Impact of pod and seed photosynthesis on seed filling and canopy carbon gain in soybean

Young B. Cho, Samantha S. Stutz, Sarah I. Jones, Yu Wang, Elena A. Pelech and Donald R. Ort*

Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL USA.

*Correspondence: Donald R. Ort, University of Illinois at Urbana-Champaign, 1206 W Gregory Drive, Urbana, IL 61801, USA

Email: d-ort@illinois.edu

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One-sentence summary

Photosynthetic efficiency of soybean pods and seeds contribute to the seed weight and the overall carbon gain of the canopy by compensating for carbon loss, with implications for yield.

ABSTRACT

There is a limited understanding of the carbon assimilation capacity of non-foliar green tissues and its impact on yield and seed quality since most photosynthesis research focuses on leaf photosynthesis. In this study, we investigate the photosynthetic efficiency of soybean (*Glycine max*) pods and seeds in a field setting and evaluate its effect on mature seed weight and composition. We demonstrate that soybean pod and seed photosynthesis contributes 13-14% of the mature seed weight. Carbon assimilation by soybean pod and seed photosynthesis can compensate for 81% of carbon loss through the respiration of the same tissues, and our model predicts that soybean pod and seed photosynthesis contributes up to 9% of the total daily carbon gain of the canopy. Chlorophyll fluorescence shows that the operating efficiency of Photosystem II in immature soybean seeds peaks at the 10-100mg seed weight stage, while that of immature pods peaks at the 75-100mg stage. This study provides quantitative information about the efficiency of soybean pod and seed photosynthesis during tissue development and its impact on yield.

INTRODUCTION

Photosynthesis converts light energy to transform CO_2 into soluble carbohydrates, which are then utilized for plant growth and maintenance (Stirbet et al., 2020). Most research has focused on leaf photosynthesis with a minimal understanding of potential carbon assimilation in non-foliar green tissue and its contribution to yield and seed quality. Chlorophyll-containing seeds (Schwender et al., 2004), ears of wheat (*Triticum aestivum*) (Maydup et al., 2010), and husks of maize (*Zea mays*) (Pengelly et al., 2011), along with other green non-foliar tissues, such as stems, all perform photosynthesis, possibly providing an additional important source of photoassimilates for the plant. Non-foliar photosynthesis is a photosynthetic process similar to that of the foliar mesophyll cell (Simkin et al., 2020), except that there are potentially two important sources of CO_2 for non-foliar photosynthesis. Both foliar and non-foliar photosynthesis utilize ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), which assimilates atmospheric CO_2 that enters the cells through the stomata. In contrast to foliar tissue, some non-foliar tissues fix a larger portion of CO_2 that is released by mitochondrial respiration and then

refixed by Rubisco (Aschan and Pfanz, 2003; Millar et al., 2011). Stomata are found in varying numbers on non-foliar tissues; however, their function and the amount of photosynthesis that relies on CO_2 from the atmosphere through these stomata have yet to be determined (Simkin et al., 2020).

The embryos of many plants (e.g., Arabidopsis (*Arabidopsis thaliana*), chickpea (*Cicer arietinum*), coffee (*Coffea arabica*), cotton (*Gossypium hirsutum*), soybean (*Glycine max*), oilseed rape (*Brassica napus*), pea (*Pisum sativum*), broad bean (*Vicia faba*)) contain considerable amounts of chlorophyll, although the level of chlorophyll depends on the developmental stage (Yakovlev and Zhukova, 1980; Simkin et al., 2010; Puthur et al., 2013; Smolikova and Medvedev, 2016). Green embryos, known as chloroembryos (Palanisamy and Vivekanandan, 1986), have all the photosynthetic complexes such as Photosystem I (PSI), Photosystem II (PSII), cytochrome b6f complex, and ATP synthase (Weber et al., 2005; Allorent et al., 2015; Kohzuma et al., 2017). Rubisco is known to be active in seeds of soybean (Allen et al., 2009), oilseed rape (Hills, 2004; Ruuska et al., 2004), broad bean, and fenugreek (*Trigonella foenum-graecum*) (Willmer and Johnston, 1976). Seed photosynthesis contributes to the accumulation of storage lipids in oilseed rape (Eastmond et al., 1996; Ruuska et al., 2004), and the rapid production of ATP and NADPH for the synthesis of complex carbohydrates, fatty acids, and proteins has also been linked to photosynthesis in some chloroembryos (Asokanthan et al., 1997; Wu et al., 2014).

The tissue surrounding a seed, such as a pod in legumes, may also photosynthesize, and the rate depends heavily on the species and how much light its seed-related tissues receive, which is determined by plant and canopy architecture. Rubisco activity has been detected in the pod walls of peas; however, Rubisco concentrations are 10–100 times lower than that seen in leaf tissue (Hedley et al., 1975). The photosynthetic activity of the wheat ear substantially contributes to the pool of carbohydrates translocated to the developing grains during the post-anthesis stages (Tambussi et al., 2005; Tambussi et al., 2007; Maydup et al., 2010; Sanchez-Bragado et al., 2014). Although the wheat ear net CO_2 assimilation rate is lower on an area basis than that of the flag leaf (Tambussi et al., 2005; Tambussi et al., 2007), wheat ear photosynthesis can contribute up to 70% of the individual grain weight yield component in a wide range of genotypes (Maydup et al., 2010) and contrasting environments (Sanchez-Bragado et al., 2014). Shading research in

barley (*Hordeum vulgare*) demonstrates a large contribution of the ear to grain weight (up to 50%) and thus yield, similar to wheat (Bort et al., 1994). Despite that only a fraction of the above canopy photosynthetic photon flux density (PPFD) reaches legume embryos, seed coats, and pods, all three tissues have relatively high rates of electron transport (Allen et al., 2009; Tschiersch et al., 2011). Both immature soybean pods and seeds are green and capable of photosynthesis but unlikely to experience positive net rates of photosynthesis (Sambo et al., 1977) due to high rates of respiration.

The most frequent method for determining the contribution of non-foliar photosynthesis is the suppression of photosynthesis through light exclusion (i.e., covering) or herbicide treatment (Sanchez-Bragado et al., 2016). Seeds that develop in foil-wrapped *Arabidopsis* siliques have lower oil and starch, as well as lower overall seed weight, than seeds that develop in siliques exposed to light (Liu et al., 2017). Other *Arabidopsis* studies using chemical inhibitors of embryonic photosynthesis (Allorent et al., 2015) or inducible photosynthesis-deficient mutants (Sela et al., 2020) report that final seed lipid and protein content is unaffected but that germination and early growth from the seeds are reduced.

Here, we investigate the photosynthetic capacity of soybean pods and seeds during development and evaluate the impact of pod and seed photosynthesis on seed weight and composition under field conditions, covering developing soybean pods with aluminum foil for 6-8 weeks (starting from less than 2 cm in pod length to fully grown and desiccated). Our goal is to determine whether pod and seed photosynthesis in soybean contributes significantly to the mature seed and to the plant overall.

RESULTS

Pod and seed photosynthesis contributes 13-14% of the seed weight under Illinois field conditions

To understand the effect of pod and seed photosynthesis on the seed weight and composition of soybean under field conditions, we conducted field experiments on covered pods. We covered pods of Clark (maturity group IV) soybean with foil at an early stage of

development to block light from reaching the pods and seeds and then measured the weight and composition of the mature soybean seeds that developed in those pods compared to uncovered pods (Figure 1A and B). The results for the cultivar Clark showed that covering the pods with foil during development significantly reduced the final weight of the seeds inside (14.3 g) compared to seeds from uncovered pods (16.6 g), a decrease of 13% (Figure 1C). Similar results were found with another cultivar in the field, Williams 82 (maturity group III), in which seeds from foil-covered pods showed a significant reduction in final seed weight (14.5 g) compared to seeds from uncovered pods (16.9 g), a decrease of 14% (Figure 2B).

We compared the efficiency of photosystem II between immature covered and uncovered pods and seeds of Williams 82 from the field using chlorophyll fluorescence. We collected green pods and seeds at 100-200 mg (average fresh seed weight). The foil-covered pods and seeds were visibly much paler (Supplemental Figure S1A and D), confirmed by the chlorophyll fluorescence images (Supplemental Figure S1B and E). The operating efficiency of photosystem II (F_q'/F_m') was significantly reduced in both the seeds and the pods that had developed in foil, compared to the uncovered seeds and pods (Supplemental Figure S1C and F). The temperature and humidity of the air space inside the foil cover and immediately outside were measured and found to have no significant difference (Supplemental Figure S2), indicating temperature and humidity did not affect the change in seed weight.

The effect of covering on the concentrations of protein and oil was not consistent in the two genetic backgrounds. Seeds from covered pods had a 5% decrease in oil and a 5% increase in protein compared to seeds from uncovered pods in Clark (Figure 1D and E), but no significant difference was found in Williams 82 (Figure 2C and D). No seed composition parameter evaluated here (Supplemental Figures S3 and S4) appeared consistently affected by the foil-covering across the cultivars tested.

Pod and seed photosynthesis can compensate for 81% of carbon loss from respiration of pod and seed under field conditions

We measured rates of net photosynthesis and dark respiration (R_{dark}) in pods during development (Figure 3A) using the LI-6800 (LICOR Biosciences Inc, Lincoln, NE, USA), allowing illumination on both sides of the pod (Figure 3B). For the determination of gross photosynthesis, the rate of respiration in the light was assumed to be equal to the rate of respiration in the dark. Measured rates of dark respiration were significantly higher in uncovered pods (10.7 μmol CO_2 m^{-2} s^{-1}) compared to covered pods (8.3 μmol CO_2 m^{-2} s^{-1}) (Table 1).

We estimated the rate of gross photosynthesis, the true rate of photosynthesis, taking into account the rate of respiration, by adding R_{dark} to the rate of net photosynthesis at each PPFD (Wittmann et al., 2006). Rates of net photosynthesis were low in illuminated pods that developed in the dark for 3-4 weeks and were substantially different across PPFD levels, following a pattern typical for light-response curves in leaves (Figure 3C). The highest measured rates of net and gross photosynthesis occurred at 2500 μmol quanta m^{-2} s^{-1} for uncovered and 750 μmol quanta m^{-2} s^{-1} for covered pods (Figure 3D). Measured net photosynthesis in uncovered pods peaked at a rate of 0.48 μmol CO_2 m^{-2} s^{-1}, whereas measured net photosynthesis in covered pods peaked at a rate of -5.9 μmol CO_2 m^{-2} s^{-1}. In uncovered pods, measured rates of gross photosynthesis peaked at 11.2 μmol CO_2 m^{-2} s^{-1}, and covered pods peaked at a rate of 2.4 μmol CO_2 m^{-2} s^{-1}. Rates of saturating photosynthesis were modeled using the R package 'Photosynthesis' (Stinziano et al., 2020); the modeled rates of saturating gross photosynthesis were similar to measured rates of gross photosynthesis. Modeled saturating rates of gross photosynthesis were 11.4 μmol CO_2 m^{-2} s^{-1} and 2.5 μmol CO_2 m^{-2} s^{-1} in uncovered and covered pods, respectively (Table 1). The maximum quantum efficiency (ΦCO_2), estimated from modeling, was ten times higher in uncovered pods than in covered pods (Table 1).

Canopy incident light for this experiment was considered to be 300 μmol quanta m^{-2} s^{-1} (close to 260 μmol quanta m^{-2} s^{-1} measured by Allen et al., 2009) for uncovered and 0 μmol quanta m^{-2} s^{-1} for covered pods. The rates of net photosynthesis at canopy incident light were measured as -2 μmol CO_2 m^{-2} s^{-1} for uncovered and -8.3 μmol CO_2 m^{-2} s^{-1} for covered pods (Table 1). The rates of gross photosynthesis in these pods at canopy incident light levels were measured as 8.7 μmol CO_2 m^{-2} s^{-1} and 0 μmol CO_2 m^{-2} s^{-1} (Table 1) for uncovered and covered, respectively. While uncovered pods at the canopy light condition of 300 μmol quanta m^{-2} s^{-1}

were still a net carbon loss for the plant, pod and seed photosynthesis was nevertheless able to compensate for 81% of the carbon lost through respiration.

A canopy model projects that pod and seed photosynthesis contributes up to 9% of total gross canopy photosynthesis

Soybean pods are mostly located under the canopy, where various light environments are present depending on height, developmental stage and time of day. To estimate the contribution of pod photosynthesis to overall canopy photosynthesis of soybean, model simulation studies were performed using a multilayer canopy model (Campbell & Norman, 1998; Drewry et al., 2010). The measured leaf area index was 9.52 ± 0.20 (SE), and the pod area index was 1.30 ± 0.14 (SE); the leaves were mainly concentrated in the middle of the canopy, while the pods were more evenly distributed throughout the canopy (Figure 4A, Supplemental Table S1). Simulation of canopy photosynthesis over the whole day of 17 August 2022 (R6 stage, full-length filled pods and fully green plant) indicated that pod photosynthesis contributed about 9.6% to the overall gross assimilation of CO_2 of the soybean canopy (Figure 4B). The simulated proportion of pod contribution was greater in the early morning and late afternoon and slightly less at noon (8.1%).

Efficiency of seed and pod photosynthesis peaks by the 100 mg developmental stage under field conditions

We investigated the photosynthetic efficiency of immature seeds from field-grown Williams 82 soybeans during their development (Figure 5A). We collected immature pods and measured seed fresh weight to separate seeds into seven different developmental stages (Figure 5B), then measured the operating efficiency of photosystem II (F_q'/F_m') of the seeds (Figure 5C). The operating efficiency of photosystem II (F_q'/F_m') of the seeds started at 0.323 in the 5-10 mg stage, increased in the 10-25 mg stage (0.393), and remained steady before decreasing (0.363) in the 75-100 mg developmental stage (Figure 5D). The chlorophyll concentration of seeds started at 0.426 mg/g DW in the 5-10 mg stage, decreased in the 10-25 mg stage (0.287 mg/g DW), then

increased and remained steady until the 75-100 mg stage (0.432 mg/g DW), after which it decreased (Figure 5E). The ratio of chlorophyll a to b increased across the sequential developmental stages (Figure 5F).

We also investigated the photosynthetic efficiency of the corresponding developing pods by measuring the efficiency of photosystem II in the same way after the seeds had been removed. The developmental stages of pods were labeled based on the average weight of seeds inside the pods (Figure 6B). The operating efficiency of photosystem II of the pods started at 0.291 in the 5-10 mg stage, decreased in the 10-25 mg stage (0.273), then rose to a peak at the 75-100 mg stage (0.3) (Figure 6D). The concentration of chlorophyll in these pods started at 0.63 mg/g DW in the 5-10 mg stage, then gradually decreased until the 75-100 mg stage (0.355 mg/g DW), after which it rose slightly (Figure 6E). The ratio of chlorophyll a to b remained similar across the sequential pod developmental stages (Figure 6F).

Photosynthesis-related genes were actively expressed in the early stages of seed development

We explored which photosynthesis genes were expressed in early soybean seed development using previously published high-throughput transcriptome profiling (RNA-seq) with the cultivar Williams 43 (Jones et al., 2013; Cho et al., 2019). The expression levels of photosynthesis-related genes were disproportionately high in the very young seeds, mostly over 50 RPKM (Reads Per Kilobase per Million mapped reads), whereas most genes in general (>98%) were expressed lower than 50 RPKM in the data. The gene expression levels were mostly high in the early stages (a few days after flowering) and peaked at 5-6 mg in whole seeds, including genes related to Rubisco, the light reactions, the photosynthetic carbon metabolism reactions, and chlorophyll synthesis (Figure 7, Supplemental Figures S5 and S6). At later stages (100-200 mg and older), photosynthesis-related genes were actively expressed but mostly decreased in the cotyledon tissues.

Interestingly, the gene expressions of photosynthesis-related genes (Figure 7) were active ahead of the peak photosynthetic efficiency of the seeds at the 10-100 mg stages, as shown by

chlorophyll fluorescence (Figure 5C). Expression levels of genes related to chlorophyll cycle pathways peaked before and after the 5-6 mg stage (Supplemental Figure S6C). This occurrence may explain the decrease of chlorophyll levels at the 10-25 mg stage (Figure 5E). Chlorophyll-related genes were expressed highly in different developmental stages depending on their functions. Chlorophyll synthesis-related genes were highly expressed in the early stages and then decreased after 5-6 mg, while chlorophyll degradation and heme synthesis-related genes were active at the latest developmental stages when the seed had turned yellow and was drying down (Supplemental Figure S6). These data suggest that the efficiency of photosynthesis and the level of chlorophyll are tightly connected with gene expression during seed development.

DISCUSSION

In this study, we evaluated the effect of soybean pod and seed photosynthesis on seed weight and composition in a field setting and also investigated the photosynthetic efficiency and rate during seed development. This study demonstrated that soybean pod and seed photosynthesis contributes 13-14% of the seed weight in the field in Illinois, which is a major soybean growing area (Figures 1-2). The carbon assimilation by pod and seed photosynthesis at under-canopy light levels can compensate for 81% of carbon loss through the respiration of the same tissue (Figure 3). A multilayer canopy model predicts that pod and seed photosynthesis contributes up to 9% of the daily total carbon assimilation of the soybean canopy (Figure 4). Chlorophyll fluorescence showed that the operating efficiency of PSII in immature seeds peaked at the 10-100 mg seed weight stages, while that of immature pods peaked at the 75-100 mg seed weight stage (Figure 6). Previously, there was little understanding of potential carbon assimilation in seed and pod tissues and its impact on yield and seed quality.

Despite having low rates of net photosynthesis, uncovered soybean pods have the ability to fix more CO_2 than is lost through dark respiration at light levels greater than 1000 μmol quanta m^{-2} s^{-1}. However, under canopy light conditions at 300 μmol quanta m^{-2} s^{-1}, the rate of net photosynthesis in uncovered pods was measured at -2 μmol CO_2 m^{-2} s^{-1}, and gross photosynthesis was measured at 8.7 μmol CO_2 m^{-2} s^{-1} (Figure 3 and Table 1). While uncovered pods under canopy light conditions were still a net carbon loss for the plant, our data showed that

pod photosynthesis was able to compensate for 81% of the carbon lost through respiration under normal field within canopy light levels. The ΦCO_2 of pods in Williams (0.06) is similar to the ΦCO_2 of leaves in Clark (0.051-0.068, from Slattery et al. 2017), indicating that pods and leaves have a similar quantum efficiency for carbon assimilation. In contrast, the gross photosynthesis at saturating light in pods was about 3 times lower than that in the leaves (11.4 vs 35.7 μmol CO_2 m^{-2} s^{-1}). The capacity of photosynthesis on an area basis is much higher in leaves, while the quantum efficiency of carbon assimilation is similar between the two tissues.

Covering young soybean pods with foil and allowing the seeds to develop to maturity in the foil, thereby reducing the input from photosynthesis, resulted in seeds with a lower weight (g/100 seeds) than uncovered seeds by 13-14%. This effect was seen in two different cultivars of soy that were grown in the Illinois field (Figures 1 and 2). From this, we conclude that seed and pod photosynthesis contributes to soybean seed weight under field conditions, similar to results found for wheat ears (Maydup et al., 2010) and barley ears (Bort et al., 1994). The relative importance of these non-foliar assimilates varies depending on the species, cultivar, and environment (Tambussi et al., 2007). From our data, we conclude that green soybean pods and seeds are photoheterotrophic, meaning that seeds and pods are sink tissues, but also that their photosynthesis substantially contributes to carbon gain, being responsible for 9% of total daily seed carbon according to our model.

Our model estimated the contribution of pod and seed photosynthesis from the parameters of a maturity group III variety measured at the SoyFACE field in Illinois on 17 August 2022 (93 days after planting), when the deepest canopy was present during the development of soybean. The angle of the sun during early morning and late afternoon allows a greater proportion of light to reach the pods (Supplemental Table S2), which in turn leads to a higher proportion of pod contribution during those times (Figure 5). Pods under the canopy can get more light before or after the development of the densest canopy; thus, our model likely tends to underestimate the contribution of pod and seed photosynthesis to canopy photosynthesis. We set the pod angle distribution parameter in the multilayer canopy model at zero, thus assuming all pods are vertical for convenience, which is another factor by which our model may underestimate the contribution of pod and seed photosynthesis because the angle of pods in the field is greater than zero. Our model was able to parameterize the sun leaf angle allowing better

light penetration into the canopy in the morning and afternoon, leading to the proportion of pod contribution being greater in the early morning and late afternoon than at noon. Three-dimensional (3D) canopy photosynthesis models can accurately simulate the intricate 3D architecture and microclimate under the canopy during plant development (Watanabe et al., 2005; Zheng et al., 2008; Song et al., 2013; Wang et al., 2017; Wang et al., 2020).

We cannot exclude the possibility that limiting light from the developing pods and seeds may have impacts not directly related to photosynthetic carbon gain. One possibility is that the foil covering interferes with the development of plastids, which play a critical role in seed development as a source of energy for oil synthesis and starch storage. Despite the low levels of available light inside the seed, soybean seed photosynthetic activity is surprisingly high, possibly due to the unique nature of the chloroplasts present in the seed (Borisjuk and Rolletschek, 2009). These chloroplasts have chlorophyll-protein complexes similar to those found in leaf chloroplasts but with a higher proportion of granal stacking (Saito et al., 1989; Asokanthan et al., 1997), which allows for distinct light harvesting properties and low saturation levels for photosynthetic electron transport (Borisjuk et al., 2005). The seed chloroplasts conduct light reactions to generate ATP/NADPH, which can fuel the biosynthesis of seed oil (Ruuska et al., 2004; Schwender et al., 2004). Furthermore, a recent study showed that the chloroplasts of bean fruit mesocarp cells differentiate into amyloplasts, which allows for the storage of surplus sucrose for starch synthesis, later used by the seeds when the sucrose supply is no longer sufficient (Belmont et al., 2022).

Recent studies using different methods to block pod and seed photosynthesis in *Arabidopsis* have found that mature seed lipid and protein are not affected, whether the photosynthesis is blocked by chemicals (Allorent et al. 2015) or by inducible mutations (Sela et al. 2020). In contrast, Liu et al. (2017), who wrapped developing *Arabidopsis* siliques in foil, observed a decrease in oil bodies in the dark-grown embryos, and in other species, such as *B. napus*, reduced pod and seed photosynthesis has a negative impact on the amount of seed oil (Ruuska et al. 2004; Schwender et al., 2004). We evaluated the effect of pod and seed photosynthesis on soybean seed composition however could not conclude that light levels and related pod and seed photosynthesis affect soybean seed composition (Figures 1 and 2). Numerous studies have reported that soybean seeds at the top of the canopy contain more protein

and less oil than seeds at the bottom of the canopy (Collins & Carter, 1956; Escalante & Wilcox, 1993a and 1993b; Huber et al., 2016). We collected seeds from the four uppermost nodes (designated as the upper layer of the canopy) separately from the rest of the nodes (designated as the lower layer of the canopy). The results for both of the cultivars studied here (Clark and Williams 82) showed that covering the pods with foil during development significantly reduced the final seed weight, compared to seeds from uncovered pods wherever they were located in the canopy (Supplemental Figures S7 and S8). In contrast to the seed weight, the protein and oil concentrations were unaffected by the foil-covering and instead related only to the position of the pod in the canopy. However, as we did not track the position (upper or lower) of the foil-covered pods, we cannot say for certain that the foil-covering had no effect on seed composition.

This study investigated the photosynthetic efficiency of developing soybean pods and seeds by measuring F_q'/F_m', representing the operating efficiency of PSII photochemistry, which is indicative of functional electron transport. Modulated chlorophyll fluorescence assesses the photosynthetic efficiency indirectly but more easily and quickly than other methods, such as gas exchange, and has been previously used for this purpose in wheat ear studies (Tambussi et al., 2005; Maydup et al., 2012). Our results showed that seed photosynthesis peaked at the 10-100 mg developmental stages and that pod photosynthesis peaked at the 75-100 mg developmental stage in Illinois field conditions (Figures 5 and 6). Additionally, the expression levels of photosynthesis-related genes were high in the early stages of seed development, even earlier than were studied here with chlorophyll fluorescence, and peaked at the 5-6 mg developmental stage, then decreased with time (Figure 7). The expression of chlorophyll synthesis genes at older seed stages, such as 100-200 mg, was observed to be synchronized with the fluorescence measurements and chlorophyll amounts in this study (Figures 5-7). These observations suggest, unsurprisingly, that the efficiency of photosynthesis and the production of chlorophyll are tightly connected with gene expression during seed development, similar to leaf photosynthesis (Foyer et al., 2012; Hibberd and Covshoff, 2010).

Multinational efforts have been made to improve photosynthesis over the past decade, with the premise that crop production improvements must be swift and substantial due to the plateauing of yields of important crops in the face of rapidly growing agricultural demand (Long and Ort, 2010). Several studies reported improved photosynthesis and increased yield in model

plants (Kromdjik et al., 2016; South et al., 2019) and crops (Yoon et al., 2020; de Souza et al., 2022). One way to improve photosynthesis in crops is to engineer a better light distribution under the canopy so that photosynthetic tissues receive more light and can operate at higher efficiency (Ort et al., 2015). Improved light distribution inside a canopy could also increase the light use efficiency of non-foliar tissues such as soybean pods and seeds. Although leaves are the most important photosynthetic tissue, this study and others (Simkin et al., 2020; Lawson and Miliken, 2023) suggest that improved efficiency of non-foliar tissues, such as soybean pods and seeds, can also contribute to improved yield of important crops.

In summary, we demonstrated that pod and seed photosynthesis contributed a significant portion of carbon assimilation and seed weight in soybean–9% of total daily carbon assimilation according to our model and 13-14% of seed weight, which is a vital component of yield. These data suggest that pod and seed photosynthesis plays an important role in soybean yield, which merits further study in the context of improving global food supplies in a sustainable way.

MATERIALS AND METHODS

Field conditions

Two cultivars of soybean [*Glycine max* (Williams 82 PI518671 and Clark PI548533)] were planted in 1.5-m rows using standard agronomic practices at the University of Illinois Energy Farm field station (40.11°N, 88.21°W, Urbana, IL, USA) on 27 May 2021. Each row (1 block) consisted of ~10 plants spaced 3.8 cm apart. There were 4 blocks for Clark and 8 blocks for Williams 82.

Treatment and sample collection

A cluster of young pods (smaller than 2 cm, the earliest in their development that we were able to wrap with foil without risk of damage) was carefully wrapped in aluminum foil with the intent to block all light from reaching the pods, while still allowing them to grow. Multiple clusters of pods were covered with foil per block in the field, while leaving the majority of the

pods on the plant uncovered. Pods remained covered in foil for at least 3 weeks before collection (for immature tissues) or until maturity (6-8 weeks). Immature pods were collected and opened, and the seeds were removed. The immature seeds were weighed to determine the developmental stage (fresh weight range); pods were labeled by the fresh weight of the seeds inside them (using the stage of the majority of the seeds if not all were the same). Foil-covered seeds and pods were kept in the dark as much as possible during the procedure. For the Williams 82 field-grown developmental series, immature seeds (and their pods) were collected at 7 fresh weight ranges: 5-10 mg, 10-25 mg, 25-50 mg, 50-75 mg, 75-100 mg, 100-200 mg, 200-300 mg. Dry pods were harvested at maturity and shelled, and the seeds were further dried in the oven overnight at 50 ℃ or air-dried for at least 2 weeks before further measurements.

Mature seed harvest and non-destructive near-infrared (NIR) spectroscopy for seed composition

From the field, both Clark and Williams 82 pods were harvested at maturity on 4 October 2021. Uncovered and foil-covered pods were collected separately. Each block for each genotype contained approximately 10 plants, and seeds were pooled from plants within the same block/genotype/treatment. Uncovered pods were initially divided into ‘Up’ (harvested from the upper four nodes of the plant) and ‘Low’ (harvested from the rest of the plant). For Clark, seeds from foil-covered pods were obtained from all 4 blocks (one block was removed from data analysis as an outlier); for Williams 82, seeds from foil-covered pods were obtained from 5 of 8 blocks. Uncovered and foil-covered seeds were further dried in the oven overnight at 50 ℃, then air-dried for at least 2 weeks before NIR measurement. For seeds from uncovered pods, total weight and the weight of 100 randomly-selected seeds were obtained from each block (divided into ‘Up’ and ‘Low’); from this, an approximate seed count was calculated. For seeds from foil-covered pods, the exact number of seeds per block was counted and weighed.

Near-infrared (NIR) spectroscopy was performed using the Perten DA7250 (PerkinElmer, Waltham, MA, USA) with the company settings for whole soybean seeds, non-destructively measuring 28 components including protein, oil, 5 fatty acids, and 18 amino acids. The mirror cup was used with all samples. For the Clark and Williams 82 field-grown seeds from

uncovered pods, 20 replicates of 10 seeds each were measured per block (200 different seeds per block), half from the 'Up' seeds and half from the 'Low' seeds. 'Up' and 'Low' data were later combined for analysis. For the Clark seeds from foil-covered pods, 4 blocks yielded 1 to 3 replicates each, for a total of 7 replicates; each replicate had 10 seeds. For the Williams 82 seeds from foil-covered pods, 5 blocks yielded 1 to 5 replicates each, for a total of 11 replicates; each replicate had 10 seeds.

Pod photosynthesis measurements

Photosynthesis on pods still attached to the plants was measured between 7 September and 15 September 2021 at the UI Energy Farm. Pods were measured using an LI-6800 (LICOR Biosciences Inc, Lincoln, NE, USA) fitted with a clear-top chamber (LI-6800-12A) using the 3 cm X 3 cm aperture insert. Small light sources (LI-6800-02) were fitted to the top and bottom of the clear-top chamber to allow illumination on both sides of the pod (Fig 3A). Pods were placed flat in the chamber to allow for full illumination on both sides of the pod. Pods were photographed, and projected pod area was estimated using ImageJ (US National Institutes of Health, Bethesda, MD, USA). The pod was allowed to acclimate in the chamber for at least 15 minutes or until the rate of photosynthesis was stable. Reference CO_2 was set to 400 μmol mol^{-1}, PPFD set to 2500 μmol quanta m^{-2} s^{-1}, energy balance set to 30°C, and relative humidity set to 50%. The chamber was set to an overpressure of 0.1 kPa with a flow rate of 1100 μmol s^{-1}. Rates of net photosynthesis were measured across a light-response curve which consisted of the following PPFDs for both light sources: 2500, 2000, 1600, 1200, 900, 750, 600, 500, 400, 300, 200, 120, 60, 20, 0, 0, 0, 0 μmol quanta m^{-2} s^{-1} with a minimum wait time of 60 s and a maximum wait time of 90 s that the LI-6800 was set to match before each measurement. We assumed the rate of respiration in the light was equal to the rate of dark respiration (R_{dark}). Gross photosynthesis, the true rate of photosynthesis, was estimated by adding R_{dark} to the rate of net photosynthesis at each PPFD (Wittmann et al., 2006). Transmittance through pods is higher than transmittance through leaves (Allen et al., 2009); however, the transmittance through the entire seed is low. Most of the area taken up by the pod in the chamber included the seeds, leading us to

choose to present PPFD on a single-sided basis as light is unlikely to pass through the seed. Light-response curves were measured on five covered and five uncovered Williams 82 pods.

Leaf area index (LAI) measurements

Leaf area index was measured on 17 August 2022 using the Sunscan Canopy Analysis System (DeltaT Devices, Cambridge, UK) at 12 cm height increments within the soybean canopy. Six rows of soybean were measured, where four replicate positions were averaged for each increment. The Ellipsoidal Leaf Angle Distribution Parameter (ELADP) was set to 0.81, and absorption was set to 0.85.

Canopy model

To estimate the contribution of pod photosynthesis to the overall canopy photosynthesis of soybean, model simulation studies were performed using a multilayer canopy model (Campbell & Norman, 1998; Drewry et al., 2010). Leaf area indexes were measured from the SoyFACE field in Illinois on 17 August 2022. Pod area indexes were estimated by measuring plant density, pod area, and number of pods per plant (Supplemental Table S1). The leaf angle distribution parameter was set as 0.81 (Campbell & Norman, 1998), and the pod angle distribution parameter was set as 0, assuming all pods are vertical.

Chlorophyll fluorescence for photosystem II operating efficiency

F_q'/F_m' was measured by a chlorophyll fluorescence (CF) imager (CF Imager, Technologica, UK). Pods (with seeds removed) were exposed to 260 μmol quanta m^{-2} s^{-1} (light intensity under canopy from Allen et al., 2009) for 10 min until stabilized, then subjected to a burst of 6100 μmol quanta m^{-2} s^{-1} for 800 ms. Seeds were exposed to 78 μmol quanta m^{-2} s^{-1} (light intensity inside pods from Allen et al., 2009) for 10 min until stabilized then subjected to a burst of 6100 μmol quanta m^{-2} s^{-1} for 800 ms. After the burst, F' and F_m' were automatically

measured by the software. The images were manually adjusted, and F_q'/F_m' was calculated. For the Williams 82 field-grown developmental series, each of the 7 immature seed stages included 41 to 48 individual seeds, and each of the 7 immature pod stages included 32 to 42 pod halves across 7 blocks (1 block was not used). Seeds were removed from the pods prior to imaging. One-half of the pod was imaged, interior side down. Seeds and pods were collected and imaged over two days, 25 to 26 August 2021. Immature Williams 82 field-grown foil-covered pods were collected and imaged over two days, 8 and 10 September 2021. Four immature foil-covered pods (7 pod-halves) and their 12 seeds (100-200 mg fresh weight) were imaged separately. For comparison, 5 immature uncovered pods (7 pod-halves) and their 15 seeds (100-200 mg fresh weight) collected at the same time were imaged separately.

Chlorophyll measurement

Separated immature pod and seed samples were frozen in liquid nitrogen for at least 10 minutes and stored in the freezer (-80 °C) until they were lyophilized. Chlorophyll content was determined using 100% (v/v) ethanol extraction (Ritchie, 2006) and microplate spectrometer (Warren et al., 2008). For the Williams 82 field-grown developmental series, each of the 7 immature seed stages included 4 to 10 replicates (containing 3 to 4 individual seeds each) from 2 to 4 (of 8) different blocks. Each of the 7 immature pod stages included 6 to 12 replicates (an individual pod-half as a replicate) from 3 to 6 (of 8) different blocks. Lyophilized seeds were weighed, and weight was considered when calculating the amount of chlorophyll present.

Transcriptome data analysis

All raw high-throughput transcriptome (RNA-Seq) data were downloaded from the Gene Expression Omnibus (GEO, GSE42871 and GSE123655); see Supplemental Dataset S1 for the list of samples. Photosynthesis-related soybean gene models were chosen based on a keyword search of annotations at Phytozome (https://phytozome-next.jgi.doe.gov/).

Temperature and humidity measurements

Temperature and humidity were measured inside 17 foil-covered packets surrounding pods in the field using SRH77A Temperature / Humidity Thermistor Instrument (Cooper-Atkins, CT, USA). An equal number of measurements were taken from equivalent pods that were uncovered. Field measurements were taken on 10 September 2021.

Statistical analysis

The statistical analyses were done with R (version 4.0.3). The biomass, photosynthesis estimated from fluorescence, and chlorophyll levels from field trials were analyzed in a mixed model analysis of variance (ANOVA) followed by a *post-hoc* Tukey test (α=0.05). Block was considered as the random effect for all analyses, while foil-covering treatment was considered as the fixed effect. For the light-response curves, we used the lme4 R package (Bates et al., 2015) to perform linear mixed effects analyses of the relationship between the physiological response variables (net and gross photosynthesis) and treatment (covered vs. uncovered). We set treatment and PPFD as fixed effects. We structured the model to allow for random intercepts for individual pods. Rates of saturating gross photosynthesis, dark respiration and φCO_2 were modeled using the 'photosynthesis' R package (Stinziano et al. 2020). Saturating rates of net and gross photosynthesis and measured rates of dark respiration were compared in covered and uncovered pods using independent t-tests. If data were not normally distributed, non-parametric analysis (Wilcoxon rank test) was conducted by R program (α=0.05).

Data availability

Raw data available in the Supplemental Dataset S1 File.

Supplemental Data

Supplemental Figure S1. The efficiency of photosystem II of covered and uncovered pods under field conditions (Williams 82).

Supplemental Figure S2. Temperature and humidity around covered and uncovered pods.

Supplemental Figure S3. Seed composition of covered and uncovered seeds under field conditions (Clark).

Supplemental Figure S4. Seed composition of covered and uncovered (lower or upper position) seeds under field conditions (Wm82).

Supplemental Figure S5. Expression of genes annotated as Rubisco, Photosystem I, Photosystem II, light harvesting complex, cytochrome b6 f-related, fructose bisphophatase and -aldolase (Calvin cycle), ferredoxin-related, ATPase.

Supplemental Figure S6. Expression of genes annotated as chlorophyll synthesis.

Supplemental Figure S7. Seed composition of covered and uncovered seeds under field conditions (Clark).

Supplemental Figure S8. Seed composition of covered and uncovered seeds under field conditions (Wm82).

Supplemental Table S1. Values, units and descriptions of the parameters in the multilayer canopy model of soybean.

Supplemental Table S2. The total light absorbed by leaves and pods of the soybean canopy.

Supplemental Dataset S1.

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TABLES

Table 1. Pod photosynthetic parameters for Williams 82, measured 7 September through 16 September 2021 at the University of Illinois Energy Farm in Urbana, IL, USA. Different letters represent significant differences at the $p<0.05$ level obtained from independent t-tests. Asterisk (*) indicates model parameters were estimated using the R package 'Photosynthesis' (Stinziano et al., 2020).

	Uncovered	Covered
Respiration in the dark ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	10.7[a]	8.3[b]
Gross photosynthesis at light saturation* ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	11.4[a]	2.5[b]
ΦCO_2 gross photosynthesis at light saturation*	0.06[a]	0.007[b]
Net photosynthesis at incident light ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	-2 (at 300 μmol quanta $m^{-2}\ s^{-1}$)	-8.3 (at 0 μmol quanta $m^{-2}\ s^{-1}$)
Gross photosynthesis at incident light ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	8.7 (at 300 μmol quanta $m^{-2}\ s^{-1}$)	0 (at 0 μmol quanta $m^{-2}\ s^{-1}$)

FIGURE LEGENDS

Figure 1. Seed composition of covered and uncovered seeds under field conditions (Clark). The box plots show the median (central line), the lower and upper quartiles (box), and the minimum and maximum values (whiskers). Each dot represents a value (n= 3 blocks). N.s., not significantly different (α=0.05). ANOVA is used with linear mixed model (random effect = block, fixed effect = cover). Least squares mean is used to compare. Covering affects seed weight, oil and protein percentage.
A. Picture of soybean in the field (Clark).
B. Representation of aluminum foil that was used to cover pods, when the length of pod was shorter than 2 cm.
C. Seed weight, g/100 seeds. Seeds from covered pods: 14.3g ± 0.4 (SE). Seeds from uncovered pods: 16.6g ± 0.4 (SE). Seeds from covered pods have a 13% decrease in weight compared to seeds from uncovered pods.
D. Oil, percent. Seeds from covered pods: 18.7%. Seeds from uncovered pods: 19.8%. Seeds from covered pods have a 5% decrease in oil compared to seeds from uncovered pods.
E. Protein, percent. Seeds from covered pods: 46.1%. Seeds from uncovered pods: 43.7%. Seeds from covered pods have an 5% increase in protein compared to seeds from uncovered pods

Figure 2. Seed composition of covered and uncovered seeds under field conditions (Wm82). The box plots show the median (central line), the lower and upper quartiles (box), and the minimum and maximum values (whiskers). Each dot represents a value (n= 5 blocks for covered and 8 blocks for uncovered). ANOVA is used with linear mixed model (random effect = block, fixed effect = cover, α=0.05). N.s., not significantly different (α=0.05). Least squares mean is used to compare. Covering affects seed weight but not oil or protein percentage.
A. Picture of soybean at the field (Wm82).

B. Seed weight, g/100 seeds. Seeds from covered pods: 14.5g ± 0.6 (SE). Seeds from uncovered pods: 16.9g ± 0.4 (SE). Seeds from covered pods have a 14% decrease in weight compared to seeds from uncovered pods.
C. Oil, percent. Seeds from covered pods: 20.5%. Seeds from uncovered pods: 20.9%. There is no significant difference in oil between the seeds from the covered and the uncovered pods.
D. Protein, percent. Seeds from covered pods: 43.2%. Seeds from uncovered pods: 43.8%. There is no significant difference in protein between the seeds from the covered and uncovered pods.

Figure 3. Rates of net photosynthesis and dark respiration in pods. Small leaf and needle chamber with the small light sources to allow illumination of the pod from the top and bottom (A). Pods were placed horizontally in the chamber to allow full illumination and to estimate pod area (B). Rates of net (C) and gross (D) photosynthesis of covered (white) and uncovered (black) pods under field conditions (Williams 82). Each dot represents a value (n=5) ±SE. We assumed that the seeds greatly inhibited the transmittance of light through the pod and used photosynthetic photon flux density (PPFD) for a single-side.

Figure 4. Canopy model of soybean photosynthesis. A. Leaf area index and pot area index at each layer of canopy from SoyFACE soybean field. Layer1 is the top and level 8 is the bottom. B. Predicted diurnal gross CO2 assimilation of leaf and pod in the soy canopy on 17 August 2022.

Figure 5. Photosynthetic activity of seeds in seven developmental stages under field conditions: 5-10 mg, 10-25 mg, 25-50 mg, 50-75 mg, 75-100 mg, 100-200 mg, and 200-300 mg, based on the fresh weight of the seeds.
A. Picture of soybean (Williams 82) plant at the 2021 Illinois field.
B. Picture of collected soybean seeds with fresh weight range shown to the right. 5-10 mg seeds are on the top row with successively larger seeds in the lower rows.
C. Chlorophyll fluorescence (CF) image of photosystem II operating efficiency (Fq'/Fm') of seeds. This is the same plate shown in B.
D. Average value of Fq'/Fm' with error bars representing standard error (n=41-48).
E. Average value of total chlorophyll level with error bars representing standard error (n=4-10).
F. Average value of chlorophyll a/b ratio with error bars representing standard error (n=4-10).

Figure 6. Photosynthetic activity of pods in seven developmental stages under field conditions: 5-10 mg, 10-25 mg, 25-50 mg, 50-75 mg, 75-100 mg, 100-200 mg, 200-300 mg. Cultivar is Williams 82.
A. Picture of soybean plant at the 2021 Illinois field.
B. Picture of collected soybean pods. The fresh weight range of the seeds inside the pods is shown to the left and right.
C. Chlorophyll fluorescence (CF) image of photosystem II operating efficiency (Fq'/Fm') of pods. This is the same plate shown in B.

D. Average value of Fq'/Fm' with error bars representing standard error (n=32-42).
E. Average value of total chlorophyll level with error bars representing standard error (n=6-12).
F. Average value of chlorophyll a/b ratio with error bars representing standard error (n=6-12).

Figure 7**.** Expression of photosynthesis related genes during seed development. A-B. Rubisco genes (dark reaction); C-D. Light harvesting complex genes (light reaction); E-F. Mg-chelatase genes (chlorophyll synthesis); G-H. fructose bisphophatase and -aldolase (Calvin cycle) genes. Developmental stages for whole seed (WS): 1=4 Days After Fertilization (DAF) WS; 2=12-14 DAF WS; 3=22-24 DAF WS; 4=5-6mg WS. Developmental stages for cotyledon: 4=5-6mg cotyledon; 5=100-200mg cotyledon; 6=400-500mg cotyledon; 7=200-300mg yellow cotyledon; 8=dry cotyledon. Reads Per Kilobase per Million mapped reads (RPKM).

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