

## MINERAL DEFICIENCY STRESS

**Responses to N Deficiency in Stay Green and Non-Stay Green Argentinean Hybrids of Maize**

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**Abstract**

Breeding has developed better yielding maize hybrids for low N environments, which also have delayed leaf senescence ('stay green' trait, SG). Here, we studied whether the SG trait can further improve yield of modern hybrids under N-limiting conditions. In two field experiments, four maize hybrids with different senescence behaviour were grown under three N fertilization levels, from 0 to 200 kg N ha<sup>-1</sup> (N0, N100 and N200). After silking, hybrids differed for senescence depending on the canopy layer ( $P < 0.05$ ): the SG AX878 only delayed senescence at the mid and upper canopy layers while the SG NK880 delayed senescence of all layers. Across N doses, higher yields were achieved by both SG hybrids, AX878 and NK880 ( $P < 0.05$ ) but yield was not only determined by senescence behaviour. Kernel weight (KW) response to N availability was larger for SGs than for their non-'stay green' counterparts. Delayed senescence in SG hybrids was not related to higher post-silking N uptake but to higher ( $P < 0.05$ ) %N in leaves and lower ( $P < 0.05$ ) %N in kernels at harvest (below the critical 1.1 % under N deficiency). Across N levels, KW positively related to N content per kernel, with a steeper slope ( $P < 0.05$ ) for the SG hybrids. Taken together, our results suggest that a condition where N limits kernel growth, in a scenario of saturating C availability, may be common to stay green genotypes of maize.

**Introduction**

In most agricultural soils N availability is often limiting, and plants might experience N deficiency if additional N is not supplied through fertilization. Therefore, the use of N fertilizers has increased dramatically in the last eight decades, from 1.3 millions of metric tons in 1930 up to 90 MMt in 2004 (Good et al. 2004). Nevertheless, the use of N fertilizers is unaffordable for poor farmers and has several negative environmental impacts (e.g. Peñuelas et al. 2012, Fowler et al. 2013). Increasing N use efficiency by crops may help to reduce fertilizers needs.

In maize, breeding has produced higher yielding genotypes for low N environments (Rajcan and Tollenaar 1999, Ding et al. 2005, Echarte et al. 2008, Ciampitti and Vyn 2012). Nonetheless, genetic variability for yield response to N availability is still high (Wang et al. 1999). Moreover, in spite of the fact that most breeding programmes are developed under moderate-high levels of fertilization

(Bänziger et al. 2006), genotype  $\times$  environment interactions have been reported for yield performance across N levels (Bänziger and Lafitte 1997, Gallais and Hirel 2004, Gallais and Coque 2005, Paponov et al. 2005, Hirel et al. 2007, Worku et al. 2007). A deeper knowledge of physiological traits underlying this genotypic variability may contribute to elucidate target traits for breeding.

Among traits that have changed during breeding, some are particularly important concerning N dynamics within the plant and could be a target for further improvement. Canopy senescence is delayed (the 'stay green' trait, SG) in modern hybrids (Ding et al. 2005, Duvick 2005, Echarte et al. 2008). This implies a longer period of photosynthetic activity, but, at the same time, a longer N retention in leaves during grain filling. N deficiency reduces net crop photosynthesis (Uhart and Andrade 1995, Hirel et al. 2007, Echarte et al. 2008) and accelerates leaf senescence (Uhart and Andrade 1995, Ding et al. 2005), all of which could be alleviated by selecting for genotypes with further

delayed leaf senescence, compared to reference modern hybrids.

On the other hand, breeding has also reduced N concentration in kernels, with modern hybrids reaching values of 1.2 % (Ciampitti and Vyn 2012), close to the lower boundary of the effective ratio between ear dry matter (DM) and N content, reported to be in the range from 1.1 % to 1.4 % (Ciampitti and Vyn 2011). Below this critical value, kernel weight (KW) is affected directly by N shortage through alterations in the activity of specific enzymes involved in C utilization by kernels (Cazetta *et al.* 1999, Below *et al.* 2000). Hence, further reductions in kernel N concentration through breeding may lead to physiological constraints to growing kernels.

These two changes brought about by breeding, that is senescence delay and lower kernel N concentration, seem to remain linked in modern maize hybrids, as several reports show that delayed senescence is related to lower kernel N concentration (He *et al.* 2001, Mi *et al.* 2003, Subedi and Ma 2005, Kosgey *et al.* 2013, Acciaresi *et al.* 2014, Antonietta *et al.* 2014). Nonetheless, the physiological processes underlying this are still not clear. A negative relationship has been reported between N remobilization and N uptake after silking (Gallais and Hirel 2004), and this has been explained as a trade-off between maintaining photosynthetic activity and root N uptake vs. protein degradation and N remobilization from leaves to kernels. However, several studies show that senescence delay is not necessarily linked to higher post-silking N accumulation in maize (Martin *et al.* 2005, Subedi and Ma 2005, Antonietta *et al.* 2014). In these cases, N concentration in kernels may be reduced because limited remobilization is not compensated for by higher post-silking N accumulation (Antonietta *et al.* 2014).

Among modern genotypes, the delayed senescence trait has been related to higher yields under low N environments in maize populations (Bänziger *et al.* 1999, 2002). However, few studies analysed the impact of the SG trait on yield in modern maize hybrids under field conditions and with limited N availability. Some works report no advantage of SG hybrids under low N (He *et al.* 2001) while others report that SG hybrids outyield non-SG hybrids under N limitations (Mi *et al.* 2003). Thus, the main hypotheses to be addressed in the present work were that in low N environments, modern maize hybrids showing the SG trait (i) achieve higher yields, through (ii) higher post-silking C accumulation and (iii) higher post-silking N uptake.

## Materials and Methods

### Experimental design and crop management

Two field experiments were conducted in 2010–2011 (Exp. I) and 2011–2012 (Exp. II) at the Experimental Field of

Facultad de Ciencias Agrarias y Forestales (Universidad Nacional de La Plata) (34° 54' 24" S; 57° 55' 56" W, Argentina). Treatments (N levels and genotypes) were arranged in a split-plot design where N level was randomized in the main plot and genotypes were randomized in each subplot. Subplots consisted of 4 rows, 0.7 m apart and 7 m long (19.6 m<sup>2</sup>). Treatments (combinations of N × hybrid) were laid out in four blocks.

In the main plots, three N levels were achieved by varying urea doses, hereafter termed N0, N100 and N200. In N0, no urea was applied. In N100, 100 kg N ha<sup>-1</sup> was applied at V3 (Ritchie *et al.* 1996), while in N200, 200 kg N ha<sup>-1</sup> was split in two equal doses, at the V3 and V6 stages. In the N200 treatment, ammonia volatilization might have increased at the V6 stage, compared to V3, due to higher soil temperatures. However, for a typical argiudol such as that used here, N losses of broadcast urea are reported to be considerably low, ranging from 2.6 % to 13.3 % of the N applied depending on N rate (from 0 to 210 kg N ha<sup>-1</sup>) and the time of fertilization (planting or V6) (Rozas *et al.* 1999). In any case, to minimize N losses through volatilization, irrigation was applied after each fertilizer application.

In the subplots, four maize hybrids belonging to different breeding programmes were sown: NK880, AX878, AW190 and DK682. These hybrids were chosen based on their different senescence behaviour but similar days to silking and to physiological maturity. Previous observations (unpublished) showed that under field conditions in La Plata, the NK880 and AX878 hybrids (hereafter referred to as 'stay green', SG) show a functional delay in senescence compared with the DK682 and AW190 hybrids (non-'stay green', NSG).

The preceding crop was maize. The soil was a typical argiudol (USDA classification) with a fine texture and an underlying layer (40 cm deep) of expandable clay. Soil samples were taken to assess organic matter and total N content in this field. Organic matter was 2.975 % between 0 and 20 cm, 2.045 % between 20 and 40 cm and 1.715 % between 40 and 60 cm. Total N in these same samples was 0.16 % between 0 and 20 cm, 0.13 % between 20 and 40 cm and 0.10 % between 40 and 60 cm. Total P content in the upper 20 cm of soil was 12 kg P ha<sup>-1</sup>.

In both experiments, all treatments received 45 kg P ha<sup>-1</sup> and 18 kg N ha<sup>-1</sup> as diammonium phosphate which was incorporated with a disc harrow at sowing. Seeds were sown manually on 12 November 2010 (Exp. I) and 26 October 2011 (Exp. II). Three seeds were placed in each hill, and seedlings were later thinned at the V2 stage (Ritchie *et al.* 1996) to obtain a final plant density of 8 pl m<sup>-2</sup>. Seedling emergence took place ca. 7 days after sowing. Plots were irrigated as needed from emergence to maturity to prevent water deficit and were maintained free

of weeds by application of herbicides when needed. Silking took place on January 31st in Exp. I and on January 17th in Exp. II. Differences in silking date between treatments or hybrids were of 3 days at most. Thermal time computations started at sowing, using mean daily air temperature and a base temperature of 8 °C (Ritchie and NeSmith 1991). In both experiments, mean temperatures after silking were always lower than the optimum temperature for maize growth (34 °C, Wilkens and Singh 2003). For this reason, we used a simple linear model to calculate thermal time, which was expressed as the sum of °C day<sup>-1</sup> (°Cd).

### Plant sampling

Destructive samplings were made at silking and physiological maturity for DM determinations. In each destructive sampling, two adjacent plants from a central row of each subplot (eight plants per treatment) were harvested. Sampling of adjacent plants reduced the potential biases arising from human plant selection. Moreover, the plants sampled were flanked by visually equivalent plants in the same row and in the rows on their sides, thereby avoiding the inclusion of dominant or dominated plants in our samples. In each destructive sampling, green area per leaf and leaf chlorophyll content were determined for each individual leaf. Green area per leaf was measured using a Licor 3000 (Li-Cor, Lincoln, Nebraska, USA) area meter. Leaf chlorophyll content was estimated with a non-destructive chlorophyll meter (SPAD-502, Minolta, Osaka, Japan) in leaves where the dry area was <50 % of total leaf area. Five measurements alongside each leaf were averaged to obtain a single SPAD value per leaf. A 'zero' SPAD value was assigned for leaves where the dry area represented more than 50 % of total leaf area, as well as for completely senesced leaves.

Dry matter measurements were made on the same plants. Plants were dissected in three parts: (i) stalks with leaf sheaths and tassels, (ii) leaf blades and (iii) ears. All parts were dried in a forced-air oven at 60 °C to constant weight. Net DM accumulation during grain filling was calculated as the difference between total DM at physiological maturity and total DM at silking.

The ears of 20 plants per subplot (80 plants per treatment) were hand harvested at maturity to estimate average yield ha<sup>-1</sup>. No barren plants were found in any of the experiments. A threshing machine was used to separate grains from the cob. Grains were weighed, and an aliquot was oven-dried to constant weight to calculate the percentage of grain moisture. Yield data are expressed as grain dry weight (g) per plant. Mean individual KW was determined in ears of two plants per subplot threshed manually, by counting and weighting the total number of kernels per plant. Kernel number per plant (KNP) was estimated on

the basis of KW of two plants per subplot, and grain yield of 20 plants per subplot sampled at final harvest, essentially as in Echarte et al. (2000).

### Progression of canopy senescence

In each experiment, two plants per subplot (eight plants per treatment) were tagged at silking for non-destructive determinations. Senescence progression was recorded weekly (or two times a week after 30 days after silking, DAS) in tagged plants. Senescence was estimated visually in terms of percentage of senescent tissue in each leaf of the plant. Green leaf area (GLA) was then calculated as the difference between leaf area measured in the first destructive sampling at around silking (described above), and dead leaf area estimated visually. GLA per plant was calculated by adding up the green area of each leaf. Data for GLA were separated into three canopy layers, each comprising approximately 30–40 % of total leaf area: an upper layer (leaves from the top of the plant up to the leaf above the ear), a mid layer (the ear leaf and two leaves below) and a lower layer (from three leaves below the ear to the base of the plant).

### Plant nitrogen (N) determinations

Plant N content was measured in the N0 and N200 treatments. Total N concentration was analysed by micro Kjeldahl analysis (LANAIS N-15; Universidad Nacional del Sur, Bahía Blanca, Argentina) in samples taken around silking and near physiological maturity. At silking, plants were divided into: (i) stem + leaf sheaths + tassel, (ii) ears and (iii) leaves, whereas at physiological maturity, ears were dissected into kernels and cobs+husks. Total N content (Nc) was calculated as the product of N concentration by dry weight of each compartment. N uptake during the post-silking period was calculated as the difference in plant Nc between the last (maturity) and the first (silking) sampling dates. Stover N remobilization was calculated as the difference between stover (leaves + stem) Nc at silking and stover Nc at physiological maturity.

### Data analysis

Data were analysed with the STATISTICA 7.1 software (StatSoft, Inc., Tulsa, Oklahoma, USA). Year and treatment effects and interaction between them were analysed by analysis of variance (ANOVA). The Levene's test was used to corroborate the assumptions of the ANOVA. For all of the dependent variables analysed, year (experiment), N level, hybrid and block were considered as fixed factors. When factors or their interactions were significant, means were compared by the LSD test ( $P = 0.05$ ) and results from this

comparisons are described in the text. To assess the significance of linear regressions, the *F*-test was used ( $P < 0.05$ ). The intercepts and slopes of linear regressions from different treatments were compared using the dummy variable analysis.

## Results

### Meteorological data and phenological variation among experiments

Sowing date occurred 17 days earlier in Exp. II compared to Exp. I, which resulted in moderate differences in meteorological conditions during specific phenological events. Cumulative thermal time from sowing to silking and total crop cycle length were very similar among experiments (1137–1078°Cd and 2028–2110°Cd in experiments I and II, respectively). The later sowing date in Exp. I led to higher average mean temperatures from sowing to silking (22.0 vs. 20.8 °C in experiments I and II, respectively, Fig. 1a) but to lower (–6 %) cumulative solar radiation (1660 vs. 1775 MJ m<sup>-2</sup> in experiments I and II, respectively, Fig. 1b). By contrast, from silking up to physiological maturity, average mean temperatures were lower in Exp. I (20.4 vs. 23.6 °C in experiments I and II, Fig. 1a) while cumulative solar radiation was 6 % higher (1165 vs. 1094 MJ m<sup>-2</sup> in experiments I and II, Fig. 1b). In both experiments, maximum temperatures were always below 34 °C, while minimum temperatures dropped below 10°C only very late in the crop cycle (after 140 days after emergence).

### Progression of canopy senescence

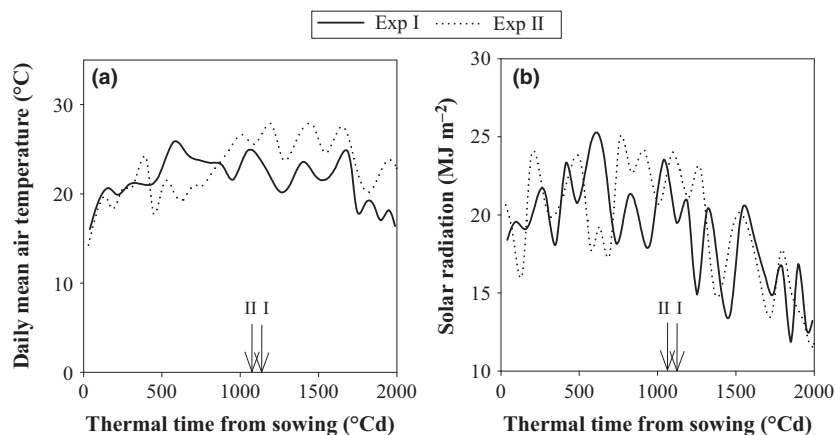
N deficiency significantly reduced total GLA per plant at silking (Table 1). As shown by the significant  $E \times N$  interaction, this N effect was more pronounced in Exp II, where GLA (averaged across hybrids) was only 86 % of

the GLA achieved in Exp. I. Moreover, GLA at silking varied between hybrids: the AW190 hybrid had in general higher GLA ( $P < 0.05$ ) than the rest of the hybrids, although these differences were clearer with increasing N doses.

In both experiments, and at all time points from silking to physiological maturity, GLA per plant was significantly different among canopy layers, N levels and hybrids (Table S1). A significant  $N \times$  canopy layer interaction was detected at mid grain filling in Exp. I, and at all times after silking in Exp. II, because N deficiency (N0) accelerated senescence of lower leaves more than that of the middle or upper canopy layers (Figs 2 & 3, Table S1).

Hybrid differences depended on canopy layers, as shown by the significant  $L \times H$  interaction at early and mid stages of grain filling (Table S1). Some hybrids showed a similar GLA at certain canopy layers while differing in others. For instance, under N deficiency (N0), the SG hybrid NK880 showed the highest GLA values of all hybrids at the lower layer in both experiments (Figs 2g & 3g), whereas a similar GLA was found for NK880 and AX878 at the upper layer (Figs 2a & 3a). This difference between AX878 and NK880 was maintained across N levels: at N100 and N200, GLA progression was quite similar in these hybrids at the upper layer (Figs 2b,c & 3b,c) while at the lower layer NK880 maintained comparatively higher GLA values than AX878 (Figs 2h,i & 3h,i). A similar behaviour of GLA was observed in the AW190 hybrid, which maintained high GLA values at the upper layer (particularly in Exp. I), but not at the lower one. In contrast to the variable response of GLA depending on canopy layer observed in AX878 and AW190, the DK682 hybrid showed a constant NSG behaviour of GLA across experiments, canopy layers and N levels, *that is* GLA of all canopy layers declined faster in DK682 than in the rest of the hybrids.

Similar hybrid responses were found for leaf chlorophyll content across experiments, N levels and canopy layers



**Fig. 1** Daily mean air temperature (a) and solar radiation (b) as a function of thermal time from sowing. Arrows indicate the R1 stage in each experiment.

**Table 1** Green leaf area per plant at silking (GLA, m<sup>2</sup> pl<sup>-1</sup>), post-silking dry matter (DM) accumulation (g pl<sup>-1</sup>), total DM at harvest (g pl<sup>-1</sup>), yield per plant (g pl<sup>-1</sup>), kernel number per plant (KNP) and kernel weight (KW, mg kernel<sup>-1</sup>) in each treatment (N level × Hybrid) and in experiments I and II

Exp.	N level	Hybrid	GLA at silking	Post-silking DM accumulation	Total DM	Yield	KNP	KW
I	N0	NK880	0.453	127	233	110	479	230
		AX878	0.475	113	228	110	479	232
		AW190	0.463	121	224	106	447	241
		DK682	0.392	145	204	98	426	230
	N100	NK880	0.526	172	294	135	478	278
		AX878	0.516	135	259	133	492	270
		AW190	0.538	108	229	131	492	256
		DK682	0.416	164	264	130	483	259
	N200	NK880	0.475	158	271	142	507	280
		AX878	0.508	157	271	125	460	273
		AW190	0.555	144	266	136	553	247
		DK682	0.475	135	250	122	480	254
II	N0	NK880	0.382	90	253	91	400	225
		AX878	0.381	110	254	97	374	257
		AW190	0.388	48	217	85	349	244
		DK682	0.387	71	197	92	352	262
	N100	NK880	0.504	97	299	138	488	284
		AX878	0.437	108	287	145	451	323
		AW190	0.507	94	302	132	486	271
		DK682	0.428	78	251	131	460	288
	N200	NK880	0.511	94	303	143	483	297
		AX878	0.486	87	272	160	480	334
		AW190	0.571	76	297	129	485	267
		DK682	0.517	103	308	141	462	306
P (Exp)		+	***	*	ns	***	***	
P (Nitrogen)		***	+	***	***	***	***	
P (Hybrid)		**	+	+	*	ns	**	
P (E × N)		*	ns	ns	***	*	ns	
P (E × H)		ns	ns	ns	+	ns	*	
P (N × H)		ns	ns	ns	ns	ns	ns	
P (E × N × H)		ns	+	ns	ns	ns	ns	
LSD (N × H)		0.066		32	41	15	59	27

P values obtained from ANOVA and LSD values ( $P < 0.05$ ) for  $N \times H$  comparisons are shown after mean values for each treatment (ns: non-significant; + $P < 0.10$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ).

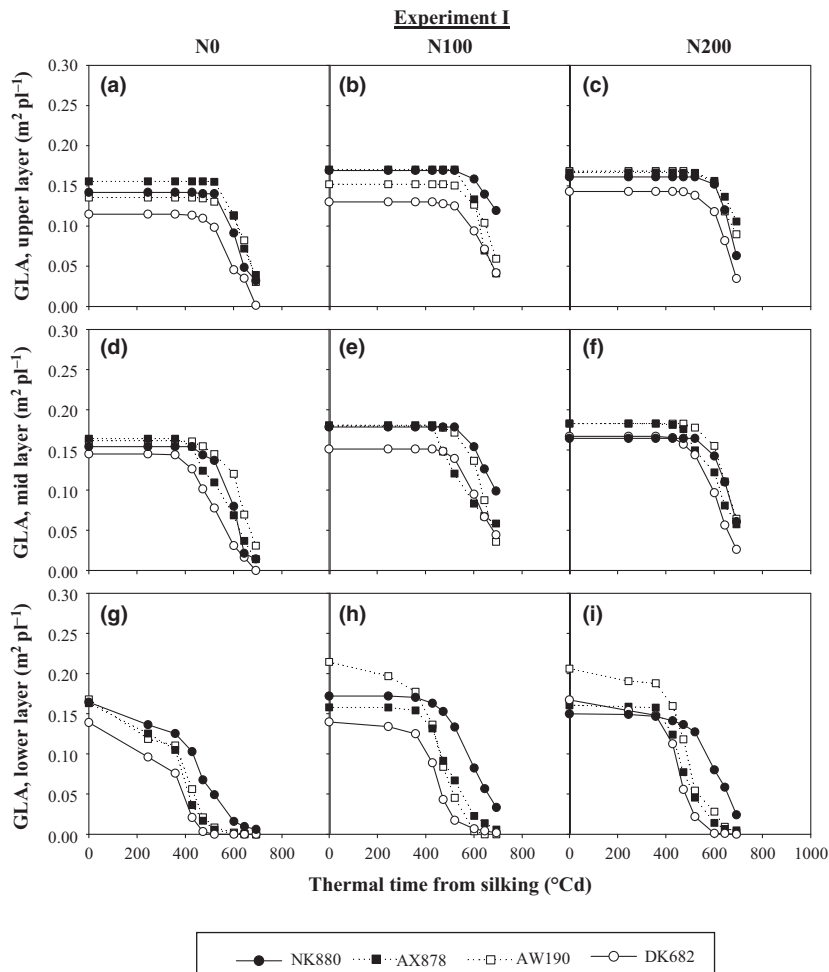
(Figs S1 & S2). At late stages of grain filling, and specially at N0, the SG hybrid NK880 showed high SPAD values at all canopy layers while the AX878 hybrid combined high SPAD values at the upper canopy layer with lower values (comparable to the NSG hybrid DK682) in basal leaves. DK682 had the lowest chlorophyll contents at all canopy layers and N levels tested.

Overall, on the basis of GLA dynamics and chlorophyll content, NK880 showed a strong SG character at all canopy layers and N levels tested, whereas AX878 showed a SG character at the mid and upper canopy layers but relatively early senescence of the lower leaves. By comparison, the behaviour of AW190 varied between years, being very similar to AX878 in Exp. I but showing a faster senescence (especially at the upper canopy layer) in Exp. II. In particular, at late stages of grain filling, SPAD values at the upper

canopy layer were lower in AW190 than in AX878. Finally, DK682 was strongly NSG, particularly at N0.

#### Dry matter accumulation, yield and yield components

Consistently with the N effects detected for GLA achieved at silking, post-silking DM accumulation was particularly lower in Exp. II ( $P < 0.001$ ) and was reduced in the N0 treatment ( $P < 0.1$ ) (Table 1). As indicated by the significant  $E \times N \times H$  interaction ( $P < 0.1$ ), hybrid differences were not related to senescence behaviour in Exp. I, but they did in Exp. II. For instance, at the N0 and N100 treatments in Exp. I, the strong SG NK880 and the NSG DK682 achieved the highest values of post-silking DM accumulation, in spite of accelerated senescence in DK682. In contrast, in Exp. II (where plants achieved lower GLA at



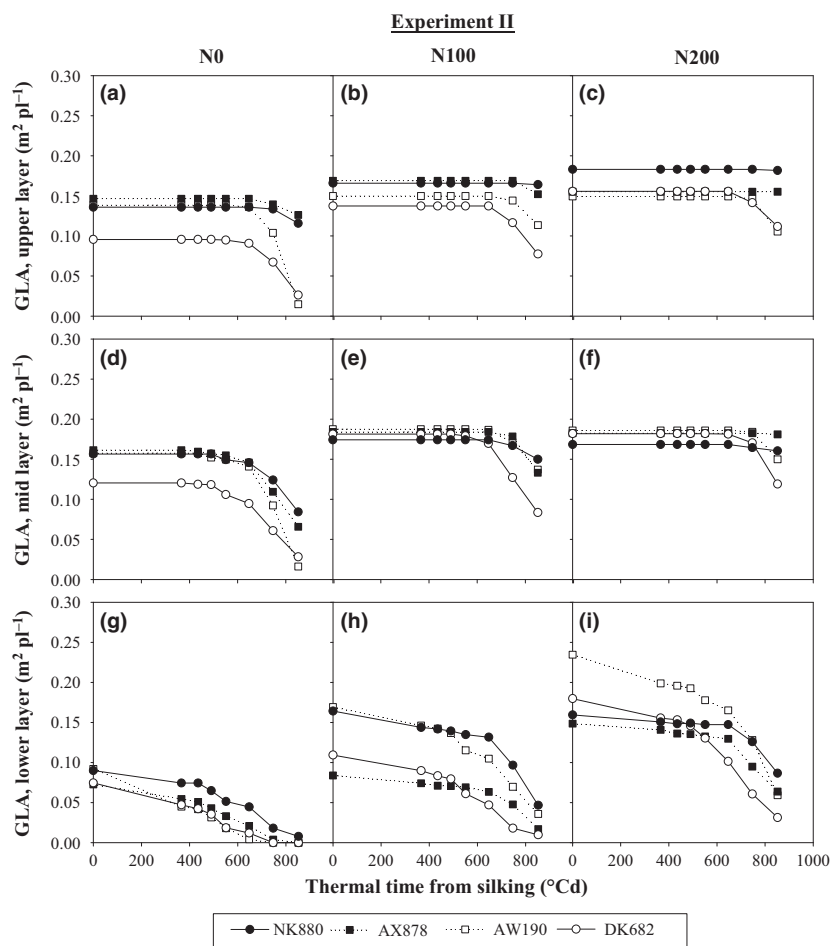
**Fig. 2** Green leaf area (GLA) per plant from silking to physiological maturity in Exp. I, in each N level: N0 (a, d, g), N100 (b, e, h) and N200 (c, f, i). In each treatment, GLA is shown separately for the upper (a, b, c), mid (d, e, f) and lower (g, h, i) canopy layer.

silking, and hence, lower leaf area indexes) hybrid differences coincided with the observed GLA progression as the maximum values for post-silking DM accumulation in the N0 treatment where achieved by both SG hybrids AX878 and NK880 (Table 1). At N200 in Exp. I, and N100 and N200 in Exp. II, post-silking DM accumulation was quite similar in all hybrids. Total DM at harvest was significantly lower in N0 compared to either N100 or N200 (Table 1). Hybrid differences were detected for total DM at harvest ( $P < 0.1$ ) because, most notably at N0, both SG hybrids NK880 and AX878 achieved higher values of plant DM than AW190 and the NSG DK682 (Table 1). As observed in post-silking DM accumulation, these differences were more pronounced in Exp. II.

In both experiments, yield was significantly higher in N100 compared to N0 (25–50 % higher in experiments I and II), but there was no consistent yield increase from further adding an extra 100 kg N Ha<sup>-1</sup> (compare N100 with N200, Table 1). A similar response was found for both yield components, KNP and KW, which significantly increased with fertilization in N100 but showed no changes

between N100 and N200 (Table 1). From the data presented in Table 1, it can be seen that, at N0, yield related to KNP ( $r^2 = 0.86$ ,  $P < 0.001$ ) but not to KW ( $r^2 = 0.11$ , NS). By contrast, at N100 and N200, yield related mainly to KW ( $r^2 = 0.77$ ,  $P < 0.01$  and  $r^2 = 0.70$ ,  $P < 0.01$  for N100 and N200, respectively) while a non-significant relation was found for KNP ( $r^2 = 0.29$ , NS and  $r^2 = 0.05$ , NS, for N100 and N200, respectively). A significant  $E \times N$  effect was detected for yield due to lower yields at the N0 level in Exp. II which, in turn, were explained by lower KNP achieved at N0 (Table 1).

Across experiments and N levels, hybrids differed significantly for yield ( $P < 0.05$ ): both SG hybrids AX878 and NK880 achieved the highest yields (average yields of 126.5 and 128.3 g m<sup>-2</sup>, respectively), while the lowest yields were achieved by AW190 and DK682 (119.8 and 119 g m<sup>-2</sup>). Nonetheless, an  $E \times H$  interaction for yield was also detected ( $P < 0.1$ ): in Exp. I, yields were highest in the strong SG NK880 and lowest in the NSG DK682, with intermediate values in both AX878 and AW190 (Table 1). In Exp. II, this ranking changed because yields



**Fig. 3** Green leaf area (GLA) per plant from silking to physiological maturity in Exp. II, in each N level: N0 (a, d, g), N100 (b, e, h) and N200 (c, f, i). In each treatment, GLA is shown separately for the upper (a, b, c), mid (d, e, f) and lower (g, h, i) canopy layer.

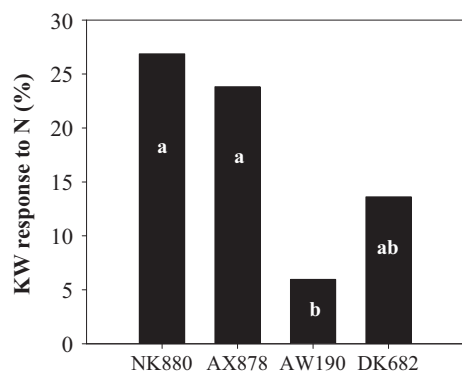
were highest in AX878 and lowest in AW190, with intermediate values for both NK880 and DK682 (Table 1), implying that yield differences were not only dependent on senescence behaviour. In addition,  $E \times H$  interaction was also detected for KW ( $P < 0.05$ ), which did not differ between hybrids in Exp. I but it did in Exp. II, where it was highest in AX878 and lowest in AW190, with intermediate values for NK880 and DK682. Overall, in both experiments, the response of KW to N fertilization (calculated as the per cent increase from N0 to N200, taking as a reference N0) differed between hybrids ( $P < 0.05$ ): both SG hybrids NK880 and AX878 showed larger changes in KW, the NSG DK682 showed intermediate values, with the smallest changes for AW190 (Fig. 4).

#### N accumulation and partitioning at silking and physiological maturity

To evaluate the effects of N availability, plant N content (Nc) was analysed in N0 and N200, which was taken as a reference of a non-stress situation. Plant Nc achieved at silking was significantly increased by fertilization (Table 2). This

effect was also significant across plant compartments, that is leaves, ears (except for Exp. I) and stems (data not shown), while no hybrid differences were detected for Nc in these compartments (except for a higher Nc in leaves of DK682 at silking in Exp. II, data not shown). Post-silking N uptake also increased with fertilization ( $P < 0.05$ , Table 2) and varied between hybrids ( $P < 0.1$ ). In spite of its NSG trait, the DK682 hybrid showed the highest post-silking N uptake across experiments, which in turn means that the SG trait was not linked to higher post-silking N uptake compared with a reference genotype such as DK682. Plant Nc at harvest increased with fertilization ( $P < 0.05$ ), but no hybrid differences were detected for this variable (Table 2).

The amount of N remobilized from vegetative organs increased with fertilization (Table 2) and  $E \times H$  interaction was found: in Exp. I, stover N remobilization was highest in the NSG AW190 while in Exp. II, the NSG DK682 showed the highest values of stover N remobilization (Table 2). N concentration (%) in leaves at harvest increased dramatically with fertilization, being 83–101 % higher in N200 compared with N0 ( $P < 0.05$ ), and hybrid differences were also detected ( $P < 0.1$ ), with the SG



**Fig. 4** Kernel weight response to N availability, calculated as:  $100 \times (KW_{N200} - KW_{N0}) / KW_{N0}$ . ANOVA detected significant effects for Experiment (not shown) and Hybrid while  $E \times H$  was non-significant. Same letters indicate homogenous groups according to the LSD test ( $P < 0.05$ ).

hybrids having higher %N in leaves at harvest independently of the N treatment (Table 2). By contrast, %N in stems at harvest showed a slight increase with fertilization (19–13 %) ( $P < 0.05$ , Table 2) and did not differ between hybrids.

#### Relationship between kernel N content and kernel weight

N concentration in kernels at harvest increased with fertilization ( $P < 0.05$ ) and differed between hybrids ( $P < 0.05$ , Table 2): the NSG hybrid DK682 achieved the highest %N in kernels, both at N0 and at N200. In fact, in both experiments, kernel %N in N0 was below 1.15 % in all but the NSG DK682 hybrid. A significant correlation was found between N content (mg) in kernels at harvest and final KW (Fig. 5). Because of the different senescence behaviour of the hybrids, this correlation was analysed separately for the SG hybrids, NK880 and AX878 ( $r^2 = 0.89$ ,  $P < 0.001$ ,  $n = 8$ ) and for the NSG hybrids, AW190 and DK682 ( $r^2 = 0.76$ ,  $P < 0.01$ ,  $n = 8$ ). Comparing both regressions, the slopes of these regressions were larger for the SG than for the NSG hybrids ( $P < 0.05$ ): increases in kernel N led to higher increases of KW in AX878 and NK880 (37 mg dry weight per mg of kernel N) than in AW190 and DK682 (20 mg of dry weight per mg of kernel N). This is consistent with the greater response of KW to fertilization in NK880 and AX878 compared to AW190 and DK682 shown in Figure 4.

## Discussion

### Canopy senescence

In the present work, hybrid differences for canopy senescence were maintained independently of the N doses (see the lack of interaction  $N \times H$  in Table S1), which is in line

with the conservative senescence behaviour reported for these same hybrids across different planting densities (Antoniotta et al. 2014). As indicated by  $L \times N$  interaction (Table S1), the effects of N deficiency on senescence acceleration are more pronounced in the lower leaves of the canopy (Wolfe et al. 1988). This differential response between canopy layers may reflect an ontogenic hierarchy, where older (lower) leaves are more responsive to senescence-accelerating signals, such as ethylene (Lim et al. 2007). By contrast, other senescence-accelerating treatments, such as an impairment of pollination, show lack of interaction between treatment and leaf position, that is senescence is accelerated at all canopy layers (Sadras et al. 2000). This apparent controversy may relate to the nature of the senescence signal, an increase in leaf soluble sugars due to pollination impairment (Sadras et al. 2000) having a more systemic effect than signals associated with natural senescence occurring under N shortage (Criado et al. 2007, Lim et al. 2007).

Compared to the NSG reference (DK682), the strongly SG NK880 delayed senescence at all canopy layers (e.g. compare both hybrids at the upper and lower canopy layers in Fig. 2a,g). By contrast, the SG AX878 maintained a SG behaviour at the upper canopy layer, but did not differ from the NSG DK682 at the lower canopy layer (e.g. Fig. 2g). A similar canopy layer-dependent hybrid behaviour was reported for these same hybrids across planting densities (Antoniotta et al. 2014). Thus, NK880 behaves as a SG at all canopy layers, and this may be due to rather qualitative (non-plastic) alterations in the signal transduction pathways regulating senescence. In contrast, AX878 shows more plasticity, with its SG character restricted to certain developmental scenarios. Such a contrasting hybrid behaviour may also cause differences in N availability for kernels at early stages of development, given the importance of lower leaves as an early source of remobilized N under N shortage (Masclaux-Daubresse et al. 2010).

Overall, senescence behaviour could not be predicted through GLA achieved at silking which was higher for the NSG AW190 (Table 1). Muchow (1988) proposed the concept of a minimum specific leaf N (SLN, around  $1 \text{ g N m}^{-2}$  leaf) above which leaf expansion does not respond to increases in leaf N content. Nonetheless, in this paper, genotypic differences were more pronounced at high N availabilities, after the minimum  $1 \text{ g N m}^{-2}$  leaf was achieved (data not shown). D'Andrea et al. (2009) also reported larger genotypic differences at high N availability suggesting that, genotypic variation for leaf expansion is expressed more intensely after the minimum SLN has been achieved.

### Dry matter accumulation, yield and yield components

After silking, delayed canopy senescence may lead to higher post-silking C accumulation. However, at high leaf area



**Table 2** Plant N content (Nc, g pl<sup>-1</sup>) at silking, post-silking N uptake (g pl<sup>-1</sup>), plant Nc at harvest (g pl<sup>-1</sup>), stover N remobilization from silking to harvest (g pl<sup>-1</sup>) and N concentration (%) in leaves, kernels and stems at harvest in each treatment (N level × Hybrid) and in experiments I and II

N level	Hybrid	Nc plant silking	Post-silking N uptake	Nc plant harvest	Stover N Remob.	Harvest		
						N% leaves	N% kernels	N% stems
N0	NK880	1.36	0.58	1.94	0.65	0.80	0.98	0.41
	AX878	1.49	0.31	1.80	0.82	0.72	0.98	0.39
	AW190	1.46	0.31	1.77	0.83	0.82	0.98	0.41
	DK682	1.01	0.93	1.95	0.48	0.69	1.15	0.39
N200	NK880	1.95	0.65	2.60	0.91	1.65	1.07	0.42
	AX878	1.82	1.01	2.84	0.90	1.46	1.27	0.51
	AW190	1.97	1.23	3.20	1.13	1.16	1.50	0.53
	DK682	1.94	1.43	3.37	1.01	1.30	1.64	0.44
N0	NK880	1.33	0.44	1.78	0.31	0.70	1.03	0.32
	AX878	1.41	0.92	2.33	0.23	0.79	1.11	0.36
	AW190	0.93	0.70	1.63	0.10	0.55	1.12	0.31
	DK682	1.42	1.25	2.67	0.52	0.52	1.69	0.27
N200	NK880	2.59	1.22	3.81	0.69	1.39	1.53	0.35
	AX878	2.14	1.25	3.39	0.40	1.42	1.39	0.46
	AW190	2.38	1.06	3.44	0.61	1.15	1.61	0.27
	DK682	2.71	1.10	3.81	0.86	1.19	1.70	0.35
P (Exp)	*	ns	**	***	ns	***	***	***
P (Nitrogen)	***	**	***	***	***	***	***	*
P (Hybrid)	ns	+	ns	ns	+	***	ns	ns
P (E × N)	**	ns	ns	ns	ns	ns	ns	ns
P (E × H)	ns	ns	ns	ns	***	ns	ns	ns
P (N × H)	ns	ns	ns	ns	ns	ns	ns	ns
P (E × N × H)	ns	ns	ns	ns	ns	ns	*	ns
LSD (N × H)	0.43	0.54	0.65	0.19	0.31	0.18	0.18	0.11

P values obtained from ANOVA and LSD values ( $P < 0.05$ ) for  $N \times H$  comparisons are shown (ns: non-significant; + $P < 0.10$ ; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ).

indexes, PAR interception by the canopy can be above 95 % until late stages of grain filling even in early senescent genotypes (Acciaresi et al. 2014), a situation that may have taken place in our Exp. I. By contrast, in Exp. II, where GLA achieved by silking in the N0 treatment was significantly lower, a delay in canopy senescence may result in higher post-silking DM accumulation, as was observed in the SG hybrids AX878 and NK880.

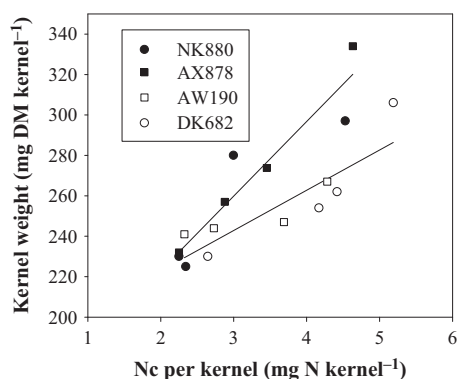
In this paper, fertilization with 100 kg N ha<sup>-1</sup> significantly increased grain yield compared to the non-fertilized treatment, but increasing fertilization rates from 100 to 200 kg N ha<sup>-1</sup> had no further effect on yield. Overall, N stress was mild considering that at the N0 level crop N extraction (averaged across hybrids) reached 149–168 kg N ha<sup>-1</sup> (calculated from Table 2). The detrimental effects of N shortage on yield were due both to lower KNP and KW, although the relation between yield and each yield component varied depending on N level. As reported previously, under N limitations, yield was mostly related to KNP (Paponov et al. 2005, D'Andrea et al. 2008, 2009, Worku et al. 2012). By contrast, at moderate (N100) and high (N200) N levels, variations in yield related mainly to KW, in line with previous reports (Lafitte and Edmeades 1995,

D'Andrea et al. 2008, Worku et al. 2012). The increasing importance of KW determining yield at moderate–high N level is consistent with the linear *plus* plateau function which describes the response of kernel number to increasing N availabilities (Uhart and Andrade 1995).

In the present paper, and across experiments and N levels, higher yields were obtained by both SG hybrids, AX878 and NK880 (Table 1). Averaging the SG and NSG pairs of hybrids (NK880 and AX878 vs. AW190 and DK682), the SG character led to a yield advantage of 8, 3 and 3 % for N0, N100 and N200 in Exp. I, and 6 %, 8 % and 12% for N0, N100 and N200 in Exp. II. Consistently in both experiments, KW response to fertilization was larger in the SGs AX878 and NK880 than in their NSG counterparts (Fig. 4).

### N accumulation and partitioning

Several reports for sorghum (Borrell and Hammer 2000, van Oosterom et al. 2010) and maize (Ma and Dwyer 1998, Rajcan and Tollenaar 1999, Pommel et al. 2006) show that SG hybrids accumulate more N during the post-silking period. However, at least for maize, these results are based on comparisons between hybrids belonging to different



**Fig. 5** Relationship between N content per kernel at harvest ( $\text{mg N kernel}^{-1}$ ) and kernel weight ( $\text{mg DM kernel}^{-1}$ ) analysed separately depending on the senescence behaviour of the hybrids (in each regression line,  $n = 8$ ). Each point represents a treatment (N level  $\times$  Hybrid) in each Exp (N analyses were performed only for N0 and N200 treatments). For the 'stay green' hybrids, NK880 and AX878,  $r^2 = 0.89$ ,  $P < 0.001$ , and parameters obtained from the fitted equation are  $y = 37x + 149$ . For the non-'stay green' hybrids, AW190 and DK682,  $r^2 = 0.76$ ,  $P < 0.01$  and parameters obtained from the fitted equation are  $y = 20x + 183$ . The parameters of both regressions were statistically compared, and significant differences were detected for both the intercepts ( $P < 0.001$ ) and the slopes ( $P < 0.05$ ).

breeding eras: higher post-silking N uptake for SG hybrids of maize could be related to the fact that newer hybrids have higher N uptake on a per unit area basis (Ciampitti and Vyn 2012) and a longer post-silking period (Duvick 2005) rather than to the SG trait itself.

Higher post-silking N uptake has been interpreted as evidence that the SG trait is a direct consequence of an improved N balance: those genotypes accumulating more N during the post-silking period would be able to meet N demand from kernels without remobilizing excessive amounts of N from leaves, thereby delaying senescence. However, several works (Martin et al. 2005, Subedi and Ma 2005, Antonietta et al. 2014) and the results presented here indicate that the SG trait is not always linked to higher post-silking N accumulation.

Within the N supply–demand framework, it is also expected that post-silking N accumulation will be negatively related to stover N remobilization: lower N uptake should be compensated for by increasing the amount of stover N remobilized to grains (Gallais and Hirel 2004). However, this was not the case for the results presented here because, within each N level, there was no relationship between the amount of stover N remobilized and post-silking N uptake (Table 2). This may be related to lower leaf N remobilization efficiency in SG genotypes (Mi et al. 2003), as leaf N concentration at harvest was higher in the SG hybrids, in this (Table 2) and previous works (e.g. He et al. 2001, Pommel et al. 2006). Hence, N remobilization

is not simply the result of a supply–demand balance; substantial regulation of this process may take place in (and be controlled by) the leaves themselves. In support of this view, Christensen et al. (1981) found that the remobilization of N from leaves was not dependent on the presence of an ear. More recently, Martin et al. (2006) reported that over-expression of an enzyme related to N remobilization (leaf glutamine synthetase 1-3) increased grain number, grain yield and, particularly, kernel Nc in maize.

#### Relationship between kernel N content and kernel weight

Ciampitti and Vyn (2011) reported that the effective ratio between kernel DM and kernel N content ranges between 1.1 % and 1.4%. In this work, the range of kernel %N extended beyond these upper and lower limits, reaching as high as 1.7 % and as low as 0.98 % depending on N level and hybrid (Table 2). Hence, plants might have experienced situations where N limited kernel growth, resulting in a significant and positive relation between kernel N content and KW (Fig. 5). By contrast, Worku et al. (2012) found a positive relation between the C/N ratio and yield at %N in kernels higher than 2.38 %, which suggests yield was C limited at these N contents.

Consistently across experiments and N doses, SG hybrids appeared to have lower %N in kernels (Table 2) which is in line with previous reports comparing modern maize hybrids with contrasting senescent behaviour (e.g. He et al. 2001, Mi et al. 2003, Subedi and Ma 2005, Kosgey et al. 2013, Ning et al. 2013, Antonietta et al. 2014). As kernel N is involved in kernel C metabolism through direct effects on the activity of several enzymes (invertase, sucrose synthase and aspartate aminotransaminase, Cazetta et al. 1999), lower %N in kernels of SGs may result in weaker sink strength. In line with this, Gong et al. (2005) reported a slower rate of export of labelled  $^{14}\text{C}$  from flag leaves to grains in a stay green hybrid of wheat compared with a conventional cultivar. The higher response of KW to kernel N found for the SG hybrids in the present paper implies that KW was limited by N availability in these hybrids, and that C availability for kernel growth was higher for NK880 and AX878 than for the NSG AW190 and DK682. Taken together, our results suggest that a condition where N limits kernel growth, in a scenario of saturating C availability, may be common to stay green genotypes of maize.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1** ANOVA results for green leaf area (GLA) at different thermal times after silking in experiments I and II.

**Fig. S1** SPAD readings at different stages from silking to physiological maturity in Exp. I, at different N levels: N0 (a, d, g), N100 (b, e, h) and N200 (c, f, i).

**Fig. S2** SPAD readings at different stages from silking to physiological maturity in Exp. II, at different N levels: N0 (a, d, g), N100 (b, e, h) and N200 (c, f, i).