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RESEARCH ARTICLE

PREDICTION MODELS FOR NON-DESTRUCTIVE ESTIMATION OF TOTAL CHLOROPHYLL CONTENT IN SUGARCANE

Krishnapriya Vengavasi*, R. Arunkumar, R. Gomathi and S. Vasantha

Abstract

Total chlorophyll content of sugarcane is an important indicator of plant health, directly correlated to the photosynthetic potential of the crop. With recent technological advancements, portable chlorophyll meters have largely replaced biochemical chlorophyll estimation, requiring laborious extraction procedure with solvents like acetone and dimethyl sulphoxide. Chlorophyll meters determine only '*greenness'* index, which has to be converted into scientifically standard units in order to make the data comprehensive. Prediction models for inter-conversion of chlorophyll units are available for crops like rice, wheat, sorghum, barley, maize, etc., but not for sugarcane till date. In the present study, total chlorophyll content was recorded in diverse sugarcane germplasm and commercial hybrids using both non-destructive and destructive sampling methods. A strong positive correlation was observed between meter readings (SPAD and CCI) with total chlorophyll content estimated using 80% acetone (r = 0.800 and 0.793) and dimethyl sulphoxide (r = 0.915 and 0.868). Regression models for the best fit curve between meter reading and extracted chlorophyll values of the tested sugarcane germplasm and hybrids were non-linear, polynomial equations of the second order. The model developed was validated in an independent experiment wherein sugarcane variety Co 86032 was subjected to increasing nitrogen levels. Highly significant linear regression was found between observed and predicted values of all estimates of total chlorophyll content with almost negligible prediction error. Thus, the model calibrated and validated for sugarcane germplasm and commercial hybrids would be a small yet significant step towards aiding high-throughput phenotyping in sugarcane thereby accelerating crop improvement programmes.

Keywords: Acetone; CCI; Chlorophyll; DMSO; SPAD; Sugarcane

Introduction

Chlorophyll content in leaves is one of the most essential physiological traits which gives basic information about the crop's potential to photosynthesise and thereby indirectly correlated to overall growth and development. Classical experiments emphasised the importance of chlorophyll content estimation by destructive sampling which required rigorous maceration of the leaf tissue with solvents like acetone, methanol and ethanol to extract the pigments. Extraction techniques involving better solvents like dimethyl sulphoxide and dimethyl formamide completely bleached the chlorophyll pigments from the leaves and hence was widely accepted by researchers as it reduced the burden of manual labour for tissue homogenisation (Holm-Hansen 1978; Barnes et al. 1992; Porra et al. 1989; Porra 2002; Ritchie 2006). In the recent times, with technological advancements leading to high-throughput phenotyping, portable chlorophyll meters have largely replaced the biochemical assays. One of the earliest of such hand-held meters was designed by the Soil-Plant Analysis Development unit of Minolta Camera Co. Japan (Rodriguez and Miller 2000), popularly called as the SPAD meter. The principle of chlorophyll estimation in the portable meter is measurement of the ratio of light

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intensities transmitted from the leaf in response to sequential illumination at two wavelengths i.e. red (650 nm) and infrared (940 nm). Many such meters are commercially available today with slight variation in wavelength of emitted light and the unit in which the measured chlorophyll is expressed. For example, certain meters give the ratio of transmittance at 940 nm to 660 nm, called the Chlorophyll Content Index (CCI) (Richardson et al. 2002). In essence, the relative chlorophyll content or the '*greenness'* index in the leaf is only being captured by non-destructive sampling. Converting these ratios or indices into scientifically standard units of chlorophyll content would require comparison of methodologies across crop species, varieties and a wide range of leaf greenness *per se*. Development and validation of such models/equations have been attempted in several agricultural crops including rice (*Oryza sativa*) (Monje and Bugbee 1992; Yuan et al. 2016), wheat (*Triticum aestivum*) (Monje and Bugbee 1992; Castelli et al. 1996; Samsone et al. 2007; Uddling et al. 2007; Zhu et al. 2012), sorghum (*Sorghum bicolor*) (Yamamoto et al. 2002), barley (*Hordeum vulgare*) and maize (*Zea mays*) (Castelli et al. 1996; Zhu et al. 2012), soybean (*Glycine max*) (Monje and Bugbee 1992; Castelli et al. 1996), pigeon pea (*Cajanus cajan*) (Yamamoto et al. 2002), field bean (*Phaseolus vulgaris*) (Samsoneet al. 2007), potato (*Solanum tuberosum*) (Uddlinget al. 2007; Zhu et al. 2012), muskmelon (*Cucumis melo*) (Azia and Stewart 2001) and *Amaranthus vlitus* (Kapotis et al. 2003). This relationship has also been worked out in some fruit trees (Schaper and Chacko 1991) and endangered plant species (Hawkins et al. 2009). Parry et al. (2014) have given an exhaustive review and analysis of the relationship between destructive and non-destructive chlorophyll sampling in 22 plant species including agricultural crops *viz*. rice, wheat, maize, sorghum, barley, soybean, tomato (*Solanum lycopersicum*), pea (*Pisum sativum*), lettuce (*Lactuca sativa*) and pepper (*Capsicum* sp.).

Sugarcane (*Saccharum* sp.) is a cash crop cultivated mainly for its commercial value in the form of sugar and bio-fuel, predominantly grown in Brazil, India and China. As in other plant species, chlorophyll content in sugarcane influences photosynthesis and thereby biomass accumulation, partitioning and cane productivity. Limited reports are available as on date about the relationship between SPAD and extracted chlorophyll in sugarcane under natural field condition (Radhamani and Kannan 2016) or when subjected to water stress (Jangpromma et al. 2010; Silva et al. 2013). Though these papers report positive correlation between meter recorded indices and extracted chlorophyll values, a reliable model and/or equation to convert one unit to the other is yet to be established in sugarcane. Development and validation of such a statistical model to estimate the leaf chlorophyll content in sugarcane by rapid non-destructive sampling methods is essential so as to reduce the resources and time involved in extraction procedures. The objective of this investigation was to develop such a model, with regression equations which would be valid across the sugarcane germplasm and commercial hybrids. In the era of high-throughput phenotyping or '*phenomics'*, such rapid non-destructive techniques which could act as proxies to time- and resource-consuming biochemical assays, are most essential to accelerate varietal selection in breeding programmes.

Materials and Methods

Experimental details

Data for deriving the relationship between methods of chlorophyll estimation in sugarcane was recorded in five independent experiments at ICAR-Sugarcane Breeding Institute, Coimbatore during the cropping season 2018-19. Experiment

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I comprised 14 sugarcane germplasm clones [*Saccharum officinarum*: Sarawak Unknown, Cebu Light Purple, Bontha, Hawaii Original 24; *S. spontaneum*: US 59-1-1; *S. barberi*: Katha Coimbatore, White Pindaria, *S. sinense*: Reha; *S. robustum*: NG 77-237; *S. robustum* inter-specific hybrid: GUK 14-7; *Pennisetum sp*.: IS 76-166; *Erianthus arundinaceus*: IJ 76-503; *E. bengalensis*: IND 82-281; *Saccharum*×Bamboo inter-generic hybrid: GUK 14-530] raised in polybags of 3 feet height and 2 feet diameter containing 5 kg of potting mixture (red soil:farm yard manure:sand::2:2:1). Chlorophyll estimation was carried out in the 4-month old crop. Experiment II was screening for water use efficiency with 18 hybrids (Co 10026, Co 15018, Co 16018, Co 85019, Co 15015, Co 12009, Co 95020, Co 13014, CoM 0265, Co 86032, Co 09004, Co 0212, Co 15021, Co 15007, Co 8021, Co 11015, Co 14025, Co 14002) grown in field condition with all recommended package of practices, except irrigation which was limited by 50% of the volume required based on pan evaporation rates. Chlorophyll in the leaves was recorded in 8-month old crop. Experiment III included 12 commercial hybrids suitable for cultivation in the tropical and sub-tropical zones of India *viz*., tropical: Co 86032, Co 0212, Co 14012, Co 06022, Co 11015, Co 13006 and sub-tropical: Co 0238, Co 15027, Co 15023, Co 98014, BO 91, CoLk 8102. Experiment IV included 10 sugarcane hybrids in the drought screening programme of advanced varietal trial (AVT: Co 13002, Co 13003, Co 13004, Co 13006, Co 13008, Co 13009, Co 13013, Co 13014, Co 13018, Co 13020) and two standard checks Co 86032 and Co 99004. Drought was imposed from 60 to 150 days after planting by withholding the irrigation, to select clones that could sustain growth and yield under severe drought. Plants in experiments III and IV were grown in the field following the recommended package of practices and chlorophyll estimation was done in the 6-month old crop. In experiment V, sugarcane variety Co 86032 was subjected to increasing levels of nitrogen in semi-hydroponic culture under glasshouse condition with controlled environment. Single budded setts of Co 86032 raised in polybags were transferred to porcelain pots containing 4 kg gravel chips and nutrient solution (1mM $CaCl_2$, 4 mM $MgSO_4$, 2 mM H_3PO_4, 2 mM KCl, 100 µM EDTA-FeNa, 10 µM H_3BO_3, 2 µM $MnCl_2$, 5 µM $ZnSO_4$, 2 µM $CuSO_4$ and 0.2 µM MoO_4). The sole source of nitrogen $Ca(NO_3)_2$ was added to the solution to create 12 varying levels of nitrogen (0, 5, 10, 25, 50, 75, 100, 250, 500, 750, 1000, 2000 µM). The solution pH was adjusted between 6.8 to 7.0, and added to the pots every alternate day. The chlorophyll measurement was recorded in 4-month old crop when the deficiency symptoms were visible.

Total chlorophyll estimation

Total chlorophyll content was recorded in sugarcane crop in the physiologically active leaf (+3) i.e., third fully opened leaf with visible dewlap. An area of approximately 60 cm^2 was marked in the mid portion of the leaf for measurement of chlorophyll by non-destructive as well as destructive sampling. Non-destructive measurement was recorded from 0900 to 1200 h using portable hand-held chlorophyll meter (MC-100, Apogee Instruments, Utah, USA) independently for the two units (i) SPAD and (ii) CCI. Average of five readings recorded in the 60 cm^2 leaf area excluding midrib was taken for individual leaf sample. For destructive sampling, the entire 60 cm^2 leaf area excluding midrib was cut into uniform squares (approximately 0.25 mm^2), from which 100 mg representative tissue was used to determine (iii) total chlorophyll extracted with 80% acetone according to Arnon (1949) (C_ACE) and (iv) total chlorophyll extracted with dimethyl sulphoxide by the method given by Hiscox and Israelstam (1979) (C_DMSO). Three technical replicates were taken for the destructive method

Table 1. Chlorophyll content in sugarcane germplasm

	Non-destructive measurement		Destructive measurement	
Genotype	SPAD	CCI	Chlorophyll content (mg g^{-1} FW) in acetone (C_ACE)	Chlorophyll content (mg g^{-1} FW) in DMSO (C_DMSO)
Sarawak Unknown	38.43	19.12	2.288	2.032
Cebu Light Purple	40.70	33.08	2.234	1.978
Bontha	36.90	18.52	1.906	1.893
Hawaii Original 24	36.67	19.24	1.955	1.880
US 59-1-1	32.37	14.34	1.705	1.314
Katha Coimbatore	26.67	9.64	1.170	1.164
White Pindaria	41.50	29.50	2.271	2.089
Reha	43.17	26.47	2.156	2.084
NG 77-237	31.30	16.50	1.964	1.421
GUK 14-7	25.93	11.07	1.227	1.087
IS 76-166	34.10	18.30	1.811	1.722
IJ 76-503	28.77	12.60	1.635	1.399
IND 82-281	37.90	20.60	1.962	1.551
GUK 14-530	46.43	30.65	2.388	2.382
Mean	35.77	19.97	1.905	1.714
CV (%)	9.13	9.55	9.16	9.22
MSE	10.657	3.642	0.030	0.025
LSD	5.460	3.192	0.292	0.264
F value	10.877	44.958	13.759	18.686
p value	9.8E-08***	3.0E-15***	7.1E-09***	2.0E-10***

Data for non-destructive measurement is the average of five meter readings, and three technical replicates for destructive measurement. CV, MSE and LSD denote coefficient of variation, mean square error and least significant difference, respectively. F and *p* values from analysis of variance indicate significant ($\alpha = 0.001$) difference among genotypes.

of chlorophyll estimation, which was expressed as mg g^{-1} fresh weight (FW).

Statistical data analysis

Initial equations for calibration model was developed by recording the above four traits *viz*., SPAD, CCI, C_ACE and C_DMSO in sugarcane hybrids in experiments I to IV. The statistical models developed from these experiments were validated by recording the same four traits in experiment V. The total chlorophyll values predicted from SPAD and CCI using the calibration equation was compared against the observed values to validate the developed model. Prediction or forecast error was calculated according to Kumar and Bhar (2005) with slight modification as below.

$$\text{Prediction error} = \frac{[\text{Observed reading} - \text{Predicted reading}]}{\text{Observed reading}}$$

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Data analysis for variance, correlation, regression, model development and validation was carried out using the statistical software R (version 3.6.0) installed with '*agricolae'* package. Graphs were plotted in Microsoft Excel 2016.

Table 2. Chlorophyll content in commercial sugarcane hybrids grown with irrigation limited by 50% volume based on pan evaporation rates

	Non-destructive measurement		Destructive measurement	
Clone	SPAD	CCI	Chlorophyll content (mg g^{-1} FW) in acetone (C_ACE)	Chlorophyll content (mg g^{-1} FW) in DMSO (C_DMSO)
Co 10026	25.56	10.02	1.098	1.299
Co 15018	26.76	10.36	1.424	1.054
Co 16018	26.08	9.24	1.112	1.101
Co 85019	31.54	17.22	1.652	1.342
Co 15015	34.86	18.58	1.460	1.484
Co 12009	33.74	15.50	1.688	1.846
Co 95020	32.24	14.28	1.638	1.417
Co 13014	35.68	14.86	1.522	1.784
CoM 0265	25.86	10.10	1.420	0.893
Co 86032	31.30	16.04	2.112	1.858
Co 09004	39.22	22.32	2.688	2.227
Co 0212	28.10	12.06	1.424	1.400
Co 15021	33.38	15.82	1.888	1.796
Co 15007	41.18	27.38	2.178	1.898
Co 8021	40.06	27.22	2.612	2.438
Co 11015	43.02	27.64	2.123	2.085
Co 14025	28.08	11.50	1.489	1.307
Co 14002	43.44	27.88	2.094	2.289
Mean	33.34	17.11	1.757	1.640
CV (%)	9.14	9.62	9.29	9.31
MSE	9.284	2.708	0.027	0.023
LSD	5.045	2.725	0.270	0.253
F value	11.807	48.634	23.850	26.026
p value	5.1E-10***	2.2E-16***	1.2E-14***	3.0E-15***

Data for non-destructive measurement is the average of five meter readings, and three technical replicates for destructive measurement. CV, MSE and LSD denote coefficient of variation, mean square error and least significant difference, respectively. F and *p* values from analysis of variance indicate significant (α = 0.001) difference among clones.

Table 3. Chlorophyll content in commercial sugarcane hybrids adapted to tropical and sub-tropical zones of India

	Non-destructive measurement		Destructive measurement	
Clone	SPAD	CCI	Chlorophyll content (mg g^{-1} FW) in acetone (C_ACE)	Chlorophyll content (mg g^{-1} FW) in DMSO (C_DMSO)
Tropical clones				
Co 86032	29.24	13.00	1.527	1.412
Co 0212	26.07	10.45	1.388	1.237
Co 14012	28.73	12.72	1.613	1.337
Co 06022	31.26	16.64	1.971	1.569
Co 11015	24.95	9.32	1.400	1.108
Co 13006	32.06	15.39	1.823	1.694
Sub-tropical clones				
Co 0238	28.48	11.14	1.594	1.406
Co 15027	29.81	13.39	1.895	1.586
Co 15023	31.25	14.83	2.241	1.671
Co 98014	28.25	11.61	1.970	1.391
BO 91	25.50	9.91	1.647	1.082
CoLk 8102	31.72	14.37	1.720	1.658
Mean	28.94	12.73	1.732	1.429
CV (%)	9.03	9.13	9.09	9.09
MSE	6.829	1.352	0.025	0.017
LSD	4.404	1.960	0.265	0.219
F value	2.625	11.814	7.908	8.026
p value	2.3E-02***	3.5E-07***	1.3E-05***	1.2E-05***

Data for non-destructive measurement is the average of five meter readings, and three technical replicates for destructive measurement. CV, MSE and LSD denote coefficient of variation, mean square error and least significant difference, respectively. F and *p* values from analysis of variance indicate significant (α = 0.001) difference among clones.

Results and Discussion

Variation in total chlorophyll content recorded in sugarcane germplasm

Significant variation was observed in the total chlorophyll content of sugarcane germplasm (Table 1). Among the genotypes tested, GUK 14-530 which is a hybrid between *Saccharum* and bamboo recorded the highest values for SPAD (46.43), CCI (30.65), C_ACE (2.388 mg g^{-1} FW) and C_DMSO (2.382 mg g^{-1} FW). *S. robustum* inter-specific hybrid GUK 14-7 recorded the least SPAD value (25.93) and C_DMSO (1.087 mg g^{-1} FW), while lowest CCI (9.64) and C_ACE (1.170

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mg g^{-1} FW) was recorded in *S. barberi* clone Katha Coimbatore.

Variation in total chlorophyll content of commercial sugarcane hybrids

Among the sugarcane hybrids grown with irrigation limited by 50% volume based on pan evaporation rates, highest indices of SPAD (43.44) and CCI (27.88) was recorded in Co 14002 (Table 2). C_ACE was highest in Co 09004 (2.688 mg g^{-1} FW), while C_DMSO was maximum (2.438 mg g^{-1} FW) in Co 8021. Co 10026 showed least SPAD (25.56) and C_ACE (1.098 mg g^{-1} FW), while CCI (9.24) and C_DMSO (1.101 mg g^{-1} FW) was lowest in Co 16018. Results from experiment III revealed significant variation in total chlorophyll content among hybrids adapted to tropical and sub-tropical zones (Table 3). Highest SPAD (32.06) and C_DMSO (1.694 mg g^{-1} FW) was observed in Co 13006, while highest CCI and C_ACE was recorded in Co 06022 (16.64) and Co 15023 (2.241 mg g^{-1} FW), respectively. Lowest values for SPAD (25.50), CCI (9.91) and C_DMSO (1.082 mg g^{-1} FW) was observed in BO 91, while Co 0212 recorded least C_ACE (1.388 mg g^{-1} FW). With regard to sugarcane hybrids in the advanced varietal trial for drought screening, SPAD and CCI values varied from 24.18 to 30.24 and 10.31 to 15.01, respectively (Table 4). Similarly, C_ACE ranged between 1.306 mg g^{-1} FW (Co 13006) to 2.053 mg g^{-1} FW (Co 13002), while C_DMSO varied from 0.939 mg g^{-1} FW in Co 13013 to 1.671 mg g^{-1} FW in Co 13020. Wide variability has been reported in total chlorophyll content of commercial sugarcane hybrids measured in terms of both SPAD and extracted chlorophyll values (Vasantha and Rajalakshmi 2009; Radhamani and Kannan 2013; Vasantha et al. 2017; Kohila and Gomathi 2018).

Model calibration

Use of chlorophyll meter index as a proxy for actual chlorophyll content in sugarcane leaf, and thereby assessing the overall plant health under various biotic and abiotic stress conditions has been commonly practiced in the recent years (Silva et al. 2007; Radhamani and Kannan 2013; Vasantha et al. 2017; Kohila and Gomathi 2018). SPAD was used to assess leaf senescence in sugarcane cultivar RB867515, which had potential applications for biofuel production (Martins et al. 2016). Correlation between the different estimates of total chlorophyll content in sugarcane viz., SPAD, CCI, C_ACE and C_DMSO was positive and highly significant, ranging from 0.793 to 0.953 (Table 5). Jangpromma et al. (2010) reported a significant correlation (r = 0.78) between SPAD index and chlorophyll content extracted with dimethyl formamide, although the number of sugarcane hybrids were limited to 10. The correlation coefficient seemed to increase during drought imposition (r = 0.90) and recovery (r = 0.98), although number of data points was low to establish a strong relationship. Significant correlation (r = 0.90) between SPAD meter reading and total chlorophyll content was observed among 24 commercial sugarcane hybrids (Radhamani and Kannan 2013). Silva et al. (2013) reported significant correlation (r = 0.68) between SPAD and total chlorophyll content in drought susceptible sugarcane cultivar, but this relationship did not hold true for tolerant cultivar. Iron being an important constituent of the chlorophyll molecule, metabolically active iron content in sugarcane leaves was also positively correlated to SPAD meter reading as well as to total chlorophyll content (Radhamani and Kannan 2013), stressing upon the use of non-destructive methods for chlorophyll estimation as a time and resource saving exercise. In the present study, the experiments were chosen carefully so as to include a wide variability of sugarcane germplasm as well as commercial hybrids, grown with recommended package of practices (Exp I and III) subjected

Table 4. Chlorophyll content in commercial sugarcane hybrids in the advanced varietal trial for drought screening

	Non-destructive measurement		Destructive measurement	
Clone	SPAD	CCI	Chlorophyll content (mg g^{-1} FW) in acetone (C_ACE)	Chlorophyll content (mg g^{-1} FW) in DMSO (C_DMSO)
Co 13002	30.08	13.73	2.053	1.636
Co 13003	29.12	12.53	1.432	1.257
Co 13004	26.11	10.86	1.351	1.171
Co 13006	28.47	10.87	1.306	1.238
Co 13008	29.30	13.59	1.715	1.532
Co 13009	26.39	10.35	1.401	1.113
Co 13013	24.81	10.36	1.139	0.939
Co 13014	26.86	10.87	1.335	1.136
Co 13018	30.24	15.01	1.918	1.524
Co 13020	33.14	15.47	1.929	1.671
Co 86032	24.18	10.31	1.371	1.219
Co 99004	30.18	12.68	1.710	1.333
Mean	28.24	12.22	1.555	1.314
CV (%)	9.04	9.10	9.15	9.12
MSE	6.511	1.236	0.020	0.014
LSD	4.300	1.874	0.240	0.202
F value	3.151	8.598	13.034	10.895
p value	9.1E-03***	6.4E-06***	1.4E-07***	7.5E-07***

Data for non-destructive measurement is the average of five meter readings, and three technical replicates for destructive measurement. CV, MSE and LSD denote coefficient of variation, mean square error and least significant difference, respectively. F and *p* values from analysis of variance indicate significant ($\alpha = 0.001$) difference among clones.

to limited (Exp II) and severe water stress (Exp IV). Regression models for the best fit curve between meter reading and extracted chlorophyll values of the tested sugarcane clones were non-linear, polynomial equations of the second order, in concurrence with reports in wheat, rice and vsoybean (Monje and Bugbee 1992), muskmelon (Azia and Stewart 2001), maize (Gitelson and Merzlyak 2004), wheat and potato (Uddling et al. 2007). The data from experiments I to IV were pooled to derive the regression models. Coefficient of determination (R^2) among the traits observed was statistically significant as presented in Figures 1 and 2. Regression between the two indices SPAD and CCI (Fig. 1A and 2A) also fitted a non-linear, polynomial curve in agreement to Richardson et al. (2002). Few reports indicate a linear relationship between SPAD and total chlorophyll content in Augustine grass (Rodriguez and Miller 2000), sorghum and pigeon pea (Yamamoto et al. 2002), but in the present investigation non-linear polynomial equations emerged as the best

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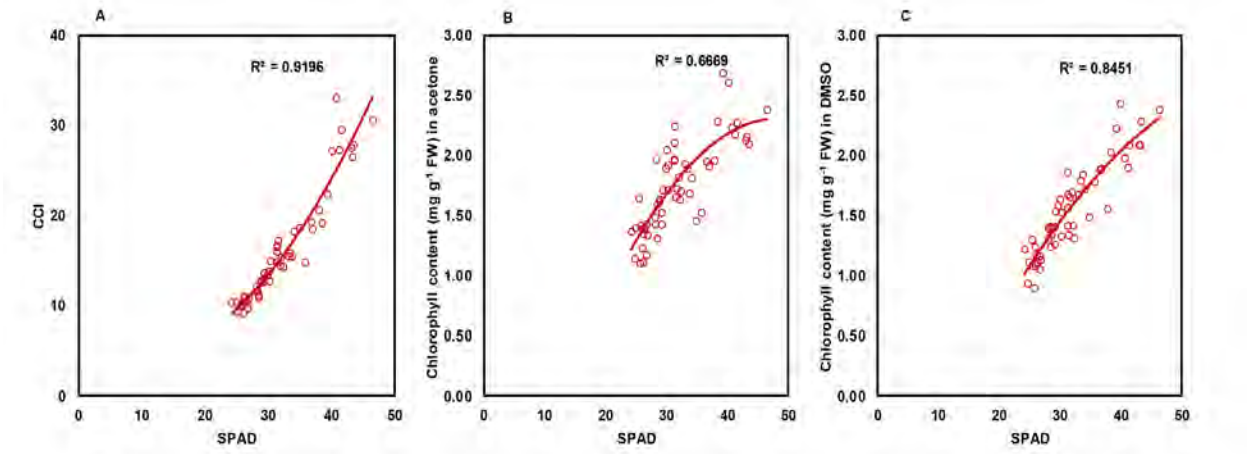

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Trait	Prediction equation from SPAD	R^2	Adjusted R^2	*p* – value
CCI	CCI = 0.02 $SPAD^2$ – 0.3309 SPAD + 5.3794	0.9196	0.9166	2.20 E-16
Chlorophyll content (mg g^{-1} FW) in acetone (C_ACE)	C_ACE = – 0.0019 $SPAD^2$ + 0.1825 SPAD – 2.0903	0.6669	0.6543	2.24 E-13
Chlorophyll content (mg g^{-1} FW) in DMSO (C_DMSO)	C_DMSO = – 0.001 $SPAD^2$ + 0.129 SPAD – 1.5261	0.8451	0.8392	2.20 E-16

Fig. 1. Polynomial regression models (n = 56) of(A) SPAD against CCI, (B) SPAD against chlorophyll content in acetone and (C) SPAD against chlorophyll content in DMSO observed in germplasm and commercial hybrids of sugarcane, (D) equations to predict CCI, C_ACE and C_DMSO from SPAD values. Calculated and adjusted coefficient of determination (R2) and p values indicate statistical significance (α = 0.001) of the predicted models

fit. Similarly, Castelli et al. (1996) demonstrated that among linear, quadratic and exponential curve fitting methods, polynomial exponential function best described the relationship between SPAD and total chlorophyll content recorded in wheat, maize, soybean and tobacco (*Nicotiana tabacum*). Hence, the model calibrated in sugarcane is in line with other major crops.

Table 5. Correlation between estimates of total chlorophyll content in sugarcane by non-destructive and destructive sampling

	SPAD	CCI	C_ACE	C_ DMSO
SPAD		0.953	0.800	0.915
CCI	1.04E-29***		0.793	0.868
C_ACE	1.36E-13***	3.20E-13***		0.869
C_ DMSO	5.60E-23***	3.91E-18***	3.85E-18***	

Data in upper panel denotes Pearson's correlation coefficient (r) between the traits, lower panel denotes p value indicating statistical significance (α = 0.001) of r (n=56).

Model validation

To validate the accuracy of model prediction, chlorophyll measurement by both destructive and non-destructive sampling was done in sugarcane variety Co 86032 raised in semi-hydroponic culture conditions subjected to increasing nitrogen levels (Table 6). This experiment was intended as to obtain wide range of total chlorophyll values as the leaves showed slight to severe yellowing symptoms due to varying nitrogen levels. Nitrogen fertilisation to sugarcane had a greater effect to

Table 6. Chlorophyll content in sugarcane variety Co 86032 subjected to increasing nitrogen levels

	Non-destructive measurement		Destructive measurement	
Nitrogen level	SPAD	CCI	Chlorophyll content (mg g^{-1} FW) in acetone (C_ACE)	Chlorophyll content (mg g^{-1} FW) in DMSO (C_DMSO)
0 µM	14.23	1.58	0.439	0.172
5 µM	19.11	4.37	0.796	0.403
10 µM	21.29	6.13	0.973	0.637
25 µM	21.96	6.61	1.016	0.706
50 µM	22.23	6.81	1.034	0.733
75 µM	29.05	12.25	1.457	1.353
100 µM	32.00	14.65	1.618	1.578
250 µM	33.30	15.89	1.697	1.668
500 µM	37.29	20.67	1.978	1.916
750 µM	37.88	21.40	2.019	1.948
1000 µM	38.59	22.79	2.094	1.986
2000 µM	41.99	27.63	2.344	2.145
Mean	29.08	13.40	1.455	1.271
CV (%)	9.39	10.50	9.67	10.17
MSE	7.460	1.981	0.020	0.017
LSD	4.603	2.372	0.237	0.218
F value	33.146	107.410	54.262	87.823
p value	8.1E-12***	2.2E-16***	3.2E-14***	2.2E-16***

Data for non-destructive measurement is the average of five meter readings, and three technical replicates for destructive measurement. CV, MSE and LSD denote coefficient of variation, mean square error and least significant difference, respectively. F and *p* values from analysis of variance indicate significant (α = 0.001) difference among nitrogen levels.

SPAD index as well as total chlorophyll content (Silva et al. 2017), hence gradually decreasing the dose of nitrogen application would yield in visible chlorosis symptoms. In the present study, with increasing nitrogen level SPAD and CCI values showed an increasing trend from 14.23 to 41.99 and 1.58 to 27.63, respectively. Similarly, lowest C_ACE (0.439 mg g^{-1} FW) and C_DMSO (0.172 0.439 mg g^{-1} FW) was recorded at 0 µM nitrogen level, whereas 2000 µM nitrogen showed highest values of C_ACE (2.344 0.439 mg g^{-1} FW) and C_DMSO (2.145 0.439 mg g^{-1} FW). Highly significant linear regression was found between observed and predicted values of all estimates of total chlorophyll content with almost negligible prediction error (Fig. 3). Thus, the equations developed could be employed for rapid estimation of total chlorophyll content in sugarcane germplasm and commercial hybrids.

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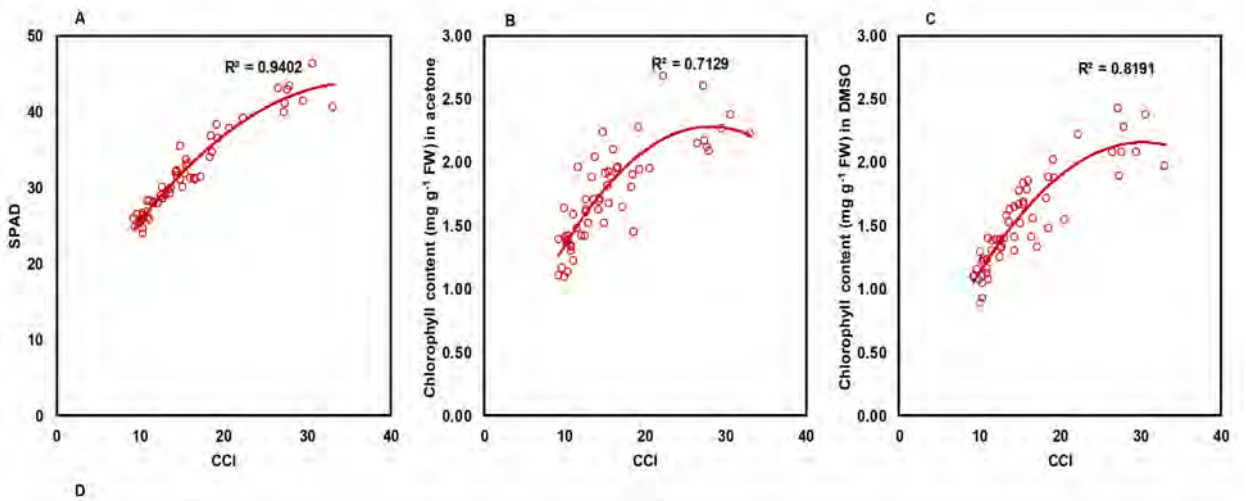

D

Trait	Prediction equation from CCI	R^2	Adjusted R^2	*p* – value
SPAD	SPAD = – 0.0263 CCI^2 + 1.9116 CCI + 9.2291	0.9402	0.9379	2.20 E-16
Chlorophyll content (mg g^{-1} FW) in acetone (C_ACE)	C_ACE = – 0.0029 CCI^2 + 0.1608 CCI + 0.0225	0.7129	0.7021	4.33 E-15
Chlorophyll content (mg g^{-1} FW) in DMSO (C_DMSO)	C_DMSO = – 0.0025 CCI^2 + 0.1512 CCI – 0.1323	0.8191	0.8123	2.20 E-16

Fig. 2. Polynomial regression models (n = 56) of (A) CCI against SPAD, (B) CCI against chlorophyll content in acetone and (C) CCI against chlorophyll content in DMSO observed in germplasm and commercial hybrids of sugarcane, (D) equations to predict SPAD, C_ACE and C_DMSO from CCI values.Calculated and adjusted coefficient of determination (R2) and p values indicate statistical significance (α = 0.001) of the predicted models

Conclusion

Non-destructive methods to assess plant health is in vogue in order to save resources, time and labour. This includes developing alternative, rapid techniques to substitute for chlorophyll extraction with solvents which require more time and effort. With this intention, keeping in view the need to interpret total chlorophyll in sugarcane not as relative indices, but as the actual content in the leaf, following equations (Eqs. i to iii for SPAD, eqs. IV to VI for CCI recorded using portable meters) have been developed and validated across diverse germplasm, commercial hybrids, crop age and/or stress condition.

$$CCI = 0.02SPAD^2 - 0.3309\ SPAD + 5.3794 \rightarrow \text{Eq. i}$$

$$C\_ACE = -0.0019SPAD^2 + 0.1825\ SPAD - 2.0903 \rightarrow \text{Eq. ii}$$

$$C\_DMSO = -0.001SPAD^2 + 0.129\ SPAD - 1.5261 \rightarrow \text{Eq. iii}$$

$$SPAD = -0.0263CCI^2 + 1.9116\ CCI + 9.2291 \rightarrow \text{Eq. iv}$$

$$C\_ACE = -0.0029CCI^2 + 0.1608\ CCI + 0.0225 \rightarrow \text{Eq. v}$$

$$C\_DMSO = -0.0025CCI^2 + 0.1512\ CCI - 0.1323 \rightarrow \text{Eq. vi}$$

Alongside these equations, important precautions to follow during non-destructive sampling is also emphasised i.e., optimum time of day for sampling (0900 to 1200 h), physiologically active leaf (+3 i.e., third fully opened leaf with visible dewlap), optimum leaf area (60 cm^2 laminar area in the middle portion of leaf, excluding mid-rib) and technical replicates (minimum five). Though total leaf chlorophyll content is only one of the several physiological traits important for crop selection, models developed herein would definitely aid high-throughput phenotyping in sugarcane for accelerating crop improvement programmes.

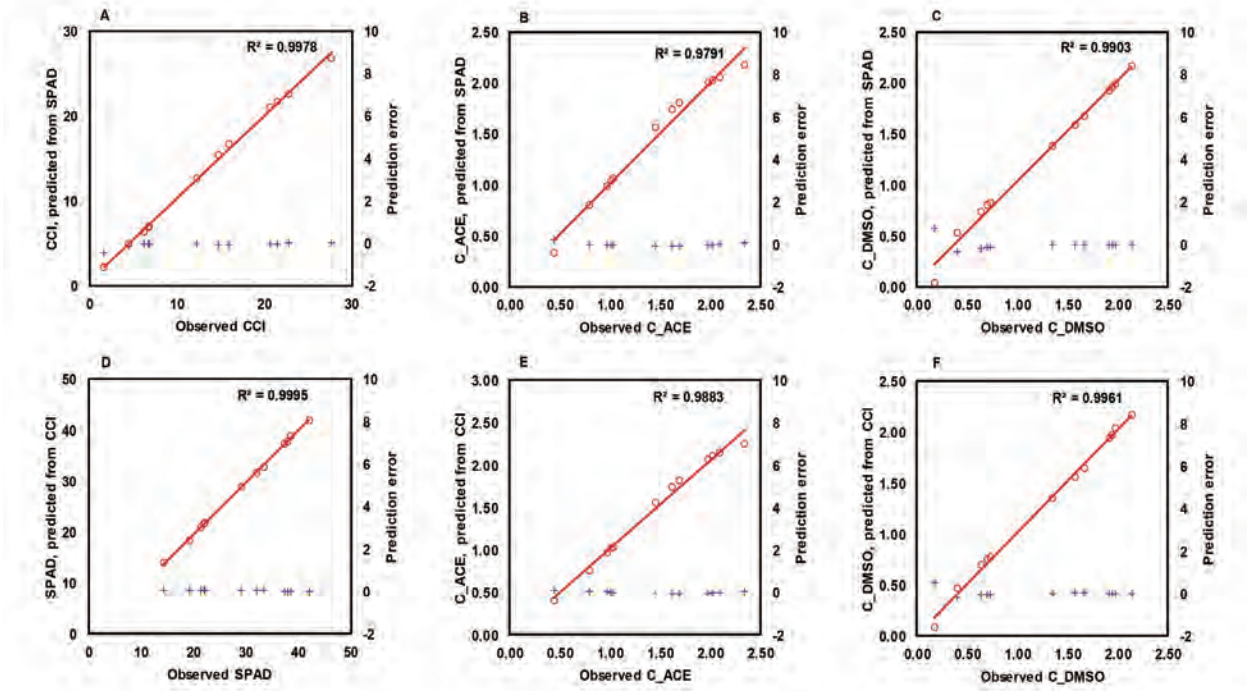

Fig. 3. Validation ofobserved against predicted values (n = 12) of chlorophyll content in sugarcane; (A) Observed CCI vs CCI predicted from SPAD, (B) observed C_ACE vs C_ACE predicted from SPAD, (C) observed C_DMSO vs C_DMSO predicted from SPAD, (D) observed SPAD vs SPAD predicted from CCI, (E) observed C_ACE vs C_ACE predicted from CCI and (F) observed C_DMSO vs C_DMSO predicted from CCI. Purple plus symbol indicates prediction error plotted in secondary axis

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