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Contrasting Properties of Flag Leaf Greenness in Ancient Wheat Species and Modern Bread Wheat

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ABSTRACT

A genotype with an ability to retain green leaf area for longer than an ordinary genotype is called a 'stay-green' phenotype. Such phenotypes may be able to assimilate more carbon during the post-anthesis grain-filling period. A field experiment was conducted to study genotypic differences of stay-green properties of ten genotypes belong to three ancient wheat species (Einkorn, Emmer and Spelt) and bread wheat in 2012 and another field experiment was conducted to identify the effect of nitrogen fertilizer on stay-green properties of the same genotypes in 2014 at University of Nottingham farms, UK. Flag leaf greenness was measured as visual greenness score and SPAD values in both experiments while NDVI was recorded only in 2014. Visual greenness score was plotted against thermal time (the base temperature at anthesis □) using a four-parameter logistic model to calculate green area duration and thermal time at maximum senescence rate. Aboveground biomass, grain yield and plant nitrogen uptake (excluding roots) were recorded at harvest. Genotypic variation was observed for all measured parametersin 2012 where the delayed onset of flag leaf senescence, slow senescence rate and prolonged leaf greenness were observed in Spelt cultivar Oberkulmer. Further, measured parameters were positively influenced by the increased level of nitrogen fertilizer in 2014. Above ground biomass was high in Spelt followed by Emmer, Bread Wheat and Einkorn genotypes at maturity. However, the highest grain yield was recorded in bread wheat genotypes compared to others suggesting its ability to convert more biomass towards the grain production. Green area duration was associated with aboveground biomass and plant nitrogen uptake in 2012. Therefore, favourable stay-green traits in Spelt, associated with N uptake, could be introduced to bread wheat through breeding programs in the future.

1. Introduction

A genotype with an ability to retain leaf green area for longer than an ordinary genotype is called a 'stay-green' phenotype [1, 2]. It is identified as a beneficial trait which helps cereals to adapt to water-deficient environments [3]. There are two types of 'stay-green' among cereal crops: 'functional stay-green' and 'non-functional stay-green'. In functional stay-green, plants assimilate more C through active photosynthesis during post-anthesis grain filling while in non-functional stay-green, leaves remain in green due to the lesions of chlorophyll catabolism, which are lacking photosynthetic capacity [4]. A direct association in stay-green phenotypes with grain yield in sorghum is reported [4]. In maize, stay-green varieties prolong the active photosynthesis under both high and low nitrogen (N) conditions; hence dry matter accumulation and grain yield are higher than the other varieties [5]. The relationship between a long and functioning stay of flag leaf greenness and wheat crop production is documented [6, 7, 8]. Post-anthesis flag leaf senescence is controlled by N supply and demand [9]. As a result of imbalance N supply and demand, the senescence rate may be accelerated [10]. Increased temperature induces senescence onset [11], while water and nutrient deficiencies, especially N, may harm flag leaf greenness [12]. Nitrogen uptake efficiency (NUpE)

is significantly influenced by water availability during the post-anthesis grain-filling period. Limited water supply may disturb the balance of N supply and demand; hence accelerated leaf senescence can be seen [13]. During the post-anthesis period, carbon assimilation is determined by the date of onset and senescence rate [14]. Carbon is as important as N since the plant needs energy and favourable structure (shoot and root) for N uptake during the post-anthesis period [15, 16]. Therefore, C and N metabolisms are equally important for the senescence process [17]. It is possible to produce more C assimilates due to prolonging photosynthesis. Therefore, remobilization of C and N is crucial in the senescence process.

Some studies suggest that storage capacity of N in the canopy controlled N remobilization during grain filling [18] rather than demand for N from the grains.Further, Nremobilization was halted by the limited capacity of sink organs [15] If post-anthesis N remobilization is a sink-driven process, delayed senescence and post-anthesis N uptake would have to contribute less N for grain filling while if it is a source-driven process, grain N concentration should be high. Under field conditions, at low N, winter wheat showed a positive correlation with the onset of senescence and NUtE suggesting that N remobilization is a source-driven process under N limited conditions [8]. The relationships between leaf senescence, N remobilization, grain yield, and grain N content are complicated and not easy to manipulate [19]. Based on quantitative trait loci (QTLs) analysis of doubled haploid mapping population, it was found that depending on the environment, delaying flag leaf senescence is associated with increase grain yield or grain protein content [6]. Further, they suggested that the effect of delaying flag leaf senescence on grain yield and quality might be influenced by post-anthesis nitrogen availability to the crop [6].

The present study was conducted to estimate and compare the flag leaf greenness of ancient wheat species and the effect of varying N fertilizer regimes on flag leaf greenness changes under field conditions. The expected outcome of the research could be used to improve the stay-green characteristics of modern bread wheat through the possible introduction of them into wheat breeding programs.

2. Materials and Methods

2.1 Experimental site and design

Two field experiments were conducted to study stay-green properties of ancient wheat species and modern bread wheat in 2011-2012 (2012 referred hereafter) and 2013-2014 (2014 referred hereafter) seasons at University of Nottingham Farms, Leicestershire, UK(52^0 50' N, 1^0 15' W). The wheat species tested were cultivated Einkorn (*Triticum monococcum* L.), cultivated Emmer (*T. dicoccum*L.) and Spelt (*T. spelta* L.), together with modern bread wheat (*T. aestivum*L.). Ten genotypes were used; three Einkorn (1, 2 and 3), two Emmer (1 and 2), three cultivars (cv) of Spelt (SB, Oberkulmer and Tauro) and two cultivars of modern bread wheat (Xi 19 and JB Diego).However, three Einkorn genotypes were excluded from the field experiment in 2014 due to their poor germination.

The field experiment in 2012 was arranged according to the randomized complete block design with four replicates. Seeds were sown on 18th October 2011. The soil was a sandy loam with pH 7.6 containing 67.8 mg l^{-1} of P, 510, mg l^{-1} of K and 241 mg l^{-1} of Mg in the top 30 cm and 78.2 kg N ha^{-1} at 90 cm in February 2012.The length and width of each plot were 24 m and 1.625 m, respectively while row width was 0.125 m. Each plot was divided into two halves, and the first half was used to collect the quadrat sample while the second half was combined at maturity. Fertilizer N, a total of 140 kg N ha^{-1}, was applied as ammonium nitrate (NH_4NO_3, 34.5 N %) in three splits at early tillering, stem elongation and flag leaf emergence at 40, 40 and 60kg N ha^{-1}, respectively.All plots received plant growth regulators (PGR) at 0.2 l ha^{-1} at early tillering and stem elongation.

The sowing date of the 2014 trail was on 19th November 2013, and soil type of the experimental site was sandy loamy with pH of 6.8. On average, P, K and Mg availability of the soil in top 30 cm of the depth was 72 mg l^{-1}, 216.7 mg l^{-1} and 221 mg l^{-1}, respectively. Soil mineral N availability in top 90 cm was 73.9, 74.9 and 65.8 kg N ha^{-1} in first, second and third blocks, respectively in February 2014. A split-plot design was used in the 2014 experiment where N treatment was randomized on the main plot and genotypes on the sub-plot with three replicates. Three N regimes equal to zero N (no fertilizer N applied; NN), 100 kg N ha^{-1} (Low N; LN) and 150 kg N ha^{-1} (High N; HN) were used, based on the results of soil mineral N analysis in February in 2014.NH_4NO_3 was applied in two splits at 40, and 60 kg N ha^{-1} at early tillering and stem elongation for LN treatment while 40, 80 and 30 kgN ha^{-1} was applied at early tillering, stem elongation and flag leaf emergence for HN treatment. The size of the subplot was 12 m x 1.625 m.During stem elongation to flag leaf emergence, all plots were treated with PGR at the rate of 1 l ha^{-1}.Best agricultural practices recommended for UK wheat production was followed by crop management.

The annual average temperature of the experimental sites was 10.3℃ and 11℃, which was 5.6% and 11% greater than the Long-term mean (LTM) of 9.7℃, in 2012 and 2014, respectively. Annual rainfall of the area in 2012 and 2014 was 718 mm and 589 mm while LTM is 604. The total incident solar radiation from sowing to harvest for 2012 and 2014 was 2863 and 2836 MJ m^{-2}, respectively. Solar radiation from anthesis to the end of grain filling (June to August) was high in 2014 than in 2012. Further, it was 10% less than the LTM in 2012 while 14% higher than the LTM in 2014. Therefore, the 2014 season had more solar radiation and brighter conditions from anthesis to the end of grain filling compared to the 2012 season.

2.2 Canopy persistence and stay-green

Flag leaf greenness was recorded starting from anthesis. Two approaches were used to assess canopy greenness; evaluating flag leafgreenness as visual scoring and SPAD values. A scale ranging from 10 (fully green) to 0 (fully senesced) was used to estimate greenness based on the whole canopy (Figure 1). Then visual greenness score was plotted against thermal time (the base temperature at anthesis ℃) using a four-parameter logistic model (Equation 1) (nonlinear regression).GenStat 15th edition was used to estimate four parameters (VSN International, UK).

$$Y = A + \frac{C}{1 + e^{-B(t-M)}} \quad \text{Equation 1}$$

Where Y is greenness score, A is lower asymptote in the unit of Y axis, C is the difference between upper and lower asymptote in the unit of Y axis, B is doubled relative senescence rate at the thermal time M, M is the thermal time when the absolute senescence rate is at maximum and t; accumulated thermal time after anthesis in degree days (℃d) [20]. Green area duration was estimated by calculating the trapezoidal area between assessment dates (area under the curve).

A soil plant analysis development meter (SPAD-502, Minolta, Osaka, Japan) was used to measure chlorophyll concentration index of the green leaves.The fully developed, the uppermost leaf was used to take SPAD measurements, avoiding the midrib due to thickness and paleness, which could affect readings, and the average across the leaf was recorded. Ten leaves were used to measure SPAD per plot, and the average value was used as a mean value per genotype. Flag leaf SPAD values were taken throughout the growing season.

Figure 1: Different stages of flag leaf greenness (10 = fully green) to (0 = fully senesced)

(Modified image based on[8])

2.3 The normalized difference vegetation index (NDVI)

The normalized difference vegetation index (NDVI) was measured in the field experiment in 2014 using a Green Seeker Handheld Crop Sensor (Model HCS-100). It was continued from the beginning of the stem elongation until flag leaf senescence at weekly intervals. Average NDVI along the sub-plot was taken above the canopy.

2.4 Aboveground biomass, grain yield and total plant nitrogen uptake at harvest

Grain yield and aboveground biomass were measured at harvest while plant nitrogen uptake (N_{shoot}) (except roots) was calculated based on the

nitrogen content (%) of the plant dry matter. Pre-harvest sampling was done at maturity when all plants were fully senesced using a quadrat of 0.25 m^2. The sample was carefully placed in pre-labelled paper sacks in the field to avoid grain losses during transport. Spikes of all uprooted plants were put inside the bag. In the laboratory, all roots were separated and discarded. Then, the fresh weight of the quadrat sample was recorded before being oven-dried to obtain a dry weight. The dried spikes were hand threshed carefully, and grains were separated from the chaff. The grains were re-dried to achieve dry weight of the grain sample.The difference between the dry weight of the spikes and grains was taken as chaff dry weight.

Plant materials were ground to achieve a particle size of < 200 µm. Then, 45 to 50 mg of samples were weighed and encapsulated in tin capsules. The encapsulated samples were then analyzed for N% according to the Dumas method using a Fisons NA-2000 elemental analyzer (Fisons, Ipswich, UK) calibrated against Methyl-N standard (N content = 9.28%). N content was presented as a percentage. Plant N uptake (N_{shoot}) was calculated using Equations 2 (except roots).

$$\text{N (shoot)(g N)} = \text{Plant DW (g) x N content of the plant} \qquad \text{Equation 2}$$

2.5 Chlorophyll extraction

The fully developed flag leaf was used to take samples for chlorophyll extraction, and at the same time, the SPAD value was recorded. The leaf samples were taken using cork borer, 1.4 cm in diameter. Collected samples were temporarily stored in liquid N before being stored in -80℃ until extraction. Two leaf discs were ground using a pestle and mortar with 50 ml of 80% acetone. The ground leaf sample was centrifuged for 5 minutes at 3000 rpm to remove debris. A spectrophotometer (Cary 50) was used to analyzethe chlorophyll content of the sample. Chlorophyll (a + b) is calculated as in [21] in 80% acetone; (µg/ml) (Equation 3).

$$\text{Chl a} = 12.25 \text{ x } (A663.6) - 2.55 \text{ x } (A646.6)$$

$$\text{Chl a} = 20.31 \text{ x } (A646.6) - 4.91 \text{ x } (A663.6)$$

$$\text{Chl a + b} = 17.768 \text{ x } (A646.6) + 7.34 \text{ x } (A663.6) \qquad \text{Equation 3}$$

2.6 Statistical analysis

Genotypic differences and N fertilizer effect on aboveground biomass, grain yield, N uptake of the plants, flag leaf greenness, SPAD and NDVI values were tested according to the analysis of variance. All the phenotypic data were subjected to a normality test before applying parametric statistical analysis. Variations among the data generated from the four-parametric logistic model were analyzed. Pearson correlations between different traits were computed using the average values across the replicates. Duncan Multiple Range Test was used to separate means at 5% probability level. Simple linear regression was used to develop the relationship between SPAD and actual chlorophyll content of the genotypes. Data were analyzed using GenStat 15th edition [22], and curve fitting of greenness score was performed using GraphPadPrism v6.05.

3. Results and Discussion

Two major breeding targets of modern bread wheat programs are high yield and grain protein concentration. However, there is a negative relationship between grain yield and grain protein content due to the genetic relationship between the traits [23]. Hence, one of the key challenges faced by wheat breeders are increasing grain yield without decreasing grain protein content. Physiological traits that positively influence post-anthesis N uptake can be used to increase grain protein content without affecting grain yield [24]. Post-anthesis flag leaf senescence was identified as one of the potential physiological traits that might improve both grain yield and grain protein concentration of bread wheat [24]. Flag leaf associated properties related to stay-green of wheat were evaluated in the present study. Flag leaf greenness was measured as visual greenness score,and SPAD value in both experiments and NDVI was recorded only in 2014 experiment.

3.1 Canopy persistence and stay-green

Calibration curves for SPAD values based on actual chlorophyll content of the genotypes were developed. Figure 2 shows the results of a simple linear regression between SPAD and chlorophyll concentration [chl] (a+b) of flag leaf of ten genotypes belong to four wheat species. According to the results of the present study, it was found that the strength of the relationship between actual [chl] and SPAD values varied depending on the wheat species. Previous studies have found that SPAD readings explained more than 93% of the variation of chlorophyll concentration in rice, wheat and soybean [25] while extractable chlorophyll for 11 food crop species were highly correlated with SPAD values [26]. Under greenhouse conditions, the relationship between chlorophyll concentration of St. Augustine grass and SPAD values were significant (r = 0.89), but the correlation in the field study was not as strong as in the greenhouse [27]. A linear relationship between chlorophyll concentration and SPAD for sorghum (*Sorghum*

bicolor) and pigeon pea (*Cajanus cajan*) has been reported [28].

SPAD values can be used as an indirect measurement of chlorophyll content since it corresponds to the amount of chlorophyll and nitrogen present in leaves [29]. Rate of chlorophyll loss can be successfully assessed by using SPAD [30]. The majority of leaf N is contained in chlorophyll molecules, and therefore the relationship between chlorophyll content and leaf N is reliable [31]. However, in contrast, it was suggested that the relationship between chlorophyll concentration and leaf N might be nonlinear due to non-chlorophyll N accumulated in the leaf as nitrate (NO^{3-}) [32]. Further, in some plant species including birch (*Betula pendula*), wheat (*Triticum aestivum*) and potato (*Solanum tuberosum*) a nonlinear relationship between chlorophyll concentrationand SPAD was found. This might be due to non-uniform distributionn of chlorophyll in leaf lamina [33].

Only ten leaf samples per genotype were used to extract chlorophyll due to limited time available in the present experiment. By increasing the number of samples, a stronger relationship between SPAD and actual [chl] might be achieved. Also, specific leaf area [34] significantly affected this relationship. The irradiance has a significant effect on SPAD values by changing the orientation of the chloroplasts within the cell [35].

According to their results, the highest SPAD value was observed at low irradiance under glasshouse conditions while the lowest was measured at the high irradiance in the middle of the day. Most of the SPAD readings in this study, under field conditions, have been taken in the middle of the day. Therefore, irradiance should be considered when the SPAD reading is to be used to estimate crop N status.

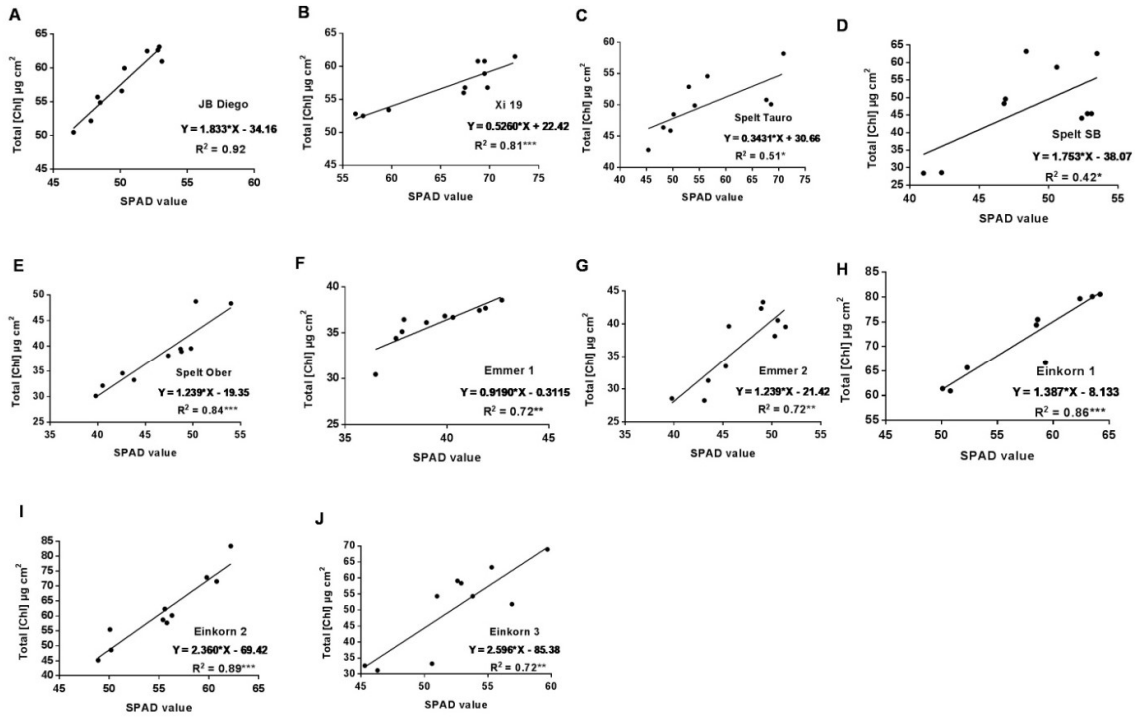

Figure 2: Simple linear regression between Chlorophyll concentration and SPAD values of (A) JB Diego (B) Xi 19 (C) SpeltTauro (D) Spelt SB (E) SpeltOberkulmer (F) Emmer 1 (G) Emmer 2 (H) Einkorn 1 (I) Einkorn 2 and (J) Einkorn 3

SPAD values of bread wheat cv. Xi 19 and cv. JB Diego declined rapidly when compared to ancient wheat species. SPAD values of Spelt genotypes at the late-grain filling period was higher than all other genotypes (*P<0.001*) in the 2012 field experiment (Figure 3). In 2014, SPAD values were measured four times after anthesis.At all sampling points,SPAD values were significantly affected by N level and genotype (GT). Bread wheat had the highest SPAD followed by Spelt and Emmer for all N levels.Genotypes with stay-green properties recorded high SPAD values than others. The relationship may vary from species to species depending on chlorophyll content and N status.

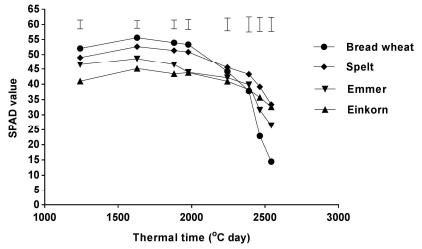

Figure 3: Observed values of SPAD throughout the growth period of bread wheat, Spelt, Emmer and Einkorn against thermal time (℃d)in 2012 experiment (error bars represent SED for species)

3.2 Visual greenness score

At anthesis, none of the genotypes had started to senesce; hence the greenness score was 10 for all genotypes, but over time, all genotypes showed a decline of flag leaf greenness. Visual greenness score was significantly different between genotypes at all sampling points (*P<0.001*) in the 2012 experiment. Spelt genotypes showed the slowest decline in leaf greenness throughout the assessment period (Figure 4). Bread wheat, Emmer and Einkorn completed flag leaf senescence four to five days earlier than Spelt genotypes.A strong positive linear relationship was found between SPAD readings and visual greenness score during the post-anthesis grain filling period for all ten genotypes (r = 0.96, *P<0.001*).

The timing of rapid flag leaf senescence (M) which describe the thermal time (℃d) when the absolute rate of flag leaf senescence is maximum, was higher in Spelt cv. Oberkulmer followed by bread wheat cv. JB Diego. Statistical differences between Spelt cv. Oberkulmer and bread wheat cv. JB Diego was significant though Spelt cv. SB and cv. Tauro were not different either from bread wheat cv. JB Diego or Xi 19 in 2012 field experiment (*Table 1 in supplementary data*). A logistic model can be used to simulate the flag leafsenescence area with thermal time from flag leaf emergence. It waspropose that more variance can be accounted for by fitting a modified Gomperz model to the green area of flag leaf over time from flag leaf emergence than a logistic model [36]. However, in the presentstudy, a standard logistic model fitted to observed values of senescence score accounted for more than 99% of the variance for seven out of ten genotypes in the 2012 field experiment (Table 1). Previous studies on senescence used a monomolecular-logistic equation describing leaf senescence kinetics during post-anthesis as a two-phase process [8].

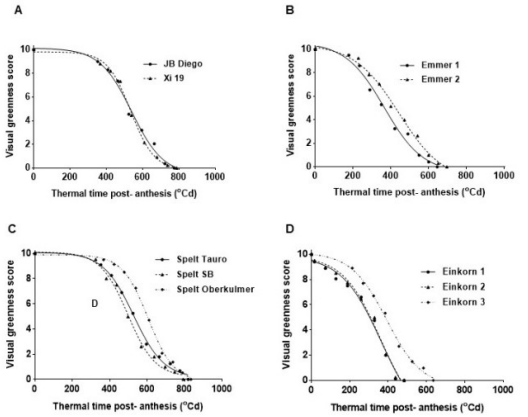

Figure 4: Observed values and fitted curves for post-anthesis flag leaf greenness in the mean of the visual score against thermal time(℃d) for (A) Bread wheat cv. JB Diego and cv. Xi 19 (B) Emmer 1 and 2 (C) Spelt cv. Tauro, cv. SB and Oberkulmer (D) Einkorn 1, 2 and 3 in 2012 experiment

Post-anthesis green area duration was significantly different among genotypes (*P<0.001*) (Figure 5)where Spelt cv. Oberkulmer had the highest green area duration, followed by Spelt cv. Tauro in 2012. Green area duration of Spelt cv. Oberkulmer and Tauro were statistically different between each other.Similar to the 2012 experiment, high green area duration and delayed onset of senescence (M) in 2014 experiment were observed in SpeltOberkulmer under NN, LN and HN conditions.

Table 1: Parameters of the Nonlinear regression model developed for flag leaf greenness in 2012 experiment [Response variable is flag leaf greenness score and Explanatory variable is thermal time after anthesis (℃d)]

GT	Parameters of nonlinear-logistic regression			
	MS	F	% of variance	SE
JB Diego	38.84	< 0.001	98.5	0.463
Xi 19	42.12	< 0.001	99.5	0.292
Spelt Tau	42.31	< 0.001	99.5	0.256
Spelt SB	44.95	< 0.001	99.1	0.374
Spelt Ober	48.40	< 0.001	99.7	0.202
Emmer 1	42.80	< 0.001	98.3	0.492
Emmer 2	41.19	< 0.001	99.2	0.340
Einkorn 1	42.73	< 0.001	97.9	0.548
Einkorn 2	43.53	< 0.001	98.5	0.471
Einkorn 3	36.22	< 0.001	99.4	0.281

MS, Mean square; F, probability level; SE, Standard error of observations

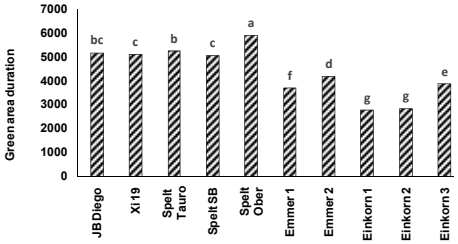

Figure 5:Green area duration of ten genotypes in 2012 field experiment. Columns with the same letters are not significantly different at 5% probability level according to DMRT.

High green area duration and slow senescence rate of Spelt cv.Oberkulmer can be partially explained by high N uptake efficiency of the plant. Further, efficient N uptake of Spelt might be associated with deep root system with high total root length (*unpublished data*). It was found that under controlled environment conditions, Spelt seedlings produced narrow root system with more seminal roots, longer seminal roots, hightotal root length and smaller tip and emergence angles [37]. It is proposed that the mature root system could be deep and narrow, while having the ability to penetrate the heavy soil and forage nutrients and water from the bottom layers of the soil horizon.

Figure 6 shows fitted curves for seven genotypes in 2014 under three N regimes. Emmer genotypes together with bread wheat cv. JB Diego at NN started to senesce before anthesis while greenness score of all other genotypes wasten at LN and HN.The timing of rapid flag leaf senescence (M) was significantly high in Spelt cv. Oberkulmer at any level of N though other Spelt genotypes had relatively low value for M. Timing of rapid flag leaf senescence of Spelt cv. Tauro was not statistically different from either bread wheat cv. Xi 19 at HN while bread wheat cv. JB Diego at NN (*Table 2 in supplementary data*).

Flag leaf senescence is a complex phenomenon controlled by genetics and the environment [7]. Both genetic [7] and environmental conditions have significant effects on senescence dynamics by recycling nutrients from vegetative parts towards reproductive organs [38]. Post-anthesis flag leaf senescence was affected by the balance between N supply, and N demandsdemand [9]. Accelerated or delayed senescence may result from an imbalance of N supply and demand [10].

According to the experiment in 2012, flag leaf of none of the genotypes started to senescence before anthesis. However, initial senescence rate of Bread Wheat, Emmer and Einkornwas greater than Spelt genotypes. Further, the senescence rate of Spelt genotypes was much slower than the others. Especially, the timing of rapid flag leaf senescence, which describes the thermal time (℃d) when the absolute rate of flag leaf senescence is maximum, was higher in Spelt cv. Oberkulmer (606 ℃d). Stay-green genotypes usually have a delayed onset of senescence [30] and slow senescence rate. Bread wheat, Emmer and Einkorn completed flag leaf senescence four to five days earlier than Spelt genotypes. It was reported that the rate of greenness declining in the flag leaf of Einkorn was greater than Emmer, spelt and bread wheat [39].

In 2014, Emmer genotypes together with bread wheat cv. JB Diego started flag leaf senescence before anthesis at NN. Flag leaf senescence completed six to seven days earlier at NN than HN, with little difference between LN and HN. Lack of N accelerates flag leaf senescence due to remobilization of N and other nutrients from flag leaf to grain [40]. Early senescence of Emmer genotypes and bread wheat cv. JB Diego might be associated with low nitrogen content in the soil. It has been further accelerated by high temperature during grain filling period and below-average rainfall. Green area duration of the genotypes increased with additional N supply in 2014.

During senescence, N the vegetative parts of the plant areremobilized to fill the grains. In wheat, 40-90% of N in grains was from the leaf which was remobilized during the senescence process. It was also found that more than 70% of the remobilized N was stored during the pre-anthesis period in wheat [41]. About 75% of N in winter wheat also remobilized from the leaf [42]. The date of onset and senescence rate determine C assimilation during post-anthesis grain filling [14]. Favourable shoot and root structures are important as same as energy for plants to uptake more N at post-anthesis grain-filling [6, 15],. Hence C and N metabolism is equally important for the senescence process [17]. Delaying senescence provides an opportunity to produce more C assimilates by prolonging photosynthesis. However, delayed senescence is not always beneficial in yield if straw has more unused carbohydrate when the season ends. Therefore, remobilization of C and N is crucial in the senescence process. The role of stay-green traits in increasing or maintaining high grain yield was discussed in previous studies [17, 30]. However; some research evidenced a negative relationship between stay-green traits and grain yield in rice [4]. Similarly in wheat, it was reported that delayed senescence of hybrid winter wheat cv. XN901 due to strong hybrid vigour which left more C in the straw at maturity. Further, as a result of long post-anthesis grain filling period of cv. XN901 grain yield increased significantly but not HI when compared with ordinary cv. Shaan 229 [43].

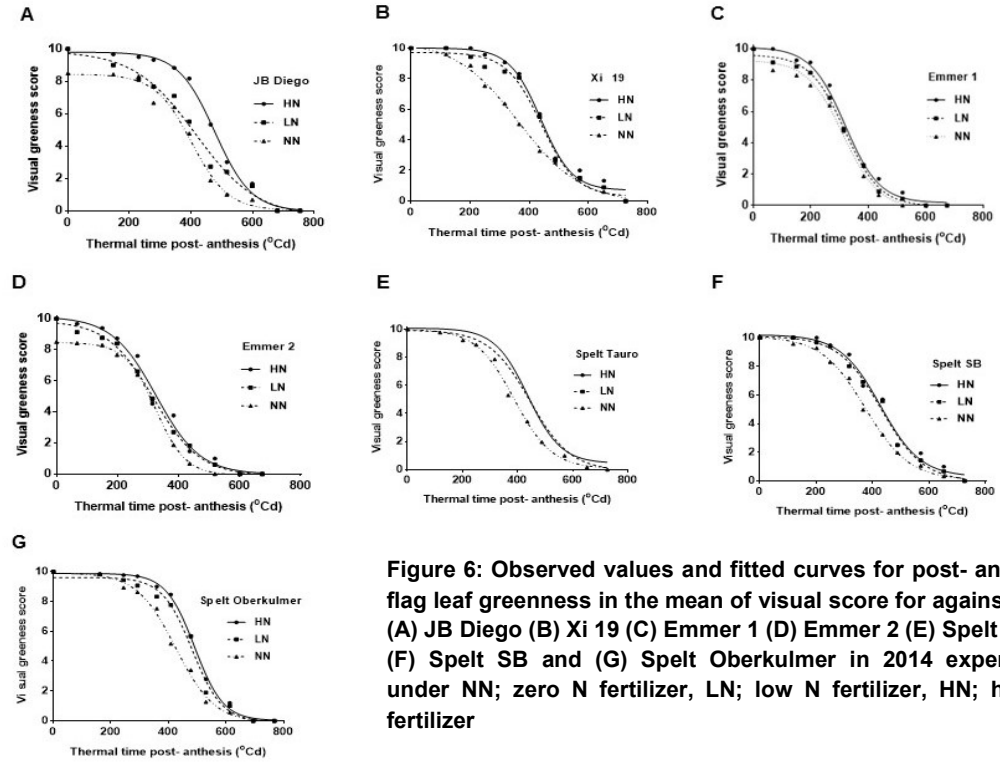

Figure 6: Observed values and fitted curves for post- anthesis flag leaf greenness in the mean of visual score for against (°Cd) (A) JB Diego (B) Xi 19 (C) Emmer 1 (D) Emmer 2 (E) Spelt Tauro (F) Spelt SB and (G) Spelt Oberkulmer in 2014 experiment under NN; zero N fertilizer, LN; low N fertilizer, HN; high N fertilizer

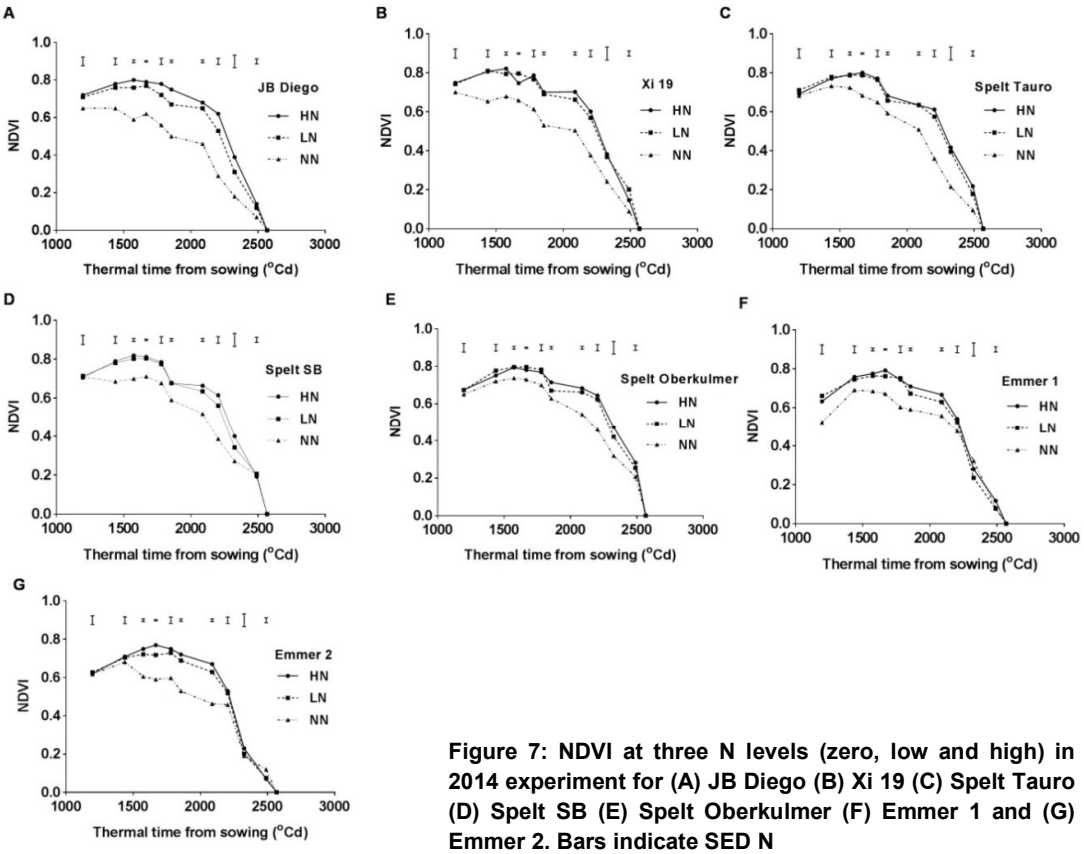

Figure 7: NDVI at three N levels (zero, low and high) in 2014 experiment for (A) JB Diego (B) Xi 19 (C) Spelt Tauro (D) Spelt SB (E) Spelt Oberkulmer (F) Emmer 1 and (G) Emmer 2. Bars indicate SED N

3.3 NDVI of the genotypes

Canopy NDVI was taken from GS31 until flag leaf fully senesced in 2014. At GS31 and GS61, only genotype and species were significant for NDVI (*P<0.001*). However, GT, N and GT x N effects were significant for post-anthesis NDVI (Figure 7). NDVI of all Spelt genotypes were significantly higher than bread wheat and Emmer during the late-grain filling period, especially Spelt cv. Oberkulmer. A strong linear relationship was found between SPAD readings and NDVI. The relationship between visual greenness score of flag leaf and post-anthesis NDVI for all species under any N level was also significant.

Normalized difference vegetation index (NDVI) was significantly affected by increased plant density, N treatment and growth stage. Increase in greenness may result in low reflectance of red waves due to increasing absorption as a result of high density of pigments per unit area while the opposite effect was observed for near-infrared reflectance [44]. NDVI was higher under HN treated plots than LN and NN plots for almost all of the genotypes in our study and had a strong relationship between SPAD readings and the visual score of flag leaf senescence. NDVI can be used to identify the stay-green genotypes successfully [45, 46]. The genotypes which can maintain maximum greenness close to maturity had a strong relationship with grain yield under stress environments [45].

3.4 Aboveground biomass, grain yield and plant nitrogen uptake (N_{shoot})

Genotypic effect on aboveground biomass (*P < 0.001*) was significant in 2012 experiment but not in 2014. However, N level was significant in 2014 (*P < 0.001*). The highest aboveground was recorded in Emmer 1 in 2012 though it was not significantly different from Spelt and bread wheat genotypes (Table 2). This was partially consistent with previous findings that the biomass production and yield of Spelt and bread wheat were either similar or bread wheat was higher under favourable conditions [46]. Averaged across the species, aboveground biomass was highest in Spelt followed by Emmer, bread wheat and Einkorn at harvest (*P < 0.001*). It is noted that Spelt and Emmer had increased aboveground biomass accumulation late in the growing season when compared to the other two species. In general, aboveground biomass was increased with N application in 2014. Aboveground biomass of bread wheat, Spelt and Emmer was not significantly different at harvest in 2014 while N supply increased aboveground biomass of all genotypes. Non-significant genotypic variation among genotypes in 2014 experiment may be due to narrow genetic background, where Einkorn was not included in the study. The grain yield of modern bread wheat was significantly high in both experiments due to improved harvest index and enhanced N application. Biomass partitioning towards grain production is poor in Spelt and Emmer despite of high aboveground biomass production at harvest.

Table 2: Aboveground biomass production (g m^{-2}) in 2012 and 2014 field experiments at harvest. In 2012 all genotypes had optimum nitrogen fertilizer level (200 kgN ha^{-1}) and in 2014 genotypes received three different N levels as zero N (NN), low N (100 kgN ha^{-1}, LN) and high N (150 kgN ha^{-1}, HN)

GT	Aboveground biomass at harvest (g m^{-2})			
	2012	2014		
		NN	LN	HN
JB Diego	1715.49	1623.32	2335.44	2477.85
Xi 19	1643.01	1552.17	1933.05	1872.54
Spelt Tau	1742.13	1669.04	1851.53	2302.22
Spelt SB	1691.68	1866.33	2073.56	2058.32
Spelt Ober	1719.59	1616.21	1904.72	2103.67
Emmer 1	1773.21	1667.05	2056.66	1783.87
Emmer 2	1597.66	1880.54	1951.09	1979.92
Einkorn 1	1248.18	-	-	-
Einkorn 2	1342.82	-	-	-
Einkorn 3	1030.47	-	-	-
SED; GT (df)	168.1***	127.644(6)*		
N (df)		50.211 (2)**		
GT x N (df)		210.835 (20)^{NS}		

***Significant at *P < 0.001*, **significant at *P < 0.01*, *significant at *P < 0.05*, *NS* - Not significant**

N_{shoot} at harvest varied significantly between genotypes where Emmer and Spelt genotypes had high N_{shoot} while bread wheat and Einkorn genotypes recorded the low values (*P < 0.001*). Additionally, Emmer 2, as a genotype, had the highest N_{shoot}, which is 48% higher than Einkorn 3. When averaged across the species, the highest N_{shoot} was observed in Emmer followed by Spelt, bread wheat and Einkorn in 2012.

N_{shoot} differed significantly between genotypes (*P < 0.05*) and N treatment (*P < 0.001*) in 2014. Spelt cv. Oberkulmer had the most N_{shoot} at NN while Emmer 2 uptake most N at HN. N treatment (*P < 0.001*) was highly significant for N_{shoot} of the species (*P < 0.05*), where Spelt and Emmer had higher N_{shoot} than bread wheat regardless of the N

treatment. Grain yield significantly differed between genotype ($P < 0.001$) for both experiments where bread wheat cv. JB Diego produced the highest grain yield while Einkorn species had less grain yield. N application ($P < 0.05$) significantly increased grain yield in 2014 (data not shown).

3.5. Relationship between green area duration, aboveground biomass production and plant N uptake(N_{shoot})

There was a significant correlation between green area duration and aboveground biomass production at maturity in 2012 (r = 0.66, $P < 0.05$) while explaining more than 37% of the aboveground biomass production variation. Leaf senescence process can be divided into two phases; full functionality phase and rapid senescence phase [48]. Genotypes with a long fully functionality phase produce more assimilates due to active photosynthesis, increasing biomass production and grain yield. Through plant uptake, the supply of N during grain filling may delay the onset of senescence and N remobilization from leaves to grains. On the other hand, when grain N demand exceeded by plant N uptake acceleration of N remobilization may be occurred [49].

Several co-located QTLs of stay-green traits in some environments with seminal root angle and seedling root number for the yield of a population derived from a cross between stay-green cultivar of SeriM82 and senescent cultivar of Hartog of wheat, which was grown in eight environments under drought treatments. Further, some more stay green QTLs were co-located with the only yield QTLs suggests that genetic regions associated with seminal root angle and seedling root number are not only accountable for the high yielding of stay-green phenotype [1]. Some pieces of evidence are available to suggest the relationship betweenstay-green QTLs and root system architecture in sorghum. Post-anthesis water acquisition improved through favourable root traits may be involved in stay-green properties of stay-green genotypes of sorghum [3].

However, the relationship between green area duration and aboveground biomass production of the genotypes in 2014 experiment was not significant, but a positive trend was identified under HN (r = 0.55, $P<0.2$). This may be due to the narrow genetic background of the genotypes used in the 2014 experiment, where three Einkorn genotypes were neglected from the study due to poor seed germination and seedling growth.

The stay-green mutants of durum wheat had a higher NUE than wild types but grain N content was not different. Therefore, the grain protein concentration of mutant was lower than the wild type. There is a possibility to break the negative relationship between grain yield and grain protein concentration by improving storage capacity of non-protein N in leaves and stems [17]. Indirectly, this reducesthe detrimental effects of excess N fertilizer on the environment by reducing leaching, denitrification and volatilization. Stay long period in green colour indicates more N stored in the leaves of ancient wheat plants.

When considering green area duration of the genotypes at post-anthesis grain filling, it was observed that Spelt had the highest green area duration due to delayed onset of senescence (m) in 2012 experiment. High aboveground biomass production might be due to high green are duration and delayed onset of senescence. Not only that, there may be a positive relationship between high green area duration and delayed onset of senescence and total N_{shoot} of Speltgenotypes. Further, it was suggested that Speltcan utilize nutrients more efficiently when grown in low input conditions [50]. Another study on recombinant inbred line mapping population of wheat x Spelt showed that larger grain is due to early anthesis and delayed flag leaf senescence [51]. The grain filling rate, the water content of the grain and maximum water content may be affected by early anthesis and delayed but fast flag leaf senescence.

4. Conclusion

Delayed onset of flag leaf senescence, slow senescence rate and prolonged leaf greenness were observed in Spelt genotypes, especially in cv. Oberkulmer when compared to the other genotypesand positively influenced when increased the level of nitrogen fertilizer. SPAD declining, visual senescence score and NDVI during post-anthesis grain filling period have a strong relationship with aboveground biomass production at harvest. High aboveground biomass production and plant nitrogen uptake of Speltpartially be explained by delayed onset of flag leaf senescence, slow senescence rate and prolonged leaf greenness. It is proposed that as an ancient wheat species,Speltcarriesimportant traits related to flag leaf greenness which eventually enhanced plant N uptake and biomass production. Therefore, it could be used as genetic materials to improve modern bread wheat.

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Supplemental Data

Table 1: Estimated parameters for the logistic model for flag leaf greenness of ten genotypes in 2012 experiment. Y; greenness score, A; lower asymptote in the unit of Y axis, C; the difference between upper and lower asymptote in the unit of Y axis, B; doubled relative senescence rate at the time M, M; the time when the absolute senescence rate is at maximum, t; accumulated thermal time after anthesis in degree days (□d). R^2 shows the percentage of fitness of absolute values to the logistic model.

GT	Y=A+ C/(1+e^(-B(t-M)))				R^2 (%)	Green area duration
	A	C	B	M		
JB Diego	-0.56	10.68	-0.0115	539.80	99.0	5166
Xi 19	-0.18	10.00	-0.0155	534.90	99.6	5091
Spelt Tauro	0.15	9.92	-0.0127	526.00	99.5	5240
Spelt SB	0.19	9.92	-0.0138	496.50	99.1	5048
Spelt Ober	-0.24	10.12	-0.0146	606.40	99.2	5886
Emmer 1	-0.47	11.06	-0.0103	364.20	98.3	3690
Emmer 2	-1.21	11.65	-0.0087	440.20	99.2	4163
Einkorn 1	-6.21	16.51	-0.0083	399.10	97.9	2762
Einkorn 2	-3.54	13.54	-0.0106	363.20	98.5	2825
Einkorn 3	-0.28	10.42	-0.0122	392.20	99.6	3860
SED (*df* = 27)	0.906***	1.086***	0.001252***	15.341***	*NA*	62.9***

***Significant at *P*< 0.001, *NA*; not applicable**

Table 2: Estimated parameters for the logistic model for flag leaf greenness of seven genotypes in 2014 experiment under three N regimes. Y; greenness score, A; lower asymptote in the unit of Y axis, C; the difference between upper and lower asymptote in the unit of Y axis, B; doubled relative senescence rate at the time M, M; the time when the absolute senescence rate is at maximum (or time at ½ C), t; accumulated thermal time after anthesis in degree days (^{0}Cd)

GT	Y=A + C/(1+e^(-B(t-M)))											
	A			B			C			M		
	HN	LN	NN	HN	LN	NN	HN	LN	NN	HN	LN	NN
JB Diego	-0.051	-0.377	-0.026	-0.01689	-0.00901	-0.00833	8.41	10.25	10.96	401.50	414.40	381.80
Xi 19	0.693	0.290	-0.147	-0.01899	-0.01818	-0.00929	9.31	9.44	10.66	439.00	440.40	363.00
Spelt Tauro	0.443	-0.109	0.069	-0.01665	-0.01382	-0.01424	9.64	10.04	9.95	437.30	444.80	386.90
Spelt SB	0.321	-0.037	0.006	-0.01436	-0.01337	-0.01359	9.88	10.13	10.03	426.90	429.60	377.50
Spelt Oberkulmer	0.015	-0.078	-0.138	-0.02010	-0.02006	-0.01488	9.86	9.71	10.07	490.60	481.90	425.10
Emmer 1	-0.093	-0.096	-0.087	-0.02004	-0.01738	-0.01607	8.55	9.59	9.50	324.57	330.10	312.10
Emmer 2	-0.014	-0.118	-0.083	-0.01999	-0.01314	-0.01518	8.65	10.00	9.79	322.80	301.00	255.80
SED GT (*df* = *36)*		0.098***			0.00112***			0.155*			3.87***	
N (*df* = 4)		0.059**			0.00072*			0.077*			2.38***	
GT x N (*df* = 39.7)		0.167*			0.00193***			0.261***			6.66***	

*** **Significant at *P < 0.001*, **significant at *P < 0.01*, *significant at *P < 0.05***