

SPAD GREENNESS TO ESTIMATE GENOTYPIC VARIATION IN FLAG LEAF CHLOROPHYLL IN SPRING WHEAT UNDER MEDITERRANEAN CONDITIONS

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ABSTRACT

Leaf chlorophyll (Chl) is emphasized as an indicator for photosynthesis in wheat (*Triticum aestivum* L.). SPAD greenness meters are used to predict extractable Chl, but few studies have evaluated relationships between flag leaf greenness and Chl among wheat genotypes. Sixteen spring wheat genotypes with similar development patterns were studied in eight environments (2 years, 2 irrigation treatments and 2 sowing times) to investigate the precision of the SPAD-502 meter to predict Chl content/concentration. Relationships of Chl with SPAD greenness generally best fit linear and quadratic models. Relationships of SPAD greenness with Chl concentration were weak and inconsistent. Strongest associations were observed with Chl_a content (highest $R^2 = 0.71$ under late sowing), whereas associations with Chl_b content were weak (highest $R^2 = 0.46$) or insignificant. Relationships between SPAD greenness and total Chl content ranged from low ($R^2 = 0.24$, $p = 0.054$) to moderate ($R^2 = 0.64$, $p < 0.001$) under rainfed conventional-sowing and late-sowing conditions in the second year, respectively. SPAD greenness can be used to diagnose spring wheat genotypes differing in flag leaf Chl_a and total Chl content under warm Mediterranean conditions, but may not always applicable in cooler Mediterranean conditions, where genetic variability, especially in Chl_b, was not expressed adequately.

Key words: Chlorophyll meter, Chlorophyll content, Chlorophyll concentration, Flag leaf, Spring wheat, drought

INTRODUCTION

Chlorophyll *a* and chlorophyll *b* are essential pigments of the plant photosystems (Richardson et al., 2002). In wheat (*Triticum aestivum* L.), chlorophyll content is positively correlated with photosynthetic rate (Evans, 1983). Leaf chlorophyll (measured by extraction and quantification of pigment content or with optical chlorophyll meters) is increasingly emphasized as an indicator for photosynthetic capacity and its stability during senescence (the 'stay green' phenotype) under both stress and non-stress conditions, and therefore for yield in wheat (Reynolds et al., 1994; Araus et al., 2008; Parry et al., 2011; Xiao et al., 2012).

Traditional extraction-based methods of measuring chlorophyll *a* (Chl_a), chlorophyll *b* (Chl_b) and total chlorophyll (Chl_{a+b}) in leaves are destructive and time consuming (Richardson et al., 2002). Therefore, handheld optical chlorophyll meters, such as SPAD (soil plant analysis development) meters, based on the absorbance, reflectance and/or transmittance of radiation by the intact

leaf, have been developed. SPAD chlorophyll meters determine the relative amount of total leaf chlorophyll in intact leaves and produce values that express relative chlorophyll content (SPAD greenness), but not of absolute chlorophyll amount per unit area (content) nor per mass (concentration) of leaf tissue. Taking SPAD readings is easy, rapid and non-destructive; therefore, use of SPAD greenness meters has increased dramatically in recent years.

SPAD readings have been used to predict chlorophyll content in a large number of crop species, such as corn (Markwell et al., 1995), sorghum and pigeonpea (Yamamoto et al., 2002), soybean (Fritschi and Ray, 2007), wheat and potato (Uddling et al., 2007) and basil (Ruiz-Espinoza et al., 2010). A number of studies, restricted to one or a small number of genotypes, have reported a curvilinear relationship between extractable chlorophyll and SPAD values in wheat (Monje and Bugbee, 1992; Castelli et al., 1996; Richardson et al., 2002; Uddling et al., 2007). However, Castelli et al.

(1996) reported that monocots (wheat and maize) and dicots (tobacco and soybean) show distinct patterns in terms of the relationship between non-destructive readings and analytical results obtained by solvent extraction.

Indeed, the SPAD value depends not only on extractable chlorophyll concentrations, but also on other aspects of leaf optics, which may be affected by a variety of environmental and biological factors (Palta, 1990; Hoel and Solhaug, 1998; Markwell et al., 1995; Martínez and Guiamet, 2004). The non-homogeneous distribution of chlorophyll molecules within the leaf and/or differential scattering and reflection of radiation can alter the relationship between extractable chlorophyll and SPAD greenness (Monje and Bugbee, 1992; Markwell et al., 1995; Uddling et al., 2007).

The lack of a consistent relationship between chlorophyll estimated with an extraction method and by the greenness meter for different genotypes may limit the potential use of SPAD as a complementary selection trait. The relationship between chlorophyll content/concentration determined with an extraction method and by a SPAD chlorophyll meter have been verified depending on sowing time and irrigation treatment among wheat genotypes

In synthetic hexaploid wheat the variation in SPAD greenness is not completely explained by changes in chlorophyll concentration (Del Blanco et al., 2000). Thus it is necessary to verify the relationship between extractable chlorophyll and SPAD greenness in different genotypes under different environmental conditions to enable breeders to select more easily for genotypes differing in chlorophyll content or concentration, when the source capacity is to be improved. This objective is particularly important if not only chlorophyll content/concentration, but also chlorophyll components (Chl_a , Chl_b , Chl_{a+b} and ratio of Chl_a to Chl_b [$\text{Chl}_{a/b}$]), are to be considered.

The objective of this study was to examine if SPAD greenness could be used as a rapid screening method to predict genotypic variation in wheat for extractable leaf chlorophyll content/concentration (Chl_a , Chl_b and Chl_{a+b}) and $\text{Chl}_{a/b}$, and to assess the stability of these relationships in different environments. To eliminate possible phenotypic differences in chlorophyll content, genotypes with similar or known development patterns were used.

MATERIALS AND METHODS

Plant materials and cultural practices

Field experiments were conducted during the 2011/2012 and 2012/2013 growing seasons at the experimental field of the Faculty of Agriculture, University of Cukurova, Balcalı, Adana (37°00' N, 35°21' E, 29 m above sea level), Turkey. The soil type is a fine loamy, montmorillonitic typic xerofluvent, low in organic matter and slightly alkaline (pH 7.1–7.6).

Sixteen unrelated spring bread wheat (*Triticum aestivum*) genotypes with a similar developmental pattern

selected for diversity in leaf traits were used. The widely adapted genotypes comprised commercial cultivars from Turkey (Adana-99, Balatlıla, Cemre, Cumakalesi, Genç-99, Karacadağ-98, Meta-2002 and Özkan), ICARDA (Cham-6 and Siete Cerros), Israel (Dariel and Galil), Italy (Colfiorito), Spain (Mané-Nick) and Pakistan (Inqilab-91 and V-3010).

In each growing season (2011/2012 and 2012/2013) in addition to conventional (optimal) sowing time (CS) and late sowing (LS) times were used to expose the cultivars to lower and higher temperatures. Each replication of the sowing times (main plots) was nested on the same field with two irrigation treatments (rainfed, RF; and irrigated, IR) as subplots and genotypes as mini-plots in a split-split plot randomised design of four replications.

Except for sowing time and irrigation treatment, the growing conditions were maintained as similar as possible through fertilization and pest control. The first-year sowings were on 25 November 2011 and 8 March 2012, and the second-year sowings were on 29 November 2012 and 8 March 2013, for CS and LS, respectively. Phosphorus ($40 \text{ kg ha}^{-1} \text{ P}_2\text{O}_5$) was applied before sowing in the form of triple super phosphate. Nitrogen was applied as ammonium nitrate in three split doses ($40 + 80 + 40 \text{ kg N ha}^{-1}$) at Zadok's (Zadoks et al., 1974) growth stages (ZGS) 00, 20 and 30. The sowing density was $450 \text{ viable seeds m}^{-2}$. Plots consisted of 8 rows, each of which was 6.0 m long with a row spacing of 0.15 m.

The irrigation applications were initiated on 6 April 2012 with CS and 12 May 2012 with LS in the first year. The respective dates in the second year were 19 March 2013 and 1 May 2013. Water supplies between irrigated and rainfed conditions in first year were similar. First-year water supply (rainfall + irrigation) until sampling time was 671 and 682 mm for RF and IR, respectively, and 255 and 255 mm in CS and LS, respectively. The respective water supply in the second year was 459, 501, 169 and 199 mm.

Measurement of SPAD greenness and extractable leaf chlorophyll

SPAD greenness and extractable leaf chlorophyll were measured on flag leaves. Sampling was performed before the onset of leaf senescence at the end of anthesis (2012 CS), at the beginning of anthesis (2012 LS and 2013 CS), and of heading (2013 LS) 1 week (in LS) or 2 weeks (in CS) after initiation of the irrigation regimes. Five healthy flag leaf blades were sampled at random from the central rows of each plot and transported in cool isolation boxes to the laboratory. The leaves were kept in a refrigerator before processing. Samples from one replication were processed within approximately 2 h from collection in the field.

In the laboratory, at first SPAD chlorophyll was measured on sampled fresh leaves using a hand-held SPAD-502 Chlorophyll Meter (Minolta Camera Co., Osaka, Japan), which generates a value predictive of chlorophyll concentration (Minolta Camera Co. 1989).

The SPAD-502 meter measures transmittance at 650 nm (red light is strongly absorbed by chlorophyll) and 940 nm (near-infrared light is a 'reference wavelength' that is used to adjust for differences in leaf structure) of radiation through the leaf, and calculates a relative SPAD meter value that is termed SPAD greenness. The average of three readings from the center of each leaf blade was used as a SPAD greenness score per leaf (5×3 readings per plot). The midpoint of the leaf is the best position in winter wheat plants on which to take chlorophyll meter readings (Hoel and Solhaug, 1998). The adaxial side of the leaves was always placed toward the emitting window of the instrument and major veins were avoided.

Following each SPAD reading, the amount of chlorophylls (Chl_a and Chl_b) was determined in two leaf disks of 8 mm diameter taken from an identical position of the same leaves. After the disks were weighed, pigments were thoroughly extracted in 80% (v/v) acetone using a glass mortar, and the homogenate was then filtered. Absorbance was measured at the relevant wavelength with a spectrophotometer (Shimadzu, UV-1208, Kyoto, Japan). Chlorophyll content/concentration was calculated on a leaf area/fresh weight basis using the equations of Porra et al. (1989). These equations are an improvement over the widely used equations of Arnon (1949).

Data analysis

Although irrigation was initiated a short time prior to sampling for SPAD greenness and extractable leaf chlorophyll measurement, and differences in water supply between irrigation treatments were small, analysis of variance (ANOVA) of the traits was carried out first on the basis of the experimental design used (a randomized split-split plot) with sowing date as the main factor, irrigation as a sub-plot, and genotype as a mini-plot with four replications. Subsequently, analyses of variance for each growing condition were performed separately to obtain a clearer picture for flag leaf traits of the genotypes. Least significant difference (LSD) values were calculated at the 0.05 level of probability. A regression was used when appropriate. The SPSS (1999) package was used for all analyses.

RESULTS AND DISCUSSION

Development of genotypes

As a result of the small pre-anthesis differences in water supply between the irrigation treatments, no effect of irrigation (and its interaction with genotype) on development of the wheat genotypes was observed (data not shown). Under CS, anthesis (ZGS 60, averaged over genotypes) occurred 138 and 123 days after sowing in 2012 and 2013, respectively. The respective values under LS were 69 and 66 days. When the relative thermal duration (determined as cumulative growing degree-days from sowing to sampling date, base temperature 0 °C) was considered, acceleration of anthesis by LS was in a narrow range from 1376 to 1149 °C days (16%) and from 1439 to 1151 °C days (20%) in 2012 and 2013, respectively. These

results indicated the overwhelming effect of growing temperature on development.

Despite the obvious genotypic trends, the development of most genotypes was very similar. Only the local Turkish cultivar Cumakalesi was consistently the latest to flower and Karacadağ-98 tended to be the earliest to anthesis. Development of the remaining genotypes was tightly clustered in the first year, especially with LS. Genotypic differences in the second year (particularly with LS) were more pronounced, but ZGS values changed only within a narrow range of development such as from heading (ZGS 57) to completion of anthesis (ZGS 67).

It is well known that the growth stage also affects chlorophyll pigments, and therefore SPAD readings (Lopes and Reynolds, 2012). The similar (or known) developmental pattern of each genotype in the present study allowed valid comparison of SPAD greenness and extractable chlorophylls among the genotypes.

Flag leaf SPAD greenness and extractable chlorophylls

The results of ANOVA over years showed consistently highly significant genotypic effects, whereas other effects were inconsistent (sowing time), negligible (irrigation) or extremely variable (interactions) (data not shown). To obtain a clearer picture, the maximum, minimum and mean values for flag leaf traits of each genotype under each conditions (two sowing times and two irrigation treatments in the 2 study years) and LSD values calculated based on the results of ANOVA for individual conditions (hereafter termed 'environments') are presented separately in Table 1. With a few exceptions for chlorophyll concentration and content under certain conditions, genotypic differences for SPAD greenness and extractable chlorophylls were significant (Table 1) and more or less consistent (data not shown). Here emphasis is placed more on the extent of genotypic changes rather than on genotypic differences *per se*.

Senescing leaves in wheat suffer the loss of chlorophylls, especially Chl_a (Reynolds et al., 2000; Zhang et al., 2006). Thus the leaf age affects chlorophyll pigments, and therefore SPAD readings (Lopes and Reynolds, 2012). Except only one cultivar, the similar developmental pattern and similar leaf age of the genotypes in the present study allowed valid comparison of SPAD greenness and extractable chlorophylls among the genotypes.

SPAD greenness

Although highly significant differences among genotypes were observed in all environments, SPAD greenness values changed within rather narrow ranges (mean change 23%) (Table 1). The highest change of 40% occurred from 38.5 to 53.9 in the first year in the LS rainfed environment (2012 LSRF), and the lowest changes (17%) from 44.6 to 52.0 and 44.5 to 52.2 were observed in 2013 in the CS rainfed and CS irrigated environments (2013 CSRF and CSIR), respectively. Changes in the other environments were more or less similar.

Table 1. Maximum, minimum and mean flag leaf SPAD greenness, chlorophyll *a* (Chl_a) and chlorophyll *b* (Chl_b) concentration (mg g⁻¹), and Chl_{a/b} ratio of 16 spring wheat genotypes grown under two sowing times (CS, conventional; LS, late) and irrigation treatments (RF, rainfed; IR, irrigated) during the 2011/2012 (2012) and 2012/2013 (2013) crop growing seasons at Adana, Turkey.

Values	2012					2013				
	SPAD	Chl _a	Chl _b	Chl _{a+b}	Chl a/b	SPAD	Chl _a	Chl _b	Chl _{a+b}	Chl a/b
			CSRf					CSRf		
Maximum	51.6	2.53	0.75	3.28	3.70	52.0	2.36	0.67	3.01	3.95
Minimum	42.6	1.85	0.55	2.39	2.90	44.6	1.95	0.53	2.48	3.35
Mean	45.8	2.16	0.67	2.82	3.26	46.5	2.13	0.59	2.72	3.66
LSD _{0.05}	4.04	0.255	0.101	0.337	0.343	3.41	ns	ns	ns	ns
			CSIR					CSIR		
Maximum	47.7	2.20	0.69	2.85	3.90	52.2	2.25	0.68	2.93	3.75
Minimum	39.1	1.65	0.45	2.10	3.08	44.5	1.87	0.51	2.38	3.30
Mean	45.8	2.03	0.59	2.62	3.48	47.0	2.09	0.60	2.69	3.51
LSD _{0.05}	2.90	ns	0.090	0.393	0.398	2.83	ns	ns	ns	ns
			LSRF					LSRF		
Maximum	53.9	2.54	0.85	3.39	3.80	56.2	2.32	0.81	3.06	3.30
Minimum	38.5	1.78	0.54	2.42	2.35	46.4	1.67	0.55	2.26	2.68
Mean	45.8	2.12	0.70	2.81	3.10	50.3	1.95	0.66	2.62	2.98
LSD _{0.05}	2.99	0.247	0.110	0.315	0.448	3.73	0.331	0.127	0.439	0.355
			LSIR					LSIR		
Maximum	51.8	2.66	1.04	3.63	3.65	53.8	1.97	0.62	2.59	3.38
Minimum	42.2	1.95	0.57	2.55	2.53	44.4	1.50	0.49	2.01	2.95
Mean	46.9	2.28	0.74	3.02	3.13	48.7	1.79	0.58	2.37	3.16
LSD _{0.05}	3.01	0.346	0.135	0.446	0.439	4.97	0.243	ns	ns	ns

ns, not significant at the 0.05 probability level.

SPAD values measured in this study were comparable to those observed in previous studies of spring wheat grown under irrigated and non-limited nitrogen conditions (Del Blanco et al., 2000; Gutiérrez-Rodríguez et al., 2000; Babar et al., 2006) and were near the upper limit of calibration studies in wheat (Monje and Bugbee, 1992; Castelli et al., 1996; Richardson et al., 2002; Uddling et al., 2007). Despite the narrow range within higher values of SPAD, significant and consistent genotypic differences were found. This further promised analysis of relationships SPAD with extracted chlorophylls.

Extractable chlorophylls

Except for occasional cases, such as for both pigments in 2013 CSRf and CSIR, for Chl_a in 2012 CSIR, and for Chl_b in 2013 LSIR, genotypic differences for extractable concentration of flag leaf Chl_a, Chl_b, and Chl_{a+b} were generally significant (Table 1). Differences were not evident for both pigments (Chl_a, Chl_b, and therefore Chl_{a+b}) in the 2013 CSRf and for Chl_b in the 2013 LSIR environments.

The highest genotypic range in chlorophyll content was observed in 2012 in the LSIR environment, in which Chl content ranged from 318 to 481 mg m⁻² for Chl_a, from 96 to 193 mg m⁻² for Chl_b, and from 427 to 674 mg m⁻² for Chl_{a+b} (data not shown, but can be seen in Figure 2). Genotypic differences in Chl_{a/b} were not evident in three environments (CSRf, CSIR and LSIR) in 2013. The extent of significant genotypic differences in the other environments changed in a wide range from 23% (2013 LSRf) to 62% (2012 LSRf). Changes shown in Table 1 were largely similar when the two genotypes that exhibit

early (Karacadağ-98) and late (Cumakalesi) development were not considered.

One of the most remarkable results in the present study was the trend for declining Chl_{a/b} under LS warm conditions in both years due to increased Chl_b. The ratio of Chl_a to Chl_b is known to change with environmental factors such as light. Lichtenthaler and Wellburn (1983) reported that plants exposed to high irradiance show high Chl_{a/b} ratios, whereas plants growing in environments with reduced irradiance have a lower Chl_{a/b} ratios. The variation in Chl_{a/b} ratio in the current study seem not to be due to reduced radiation, as light intensity above the canopy constantly increases during the spring months, when late-sown crops are grown. The reduction Chl_{a/b} under LS conditions may be caused by increased growing temperatures during the flag leaf expansion. For a precise interpretation, temperature and irradiance measurements at the flag leaf level in the canopy during flag leaf expansion are needed.

Relationship of SPAD greenness with chlorophyll concentration and Chl_{a/b}

The linear, quadratic and exponential (data not shown) relationships among chlorophyll concentration and Chl_{a/b} with SPAD greenness were generally similar. The linear and quadratic regression coefficients of determination (*R*²) for flag leaf chlorophyll concentration (Chl_a, Chl_b, and Chl_{a+b} per unit fresh weight) and Chl_{a/b} with SPAD greenness are illustrated in Table 2. The degree of quadratic fit was improved only in two environments, namely in 2012 LSRf and 2013 CSRf for Chl_a and Chl_b, respectively.

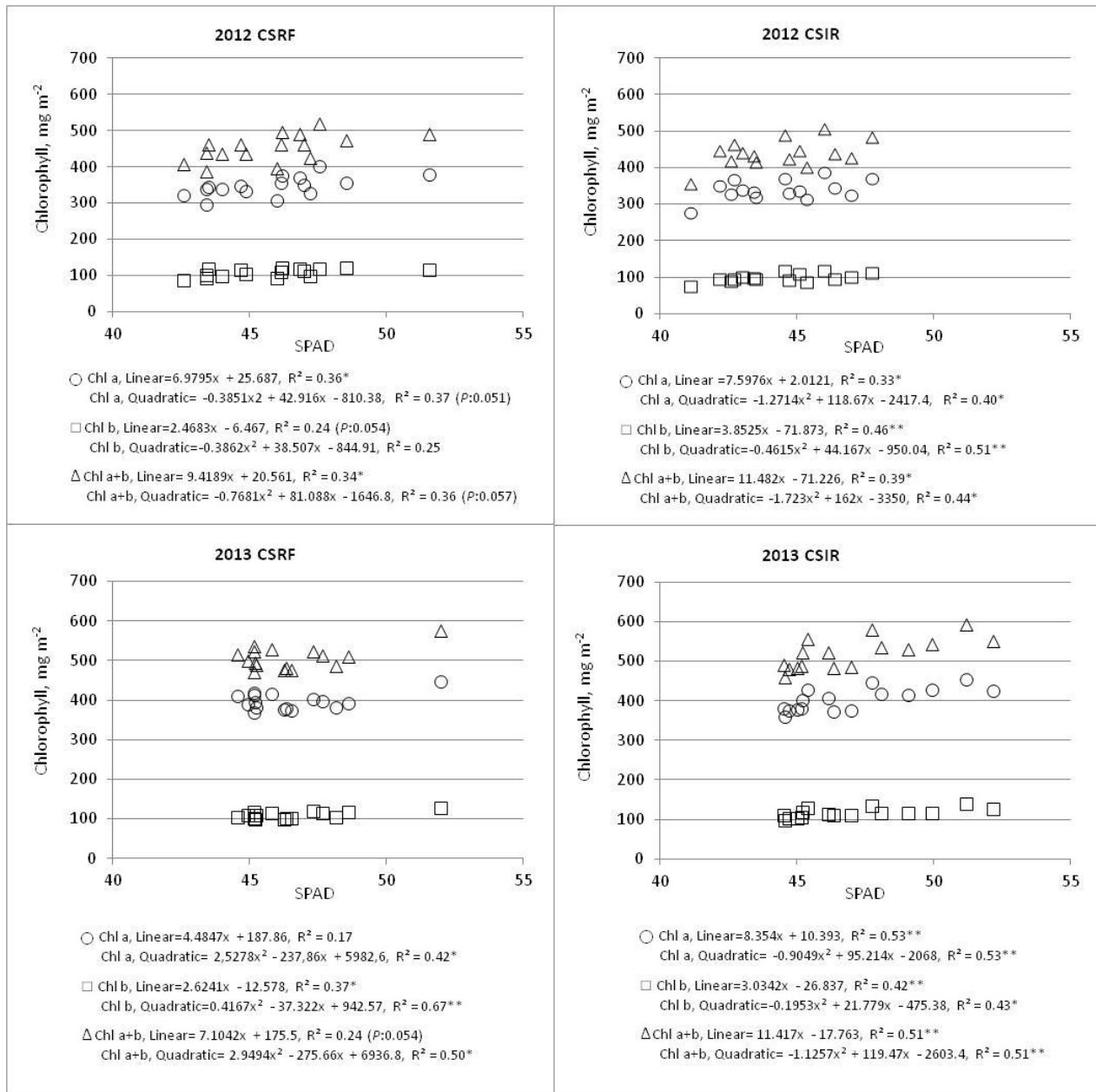


Figure 1. Relationship between flag leaf extracted chlorophyll (Chl) content and SPAD greenness for 16 spring wheat genotypes grown under a conventional sowing (CS) time and two irrigation treatments (RF, rainfed; IR, irrigated) during the 2011/2012 (2012) and 2012/2013 (2013) crop growing seasons at Adana, Turkey. *, ** significance at 0.05 and 0.01 probability levels, respectively.

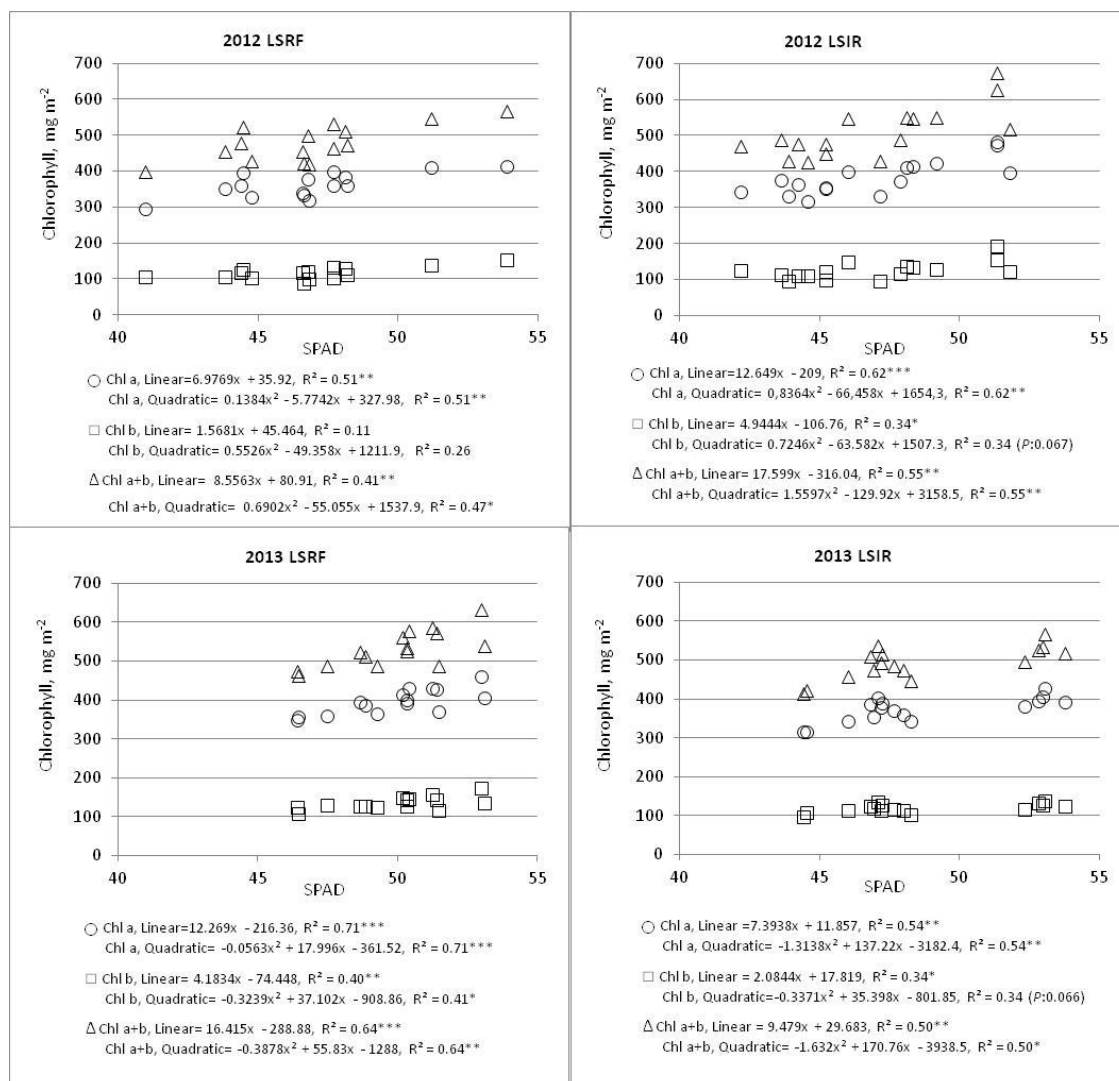


Figure 2. Relationship between flag leaf extracted chlorophyll (Chl) content and SPAD greenness for 16 spring wheat genotypes grown under a late sowing (LS) time and two irrigation treatments (RF, rainfed; IR, irrigated) during the 2011/2012 (2012) and 2012/2013 (2013) crop growing seasons at Adana, Turkey. *, **, *** significance at 0.05, 0.01 and 0.001 probability levels, respectively.

The results of this study further suggest that the usefulness of SPAD readings in typical Mediterranean wheat growing conditions is questionable and, for prediction purposes, growing conditions are an important consideration and the date (growing conditions) cannot be pooled. Complex optical properties due to factors other than the chlorophyll amount in the leaf, which change with growing environment, seem problematic for application of SPAD greenness.

CONCLUSIONS

Genotypic differences in flag leaf SPAD greenness and extractable chlorophylls among spring wheat genotypes with similar development provided the opportunity to assess the accuracy and precision of SPAD readings to estimate genotypic variation in flag leaf chlorophyll amounts independent of phenology (leaf age).

The ability of the SPAD-502 meter to estimate genotypic differences in chlorophyll content per unit flag

leaf area was satisfactory as long as the leaves developed under warm (and irrigated) conditions. It might be due to the fact that cell under warm condition perform well having more chlorophyll contents of leaf morphology. Secondly the genetic variation could be the main reason of high chlorophyll contents of some varieties performing well under Mediterranean conditions. However, the SPAD meter was less reliable for estimation of genotypic variation in leaf chlorophyll under the typical cooler wheat-growing conditions of the Mediterranean, where genetic variability, especially in Chl_b, was not expressed adequately. Applicability of the SPAD-502 meter also might be restricted when the objective is to assess the capacity of the photosynthetic antenna system, as SPAD greenness was generally a better indicator of Chl_a and Chl_{a+b} than Chl_b. This is the one of the main drawback of SPAD meter in determining the photosynthetic antenna system of plants. Therefore instrument must be updated to determine the both chlorophyll contents as well as light harvesting capacity of leaf. Further research is also

indispensable at cellular level to understand the efficiency of SPAD for putting a theoretical conclusions. In spite of these limitations, chlorophyll meters are valuable instruments if they are properly calibrated with extractable chlorophyll measurements for a given set of growing conditions.

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