Association of chlorophyll content and spad chlorophyll in diverse sweet sorghum cultivars under different environments

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ABSTRACT: Sweet sorghum (*Sorghum bicolor* (L.) Moench) can be an effective crop for bio-ethanol production due to its adaptability and high sugar yields. Like many other crops, chlorophyll content is related to high productivity and can be measured directly and indirectly. However, literature is lacking concerning non-destructive measurement of chlorophyll in sweet sorghum. The objectives of this study were to determine the most effective technique and best plant age for estimation of chlorophyll content by SPAD chlorophyll meter readings (SCMR). Chlorophyll content and SCMR were taken at 10 day intervals between 40 and 100 days after planting (DAP) at the Field Crops Research Station, Khon Kaen University (KKU) and at the National Corn and Sorghum Research Center, Nakhon Rachsima (NCSRC). SCMR and chlorophyll content were significantly correlated for all test dates at KKU (r = 0.49 to 0.64) but only moderately correlated at 40 DAP (r = 0.48), 90 DAP (r = 0.33) and 100 DAP (r = 0.32) for the NCSRC location. SCMR values had higher correlation between locations and across sample times than leaf chlorophyll content. Differences among entries were significant at all plant ages for SCMR at KKU but for only 70, 80, 90, and 100 DAP for NCSRC. Differences among genotypes were also observed for chlorophyll content at both locations for 80, 90 and 100 DAP. The SPAD chlorophyll meter is a useful tool for indirectly measuring leaf chlorophyll content in sweet sorghum, and has several advantages over direct measurement, including reduced labor and better reproducibility.
Keywords: Chlorophyll content, SCMR, *Sorghum bicolor* L., correlation, indirect selection

Introduction

Sweet sorghum (*Sorghum bicolor* L. Moench) produces juice with 10-20% soluble sugar that can be extracted from the stalks and be directly fermented to bio-ethanol (Reddy et al., 2005). Sweet sorghum can be grown in a wide range of environments in the Americas, Africa and Asia (Rao et al., 2013a). Sweet sorghum has many desirable characteristics such as wide adaptability, rapid growth (Reddy, 2008), drought tolerance (Tesso et al., 2005), waterlogging tolerance, salinity resistance (Reddy and Reddy, 2003; Almodares et al., 2008) and high biomass productivity (Almodares and Hadi, 2009; Wu et al., 2010). There are diverse genotypes of sweet sorghum around the world; however the initial targets in selection of either pure line or hybrid sweet sorghum are high stalk yield, and high juice brix value, which will lead to high ethanol yield.

There is a need to select for high yields through indirect methods. For example, plant height and stalk diameter have been correlated with stalk yield (El-Lattief, 2011; Alhajturki et al., 2012; Codesido et al., 2013; Rao et al., 2013), and brix and sugar content have been correlated with ethanol yield (Dutra et al., 2013). These plant characteristics are related to photosynthesis rate and the accumulation of photosynthate, which is highly dependent upon chlorophyll content in leaves. Chlorophyll content in leaves can be measured directly (Moran, 1981), however, this method is destructive to the plant, costly, and time consuming for a large of number of samples

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(Ruttanaprasert et al., 2012). Therefore, indirect measurement of chlorophyll content in plant leaves is desirable.

Soil Plant Analysis Development (SPAD) chlorophyll meter reading (SCMR) is one alternative method for non-destructively measuring chlorophyll content in plant leaves. The Minolta SPAD-502 meter (Tokyo, Japan) has been developed to assess crop health and has been widely used by scientists conducting experiments in different areas of plant sciences (Markwell et al., 1995). High correlation between SCMR values and chlorophyll content have been reported in many crops such as grain sorghum (*S. bicolor* (L) Moench) (Xu et al., 2000), sugarcane (*Saccharum officinarum* L) (Jangpromma et al., 2010), Jerusalem artichoke (*Helianthus tuberosus* L) (Ruttanaprasert et al., 2012), maize (*Zea mays* L) and soybean (*Glycine max* (L) Merr.) (Markwell et al., 1995), cotton (*Gossypium hirsutum* L) (Wu et al., 1998), rice (*Oryza sativa* L) (Jinwen et al., 2009), potato (*Solanum tuberosum* L) (Bindi et al., 2002), wheat (*Triticum aestivum* L) (Ommen et al., 1999; Udding et al., 2007), and peanut (*Arachis hypogaea* L.) (Arunyanark et al., 2009). However, Barwinsky and Remphrey (2009) reported that chlorophyll content was not correlated to SCMR in Amur maple (*Acer ginnala* Maxim) for tolerance to lime-induced iron chlorosis. In sweet sorghum, literature is lacking for the comparison of chlorophyll content in leaves to SCMR. The objectives of this study were to determine chlorophyll content at different plant stages and determine the association between chlorophyll content and SCMR for sweet sorghum. The SCMR technique would be an extremely useful non-destructive and efficient tool for plant breeders and for physiological studies if chlorophyll content and SCMR are closely correlated across genotypes and plant ages.

Material and methods

Plant materials and experimental sites

Twenty-six sweet sorghum genotypes were divided into two groups (i.e. eleven pure lines; KKU a-11, 14, 48, 53, 139, KKU10, BJ248, Theis, Keller, Urja and Suwan sweet extra and fifteen F_1 hybrids sweet sorghum; KKU A-11 × KKU40, KKU A-11 × BJ248, KKU A-11 × Theis, KKU A-14 × KKU40, KKU A-14 × BJ248, KKU A-14 × Theis, KKU A-48 × KKU40, KKU A-48 × BJ248, KKU A-48 × Theis, KKU A-53 × KKU40, KKU A-53 × BJ248, KKU A-53 × Theis, KKU A-139 × KKU40, KKU A-139 × BJ248 and KKU A-139 × Theis). The hybrids were crossed between five female and three male parental lines in 2013. The experiments were conducted in two environments: the Field Crops Research Station, Khon Kaen University (KKU), Khon Kaen, Thailand (latitude 16°26'N and longitude 102°50'E, altitude 190 m above sea level, average rainfall 1,327 mm/yr, average temperature 27.8 °C, relative humidity 85.4% and soil texture is sand) and the National Corn and Sorghum Research Center (NCSRC), Nakhon Rachsima, Thailand (latitude 14.5°0'N and longitude 101°0'E, altitude 388 m above sea level, rainfall 1,000-2,000 mm/yr, average temperature 30 °C, relative humidity 85 % and soil texture is clay). The twenty-six sweet sorghum genotypes were planted on 22 August, 2013 and 9 September, 2013 at KKU and NCSRC respectively.

Experimental design and management

The experiments were arranged in a randomized complete block design with three replications, four rows in each plot and 4 m in length with spacing 75 cm between rows and 10 cm between plants, resulting in a theoretical population of 133,333 plants/ha. Fertilizer was applied two times: basal fertilizer 15-15-15 (N-P_2O_5-K_2O) was applied at the rate 156.25 kg/ha at each

location at planting, and top dressing was applied 30 days after planting (DAP) at the same rate. Weeds were extracted manually prior to fertilization. Carbosulfan 5G (2,2-dimethyl-3H-1-benzofuran-7-yl) N-(dibutylamino)sulfanyl-N-methylcarbamate) was used for insect protection (shoot fly; *Atherigona soccata*) at planting and 30 days after planting. Sweet sorghum heads were covered by mesh to prevent bird predation. At NCSRC the irrigation was used by furrow method once a week, sprinkler was used at the Field Crops Research Station once a week and both locations were not fungicide application used.

Data collection

The SPAD chlorophyll meter (Minolta SPAD-502 meter, Tokyo, Japan) was recorded SCMR values. SCMR was measured at 10-d intervals from 40 to 100 DAP. At 40 and 50 DAP; SCMR was recorded at the first fully expanded leaf from the top of the plant and at three positions in each leaf (within 10 cm from base of leaf, mid leaf, within 10 cm of tip of leaf). Five plants were recorded within each plot. For readings from 60 to 100 DAP, SCMR was recorded at the third leaf below the flag leaf at the three positions described above in each leaf, 5 plants/plot.

Chlorophyll content was recorded during the same time period and positions on the leaf as SCMR from leaf discs (1 cm^2) collected and soaked in 5 ml N,N-dimethylformamide as a solvent for chlorophyll extraction. The test tubes were kept in the dark 24 hr. Chlorophyll content was then measured using a UV-visible spectrophotometer; (Themo Spectonic Genesis 10) at 647 and 664 nm for measuring chlorophyll a and b, as described by Moran et al. (1981). Field measurements of SCMR or sample collection for leaf chlorophyll content were all performed between 9:00-11:00 AM (local time).

Data analyses

The data were analyzed using SAS v. 9.3 (SAS Institute, Cary, NC). Box and whisker plots of the data were constructed using PROC BOXPLOT in SAS. Analysis of variance was conducted using the PROC GLIMMIX procedure in SAS with genotypes, location and days after planting (DAP) as fixed effects, with all interactions included. Replication, nested within location, was treated as a random effect. Individual plots were identified as subjects within the RANDOM statement to account for repeated measures. Tests for simple effects within interactions (for example, cultivar differences at a specific DAP) were computed using the SLICE and SLICEDIFF options within the LSMEANS statement. Significant differences between LS means were determined using Tukey's HSD at $\alpha = 0.05$. Correlations were calculated using PROC CORR of SAS. Both Pearson and Spearman correlations were calculated, but results were highly similar so only Pearson correlations are presented here.

Results

Analysis of variance for SCMR and chlorophyll content

SCMR was affected by genotype, location and DAP; however, the genotype × location and genotype × location × DAP interactions were not significant (**Table 1**). The SCMR values tended to be slightly greater at NCSRC than at KKU. At KKU differences among the 26 genotypes were observed at all measurement dates. However, at NCSRC the differences among genotypes were not significant at 40, 50 or 60 DAP (data not show). At KKU the mean SCMR peaked at 70 DAP (51.39) and then declined to an average almost as low as at 40 DAP, but with a broader range of readings (data not show). At NCSRC the mean SCMR value also peaked at 70 DAP but remained unchanged

at 80 DAP. It only declined slightly at 90 and 100 DAP (data not show).

All main factors and all interactions were significant for leaf chlorophyll content (**Table 1**). As with the SCMR, chlorophyll content tended to be greater at NCSRC than at KKU. At KKU differences among genotypes for chlorophyll content were not significant at 40 or 70 DAP. At NCSRC, differences among genotypes were not significant at 40, 50 or 60 DAP (data not show). At KKU, leaf chlorophyll content peaked at 80 and 90 DAP, and then decreased sharply at 100 DAP (data not show). At NCSRC leaf chlorophyll content also peaked at 80 and 90 DAP, but only decreased slightly at 100 DAP. Leaf chlorophyll content also increased sharply between 40 and 50 DAP, and then decreased at 60 DAP before increasing again at 70 DAP (data not show).

Correlations across sampling dates

At KKU, SCMR taken on any particular day were correlated with SPAD readings taken on all other days. The highest correlations were between 40 and 50 DAP (r = 0.807), and between 90 and 100 DAP (r = 0.751) (**Table 2**). The lowest correlation was between 40 and 70 DAP (r = 0.351), though this was still significant ($p < 0.01$). A different pattern was observed at NCSRC. Between 60 and 100 DAP, SPAD readings between any given date were correlated. The highest correlation was between 80 and 90 DAP (r = 0.564). However, SPAD readings at 40 and 50 DAP did not correlate with readings taken on later days. The SPAD readings at 40 and 50 DAP were correlated at r = 0.308 (**Table 2**).

Leaf chlorophyll content was correlated between all dates at KKU, though the correlation coefficients are generally lower than for those observed for SPAD meter readings (**Table 3**). The highest correlations were between 70 and 80 DAP (r = 0.587) and between 40 and 50 DAP (r = 0.509). At NCSRC, most leaf chlorophyll measurements did not correlate well with those taken on other dates. The highest correlation was between 70 and 90 DAP (r = 0.405).

Correlation between locations

At 40 and 50 DAP, SCMR values were not correlated between KKU and NCSRC, but at later dates they tended to be highly correlated between the two locations (r = 0.600; **Table 4**). Leaf chlorophyll content measurements were not correlated between locations, except at 70 DAP (r = 0.436; **Table 4**).

Correlation between SCMR and leaf chlorophyll content

At KKU, SCMR and leaf chlorophyll content were highly correlated at all sampling dates, from 40 to 100 DAP. The correlations ranged from r = 0.491 (at 100 DAP) to r = 0.639 (at 40 and 70 DAP; **Table 4**). Correlations were not as high at NCSRC; the highest correlation was r = 0.483 at 40 DAP. SPAD chlorophyll meter reading and leaf chlorophyll content were not correlated at 60 or 70 DAP at NCSRC (**Table 4**).

Discussion

Chlorophyll is a necessary pigment for photosynthesis in plants and its content in plant leave extracts can be measured directly by a spectrophotometer. This method is time consuming and laborious. SPAD chlorophyll meter reading (SCMR) was developed to indirectly measure chlorophyll content in plant leaves. However, we found the interaction between genotype and DAP, each genotype had different both SCMR and chlorophyll content in various DAP. For the recommendation about measuring the SCMR and

chlorophyll content that should be recorded at suitable growth stages each genotype due to the SCMR and chlorophyll content varied on growth stage. Chlorophyll content and SCMR have been reported to be correlated for other crops. Rattanaprasert et al. (2013) found significant correlation between chlorophyll content and SCMR in three Jerusalem artichoke cultivars at 30, 60 and 90 DAP (ranging from r = 0.84 to 0.93**). Yamamoto et al. (2002) found significant correlation between chlorophyll content and SCMR in pigeon pea (*Cajanus cajan* (L.) Millsp.) at the vegetative and ripening stages (r = 0.91** and 0.96** respectively). For peanut, Arunyanart et al. (2008) reported highly significant and positive correlations between chlorophyll content and SCMR (ranging from r = 0.67 to 0.93 for 30, 60 and 90 DAP). Significant correlations were maintained for peanut even under drought stress (r = 0.76 to 0.96) (Aranyanart et al., 2009). In species more closely related to sweet sorghum, significant correlations between SCMR and chlorophyll content were reported in maize (Markwell et al. 1995), in wheat (r = 0.90) (Udding et al. (2007)) and in sugarcane under stress and non-stress conditions (Jangpromma et al. 2010; Garkar et al. (2011). Significant correlations have also been reported with grain sorghum (Xu et al., 2000; Yamamoto et al., 2002), however there are no reports for sweet sorghum. The correlation between SCMR and chlorophyll was significant and consistent at the KKU location in our study but much less significant at NCSRC. The variation among genotypes at the NCSRC location for chlorophyll content was minimal and resulted in non-significant mean separations across all sampling dates (data not shown). However, significant variations occurred among genotypes for SCMR at this location.

Temperatures and relative humidity between the two locations were similar. Though rainfall was different between locations other studies did not show lack of correlation between SCMR and chlorophyll under different water regimes (Aranyanart et al., 2009; Jangpromma et al., 2010; Garkar et al., 2011). Nonetheless, during October, when the 40 and 50 DAP samples were taken, NCSRC received 231 mm more rain than KKU. This could partly explain the lack of correlation between the two locations at these two sampling dates for both measured traits. The soils were also quite different. KKU had sandy soil with a pH of 5.5 while NCSRC had a clay soil with a pH of 7.0. Given the heavier soil texture and excessive rainfall at NCSRC during September and October, it is possible that the plants at this location were suffering some waterlogging stress early in the season. This could also explain why the SPAD readings on 40 and 50 DAP did not correlate with readings taken later in the season at this location.

SCMR was much more consistent across sample times (**Table 2**) and between locations after 50 DAP (**Table 4**) for the 26 entries indicating that this method could separate genotypes much better than via chlorophyll readings. Based on the higher correlations between SCMR and chlorophyll at KKU, it would appear that 70 DAP would be recommended for evaluation for this group of material. Since maturity of sweet sorghum lines can vary greatly, evaluations should be grouped by maturity when testing lines for selection. The number of days to flowering for this group ranged from 65-75 d, and was not different between locations (Bunphan et al., 2015). Despite the plant age, pure line Swan Sweet Extra was consistently among the top three highest in SCMR across locations and sample times and A-11 × KKU40 was consistently the highest hybrid (data not shown). These two are also among the highest in chlorophyll content. Thus selection based on SCMR should be effective for future breeding programs. Selection experiments would be recommended to verify these conclusions however.

Table 1 Analysis of variance results (F-statistics) for SPAD chlorophyll meter reading (SCMR) and leaf chlorophyll content of 26 sweet sorghum cultivars at two locations in Thailand

		SCMR	Chlorophyll
Effect	DF†	*F*	
Genotype	25/104	6.84 ***	7.77 ***
Location	1/104	683.03 ***	1701.28 ***
Days after planting (DAP)	6/624	680.87 ***	415.70 ***
Genotype *Location	25/104	1.16 ns	2.66 ***
Genotype *DAP	150/624	1.98 ***	1.61 ***
Location*DAP	6/624	86.78 ***	127.10 ***
Genotype*Location*DAP	150/624	1.04 ns	1.58 ***

† DF = numerator/denominator degrees of freedom

*** = significant at α = 0.001, ns = not significant

Table 2 Pearson correlations (r) between SPAD chlorophyll meter readings taken on 26 sweet sorghum genotypes at different days after planting (DAP) at two locations in Thailand (n = 78).

Location: KKU						
DAP	40	50	60	70	80	90
50	0.807 ***					
60	0.483 ***	0.638 ***				
70	0.351 **	0.500 ***	0.655 ***			
80	0.526 ***	0.601 ***	0.645 ***	0.556 ***		
90	0.406 ***	0.524 ***	0.588 ***	0.371 ***	0.658 ***	
100	0.485 ***	0.561 ***	0.596 ***	0.457 ***	0.649 ***	0.751 ***
Location: NCSRC						
DAP	40	50	60	70	80	90
50	0.308 **					
60	0.024 ns	0.008 ns				
70	0.088 ns	- 0.053 ns	0.570 ***			
80	0.163 ns	0.122 ns	0.377 ***	0.510 ***		
90	0.179 ns	0.189 ns	0.442 ***	0.481 ***	0.564 ***	
100	0.104 ns	0.014 ns	0.363 **	0.525 ***	0.502 ***	0.497 ***

** = significant at α = 0.01, *** = significant at α = 0.001, ns = not significant.

Table 3 Pearson correlations (r) between leaf chlorophyll content measured on 26 sweet sorghum genotypes at different days after planting (DAP) at two locations in Thailand (n = 78).

Location: KKU						
DAP	40	50	60	70	80	90
50	0.509 ***					
60	0.378 ***	0.315 **				
70	0.411 ***	0.357 **	0.456 ***			
80	0.429 ***	0.317 **	0.515 ***	0.587 ***		
90	0.353 **	0.497 ***	0.328 **	0.241 *	0.363 **	
100	0.255 *	0.460 ***	0.354 **	0.257 *	0.320 **	0.360 **
Location: NCSRC						
	40	50	60	70	80	90
50	0.093 ns					
60	-0.101 ns	0.337 **				
70	0.259 *	0.284 *	0.155 ns			
80	0.013 ns	0.144 ns	0.024 ns	0.216 ns		
90	-0.050 ns	0.155 ns	0.082 ns	0.405 ***	0.327 **	
100	0.002 ns	0.181 ns	0.291 **	0.179 ns	-0.061 ns	0.107 ns

* = significant at α = 0.05, ** = significant at α = 0.01, *** = significant at α = 0.001, ns = not significant

Table 4 (a) Pearson correlations (r) between two locations in Thailand (KKU and NCSRC) for SPAD chlorophyll meter reading (SCMR) and leaf chlorophyll content measured on 26 sweet sorghum entries at different days after planting (DAP; n = 26) and (b) correlations (r) between SPAD chlorophyll meter reading (SCMR) and leaf chlorophyll content measured on 26 sweet sorghum genotypes at different days after planting (DAP) at two locations in Thailand (n = 78)

DAP	Variable [a]		Location [b]	
	SCMR	Chlorophyll	KKU	NCSRC
			r	
40	0.236 ns	0.187 ns	0.639 ***	0.483 ***
50	0.275 ns	0.290 ns	0.533 ***	0.289 *
60	0.760 ***	0.300 ns	0.630 ***	0.138 ns
70	0.609 ***	0.436 *	0.639 ***	0.221 ns
80	0.604 **	0.380 ns	0.499 ***	0.240 *
90	0.697 ***	0.270 ns	0.642 ***	0.328 **
100	0.600 **	0.127 ns	0.491 ***	0.321 **

* = significant at α = 0.05, ** = significant at α = 0.01, *** = significant at α = 0.001, ns = not significant

Conclusions

SCMR appears to be an effective tool to evaluate chlorophyll content non-destructively as has been reported in other crops. SCMR was positively correlated with chlorophyll content at each plant age at the KKU location but was not as significant or consistent at NCSRC. SCMR was able to detect genotype variation and was more consistent than chlorophyll content across sampling times and locations. SCMR could be suggested to measure chlorophyll content because it has more advantage than directly measuring by spectrophotometer because it has low-cost, is non-destructive, and more repeatable. More individual measurements can be taken using the SPAD meter, further reducing experimental error. Recommended plant ages for effectively measuring SCMR would be from 50 to 70 days after planting and depending on maturity of sweet sorghum genotypes.

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