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Chlorophyll Content and SPAD Value Relationships between Varying Nitrogen Application and Cultivar in Rice

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The SPAD-502 meter is a portable tool that is extensively used for the rapid, accurate and non-destructive measurement of leaf chlorophyll concentrations. Measurements with the SPAD-502 meter produce relative SPAD meter values that are proportional to the amount of chlorophyll present in the leaf. In order to convert these values into absolute units of chlorophyll concentration, calibration curves must be derived and utilized. Here, we present calibration equations for rice that can be used to convert SPAD values into total chlorophyll per unit leaf area (mg cm^{-2}; y = 0.178x - 3.531) or per unit fresh weight of leaf tissue (3.53 mg g^{-1}). These relationships were derived using leaf analysis of two rice genotypes (cv. 'Manaswinee' and cv. 'Hasanta') raised with three nutrient management practices (100% Soil Test Based Nitrogen Recommendation (STBNR), 75% STBNR + *in situ* green manuring (GM) of *dhaincha* and 50% STBNR + *in situ* green manuring of *dhaincha*) under puddled transplanting condition during *Kharif* seasons of 2018 and 2019 at Agronomy Main Research Farm, OUAT, Bhubaneswar, Odisha, India. Chlorophyll concentrations were determined at active tillering, heading and physiological maturity stages. Our results revealed that the converted SPAD values differed from photometric measurements of solvent-extracted chlorophyll by regression coefficient (r) values ranging from 0.711 to 0.862 and the relationship was linear using different models. Application of 75% Soil Test Based Nitrogen Recommendation (75 kg N ha^{-1}) with green manuring of *dhaincha* in rice showed the maximum linear regression coefficient (r=0.862) in rice genotype 'Hasanta' at the heading stage.

(***Key words:*** *Chlorophyll a, Chlorophyll b, Total chlorophyll, Linear model, Rice varieties, SPAD value*)

The Soil Plant Analysis Development (SPAD) chlorophyll meter is one of the most commonly used diagnostic tools to measure crop nitrogen status. It measures the leaf transmittance in red light at 650 nm (at which chlorophyll absorbs) and in near-infrared light at 940 nm (for the correction of leaf thickness). The ratio of these two transmission values is referred to as SPAD reading or SPAD value (Hoel and Solhaug, 1998) and calculates a relative SPAD meter value that should ''correspond to the amount of chlorophyll present in the sample leaf'' (Minolta, 1989). Chlorophyll's role is to absorb light for photosynthesis. There are two main types of chlorophyll: a and b. Chlorophyll a's central role is as an electron donor in the electron transport chain. Chlorophyll b's role is to give organisms the ability to absorb higher frequency blue light for use in photosynthesis. The leaf chlorophyll concentration is usually determined by extraction from leaf samples and subsequent spectrophotometric measurements (Arnon, 1949; Porra *et al*., 1989). Such *in vitro* determinations are destructive, expensive, and time consuming, and may therefore not be applicable for all purposes. Chlorophyll meters are widely used to guide nitrogen (N) management by monitoring leaf N status in agricultural systems. Using an SPAD meter to assess leaf chlorophyll concentration has become common, but calibrating SPAD readings into direct units of chlorophyll concentration is still difficult, and an understanding of the relationship between these two parameters is necessary (Markwell *et al*., 1995). The relationship between SPAD readings and chlorophyll content per leaf area has been found to vary widely among species,

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in some cases even within the same species (Uddling *et al*., 2007). Approximately 80% of leaf N is allocated to chloroplasts and approximately 50% of leaf N is invested in photosynthetic proteins in leaves. However, only 0.5 to 1.5% of leaf N is allocated to chlorophyll depending on the plant's growth environment and species (Evans, 1989; Le Roux *et al*., 1999). Knowing the effects of leaf characteristics and environmental factors on SPAD readings and the relationship between chlorophyll content and leaf N content per leaf area will be important questions when the SPAD-502 is used to guide N management practices in agriculture systems. The objectives of this study were (1) to estimate the variations in SPAD readings and chlorophyll content per unit leaf area within rice varieties, and (2) to identify the relationships between SPAD values and chlorophyll content.

MATERIALS AND METHODS

The field experiment was conducted at Agronomy Main Research Farm, College of Agriculture, Odisha University of Agriculture and Technology (OUAT), Bhubaneswar, Odisha during *Kharif* seasons of 2018 and 2019 to assess the chlorophyll content and SPAD value of two rice varieties under three nutrient management practices. The experimental site is located at 20° 15′N latitude and 85° 52′E longitude with an elevation of 25.9 m above mean sea level and about 64 km away from the Bay of Bengal. The climate of Bhubaneswar is characterized by hot, moist and sub-humid with hot summer and mild winter. The rainfall is monsoonal with unimodal distribution. The soil of the experimental site was sandy loam in texture. In the study, three nutrient management (N_1: 100% Soil Test Based Nitrogen Recommendation (STBNR), N_2: 75% STBNR + *in situ* green manuring (*dhaincha*) and N_3: 50% STBNR+ *in situ* green manuring of *dhaincha*) and two rice varieties (V_1: 'Manaswinee' of 125 d duration and V_2: 'Hasanta' of 145 d duration) were tried in Randomized Complete Block Design (RCBD) with four replications. In 100% Soil Test Based Nitrogen Recommendation (STBNR), 100 kg nitrogen ha^{-1} was applied. Sowing of *dhaincha* was undertaken on 12 June during both the years (2018 and 2019). Nursery raising for rice was done on 28 June and 29 June of the respective seasons. Transplanting was done on 25 July and 3 August of respective seasons. Rice cv. 'Manaswinee' was harvested on 15 and 16 November and cv. 'Hasanta' on 28 and 29 November, respectively, during 2018 and 2019.

SPAD values were recorded at active tillering, heading and physiological maturity stage by using SPAD 502, Minolta Camera Co Ltd, Osaka, Japan. The youngest fully expanded leaf of a representative hill was used for SPAD measurement. Readings were taken on one side of the midrib of the leaf blade, midway between the leaf base and tip. A mean of 15 readings per plot was taken as the measured SPAD value. The chlorophyll content was measured by colorimetric method at active tillering, heading and physiological maturity stages. The fresh leaf tissues (0.1 g) were cut into small pieces and incubated in 10 ml of Dimethyl Sulfoxide (DMSO) in dark at room temperature for 24 h. After the incubation period, the sample was kept in a boiling water bath at 65°C for five minutes. Later, absorbance (A) was measured at 645 nm and 663 nm by UV-VIS Systronics Spectrophotometer. Care was taken to make the final volume to 10 ml with DMSO, wherever the volume was reduced during boiling. The concentration of chlorophyll a, chlorophyll b and total chlorophyll were calculated using the formulae given by Arnon (1949) as follows.

Chlorophyll a = $[(12.7 \times A_{663}) - (2.69 \times A_{645})] \times [V/(1000 \text{ x } W)]$ mg g^{-1} fresh weight

Chlorophyll b = $[(22.9 \times A_{645}) - (4.68 \times A_{663})] \times [V/(1000 \text{ x } W)]$ mg g^{-1} fresh weight

Total Chlorophyll = $[(20.2 \times A_{645}) + (8.02 \times A_{663})] \times [V/(1000 \text{ x } W)]$ mg g^{-1} fresh weight

Statistical analysis

Analyses of Variance (ANOVA) were conducted to differentiate between the SPAD values and absolute chlorophyll concentration using CropStat 7.2. The mean values were compared using one-way analysis of variance with a Tukey test ($P < 0.05$). Regression analyses were performed to test the form of relationship between parameters using Microsoft Excel data analysis tool. All regressions were fitted by both linear and power models, and the model with the higher regression coefficient value was selected. The significance of regression coefficients was tested by the Shapiro-Wilk method ($P < 0.05$).

RESULTS AND DISCUSSION

SPAD value

The principle of chlorophyll meter-based method is that leaf N status is reflected in leaf greenness, which is reflected by values such as SPAD. The SPAD values at different growth stages of rice followed the same trend of chlorophyll content and were, on an average, 38.8, 40.1 and 35.2 at active tillering, heading and physiological maturity stages, respectively. At the active tillering and heading stages, application of 75% STBNR + GM recorded the maximum SPAD values, which was at par with 100% STBNR, whereas application of 50% STBNR + GM recorded significantly lower values (Table 1). Our findings indicate that the threshold SPAD value for N application varies within the species. Linear regression analysis showed that chlorophyll accumulation at heading was more strongly related to SPAD values and finally with N accumulation and grain filling because of adequate availability of nutrients in combination treatments and increased N remobilization and more greenness of leaf as reported by Iqbal *et al*. (2021). Swain and Sandip (2010) also reported an increase in SPAD values with an increase in nitrogen levels from 0 to 150 kg ha^{-1}. At physiological maturity, a different trend was noted *i.e*., integrated use of 75% STBNR + GM or 50% STBNR + GM recorded similar SPAD values, whereas sole application of inorganic fertilizers *i.e*., 100% STBNR recorded the least value for the reason stated earlier. Irrespective of stages, rice cv. 'Manaswinee' had higher SPAD values than cv. 'Hasanta' in 2019, but the difference was not significant in 2018. Rice cv. 'Manaswinee' recorded the mean SPAD values of 39.6, 41.2 and 36.1 at active tillering, heading and physiological maturity stages as against 37.9, 39.1 and 34.2, respectively, in the case of rice cv. 'Hasanta'. Varietal differences in SPAD value have been also reported by Swain and Sandip (2010) and they observed higher SPAD values in rice cv. 'Swarna' than cv. 'Lalat' throughout the crop growth stages. Hassan *et al*. (2009) reported significant variations among rice varieties of Bangladesh for their SPAD values.

Total chlorophyll (C_{a+b}) content

The mean total chlorophyll content was the maximum of 3.59 mg g^{-1} fresh weight (FW) at the heading stage followed by 2.80 mg g^{-1} FW at the active tillering stage and 1.89 mg g^{-1} FW at physiological maturity (Table 2). This might be due to the fact that the allocation ratio of leaf N to chlorophyll is influenced by N supplementation conditions at the panicle initiation stage by the 3rd split application of N as chlorophyll synthesis and protein are also associated with leaf N concentration and leaf chlorophyll content is directly related to N uptake (Iqbal *et al*., 2021). The study is in close proximity to the findings of Xiong *et al*. (2015). The low chlorophyll content at physiological maturity was due to the aging of plants. Swain and Sandip (2010) reported a continuous decrease in chlorophyll content in rice with advancement in time even after the application of nitrogenous fertilizer.

Table 1. *Effect of nutrient management and variety on SPAD value of rice at different growth stages*

Treatment	Active tillering			Heading			Physiological maturity		
	2018	2019	Pooled mean	2018	2019	Pooled mean	2018	2019	Pooled mean
Nutrient management									
100% STBNR	39.9	39.7	39.8	40.6	40.5	40.5	33.7	32.2	32.9
75% STBNR+GM	40.2	41.1	40.6	41.9	42.2	42.1	39.1	37.3	38.2
50% STBNR+GM	36.1	35.6	35.9	38.8	36.9	37.9	34.6	34.2	34.4
SE(m)±	0.76	0.84	0.57	0.74	1.08	0.65	1.36	1.03	0.85
CD (0.05)	2.3	2.5	1.6	2.2	3.3	1.9	4.1	3.1	2.5
Rice variety									
Manaswinee	39.3	40.0	39.6	41.1	41.2	41.2	36.4	35.9	36.1
Hasanta	38.1	37.7	37.9	39.7	38.5	39.1	35.3	33.2	34.2
SE(m)±	0.62	0.68	0.46	0.60	0.88	0.53	1.11	0.84	0.70
CD (0.05)	NS	2.1	1.3	NS	2.7	1.5	NS	2.5	NS
Mean	38.7	38.8	38.8	40.4	39.9	40.1	35.8	34.6	35.2

Table 2. *Effect of nutrient management and variety on total chlorophyll (mg g^{-1} FW) of rice at different growth stages*

Treatment	Active tillering			Heading			Physiological maturity		
	2018	2019	Pooled mean	2018	2019	Pooled mean	2018	2019	Pooled mean
Nutrient management									
100% STBNR	39.9	39.7	39.8	40.6	40.5	40.5	33.7	32.2	32.9
75% STBNR+GM	40.2	41.1	40.6	41.9	42.2	42.1	39.1	37.3	38.2
50% STBNR+GM	36.1	35.6	35.9	38.8	36.9	37.9	34.6	34.2	34.4
SE(m)±	0.092	0.135	0.082	0.122	0.185	0.111	0.176	0.137	0.112
CD (0.05)	0.28	0.41	0.24	0.37	0.56	0.32	NS	0.41	0.32
Rice variety									
Manaswinee	2.89	3.01	2.95	3.73	3.78	3.75	2.00	2.06	2.03
Hasanta	2.72	2.57	2.64	3.55	3.32	3.44	1.89	1.61	1.75
SE(m)±	0.075	0.110	0.067	0.100	0.151	0.091	0.144	0.112	0.091
CD (0.05)	NS	0.33	0.19	NS	0.46	0.26	NS	0.34	0.26
Mean	2.81	2.79	2.80	3.64	3.55	3.59	1.95	1.83	1.89

Application of 75% STBNR + GM recorded the maximum total chlorophyll content at the active tillering stage during both the years (3.11 and 3.24 mg g^{-1} FW), being at par with 100% STBNR, while the values were less with 50% STBNR + GM. A similar trend was noted at the heading stage. Pooled over years, 75% STBNR + GM recorded the maximum total chlorophyll content of 3.84 mg g^{-1} FW, being at par with 100% STBNR and significantly superior to 50% STBNR + GM. During 2019, rice cv. 'Manaswinee' recorded higher total chlorophyll content than cv. 'Hasanta', whereas the difference was not significant during 2018, at both the growth stages. Pooled over years, total chlorophyll content of cv. 'Manaswinee' (2.95 and 3.75 mg g^{-1} FW at active tillering and heading), was higher than cv. 'Hasanta'. Our outcomes are consistence with Iqbal *et al*. (2021) who reported that co-incorporation of manure and inorganic fertilizer improved leaf physiological traits in rice. Preferably the improvement in chlorophyll content during the grain filling period was due to quicker release of nutrients from synthetic fertilizer accompanied by a slow and steady release of nutrients from organic fertilizer across the entire growth period and these findings highlight the importance of chlorophyll a, as it is the primary photosynthetic pigment.

At physiological maturity, 75% STBNR + GM recorded the maximum total chlorophyll content and was superior to the other two nutrient management practices. Application of 75% STBNR + GM recorded the maximum total chlorophyll content of 2.24 mg g^{-1} FW. During 2019, rice cv. 'Manaswinee' recorded significantly higher total chlorophyll content than cv. 'Hasanta', whereas the differences were not significant during 2018. Pooled over the years, 'Manaswinee' recorded total chlorophyll content of 2.03 mg g^{-1} FW and proved significantly superior to cv 'Hasanta'. Hassan *et al*. (2009) reported significant variations among the rice varieties, Badiruzzaman, Golapi, Laichi, Banshfulchikon, Malanchi, Barud, Kamargachikon and Tulshimala of Bangladesh for their chlorophyll a, chlorophyll b and total chlorophyll content.

Chlorophyll a and b content

The nutrient management practices and rice varieties influenced the chlorophyll a and b content of rice at different stages (Tables 3 and 4). The mean chlorophyll a and b content was the maximum at heading followed by active tillering and physiological maturity stage. This might be due to fact that the allocation ratio of leaf N to chlorophyll is affected by N supplementation conditions as stated earlier. Integrated use of 75% STBNR + GM also recorded the maximum content of chlorophyll a during all three developmental stages and is consistent with Iqbal *et al*. (2021), who reported that co-incorporation of manure and inorganic fertilizer improved leaf physiological traits in rice and were directly related to N uptake. Whereas, chlorophyll b was the maximum when the inorganic fertilizer was applied alone (100% STBNR) and was at par with integrated use of green manure along with inorganic

Table 3. *Effect of nutrient management and variety on chlorophyll a (mg g^{-1} FW) content of rice at different growth stages*

Treatment	Active tillering			Heading			Physiological maturity		
	2018	2019	Pooled mean	2018	2019	Pooled mean	2018	2019	Pooled mean
Nutrient management									
100% STBNR	2.13	1.94	2.04	2.43	2.35	2.39	1.16	0.99	1.07
75% STBNR+GM	2.23	2.32	2.27	2.52	2.51	2.52	1.58	1.43	1.51
50% STBNR+GM	1.58	1.57	1.57	2.05	2.10	2.08	1.19	1.26	1.23
SE(m)±	0.094	0.099	0.068	0.105	0.137	0.087	0.073	0.089	0.058
CD (0.05)	0.28	0.30	0.20	0.32	NS	0.25	0.22	0.27	0.17
Rice variety									
Manaswinee	2.02	2.07	2.04	2.43	2.51	2.47	1.35	1.42	1.38
Hasanta	1.94	1.82	1.88	2.23	2.13	2.18	1.27	1.04	1.16
SE(m)±	0.077	0.081	0.056	0.086	0.112	0.07	0.060	0.073	0.047
CD (0.05)	NS	0.24	0.16	NS	0.34	0.204	NS	0.22	0.14
Mean	1.98	1.94	1.96	2.33	2.32	2.33	1.31	1.23	1.27

Table 4. *Effect of nutrient management and variety on chlorophyll b (mg g^{-1} FW) of rice at different growth stages*

Treatment	Active tillering			Heading			Physiological maturity		
	2018	2019	Pooled mean	2018	2019	Pooled mean	2018	2019	Pooled mean
Nutrient management									
100% STBNR	0.91	0.97	0.94	1.33	1.43	1.38	0.55	0.67	0.61
75% STBNR+GM	0.88	0.93	0.91	1.34	1.31	1.32	0.73	0.73	0.73
50% STBNR+GM	0.69	0.64	0.66	1.25	0.94	1.10	0.65	0.43	0.54
SE(m)±	0.071	0.079	0.053	0.098	0.133	0.083	0.017	0.078	0.070
CD (0.05)	NS	0.24	0.15	NS	0.40	0.24	NS	0.23	NS
Rice variety									
Manaswinee	0.87	0.95	0.91	1.30	1.27	1.28	0.67	0.65	0.66
Hasanta	0.78	0.75	0.77	1.32	1.19	1.25	0.62	0.57	0.59
SE(m)±	0.058	0.064	0.043	0.080	0.109	0.067	0.095	0.064	0.057
CD (0.05)	NS	0.19	0.13	NS	NS	NS	NS	NS	NS
Mean	0.83	0.85	0.84	1.31	1.23	1.27	0.64	0.61	0.63

fertilizer (75% STBNR + GM) during active tillering and heading stages. Numerically higher values in 100% STBNR might be due to readily available plant nitrogen from inorganic sources during the initial growth stages. At physiological maturity, application of 75% STBNR + GM recorded the maximum chlorophyll b content and 100% STBNR recorded a statistically similar value, whereas 50% STBNR + green manuring recorded a significantly lower value.

Rice cv. 'Manaswinee' recorded higher chlorophyll a and b content at the active tillering stage during both the years, but the difference between the two cultivars was significant only during 2019 and was superior to rice cv. 'Hasanta'. Hassan *et al*. (2009) and Vanisri *et al*. (2017) have also reported varietal differences in chlorophyll a and b content of rice for their genetic makeup.

Relationship between SPAD values and chlorophyll content

The relationship between SPAD meter readings and chlorophyll content was linear (Table 5 and Fig. 1). The value of r indicated a good fit between the two parameters. The r values in linear models ranged

Table 5. *Linear models for each growth stage of two rice cultivars*

Variety	Growth stage	Model	No of samples	SPAD value	r
Manaswinee	Active tillering	y = 0.152x - 3.069	12	32.1-45.0	0.813
	Heading	y = 0.119x - 1.179	12	35.8 - 46.9	0.785
	Physiological maturity	y = 0.110x - 1.953	12	29.7 - 43.6	0.764
Hasanta	Active tillering	y = 0.155x - 3.261	12	31.7 - 41.3	0.711
	Heading	y = 0.178x - 3.531	12	31.6 - 44.8	0.862
	Physiological maturity	y = 0.120x - 2.379	12	30.6 - 42.6	0.811

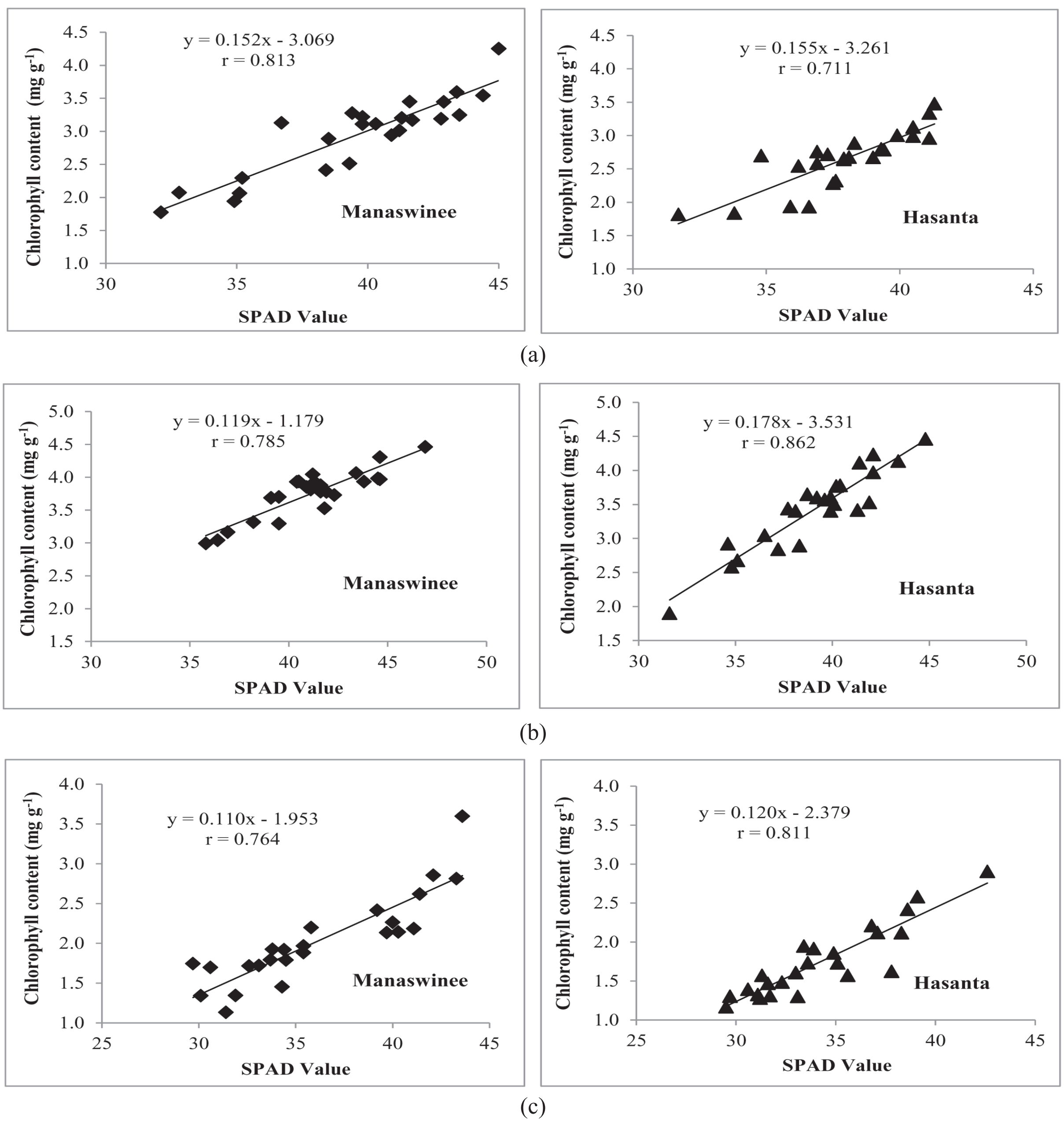

Fig. 1. *Relationship between SPAD value and leaf blade total chlorophyll content in rice cv. 'Manaswinee' and 'Hasanta' at (a) active tillering stage, (b) heading and (c) physiological maturity stages*

from 0.711 to 0.862. A similar type of relationship for rice cv. 'Nipponbare' and 'Kinuhikari' grown under varying fertilizer application schedules was reported by Wakiyama (2016). Monje and Bugbee (1992) and Hassan *et al*. (2009) also reported linear relationship between SPAD values and chlorophyll content and the chlorophyll content is linked strongly to leaf nitrogen content (Bojovic and Marcovic, 2009).

The linear model was the best fit and explained the relationship between chlorophyll content and SPAD readings. Application of 75% Soil Test Based Nitrogen Recommendation (75 kg ha^{-1}) + green manuring of *dhaincha* in rice recorded the maximum content of chlorophyll a, chlorophyll b, total chlorophyll and SPAD values. The two cultivars differed in maturity duration and cv. 'Manaswinee' had higher values of these four parameters than cv. 'Hasanta'.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors. All the authors were equally and actively involved in this article. Stuti Debapriya Behera was involved in the field work and laboratory work. L.M. Garnayak and B. Behera have designed the experiment and helped in laboratory work. R.K. Panda helped in the preparation of the manuscript.

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