

## RESEARCH PAPER

# Stay-green in spring wheat can be determined by spectral reflectance measurements (normalized difference vegetation index) independently from phenology

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## Abstract

The green area displayed by a crop is a good indicator of its photosynthetic capacity, while chlorophyll retention or 'stay-green' is regarded as a key indicator of stress adaptation. Remote-sensing methods were tested to estimate these parameters in diverse wheat genotypes under different growing conditions. Two wheat populations (a diverse set of 294 advanced lines and a recombinant inbred line population of 169 sister lines derived from the cross between Seri and Babax) were grown in Mexico under three environments: drought, heat, and heat combined with drought. In the two populations studied here, a moderate heritable expression of stay-green was found—when the normalized difference vegetation index (NDVI) at physiological maturity was estimated using the regression of NDVI over time from the mid-stages of grain-filling to physiological maturity—and for the rate of senescence during the same period. Under heat and heat combined with drought environments, stay-green calculated as NDVI at physiological maturity and the rate of senescence, showed positive and negative correlations with yield, respectively. Moreover, stay-green calculated as an estimation of NDVI at physiological maturity and the rate of senescence regressed on degree days give an independent measurement of stay-green without the confounding effect of phenology. On average, in both populations under heat and heat combined with drought environments CTg<sub>f</sub> and stay-green variables accounted for around 30% of yield variability in multiple regression analysis. It is concluded that stay-green traits may provide cumulative effects, together with other traits, to improve adaptation under stress further.

**Key words:** Chlorophyll loss, NDVI, maturity, SPAD, *Triticum aestivum*.

## Introduction

Heat and drought stress are two of the main factors which limit the productivity of staple crops worldwide. Without the CO<sub>2</sub> fertilization effect, a 0.5 °C increase in average temperature is predicted to reduce wheat yield by 0.45 ton ha<sup>-1</sup> in India, and rain-fed wheat yield by 4–7% in China by 2050 (Easterling *et al.*, 2007), two of the world's major wheat producing countries (FAO, 2009). In the context of wheat improvement for drought and heat adaptation, strategic trait-based crossing has combined complementary stress-adaptive traits in a new generation of progeny that, when compared with conventionally bred lines, showed superior performance in Mexico (Reynolds *et al.*, 2009).

However, many physiological and morphological traits are yet to be fully exploited in wheat breeding, often because suitable phenotyping methods have not been defined. In the current study, the value of the stay-green phenotype in heat- and drought stressed environments was addressed and different quantitative phenotyping methodologies were tested.

Senescence is a genetically programmed and environmentally influenced process resulting in the destruction of chlorophyll and the remobilization of nutrients to younger or reproductive parts of plants (Vijayalakshmi *et al.*, 2010). Four or five classes of delayed senescence or 'stay-green'

have been described in the literature (Thomas and Smart, 1993; Thomas and Howarth, 2000) and the stay-green phenotype has shown proven utility to improve yields under abiotic stress (Borrell *et al.*, 2000a; Verma *et al.*, 2004; Harris *et al.*, 2007; Kumari *et al.*, 2007; Vijayalakshmi *et al.*, 2010) and also under biotic stress, like spot blotch (Joshi *et al.*, 2007). Specifically, stay-green expression in sorghum has been shown to have a significant yield advantage under post-anthesis drought compared with hybrids not possessing this trait (Borrell *et al.*, 2000a). More recently, Bogard *et al.* (2011) has shown that delaying leaf senescence was associated with increased grain yield or grain protein concentration, but this depended largely on the type of environment considered. Reports of stay-green heritability have been highly variable, for example, it was not particularly high in wheat (Tao *et al.*, 2000) whereas others have shown high heritability for a gene related to stay-green expression in the same species (Silva *et al.*, 2000). In sorghum and maize, high heritability for stay-green has been reported (Subudhi *et al.*, 2000; Bekavac *et al.*, 2007). This variation is probably due to the utilization of different methods to assess stay-green. For instance, to identify the stay-green phenotype visual observations have been used, but also retention of green leaf area and rate of chlorophyll loss with SPAD have been reported to be useful (Borrell *et al.*, 2000a, b; Haussmann *et al.*, 2002; Verma *et al.*, 2004; Harris *et al.*, 2007). Harris *et al.* (2007) identified stay-green sorghum using the onset of leaf senescence, the absolute and relative rate of leaf senescence, and green leaf area and SPAD chlorophyll content at maturity. The onset of senescence was generally delayed in the stay-green genotypes (Harris *et al.*, 2007). There is evidence for an association between different measurements of stay-green, for example, genotypes with a later onset of senescence may subsequently senesce more rapidly (Bogard *et al.*, 2011). Moreover, senescence kinetics in the post-anthesis period in wheat has been described using non-linear models like the Gompertz models (Pepler *et al.*, 2005; Vijayalakshmi *et al.*, 2010).

A challenge associated with the measurement of stay-green is the lack of control of phenology where both early and late genotypes are evaluated or where information on phenology is simply not considered. Also, very often the spikes are ignored and these are known to contribute to grain yield under source limitations (Maydup *et al.*, 2010). In the current study, these difficulties were addressed in two ways, firstly by using wheat populations that were specially developed to control phenology like the Seri/Babax population (Pinto *et al.*, 2010) and a diverse set of wheat elite lines (carefully selected to obtain a minimum range of variation in phenology), obtained from CIMMYT (International Maize and Wheat Improvement Centre) nurseries (Lopes and Reynolds, 2010a); secondly, by using integrative methods to estimate stay-green expression in the entire wheat plot (i.e. with a GreenSeeker spectral sensor which measures normalized difference vegetation index, commonly known as NDVI). The specific objectives of the study were: (i) to show that NDVI can be used to determine the rate

and pattern of senescence from the crop canopy; (ii) to test whether regression analysis of NDVI decay during grain-filling can be used to estimate a heritable value of stay-green expression at maturity, independently from the confounding effects of phenology; (iii) to determine how heat and drought stress interact with stay-green expression; and (iv) to discuss how selection for stay-green may be applied in adapting wheat to climate change.

## Materials and methods

### Plant material and field trials

Two populations were used in this study: population 1 consisted of a group of 294 wheat elite lines, obtained from CIMMYT (International Maize and Wheat Improvement Centre) nurseries (26th, 27th and 28th ESWYT, Elite Spring Wheat Yield Trial, 1–16th SAWYT, Semiarid Wheat Yield Trial, and from the HTWYT, High Temperature Wheat Yield Trial) released in the past 30 years and these include several synthetically derived wheat material. Population 2 consisted of a recombinant inbred line (RIL) population of 169 sister lines derived from a reciprocal cross between the related elite lines: semi-dwarf spring wheat variety Seri M82 from the ‘Veery’ cross (KVZ/BUHO//KAL/BB) and a fixed line (Babax) derived from the ‘Babax’ cross (BOW/NAC//VEE/3/BJY/COC). All trials were sown in two-replicate alpha-lattice designs in the Yaqui Valley, Mexico at CIMMYT’s Experimental Station, Norman E. Borlaug (CENEB) near Ciudad Obregon located in north-western Mexico (27°25′ N 109°54′ W, 38 m above sea level). The site is a high radiation, irrigated environment (Table 1). The soil is a Typic Calciorthid, low in organic matter (0.76%) and slightly alkaline (pH 7.7) with a plant-available water-holding capacity of about 200 mm. Soil analyses conducted previously at various profiles indicated that there were no problems associated with mineral deficiencies or toxicities or with salinity problems (Olivares-Villegas *et al.*, 2007). Four field trials per population were sown in the 2009–2010 cycle: one control environment with full irrigation with more than 500 mm of water applied; one under terminal drought (total crop water supply was

**Table 1.** Growing conditions and yields of the trials performed during this study

Emergence date, days from sowing to heading (DH), rainfall, number of irrigations, amount of water applied per irrigation, Max/min temperature (Temp), radiation (Rad), max/min relative humidity (RH), total available water at 0–120 cm of soil depth during grain-filling and yields are shown for populations 1 and 2 (POP 1 and POP 2) grown under drought, heat, and heat plus drought (H+D) conditions. See Supplementary Table S1 at JXB online for monthly measurements.

	Drought	Heat	H+D
Emergence date	05/12/2009	01/03/2010	01/03/2010
DH: POP 1(d)	73.2	53.2	52.4
DH: POP 2 (d)	71.4	53.2	52.4
Rainfall (mm)	31.6	31.6	31.6
Number of irrigations	2	6	4
Irrigation (mm)	70	100	100
Temp (max/min °C)	27.4/9.4	28.3/10.8	28.3/10.8
Rad (MJ m <sup>-2</sup> d <sup>-1</sup> )	19.0	23.3	23.3
RH (max/min %)	88.2/28.7	85.9/25.2	85.9/25.2
Total H <sub>2</sub> O: 0–120 cm (mm)	<200	>600	<450
Yield: POP 1(g m <sup>-2</sup> )	370	400	340
Yield: POP 2 (g m <sup>-2</sup> )	386	351	267

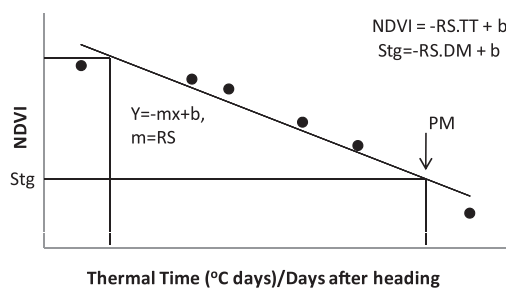
less than 200 mm); one under high environmental temperatures due to a delayed planting date, but irrigated throughout the crop cycle (total crop water supply >600 mm); another under high environmental temperatures due to a delayed planting date, but with reduced irrigation leading to mild pre- and post-anthesis drought (<450 mm) (for details see Table 1). Appropriate fertilization, weed, disease, and pest control were implemented to minimize other yield limitations. Plots comprised one 80 cm raised bed spaced at 60 cm between centres and comprising two rows per bed.

#### Field trait measurements and calculations

Grain yield (machine harvested) was determined using standard protocols (Sayre *et al.*, 1997). Days to heading (DH) was recorded as the number of days for more than 50% of plants to exhibit heads out (Zadocks stage 59; Zadocks *et al.*, 1974), and days to physiological maturity (PM) was recorded when 50% of the spikes in a plot showed a total loss of green colour (Zadocks stage 89; Zadocks *et al.*, 1974). Canopy temperature was measured at the mid-grain-filling stage (CTgf) using a portable infrared thermometer (Mikron M90 Series, Mikron Infrared Instrument Co., Inc., Oakland, NJ, USA) and chlorophyll content at anthesis was measured with a SPAD-502 Minolta (Spectrum Technologies Inc., Plainfield, IL, USA) in three flag leaves per plot. CTgf was measured between 13.00–14.00 h of fine windless and cloudless days. SPAD meters use red and near infra-red emitting diodes which pass light through the leaf. Chlorophyll absorbance is measured at 650 nm and wavelength peaking at 940 nm was used to measure non-chlorophyll absorbance (cell walls, etc). Normalized difference vegetative index (NDVI) is calculated using measurements taken at ~660 nm and ~770 nm  $[(R770-R660)/(R770+R660)]$ , ([http://www.ntechindustries.com/lit/gS/GS\\_Vegetation\\_Indices.pdf](http://www.ntechindustries.com/lit/gS/GS_Vegetation_Indices.pdf)). All NDVI measurements were taken with a GreenSeeker sensor (Optical Sensor Unit, 2002 Ntech Industries, Inc., Ukiah, CA, USA). The instrument records the reflectance in one bed per plot at speeds of 10–20 times plot<sup>-1</sup>; the distance between the GreenSeeker and the plot was kept constant at around 50 cm, measuring only one row of the plot to avoid pointing the soil at late grain-filling stages. NDVI measurements were taken approximately once a week during grain-filling (starting in the mid-stages of grain-filling and ending when all plots attained physiological maturity). Calculation of stay-green was obtained by two different methods: (i) estimation of NDVI at physiological maturity; and (ii) and the rate of senescence (RS). For the estimation of NDVI at physiological maturity, the regression equation obtained from the NDVI decay during grain-filling against days after heading was used by introducing days to physiological maturity in the equation and calculating the corresponding NDVI (Fig. 1). This calculation was based on Harris *et al.* (2007) observations of highly significant correlations between predicted SPAD at maturity and SPAD taken at maturity as a measurement of stay-green. Moreover, higher NDVI values estimated at physiological maturity correspond to a stay-green phenotype, whereas low NDVI values correspond to a senescent phenotype. The RS was calculated as the slope of the linear NDVI decline over thermal time as shown in Fig. 1 (adapted from Bogard *et al.* 2011). Regarding RS, stay-green phenotypes are defined here as those showing smaller rates of senescence. Both measurements of stay-green were not confounded by phenology: for rate of senescence, degree days were used instead of days to heading or anthesis; NDVI at physiological maturity is estimated for each genotype exactly at physiological maturity. For population 2, only two measurements of NDVI were taken under full irrigation during grain-filling and to calculate both RS and NDVI at physiological maturity only these two points were used for regression analysis.

#### Statistical analysis

The adjusted means for each environment and genotype were obtained using the mixed models theory and conducted with the MIXED procedures from the SAS Institute (2004) considering the effects of replications and blocks within replications as random



**Fig. 1.** Diagram illustrating calculations of rate of senescence (RS) and greenness of the plot at physiological maturity (Stg). The rate of senescence (RS) was determined as the slope of the NDVI decay against thermal time (TT). The greenness of the plot at maturity (Stg) was estimated using the slope of NDVI decay against days after heading, and the corresponding day of physiological maturity (PM) was substituted in the equation to estimate the NDVI value (greenness of the plot) at maturity for each genotype.

and genotypes as fixed. Principal component analysis was performed using a mean of all sites for each genotype and trait using the PRINCOMP procedure (SAS Institute, 2004). Broad sense heritability ( $H^2$ ) was estimated for each trait individually in each environment and across all environments as:

$$H^2 = \sigma_g^2 / [\sigma_g^2 + (\sigma_{ge}^2 / e) + (\sigma^2 / re)]$$

where  $r$  = number of repetitions,  $e$  = number of environments,  $\sigma^2$  = error variance,  $\sigma_g^2$  = genotypic variance, and  $\sigma_{ge}^2$  = genotype by environment interaction variance.

Phenotypic correlations were calculated using the CORR procedure and multiple regression analysis was obtained with the STEPWISE procedure using canopy temperature at grain-filling, SPAD, stay-green associated traits (NDVI at physiological maturity and rate of senescence), and days to heading and to maturity (SAS Institute, 2004).

## Results

### Environmental characterization and overall analysis of yield and physiological traits

Both populations were grown under four different conditions in Mexico, including: a full irrigation control; a drought treatment where irrigation was withheld after germination; a late sowing treatment to increase temperatures during grain-filling with full irrigation; and a late sowing treatment with reduced irrigation such that mild drought during grain-filling also coincided with warm temperatures (Table 1). Measurements were taken in all environments but are only presented for the drought, heat, and heat combined with drought environments since these are the conditions where stay-green is expected to be of most importance (Borrell *et al.*, 2000a; Verma *et al.*, 2004; Harris *et al.*, 2007; Kumari *et al.*, 2007; Vijayalakshmi *et al.*, 2010).

Temperatures at the experimental station where the trials were conducted are mild during the winter and progressively increase after March (see long-term temperature data in Supplementary Table 1 at *JXB* online). The increase in

temperature causes considerable stress during the final stages of grain-filling, especially in the late-sowing trials, with average maxima temperatures above 30 °C. The heat and drought stress combinations reduced the average yield of the populations by between 40% and 60% of the control value (Table 1). Days to heading was accelerated under stress, especially at warmer temperature (Table 1). Yields, phenology associated traits (days to heading, DH, and days to maturity, DM), canopy temperature at mid-grain-filling (CTgf), chlorophyll content measured at around anthesis (SPAD), the rate of senescence (RS), and estimated NDVI at physiological maturity (Stg) were measured in both populations and in all environments (Table 2). All traits measured showed significant genotype, environment, and genotype×environment interaction effects (Table 2). Overall (using means of each genotype in all environments), grain yield was negatively associated with days to heading (DH), days to maturity (DM), rate of senescence (RS), and canopy temperature at grain-filling (CTgf), but positively with NDVI estimated at physiological maturity (Stg) (Table 2). These results were consistent in both populations (Table 2). Analysis including all environments showed high heritability for most traits except for RS, Stg in population 2, and CTgf in both populations where heritability were low to moderate.

#### *Contribution of stay-green expression to yield, under drought and heat environments*

In both populations, genotype effects for all traits measured were significant in all environments tested: irrigated, drought, heat, and heat combined with drought (Table 3).

As explained above, stay-green was calculated in two ways: (i) NDVI estimated at physiological maturity (Stg) and (ii) rate of senescence (RS); heritability for both stay-green traits measured in each environment were moderate to high in population 1 whereas low to moderate in population 2 (Table 4). Stg was correlated positively with yield under heat and heat combined with drought environments in both populations (Table 4). RS was negatively associated with yield under drought, heat, and heat combined with drought, in population 1 (Table 4). However, RS was poorly correlated with yield in population 2 in several environments and associations were only significant under heat (Table 4). In order to show that measurements of Stg and RS were not biased by phenology, correlations of these two traits were determined using a subset of lines showing a very narrow range of phenology (three days). In Table 4 it is shown that correlations between stay-green traits were still significant within a group of lines maturing in three days.

#### *Associations between traits under drought and heat environments*

PCA analyses for populations 1 and 2 in all three environments are shown in Figs 2 and 3, respectively. Consistent patterns were observed between YLD and DH/DM (opposite directions) in both populations and environments (Figs 2, 3). This was also confirmed by negative associations (phenotypic) between yield and DH/DM (Tables 5, 6). The Stg vector showed less than a 90° angle with the yield vector under heat and heat combined with drought environments in both populations (Figs 2B, C, 3B, C), confirming results from phenotypic correlations (positive, Table 4). The RS

**Table 2.** Average (Avg), significance of genetic (Genotype), environmental (Env) and genotype by environment interaction (G×E) effects, heritability ( $H^2$ ), and correlation of each trait with yield (CORR YLD) for yield (YLD), days to heading (DH), days to maturity (DM), canopy temperature measured at grain filling stage (CTgf), stay-green (Stg) measured as the estimated NDVI value at maturity, rate of senescence calculated using the slope of NDVI decay (RS), and leaf chlorophyll content in SPAD units at around anthesis, all measured in population (POP) 1 (294 genotypes) and population (POP) 2 (169 genotypes) grown in 3 environments (drought, heat and heat combined with drought)

Figures in bold indicate significant phenotypic correlations with yield (at  $P < 0.05$ ) using means of each genotype across all environments ( $n = 294$  and  $169$  for POP 1 and 2, respectively). \*\*\* significant at  $P < 0.0001$ , \*\* significant at  $P < 0.001$ , significant at  $P < 0.01$ . NS, non significant. § Correlation with yield was significant at  $P = 0.06$ .

		Yield ( $\text{g m}^{-2}$ )	DH (d)	DM (d)	CTgf ( $^{\circ}\text{C}$ )	Stg (NDVI units)	RS (NDVI units $\text{d}^{-1}$ )	SPAD
POP 1	<i>n</i>	294	294	294	294	294	294	294
	Avg	370.8	59.6	97.3	30.8	0.2	-0.001	46.4
	Genotype	***	***	***	***	***	***	***
	Env	***	***	***	***	***	***	***
	G×E	***	***	***	**	***	***	*
	$H^2$	0.67	0.87	0.73	0.38	0.60	0.22	0.81
	CORR YLD		<b>-0.37</b>	<b>-0.31</b>	<b>-0.37</b>	<b>+0.32</b>	<b>-0.37</b>	0.02
POP 2	<i>n</i>	169	169	169	169	169	169	169
	Avg	335.0	59.0	93.7	29.9	0.25	-0.001	47.3
	Genotype	***	***	***	***	***	***	***
	Env	***	***	***	*	***	***	***
	G×E	***	***	***	NS	***	***	***
	$H^2$	0.66	0.97	0.92	0.34	0.13	0.00	0.42
	CORR YLD		<b>-0.47</b>	<b>-0.44</b>	<b>-0.29</b>	<b>+0.23</b>	-0.14 <sup>§</sup>	-0.04

**Table 3.** Significance of fixed effects in the analysis of variance of population 1 and 2 (POP1 and 2) in each environment (drought, heat and heat combined with drought) for several traits including: yield (YLD in  $\text{gm}^{-2}$ ), chlorophyll content in SPAD units (SPAD), canopy temperature measured at grain filling (CTgf in  $^{\circ}\text{C}$ ), days to heading (DH in days), days to maturity (DM in days), rate of senescence (RS NDVI units  $\text{day}^{-1}$ ) and NDVI estimated at maturity (Stg in NDVI units). Average of all genotypes (Avg), highest and lowest (max and min, respectively) reported values are shown. \*\*\* significant at  $P < 0.0001$ , \*\* significant at  $P < 0.001$ , significant at  $P < 0.01$ . NS, non significant.

Trait	FixedEffect	Df	Drought		Heat		HD	
			POP1	POP2	POP1	POP2	POP1	POP2
YLD	Rep	1/1	NS	NS	NS	NS	NS	NS
	Genotype	293/168	***	***	***	***	***	***
	Avg		371.2	386.0	401.2	351.3	340.0	267.8
	Max		466.5	509.0	507.2	433.5	451.3	357.3
	Min		247.9	240.6	235.3	279.5	201.3	152.4
SPAD	Rep	1/1	NS	NS	NS	*	NS	*
	Genotype	293/168	***	***	***	***	***	***
	Avg		48.8	51.3	45.0	47.5	46.8	44.7
	Max		55.6	55.9	52.6	54.1	54.7	53.4
	Min		40.3	45.5	36.4	38.5	38.3	32.3
CTgf	Rep	1/1	NS	NS	*	*	NS	***
	Genotype	293/168	***	*	***	***	*	*
	Avg		28.3	29.3	31.5	31.2	32.6	29.2
	Max		30.7	30.8	32.9	32.1	35.0	30.9
	Min		27.1	27.7	30.5	29.9	24.7	28.0
DH	Rep	1/1	NS	*	*	NS	NS	*
	Genotype	293/168	***	***	***	***	*	***
	Avg		73.2	71.4	53.2	53.2	52.4	52.4
	Max		79.5	75.4	56.2	56.6	55.5	56.5
	Min		67.6	67.1	48.3	49.0	48.3	48.1
DM	Rep	1/1	NS	*	NS	NS	NS	*
	Genotype	293/168	***	***	***	***	*	***
	Avg		112.3	114.7	89.7	84.2	83.0	82.1
	Max		117.4	120.2	93.8	88.0	87.0	86.0
	Min		107.5	107.8	82.2	78.0	77.5	77.3
RS	Rep	1/1	NS	NS	NS	*	NS	NS
	Genotype	293/168	***	*	***	*	***	***
	Avg		-0.0015	-0.0015	-0.0011	-0.0011	-0.0010	-0.0009
	Max		-0.0011	-0.0011	-0.0006	-0.0009	-0.0006	-0.0006
	Min		-0.0020	-0.0018	-0.0015	-0.0013	-0.0015	-0.0013
Stg	Rep	1/1	NS	NS	NS	***	NS	NS
	Genotype	293/168	***	*	***	*	***	***
	Avg		0.35	0.19	0.19	0.31	0.17	0.24
	Max		0.52	0.44	0.41	0.41	0.27	0.33
	Min		0.24	0.00	0.03	0.23	0.08	0.17

vector showed more than a  $90^{\circ}$  angle with the yield vector (Figs 2, 3) confirming the negative associations observed by phenotypic correlations (Table 4 for both populations). Finally, CTgf vectors showed more than a  $90^{\circ}$  angle with Stg (Figs 2B, 3C) whereas it was lower than  $90^{\circ}$  between CTgf and RS vectors (Figs 2C, 3C), particularly in the heat combined with drought environments. Correlations between Stg with CTgf were negative and significant under heat in population 1 and under heat combined with drought in population 2 (Tables 5, 6). RS with CTgf were positively correlated under heat combined with drought in population 1 only (Tables 5, 6), but not significant in population 2. Finally, all traits were used as yield-predicting variables in multiple regression analysis (Table 7). Around 30% and 20% of yield variability was explained by the variables included in the model (CTgf, RS, Stg, and SPAD), when

using means of all environments for each genotype in populations 1 and 2, respectively (data not shown). Multiple regression analysis was also used for the same traits in each stress environment, individually and for each population (Table 7). For most environments, SPAD explain very little yield variability and was only added to the model in a few environments within populations 1 and 2 (Table 7). Stay-green traits (RS and Stg) explained, on average, 8% of yield variability in the heat environments (heat and heat combined with drought) in both populations (Table 7). CTgf was the best predicting yield variable in population 2, whereas stay-green variables were better than CTgf to explain yield in population 1 (Table 7). On average, in both populations under heat and heat combined with drought environments CTgf and stay-green variables accounted for around 30% of yield variability (Table 7).

**Table 4.** Heritability ( $H^2$ ) of stay-green traits including estimated NDVI at maturity (Stg), and rate of senescence (RS) measured in two populations (POP 1 and POP 2) grown in drought, heat, and heat combined with drought environments

Phenotypic correlations with yield with Pearson correlation coefficients ( $r$ ) of stay-green traits with yield are shown (Stg YLD and RS YLD) and corresponding  $P$  values are shown in each environment. Significant correlations of stay-green traits and yield are highlighted in bold figures.

	Drought	Heat	Heat+Drought	HEAT ENVs <sup>a</sup>
<b>POP 1</b>				
Stg $H^2$	0.6	0.74	0.7	
RS $H^2$	0.82	0.78	0.49	
Stg YLD ( $r$ )	-0.11	<b>0.26</b>	<b>0.36</b>	<b>0.22</b>
$P$	0.07	<0.0001	<0.0001	<0.01
RS YLD ( $r$ )	<b>-0.28</b>	<b>-0.36</b>	<b>-0.21</b>	<b>-0.30</b>
$P$	<0.0001	<0.0001	<0.001	<0.0001
<b>POP 2</b>				
Stg $H^2$	0.64	0.39	0.31	
RS $H^2$	0.29	0.31	0.43	
Stg YLD ( $r$ )	0.14	<b>0.36</b>	<b>0.4</b>	<b>0.22</b>
$P$	0.06	<0.0001	<0.0001	<0.05
RS YLD ( $r$ )	-0.08	<b>-0.23</b>	-0.09	<b>-0.20</b>
$P$	0.29	<0.001	0.26	<0.05

<sup>a</sup> Correlations were obtained in a subset of lines of each population ( $n=152$  in population 1 and  $n=113$  in population 2) showing only a 3 d range of phenology in Heat ENVs (heat and heat+drought).

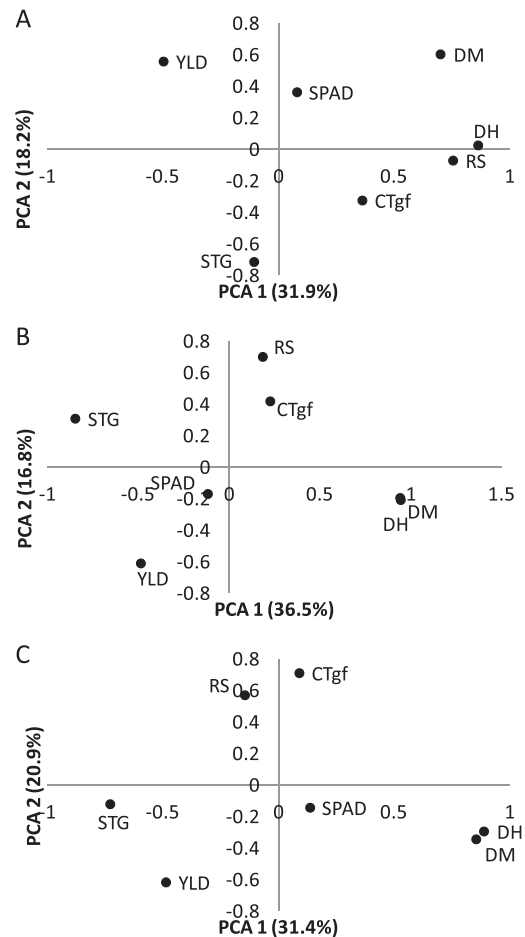
## Discussion

### Usefulness of NDVI to identify stay-green expression in spring wheat

NDVI and other indices from multispectral radiometers have been proposed as a means of estimating chlorophyll (Munden *et al.*, 1994), biomass (Hansen and Schjoerring, 2003; Babar *et al.*, 2006; Marti *et al.*, 2007), ground-cover (Boissard *et al.*, 1992; Mullan and Reynolds, 2010), nitrogen status (Wright *et al.*, 2005), and yield in wheat and other cereals (Filella *et al.*, 1995; Aparicio *et al.*, 2000; Royo *et al.*, 2003). Many other studies have shown that the GreenSeeker sensor can be useful to determine and integrate remotely the total greenness of wheat plots (Hansen and Schjoerring, 2003; Babar *et al.*, 2006; Marti *et al.*, 2007). The NDVI also gives a continuous measurement of stay-green and can substitute discrete scores obtained by subjective visual observations. Moreover, this equipment (GreenSeeker) is not sensitive to weather changes (since it uses internal light) like other passive multispectral radiometers do (Hansen and Schjoerring, 2003).

### Contributions of the stay-green expression to yield under drought and heat environments

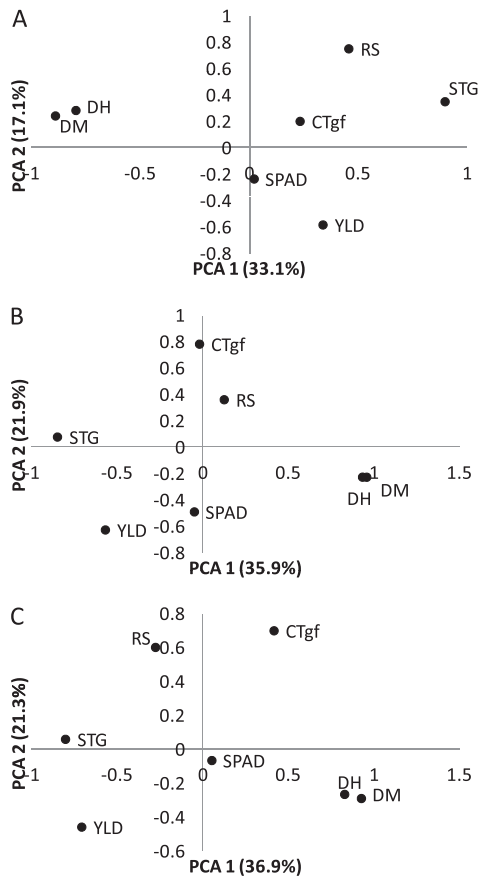
The results presented here indicated that stay-green was correlated with yield under heat and heat combined with drought either based on the estimation of NDVI at physiological maturity (Stg) or on the rate of senescence (RS). The NDVI at physiological maturity, which provides



**Fig. 2.** PCA analysis of physiological and yield traits measured in population 1 under drought (A), heat (B), and heat combined with drought (C). Traits included in the PCA: yield (YLD), chlorophyll content in SPAD units (SPAD), canopy temperature measured at grain filling (CTgf), rate of senescence (RS), and NDVI estimated at maturity (Stg).

information on how much greenness a genotype can maintain close to maturity, was positively associated with yield under heat and heat combined with drought environments. For the RS, which provides information on how fast a genotype loses chlorophyll, negative associations with yield were observed in both populations. This was expected, as decreased rates of senescence are an attribute of stay-green phenotypes (Harris *et al.*, 2007). On the other hand, the onset of senescence was determined in a sub-set of genotypes where enough data points were available and delayed senescence was associated with the stay-green expression (data not shown). This is in agreement with previous results in sorghum shown by Harris *et al.* (2007).

While significant correlations between stay-green attributes and yield were observed, Pearson correlation coefficients between these traits were low to moderate in both populations. Moreover, under full irrigation, significant correlations were not observed (data not shown). Despite the relatively low correlations, stay-green traits (RS and Stg) have been used as part of multiple regression analysis and together with canopy temperature, accounted for 30%



**Fig. 3.** PCA analysis of physiological and yield traits measured in population 2 under drought (A), heat (B), and heat combined with drought (C). Traits included in the PCA: yield (YLD), chlorophyll content in SPAD units (SPAD), canopy temperature measured at grain filling (CTgf), rate of senescence (RS), and NDVI estimated at maturity (Stg).

of yield variability under heat and heat combined with drought environments. This shows the value of stay-green to obtain cumulative effects together with other traits to further improve adaptation under stress.

#### Physiological basis for stay-green

A few tentative physiological explanations for the stay-green phenotype have been attributed to higher cytokinin levels and/or reduced ethylene production or perception (Thomas and Howarth, 2000) or higher N assimilation (Borrell *et al.*, 2001; and reviewed in Hirel *et al.* 2007). Moreover, differences in N remobilization may be a part of the physiological basis for genetic differences in stay-green (Van Oosterom *et al.*, 2010). Other hypotheses have been proposed, for example, Christopher *et al.* (2008) postulated that a stay-green genotype was able to extract a small amount of extra soil moisture from deep in the profile late in the season. This stay-green genotype had a narrower root system which conferred a yield advantage in areas where moisture was available in deep non-constrained soils (Christopher *et al.*, 2008). Canopy temperature can be used

**Table 5.** Pearson correlation coefficients and probabilities of associations between traits measured in Population 1 using means of each genotype ( $n = 294$ ) grown under heat and heat combined with drought environments

Traits included: Stay-green estimated with NDVI at maturity, STG, rate of senescence, RS, yield, YLD, days to heading, DH, days to maturity, DM, canopy temperature at grain filling, CTgf, and leaf chlorophyll content at anthesis, SPAD). Lower triangle contains correlations of traits under heat and upper triangle contains correlations of traits under heat combined with drought. Bolt figures indicate significant correlations.

POP 1	STG	RS	YLD	DH	DM	CTgf	SPAD
STG		0.06	<b>0.36</b>	<b>-0.48</b>	<b>-0.42</b>	-0.05	0.00
		0.28	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	0.44	0.95
RS	<b>0.16</b>		<b>-0.21</b>	<b>-0.15</b>	<b>-0.17</b>	<b>0.19</b>	<b>-0.19</b>
	<b>&lt;0.01</b>		<b>&lt;0.001</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>	<b>&lt;0.01</b>
YLD	<b>0.26</b>	<b>-0.36</b>		<b>-0.23</b>	<b>-0.18</b>	<b>-0.32</b>	0.02
	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>		<b>&lt;0.0001</b>	<b>&lt;0.01</b>	<b>&lt;0.0001</b>	0.77
DH	<b>-0.75</b>	<b>0.15</b>	<b>-0.29</b>		<b>0.93</b>	-0.07	0.09
	<b>&lt;0.0001</b>	<b>&lt;0.01</b>	<b>&lt;0.0001</b>		<b>&lt;0.0001</b>	0.24	0.12
DM	<b>-0.77</b>	<b>0.15</b>	<b>-0.29</b>	<b>0.98</b>		-0.10	0.08
	<b>&lt;0.0001</b>	<b>&lt;0.01</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>		0.09	0.19
CTgf	<b>-0.21</b>	0.02	<b>-0.22</b>	0.04	0.03		0.05
	<b>&lt;0.001</b>	0.79	<b>&lt;0.001</b>	0.53	0.56		0.43
SPAD	0.07	-0.06	0.10	-0.06	-0.05	0.03	
	0.25	0.28	0.08	0.30	0.36	0.57	

**Table 6.** Pearson correlation coefficients and probabilities of associations between traits measured in Population 2 using means of each genotype ( $n = 169$ ) grown under heat and heat combined with drought environments

Traits included: Stay-green estimated with NDVI at maturity, STG, rate of senescence, RS, yield, YLD, days to heading, DH, days to maturity, DM, canopy temperature at grain filling, CTgf, and leaf chlorophyll content at anthesis, SPAD). Lower triangle contains correlations of traits under heat and upper triangle contains correlations of traits under heat combined with drought. Bolt figures indicate significant correlations.

POP 2	STG	RS	YLD	DH	DM	CTgf	SPAD
STG		0.13	<b>0.40</b>	<b>-0.50</b>	<b>-0.79</b>	<b>-0.23</b>	-0.03
		0.08	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.001</b>	0.74
RS	0.08		-0.09	<b>-0.27</b>	<b>-0.38</b>	0.13	-0.06
	0.31		0.26	<b>&lt;0.001</b>	<b>&lt;0.0001</b>	0.08	0.46
YLD	<b>0.36</b>	<b>-0.23</b>		<b>-0.48</b>	<b>-0.47</b>	<b>-0.54</b>	0.06
	<b>&lt;0.0001</b>	<b>&lt;0.001</b>		<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>	0.45
DH	<b>-0.70</b>	0.08	<b>-0.36</b>		<b>0.82</b>	0.11	0.04
	<b>&lt;0.0001</b>	0.30	<b>&lt;0.0001</b>		<b>&lt;0.0001</b>	0.17	0.60
DM	<b>-0.77</b>	0.08	<b>-0.38</b>	<b>0.96</b>		0.15	0.04
	<b>&lt;0.0001</b>	0.31	<b>&lt;0.0001</b>	<b>&lt;0.0001</b>		0.06	0.62
CTgf	-0.01	0.00	<b>-0.45</b>	<b>-0.19</b>	<b>-0.20</b>		0.09
	0.90	1.00	<b>&lt;0.0001</b>	<b>&lt;0.05</b>	<b>&lt;0.001</b>		0.22
SPAD	0.05	<b>-0.34</b>	<b>0.19</b>	0.05	0.06	-0.11	
	0.52	<b>&lt;0.0001</b>	<b>&lt;0.05</b>	0.54	0.46	0.14	

as a surrogate of root functionality under drought (Lopes and Reynolds, 2010b). In this study, the relationship observed between stay-green and canopy temperature would confirm a link between roots and stay-green expression and also the functionality of stay-green in terms of gas

**Table 7.** Multiple regression for yield using predicting variables associated with canopy temperature at the grain-filling stage (CTgf), stay-green [rate of senescence (RS), and NDVI at maturity (Stg)], and chlorophyll concentration (SPAD)

Yield variability accounted for by the variables included in the model is shown in percentage from  $R^2$  and total variability explained by the best significant model (at  $P < 0.0001$ ) is also shown (TOTAL). Multiple regressions were determined for each environment separately (drought, heat, and drought combined with heat). Estimated regression parameters (Est) and standard error of Est (STD) are shown for each trait included in the model. (–) is used if a particular trait was not significantly included in the model ( $P < 0.05$ ) to explain yield.

Trait	Drought			Heat			HD		
	Est	STD	$R^2$	Est	STD	$R^2$	Est	STD	$R^2$
<b>POP 1</b>									
CTgf	–25.3	4.5	11.2	–16.7	5.8	2.1	–13.8	2.6	9.2
RS	–65785	14859	5.4	–188317	23694	13.2	–55430	15338	3.4
Stg	–85.1	53.5	0.7	210.1	37.5	10.3	223.2	31.6	12.9
SPAD	2.6	0.9	2.6	–	–	–	–	–	–
TOTAL			19.9			25.6			25.5
<b>POP2</b>									
CTgf	–	–	–	190.7	79.8	12.9	–38.0	4.9	29.3
RS	–117842	33260	6.9	–58647	23737	1.8	–	–	–
Stg	194.1	50.0	2.0	190.7	79.8	2.7	366.4	78.0	8.1
SPAD	–	–	–	1.3	0.8	4.3	1.3	0.7	1.2
TOTAL			8.9			21.7			38.6

exchange. Here, the associations between canopy temperature and stay-green traits were not consistent across all environments. Negative associations between Stg (NDVI at physiological maturity) and CTgf were observed under heat in population 1 and under heat combined with drought in population 2. At the same time the RS was positively associated with CTgf only under heat in population 2. These relationships between stay-green and canopy temperature would explain a better capacity to use water by the stay-green genotypes under more stressful environments. Moreover, cooler canopies associated with stay-green expression suggested that the stay-green trait was functional, at least in terms of gas exchange. However, the associations observed in this study (lack of significance in many environments) do not confirm with confidence the former hypothesis. The lack of significant correlations between CTgf and stay-green in several environments is indicative of the role that other factors may play in stay-green expression, and examples of such factors are given above (see introduction in this sub-section).

## Conclusions and implications to breeding

The results presented in this study suggest a simple and integrated way to measure stay-green in large sets of germplasm using a GreenSeeker sensor to measure NDVI during the grain-filling stage in wheat plots. The precision of estimation of these traits will increase with the number of NDVI measurements taken and, probably after mid-grain-filling, two weekly measurements should be taken under stressed environments. The rate of senescence and estimated

NDVI at physiological maturity showed moderate heritability and were calculated independently from phenology. The rate of senescence and estimated NDVI at physiological maturity correlated with yield, but the advantage was clearer under stressful environments with no effect under full irrigation. Cumulative effects to improve stress adaptation may be achieved by introgressing low canopy temperature and stay-green expression traits into new wheat lines.

## Supplementary data

Supplementary data can be found at *JXB* online.

**Supplementary Table S1.** Monthly means of air temperature (maximum, minimum, and average), solar radiation, and rainfall during the 2009–2010 season in Mexico.

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