# Photosynthetic Carbon Fixation and Crops

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## Summary

Photosynthesis in higher plants is the process of transferring energy from light to a chemical form, and using it to capture or fix carbon dioxide ($CO_2$) from the air into organic carbon.  This article focuses on the last step, the photosynthetic fixation process, and starts by outlining the main features of C3, C4 and CAM plants.  The main form of fixation is C3, found in most plants, and is catalyzed by the enzyme RUBISCO.  The two other photosynthetic types are physiological adaptations to reduce photorespiration and to allow function in drought and stress.  Photorespiration occurs in a low $CO_2$ environment.  Generally, C3 photosynthesis is an excellent compromise of photosynthetic efficiency with some photorespiration in temperate conditions.  C4 photosynthesis minimizes photorespiration by splitting initial fixation into a 4-carbon acid step first, spatially separated from RUBISCO by leaf anatomical features, before later re-fixation in the C3 pathway.  C4 photosynthesis is suited to maintained photosynthesis and productivity for plants that have evolved in drier and warmer environments.  CAM photosynthesis splits initial fixation in time: during the night $CO_2$ is fixed first to a 4-carbon acid, followed later by C3 photosynthesis during the day when stomata are closed.  A comparison of the efficiencies of C3 and C4 fixation modes in various environmental conditions is then presented.  Two C4 crops, maize (corn) and sugar cane, have high rates of photosynthesis and high yields, which has lead researchers to attempt to shift C4 metabolism into C3 crops.  The progress in converting certain C3 crops into C4 is discussed.  For readers interested in field measurements to assess photosynthesis and growth in single plants or field plots, an overview of methods follows.  Finally, the role of photosynthesis in variety development and improved crop performance is presented.

## Photosynthesis and Plants

The defining feature of the plant kingdom is that plants are able to derive their own carbon (C) source directly from inorganic carbon dioxide ($CO_2$) in the air, by chemically fixing it into an organic form.  Lower forms of life resort to specialized chemical fixation, while animals depend on organic forms of C already assimilated by plants or other animals.  The plant process is called **PHOTOSYNTHESIS**, a term that encompasses light energy and making something useful.  Energy cannot be created or destroyed, so photosynthesis transfers light energy (irradiance) from the sun to usable chemical forms in the plant (NADH, NADPH, ATP) and organic C building blocks.  In crops the harvested forms of fixed C are carbohydrates like starch in grain and tubers, lipids, and various other carbohydrates like sugars (sucrose, fructose) after food processing or industrial refining.  Lesser amounts of other saccharides (oligosaccharides) are also found, as are storage forms such as fructans (a fructose polymer) in grass stems, and structural forms of C that are essentially indigestible to humans (hemicellulose, cellulose, lignin), but are readily digested by grazing animals or other organisms.

Photosynthesis is divided into multiple steps[1,4]: initial capture of solar radiation by leaf pigments, transfer of

this energy for water splitting, generation of NADH (reducing power like a battery) and ATP (energy stored in the phosphate bond), capture of $CO_2$ entering through leaf stomata, and fixation of C to an organic form within plant cells.

The goal of this article is to focus on the last C fixation step by covering C3, C4 and CAM forms of photosynthetic fixation.  Brief discussions are included on the relative efficiencies of each photosynthetic form, opportunities to improve photosynthetic efficiency such as putting C4 into C3 plants, methods to measure photosynthesis in crops, and using photosynthesis to improve yield.

## Variations of Photosynthetic Pathways and Metabolism

### *The main theme of C3 photosynthesis*

The basic photosynthetic pathway, the photosynthetic carbon reduction (PCR) or Calvin-Benson cycle, is C3, in which the initial product of $CO_2$ fixation is a 3-carbon molecule (3C) organic acid (**FIGURE 1, 2**).

This means of fixation is found in the majority of plants. The key enzyme involved is called RUBISCO. Since the basis of life hinges on this enzyme and its associated pathways[6,7], the discovery of C3 fixation metabolism was judged worthy of a Nobel prize in 1961. Although parts of photosynthesis were already known prior to 1946 (the Hill reaction, the Emerson effect, chlorophyll, overall carbon acquisition and starch production, etc.), the main $CO_2$ fixing pathway was only resolved after 1945 in Melvin Calvin's laboratory after following ^{14}C radioactive tracers in photosynthesizing *Chlorella* (algae) suspension[7]. The PCR cycle has 13 steps, with 11 enzymes catalyzing $CO_2$ assimilation. The cycle can be divided into three unequal stages: first carboxylation (the addition of $CO_2$) to a 5C sugar to make two 3C molecules; second, reduction of the 3C products; and third, 11 steps to regenerate the 5C sugar.

In the PCR cycle, $CO_2$ is added to an acceptor molecule, the phosphate activated 5C sugar ribulose 1,5-bisphosphate (RuBP). This reaction is catalyzed by the enzyme RUBISCO, the common scientific acronym for ribulose bisphosphate carboxylase-oxygenase. The product of the reaction is 3-phosphoglyceric acid (3-PGA). To fully account for all precursors and products in the pathway (stoichiometry), three molecules of $CO_2$ are added to three molecules of the 5C sugar to make 18C in total, resulting in six molecules of the 3C product, 3-PGA. The six 3-PGA molecules are then reduced via two steps to six molecules of another 3C compound, glyceraldehyde-3-phosphate (GAP). In the regeneration stage, one molecule of GAP is used for biosynthesis and energy, and five molecules of GAP (15C total) are moved through 11 regeneration steps to make three molecules of the 5C acceptor, ribulose 1,5-bisphosphate (15C total). Then the cycle turns again (**FIGURE 1**).

Simply stated, the empirical equation of the carboxylation step catalyzed by the enzyme RUBISCO is:

$$CO_2 \ + \ \text{ribulose 1,5-bisphosphate} \ \rightarrow \ 2 \ \text{3-phosphoglyceric acid}$$
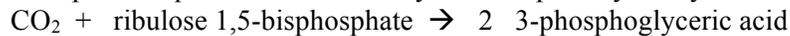
The balanced stoichiometric equation is:

$$3 \ CO_2 \ + \ 3 \ \text{ribulose 1,5-bisphosphate} \ \rightarrow \ 6 \ \text{3-phosphoglyceric acid}$$
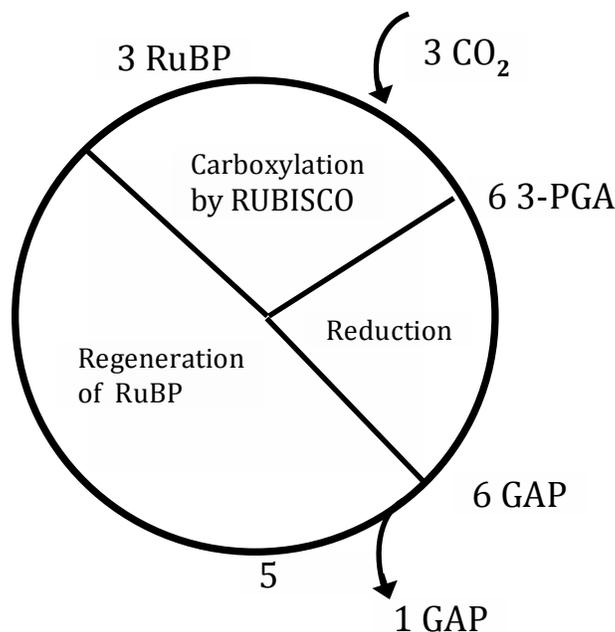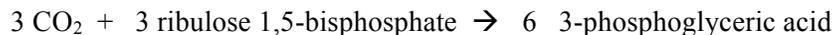


**FIGURE 1. The photosynthetic carbon reduction (PCR) cycle, with initial carboxylation by the enzyme RUBISCO of ribulose 1,5-bisphosphate (RuBP) to 3-phosphoglyceric acid (3-PGA); glyceraldehyde-3-phosphate (GAP). Modified from figure 12.40 of Buchanan et al.[4].**

### So what is the fate of the 3C compounds (triose phosphates) made in photosynthesis?

The 3-PGA enters the general triose phosphate pool in chloroplasts where photosynthesis is occurring[4]. There, with cell cytosol and amyloplasts, these pools supply various metabolic pathways via phospho*enol*pyruvate (PEP) and pyruvate (for glycolysis and energy production). The triose phosphate pool can interact with the pentose (5C) phosphate pool, and contains 3C, 4C, 5C and 7C phosphorylated members that interact with various metabolic pathways.

Triose phosphates in chloroplasts can be converted into a hexose phosphate and then polymerized into starch. Starch grains are short term storage of a day's photosynthate that reach their maximum size in early evening. During the night they are broken down to supply glucose and other hexoses for metabolism of the chloroplast and cell cytosol. The hexose pool consists of three inter-convertible molecules kept at equilibrium, glucose 1-phosphate, glucose 6-phosphate, and fructose 6-phospate. Sucrose is the main form of sugar transported from leaves to other parts of the plant. When sucrose arrives at more distant locations, it is split into its component smaller sugar molecules, glucose and fructose, which then enter the hexose pool of the distant cell/s. These pools are also used for long term storage of carbohydrate, such as starch grains in amyloplasts in cells of storage organs, for example, potato tubers or seeds. In general, C can enter the leaf cell's hexose pool after photosynthate triose P is run through a reverse form of glycolysis, or from any of the following: gluconeogenesis (this pathway makes glucose from 4C organic acids, the 4C compound oxaloacetate resulting from the breakdown of oil/fat reserves); adding phosphates to free hexose sugars; or breaking down starch and sucrose. The hexose phosphate pool supplies sucrose synthesis, starch synthesis, cell wall synthesis, glycolysis (breaking down glucose to energy), and the pentose phosphate pathway (breaking down glucose to a 5C sugar ribose and energy).

### C3 photosynthesis and the dilemma of photorespiration

When a leaf has access to sufficient $CO_2$, RUBISCO fixes C and two molecules of triose phosphate result. However, RUBSCO also has affinity for oxygen ($O_2$), and when $CO_2$ concentrations are low, the enzyme participates in oxygenation, not carboxylation. Instead of producing two molecules of 3-PGA, RUBISCO now produces one molecule of 3-PGA and one molecule of a 2C compound, 2-phosphoglycerate, thus reducing the initial C fixing efficiency. The 2C compound, 2-phosphoglycerate, is converted to 2C glycolate, eliminated from the chloroplast and dissipated through a photorespiratory cycle involving another plastid of the cell (the peroxisome), converted to the 2C compound glyoxylate, then to a 2C amino acid glycine followed by a 3C amino acid serine in the mitochondria. The 3C amino acid is then reshuffled to 3C organic acid glycerate before it re-enters the chloroplast to be phosphorylated for the triose phosphate pool. All these extra steps comprise the photorespiratory cycle that overall lowers the net efficiency of photosynthesis.

### C4 photosynthesis

Some plants that have evolved in higher temperature, higher solar radiation, or regions of frequent drought or salt stress, have C4 photosynthesis[16]. The additional and initial steps of C4 fixation were discovered in sugar cane leaves in the mid-1960s by Kortschack (Hawaii), Karpilov (Russia) and in Australia by Hatch and Slack[21]. Crop examples with C4 photosynthesis are maize (corn), sorghum, millets, and C4 grasses from warm grassland ecosystems. Dicots can also have C4 photosynthesis (e.g. Chenopodeacae) although less frequently[20]. A very approachable review article on C4 plant genera is by Sage[39] – "what plant has C4, what C4 does for the plant, where the plant grows". About 500 plant genera (the genus is the first part of the latin name of a species) of 4500 total in the world have C4 members, with 401 being monocots (mainly grasses, sedges) and 86 being dicots.

The defining feature of <u>**C4 photosynthesis is C fixation split in space**</u>, with initial C fixation into a 4C organic acid by the enzyme phospho-*enol*-pyruvate carboxylase, known as PEP carboxylase, in the chloroplasts of mesophyll cells[1]. This acid is then transported from mesophyll cells into a separate specialized ring of cells surrounding a leaf vein, known as Krantz (wreath/ring) anatomy or the bundle sheath cells. In the bundle sheath cells, the C4 acid is broken down to a C3 compound with the release of $CO_2$. As a result, the $CO_2$ concentration is increased around RUBISCO

situated in chloroplasts of the bundle sheath cells. The bundle sheath cells are suberized (water/gas-proofed) and have low oxygen concentration. The plant can then fix $CO_2$ using RUBISCO in an almost zero oxygen environment. In the bundle sheath cell environment RUBISCO is not blocked by oxygen and photorespiration is minimized. The comparison between C3 and C4 photosynthesis is illustrated in **FIGURE 2**.

## C3 photosynthesis in the mesophyll cell

## C4 photosynthesis split in space between mesophyll and bundle sheath cell
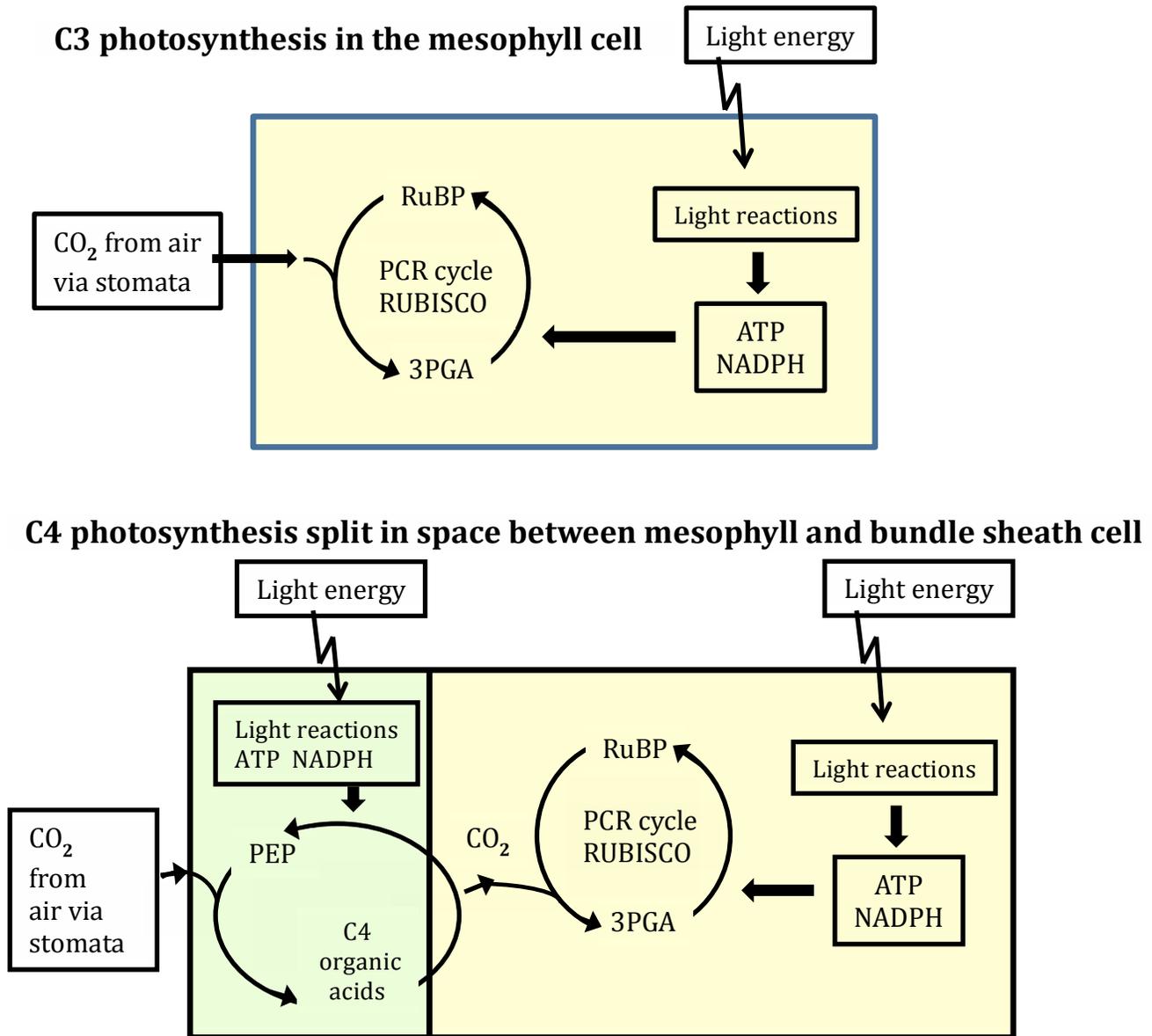
**FIGURE 2. Comparison of basic photosynthesis, C3, which occurs in mesophyll cells of leaves, to the C4 type, which is split in space between the two cell types, mesophyll and bundle sheath. Abbreviations: Adenosine triphosphate (ATP); photosynthetic carbon reduction (PCR) cycle; phospho*enol*pyruvate (PEP); 3-phosphoglyceric acid (3-PGA); reduced form of nicotinamide adenine dinucleotide phosphate (NADPH); ribulose 1,5-bisphosphate (RuBP). Adapted from Lawlor[1].**

Photosynthesis experts now agree that the main reason for C4 photosynthesis is avoidance of photorespiration and operation within a low $CO_2$ environment[15]. C4 is a photorespiratory adaptation from the C3 pathway[26].

### C4 and its subtypes

In the decade after the discovery of C4 photosynthesis, researchers identified three variations of C4 metabolism, the main one being **NADP-ME** (maize, sorghum, sugar cane), and the other two being **PEP-CK** (*Panicum, Chloris, Urochloa*), and **NAD-ME** (the dicots *Atriplex, Portulaca, Amaranthus*) and *Eleusine* millet[1,17,20]. PEP carboxylase in the mesophyll cell cytosol is the main initial C4 fixing step in all these variations. It takes a bicarbonate ion ($CO_2$ as an aqueous form, catalyzed by carbonic anhydrase) and very rapidly converts this and PEP to a 4 C acid, oxaloacetate (OAA). The reaction is energetically not reversible and since PEP carboxylase has no affinity for oxygen the process is very efficient at fixing $CO_2$ at very low concentrations. The fate of OAA follows three different routes depending on C4 subtype, all of which regenerate PEP in mesophyll chloroplasts but vary in location, cell compartment, and pathway conversion of further C4/C3 products.

For **NADP-ME type** C4 plants, OAA enters and is reduced in the mesophyll cell chloroplast to form malate (4C) with the enzyme NADP malate dehydrogenase. The malate is then transported out of the chloroplast, out of the mesophyll, and into chloroplasts of the bundle sheath cells. In the bundle sheath chloroplast, malate is decarboxylated by NADP-malic enzyme to produce C3 pyruvate and $CO_2$, along with NADPH and $H^+$. The $CO_2$ then enters the C3 fixation cycle with RUBISCO, situated also in the bundle sheath chloroplast.

For the **PCK type**, OAA is converted to an amino acid aspartate that is transported out of the mesophyll cytosol, and into the cytosol of the bundle sheath cells. Aspartate is deaminated in the bundle sheath cells to give OAA, and then phosphorylated by PEPCK (PEP carboxykinase) to give PEP and $CO_2$. The $CO_2$ enters the bundle sheath chloroplast to be fixed in the PCR cycle.

For the **NAD-ME type,** OAA is converted to aspartate, which is transported out of the mesophyll cytosol, and into the cytosol of the bundle sheath cells, and into the

mitochondria of the bundle sheath cells. This aspartate is converted to OAA and then reduced to malate within the mitochondria. The malate is decarboxylated by NAD-malate dehydrogenase, and the $CO_2$ moves to the cytosol and on into the chloroplast to enter the PCR cycle for C3 fixation.

### Conditions where C4 is an advantage

C4 fixation can be more efficient at low $CO_2$ concentration, or when photosynthesis is occurring at a very high rate and the $CO_2$ concentration is low and restricting activity[1]. Such conditions exist on hot days around solar noon within a canopy when photosynthesis rates are at maximum, or when a crop is exposed to high irradiance (solar radiation), or when stomata are partially closed from drought or salinity stress. However, **C4 photosynthesis is more expensive metabolically** for reasons explained below. For C4 plants, photosynthetic productivity is maximized when growing without water stress in high irradiance conditions (mid-summer, cloudless days, sub tropics, intercontinental regions). This accounts for the high biomass and yield productivity of sugar cane and maize.

The natural distribution of C3 and C4 species is determined by climatic factors of temperature and rainfall. In hot, dry arid regions C4 species are present in greater numbers than C3 species. C4 species are abundant in grasslands and savannahs[41]. The most detailed study, and probably the most famous, is by Hattersley[22], covering all grasses in Australia, some 833 native species and 292 naturalized species. C4 grasses predominate over 80% of the Australian continent, 65% of the native species are C4 and 60% of the total grass species are C4. Similar distribution has also been found for grasses in South America[12,13], with 100% C4 in very arid and hot regions to about 30% C4 in wetter and cooler regions. In grassland ecology, the type of C4 variation can also be correlated with ecosystem and climate. Broadly speaking for Australian C4 grasses, in very dry environments (200 mm annual rainfall), 45% of C4 are NAD-ME, 45% are NADP-ME, and around 10% are PCK types[23]. NAD-ME types decrease in frequency from 45% to 20 % as rainfall increases, so NAD-ME is an adaptation well suited to dry arid conditions. In addition, NAD-ME types are more prevalent in hot conditions, as measured by minimum daily temperatures averaged over January

(mid-summer in Australia). Most of the exceptional drought tolerance is found in NAD-ME types, *Cynodon*, *Eleusine* (millet), *Tetrapogon*, *Eragrostis* (tef), *Panicum* (millet), but include salt grasses (*Spartina*) and dicots.

NADP-ME types increase from 45% to 65% of the C4s as annual rainfall increases from 200 to 800 mm (think of maize and sugar cane). While NADP-ME is suited to arid conditions, it is even better suited to moister conditions. The PCK types are not as abundant, and they increase from 10% to 15% as rainfall increases. These same C4 subtype distribution patterns are observed in North America[45], Africa and the Middle East. Types having the anatomical feature of suberized lamella of the Krantz cells (bundle sheath cells), increase from 50% to 80% of the C4s as rainfall increases.

In nature, some genera (*Flaveria, Moricandia*) have C3-C4 intermediates with some species being C4, some on a continuum of C3-C4, and others C3[31]. Kranz anatomy of the intermediates is not as marked as C4, they do have PEP carboxylase, they exhibit levels of leakage and inefficient metabolism from the Kranz anatomy, and often RUBISCO is active in the mesophyll cells. Therefore they have varying levels of C4 activity as well as photorespiration.
 So what is the relevance of the C4 subtypes? The variations in metabolism provide a view of evolution of C4 that came after C3 and CAM photosynthesis[39]. They have also been used more practically to reclassify grasses taxonomically. The C4 subtypes also differ in photorespiration efficiency and sub-cellular compartmentalization of key enzymes, so they offer a choice of manipulation possibilities for converting certain C3 crops into C4[30,40].

### Is C4 photosynthesis superior to C3?
Common mistakes students make about C4 metabolism is that all tropical crops function that way and C4 is an adaptation to high temperature. But C4 can only confer an advantage under high light intensity conditions, and conditions of severe water stress for plant species that still maintain partial stomatal opening[16,22,23]. C4 metabolism is seen in halophytes (plants which grow in saline soils or on coastal dunes). A few C4 species occur in cooler dry places, like Saskatchewan. Rice, which grows in the wet humid

and cloudy tropics, has C3 metabolism. C4 is a metabolic adaptation to operate under low $CO_2$ concentration and to reduce costly photorespiration.

Since C4 metabolism is seen in two highly productive crops, sugar cane and maize, it is worth considering the cost of metabolism (as in Lawlor[1], chapter 9). If you took a side-by-side measurement of leaf photosynthesis in a greenhouse (midsummer, at noon, 28 to 30°C) from a non-stressed maize plant (C4) and wheat plant (C3), you would expect the photosynthetic rate to be higher for maize than for wheat (28+ and 24 micro mols of $CO_2$ per $m^2$ leaf per second, for maize and wheat respectively). A demonstration of these measurements between maize and rice is included in Sheehy et al.[42]. If you then went outside on a hot bright sunny day at noon, maize photosynthesis would be even greater but wheat (or rice) photosynthesis would not change much from 24 micro moles of $CO_2$ per $m^2$ leaf per second. It is tempting to assume that the greater C4 photosynthesis in sugar cane and maize is better than C3 photosynthesis and will confer an advantage in all conditions. This was the root of the idea to shift C4 metabolism into C3 plants to improve crop growth and yield, when less was known about photosynthesis than today. Exactly how much does C4 metabolism cost and just when is it an advantage? About 3.6 ATP and 2.3 NADPH molecules are required to perform C4 metabolism above the costs of C3 metabolism, and additionally, specific anatomical features, enzymes, and metabolic transporting systems are also needed.

The most usual way of overviewing the cost of immediate photosynthesis is using **quantum yield**, the ratio of 1 $CO_2$ fixed to the amount of quanta (energy in photons) required[1,43]. Maize requires 17 quanta of light (energy from 17 photons) per molecule of $CO_2$ fixed, or 0.06 quantum yield (calculated from 1/17).

Quantum yield for C3 plants under ambient conditions is mostly 0.05 to 0.06 from Skillman[43]. When oxygen is removed (less photorespiration), C3 plant quantum yields increase to 0.10, and in elevated $CO_2$ they reach 0.12. Therefore, mean C3 (0.052) and C4 (0.057) plant quantum yields are similar at 25°C and moderate $CO_2$, where C3 plants suffers from some photorespiration and C4 plants have lower efficiency due to the extra steps (higher energy

cost) of C4 metabolism. For a range of C4 plants, quantum yields at 25°C to 35°C ranged from 0.05 to 0.08, 0.06 being the predominant value. At high $CO_2$ concentrations, both C3 and C4 are similar with quantum yields of 0.10, and the C4 steps can be omitted (they are present but not used) in the C4 plants under these conditions[1].

C4 plants can use high irradiance (high light intensity), C3 plants cannot. C4 plants are less efficient at low light intensity. The separate and initial C fixation by PEP carboxylase can capitalize on very low $CO_2$ concentrations in a different place than the PCR cycle, and can increase the concentration of $CO_2$ at RUBISCO by a factor of 3 to 8 times greater than that possible in C3 metabolism. C4 plants have only minute levels of photorespiration, they have smaller light and $CO_2$ compensation concentrations than C3 (the compensation point is the amount of light or $CO_2$ concentration for gross photosynthesis to equal photorespiration), better carboxylation efficiency and higher rates of photosynthesis[1,2]. C4 plants also require less RUBISCO enzyme per unit area of leaf. RUBISCO is a large enzyme which is turned over frequently, requiring plants to have a steady supply of nitrogen. Therefore C4 plants have a greater nitrogen efficiency for photosynthesis. C3 plants that grow well are more likely to become nitrogen limited sooner. In drought or severe stress, stomata are only partially open and C4 plants have a greater photosynthetic rate than C3 plants and consequently lower transpiration losses and higher water use efficiency ($CO_2$ fixed per water transpired).

## CAM Metabolism

Another variation of C fixation for more extreme stress is CAM metabolism, named after desert plants, members of Crassulaceae[1,3,27]. The defining feature of CAM photosynthesis is **fixation split in time in the same cell**, with stomatal opening and initial fixation by PEP carboxylase occurring at night when temperatures are lower and irradiance loads minimal, and if stress is not severe, during early hours of daylight and very late afternoon (FIGURE 3). This fixed $CO_2$ is stored in the vacuole as a C4 acid (malate, citrate). During the day when temperatures and irradiance loads are high, stomata are closed. However, photosynthesis can take place during the light hours because the C4 acids fixed during the night are decarboxylated in the cytosol to release $CO_2$, which proceeds to the PCR cycle during the day.

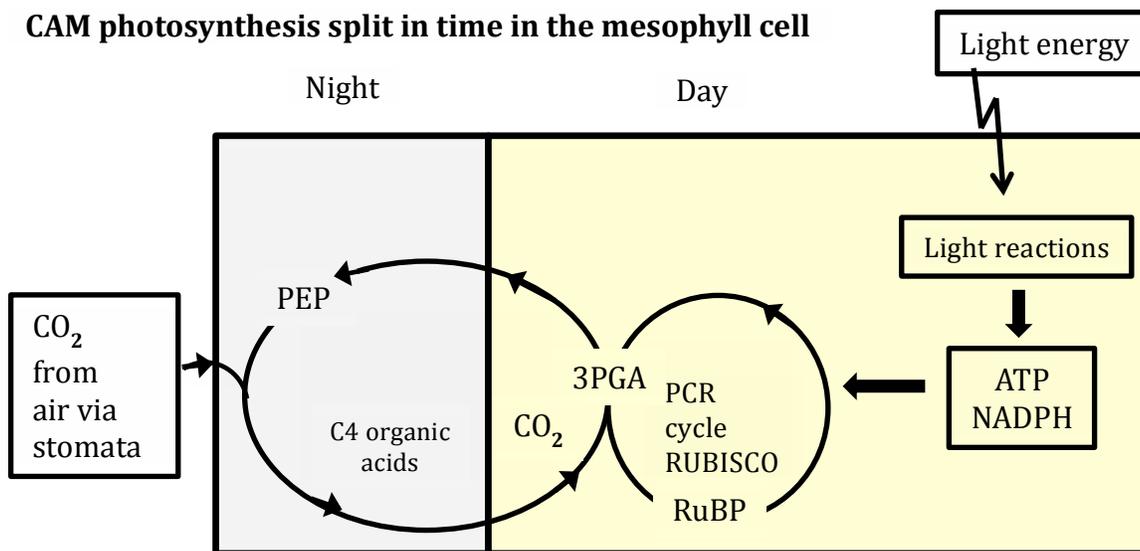## CAM photosynthesis split in time in the mesophyll cell



Figure 3. Features of CAM photosynthesis, where initial fixation and the PCR cycle are split in time in the mesophyll cell. Adenosine triphosphate (ATP); photosynthetic carbon reduction (PCR) cycle; phospho*enol*pyruvate (PEP); 3-phosphoglyceric acid (3-PGA); reduced form of nicotinamide adenine dinucleotide phosphate (NADPH); ribulose 1,5-bisphosphate (RuBP). Adapted from Lawlor[1].

The advantage of CAM metabolism over other photosynthesis is improved water use efficiency because transpirational losses are minimized during daylight hours. Plants with CAM metabolism usually are succulent, and include Crassulaceae, members of Orchidaceae and Caryophyllales. All CAM plants in their native habitat operate in severe dry environments, usually hot by day, or they are tree epiphytes (tree orchids) that experience stress. Typical plant examples that readers may recognize are Jade plants, *Kalanchoe*, the ice plant *Mesembryanthemum crystallinum*, aloes, orchids, and *Opuntia* (prickly pear cactus). Pineapple (*Ananas comosus*), a bromeliad, is one of the few economic crops with CAM metabolism.

The genus *Clusia* contains dicot trees (a tropical kind of succulent magnolia), and *Clusia minor* has varied types of CAM metabolism: CAM, CAM cycling, CAM idling, and C3. Explanations of these modes are found in a comprehensive review by Luttge[27]. CAM variation in *C. minor* is dependent on photorespiratory conditions[28] associated with intense radiation, time of day, time of year, drought, temperature, nitrogen supply, and whether a sun or shade leaf[38]. High irradiance and UV-A or blue light induce CAM, and red light (wavelengths longer than 530 nm) keep leaves in C3 photosynthesis[18]. At the same time of day, some CAM plants can have one leaf on a branch operating in CAM and another leaf on the same branch operating in C3; and some CAM plants can additionally operate C3 and CAM at different ends of the same cell. CAM plants are classified as facultative when CAM is induced by environmental factors, for example, the dry season in the subtropics (drought and high irradiance), or summer (high temperature and high irradiance). Obligate CAM plants spend their lives in CAM mode and do not switch to other forms of photosynthesis. Evolution of CAM types is thought to have occurred before C4. The quantum yields of CAM are lower than C3 and C4 photosynthesis[43], averaging about 0.033. The greater range of 0.02 to 0.12 from 6 CAM publications analyzed by Skillman demonstrates the metabolic and functional flexibility within CAM plants as they deal with water conservation and water-deficit stress[43]. There would be no advantage for crop productivity to shift CAM metabolism to C3 plants due to the lower overall productivity and metabolic cost of this form of photosynthesis.

## Placing C4 Metabolism into C3 Crops

### *Can we make C3 crops into C4 crops to improve productivity?*

For C3 crops grown in cool temperate areas, C3 appears to be the best strategy. For C3 crops grown under high temperature, high irradiance, or frequent drought stress, C4 photosynthesis offers the advantage of reduced photorespiration and overall higher photosynthetic productivity[47]. In rice for example, this crop has expanded and production now occurs in warmer, drier areas than traditional rice growing regions. C3 photosynthesis limits productivity through photorespiration losses, and this increases with temperature because the oxygenation problem at RUBISCO becomes greater. With frequent water deficiency and partially open stomata, the oxygenation problem at RUBISCO also increases. To solve world hunger, rice photosynthesis has been ear-marked for improvement by the International Rice Research Institute as a target for the next green revolution[42]. The first green revolution resulted in yield advances from short straw, day neutrality (wheat) and nitrogen response, and more lately, from hybrid rice production.

Researchers have attempted to convert C3 crops to C4 photosynthesis for several decades (reviewed by Brown and Bouton[11] on conventional crossing, and by Peterhansel et al.[34] for molecular genetics approaches). Initially, a C3 plant was crossed with a close relative that was either C4 or a C3-C4 intermediate. Progeny from these crosses inherited various forms of C3 and C4 photosynthesis, but resulting photosynthesis and water use efficiency were small compared to the C3 parent. No evidence exists of a key single gene to trigger an entire C4 cycle. Rather, Brown and Bouton concluded that a complete pathway of working enzymes (all inherited independently) together with substantial modifications to leaf anatomical features were required for a consistent and productive C4 plant[11].

Entry of intermediates from one cell compartment to another is through special carriers in plastid membranes, known as transporters. Passage of intermediates within the cytosol from cell to cell is via diffusion. Enzymes have specific and different

affinities for substrate (input) and product (output). Substrate and product concentrations are within controlled ranges, and their flux (movement and concentration of metabolite and energy intermediates from location to location) is tightly controlled[15]. Making a plant C4 is more than just adding the enzymes carbonic anhydrase (to make bicarbonate) and PEP carboxylase to any leaf cell. Interestingly, plants already have most of the enzymes necessary for C4 metabolism, but the enzymes are present in reduced amounts and they are not regulated in a C4 manner, and so they produce zero C4 in the cells of choice[25]. In fact, cells around stem veins and developing grain (rice) have a version of C4 enzymes, but they are not used in C4 photosynthesis, rather they recycle C coming from other places or from respiration[29]. When leaves lack necessary anatomical features such as closely spaced veins, Kranz anatomy, and cell suberization, C4 metabolism is grossly inefficient and photosynthesis is not increased.

The first technical difficulty is choosing which type of C4 to shift, with its several pathways and enzymes involved so photorespiration is taken into account[35]. The key enzymes like PEP carboxylase and RUBISCO are large and complex with multiple subunits, they are continually turned over in the cell, and they require numerous other proteins (chaperonins) to assemble them[1]. This means that enzymes plus their assembly mechanisms all need genes encoded into the DNA. And not all the DNA is nuclear, some of this is chloroplastic DNA, although the control of everything is inherited from nuclear DNA. The second difficulty is ensuring sufficient C4 activity in the absence C4 anatomical features. The third difficulty is placing the required C4 enzymes where they should be functioning in a target cell compartment, and with the original C3 enzymes elsewhere in the cell now switched off[30]. For greatest efficiency, no RUBISCO should be present or active in the same cells containing PEP carboxylase.

With genetic engineering, ability to transform plants at the nuclear and plastid (chloroplast) level opened up a new world of possibilities for novel C4 crops[30,35]. The main method has been to target PEP carboxylase and over-express this enzyme (make lots of the enzyme) by genetic engineering in tobacco, potato, rice and *Arabidopsis*. In most of these endeavors, the maize version of PEP carboxylase was used, and its gene was driven with a 35S promoter, so the enzyme was produced constantly. Effects varied: more efficient use of $CO_2$, excess malate production (the C4 acid), less oxygen inhibition of photosynthesis, improved dark respiration, a neutral state of no effect, negative effects such as chlorosis, less sugars and more acids, reduced phosphorylated metabolites, and even stunted growth. The enzyme NADP-ME has also been overexpressed to remove the malate bottle neck and it did improve efficiency. When both enzymes together have been overexpressed in the same cell, results were promising in one species and neutral (no improved photosynthesis) in another. PEP carboxykinase (PCK) from the C4 *Urochloa* has also been expressed in rice, with and without PEP carboxylase, but overall photosynthesis has not been higher.

Most attempts to shift C4 have used two-cell systems, meaning classical C4 fixation which is split in space in mesophyll and bundle sheath cells[24,47]. A single-cell version of C4 metabolism may produce a functioning C4 pathway in C3 plants, but using four enzymes (PEP carboxylase, pyruvate orthophosphate dikinase, NADP-malate dehydrogenase, NADP-malic enzyme) has not yet produced greater plant growth[30,34,35]. Another novel approach is to go after photorespiration itself. This has been attempted in *Arabidoposis* by adding a less costly bacterial version of a photorespiration pathway within the chloroplast. Products of RUBISCO oxygenation are removed without reactive oxygen-containing compounds and expensive C and N cycling, and amazingly, photosynthesis and growth have been improved[35]. The approaches of re-designing plant RUBISCO (use of the enzyme or subunits from algae and bacteria, inheritance of the large subunits from the nucleus only, use of subunits from a C3 and C3-C4 intermediate) have not produced better photosynthesis; instead plants have suffered chlorosis or they need to operate in higher $CO_2$ concentration[19]. Research is ongoing in rice, despite such challenges.

### Measuring C Acquisition in the Field

In the laboratory, photosynthesis and C fixation was first characterized by following the appearance of individual metabolites. Such experiments give a detailed account of what is happening in a cell or leaf in a short period of time (seconds to minutes), like a snap shot. In the field, researchers are more interested in the longer term benefits from photosynthesis and, ultimately, we are all interested in increasing yield.

More C fixed means more growth and more yield. Field measurements associated with photosynthesis and C acquisition fall into five categories: gas exchange; growth analysis; isotope labeling; indirect light measures; and radiation interception. An overview of the main features of these categories is found in TABLE 1, and in Jones[2].

**TABLE 1. Methods used to assess photosynthesis and related crop productivity (after Jones[2]). An example of the measurement from published literature is listed in the far right column.**

| METHOD | PROS | CONS | REFS |
|---|---|---|---|
| **GAS EXCHANGE BY INFRARED GAS ANALYSIS (IRGA)** | | | |
| Single leaf | One or two samples per plot, takes 2 minutes per sample. Handheld chamber. | Upper canopy single leaf can overestimate canopy. Measurement is for a single point in time from a single leaf or part of a leaf. | 32 |
| Field plot | Accounts for whole canopy, $1m^2$ footprint. | Need a plot, limited by moving the chamber, or number of chambers, single point in time or day. | 8 |
| **GROWTH ANALYSIS** | | | |
| Whole season Dry weight of plant and organs over time | Shoot carbon acquisition, requires manpower, oven, dry weight. | Destructive sampling. | 5 |
| Organ comparison: Specific leaf area and weight | Detailed leaf measurements. | Destructive sampling, Usually biased to upper canopy positions. | 10, 32 |
| Leaf area duration Green leaf area | Detailed leaf measurement that looks at leaf longevity as photosynthetic productivity. | Requires methods that follow a leaf age / color. | 9 |
| **ISOTOPE LABELLING** | | | |
| C combustion and $^{14}$C radioactive | Used for immediate C fixation and its associated metabolism. Used for herbicide effects on photosynthesis. | Radioactive, methods to incorporate only the labeled input molecule, destructive sampling methodology. | 31 |
| $^{12}$C/$^{13}$C ratio | Used to assess C discrimination for performance in stress, crop history. Non-radioactive. | Destructive sampling. Can be difficult to interpret location effects. | 14 |
| **OTHER LIGHT METHODS** | | | |
| SPAD (chlorophyll) meter, leaf greenness instruments | Non destructive, rapid, can use in field, independent reading in the place of a visual rating scale. | Many measurements required for accuracy (SPAD). Calibrated to a control plot or plant for productivity studies. | 9 |
| Fluorescence | Can be very detailed in controlled conditions, less detailed in field with commercial hand-held devices. | Best precision gained in controlled conditions. Commercial field devices used to indicate damage or inefficient photosynthesis. | 38 |
| Radiation interception | Several radiation interception measurements per plot, can calculate leaf area if extinction coefficient of crop canopy is known. | Non destructive, but quality of data depend on instrument, time of day versus continuous measurements, and canopy structure. | 33 |
| Radiation use efficiency | Relates biomass productivity to solar radiation over a crop season, or whole season performance. Radiation is the energy to power photosynthesis and growth. | Requires a weather station with radiation sensor, crop radiation interception, and destructive biomass readings throughout the season. | 33 |

Measurements are influenced by light and time of day, and upper leaves tend to be light saturated. Whole canopy measurement is superior because it accounts for canopy structure and complete light use, but this needs a 1m$^3$ chamber (cuvette) per plot per sample, or measurements taken repeatedly over a period of weeks (which assesses canopy performance over time), and cannot be practically used to measure lots of plots.

## Growth analysis

Growth analysis has been in vogue since the 1960s and represents a low-cost means of assessing crop productivity in research. Crop growth rate data of the whole plant, or specific organs within the plant, are fairly intuitive and can be understood by a general audience. Here dry weight acquisition is equated with C acquisition since 90% of a plant's dry weight is made up of C. More detailed measurements such as specific leaf area and weight are used to assess leaf thickness and starch storage effects. Leaf area duration (how long the leaf lives and photosynthesizes) is useful to follow canopy productivity in stressful environments.

## Isotope labelling

Historically, the use of radioactive compounds to follow processes of photosynthesis was called tracing, or pulse labelling. A plant, group of plants, a leaf, or a liquid suspension containing chloroplasts previously extracted from leaf cells is exposed to $^{14}$C-labelled $CO_2$. The fate of this label is followed as it is incorporated into organic acids, sugars, carbohydrates and plant structural components. Over a short chase time of seconds, minutes or hours, results reveal immediate C fixation metabolism in the cellular compartments. A longer sampling period of days and weeks reveals how the C is metabolized into more distant plant organs (shoot apex, stems, roots), and the various forms of C storage products (organic acids, sugars, alcohols, oligosaccharides, polymers). Originally, the total amount of C was measured from combusting plant material, and by use of radioactive isotopes. With advances in mass spectroscopy instrumentation, radioactive tracing has been replaced by safe non-radioactive isotopes like $^{13}$C and $^{15}$N. In some experiments, dual labelling (N and C) is used to follow the fate of metabolic intermediates. Specifically for carbon, the ratio of $^{12}$C to $^{13}$C is used.

## Other light methods

A portable fluorescence meter, possibly calibrated to another photosynthesis and florescence measurement back in the lab (e.g. Roberts et al.[38]), can be used to screen field plants and plots for photosynthetic performance under water excess, water deficit stress, and herbicide injury. Any photosynthetic injury that generates chlorophyll degradation and canopy yellowing can be followed by a SPAD (soil plant analysis development) meter. The SPAD meter is usually calibrated to N concentration in leaves in N fertility studies. It is also used to measure chlorophyll content and canopy longevity (leaf area duration) in the place of visual rating scales or digital color analysis of field plots. Another method similar to the SPAD is NDVI (normalized difference vegetation index) and similar instrumentation; they measure reflectance in known wavelength bands (e.g. near infrared and red light) and these sensors are sold as hand held devices. These techniques offer an advantage over SPAD because fewer readings are needed per sample or plot to remove background noise.

Inferences of canopy performance, and hence overall photosynthetic ability, can also be made using light interception instrumentation- essentially this is the proportion of available light that can be used by the leaves of a crop at various points in the season and indicates whether the crop is operating under good fertility (rapid leaf area development) or stress (reduced leaf area development). This method has a mathematical relationship between light or radiation interception and leaf area index (amount of canopy leaf area per unit area of ground), via an extinction coefficient. Radiation interception can additionally be related to the conversion of C to biomass and yield using the calculation of radiation use efficiency, but necessitates destructive sampling for plant biomass. Radiation interception and radiation use efficiency are used to overview whole field performance, and to compare different cultivars, traits, crop types, cropping systems, or other large scale management factors over the duration of vegetative and most reproductive growth. Radiation use efficiency (g of shoot dry weight per MJ radiation intercepted per m$^2$) from seasonal data is a field assessment version of the photosynthetic quantum yield in the laboratory (molecules of $CO_2$ fixed per quantum of light).

## Photosynthesis and Improved Crop Performance, has it Happened Yet?

### *Do crop cultivars exhibit greater photosynthesis today compared to older cultivars?*

Since the invention and improvement of field portable IRGAs, one would think that plant breeders routinely screen plant material for highest photosynthesis. In practice, however, the lengthy IRGA measurement times and the confounding factors determining individual leaf photosynthetic rates limit their utility in most breeding programs. Photosynthesis measured by IRGA is a snapshot of initial fixation in the crop life, and such measurements are not linked closely enough with yield to predict high yield. The literature does contain examples where breeding programs have screened for higher leaf and canopy photosynthesis and this has resulted in new cultivars in maize, wheat and soybean[8,44]. On a seasonal basis, photosynthesis has been improved in some crops when compared to obsolete or historic cultivars, for example in short-season soybean in Canada[32]. Usually, leaf photosynthesis from side-by-side comparisons show the new cultivar to have only marginally higher photosynthetic rates than old cultivars and any definitive link to yield is not as strong as desired[8].

Few examples exist in the literature where yield has actually been improved via improved photosynthesis. Anyone with patience and time will discover that most papers that report increased photosynthesis cannot demonstrate a yield response to higher rates of leaf photosynthesis *per se*, especially when other factors such as leaf longevity, irradiance level, leaf area, rooting depth and length of grain filling periods are taken into account. Any increase in photosynthesis is therefore small. Typically the leaf photosynthesis rate measured from cultivars released over many years do not strongly correlate with a yield increase at all, as seen in rice and wheat. Often any increase can be attributed to other factors such as agronomic management (nitrogen fertilizer, pesticides) that protect the crop canopy.

Photosynthesis is but the first step of a multi-stage process of getting C into the plant, then placing C into a storage form (for example: structural, cellulose; non-structural, starch), and remobilizing stored C into sugars and re-storing C as starch (or similar carbohydrate or C containing compounds) within the grain. A high leaf photosynthesis rate is just one aspect

of a plant's performance, as with a motor vehicle's maximum speed specification (C4 at 170 mph, C3 at 120 mph). But we all know that vehicles are designed for different purposes and driving conditions, and maximum speed does not directly equate with vehicle performance, longevity, miles travelled, or freight hauled. The failure of improved leaf photosynthetic rates to correspond with improved yield means that photosynthesis is tightly regulated itself and cannot be easily moved upwards, or photosynthesis is not highly heritable with yield, or both. Many processes downstream from C fixation are frequently more limiting to yield (e.g. life cycle length, leaf area duration, harvest index) and need to be addressed first[37]. Only then can yield be gained by greater initial leaf C fixation.

Despite breeding disappointments, detailed photosynthetic measurements are used on subsets of material and experiments of smaller scope compared to large scale genotype screening. Cereals are currently being selected for higher leaf radiation use efficiency by various plant breeding organizations (e.g. CIMMYT[36]) and companies. Maize performance has been improved in stressful environments and high plant population environments[46]. Recent maize cultivars have been indirectly selected for improved biomass acquisition, greater radiation use efficiency, leaf area duration, and maintained photosynthetic duration (not higher photosynthetic rate *per se*) during reproductive growth[5]. The stay green trait, which confers greater N uptake and prolonged C fixation after flowering and greater yields in maize, sorghum and forage crops, has been measured with leaf area duration, specific leaf area (thickness) and specific leaf nitrogen, and leaf greenness. Such screening was used to improve sorghum cultivars[9,10]. But the future is bright and holds promise, and perhaps in our lifetimes we will see C3 crops taking on C4 photosynthesis with improved yields. Such a feat will certainly break the current paradigm in C3 crops that have reached a yield plateau.

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