

## Article

# Variation among Soybean Cultivars in Mesophyll Conductance and Leaf Water Use Efficiency

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**Abstract:** Improving water use efficiency (WUE) may prove a useful way to adapt crop species to drought. Since the recognition of the importance of mesophyll conductance to CO<sub>2</sub> movement from inside stomatal pores to the sites of photosynthetic carboxylation, there has been interest in how much intraspecific variation in mesophyll conductance ( $g_m$ ) exists, and how such variation may impact leaf WUE within C<sub>3</sub> species. In this study, the  $g_m$  and leaf WUE of fifteen cultivars of soybeans grown under controlled conditions were measured under standardized environmental conditions. Leaf WUE varied by a factor of 2.6 among the cultivars, and  $g_m$  varied by a factor of 8.6. However, there was no significant correlation ( $r = -0.047$ ) between  $g_m$  and leaf WUE. Leaf WUE was linearly related to the sub-stomatal CO<sub>2</sub> concentration. The value of  $g_m$  affected the ratio of maximum Rubisco carboxylation capacity calculated from the sub-stomatal CO<sub>2</sub> concentration to that calculated from the CO<sub>2</sub> concentration at the site of carboxylation. That is, variation in  $g_m$  affected the efficiency of Rubisco carboxylation, but not leaf WUE. Nevertheless, there is considerable scope for genetically improving soybean leaf water use efficiency.

**Keywords:** soybean; water use efficiency; mesophyll conductance; stomatal conductance; internal CO<sub>2</sub> concentration; photosynthesis

## 1. Introduction

With increasing limitations on the amount of water available to support agriculture, increasing the water use efficiency (WUE) of crops—the ratio of crop dry mass gained to water consumed—is a reasonable objective. Selection for high leaf WUE in wheat improved yield in dry conditions [1]. Leaf WUE is often defined as the ratio of photosynthesis to transpiration. However, the rate of transpiration is directly related to the difference in water vapor pressure between the air inside and outside the leaf (the leaf to air vapor pressure difference, LAVPD), so the LAVPD during the measurement has a large impact on WUE. Because of this, comparisons of leaf WUE often use “intrinsic” water use efficiency [2], the ratio of photosynthesis to stomatal conductance.

Limitations to C<sub>3</sub> photosynthesis at high light and at the current ambient CO<sub>2</sub> concentration have long been quantified by the maximum capacity of rubisco carboxylation ( $V_{Cmax}$ ) [3]. However, it has been recognized within the last several years that mesophyll conductance to CO<sub>2</sub> movement from inside the stomata to the site of fixation within chloroplasts ( $g_m$ ) can be a significant limitation to photosynthesis (A) [4,5]. Because of the potential of variation in  $g_m$  to affect A independently from stomatal conductance ( $g_s$ ), there is interest in determining the extent of variation in  $g_m$  within species, and its effect on leaf WUE.

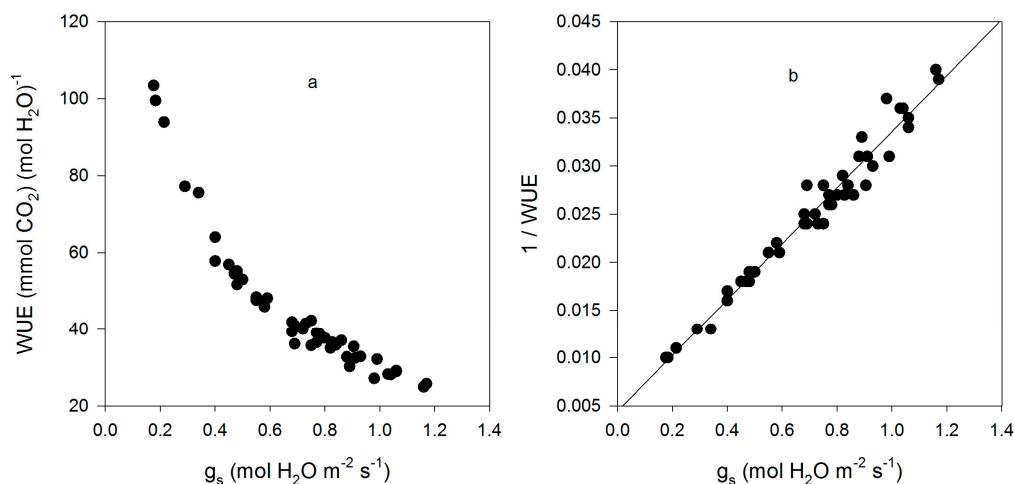
Intraspecific variation in  $g_m$  has been reported in barley [6], grape [7], tomato [8], wheat [9], and rice [10], and its relationship to leaf WUE examined. In grape and barley,  $g_m$  was positively correlated with leaf WUE. In tomato and rice, variation was found in the ratio of  $g_m$  to  $g_s$ , and that

ratio was correlated with leaf WUE. In wheat variation,  $g_m$  was correlated with  $A$ , but there was no clear relationship between  $g_m$  and leaf WUE.

Among these studies,  $g_m$  was quantified either from leaf fluorescence changes with  $CO_2$  concentration, or from carbon isotope discrimination, both of which methods are based on assumptions which are sometimes dubious [11–13]. A more rapid method for estimating  $g_m$ , with fewer assumptions, has been developed based on the response of photosynthesis to oxygen concentration [14], and was used here to screen fifteen cultivars of soybean for  $g_m$  and to test for relationships among  $g_m$ ,  $g_s$ ,  $A$ , and leaf WUE. Genetic improvement of WUE depends upon sufficient genetic variation in WUE and the identification of physiological processes which affect it, which, as indicated by the literature cited, may vary with crop species. The goals of this study were to determine whether variation in intrinsic leaf WUE or  $g_m$  occurred in soybean measured under standardized conditions, and to determine whether any variation in  $g_m$  was related to variation in intrinsic leaf WUE.

## 2. Results

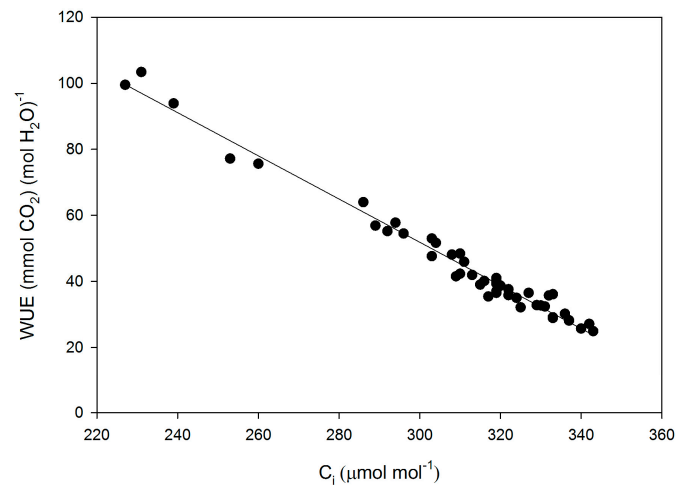
The cultivars differed significantly in  $g_s$ ,  $g_m$ , leaf intrinsic WUE, sub-stomatal  $CO_2$  concentration ( $C_i$ ), and the  $CO_2$  concentration at rubisco ( $C_c$ ), but not in  $V_{Cm}C_c$  ( $V_{Cmax}$  based on  $C_c$ ),  $V_{Cm}C_i$  ( $V_{Cmax}$  based on  $C_c$ ), or  $A$  (Table 1). For cultivars with high and low values of  $g_m$ , the values estimated using the initial slope of  $A$  vs.  $C_i$  agreed with those using the  $O_2$  effect. Intrinsic leaf WUE varied by a factor of 2.6 among the cultivars, with Fiskeby having the highest, and Ford having the lowest WUE (Table 1). Mesophyll conductance varied by a factor of 8.6 among cultivars, with Biloxi having the highest and Clark the lowest mean values. Variation in cultivar means of intrinsic WUE was due mostly to variation in  $g_s$ , which ranged from 0.38 to 1.12  $mmol\ H_2O\ m^{-2}\cdot s^{-1}$ , rather than photosynthesis, which ranged from 23.7 to 31.1  $\mu mol\ CO_2\ m^{-2}\cdot s^{-1}$ . Fiskeby, with the highest WUE, had the second lowest  $g_s$ , and Ford, with the lowest WUE, had the highest  $g_s$ . For all individual leaf measurements, intrinsic WUE was strongly negatively related to  $g_s$ , with the reciprocal of WUE linearly related to  $g_s$  (Figure 1).  $C_i$  ranged from about 225 to 345  $\mu mol\cdot mol^{-1}$  and there was a negative linear relationship between  $C_i$  and intrinsic WUE (Figure 2).



**Figure 1.** Leaf intrinsic water use efficiency (WUE) (a); and the reciprocal of leaf intrinsic WUE (b) as a function of stomatal conductance ( $g_s$ ) for all measurements made on fifteen cultivars of soybean. The linear regression of  $(1/WUE) = 0.0044 + 0.029 \times g_s$  had an  $r^2$  value of 0.965.

**Table 1.** Mean leaf gas exchange parameter values of the soybean cultivars tested. Parameters were determined for three or four leaves per cultivar.  $g_s$  is stomatal conductance in  $\text{mmol (H}_2\text{O) m}^{-2}\cdot\text{s}^{-1}$ ,  $g_m$  is mesophyll conductance in  $\text{mmol (CO}_2\text{) m}^{-2}\cdot\text{s}^{-1}$ ,  $A$  is photosynthetic rate in  $\mu\text{mol (CO}_2\text{) m}^{-2}\cdot\text{s}^{-1}$  measured at  $400 \mu\text{mol}\cdot\text{mol}^{-1}$   $\text{CO}_2$ , WUE is intrinsic water use efficiency in  $\text{mmol (CO}_2\text{) per mol (H}_2\text{O)}$ ,  $V_{\text{Cm}C_i}$  is the maximum rate of rubisco carboxylation based on  $C_i$ , and  $V_{\text{Cm}C_c}$  is the maximum rate of rubisco carboxylation based on  $C_c$ .  $C_i$  is the sub-stomatal  $\text{CO}_2$  concentration, and  $C_c$  is the  $\text{CO}_2$  concentration at rubisco, in  $\mu\text{mol}\cdot\text{mol}^{-1}$ . Probability of equal means from ANOVA is given, and the Tukey–Kramer honestly significant difference (HSD) is provided when the probability is  $<0.05$ .

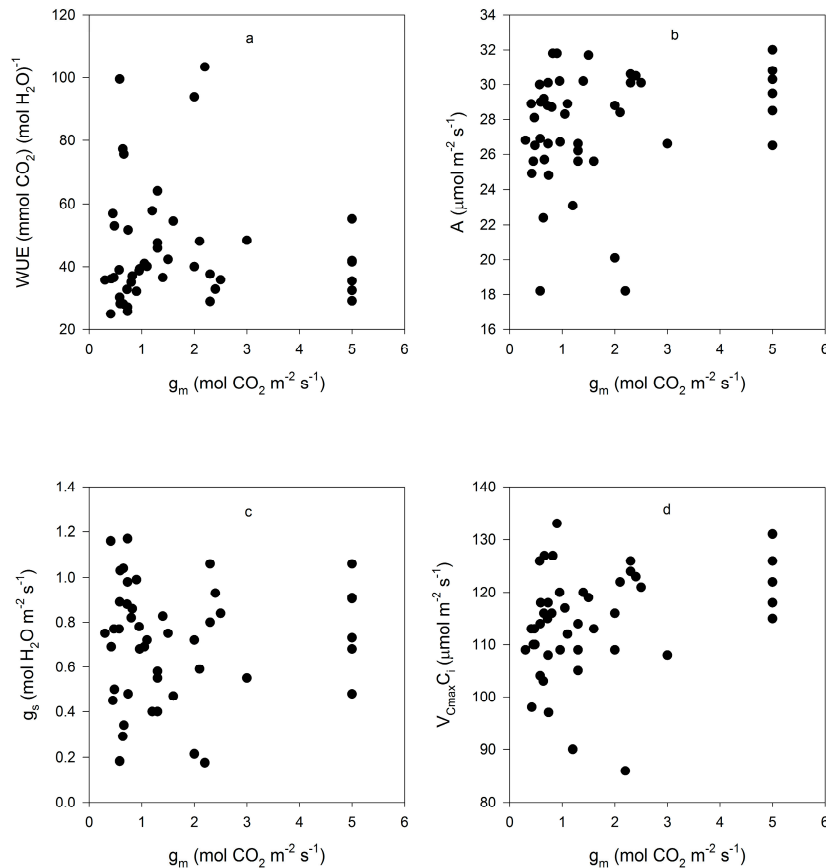
Cultivar	$g_s$	$g_m$	$A$	WUE	$V_{\text{Cm}C_i}$	$V_{\text{Cm}C_c}$	$C_i$	$C_c$
A5959	703	2.63	27.7	43.1	113	118	313	299
Biloxi	803	3.60	28.9	36.4	116	123	323	307
Chief	878	2.28	31.1	35.9	127	139	321	297
Clark	677	0.42	26.6	40.3	108	138	324	258
Essex	587	1.50	26.4	56.9	114	121	291	272
Fiskeby V	418	2.56	24.4	67.6	114	122	276	259
Ford	1123	0.60	29.4	26.2	116	138	340	288
Holt	502	2.05	25.0	63.5	105	112	287	270
Kent	820	0.78	28.0	34.7	115	132	328	291
Lincoln	725	0.77	28.4	39.1	118	137	317	277
Perry	883	2.10	30.8	35.7	121	127	322	306
PI-41	430	1.09	25.8	62.0	117	130	283	257
Ripley	380	0.76	23.7	64.0	101	118	279	242
Spencer	728	2.68	29.0	41.6	118	124	314	297
Wabash	910	1.11	28.1	31.8	114	128	333	300
Probability	0.001	0.016	0.081	0.029	0.097	0.279	0.019	0.036
HSD	592	2.79		31.1			56	65



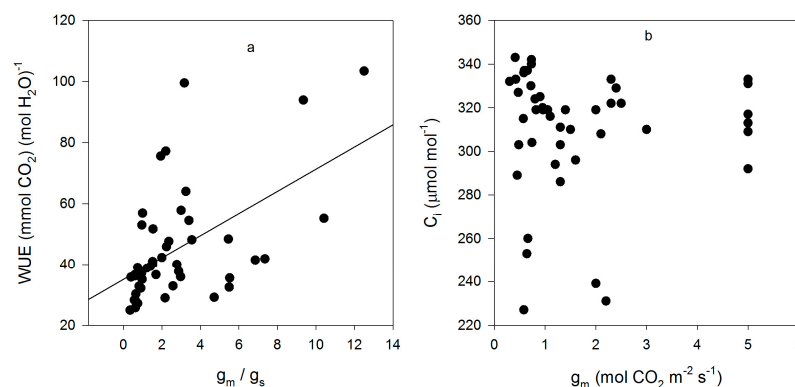
**Figure 2.** Leaf intrinsic water use efficiency (WUE) as a function of sub-stomatal  $\text{CO}_2$  concentration ( $C_i$ ) for all measurements made on fifteen cultivars of soybean. The linear regression:  $\text{WUE} = 248 - 0.654 \times C_i$  had an  $r^2$  of 0.98.

Mesophyll conductance had no significant correlation with intrinsic WUE ( $r = -0.047$ ),  $g_s$  ( $r = +0.065$ ),  $A$  ( $r = +0.234$ ), or  $V_{\text{Cmax}C_i}$  ( $r = +0.227$ ) (Figure 3). Using means for the cultivars for all of these parameters also produced no significant correlations ( $r = 0.079, 0.217, 0.040$ , and  $0.301$ , for  $g_m$  versus intrinsic WUE,  $g_s$ ,  $A$ , and  $V_{\text{Cmax}C_i}$ , respectively). The ratio of  $g_m$  to  $g_s$  in soybean had a small but significant correlation with intrinsic leaf WUE (Figure 4), but there was no significant correlation between  $C_i$  and  $g_m$  (Figure 4). The ratio of the maximum capacity of rubisco carboxylation modelled based on  $C_i$  to that that based on  $C_c$  decreased with mesophyll resistance, which is the reciprocal of  $g_m$ , from about 0.70 to 0.98 (Figure 5). Photosynthesis at  $C_a = 400 \mu\text{mol}\cdot\text{mol}^{-1}$  was

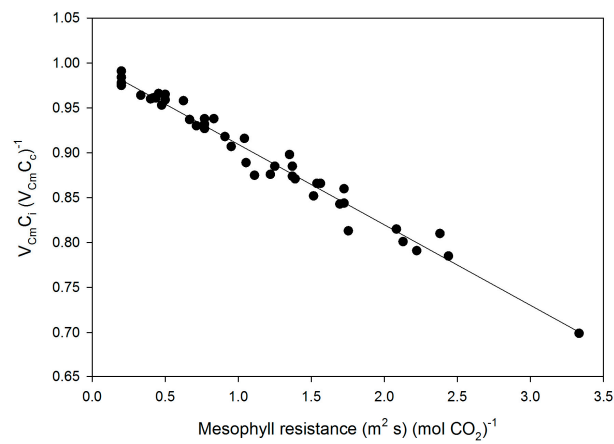
positively related to  $C_i$  (Figure 6), similar to a saturating  $A$  vs.  $C_i$  curve for an individual leaf. While there was a small but significant correlation between  $C_i$  and  $C_c$  (Figure 7), there was a wide range of  $C_i$  values for a given  $C_c$ . For example cultivar means of  $C_i$  ranged from about 275 to 325  $\mu\text{mol}\cdot\text{mol}^{-1}$  for mean  $C_c$  values of about 256 to 259  $\mu\text{mol}\cdot\text{mol}^{-1}$  (Figure 7).



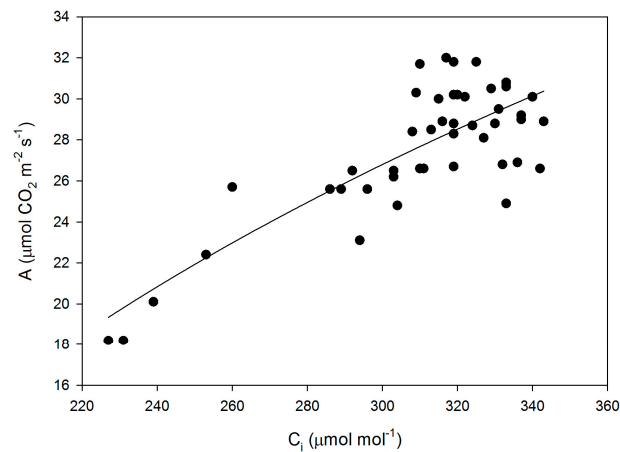
**Figure 3.** Correlations between  $g_m$  and (a) leaf intrinsic water use efficiency (WUE); (b)  $\text{CO}_2$  assimilation rate ( $A$ ); (c) stomatal conductance ( $g_s$ ); (d) and the maximum rate of rubisco carboxylation based on  $C_i$  ( $V_{C_{\max}}C_i$ ) for all measurements made on fifteen cultivars of soybean. Correlation coefficients were not significant at  $p = 0.05$ , and are given in the text.



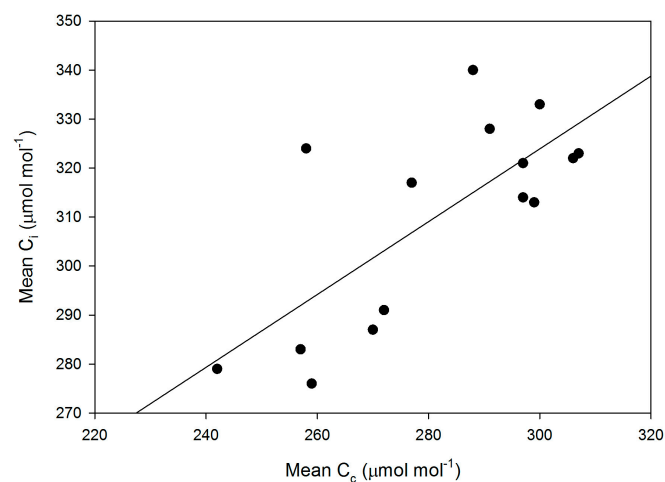
**Figure 4.** Relationships between the ratio of  $g_m$  to  $g_s$  and WUE (a); and between  $g_m$  and  $C_i$  (b) for all measurements made on fifteen cultivars of soybean. The correlation between the  $g_m$  to  $g_s$  ratio and WUE had an  $r$  value of +0.53, and that between  $g_m$  and  $C_i$  was +0.116, which was not significant at  $p = 0.05$ .



**Figure 5.** The ratio of  $V_{C_{max}}$  based on  $C_i$  to that based on  $C_c$  as a function of mesophyll resistance, the reciprocal of  $g_m$ , for all measurements made on fifteen cultivars of soybean. The linear regression:  $V_{C_m}C_i/V_{C_m}C_c = 1.00 - 0.090 \times (1/g_m)$  had an  $r^2$  of 0.976.



**Figure 6.**  $CO_2$  assimilation rate ( $A$ ) as a function of sub-stomatal  $CO_2$  concentration ( $C_i$ ) for all measurements made on leaves of fifteen cultivars of soybean. The equation  $A = -125.6 + 26.7 \ln(C_i)$  had an  $r^2$  value of 0.645.



**Figure 7.** Cultivar mean values of  $C_i$  versus mean values of  $C_c$ . The linear regression had an  $r^2$  value of 0.532.

### 3. Discussion

The linear relationship between intrinsic leaf WUE and  $C_i$  was as expected, since  $C_a$  was constant across the measurements. Carbon isotope discrimination has long been used as a surrogate for  $C_i$  in screening for leaf WUE in  $C_3$  species. It is important to know how much variation in  $g_m$  would disrupt the correlation between  $C_i$  (and WUE) and carbon isotope discrimination, which should reflect  $C_c$  rather than  $C_i$  [6]. The ranking of these soybean cultivars based on  $C_c$  or carbon isotope discrimination would not provide a reliable ranking of their  $C_i$  or intrinsic leaf WUE values (Figure 7).

While variation among cultivars in  $C_i$  was more strongly influenced by variation in  $g_s$  than  $A$ , there was still the usual [15] penalty in  $A$  associated with low  $g_s$ , low  $C_i$  and high intrinsic WUE (Figure 5). One reason for interest in  $g_m$  with regard to WUE is the possibility that high  $g_m$  might offset the penalty in  $A$  associated with low  $C_i$  and high WUE [16]. Certainly, a high  $g_m$  results in a higher value of  $A$  at a given  $C_i$ , and results in  $V_{Cm}C_i$  being closer to  $V_{Cm}C_c$  (Figure 4). However,  $g_m$  was poorly correlated with any other leaf gas exchange parameter ( $g_s$ ,  $A$ ,  $C_i$ ,  $V_{Cm}C_i$ , WUE) in this data. It is possible that the correlation between the ratio of  $g_m$  to  $g_s$  and WUE reported in rice and tomato [8,10] has little to do with variation in  $g_m$ , but simply reflects a strong relationship between  $g_s$  and WUE, as found here in soybean. Furthermore, it remains unknown how much a genetic increase in  $g_m$  might cost in terms of leaf nitrogen, which would tend to offset the resulting increased efficiency of rubisco [16]. It is thought that there is a metabolic component to  $g_m$ , and that therefore there would be some nitrogen cost to increasing  $g_m$  [16]. Reasons for cultivar differences in the operational  $C_i$  under these identical and non-stressful environmental conditions remain unknown, but  $g_s$  and the operational  $C_i$  were clearly the primary determinates of leaf intrinsic WUE among these soybean leaves.

The observed 2.95 fold range in mean  $g_s$  among soybean cultivars would have a substantial impact on canopy transpiration, even though relative differences in transpiration decrease as the scale increases from leaf to canopy [17]. Using the mean leaf boundary layer conductance of 1200 mmol ( $H_2O$ )  $m^{-2} \cdot s^{-1}$  of soybean leaves measured near midday on 9 days in Beltsville, Maryland [18], a 2.95 fold range in leaf  $g_s$  would translate into approximately a 1.45 fold range in canopy transpiration. Such a large range in canopy transpiration could have a substantial impact on the rate of development of plant water stress in the field. Thus, there is considerable scope for reduction in canopy transpiration rate and improvement in soybean WUE through breeding. However, the results of this study make it unlikely that screening soybeans for  $g_m$  values would be a profitable method of attempting to increase WUE in soybean.

It should be noted that the intrinsic leaf WUE and  $g_s$  values measured here were measured under a standardized LAVPD. “Intrinsic” leaf WUE is, of course, not really intrinsic to the leaf, but would be expected to vary with the measurement conditions, as  $A$  and  $g_s$  vary with environment. The response of  $g_s$  to LAVPD in soybean is known to vary among cultivars [19]. Fletcher et al. [20] have identified lines of soybeans differing in the response of whole plant transpiration rate to LAVPD, but did not provide information about either  $g_s$  or photosynthetic responses to LAVPD, or information on leaf WUE. Field measurements of  $g_s$  and  $A$  in response to LAVPD in several soybean cultivars [21] did not present data on operational  $C_i$ .

Our results indicated that  $g_s$  and  $C_i$  were correlated with leaf intrinsic WUE, and may be useful in selecting lines with high WUE. However, the lack of correlation between  $g_m$  and leaf intrinsic WUE indicates that  $g_m$  may not be useful in ranking lines for WUE, although both factors varied among cultivars. It will be important to assess the operational  $C_i$  of soybean lines under a range of field conditions to determine how robust the differences in leaf intrinsic WUE found here are in the field. The results presented here also indicate that carbon isotope discrimination may not reliably identify soybean lines with differences in leaf intrinsic WUE.

#### 4. Materials and Methods

Three or four plants of each of fifteen cultivars of soybean (*Glycine max* L. Merr.) obtained from the U.S. Department of Agriculture soybean germplasm collection (Table 1) were grown together in one large controlled environment chamber at the controlled environment facility of the Beltsville Agricultural Research Center, Beltsville, Maryland. All of the cultivars are adapted to the eastern United States. Some (Biloxi, Clark, Kent, PI-471938, A5959) were chosen because they had diverse responses of stomatal conductance or transpiration rate to humidity in prior studies [19,20]. The others were chosen based on my prior measurements of leaf gas exchange rates in various experiments. The chamber had 14 h of light per day at  $1000 \mu\text{mol m}^{-2}\cdot\text{s}^{-1}$  photosynthetic photon flux density (PPFD) from a combination of metal halide and high pressure sodium lamps. This gave a daily total photon flux similar to average mid-summer days at Beltsville, Maryland. The day/night air temperatures were 26/20 °C, with a dew point temperature of about 18 °C. These approximate mean conditions in Beltsville for the soybean growing season. The CO<sub>2</sub> concentration was controlled at  $400 \mu\text{mol}\cdot\text{mol}^{-1}$  during the day and  $430 \mu\text{mol}\cdot\text{mol}^{-1}$  at night by the addition of pure CO<sub>2</sub> or air scrubbed of CO<sub>2</sub>, under the control of a WMA-5 (PP Systems, Amesbury, MA, USA) infrared analyzer which sampled chamber air continuously. Plants were grown one per pot in 15 cm diameter plastic pots filled with vermiculite and were fertilized daily with a complete nutrient solution containing 14.5 mM nitrogen.

Leaf gas exchange measurements were conducted on fourth main stem trifoliolate leaves a few days after those leaves reached full area expansion. Leaf gas exchange measurements were made with a recently calibrated Li-6400 portable photosynthesis system (Li-Cor, Inc., Lincoln, NE, USA). Leaf temperature was controlled at 25 °C, the PPFD was  $1500 \mu\text{mol m}^{-2}\cdot\text{s}^{-1}$ , and the LAVPD was maintained at  $1.0 \pm 0.1$  kPa. Each leaf was measured under three conditions of CO<sub>2</sub> and O<sub>2</sub>. First, the leaf was exposed to 21% O<sub>2</sub>, and  $400 \mu\text{mol}\cdot\text{mol}^{-1}$  external CO<sub>2</sub>. The external CO<sub>2</sub> was then lowered to  $300 \mu\text{mol}\cdot\text{mol}^{-1}$  at the same O<sub>2</sub> concentration. Finally, the oxygen concentration was lowered to 2%, while the CO<sub>2</sub> concentration remained at  $300 \mu\text{mol}\cdot\text{mol}^{-1}$ . Care was taken to ensure that gas exchange rates were steady at each condition. These measurements were made on three or four replicate leaves from different plants for each cultivar. A, g<sub>s</sub>, C<sub>i</sub>, and LAVPD were calculated by the instrument software.

Intrinsic leaf WUE was calculated separately for each leaf as A/g<sub>s</sub> measured at  $400 \pm 5 \mu\text{mol}\cdot\text{mol}^{-1}$  CO<sub>2</sub>, and 21% O<sub>2</sub>, at 25 °C, and at a LAVPD of 1 kPa. Mesophyll conductance was also calculated for each leaf from the gas exchange rates at  $300 \mu\text{mol}\cdot\text{mol}^{-1}$  CO<sub>2</sub> at 21% and 2% O<sub>2</sub> using the method described in Bunce [14], with the calculation utility developed by Singh [22]. The method is based on the idea that the sensitivity of photosynthesis to oxygen depends on the concentration of CO<sub>2</sub> at the site of Rubisco, rather than the sub-stomatal CO<sub>2</sub> concentration. This method of estimating g<sub>m</sub> uses the change in photosynthetic rate between two O<sub>2</sub> concentrations at limiting CO<sub>2</sub> concentrations to indicate the CO<sub>2</sub> concentration at the site of rubisco (C<sub>c</sub>), using a standard biochemical C<sub>3</sub> photosynthesis model [14]. This method of estimating g<sub>m</sub> also provides estimates of V<sub>Cmax</sub> based on C<sub>i</sub> and based on C<sub>c</sub>. For some leaves with the highest and lowest values of g<sub>m</sub>, additional points on A vs. C<sub>i</sub> curves at 21% O<sub>2</sub> were used to calculate g<sub>m</sub> using the Sharkey et al. calculation utility [23] based on the curvature of the initial slope of the A vs. C<sub>i</sub> curve.

Analysis of variance was used to test for differences among cultivars in mean leaf gas exchange parameters. For both g<sub>s</sub> and g<sub>m</sub>, homogeneity of variance was violated, so values were log transformed prior to ANOVA. Correlations among gas exchange parameters were calculated using all data on individual leaves, because of the increased statistical power compared with using mean values, given the substantial variability among leaves with cultivars. Where correlations were significant at  $p = 0.05$ , linear or simple non-linear regressions are presented.

**Conflicts of Interest:** The author declares no conflict of interest.



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