of inhibiting photosynthesis. Whereas PGA can be recycled through the Calvin cycle, PG is converted to PGA via a coordinated series of chemical reactions in chloroplasts, peroxisomes and mitochondria (Bauwe *et al.*, 2010; Sage *et al.*, 2012). The formation and recycling of PG, known as photorespiration, can significantly limit carbon gain in plants performing $C_3$ photosynthesis, particularly under environmental conditions such as water deficit, when $CO_2$ concentration inside the leaves decline due to the reduced stomatal conductance. At current atmospheric levels of $CO_2$ and $O_2$, it is estimated that photorespiration dissipates about 25% of the organic carbon initially fixed in $C_3$ plants (Peterhansel and Maurino, 2011). Therefore, photorespiration is interpreted as wasteful process and is very likely a vestige of the high $CO_2$ and $O_2$ atmospheric concentrations (~1,000 ppm) under which the Rubisco-mediated $CO_2$ fixation initially evolved (~3.5 billion years ago) (Sage, 2004).

Over the last 40 million years ago (Mya) until the beginning of modern human era,

atmospheric levels of $CO_2$ have considerably declined (Zhang *et al.*, 2013) and this environmental change has been advocated as a major evolutive driver for terrestrial plants evolving innovative photosynthetic pathways capable of withstanding the atmospheric decline in $CO_2$ concentration. These adaptive traits are known as $CO_2$-concentrating mechanisms (CCMs), and in land plants primarily comprise the $C_4$ and the Crassulacean acid metabolism (CAM). $C_4$ and CAM plants evolved from $C_3$ ancestors, recruiting existing genes involved in anaplerotic functions to play photosynthesis-related roles (Sage, 2002; Edwards and Ogburn, 2012). Both photosynthetic behaviors independently evolved several times, with CAM occurring first whereas $C_4$ probably evolved more recently (Silvera *et al.*, 2010; Edwards and Ogburn, 2012).

**$C_4$ photosynthesis**

$C_4$ photosynthesis evolved independently more than 60 times in 19 different Angiosperm families (Sage *et al.*, 2011, 2014). The evolution of the $C_4$ metabolism involves a series of biochemical and metabolic modifications, including increased photosynthetic capacity, repositioning of the organelles, changes in enzymatic kinetics and regulatory properties, redistribution of enzymes within the tissues, among others (Sage *et al.*, 2011, 2012). Moreover, in the so-called dual-cell $C_4$ plants, the evolution of $C_4$ also involved relevant structural changes, including the development of a inner ring of bundle sheath cells (BSCs) around vascular tissues and an outer layer of mesophyll cells (MCs) in contact with the epidermis (*i.e.* Kranz anatomy) (Sage, 2004; Voznesenskaya *et al.*, 2010). However, relatively rare examples of single-cell $C_4$ species have also been found both in aquatic (e.g. *Hydrilla verticillata*, *Egeria densa*) and terrestrial environments (e.g. *Borszczowia aralocaspica*, *Bienertia cycloptera*), which are able to concentrate $CO_2$ at the active site of Rubisco due to a polarized distribution of the organelles within each photosynthetic cell (Sage, 2002; Voznesenskaya *et al.*, 2002; Edwards *et al.*, 2004).

In dual-cell $C_4$ plants, $CO_2$ is converted to bicarbonate ($HCO_3^-$) via carbonic anhydrase (CA) before being fixed by phospho*enol*pyruvate carboxylase (PEPC) in the MCs. The carboxylation catalyzed by PEPC consumes phospho*enol*pyruvate (PEP) and $HCO_3^-$ to form oxaloacetate (OAA) and inorganic phosphate (Pi), and this reaction is facilitated by the high affinity of PEPC for $CO_2$ (~60 times higher than that of Rubisco) (Edwards *et al.*, 2004). The OAA formed is converted to a four-carbon acid (malate or aspartate), and these acids are transported to the BSCs, where they are decarboxylated via NAD(P) malic enzyme (NAD-ME and/or NADP-ME), and the $CO_2$ released is refixed by Rubisco, initiating the Calvin cycle. The pyruvate (Pyr)

formed during the decarboxylation process is transported to the chloroplast of MCs, where it becomes the substrate for the enzyme pyruvate orthophosphate dikinase (PPDK) to regenerate PEP, restarting the $C_4$ cycle (Fig. 1) (Voznesenskaya *et al.*, 2002; Sage, 2004).

This spatial separation between the sites of $CO_2$ pre-fixation via PEPC and the $CO_2$ assimilation site via Rubisco provided by the Kranz anatomy results in a higher concentration of $CO_2$ near the active site of Rubisco, thereby minimizing the occurrence of photorespiration and facilitating high rates of atmospheric $CO_2$ fixation in hot and moderately dry environmental conditions (Kraybill and Martin, 1996*;* Kanai and Edwards, 1999). In agreement, $C_4$ plants are predominantly found in tropical, subtropical and hot temperate regions (Edwards *et al.*, 2010).

In line with the multiple evolutive origins of $C_4$, considerable structural, metabolic and physiological diversity can be observed among $C_4$ plants. About 22 types of Kranz anatomy variants have already been described (Freitag and Stichler, 2000; Sage *et al.*, 2011; Kadereit *et al.*, 2013; Voznesenskaya *et al.*, 2017); the atriplicoid-type anatomy, which exhibits a complete sheath around the bundles, being the most commonly found (Sage *et al.*, 2011).

In terms of decarboxylation system, two major $C_4$ types have been described: the NADP-ME and the NAD-ME types. In the NADP-ME type, the OAA produced in the MCs is reduced to malate and transported to BSC chloroplasts, where the decarboxylation takes place releasing pyruvate. NADP-ME is the primary decarboxylation system in about 43 $C_4$ lineages, including all major high-productive monocot crops such as maize (*Zea mays*), sugarcane (*Saccharum* spp.) and sorghum (*Sorghum bicolor*) (Sage, 2004; Muhaidat *et al.*, 2007). Conversely, in the NAD-ME type, most OAA is transaminated to aspartate in the cytoplasm and transported to BSC mitochondria, where the decarboxylation takes place releasing pyruvate, which in turn is transaminated to alanine (Kanai R. and Edwards, 1999; Furbank, 2011) Species from 20 lineages, mostly eudicots, utilizes NAD-ME as the major decarboxylation system, including *Cleome* species (Brautigam *et al.*, 2011), *Amaranthus hypochondriacum* (Long *et al.*, 1994), among others. A third decarboxylation enzyme, the phospho*enol*pyruvate carboxykinase (PEPCK), is usually described as playing an accessory role rather than being the exclusive decarboxylation system in $C_4$ plants, therefore coexisting with either NADP-ME or NAD-ME enzymes (Muhaidat *et al.*, 2007; Bräutigam *et al.*, 2014; Wang *et al.*, 2014).

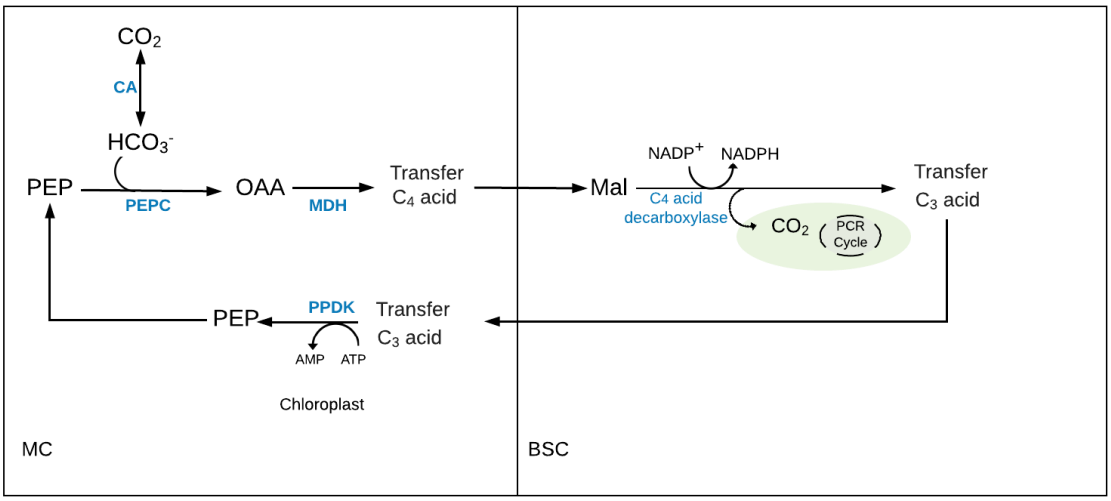


**Figure 1: A simplified view of the $C_4$ pathway.** In mesophyll cell (MC), $CO_2$ is converted to bicarbonate ($HCO_3^-$) by carbonic anhydrase (CA). $HCO_3^-$ is then combined to phospho*enol*pyruvate (PEP) by PEP carboxylase (PEPC) to form oxaloacetate (OAA), which is converted into another 4-C organic acid (malate or aspartate) that is shuttled to bundle sheath cells (BSC). Decarboxylation of the 4-C organic acid in the BSC releases $CO_2$ that initiates the photosynthetic carbon reduction cycle (PCR cycle). The 3-C organic acid released from the decarboxylation is transferred to the MCs, where it is phosphorylated to PEP by pyruvate phosphate dikinase (PPDK).

Comparative studies performed in $C_3$ and $C_4$ species of *Flaveria* or *Cleome* revealed that virtually all enzymes required for the $C_4$ pathway are present in $C_3$ plants (Brautigam *et al.*, 2011). Therefore, increased attention has been devoted to understanding how these genes were recruited as such knowledge may significantly facilitate the current endeavors of engineering $C_4$ into $C_3$ species (Aubry *et al.*, 2011). Besides possessing both $C_3$ and $C_4$ species, the genus *Flaveria* also comprises some $C_3$–$C_4$ intermediate species, which have also been intensively studied as a means to develop hypotheses about the evolution and functioning of $C_4$ photosynthesis (McKown *et al.*, 2005; Vogan *et al.*, 2007).

**Crassulacean acid metabolism (CAM)**

CAM photosynthesis is particularly frequent among vascular plants inhabiting arid and semiarid environments, being found in approximately 16,800 species of 343 genera in 34 families, including angiosperms (monocotyledons and eudicots) as well as gymnosperms (Winter and Holtum, 2002; Silvera *et al.*, 2010). Several commercially important species, such as pineapple (*Ananas comosus*), agaves (*Agave* spp.), cactus (Cactaceae), and orchids (Orchidaceae), perform CAM photosynthesis and are recognized by their high water use efficiency (WUE) and remarkable

capacity to grow under relatively dry conditions (Cushman, 2001).

In CAM plants, stomata are typically open during the night, when $CO_2$ pre-fixation via PEPC generates OAA, which is converted to malate by cytosolic malate dehydrogenase (MDH) and subsequently accumulated overnight in the vacuole. During the day, malate is transported out of the vacuoles, where it is decarboxylated by a cytosolic NADP-ME or mitochondrial NAD-ME, producing $CO_2$ for subsequent fixation via Rubisco behind closed stomata (Cushman and Borland, 2002; Lüttge, 2004) (Fig. 2). Since the atmospheric $CO_2$ uptake in CAM plants usually takes place in moments of lower evaporative demand (i.e., nighttime, dawn and dusk), fewer water molecules are lost through transpiration per carbon molecule fixed, thus providing significantly higher WUE values than $C_4$, and particularly $C_3$ plants (Osborne and Sack, 2012).

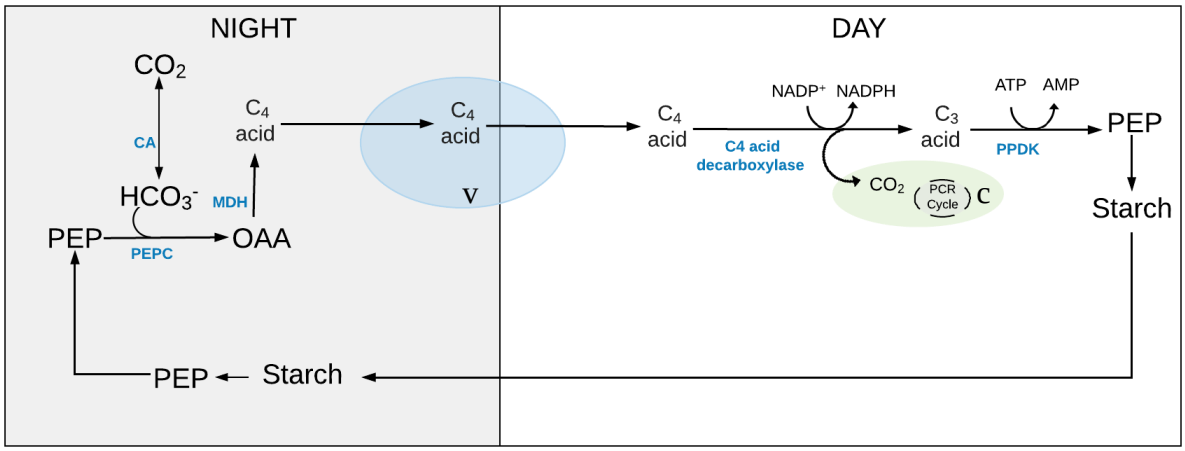


**Figure 2: A simplified view of the CAM pathway.** During the night, $CO_2$ is captured as bicarbonate ($HCO_3^-$) by phospho*enol*pyruvate carboxylase (PEPC) originating a $C_4$ acid (usually malate), which is accumulated overnight in the vacuole. During daytime, the $C_4$ acid leaves the vacuoles and is decarboxylated, releasing $CO_2$ to initiate the photosynthetic carbon reduction cycle (PCR cycle) in the chloroplast. The pyruvate (Pyr) released at the decarboxylation step is converted to triose phosphate and subsequently into starch. During the night, starch degradation contributes to the formation of PEP via pyruvate phosphate dikinase (PPDK) to reinitiate the CAM cycle. V, vacuole; C, chloroplast.

CAM photosynthesis may vary depending on the species and environmental conditions, operating in different modes according to the stomatal behavior and acid accumulation patterns (Cushman, 2001). In most CAM plants, stomata are open at night and closed during the day, and large amounts of organic acids are accumulated and consumed during the night and daytime, respectively. Representatives of this classic mode of CAM functioning include numerous species

belonging to Crassulaceae (e.g., *Kalanchoë daigremontiana*), Cactaceae (e.g., *Opuntia basilaris* and *Opuntia ficus-indica*), Bromeliaceae (e.g., *Bromelia humilis*) and Orchidaceae (e.g., *Schomburgkia humboldtiana*) families (Winter and Smith, 1996; Lüttge, 2004; Winter *et al.*, 2008).

In contrast, the so-called CAM cycling species exhibit daytime stomata opening as observed in $C_3$ plants while showing nocturnal acid accumulation derived from the refixation of nocturnally respired $CO_2$ (Herrera, 2009). When challenged with extreme drought conditions, CAM plants can also engage in a 'emergency mode' known as CAM idling, in which stomata remain closed 24 hours per day but a small diurnal fluctuation in organic acids remains due to respiratory $CO_2$ refixation (Lüttge, 2006; Freschi *et al.*, 2010). CAM idling mode does not allow additional carbon gain and consequently cannot support plant growth; however, it provides an efficient mechanism to recycle respiratory $CO_2$ behind closed stomata, thus minimizing water loss and potentially favoring a faster recovery in atmospheric $CO_2$ capture when environmental conditions become suitable to plant growth (Kraybill and Martin, 1996; Herrera, 2009).

Moreover, significant plasticity in CAM expression can be observed both within and between species. In recent years, CAM plasticity has been interpreted as a continuum trait, with constitutive plants exhibiting the lowest plasticity in CAM expression and facultative species at the opposite end. In adult constitutive plants, CAM photosynthesis is expressed even when environmental conditions are conducive to daytime $CO_2$ uptake. In contrast, the CAM behavior is an option rather than the mandatory carbon fixation mechanism in facultative species (Silvera *et al.*, 2010; Winter and Holtum, 2014). In facultative CAM plants, mature tissues can freely cycle between CAM and $C_3$ (or $C_4$) photosynthesis depending on the environmental conditions, frequently being promoted by drought and/or salinity (Winter and Holtum, 2014).

The induction of CAM in facultative species is one of the most complex metabolic adaptative responses against drought, involving extensive changes in gene expression, metabolic fluxes and stomatal behavior (Cushman and Borland, 2002). Recent studies have increasingly described facultative CAM species in plant families as diverse as Aizoaceae (*Mesembryanthemum crystallinum*), Bromeliaceae (*Guzmania monostachia*), Clusiaceae (*Clusia cylindrica*, *C. minor*, and *C. pratensis*), Talinaceae (*Talinum triangulare* ), Piperaceae (*Peperomia scandens*), Portulacaceae (*Portulaca oleracea*), among others (Medina *et al.*, 1977; Koch and Kennedy, 1982; Herrera *et al.*, 1991; Holtum *et al.*, 2004; Winter *et al.*, 2008, 2009; Winter and Holtum, 2014).

Facultative CAM has been interpreted as particularly advantageous for annual species of semi-arid regions and/or environments characterized by a wet season followed by a period of drought (Lüttge, 2004). Under wet conditions, these plants can perform $C_3$ (or $C_4$) photosynthesis favoring fast and efficient growth, whereas a slow growth mode associated with reduced water loss can be maintained through CAM under water limiting conditions (Holtum *et al.*, 2018).

$C_3$-CAM facultative plants have received considerably more attention than $C_4$-CAM species most likely due to the more limited occurrence of the later. Currently, only six species have been shown to perform facultative $C_4$-CAM photosynthesis, all of them belonging to the genus *Portulaca* (*P. oleracea, P. grandiflora, P. australis, P. pilosa, P. cyclophylla* and *P. digyna*) (Koch and Kennedy, 1980, 1982; Ku *et al.*, 1981; Guralnick and Jackson, 2001; Guralnick *et al.*, 2002; Holtum *et al.*, 2017). More extensive screening is still required to evaluate whether other $C_4$ species may also be able to display CAM-like traits when challenged with limitations in the water supply or other environmental stresses.

**$C_4$ and CAM: similarities and differences**

$C_4$ and CAM behaviors share many similarities, including the pre-fixation of $CO_2$ as organic acids via PEPC and the subsequent decarboxylation of these acids near the active site of Rubisco. However, whereas the PEPC- and Rubisco-mediated carboxylation in $C_4$ plants take place in different compartments (MCs and BSCs, respectively), the separation between both carboxylation systems in CAM plants is rather temporal (i.e., PEPC at night and Rubisco during the day, both in the MCs). In both cases, an efficient accumulation of internal $CO_2$ is achieved, as CAM plants concentrate $CO_2$ in the dark in the form of acids whereas $C_4$ species concentrate $CO_2$ in the BSCs during the day.

Although the $C_4$ and CAM behaviors share similarities, the simultaneous occurrence of both CCMs in the same tissue has been regarded by some authors as biochemically conflicting (Sage, 2002). As described by Sage (2002), these two syndromes should be considered as incompatible because they present a differential regulation in the enzymatic activation of carboxylation and decarboxylation steps; metabolic transport dynamics and different structural arrangements for each syndrome (Kranz anatomy in $C_4$ and succulence in CAM). However, the existence of *Portulaca* species that can perform both $C_4$ and CAM photosynthesis within a single leaf indicates that both these photosynthetic adaptations can operate in the same tissues, probably

depending on additional layers of control of enzymes as well as transport and regulatory proteins. Therefore, investigating these species may provide important clues on how both syndromes can co-exist within a single individual, potentially opening up a window of opportunities for the future engineering of crops constitutive or facultatively expressing both CCMs.

### *Portulaca oleracea* as a model system for investigating $C_4$/CAM compatibility

In the order Caryophyllales, eight families present $C_4$ photosynthesis (*i.e.* Aizoaceae, Amaranthaceae, Caryophyllaceae, Gisekiaceae, Molluginaceae, Nyctaginaceae, Polygonaceae, and Portulacaceae) and six families present CAM (*i.e.* Anacampserotaceae, Cactaceae, Didiereaceae, Montiaceae, Portulacaceae and Talinaceae) (Sage, 2004; Kluge and Ting, 1978; Richards, 1915; Winter, 1979; von Willert *et al*., 1992; Guralnick and Jackson, 2001; Koch and Kennedy, 1980; Martin and Zee, 1983). Both facultative and constitutive CAM have been reported in Montiaceae and Talinaceae (Herrera *et al.*, 1991; Winter and Holtum, 2011) whereas Portulacaceae contains the only known $C_4$-CAM species, all of which expressing CAM in a facultative fashion (Holtum *et al.*, 2017; Winter and Holtum, 2017)

Among the $C_4$-CAM *Portulaca* species, *P. oleracea* (commonly known as purslane) has received relatively more attention, showing drought-induced CAM expression in both leaves and stems (Mazen, 1996; Guralnick and Jackson, 2001; Guralnick *et al.*, 2002; Christin *et al.*, 2014; Winter and Holtum, 2014). This fast-growing herbaceous weed displays small succulent leaves with atriplicoid-type Kranz anatomy and large water storage cells (WSCs), exhibiting NAD-ME-type $C_4$ photosynthesis under well-watered conditions (Lara *et al.*, 2003, 2004; Voznesenskaya *et al.*, 2010; Ocampo *et al.*, 2013). Curiously, *P. oleracea* stems also present photosynthetically active cells, but lack Kranz anatomy traits, very likely performing $C_3$ photosynthesis under well-watered conditions (Kraybill and Martin, 1996; Voznesenskaya *et al.*, 2010). Therefore, this species seems to perform facultative $C_4$-CAM in the leaves and $C_3$-CAM in the stems, thereby representing a particularly interesting system for investigating the genetic, biochemical and physiological mechanisms behind such remarkable photosynthetic plasticity (Kraybill and Martin, 1996; Mazen, 1996; Lara *et al.*, 2003, 2004).

In one of the first studies on the occurrence of CAM behavior in this species, Kraybill and Martin (1996) found a significant nocturnal accumulation of titratable acidity under water deficit conditions. Distinct kinetic properties of PEPC in $C_4$- and CAM-performing *P. oleracea* were later

demonstrated, suggesting that specific PEPC isoforms would be expressed in each of these two photosynthetic behaviors (Mazen, 1996; 2000) In line with this, Christin *et al.* (2014) identified two *PPC* genes differentially expressed in well-watered and droughted *P. oleracea* plants.

Immunolocalization data obtained by using an antibody against maize PEPC, suggested that PEPC may be expressed both in MCs and WSCs of both well-watered and droughted plants (Guralnick *et al.*, 2002; Lara *et al.*, 2003, 2004). Moreover, Rubisco was shown to remain in the BSCs even after the drought-induced $C_4$-to-CAM transition, thereby implicating that an alternative model of CAM functioning may take place in *Portulaca* leaves. Based on this set of evidence, two distinct hypothetical models have been proposed for the $C_4$-CAM system in *Portulaca* leaves: (1) during CAM induction, acids accumulated overnight in both MCs and WSCs are shutlled to the BSCs for decarboxylation, which would caracterized an alternative, two-cell CAM system, herein addressed as a '$C_4$-CAM hybrid' system (Lara *et al.*, 2003, 2004); and, (2) CAM could occur independently of the $C_4$ pathway, operating as a typical single-cell CAM system in MCs and WSCs relaying on very low Rubisco activity levels in those cells (Guralnick *et al.*, 2002). It is important to highlight that both these hypotheses still lack conclusive evidence; therefore, additional information is needed to clarify how a '$C_4$-CAM hybrid' system can work in plants. Thus, *P. oleracea* offers us an opportunity to investigate in greater depth the biochemical, physiological, genetic and regulatory aspect associated with the occurrence of a '$C_4$-CAM hybrid' system in plants, potentially enabling the future application of this knowledge for the genetic improvement of species of agronomic interest.

# 6. Conclusion

Altogether, our findings reveal a remarkable photosynthetic plasticity in *P. oleracea* and start to elucidate the key components responsible for the compatibility between the $C_4$ and CAM pathway in this species. CAM was shown to be expressed in a fully facultative fashion in leaves, whereas both ontogenetic and environmental cues seem to control the induction of this photosynthetic behavior in stem tissues. Gas exchange and chlorophyll *a* fluorescence analysis in droughted plants suggested that the daytime decarboxylation of the organic acids accumulated overnight may have supplied $CO_2$ to Rubisco behind closed stomata at sufficient levels to maintain the photosynthetic apparatus integrity and functioning even after prolonged drought exposure. Overall, no marked anatomical changes were observed in leaves or stems during the CAM induction, suggesting that gene expression and metabolism reprogramming may account for most of the $C_4$-to-CAM and $C_3$-to-CAM transition in leaves and stems, respectively.

# 7. References


**Aubry S, Brown NJ, Hibberd JM**. 2011. The role of proteins in $C_3$ plants prior to their recruitment into the $C_4$ pathway. Journal of Experimental Botany **62**, 3049–3059.

**Badger MR, Price GD**. 1994. The role of carbonic anhydrase in photosynthesis. Plant physiology and plant molecular biology **45**, 369–392.

**Bauwe H, Hagemann M, Fernie AR**. 2010. Photorespiration: players, partners and origin. Trends in Plant Science **15**, 330–336.

**Brautigam A, Kajala K, Wullenweber J, *et al.*** 2011. An mRNA blueprint for $C_4$ photosynthesis derived from comparative transcriptomics of closely related $C_3$ and $C_4$ species. Plant Physiology **155**, 142–156.

**Bräutigam A, Schliesky S, Külahoglu C, Osborne CP, Weber APM**. 2014. Towards an integrative model of $C_4$ photosynthetic subtypes: insights from comparative transcriptome analysis of NAD-ME, NADP-ME, and PEPCK $C_4$ species. Journal of Experimental Botany **65**, 3579–3593.

**Chastain CJ, Failing CJ, Manandhar L, Zimmerman MA, Lakner MM, Nguyen THT**. 2011. Functional evolution of $C_4$ pyruvate, orthophosphate dikinase. Journal of Experimental Botany **62**, 3083–3091.

**Christin PA, Arakaki M, Osborne CP, *et al.*** 2014. Shared origins of a key enzyme during the evolution of $C_4$ and CAM metabolism. Journal of Experimental Botany **65**, 3609–3621.

**Chu C, Dai Z, Ku MSB, Edwards GE**. 1990. Induction of Crassulacean acid metabolism in the facultative halophyte *Mesembryanthemum crystallinum* by abscisic acid. Plant Physiology **93**, 1253–1260.

**Cushman JC**. 2001. Crassulacean acid metabolism. A plastic photosynthetic adaptation to arid environments. American Society of Plant Biologists **127**, 1439–1448.

**Cushman JC, Borland AM**. 2002. Induction of Crassulacean acid metabolism by water limitation. Plant, Cell and Environment **25**, 295–310.

**Dai Z, Ku MSB, Zhang D, Edwards GE**. 1994. Effects of growth regulators on the induction of Crassulacean acid metabolism in the facultative halophyte *Mesembryanthemum crystallinum* L. Planta **192**, 287–294.

**Eastmond PJ, Ross JD**. 1997. Evidence that the induction of crassulacean acid

metabolism by water stress in *Mesembryanthemum crystallinum* (L.) involves root signalling. Plant, Cell and Environment **20**, 1559–1565.

**Edwards GE, Franceschi VR, Voznesenskaya E V.** 2004. Single-Cell $C_4$ photosynthesis versus the dual-cell (Kranz) paradigm. Annual Review of Plant Biology **55**, 173–196.

**Edwards EJ, Ogburn RM**. 2012. Angiosperm responses to a low-$CO_2$ World: CAM and $C_4$ photosynthesis as parallel evolutionary trajectories. International Journal of Plant Sciences **173**, 724–733.

**Edwards EJ, Osborne CP, Stromberg CAE, *et al.*** 2010. The origins of $C_4$ grasslands: integrating evolutionary and ecosystem science. Science **328**, 587–591.

**Freitag H, Stichler W**. 2000. A remarkable new leaf type with unusual photosynthetic tissue in a central Asiatic genus of Chenopodiaceae. Plant Biology **2**, 154–160.

**Freschi L, Rodrigues MA, Domingues DS, Purgatto E, Sluys M Van, Magalhaes JR, Kaiser WM, Mercier H**. 2010. Nitric oxide mediates the hormonal control of Crassulacean acid metabolism expression in young pineapple plants. Plant Physiology **152**, 1971–1985.

**Furbank RT**. 2011. Evolution of the $C_4$ photosynthetic mechanism: are there really three $C_4$ acid decarboxylation types? Journal of Experimental Botany **62**, 3103–3108.

**Guralnick LJ, Edwards G, Ku MSB, Hockema B, Franceschi VR**. 2002. Photosynthetic and anatomical characteristics in the $C_4$–crassulacean acid metabolism-cycling plant, Portulaca grandiflora. **29**.

**Guralnick LJ, Jackson MD**. 2001. The occurrence and phylogenetics of Crassulacean acid metabolism in the Portulacaceae. International Journal **162**, 257–262.

**Hatch MD, Agostino A, Burnell JN**. 1988. Photosynthesis in phosphoenolpyruvate carboxykinase-type $C_4$ plants: Activity and role of mitochondria in bundle sheath cells. Archives of Biochemistry and Biophysics **261**, 357–367.

**Herrera A**. 2009. Crassulacean acid metabolism and fitness under water deficit stress: If not for carbon gain, what is facultative CAM good for? Annals of Botany **103**, 645–653.

**Herrera A, Delgado J, Paraguatey I**. 1991. Occurrence of inducible Crassulacean acid metabolism in leaves of *Talimum triangulare* (Portulacaceae). Journal of Experimental Botany **42**, 493–499.

**Holtum JAM, Aranda J, Virgo A, Gehrig HH, Winter K**. 2004. δ13C values and

crassulacean acid metabolism in *Clusia* species from Panama. Trees - Structure and Function **18**, 658–668.

**Holtum JAM, Hancock LP, Edwards EJ, Winter K**. 2017. Optional use of CAM photosynthesis in two $C_4$ species, *Portulaca cyclophylla* and *Portulaca digyna*. Journal of Plant Physiology **214**, 91–96.

**Holtum JAM, Hancock LP, Edwards EJ, Winter K**. 2018. Crassulacean acid metabolism (CAM) in the Basellaceae (Caryophyllales). ARPN Journal of Engineering and Applied Sciences **12**, 3218–3221.

**Kadereit G, Borsch T, Weising K, Freitag H**. 2013. Phylogeny of Amaranthaceae and Chenopodiaceae and the evolution of $C_4$ photosynthesis. International Journal of Plant Sciences **164**, 959–986.

**Kanai R., Edwards GE**. 1999. The biochemistry of $C_4$ photosynthesis. Biochemistry, 3–6.

**Koch KE, Kennedy RA**. 1980. Characteristics of Crassulacean *Acid Metabolism in the Succulent* $C_4$ dicot, *Portulaca oleracea* L. Plant Physiology **65**, 193–197.

**Koch K, Kennedy R**. 1982. Crassulacean acid metabolism in the succulent $C_4$ dicot, *Portulaca oleracea* L under natural environmental conditions. Plant Physiology **69**, 757–761.

**Kraybill AA, Martin CE**. 1996. Crassulacean acid metabolism in three species of the $C_4$ Genus *Portulaca*. International Journal of Plant Sciences **157**, 103–109.

**Ku SB, Shieh YJ, Reger BJ, Black CC**. 1981. Photosynthetic characteristics of *Portulaca grandiflora*, a succulent $C_4$ dicot. Plant Physiology **68**, 1073–1080.

**Lara M V., Disante KB, Podestá FE, Andreo CS, Drincovich MF**. 2003. Induction of a Crassulacean acid like metabolism in the $C_4$ succulent plant, *Portulaca oleracea* L.: Physiological and morphological changes are accompanied by specific modifications in phosphoenolpyruvate carboxylase. Photosynthesis Research **77**, 241–254.

**Lara M V., Drincovich MF, Andreo CS**. 2004. Induction of a crassulacean acid-like metabolism in the C4 succulent plant, *Portulaca oleracea* L.: Study of enzymes involved in carbon fixation and carbohydrate metabolism. Plant and Cell Physiology **45**, 618–626.

**Livak KJ, Schmittgen TD**. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods **25**, 402–408.

**Long JJ, Wang JL, Berry JO**. 1994. Cloning and analysis of the $C_4$-photosynthetic

NAD-dependent malic enzyme of amaranth mitochondria. Journal of Biological Chemistry **269**, 2827–2833.

**Ludwig M**. 2016. The roles of organic acids in $C_4$ photosynthesis. Frontiers in Plant Science **7**, 1–11.

**Lüttge U**. 2004. Ecophysiology of Crassulacean acid metabolism (CAM). Annals of Botany **93**, 629–652.

**Lüttge U**. 2006. Photosynthetic flexibility and ecophysiological plasticity: Questions and lessons from *Clusia*, the only CAM tree, in the neotropics. New Phytologist **171**, 7–25.

**Martin CE, Zee a K**. 1983. $C_3$ photosynthesis and crassulacean acid metabolism in a Kansas rock outcrop succulent, *Talinum calycinum* Engelm. (Portulacaceae). Plant Physiology **73**, 718–723.

**Maxwell K, Johnson GN**. 2000. Chlorophyll fluorescence - a practical guide. Journal of Experimental Botany **51**, 659–668.

**Mazen AMA**. 1996. Changes in properties of phosphoenolpyruvate carboxylase with induction of Crassulacean Acid Metabolism (CAM) in the $C_4$ plant *Portulaca oleracea*. Photosynthetica **38**, 385–391.

**Mazen AMA**. 2000. Changes in levels of phosphoenolpyruvate carboxylase with induction of Crassulacean acid metabolism (CAM) like behavior in the $C_4$ plant *Portulaca oleracea*. Physiol Plant **98**, 111–116.

**McKown AD, Moncalvo JM, Dengler NG**. 2005. Phylogeny of *Flaveria* (Asteraceae) and inference of $C_4$ photosynthesis evolution. American Journal of Botany **92**, 1911–1928.

**Medina E, Delgado M, Troughton JH, Medina JD**. 1977. Physiological ecology of $CO_2$ fixation in Bromeliaceae. Flora **166**, 137–152.

**Muhaidat R, Sage RF, Dengler NG**. 2007. Diversity of Kranz anatomy and biochemistry in $C_4$ eudicots. American Journal of Botany **94**, 362–381.

**Ocampo G, Koteyeva NK, Voznesenskaya E V., Edwards GE, Sage TL, Sage RF, Travis Columbus J**. 2013. Evolution of leaf anatomy and photosynthetic pathways in Portulacaceae. American Journal of Botany **100**, 2388–2402.

**Osborne CP, Sack L**. 2012. Evolution of $C_4$ plants: a new hypothesis for an interaction of $CO_2$ and water relations mediated by plant hydraulics. Philosophical Transactions of the Royal Society B: Biological Sciences **367**, 583–600.

**Peterhansel C, Maurino VG**. 2011. Photorespiration redesigned. Plant Physiology **155**, 49–55.

**Peters W, Beck E, Piepenbrock M, Lenz B, Schmitt JM**. 1997. Cytokinin as a negative effector of phosphoenolpyruvate carboxylase induction in *Mesembryanthemum crystallinum*. Journal of Plant Physiology **151**, 362–367.

**Pikart FC, Marabesi MA, Mioto PT, Gonçalves AZ, Matiz A, Alves FRR, Mercier H, Aidar MPM**. 2018. The contribution of weak CAM to the photosynthetic metabolic activities of a bromeliad species under water deficit. Plant Physiology and Biochemistry **123**, 297–303.

**Rascher U, Lüttge U**. 2002. High-resolution chlorophyll fluorescence imaging serves as a non-invasive indicator to monitor the spatio-temporal variations of metabolism during the day-night cycle and during the endogenous rhythm in continuous light. Plant Biology **4**, 671–681.

**Raven JA, Beardall J**. 2016. The ins and outs of $CO_2$. Journal of Experimental Botany **67**, 1–13.

**Sage RF**. 2002. Are Crassulacean acid metabolism and $C_4$ photosynthesis incompatible? Functional Plant Biology **29**, 775–785.

**Sage RF**. 2004. The evolution of $C_4$ photosynthesis. New Phytologist **161**, 341–370.

**Sage RF, Christin P-A, Edwards EJ**. 2011. The $C_4$ plant lineages of planet Earth. Journal of Experimental Botany **62**, 3155–3169.

**Sage RF, Khoshravesh R, Sage TL**. 2014. From proto-Kranz to $C_4$ Kranz: building the bridge to $C_4$ photosynthesis. Journal of Experimental Botany **65**, 3341–3356.

**Sage RF, Sage TL, Kocacinar F**. 2012. Photorespiration and the evolution of $C_4$ photosynthesis. Annual Review of Plant Biology **63**, 19–47.

**Santa-Cruz A, Martinez-Rodriguez MM, Perez-Alfocea F, Romero-Aranda R, Bolarin MC**. 2002. The rootstock effect on the tomato salinity response depends on the shoot genotype. Plant Science **162**, 825–831.

**Silvera K, Kurt MN, Mark Whitten W, Williams NH., Winter K, Cushman JC**. 2010. Evolution along the crassulacean acid metabolism continuum. Functional Plant Biology **37**, 995–1010.

**Taybi T, Cushman JC**. 2002. Abscisic acid signaling and protein synthesis requirements for phosphoenolpyruvate carboxylase transcript induction in the common ice plant. Journal of Plant Physiology **159**, 1235–1243.

**Vieira EA, da Cruz Centeno D, Freschi L, da Silva EA, Braga MR**. 2017. The dual strategy of the bromeliad *Pitcairnia burchellii* Mez to cope with desiccation. Environmental and Experimental Botany **143**, 135–148.

**Vogan PJ, Frohlich MW, Sage RF**. 2007. The functional significance of $C_3$-$C_4$ intermediate traits in *Heliotropium* L. (Boraginaceae): Gas exchange perspectives. Plant, Cell and Environment **30**, 1337–1345.

**Voznesenskaya E V, Franceschi VR, Kiirats O, Artyusheva EG, Freitag H, Edwards GE**. 2002. Proof of $C_4$ photosynthesis without Kranz anatomy in *Bienertia cycloptera* (Chenopodiaceae). Plant Journal **31**, 649–662.

**Voznesenskaya E V., Koteyeva NK, Edwards GE, Ocampo G**. 2010. Revealing diversity in structural and biochemical forms of $C_4$ photosynthesis and a $C_3$-$C_4$ intermediate in genus *Portulaca* L. (Portulacaceae). Journal of Experimental Botany **61**, 3647–3662.

**Voznesenskaya E V., Koteyeva NK, Edwards GE, Ocampo G**. 2017. Unique photosynthetic phenotypes in *Portulaca* (Portulacaceae): $C_3$-$C_4$ intermediates and NAD-ME $C_4$ species with Pilosoid-type Kranz anatomy. Journal of Experimental Botany **68**, 225–239.

**Wang Y, Bräutigam A, Weber APM, Zhu XG**. 2014. Three distinct biochemical subtypes of $C_4$ photosynthesis? A modelling analysis. Journal of Experimental Botany **65**, 3567–3578.

**Whitney SM, Houtz RL, Alonso H**. 2011. Advancing our understanding and capacity to engineer nature's $CO_2$-sequestering enzyme, Rubisco. Plant Physiology **155**, 27–35.

**Winter K, Garcia M, Holtum JAM**. 2008. On the nature of facultative and constitutive CAM: Environmental and developmental control of CAM expression during early growth of *Clusia, Kalanchoë,* and *Opuntia*. Journal of Experimental Botany **59**, 1829–1840.

**Winter K, Garcia M, Holtum JAM**. 2009. Canopy $CO_2$ exchange of two neotropical tree species exhibiting constitutive and facultative CAM photosynthesis, *Clusia rosea* and *Clusia cylindrica*. Journal of Experimental Botany **60**, 3167–3177.

**Winter K, Holtum JAM**. 2002. How closely do the d13 C values of Crassulacean acid metabolism plants reflect the proportion of $CO_2$ fixed during day and night? Plant Physiology **129**, 1843–1851.

**Winter K, Holtum JAM**. 2011. Induction and reversal of crassulacean acid metabolism in *Calandrinia polyandra*: Effects of soil moisture and nutrients. Functional Plant Biology **38**,

576–582.

**Winter K, Holtum JAM**. 2014. Facultative crassulacean acid metabolism (CAM) plants: Powerful tools for unravelling the functional elements of CAM photosynthesis. Journal of Experimental Botany **65**, 3425–3441.

**Winter K, Holtum JAM**. 2017. Facultative crassulacean acid metabolism (CAM) in four small $C_3$ and $C_4$ leaf-succulents. Australian Journal of Botany **65**, 103.

**Zhang YG, Pagani M, Liu Z, *et al.*** 2013. A 40-million-year history of atmospheric $CO_2$ A 40-million-year history of atmospheric $CO_2$. Royal Society **371**, 20130096.