Senescence and yield responses to plant density in stay green and earlier-senescing maize hybrids from Argentina

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ABSTRACT

Increases in maize (*Zea mays* L.) yield over the past few decades have been associated with breeding for tolerance to progressively higher plant densities. Since high plant density exacerbates interplant competition, it has been suggested that improved resource capture through delayed senescence might be advantageous in such situations. The main objectives of this work were to determine (1) the time-course of canopy senescence, (2) post-silking C and N accumulation and (3) yield responses of contemporary maize hybrids with different expression of the stay green (SG) character grown in a range of plant densities from moderate to intense crowding stress. Three experiments consisting of a combination of different plant densities (from 6 to 10 pl m^{-2}) and commercial hybrids with different timing of senescence were carried out. High density accelerated leaf senescence at the lower canopy layer. The SG hybrids delayed senescence and retained green leaves at physiological maturity at all tested densities. One of these hybrids (NK880), with a strong SG character, retained green leaves at all canopy layers, even at the lower layer exposed to limiting irradiance. Lower canopy leaves maintained high respiratory rates in NK880, while leaves of the NSG hybrid (DK682) senesced and their respiration became not detectable. At the highest tested density, the NSG DK682 achieved greater grain yields than the SG NK880. Increased density reduced kernel weight (KW), and this decrease was more pronounced for the SG NK880 (6–18% comparing 10 vs. 8 pl m^{-2}). In spite of delayed senescence in NK880, no hybrid differences were found for post-silking dry matter accumulation and plant dry matter at physiological maturity. Unexpectedly, plant nitrogen content (Nc) at harvest was similar (Exp. I) or even lower ($P < 0.05$, Exp. II) in the SG NK880. This was the result of lower net N accumulation during the post-silking period (Exp. I) or lower Nc achieved at silking (Exp. II) in the SG NK880. A strong positive relation was found between KW and N concentration in kernels, with %N in kernels being below the critical N concentration to achieve potential KW (around 1.4%) in the SG hybrid. This suggests that yield in NK880 was limited by N. In the SG genotype, N remobilization from vegetative organs did not seem to compensate for the N deficit for optimum grain growth. In summary, at high densities the NK880 hybrid displayed a strong, constitutive SG character, even if it accumulated less N, and senescence delay was not reflected in higher grain yield.

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1. Introduction

In the last decades, maize yield was improved through a tight association between changes in management practices and breeding. Plant density is one of the practices that changed most (Tollenaar and Lee, 2002; Tollenaar et al., 2006). For instance, for the U.S. corn belt, gains in yield of 110 kg ha^{-1} y^{-1} in the period 1960–2000 correlated with increases in plant density of 1000 pl ha^{-1} y^{-1} (Duvick, 2005). At the same time, during the last eight decades, breeding has mostly improved tolerance to crowding stress with no increase in potential yield per plant (Duvick, 1997; Tokatlidis and Koutroubas, 2004; Tokatlidis et al., 2005; Tollenaar and Wu, 1999). Nonetheless, there are exceptions to this, such as in Argentina, where breeding has improved not only crop yield at high densities, but also maximum grain yield per plant (at 3 pl m^{-2}) for hybrids released between 1960 and 2000 (Luque et al., 2006).

Breeding for tolerance to high plant density raises a number of issues. For example, grain yield per unit area of new hybrids is highly dependent on plant density, with smaller and more variable ranges of optimum densities than older genotypes (Fasoula and Fasoula, 2002; Tokatlidis and Koutroubas, 2004; Tokatlidis et al., 2005). This may result in yield penalties in sites where occasional stress (e.g., drought) makes it difficult to predict optimum density (Duvick, 2005; Tokatlidis et al., 2011). Moreover, at high interplant competition levels stand variability increases because,

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e.g., dominant plants show luxury consumption of nutrients to the detriment of dominated ones (Rossini et al., 2011; Tokatlidis et al., 2005; Tollenaar and Wu, 1999). Whether the trend for higher plant density continues in the future or not, improved understanding of morpho-physiological traits related to crowding stress tolerance could help in breeding efforts to enhance grain yield.

The increase in plant density decreases the amount of resources available per plant (Tollenaar et al., 2006). During the reproductive stage, crowding stress reduces kernel number per plant as kernel number fixation is related to plant growth rate around silking (Andrade et al., 2002). After kernel number is defined, grain filling relies mostly on current assimilate production (Borrás et al., 2004). In maize, remobilization of assimilates stored in vegetative tissues is usually very low (Borrás et al., 2004), and almost none in environments where remobilization is impaired (e.g., in cool climates, Maddonni et al., 1998). Higher plant density accelerates the rate of leaf senescence (Borrás et al., 2003), reducing post-silking net photosynthesis and hence, assimilate availability for kernel growth if leaf area index falls below critical values. When increases in plant density are not enough to compensate for reductions in yield per plant (i.e.), at supra-optimal densities, crowding stress results in lower yields per unit area (Echarte et al., 2000; Sarlangue et al., 2007; Tokatlidis et al., 2011; Tokatlidis and Koutroubas, 2004).

An extended post-anthesis period and longer green leaf area (GLA) duration are two of several morpho-physiological traits associated with genetic improvement of maize yield (Duvick, 2005). The stay green trait (SG) is defined as a delay in senescence in comparison with a standard reference genotype (Thomas and Howarth, 2000). Under environmental stress (drought, nitrogen deficiencies), the SG trait has been related to higher yields in maize and sorghum grown in temperate/subtropical regions (Bänziger et al., 1999, 2002; Borrell et al., 2000). Under well-watered conditions, these genotypes might be able to meet nitrogen (N) requirements by the grains with N supplied by root uptake, delaying N remobilization from vegetative parts, and thereby, delaying the onset of senescence (Ciampitti and Vyn, 2011; Rajcan and Tollenaar, 1999b). Many studies of SG genotypes compare older, earlier-senescing vs. modern, SG hybrids (e.g., Ma and Dwyer, 1998; Rajcan and Tollenaar, 1999a,b; Valentinuz and Tollenaar, 2004). Nonetheless, there is little information to address whether in modern maize hybrids the SG trait still represents a yield advantage under crowding stress conditions (i.e.)., without water limitations but with interplant competition for light and nutrients).

At higher densities plants may face a decrease in the amount of soil resources available per plant, (e.g., water and nutrients, Tollenaar et al., 2006). The SG hybrids have been reported to yield more than NSG hybrids under limiting N fertilization (Ma and Dwyer, 1998; Rajcan and Tollenaar, 1999b), and to have larger post-silking light interception (Richards, 2000). Thus, our original hypotheses were that at high plant densities, and compared to a reference NSG genotype: (1) modern SG hybrids delay canopy senescence even at high plant densities (i.e.), the SG trait is a constitutive trait; (2) post-silking C and N accumulation are higher in SG hybrids; and therefore (3) grain yield is higher in SG hybrids. In this paper, our specific objectives were to study (1) the time-course of canopy senescence, (2) post-silking C and N accumulation, and (3) yield responses of maize hybrids with different expression of the SG character grown in a range of plant densities from moderate to intense crowding stress.

2. Materials and methods

2.1. Experimental design and crop management

The experiments were conducted in La Plata (34°54′24″ S; 57°55′56″ W, Argentina), in the Experimental Field of Facultad de Ciencias Agrarias y Forestales (Universidad Nacional de La Plata). Treatments consisted of (i) plant densities (6, 8 and 10 pl m^{-2} in Exp. I; 7, 8, 9 and 10 pl m^{-2} in Exp. II; 8 and 10 pl m^{-2} in Exp. III) and (ii) genotypes. The following maize hybrids were chosen because of their different senescence behavior, but similar days to silking and crop cycle length: DK682 and AW190 (standard non stay green, NSG), and AX878 and NK880 (stay green, SG). Previous observations (unpublished) showed that under field conditions in La Plata, chlorophyll and photosynthetic electron transport rate (which is closely related to photosynthesis, Earl and Tollenaar, 1999) of the ear leaf declined more slowly after silking in NK880 and AX878 (SG) than in DK682 and AW190 (NSG).

The field was under fallow before Exp. I, while for Exps. II and III the preceding crop was maize. Treatments (combinations of density × hybrid) were laid out in three (Exp. I) or four (Exp. II) blocks. Treatments were arranged in a split-plot design where plant density was the main plot and genotypes were randomized in each subplot. Subplots consisted of 4 rows (except for Exp. I when five rows were sown) 6.5 m long and 0.7 m apart. Seeds were sown manually on October 27, 2008 (Exp. I, DK682 and NK880); October 29, 2009 (Exp. II, DK682 and NK880); and November 12, 2010 (Exp. III, DK682, AW190, AX878 and NK880). In all experiments, three seeds were placed in each hill, and seedlings were later thinned to one plant per hill at the V2 stage (Ritchie et al., 1996). Seedling emergence took place ca. 7 days after sowing and final plant densities at harvest coincided with the density planned for each treatment.

The soil was a typical argiudol (USDA classification) with a fine texture and an underlying layer (40 cm deep) of expandable clay. Typical total N content in the first 20 cm of soil was 1630 mg kg^{-1} and total P content was 5.88 mg kg^{-1}. Fertilizers were broadcast at a rate of 18 kg ha^{-1} of P (triple superphosphate, incorporated into the soil with a disc harrow) prior to sowing and 100 kg ha^{-1} of N (urea) at V7 in Exp. I; 40 kg ha^{-1} of N and 100 kg ha^{-1} of P (diammonium phosphate, DAP) at V3 and 80 kg ha^{-1} of N (urea) at V7 in Exp. II; and 18 kg ha^{-1} of N and 46 kg ha^{-1} of P (DAP) at V3 and 200 kg ha^{-1} of N (urea) in two doses (at V3 and V7) in Exp. III. These rates of N fertilizer aimed at satisfying N needs for a crop yield of 10000 kg ha^{-1} (i.e.), around the maximum yields expected for this location). Plots were irrigated as needed from emergence to maturity in order to prevent water deficit. Plots were maintained free of weeds by application of herbicides when needed. Silking took place on January 15th in Exp. I, January 10th in Exp. II and January 31st in Exp. III (Fig. 1). In all experiments, differences in silking date between the earliest and latest hybrid were 3 days at most. Thermal time computations started at silking, using mean daily air temperature and a base temperature of 8 °C (Ritchie and NeSmith, 1991). In the three 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^{-1} (°Cd).

2.2. Plant sampling

In Exps. I and II, destructive samplings were made at silking and physiological maturity for chlorophyll and dry matter determinations. In Exp. III, only one final harvest (8 plants per treatment) was made. For each destructive sampling, two adjacent plants from a central row of each subplot (6 or 8 plants per treatment in Exps. I and II respectively) were harvested. Other authors used a similar number of plants sampled per treatment to assess senescence progression, chlorophyll content and for dry mass determinations (Escobar-Gutiérrez and Combe, 2012; Martin et al., 2005; Pommel et al., 2006). The plants sampled were adjacent in the same row, thus reducing the potential biases arising from human plant selection. Moreover, the plants sampled were flanked by visually
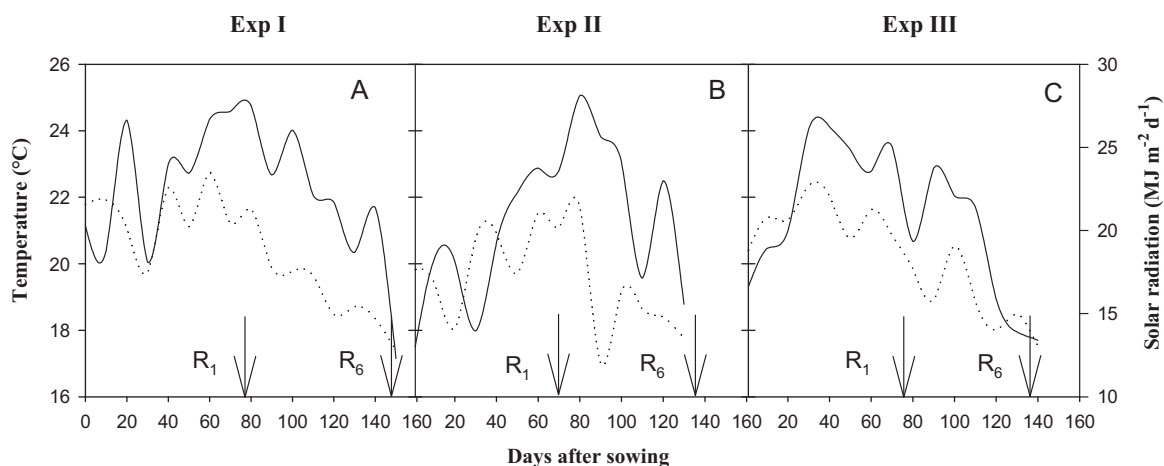
Fig. 1. Mean air temperature (solid lines) and solar radiation (dotted lines) recorded during experiments I (A), II (B) and III (C). R_1 (silking) and R_6 (physiological maturity) stages are indicated by arrows. All hybrids have very similar days to anthesis and to physiological maturity.

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. With these precautions, the difference in plant dry mass at physiological maturity between the two plants sampled in each block × treatment combination, expressed as a percentage of the subplot mean, was 10–16% for DK682, 19–20% for NK880, 11% for 6 pl m^{-2}, 16% for 7 pl m^{-2}, 16–19% for 8 pl m^{-2}, 21% for 9 pl m^{-2} and 14–19% for 10 pl m^{-2}.

2.3. Canopy senescence

In all experiments, 6 (Exp. I) or 8 (Exps. II and III) plants per treatment (two adjacent plants in a central row of each subplot) 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 below), 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).

Leaf chlorophyll content and green leaf area per leaf was determined in 6 (Exp. I) or 8 (Exp. II) plants per treatment harvested at silking and at physiological maturity. Leaves were dissected from the plant and measured. Leaf chlorophyll content (average of five measurements along each leaf) was estimated with a non destructive chlorophyll meter (SPAD 502, Minolta), and green area per leaf was measured using a Licor 3000 (Li-Cor, Nebraska, USA) area meter.

2.4. Dry matter measurements

Dry matter measurements were taken from plants harvested in each destructive sampling (at silking and at physiological maturity). 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. In the three experiments, plants that had been tagged for visual estimations of senescence progression (Section 2.2) were harvested at physiological maturity.

Net C accumulation during grain filling was calculated as the difference between total DM at physiological maturity and total DM at silking.

The ears of 60 (Exp. I) or 80 (Exp. II) plants per treatment (density × hybrid) were hand harvested at maturity to estimate average yield m^{-2}. 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 were expressed as grain dry weight (g) per m^{-2}. Mean individual kernel weight (KW) was determined in ears of 6 (Exp. I) or 8 (Exps. II and III) plants per treatment (density × hybrid) threshed manually, by counting and weighting the total number of kernels per plant. The kernel number (KN) shown in Section 3 was estimated on the basis of KW, and grain yield of the 60 or 80 plants per treatment sampled at final harvest, essentially as in Echarte et al. (2000).

2.5. Respiration measurements

In Exp. III, respiration measurements were performed on the ear leaf and the third leaf below the ear following the method described by Earl and Tollenaar (1998), except that respiration was estimated as O_2 consumption rather than CO_2 emission. Respiration measurements were taken at three stages during grain filling (110, 245 and 500 °Cd after silking). In each sampling date and for each leaf position, the leaves of at least 6 different plants were sampled (one disc per leaf) for respiration measurements. Leaf respiration was measured between 11 a.m. and 2 p.m. in discs of 11 cm^2 taken from the central portion of the leaves, avoiding the midrib. Samples were acclimated to darkness for 20 min in a water vapor-saturated atmosphere. Respiration was measured as O_2 consumption, with a Clark-type oxygen electrode (Hansatech, UK), at ambient temperature.

2.6. Nitrogen

Total N concentration was analyzed by micro Kjedahl analysis (LANAIS N-15, Universidad Nacional del Sur, Bahía Blanca, Argentina) in samples taken around silking and near physiological maturity (71 DAS in Exp. I and 66 DAS in Exp. II). 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. Nitrogen accumulation during the post
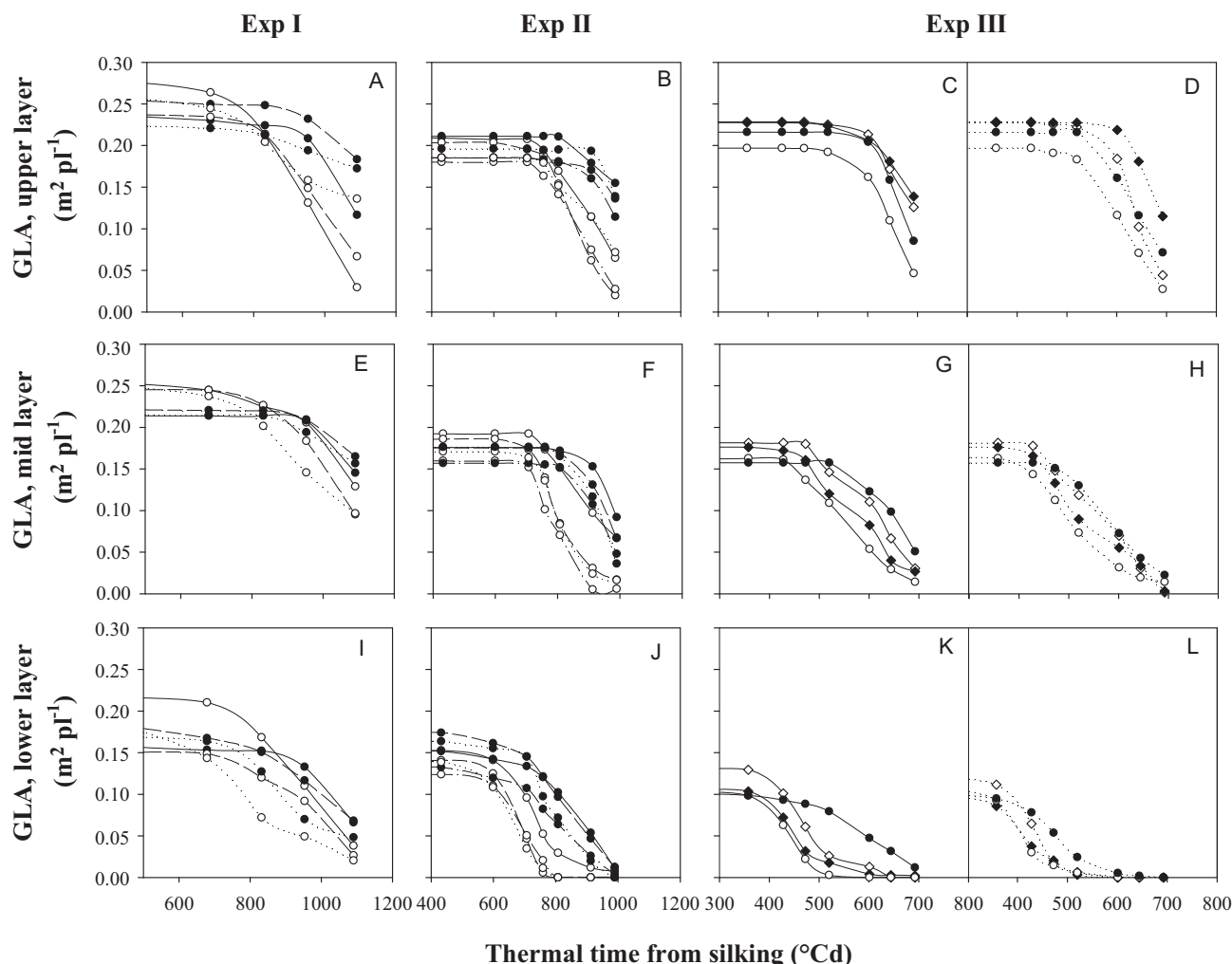
Fig. 2. Senescence progression measured as decline of green leaf area per plant (m² pl⁻¹) at the upper (A, B, C and D), mid (E, F, G and H) and lower (I, J, K and L) canopy layers, in experiments I (A, E and I), II (B, F and J) and III (C, D, G, H, K and L). For experiments I and II, densities are indicated by lines as follows: solid lines, 6 or 7 pl m⁻²; dashed lines, 8 pl m⁻²; dash–dotted lines, 9 pl m⁻²; dotted lines, 10 pl m⁻². For experiment III, panels C, G and K represent 8 pl m⁻², whereas panels D, H and L correspond to 10 pl m⁻². Hybrids are indicated by symbols: empty circles, DK682; solid circles, NK880; empty diamonds, AW190; solid diamonds, AX878.

silking period was calculated as the difference in plant Nc between the last (maturity) and the first (silking) sampling dates. Nitrogen remobilization (g) from vegetative tissues was calculated as the difference between leaf + stem Nc at silking and at physiological maturity. Nitrogen harvest index (NHI) was calculated as follows: NHI = $Nc_{kernels}/(Nc_{kernels} + Nc_{cob\ and\ husks} + Nc_{stems} + Nc_{leaves})$.

2.7. Data analysis

Data were analyzed with the STATISTICA 5.1 (StatSoft, Inc.) software. Treatment effects and interaction between treatments were analyzed by analysis of variance (ANOVA) using mixed models. Residuals were analyzed to corroborate the assumptions of the ANOVA. For all of the dependent variables analyzed, density, hybrid and block were considered as fixed factors. When factors or their interactions were significant, means were compared by the LSD test (a = 0.05). To assess the significance of linear regressions the F-test was used ($P < 0.05$).

3. Results

3.1. Meteorological conditions and phenological variables

Meteorological conditions varied between experiments. Overall, mean temperatures were higher in Exp. I than in Exps. II and

III (Fig. 1). Average temperatures during the period spanning 15 days before to 15 days after silking were higher in Exp. I, and lower in Exps. II and III (24.6, 22.9 and 22.3 °C, respectively). Post-silking average temperatures were higher in Exps. I and II (22.0 and 22.1 °C) than in Exp. III (20.8 °C). Solar radiation (as an average of the entire crop cycle) was highest in Exp. I (19.2 MJ m⁻² d⁻¹), intermediate in Exp. III (18.4 MJ m⁻² d⁻¹) and lowest in Exp. II (17.15 MJ m⁻² d⁻¹). As shown by arrows in Fig. 1, physiological maturity occurred later in Exp. I in comparison with experiments II and III. The post-silking period (up to physiological maturity) was longest in Exp. I (71 days), intermediate in Exp. II (65 days) and shortest in Exp. III (60 days).

3.2. Canopy senescence

Overall, before the onset of fast senescence, green leaf area (GLA) per plant at all canopy layers was not affected by plant density (Fig. 2, Supplementary Table 1). Later in the grain filling period, the increase in plant density decreased GLA at the lower canopy layer in Exp. I at 954 °Cd (Fig. 2I), whereas at 1091 °Cd, senescence of the upper canopy layer seemed to be delayed by the highest plant density (Fig. 2A). In Exp. II, at the lower and mid canopy layers, increased density resulted in reduced GLA towards the end of the grain filling period (Fig. 2J and F). Likewise, in Exp. III, GLA decreased with density at the lower (after 428 °Cd after silking, Fig. 2K and L),

Table 1
Grain yield (g m^{-2}), kernel number per m^2 (KN m^2), kernel weight (KW, mg kernel^{-1}), post-silking dry matter (DM) accumulation (g m^{-2}) and total DM at harvest (g m^{-2}). Same letters indicate homogenous groups ($P < 0.05$).

Exp.	Pl m^{-2}	Hybrid	Yield (g m^{-2})	KN m^{-2}	KW (mg kernel^{-1})	Post-silking DM accumulation (g m^{-2})	Total DM (g m^{-2})
	6	DK682	1094 e	3500	313	1112	1858
		NK880	1171 de	3761	314	1224	1973
	8	DK682	1277 cd	4189	307	1117	1838
		NK880	1392 ab	4719	299	1067	2261
I	10	DK682	1461 a	4811	305	1481	2384
		NK880	1327 bc	4767	281	998	2258
	P (density)		***	***	*	NS	**
	P (hybrid)		NS	*	NS	NS	NS
	P (D × H)		*	NS	NS	NS	NS
	7	DK682	1198 b	4285	280	802	2014
		NK880	1151 b	4134	282	996	2061
	8	DK682	1179 b	4388	269	741	2032
		NK880	1233 ab	4793	259	1064	2121
	9	DK682	1324 a	4971	268	815	2180
II		NK880	1180 b	4311	266	741	2043
	10	DK682	1332 a	4920	272	1067	2376
		NK880	1128 b	4927	232	936	2214
	P (density)		NS	*	+	NS	**
	P (hybrid)		**	NS	+	NS	NS
	P (D × H)		*	+	NS	NS	NS
	8	AW190	1089 ab	4503	243		2096
		AX878	1003 bc	3699	273		2124
		DK682	1045 abc	4116	254		1956
		NK880	1133 a	4091	280		1938
	10	AW190	1056 abc	4637	228		2150
III		AX878	1115 a	4405	267		2311
		DK682	1036 ab	4240	252		1827
		NK880	977 c	4160	228		2072
	P (density)		NS	*	*		***
	P (hybrid)		NS	*	+		NS
	P (D × H)		**	NS	NS		NS

NS: not significant.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.
+ $P < 0.1$.

mid (after 520 °Cd after silking, Fig. 2G and H) and upper (after 601 °Cd after silking, Fig. 2C and D) canopy layers. After the onset of fast senescence, significant hybrid effects were detected in all experiments. In Exp. I, the SG NK880 retained a higher GLA at the upper layer at 954 °Cd, and at all layers at 1091 °Cd. In Exp. II, NK880 retained a significantly larger GLA than DK682 at the lower canopy layer after 599 °Cd (Fig. 2J), and at the mid and upper canopy layers starting after 808 °Cd (Fig. 2F and B). Significant hybrid differences in the lower canopy layer were detected in Exp. III starting at 428 °Cd. In the mid and upper layers, significant differences arose earlier, at 358 °Cd. Consistently across densities, the SG NK880 retained the largest GLA at the lower and mid canopy layers, late in the grain filling period (Fig. 2K, L, G and H), whereas at the upper third of the canopy, the SG AX878 retained the largest GLA, particularly at 10 pl m^{-2} (Fig. 2C and D). Overall, GLA declined faster in DK682 than in NK880, although these differences were more noticeable in the lower and mid canopy layers, than in the upper third of the plant.

Consistent with changes in GLA during the reproductive phase, the average chlorophyll content (SPAD units) of leaves was generally higher in the SG NK880 than in the NSG DK 682, particularly at the lower third in Exp. I and at the lower and mid thirds of the canopy in Exp. II (Supplementary Fig. 1).

3.3. Yield, and yield components

At the highest plant density tested (10 pl m^{-2}), DK682 had significantly higher grain yields (g m^{-2}) than NK880 in all experiments (Table 1). However, at intermediate densities no apparent differences arose between hybrids (except at 8 pl m^{-2} in Exp. I, where

yield in NK880 was higher than in DK682). In Exp. III, among the four hybrids tested, only NK880 showed a decrease in yield at 10 pl m^{-2} (compared to 8 pl m^{-2}). For the other hybrids, no significant differences in yield were found between 8 and 10 pl m^{-2} (Table 1) except for AX878, whose yield increased at 10 pl m^{-2}. Thus, yield of the strongly SG NK880 was always penalized at a high density, compared to the reference NSG hybrid DK682.

As expected, the increase in plant density exacerbated interplant competition, reducing yield per plant (data not shown). In Exp. I, significantly higher KN was detected for the SG hybrid NK880 compared with the NSG DK682, though this difference was not evident at 10 pl m^{-2} (Table 1). Hybrids showed a similar performance in Exp. II, where higher ($P < 0.1$) KN in the SG NK880 was evident at 8 pl m^{-2} but not at 10 pl m^{-2}. In addition, a lower ($P < 0.1$) KW was detected for the SG hybrid NK880 averaged across all densities (Table 1). In Exp. III, there were significant hybrid effects for KN: AW190 had higher ($P < 0.05$) KN than the rest of the hybrids (Table 1). KW decreased at 10 pl m^{-2}, and this effect was most noticeable for the SG hybrid NK880 which, in comparison with the rest of the hybrids, showed the highest KW at 8 pl m^{-2} but the lowest KW at 10 pl m^{-2}. At 10 pl m^{-2} the highest KW was achieved by the AX878 hybrid, which showed a SG character only at the upper canopy layer (Table 1 and Fig. 2D).

3.4. Dry matter accumulation and respiration rates of leaves at mid and basal positions in the plant

In spite of the differences in senescence (Fig. 2), post-silking DM accumulation was not significantly different among hybrids in Exps. I and II (Table 1). Also, hybrid differences were not detected
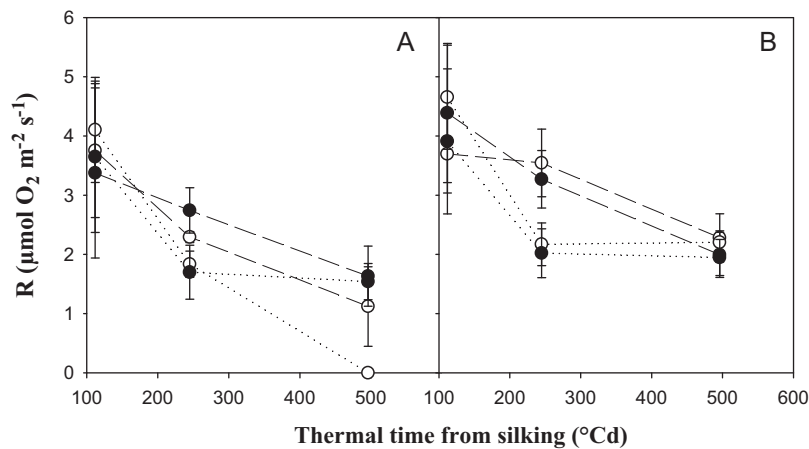
Fig. 3. Respiratory rates (on a leaf area basis) measured in Exp. III, in the third leaf below the ear (A) and in the ear leaf (B). Densities are indicated by lines: dashed lines, 8 pl m^{-2}; dotted lines, 10 pl m^{-2}. Hybrids are indicated by symbols: empty circles, DK682; solid circles, NK880. Each data point is the average of 6–8 independent measurements. Vertical bars represent the standard error of the mean.

for total DM at harvest in Exps. I, II and III (Table 1). At low-moderate plant densities (6–8 pl m^{-2}), post-silking DM accumulation was related to GLA remaining at each canopy layer late in the grain filling period (i.e.), 830 and 807 °Cd after silking in Exps. I and II, respectively), although the slope of this relationship decreased from the upper to the bottom canopy layers. At higher densities a similar top-to-bottom pattern was found, and the relationship was non-significant for the lower canopy layer (Supplementary Fig. 2).

In part, the lack of an association between delayed senescence and post-silking DM accumulation may be due to the respiratory costs of maintaining green leaves in lower canopy levels in the SG hybrid NK880. This prompted us to measure leaf respiration. Respiratory rates measured in the ear leaf and in the leaf three nodes below the ear, varied depending on phenological stage, density and hybrid (Fig. 3). Early after silking (7 DAS, 112 °Cd) no significant differences between densities or hybrids were found in the ear leaf or in the leaf three nodes below the ear. At 17 DAS (245 °Cd) density effects ($P < 0.05$) were detected for both leaf positions: respiratory rates being lower at 10 pl m^{-2}. At later stages of grain filling (34 DAS, 496 °Cd), no density effects were detected in either leaf position while hybrid effects arose in the third leaf below the ear: respiratory rates were lower ($P < 0.05$) in the NSG hybrid DK682 ($P < 0.05$). This was especially noticeable at 10 pl m^{-2}, where respiratory rates of DK682 dropped to zero as a consequence of senescence while

respiration rates in NK880 remained unchanged between 250 and 500 °Cd after silking.

3.5. Nitrogen content per unit area

Above-ground N content per unit area (Nc) at physiological maturity was lower in Exp. II than in Exp. I (Fig. 4). In Exp. I no significant differences were detected for plant Nc at physiological maturity, even though the NSG hybrid DK682 showed higher ($P < 0.1$) N uptake after silking than the SG NK880 (Table 2 and Fig. 4A). In Exp II, the NSG DK682 had higher ($P < 0.1$) Nc at silking, and higher ($P < 0.05$) Nc at physiological maturity (Table 2 and Fig. 4B).

Not only N accumulation but also N partitioning differed between densities and hybrids. In Exp. I, at physiological maturity, Nc of leaves or stems did not differ between hybrids (Table 3). In Exp. II (where plants absorbed less N, compare Fig. 4A and B), Nc of both leaves and stems at physiological maturity were higher ($P < 0.05$) in NK880 than in DK682 at all densities (Table 3). N remobilization from vegetative tissues did not differ between hybrids in Exp. I, whereas in Exp II the SG NK880 hybrid remobilized less N than DK682 at 7 and 8 pl m^{-2}, but not at higher densities (Table 3). In both experiments, the same trend was found for kernels Nc: the NSG hybrid DK682 had higher kernel Nc than NK880 across all densities (Table 3). This was the result of higher ($P < 0.05$) N
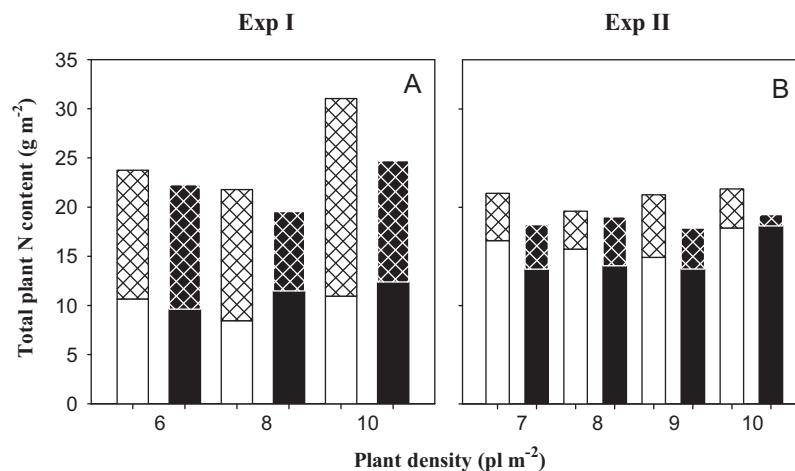

Fig. 4. Total plant N content per unit area (g m^{-2}) at silking (smoothed bars) and at physiological maturity (combined bars). Crossed bars represent post-silking N uptake. Bar color denotes the hybrid (white bars, DK682; black bars, NK880) in experiments I (A) and II (B). Significances are shown in Table 2.

Table 2
ANOVA results for N content (Nc) per unit area (g m^{-2}) at silking, post-silking N uptake and Nc at physiological maturity in experiments I and II.

Exp.		Nc silking	N uptake after silking	Nc physiological maturity
I	P (density)	NS	NS	*
	P (hybrid)	NS	+	NS
	P (D × H)	NS	NS	NS
II	P (density)	**	NS	***
	P (hybrid)	+	NS	*
	P (D × H)	NS	NS	NS

NS: not significant.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.
+ $P < 0.1$.

Table 3
Average N concentration in kernels (%) at harvest, N content (Nc, g pl^{-1}) in leaves, stems + sheaths and kernels at physiological maturity, N remobilization (g pl^{-1}) and N harvest index (NHI) in experiments I and II. Same letters indicate homogenous groups in each experiment (P < 0.05).

Exp.	Pl m^{-2}	Hybrid	N% kernels	Nc leaves (g pl^{-1})	Nc stems (g pl^{-1})	Nc kernels (g pl^{-1})	Remobilization (g pl^{-1})	NHI
I	6	DK682	1.44	0.32	0.42	3.02	0.20	0.77
		NK880	1.37	0.34	0.37	2.79	0.16	0.76
	8	DK682	1.45	0.23	0.18	2.19	0.25	0.81
		NK880	1.16	0.26	0.26	1.81	0.29	0.74
	10	DK682	1.59	0.31	0.29	2.38	0.07	0.77
		NK880	1.27	0.25	0.26	1.86	0.18	0.75
	P (density)		NS	NS	NS	**	NS	NS
	P (hybrid)		***	NS	NS	*	NS	+
	P (D × H)		NS	NS	NS	NS	NS	NS
II	7	DK682	1.35	0.29	0.24	2.37	0.88	0.78
		NK880	1.06	0.43	0.32	1.71	0.53	0.65
	8	DK682	1.29	0.18	0.19	1.93	0.92	0.79
		NK880	1.01	0.37	0.35	1.51	0.42	0.63
	9	DK682	1.34	0.22	0.17	1.84	0.64	0.78
		NK880	1.01	0.31	0.29	1.25	0.47	0.63
	10	DK682	1.30	0.18	0.18	1.70	0.74	0.78
		NK880	1.04	0.31	0.24	1.24	0.77	0.64
	P (density)		NS	*	NS	***	NS	NS
	P (hybrid)		***	***	**	***	**	**
	P (D × H)		NS	NS	NS	NS	+	NS

NS: not significant.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.
+ $P < 0.1$.

concentration (%) in kernels of DK682, compared to NK880 (Table 3); however, at 10 pl m^{-2}, lower grain yield in NK880 also contributed to its lower kernel Nc. Differences in N allocation between hybrids were reflected as differences in N harvest index (NHI). NHI tend to be higher in the NSG hybrid DK682 both in Exp. I ($P < 0.1$) and in Exp. II ($P < 0.05$). In both hybrids, KW positively related to kernel N concentration ($r^2 = 0.89$ and 0.57 for DK682 and NK880, respectively, Fig. 5), suggesting that, overall, N availability for developing kernels was less than the optimum required to reach the potential KW for each treatment.

4. Discussion

4.1. Canopy senescence and N balance

Higher plant densities accelerated senescence, particularly at the lower canopy layer. Borrás et al. (2003) reported that, at the whole plant level, the rate of green leaf area decline during the grain filling period increased with increased density. In the present paper, we examined the effects of plant density on senescence at different canopy layers. Density effects over GLA decline were mostly noticeable at the lower and mid canopy strata, whereas, at the upper canopy layer GLA loss was not consistently increased at higher density across hybrids and experiments. Senescence of each canopy
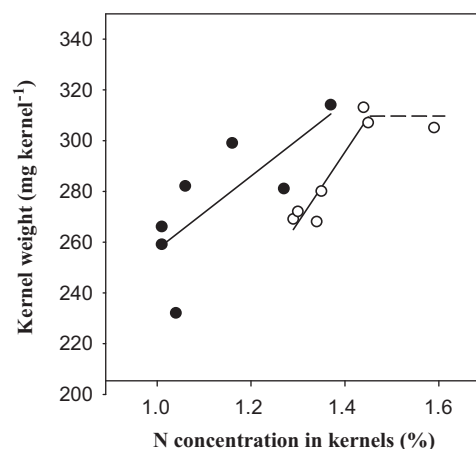

Fig. 5. Kernel weight as a function of N concentration in kernels, across all densities in experiments I and II. Each data point represents the average per treatment in each experiment (hybrid × density × experiment). Data were plotted separately for DK682 (empty circles) and NK880 (solid circles). Assuming a critical N% of 1.4, values above this threshold (only one datum for DK682) were not included in the linear regression. Solid lines represent the fitted regression; the dashed line represents the extrapolation when values above the critical kernel %N were found (only in DK682). For DK682, $r^2 = 0.89$; $n = 6$; $P < 0.01$. For NK880, $r^2 = 0.57$; $n = 7$; $P < 0.05$.

layer also varied with hybrid. For example, while DK682 always senesced faster than NK880, regardless of canopy layer, senescence of lower leaves of AX878 followed a time-course similar to DK682, but mid and upper leaves of AX878 exhibited delayed senescence (Fig. 2). Interestingly, AX878 had the highest grain yield at high density. Valentinuz and Tollenaar (2004) found that, at high plant densities (12 pl m^{-2}), modern SG hybrids with delayed senescence of mid and upper leaves out-yielded an older, NSG genotype.

Consistently, senescence was delayed in the SG NK880 compared to the NSG DK682. In sorghum (Borrell and Hammer, 2000; van Oosterom et al., 2010), and in many cases in maize (Ma and Dwyer, 1998; Rajcan and Tollenaar, 1999b) the delay of senescence in stay green genotypes is the result of increased N uptake during the grain filling period, which reduces the dependence of grain growth on leaf N remobilization and, thereby, allows for delayed senescence. However, in this work, the SG NK880 accumulated less N (especially at high density and in Exp. II) at physiological maturity in comparison with the NSG DK682, yet canopy senescence was delayed in NK880. In maize, expression of the SG trait does not seem to be necessarily associated with increased post-silking accumulation of N. For example, Martin et al. (2005) and Subedi and Ma (2005) found no significant increase of net N accumulation after silking in stay green hybrids. In cases where the stay green is not related to increased post-silking N uptake, kernel %N content may decrease (e.g., Subedi and Ma, 2005, NK880 in this study). These, and our data for NK880, raise the possibility that different physiological mechanisms might lead to a SG phenotype. On one hand, an improved post-silking N balance underlies delayed senescence in many stay green hybrids (e.g., Ma and Dwyer, 1998; Rajcan and Tollenaar, 1999b; van Oosterom et al., 2010), whereas in other cases the stay green might be caused by other physiological mechanisms. For example, in soybean (Guiamet et al., 1990; Guiamet and Gianibelli, 1994) and wheat (Spano et al., 2003) mutants, the stay green character is expressed during the grain filling period. Interestingly, compared to a wild-type genotype, these SG mutants also showed delayed senescence of detached leaves incubated in darkness, i.e., where senescence proceeds independently of changes in N supply or demand by sinks (Guiamet et al., 1990; Guiamet and Gianibelli, 1994; Spano et al., 2003). In these cases, the stay green might be the result of a blockage in the chloroplast degradation machinery, with retention of chloroplast functions. Such genotypes might interfere with the ability of leaves to increase N remobilization in response to grain demand for N. We surmise that the stay green character of NK880 is due to physiological changes that subtly impair its ability to remobilize N.

4.2. Yield and yield components, and dry matter accumulation

At high densities, delayed canopy senescence in NK880 did not result in a yield advantage. The higher yields (associated with higher KW) achieved by DK682 (compared to NK880) at high density could be related to higher assimilate availability for developing kernels. However, hybrids did not differ in post-silking DM accumulation and total DM at harvest, and assimilate availability, expressed as post-silking DM accumulation per kernel, was not significantly different between NK880 and DK682 (data not shown).

Delayed senescence in the SG hybrid did not translate into greater post-silking DM accumulation. Since the largest differences in terms of senescence were detected at the lower canopy layer, this could be due to neutral or even negative effects produced by delayed senescence of basal leaves. In dense canopies, leaves at the lower layers may be exposed to irradiance levels below the light compensation point for C$_4$ plants during most of the day. A negative relationship between total DM per plant at harvest and respiration rates of upper leaves averaged through the crop cycle has been reported (Earl and Tollenaar, 1998).

Respiration rates decrease from the upper (ear leaf and above) to the lower parts of the canopy (Earl and Tollenaar, 1998; Fig. 3). In this work, hybrids differed in respiration rates of basal leaves. While in NK880 respiration rates of the third leaf below the ear remained constant between mid and late stages of grain filling, in DK682 respiration rates at mid stages dropped to zero as a consequence of senescence. Thus, by reducing their respiration rates, senescence of shaded (lower) leaves may help to avoid unnecessary losses of C during the reproductive period. Boonman et al. (2006) found higher respiration rates of lower leaves in a SG genotype of tobacco, which resulted in negative daily rates of net C assimilation by these leaves, compared with wild type plants grown at high plant densities.

4.3. Nitrogen limitations to kernel growth

According to Ciampitti and Vyn (2011), the effective ratio between grain DM and N content ranged from 1.1% to 1.4%, which means that N concentrations below 1.4% may limit grain growth. The significant relation between N concentration and KW (Fig. 5) suggests that N played a role in KW determination, especially in the SG hybrid, which had the lowest N concentration in grains across the whole range of densities and in all experiments. Ciampitti and Vyn (2012) show that N internal efficiency (i.e.), grain yield/whole plant N uptake at physiological maturity) increased in modern hybrids of maize in part because of a decrease in kernel %N, however our data for the SG NK880 suggest that further decreases in kernel %N could negatively impact on KW.

For a given leaf N content at physiological maturity, N concentration in kernels and grain yield were lower in NK880 than in DK682 (Table 3). Borrás et al. (2003) reported that in plants where the number of grains was reduced by 50%, the amount of N exported from vegetative parts did not differ in comparison with plants where the number of sinks was not affected. Similarly, Christensen et al. (1981) reported that in intact plants as well as in plants where the ear was removed early after silking, the amount of N exported from leaves during the first half of grain filling was the same, with the stem as an alternative sink for N in earless plants. Thus, there may be some degree of source influence over N remobilization from leaves. Retention of N in leaves of the SG NK880 hybrid may reflect inhibition of N remobilization to grains at the leaf level, thereby limiting grain growth, particularly at high densities.

5. Conclusions and prospects for breeding

In this work we show that: (i) the SG trait is expressed across a range of plant densities, (ii) the expression of the SG trait can vary depending on the canopy layer considered, (iii) at high densities, highest yields are achieved in those hybrids that optimize GLA retention as a function of incident irradiance (i.e.), delayed senescence only of mid and upper canopy leaves, e.g., AX878), (iv) stay green does not necessarily reflect increased post-silking accumulation of N.

Expression of the SG trait without increased post-silking uptake of N may reduce the supply of N to grains, thereby negatively affecting their KW and final yield. Retention of N in leaves and stems of the SG hybrid even in spite of an apparent N deficit for kernel growth implies that N remobilization is not a simple response to sink demand. Thus, in some cases stay green might reflect an inability for N remobilization from vegetative parts of the plant, and this may be particularly counterproductive at moderately high plant densities. Stay green may be a surrogate trait for increased post-silking accumulation of N only if combined with yield and kernel % N measurements. Breeding programs might complement visual scoring for green leaf retention with analysis of kernel %N to

sort out genotypes impaired in their ability to remobilize enough N to grains. Understanding the regulation of, and interplay between, senescence of leaves in different canopy strata might help in efforts to breed maize hybrids better suited for high planting densities.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fcr.2013.09.016.

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