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# Physiological analysis of leaf photosynthesis of backcross-derived progenies from soybean (*Glycine max* (L.) Merrill) and *G. tomentella* Hayata

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## ABSTRACT

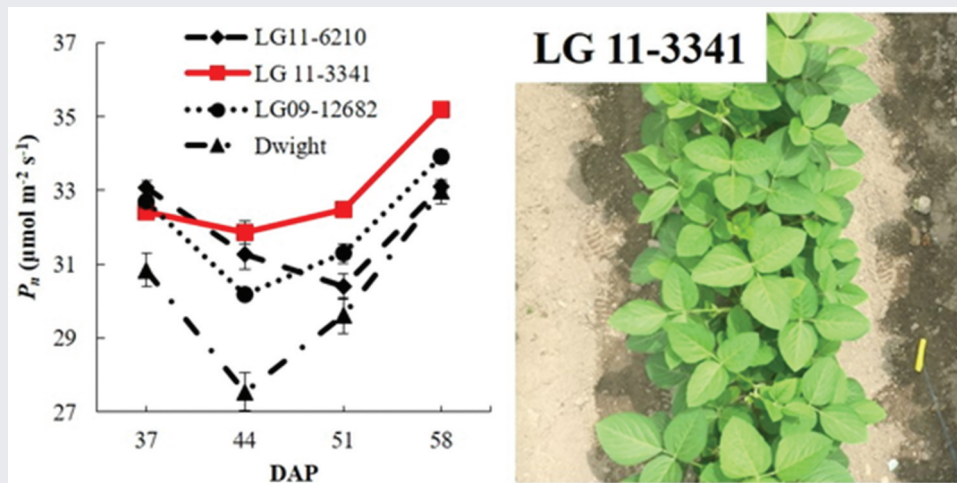
The enhancement of leaf photosynthesis is an enticing aspect for increasing crop seed yield. Using wild-related species in soybean breeding can be a potential source to enhance leaf photosynthesis. Two backcross-derived progenies of soybean (*Glycine max*) with *Glycine tomentella* Hayata were evaluated in terms of gas exchange, biomass, and seed yield. The gas exchange parameters along with Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) content, and leaf nitrogen content (LNC) were measured from flowering stage up to seed initiation stage. Results revealed significant increases of net photosynthetic rate ( $P_n$ ), mesophyll activity ( $P_n/C_i$ ), seed yield, and total aboveground dry weight (TDW) in progenies relative to Dwight. We observed significantly higher specific leaf weight (SLW) in progenies and was strongly correlated with  $P_n$  ( $r = 0.86^{***}$ ). There was no significant difference between Dwight and the progenies in stomatal conductance ( $g_s$ ), but Dwight, in fact, had higher intercellular  $CO_2$  concentrations ( $C_i$ ). It indicates that increases in  $P_n$  were associated with improved  $P_n/C_i$ . These findings suggest the potential use of soybean wild relatives in breeding to enhance soybean leaf photosynthesis.

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## KEYWORDS

Soybean; *Glycine tomentella*; net photosynthetic rate; mesophyll activity; Rubisco content; leaf nitrogen content



## 1. Introduction

A projected population of over nine billions people in 2050 requires twofold increase in the current rate of agricultural productivity (Tilman et al., 2011). Meeting the estimated demand while overcoming the exploitation of natural resources places photosynthesis at the forefront of a sustainable life (Janssen et al., 2014). Photosynthesis is a vital process for plants to grow, reproduce, and sustain all forms of life in one way or another (Lamber & Chapin, 2008). It has contributed little to the biomass enhancement so far but

improvements in its efficiency are expected to play a huge role in the efforts of agricultural productivity (Evans, 1997; Long et al., 2006; Zhu et al., 2010). Although some positive studies have been reported about photosynthetic enhancement in historic soybean germplasm (Koester et al., 2016), one of the avenues could be the utilization of soybean wild-related species.

Soybean, a major commodity crop (Hay et al., 2017), has 26 wild-related species (Singh and Nelson, 2015). Among these species, *Glycine tomentella* Hayata has

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four cytotypes, broad geographical distribution, and resistance to leaf rust (Bilgin et al., 2009; Chung & Singh, 2008; Schoen et al., 1992). In a struggle to broaden the soybean gene pool and to introduce leaf rust resistance to commercial soybean cultivars, Singh and Nelson (2015) crossed *G. tomentella* Hayata with *G. max* [cv. Dwight]. The F1 generation was backcrossed with Dwight until self-fertile progenies were developed. These progenies showed higher seed yield relative to Dwight in field trails (Begemann, 2015) and in controlled experiment (Akpertey, 2015) which suggests the lines carry some genetic factors of *G. tomentella* origin that enhanced yield potential. In fact, genetic material in wild *Glycine* species has been referred to as a locked treasure. Thus, we hypothesize that the utilization of wild-related soybean species in breeding programs may have resulted in progenies with enhanced photosynthetic capacity in addition to improvements in yield.

However, to the best of our knowledge, studies comparing photosynthetic capacity between progenies carrying genetic factors from wild-related soybean species and their cultivated parent have not been reported yet. For instance, Evans and Dunstone (1970) compared the photosynthetic capacity of modern wheat cultivars and their wild ancestor and reported lower photosynthetic rates in modern wheat. Similarly, Xue and Gao (2017) studied  $P_n$  between wild and cultivated soybean species along with Cadmium (Cd) stress; and reported that without Cd stress, there was no significant difference between them. Their assessment of photosynthetic capacity was the study of two different backgrounds whereas the question of what happens if they are crossed remains unanswered. Therefore, the objectives of this study are 1) to explore the difference and the underlying physiological mechanism in gas exchange parameters between Dwight and progenies and 2) to have an insight into the apparent seed yield and TDW enhancement in progenies.

## 2. Materials and methods

### 2.1. Plant materials and sowing procedure

We selected two backcrossed-derived progenies (BC4F6) of soybean and *G. tomentella* Hayata as candidates. These progenies were chosen based on higher  $P_n$  in our preliminary experiment in Illinois, U.S. (Figure 1) which include LG11-3341 and LG09-12682. LG11-6210 was cultivated checked as a high-yield genotype and Dwight is the recurrent soybean parent. Progenies and Dwight are indeterminate in growth. They were sown on 04, July 2016 and 27, June 2017 in a randomized complete block design (RCBD) with two replications in an experimental field at the Laboratory of Crop Science, Graduate School of Agriculture, Kyoto University (35°.2" N, 135°. 47" E). The plot size was 1 × 0.75 m and 2.4 × 2.9 m with a density of 9.52 plants per m<sup>2</sup> in 2016 and 2017, respectively. Seeds were soaked in pesticides prior to sowing in order to minimize pest damage. A 3–10–10 gm<sup>-2</sup> ratio of N, P<sub>2</sub>O<sub>5</sub>, and K<sub>2</sub>O fertilizers was used during sowing, respectively. Three seeds were planted per hill and were thinned to one immediately after unifoliate leaves fully expanded. Irrigation and pesticide applications were practiced on a regular basis.

### 2.2. Gas exchange measurements

We collected the data of  $P_n$ ,  $g_s$ , and  $C_i$  on the most recent, fully expanded, central leaflets of six randomly selected plants in each plot on days 37, 44, 51, and 58 after planting (DAP) in 2017. These measurements were conducted between 9:00 a.m. to 12:30 p.m. using an LI-6400XT portable photosynthesis system (LI-COR Bioscience; Lincoln, NE, USA). We set temperature inside the chamber to 33° C. Reference level of carbon dioxide was controlled at 400  $\mu\text{mol mol}^{-1}$  using CO<sub>2</sub> cartridges, with an airflow rate of 500  $\mu\text{mol s}^{-1}$ . Light intensity was set to 1500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  with 10% blue light. Humidity was kept in a range of 50–80% inside the chamber and

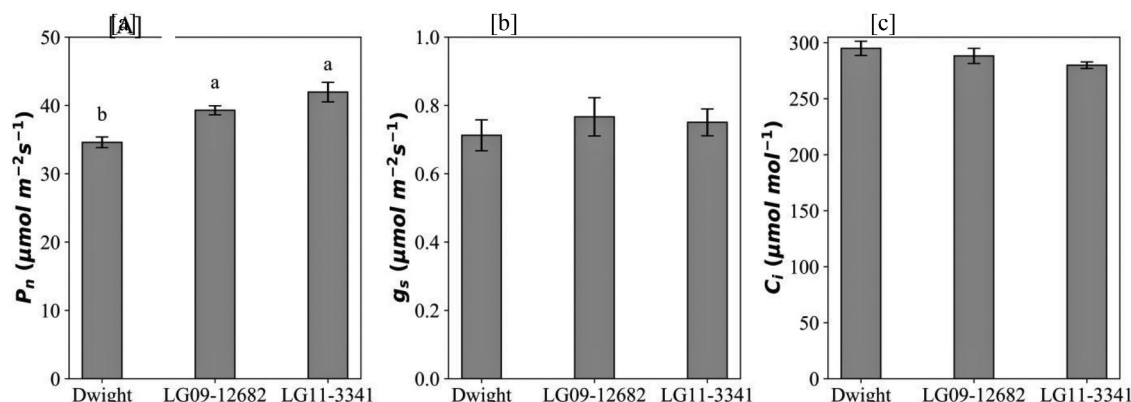


Figure 1. Genetic Variation of Gas Exchange traits between Dwight and Progenies in our preliminary observations in Illinois, U.S.A.

was adjusted to the ambient environment accordingly. Diurnal effects were minimized by a random selection of plots for all genotypes.

### 2.3. Rubisco content and LNC

Leaves were tagged with an adhesive tape after the gas exchange measurements. The tagged samples were collected, from three of the six plants in each plot where the gas exchange was measured, for Rubisco content and LNC analysis. The sampled leaves were then taken to the laboratory and four discs of 1.2 cm in diameter were removed using a leaf punching device. The discs were then immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . SDS-PAGE was used for measuring the Rubisco content (Makino et al., 1986). Leaf tissues were homogenized using chilled mortars and pestles in the presence of an extraction buffer, which consists of 50 mM HEPES-KOH, 5 mM  $\text{MgCl}_2$ , 1 mM EDTA, 0.1% (w/v) PVPP, 0.05% (v/v) Triton X-100, 5% glycerol, 4 mM amino-n-caproic acid, 0.8 mM benzamidine-HCl, and 5 mM DTT at pH 7.4 with a small amount of quartz sand. The homogenate was centrifuged at 14,500  $g$  for 6 minutes. The supernatant was used for the quantification of Rubisco content where bovine serum albumin (BSA) was assigned as a standard. The final product was read at 595 nm using spectrophotometer. After taking samples for Rubisco content, the leaf area of the remaining leaves was determined using a Leaf Area meter (LI-3100 C Area Meter). The leaves were then oven-dried at  $80^{\circ}\text{C}$  for 72 hours to determine total leaf dry weight. A Kjeldahl Digestion method was used to measure LNC. A leaf sample of 0.2 g was dissolved in 4 ml of concentrated  $\text{H}_2\text{SO}_4$ . The solution was heated until the acid became colorless. The colorless solution was then diluted with water until the total volume reached to 40 ml. We separated 20  $\mu\text{l}$  aliquots of this solution into glass tubes. The separated solution was then mixed with 2480  $\mu\text{l}$  of distilled water, 1000  $\mu\text{l}$  of Indophenol A, and 1500  $\mu\text{l}$  of Indophenol B. They were read at 635 nm wavelength

using a spectrophotometer. We adopted the LNC measurement method from Vickery (1946).

### 2.4. Stomatal density ( $N_{stomata}$ )

A lateral leaflet of the same trifoliolate, used for gas exchange, was also sampled and stored in a box with ice. The Suzuki Universal Method of Printing (SUMP) was used for printing stomatal maps. A droplet (almost 10  $\mu\text{l}$ ) of SUMP liquid (amyl acetate) was placed on a SUMP disc. The leaf was then attached to the disc and was placed between two magnets until the liquid became dry. After detaching the leaves, the discs were observed in a  $10 \times 10$  magnification light microscope (BH-32; OLYMPUS) with a mounted digital camera (FLOYD; Wraymar, Multi-Interface Digital Camera). Digital pictures were then analyzed using ImageJ (ImageJ 1.50i; Wayne Rasband, National Institute of Health, USA). We followed Tanaka and Shiraiwa (2009) for the determination of  $N_{stomata}$ .

### 2.5. Yield components

Five and ten plants per plot were harvested at full maturity (R8) for 2016 and 2017, respectively. The plants were separated into seed and stems (including pod shells). Stems and pod shells were oven-dried at  $80^{\circ}\text{C}$  for 72 hours and dry matter was measured. To determine seed yield, seed moisture was calculated and set to 14% whereas, in TDW, we reduced seed moisture to 0%.

### 2.6. Statistical analysis

Tow-way analysis of Variance (ANOVA) and Tukey Post Hoc (Tukey-HSD) test of multiple comparisons were performed on seed yield, TDW, and Harvest Index using R's built-in and Agricolae packages (De Mendiburu, Felipe, 2015) (Table 2 and Figure 5). A one-way ANOVA test was conducted on gas exchange parameters, and the difference was determined using Tukey's Post Hoc test of multiple comparison (the difference is stated in the text only). Pearson correlation test was performed

**Table 1.** Correlation coefficients between gas exchange and leaf traits in a pooled dataset.

	$P_n$	$g_s$	$C_i$	SLW	Rubisco	$P_n/C_i$	LNC
$g_s$	0.49 <sup>†</sup>						
$C_i$	-0.68**	0.24					
SLW	0.86***	0.44	-0.58*				
Rubisco	0.40	-0.27	-0.62*	0.64*			
$P_n/C_i$	0.99***	0.37	-0.78***	0.85***	0.46		
LNC	0.72**	0.16	-0.68**	0.86***	0.83***	0.74***	
Stomata	0.16	0.06	-0.11	0.46	0.76**	0.15	0.58*

$P_n$  = Net Photosynthetic Rate,  $g_s$  = Stomatal Conductance,  $C_i$  = Intercellular  $\text{CO}_2$  Concentration, SLW = Specific Leaf Weight,  $P_n/C_i$  = Mesophyll Activity, LNC = Leaf Nitrogen Content.

Values are averaged for each measurement ( $n = 16$ ).

\*\*\* =  $p < 0.000$ , \*\* =  $p < 0.001$ , \* =  $p < 0.05$ , <sup>†</sup> =  $p < 0.10$ .

**Table 2.** Analysis of Variance on seed yield, TDW, and Harvest Index (HI).

Factor			Seed Yield g plant <sup>-1</sup>	TDW g plant <sup>-1</sup>	HI
Mean	Year(Y)	2016	29.01	45.02	0.59
		2017	48.99	75.42	0.59
ANOVA	Genotype(G)	Dwight	35.32	52.96	0.60
		LG11-6210	40.23	60.07	0.61
		LG09-12682	43.58	65.84	0.60
		LG11-3341	50.19	82.27	0.56
		Y	0.000***	0.000***	0.105 <sup>n.s</sup>
ANOVA	Y x G	G	0.000***	0.000***	0.000***
		Y	0.320 <sup>n.s</sup>	0.148 <sup>n.s</sup>	0.025*
		Y x G			

TDW = Total Dry Weight, HI = Harvest Index.

\*\*\* =  $p < 0.000$ , \* =  $p < 0.05$ , n.s. = not significant.

Seed yield measured at 14% moisture content.

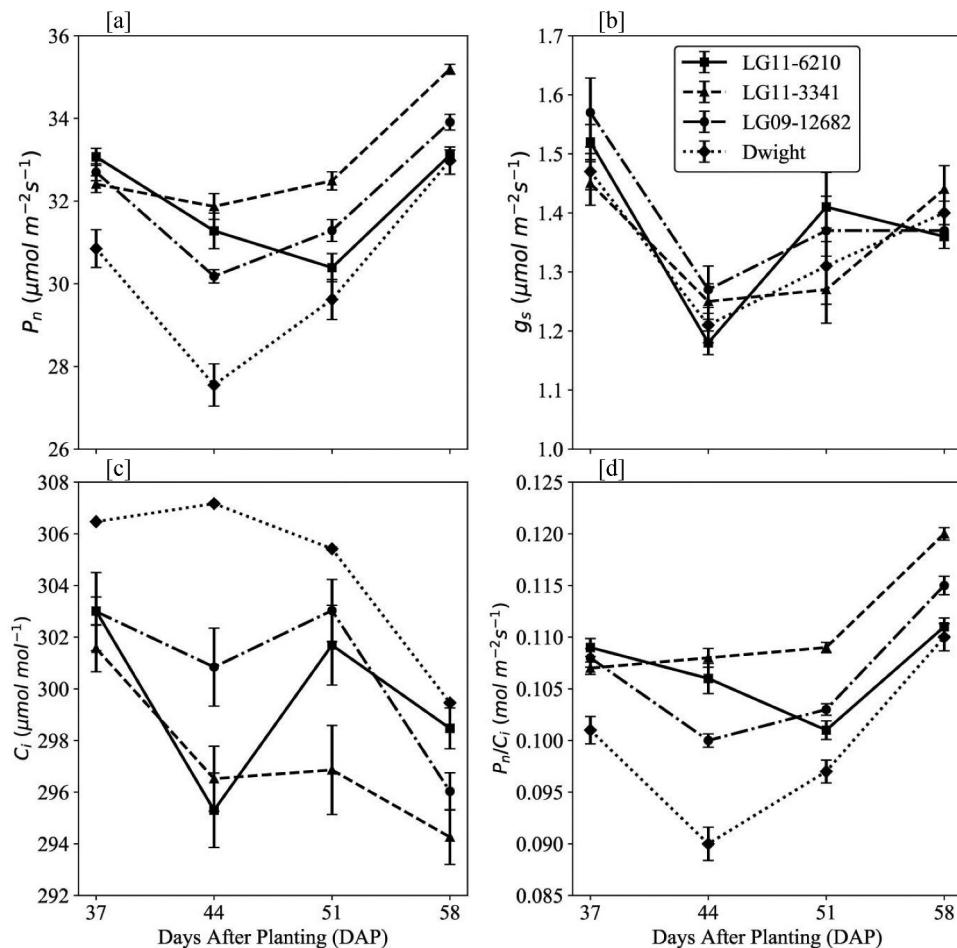
using R's Psych package (William Revelle) (Table 1). These tests were verified using Python's Penguin, Scikit-Learn, Statsmodels, and Scipy modules.

### 3. Results

#### 3.1. Gas exchange measurements

$P_n$  was significantly higher ( $p < 0.05$ ) in progenies relative to Dwight. Its values ranged from 27.55  $\mu\text{mol m}^{-2}\text{s}^{-1}$  in

Dwight on 44 DAP to 35.19  $\mu\text{mol m}^{-2}\text{s}^{-1}$  in LG11-3341 on 58 DAP. Relatively lower  $P_n$  was observed for all genotypes on 44 DAP followed by a gradual increase up to 58 DAP. LG11-6210 had increased  $P_n$  in the early reproductive stages, the difference was not significant in later growth stages though (Figure 2(a)).  $g_s$  ranged from 1.18  $\text{mol m}^{-2} \text{s}^{-1}$  in LG11-6210 on 32 DAP up to 1.57  $\text{mol m}^{-2} \text{s}^{-1}$  in LG09-12682 on 44 DAP. No significant and consistent differences in  $g_s$  were observed between Dwight and the progenies (Figure 2(b)). Unlike  $P_n$ , higher



**Figure 2.** Genetic variation of gas exchange parameters between Dwight and progenies.

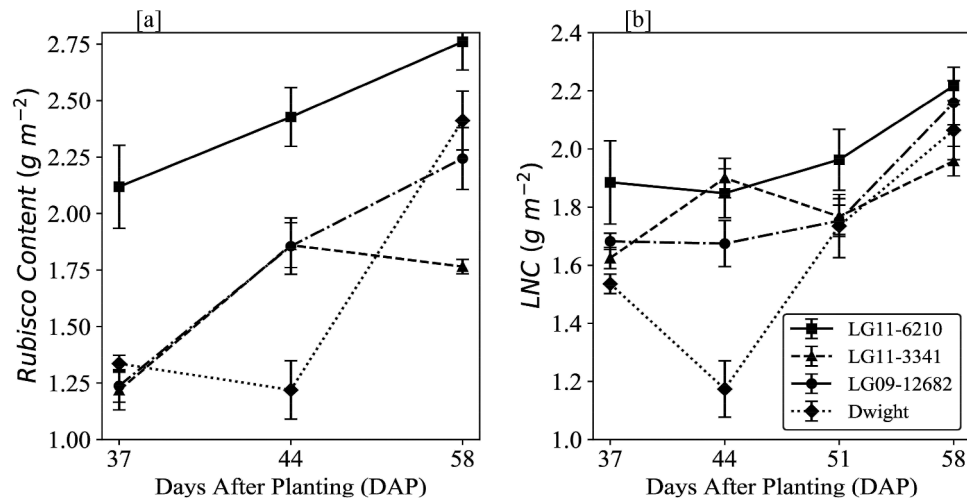


Figure 3. Variations of Rubisco and nitrogen content between Dwight and the progenies.

$C_i$  values were observed for Dwight.  $C_i$  was significantly higher ( $p < 0.05$ ) in Dwight than the progenies, ranging from  $294.26 \mu\text{mol mol}^{-1}$  for LG11-3341 on 58 DAP up to  $307.17 \mu\text{mol mol}^{-1}$  in Dwight on 44 DAP (Figure 2(c)).

### 3.2. Rubisco content and LNC

The range of Rubisco content started from  $1.21 \text{ gm}^{-2}$  in LG11-3341 on 32 DAP up to  $2.27 \text{ gm}^{-2}$  in Dwight on 58 DAP. LG11-6210 and LG09-12682 showed a linear increase in Rubisco content. LG11-3341 was characterized by an increase in Rubisco content during late flowering (44 DAP) and then a slow decrease to 58 DAP. Interestingly, Dwight followed a completely inverse pattern of LG11-3341. It was characterized by a decrease in Rubisco content on 44 DAP and then a sharp increase on 58 DAP. The checked genotype, LG11-6210, showed significantly higher Rubisco content than progenies and Dwight (Figure 3(a)).

LNC ranged from  $1.17 \text{ gm}^{-2}$  in Dwight on 44 DAP up to  $2.15 \text{ gm}^{-2}$  in LG09-12682 on 58 DAP. It varied among measurements and genotypes. In the early measurements (37–44 DAP), progenies had relatively higher LNC but the difference between progenies and Dwight was significant only on 44 DAP. In the later measurements (51–58 DAP); however, the difference was not significant between Dwight and progenies (Figure 3(b)).

### 3.3. Stomatal density ( $N_{stomata}$ )

$N_{stomata}$  ranged from  $249 \text{ mm}^{-2}$  in LG11-3341 up to  $350 \text{ mm}^{-2}$  in LG11-6210. Dwight had a linear increase in  $N_{stomata}$  over the time course, whereas fluctuations in  $N_{stomata}$  were observed in both progenies. There was no significant difference in  $N_{stomata}$  between the progenies and Dwight (Figure 4(a)).

As Table 1 shows,  $P_n$  had positive significant correlations with SLW and LNC, whereas the correlation with  $C_i$

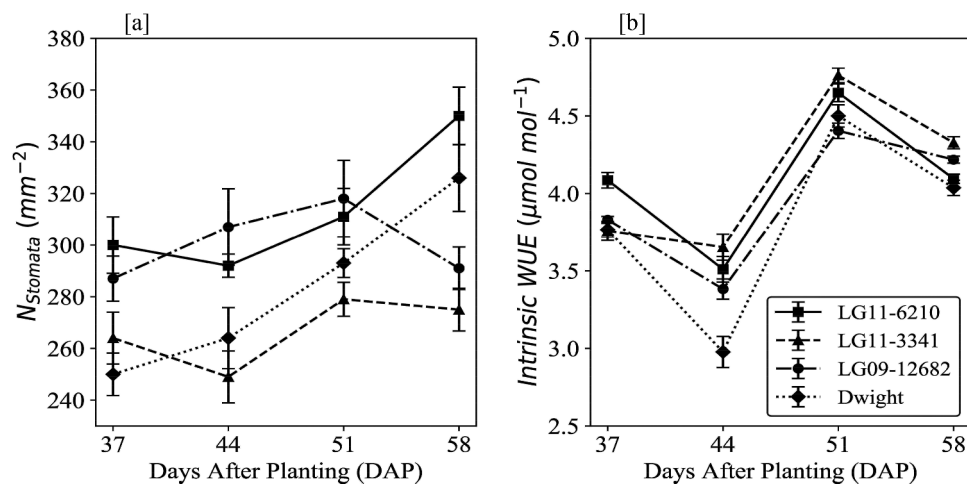


Figure 4. Variation of Stomatal Density and Intrinsic WUE between Dwight and progenies.

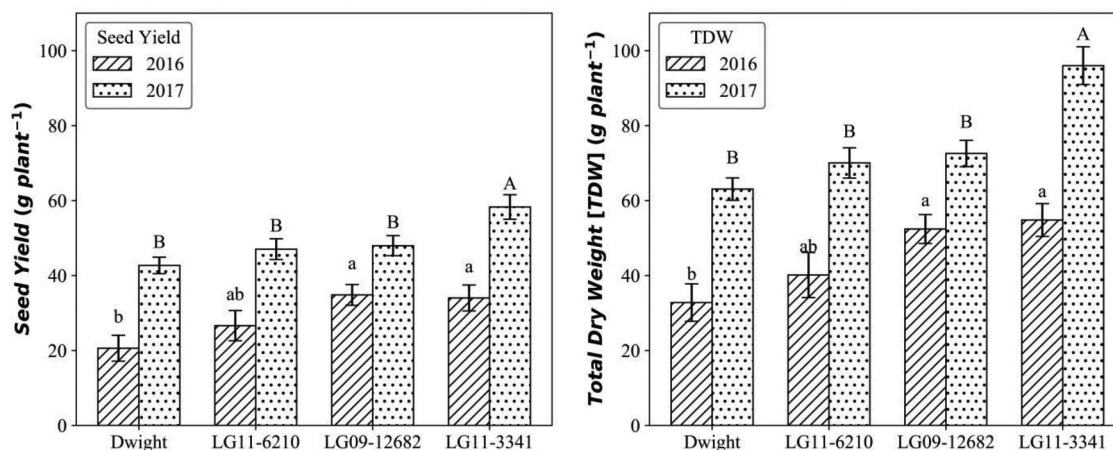


Figure 5. Variation of seed yield and total dry weight between Dwight and progenies.

was negative. Similarly,  $C_i$  had negative strong correlations with SLW, Rubisco, and LNC.  $g_s$  had a moderate correlation ( $\rho = 0.49$ ) with  $P_n$ ; however, statistical analysis showed no effect ( $\rho > 0.1$ ) of  $N_{stomata}$  on  $g_s$ ,  $C_i$ , and  $P_n$ .

### 3.4. Seed Yield and TDW

Seed yield ranged from 20.60 g plant<sup>-1</sup> in Dwight up to 34.84 g plant<sup>-1</sup> in LG09-12682 in 2016. In 2017, on the other hand, it ranged from 42.68 g plant<sup>-1</sup> in Dwight up to 58.28 g plant<sup>-1</sup> in LG11-3341. Progenies produced significantly higher seed yield comparing Dwight in 2016, whereas the difference was only significant between LG11-3341 and Dwight in 2017 (Figure 5 [left-hand side]). In 2016, TDW was as low as 32.76 g plant<sup>-1</sup> in Dwight and as high as 54.80 g plant<sup>-1</sup> in LG11-3341. Both progenies produced significantly higher TDW in this year. A steep increase in TDW was noticed in 2017 for Dwight and progenies; however, only LG11-3341 produced significantly higher TDW than Dwight (Figure 5 [right-hand side]). Table 2 shows Two-way ANOVA test we carried out on seed yield, TDW, and Harvest HI. There was significant difference between years and among genotypes in terms of seed yield and TDW; however, the difference in HI was significant among genotypes as well as when the interaction of year and genotypes was considered.

## 4. Discussion

Several studies have noted discrepancies in leaf photosynthetic capacity for crop species varying in genomic and/or cytoplasmic formation (Brodersen et al., 2008; Brown & Bouton, 1993; Carver et al., 1989; Hay et al., 2017; Izhar & Wallace, 1967; Sakoda et al., 2016). For example, lower photosynthetic rates have been reported in modern wheat cultivars compared with their wild

ancestors (Evans & Dunstone, 1970). No improvements in the photosynthetic rates in rice breeding have been noted for the last 50 years in Venezuela (Alejandro et al., 2011). Koester et al. (2016), on the other hand, reported photosynthetic enhancement of recently release soybean cultivars. Similarly, Morrison et al. (2000) reported a 0.52% annual increase in photosynthetic rates of newly released cultivars.

Our study documents the potential for utilizing soybean wild species in breeding in order to enhance soybean leaf photosynthesis. The results highlight that soybean progenies had higher  $P_n$  than Dwight (Figure 2(a)). Variations of  $P_n$  in these progenies were mainly associated with increases in mesophyll activity not only due to lower  $C_i$  per given  $g_s$  but also an apparent proxy of mesophyll activity ( $P_n/C_i$ ), which includes both CO<sub>2</sub> diffusion from intercellular spaces into and its fixation in chloroplast, followed the same pattern as  $P_n$ . Since mesophyll activity represents CO<sub>2</sub> diffusion from intercellular air spaces into and its fixation in chloroplast, it is reasonable to assume that progenies have either a higher mesophyll conductance, a proxy for CO<sub>2</sub> diffusion, or increased capacity for CO<sub>2</sub> fixation the Calvin cycle. These findings are consistent with the findings of Fischer et al. (1998). They also elucidated the physiological mechanism (Mesophyll activity or  $A_{max}/C_i$ ) for increased photosynthetic capacity and stated that 2/3 increases in  $A_{max}$  were associated with increases in  $A_{max}/C_i$  and 1/3 increase was associated with increases in  $C_i$ . The effect of  $C_i$  on  $A_{max}$  in their study was clearly linked to higher stomatal conductance in genotypes with higher photosynthetic rates. In our study; however,  $g_s$  did not differ noticeably between progenies and Dwight. Therefore, we concluded that the variations in  $P_n$  were solely linked to improvements in  $P_n/C_i$ .

There was no significant correlation between  $P_n$  and Rubisco content, and  $P_n/C_i$  and Rubisco content when all the data were pooled. This was mainly due to lower Rubisco content of LG11-3341 and LG09-12682, and its association with a higher  $P_n$ . It suggests that factors other than Rubisco content were involved in enhanced  $\text{CO}_2$  fixation in the progenies. One possibility could be the increased activity of Rubisco Activase. Since Rubisco activase is controlled by the ratio of Adenosine triphosphate (ATP) to Adenosine diphosphate (ADP) [ $ATP:ADP$ ] (Streusand & Portis, 1987), it is possible that progenies might have developed a more efficient PSII and electron transfer system resulting in better proton harvesting. Another possibility could be the higher activation status of Rubisco content. Simply put, the larger the portion of Rubisco activated, the more the  $\text{CO}_2$  is fixed.

The effect of LNC on leaf photosynthesis has been well documented in different studies (Allison et al., 1997; Boussadia et al., 2010; Sakoda et al., 2016). In this study, LNC was also significantly correlated with  $P_n$  ( $r = 0.72^{**}$ ) when the data were pooled for all the four measurements. Within each measurement, there was a significant correlation between  $P_n$  and LNC on 37–44 DAP, but no correlation was observed in the latter two measurements. Since LNC was not significantly different between progenies and Dwight in the latter two measurements (51–58 DAP), this suggests that variations in  $P_n$  were not due to variations in LNC.

It is reported that plant biomass and therefore seed yield is tightly linked to transpiration rates (Sinclair et al., 1984). Condon et al. (2004) emphasized the importance of improved water-use efficiency (WUE) for sustainable agricultural water supply. An outstanding characteristic of the progenies was the higher intrinsic water-use efficiency compared to Dwight (Ares et al., 2000). As (Figure 4(b)) clearly demonstrates, the progenies were able to maintain both a higher  $P_n$  and intrinsic WUE simultaneously. These results suggest that the progenies could further be utilized in breeding programs for the purpose of combining higher photosynthetic capacity with increased WUE.

As a result, the progenies were observed to have higher seed yield and TDW relative to Dwight. Compared to 2017, drastically lower seed yield and TDW were observed in 2016. We believe that late sowing did not allow plants to invest enough in vegetative biomass before reaching reproductive stage which in turn produced poor yield. Koutroubas et al. (1998) reported that the dry matter accumulation and nitrogen at early growth stages are essential in producing higher yield. Similarly, Specht et al. (1999) reported that

increasing seed yield potential through increasing total dry matter is considered to play a role. In this study, yield enhancements of the progenies and the difference between these two years were also related to the enhanced dry matter accumulation. The lower HI of LG11-3341 was not due to its lack of ability to convert biomass to seed yield, rather this progeny is very tall, and lodging occurs despite controlling measures.

Our results of seed yield were, as a whole, consistent with the findings of Akperterey (2015) and (Begemann, 2015); however, genotypes in general were lower performing in Akperterey's study. We had higher seed yield for all genotypes and the difference was obvious for progenies especially LG11-3341 whereas, in Akperterey's study, this progeny produced lower seed yield than Dwight. This could be related to the lodging that occurred in Akperterey (2015) experiment; we, on the other hand, managed to reduce lodging.

## 5. Conclusion

To conclude, this is the first study that reports substantially enhanced leaf photosynthetic capacity in backcross-derived progenies from soybean and its wild relative, *G. tomentella* Hayata. Studied parameters showed that enhanced leaf photosynthesis was triggered by higher  $P_n/C_i$  in progenies. LG11-3341 and LG09-12682 were characterized by consistently higher  $P_n$  than Dwight throughout the growing season. This study demonstrates that the use of *Glycine tomentella* Hayata in soybean breeding has the potential to significantly increase leaf photosynthetic capacity, as well as other important parameters such as WUE, in the resulting progenies.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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