Functional Significance of Shade-Induced Leaf Senescence in Dense Canopies: An Experimental Test Using Transgenic Tobacco

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abstract: Canopy photosynthesis models have predicted an optimal leaf area index (LAI; leaf area per unit surface area) and leaf nitrogen distribution at which whole-plant carbon gain per unit N is maximized. In this study we experimentally tested these models, using transgenic P_{SAG12}-*IPT* tobacco (SAG; *Nicotiana tabacum* L.) plants with delayed leaf senescence and therefore a greater LAI and more uniform N distribution than the wild type (WT). In a competition experiment, the increased density of surrounding WT plants caused a greater reduction in dry mass of mature SAG target plants than in that of WT target plants, indicating negative effects of delayed leaf senescence on performance at high canopy density. Vegetative SAG plants achieved a lower calculated daily carbon gain than competing WT plants because the former retained leaves with a negative carbon gain in the shaded, lower part of the canopy. Sensitivity analyses showed that the carbon gain of SAG plants would increase if these lower leaves were shed and the N reallocated from these leaves were used to form additional leaf area at the canopy top. This strategy, which is adopted by the WT, is most advantageous because it results in the shading of competing neighbors.

Keywords: carbon gain, senescence, transgenic plants, competition, canopy light gradient.

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The efficient use of resources for photosynthesis, the process driving plant growth, is considered important for plant fitness (Mooney and Gulmon 1979; Hirose 2005). Plants in dense herbaceous vegetation compete with their neighbors for available light and other resources; in particular, nitrogen often limits plant growth in such communities (Chapin 1980). Since a large fraction of leaf N is associated with the photosynthetic apparatus (Evans 1989; Evans and Seemann 1989), there is a strong positive correlation between foliar N contents and photosynthetic capacity (Field and Mooney 1986; Evans and Seemann 1989; Wright et al. 2004). In a growing canopy, plants can therefore increase their light capture and carbon gain by allocating more leaf area and N to the upper, well-illuminated part of the canopy. However, as the leaf area index (LAI; leaf area per surface area) increases, the older leaves lower in the canopy become progressively more shaded (Monsi and Saeki 1953), so their carbon balance is decreased. Carbon gain of the whole plant and ultimately fitness therefore depend not only on the deployment and distribution of leaf area and N in the canopy but also on the strategy of surrounding plants.

In the past, numerous theoretical models have been developed to approach the question of optimal strategies for the maximization of carbon gain and fitness in dense stands (reviews in Grindlay 1997; Anten 2005; Hirose 2005). Monsi and Saeki (1953) derived that the LAI is optimal if the lowest leaves in the canopy receive an amount of light equal to their light compensation point, at which point these leaves should be dropped. This approach implicitly assumed photosynthesis to be limited

only by light availability. A subsequent study (Anten et al. 1995) that included nitrogen limitation on photosynthesis derived that the optimal LAI should increase with N availability. Thus, at low N availability, plants were predicted to drop their leaves before they reach zero carbon balance. Models further derived that the optimal N distribution over the foliage should parallel the vertical light gradient in a canopy, which indeed has been frequently observed in natural stands of vegetation (Hirose 2005). This is because high leaf N contents and associated photosynthetic capacity can result in greater benefits in terms of carbon gain in leaves receiving high light than in shaded leaves (Field 1983).

While the studies made good qualitative predictions regarding general patterns of N distribution and leaf area observed in nature, quantitatively there were consistent disparities between predictions and actual values. Real values of LAI were always found to be larger and real N distributions less steep than predicted ones (see reviews in Anten 2005; Hirose 2005). In all these studies, optimal characteristics were defined as those resulting in maximum whole-stand photosynthesis. It has been recognized, however, that the optimization criterion should be applied to individual plants instead of whole stands. More recent models that incorporate competitive optimization (see Parker and Maynard Smith 1990), with trait values being considered optimal when photosynthesis of an individual is maximized relative that of its neighbors, more accurately predict actual N distribution patterns and LAIs of natural vegetation (Schieving and Poorter 1999; Anten 2002, 2005).

The studies discussed so far have treated N distribution and leaf area as static phenomena even though they arise from a process of N reallocation, leaf senescence, and growth of new leaves. Particularly under limiting N availability, reallocation may provide an important fraction of the N needed for the formation of new leaves. These dynamics were incorporated in canopy photosynthesis models, further improving the classical models (Franklin and Ågren 2002; Hikosaka 2003, 2005).

The good correspondence between these model predictions and empirical data provides evidence that plants indeed optimize their LAI and N distribution to achieve a maximal carbon gain, thereby efficiently making use of often limiting resources (Hirose 2005). They further suggest that optimization theory can be used as a theoretical framework to scale up from leaf-level properties to the structure and productivity of vegetation stands. A major shortcoming is, however, that the underlying assumption that plants maximize their fitness in this manner has not been experimentally tested, which is the goal of this study.

A straightforward way of testing whether a phenotype confers a selective advantage in a certain environment is to make use of manipulated phenotypes using genetic transformation or phenotypic manipulation (Ackerly et al. 2000). For example, it was demonstrated that there is a functional advantage for a plant to be tall in dense vegetation because manipulated plants with shorter stems were overtopped and outcompeted by their unmanipulated taller neighbors (Schmitt et al. 1995). In this study, we have chosen to make use of transgenic P_{SAG12}-*IPT* tobacco (SAG) plants with a strong delay in leaf senescence (Gan and Amasino 1995). Such plants develop a greater LAI than normally senescing wild-type (WT) plants and have a more uniform distribution of leaf N and associated photosynthetic capacity, at least when grown solitarily (Gan and Amasino 1995; Wingler et al. 1998; Jordi et al. 2000). This phenotype is caused by production of the senescence-inhibiting phytohormone cytokinin, targeted specifically to tissues at the onset of senescence (Gan and Amasino 1995).

We tested whether the higher LAI and more uniform N distribution of SAG plants resulted in a lower carbon gain in dense leaf canopies, consequently leading to inferior competitiveness compared to the WT. To this end, lifetime performance of SAG and WT plants was assessed by growing target plants of either genotype surrounded by WT plants at different densities until maturity. Vegetative and reproductive biomass and individual seed mass of the target plants were then analyzed. In a second experiment, carbon gain of vegetative SAG and WT plants was estimated by detailed analysis of the distribution of leaf area, photosynthetic capacity, respiration rate, and irradiance in dense canopies. A limiting N supply was used. Finally, the consequences of reduced N reallocation and delayed leaf senescence of the SAG plants for carbon gain and competitiveness were assessed using sensitivity analyses. The results point to an important role of shade-induced leaf senescence in reducing costs of respiration, thereby increasing carbon gain, and in making N available for leaf area formation at the top of the canopy to further increase carbon gain and effectively compete with neighboring individuals.

Material and Methods

Plant Material

The plants used in this study were wild-type (WT) tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) and transgenic hemizygous P_{SAG12}-*IPT* (SAG) tobacco (Gan and Amasino 1995; Jordi et al. 2000).

Competition Experiment with Mature Reproductive Plants

On March 24, 2000, WT and SAG seeds were sown in a mixture of potting soil (Lentse no. 2) and sand (1 : 2, v/v) in the Wageningen University greenhouse under natural daylight with additional SON-T lighting for 16 h day^{-1} at an average temperature of 20°C during the day and 18°C at night. After 3 weeks, seedlings were transplanted to 250-L containers (90 cm × 70 cm × 40 cm) filled with the same substrate. In each container, one WT or SAG target plant was placed in the middle and surrounded by either three or five WT plants (density was 6.3 and 9.5 plants m^{-2}, respectively). Plants were watered via a drip watering system. Ripened pods of target plants were harvested regularly starting 1 month before the final harvest. At the final harvest at 20 weeks after sowing, on August 8, 2000, target plants were separated into stems, leaves, roots, seeds, and pods, and dry mass of each fraction was measured after oven drying at 70°C for at least 24 h.

Competition Experiment with Vegetative Plants

Two series of plants were grown, one for analyzing competitive interactions between WT and SAG and one for establishing relationships between leaf N and photosynthetic capacity and between chlorophyll and apparent quantum yield. For the analysis of competition, SAG and WT seeds were sown on April 24, 2003, in potting soil mixed with sand (16 : 1, v/v) in the greenhouse of Utrecht University under natural daylight and at an average temperature of 25°C during the day and 20°C at night. The photosynthetic photon flux density (PPFD) directly above the canopy was 300 μmol m^{-2} s^{-1} on average but varied with time of day and cloud cover. Three weeks after sowing, a homogeneous selection of seedlings was transferred to 250-cm^3 tubes of 5.0-cm diameter arranged in plastic racks and filled with a mixture of soil, sand, clay, and perlite (16 : 1 : 1 : 18 on volume basis). The substrate contained 57 mg NH_4NO_3, 58 mg K_2HPO_4, 400 mg 17% (w/w) CaMg-carbonate (Vitasol BV, Stolwijk, Netherlands), and 100 mg slow-release micronutrient fertilizer (Micromax, Scotts, Marysville, OH) per pot. In this manner, a low amount of N relative to the other nutrients was provided, in order to induce N limitation. The WT and SAG plants were grown at 331 plants m^{-2} in a 1 : 1 mixture and in monocultures. Each stand consisted of 150 plants. To decrease light penetration from the sides, the border of all stands was made up of SAG plants, which retained their lower leaves. Plants in the outer two rows of the stands were not used for any measurements, to minimize edge effects. The tubes were placed on an irrigation mat (Maasmond-Westland, De Lier, Netherlands) that was kept moist by watering twice a day.

Plants were harvested 11 weeks after sowing, on July 7–8, 2003. Four subplots established in each stand were treated as blocks. Eight plants per genotype and per block in each stand were individually harvested using the stratified clipping method (Monsi and Saeki 1953). Leaves were assigned to one of five canopy layers, each 9 cm in height, based on the position where the petiole was attached to the stem. Green leaves, dead leaves, and stems were separated. Petioles and the youngest leaves with a lamina length of less than 5 cm were included in the stem fraction. The apex height of each individual, number of leaves in each layer, and leaf area were measured. Leaf area was determined with a leaf area meter (LI-3100, LI-COR, Lincoln, NE). The chlorophyll content per unit area was estimated on six to eight positions per leaf with a SPAD-502 meter (Minolta, Tokyo) on every second plant (for conversion of SPAD measurements to absolute chlorophyll contents, see below). The dry weight of each fraction was measured after oven drying at 70°C for at least 24 h. Dark respiration rates (R_D) were measured on leaves in each canopy layer of eight dominant plants taken from the monocultures (see below). Dominant plants were defined as reaching the highest of the five canopy layers (at least 80% of maximum plant height). Three dominant plants per genotype in each of the four blocks in each stand were randomly selected and analyzed for leaf N distribution, based on which whole-plant daily carbon gain was calculated.

One day before harvest, on July 6, the PPFD distribution in each block was measured in a horizontal plane in two positions at 10-cm height increments. Measurements were performed using a small line sensor (80 mm × 10 mm × 12 mm) composed of six GaAsP photodiodes (type G1118, Hamamatsu, Hamamatsu City, Japan) under a flat white perspex cover. The sky was completely clouded during the measurements. The PPFD was simultaneously measured in a horizontal plane directly above the canopy (I_0) with a quantum sensor (LI-185A, LI-COR) to obtain relative PPFD (Hirose and Werger 1987*b*). During the last week of the competition experiment with vegetative plants, from July 1 to July 7, 2003, the I_0 was recorded every minute with a data logger.

Relations between Leaf N and Chlorophyll Contents and Photosynthetic Characteristics

Between February and April 2003, another series of WT and SAG plants was grown in monocultures. Additional lighting of ca. 320 μmol m^{-2} s^{-1} for 16 h day^{-1} was provided by SON-T lights. Four weeks after sowing, seedlings were transferred to 65-mL pots filled with a soil : sand mixture (16 : 1; v/v) supplemented with 1 g L^{-1} slow-release complete fertilizer (Osmocote mini plus, release time 3–4

months; Scotts), 6 mL L^{-1} 125 mM K_2HPO_4, and 2 g L^{-1} 17% CaMg-carbonate (Vitasol BV). Seven weeks after sowing, plants were transferred to 3-L pots filled with the same substrate and were arranged at a density of 35 plants m^{-2}. Twelve weeks after sowing, five randomly selected plants of each genotype were transported to the laboratory for measurements on the light response of photosynthesis, organic nitrogen contents per unit leaf area (N_{LA}), and chlorophyll on leaves at different heights. Border plants were excluded from measurements.

Leaf Analysis

Total N was determined on homogenized dry material with an elemental analyzer (Carlo Erba, Model EA NA 1110, Milan). Nitrate was analyzed using salicylic acid as a reagent (Cataldo et al. 1975). Nitrate concentrations were subtracted from total N concentrations to obtain the organic N concentration. The N_{LA} of dead WT leaves was obtained by first measuring the length (l) and width (w) of eight old but still turgescent leaves from the WT monoculture and harvesting them 10 days later, when they had fully senesced (leaves were yellow and dehydrated). Leaf area was estimated from the l and w measurements using a separately determined regression: area $= 0.64 \times l \times w$ ($r^2 = 0.99$; $P < .001$; $n = 83$). Chlorophyll content was determined with a spectrophotometer on a 2-cm^2 fresh sample extracted in dimethylformamide (Inskeep and Bloom 1985). It was determined in the leaf fractions used for gas-exchange measurements and correlated with measurements by a SPAD-502 meter performed on the same samples. The following relation was found: chlorophyll $= 11.36 \times$ SPAD ($r^2 = 0.93$; $P < .001$). This relationship was used to calculate chlorophyll contents of leaves of the vegetative plants, on which only SPAD measurements had been done.

Gas-Exchange Measurements

A gas-exchange measuring system was used with leaf chambers with a 69 × 67-mm window and has been described previously (Pons and Welschen 2002). An infrared gas analyzer (LI-6262, LI-COR) was used to measure CO_2 and H_2O partial pressure. Leaf temperature was maintained at 25°C, which was very close to the average day temperature in the greenhouse; leaf-to-air vapor pressure difference was approximately 1 kPa, and CO_2 partial pressure of the air entering the leaf chambers was 38 Pa. The leaf area enclosed in the chamber was measured. Net rates of photosynthesis (A_{net}) were calculated according to von Caemmerer and Farquhar (1981). Dark respiration rates (R_D) were measured after 20 min in the dark.

Calculation of Daily Whole-Plant Carbon Gain

Daily carbon gain was calculated for individual dominant plants. Photosynthetic characteristics of leaves in each canopy layer were calculated from the relationships with N_{LA} or chlorophyll content per unit area. These relations were separately determined for SAG and WT plants but showed no significant differences between genotypes. The apparent quantum yield (Φ) was obtained from linear regression of A_{net} against PPFD in the initial linear part of the light response curve, including the respiration rate in the light, which was previously determined as $0.54R_D$ (Pärnik and Keerberg 1995; Atkin et al. 1997). Light response curves of A_{net} were fitted to a nonrectangular hyperbola (Hirose and Werger 1987*b*):

$$A_{net} = \frac{\Phi I + A_{max} - [(\Phi I + A_{max})^2 - 4\theta I \Phi A_{max}]^{0.5}}{2\theta} - 0.54R_D, \quad (1)$$

where A_{max} is the gross photosynthetic rate at saturating light intensity, I is PPFD, and θ is the convexity parameter. The average curvature factor (0.808 ± 0.018) was used because no significant correlation with either N_{LA} or chlorophyll was found.

Light extinction in the canopy is described by Beer's law (Monsi and Saeki 1953):

$$I_j = I_0 \exp(-KF_j), \quad (2)$$

where I_j is the PPFD at height j in the canopy, K is the extinction coefficient, and F_j is the cumulated LAI above height j. The value of K was determined for each block in the mixture and monocultures by fitting equation (2) to measured light and leaf area data (Hirose and Werger 1987*a*). Light was treated as diffuse only and was therefore measured under diffuse conditions. The leaf area within each canopy layer was assumed to be equally divided over five sublayers that each spanned 20% of the height of the layer. The leaves in each sublayer were assumed to receive on average the calculated amount of incident light received in the middle of that sublayer. Incident PPFD on leaves (I_j') at height j in the canopy was then calculated as

$$I_j' = \frac{KI_0 \exp(-KF_j)}{(1 - m)^{0.5}} \quad (3)$$

(Hikosaka 2003), where m is a scattering coefficient, the sum of leaf transmission and reflection, that was assumed to be 0.2 (Goudriaan 1977). Carbon gain was calculated using an average light profile measured in the greenhouse over a 24-h period based on all logged I_0 data in the week preceding harvest (July 1–7, 2003; daily irradiance 16.1

mol quanta day^{-1}) or for a representative overcast day (July 6, 2003; daily irradiance 11.1 mol quanta d^{-1}). Daily rates of gross photosynthesis in each sublayer were calculated for each individual plant by substituting I_j' from equation (3) in equation (1), integrating over 24 h, multiplying by the leaf area in each subclass, and summing the values for the five sublayers. Daily respiration rates (R) were obtained by integrating the average measured R_D over 24 h, using $0.54R_D$ during the light period (5 a.m.–10 p.m.), and multiplying by the leaf area in each canopy layer. Daily net rates of photosynthesis, or carbon gain, were obtained by subtracting respiration rates from gross photosynthesis rates in each layer. Finally, whole-plant daily rates of respiration and gross and net photosynthesis were calculated by summing rates for each layer.

Statistical Analyses

In the experiment with reproductive plants, a two-way ANOVA was used to test for differences in dry mass or its components, with genotype and density as fixed factors, followed by Tukey's *b*-test. In the experiment with young vegetative plants, a three-way ANOVA was used to test for differences between parameters, with genotype and stand as fixed factors and block as a random factor. Differences between SAG and WT in the mixture were analyzed with two-tailed Student's *t*-tests. The stand-level parameters LAI, dry mass per unit surface area, and K were analyzed using a two-way ANOVA with genotype and stand as fixed factors. Light response curves of photosynthesis and relations between chlorophyll, N_{LA}, and photosynthesis parameters were fitted using the least squares method. Before analysis, data were log-transformed when it improved homogeneity of variance, based on the Shapiro-Wilks test. Data were analyzed using the SPSS 12 statistical package.

Results

Increased Competition with Surrounding WT Plants Decreased Dry Mass of Reproductive SAG Target Plants

In the competition experiment with reproductive plants, SAG target plants produced more total dry mass than WT target plants at the lower density of surrounding WT plants but not at the higher density (fig. 1). Increased competition with surrounding WT plants therefore decreased dry mass of SAG target plants significantly more than that of WT target plants ($P < .05$, genotype × stand interaction, two-way ANOVA). The difference was mostly due to decreases in leaf and stem mass of the SAG plants, while SAG root mass was lower at both densities (table 1). Since tobacco is an annual species, seed production is an important fitness component. Total seed and seedpod mass were lower in SAG target plants than in WT target plants, and individual SAG seeds were lighter than WT seeds at both densities (table 1). Increased density of surrounding WT plants also decreased seedpod mass of SAG target plants more than that of the WT target plants, resulting in a marginally significant genotype × density interaction (table 1). These results indicate that delayed leaf senescence may affect lifetime plant performance positively at low densities but negatively at higher densities.

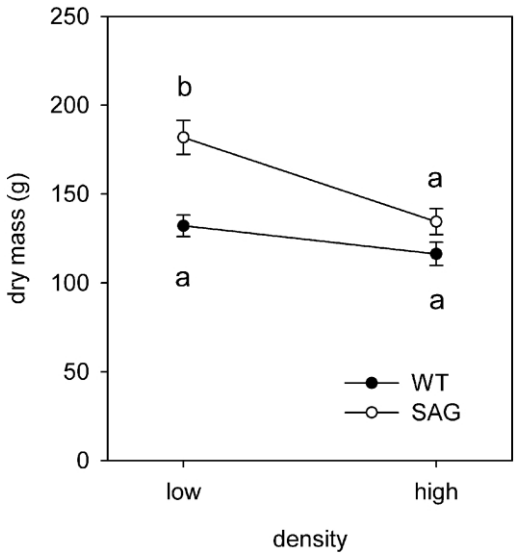

Figure 1: Total dry mass of mature wild-type (WT) and P_{SAG12}-*IPT* (SAG) target tobacco plants grown surrounded by WT plants at low and high density (6.3 and 9.5 plants m^{-2}, respectively) for 20 weeks. Data points labeled with different letters are significantly different at $\alpha < 0.05$ (Tukey's *b*-test). Error bars indicate the SE; $n = 9$.

Competition with the WT Did Not Decrease SAG Dry Mass in the Vegetative Growth Stage

In the experiment with vegetative SAG and WT plants grown in monocultures and a mixture, whole-stand shoot dry mass at harvest tended to be higher for SAG plants but was not affected by the genotype of surrounding plants (table 2). The dry mass of dead WT leaves was included in the total shoot dry mass but was underestimated by an unknown fraction because the lowest dead WT leaves in contact with the soil had partly decomposed. At the individual plant level, no significant differences between genotypes were observed in the mean shoot dry mass (table 2).

Table 1: Dry-mass partitioning of reproductive wild-type (WT) and P_{SAG12}-*IPT* (SAG) target tobacco plants surrounded by WT plants at different densities

	6.3 plants m^{-2}		9.5 plants m^{-2}		ANOVA		
	WT	SAG	WT	SAG	G	D	G × D
Dry mass (g):							
Leaves	33.3 ± 1.52^{A}	52.8 ± 2.91^{B}	30.5 ± 1.47^{A}	38.4 ± 2.62^{A}	$P < .001$	$P < .001$	$P < .05$
Stems	75.7 ± 3.69^{AB}	112 ± 6.08^{C}	67.4 ± 4.04^{A}	85.9 ± 5.07^{B}	$P < .001$	$P < .01$	$.5 < P < .10$
Roots	$6.77 \pm .49^{A}$	$4.83 \pm .57^{B}$	$5.86 \pm .51^{B}$	$3.02 \pm .24^{C}$	$P < .001$	$P < .01$	NS
Seeds	$9.23 \pm .51^{A}$	$5.37 \pm .46^{B}$	$5.92 \pm .93^{B}$	$3.08 \pm .51^{C}$	$P < .001$	$P < .001$	NS
Seedpods	$6.88 \pm .45^{A}$	$6.12 \pm .49^{A}$	$6.57 \pm .47^{A}$	$3.76 \pm .60^{B}$	$P < .01$	$P < .05$	$.5 < P < .10$
1,000-grain weight (mg)	76.2 ± 1.33^{A}	67.8 ± 1.25^{B}	$77.7 \pm .89^{A}$	65.1 ± 1.61^{B}	$P < .001$	NS	NS

Note: Plants were grown for 20 weeks. Data are means ± SE; $n = 9$. Numbers in the same row followed by a different letter are significantly different at $\alpha < 0.05$ (Tukey's *b*-test). Results of a two-way ANOVA with genotype (G) and density (D) as fixed factors are also presented. NS = not significant.

LAI, N Distribution, and Leaf Area Distribution of SAG Plants Were Different from Those of the WT in Dense Stands

SAG plants had a greater LAI and green-leaf dry mass than the WT both in monocultures and in the mixture because they retained their lower leaves (table 2). In fact, no dead leaves were observed on SAG plants at all. By contrast, in the WT a large part of the lower leaves had already senesced before harvest. Stem mass and mean height were similar between genotypes. However, a small significant reduction in height of the SAG genotype was found when only dominant plants, which had reached the highest canopy layer (80% of the height of the tallest plant), were considered. Dominant SAG plants contained more N in their shoots than dominant WT plants (table 2).

The dominant WT plants in the mixture positioned their leaf area almost exclusively in the upper two of five canopy layers (fig. 2*A*). Below that, 35% of WT plants retained living leaves in the third layer, 4% did so in the second-lowest layer, and none did so in the lowest layer. By contrast, dominant SAG plants in the mixture retained living leaves in every canopy layer, with a notably high leaf area in the lowest layer (fig. 2*A*). Their leaf area in the highest canopy layer was, however, significantly smaller compared to that of the WT, consistent with their somewhat shorter stature.

Leaf mass per unit area (LMA) was similar between genotypes in the highest canopy layer and gradually declined toward the bottom of the canopy (fig. 2*B*). In the dominant WT plants in the mixture, LMA declined more strongly with decreasing height than in SAG plants (fig. 2*B*), as did chlorophyll content per unit area (fig. 2*C*) and organic nitrogen content per unit area (N_{LA}; fig. 2*D*), showing that SAG plants indeed had a somewhat more uniform N distribution than WT plants. Dominant plants in the monocultures showed distributions of leaf area, dry mass, chlorophyll, and N similar to those of plants in the mixture (data not shown). With the larger leaf area in the upper

Table 2: Stand- and plant-level characteristics of wild-type (WT) and P_{SAG12}-*IPT* (SAG) tobacco plants grown in monoculture or a 1 : 1 mixture for 11 weeks

	Monoculture		Mixture		ANOVA		
	WT	SAG	WT	SAG	G	S	G × S
LAI ($m^2\ m^{-2}$)	$7.69 \pm .15$	$13.9 \pm .38$	$3.92 \pm .41$	$7.39 \pm .16$	$P < .001$	…	…
Dry mass (kg m^{-2})	$.553 \pm .032$	$.635 \pm .035$	$.287 \pm .024$	$.346 \pm .032$	$.05 < P < .10$	NS	NS
Dry mass (g $plant^{-1}$):							
Whole shoot	$1.68 \pm .11$	$1.92 \pm .10$	$1.74 \pm .14$	$2.09 \pm .13$	NS	NS	NS
Green leaves	$.60 \pm .04$	$.99 \pm .05$	$.60 \pm .05$	$1.03 \pm .07$	$P < .01$	NS	NS
Dead leaves	$.19 \pm .03$	0	$.18 \pm .02$	0	$P < .01$	NS	NS
Stem	$.90 \pm .05$	$.93 \pm .05$	$.95 \pm .08$	$1.06 \pm .06$	NS	NS	NS
Height (cm):							
All plants	$36.5 \pm .98$	$33.2 \pm .94$	36.4 ± 1.67	$37.1 \pm .83$	NS	$P < .01$	$.05 < P < .10$
Dominant plants	$40.1 \pm .55$	$37.7 \pm .54$	$41.6 \pm .63$	$39.4 \pm .57$	$P < .05$	$.05 < P < .10$	NS
Shoot N (mmol):							
Dominant plants	$1.54 \pm .09$	$1.69 \pm .06$	$1.55 \pm .07$	$1.80 \pm .12$	$.05 < P < .10$	NS	NS

Note: Data are means ± SE. The mean leaf area index (LAI) of four blocks, which together formed one stand, is presented. Other data are for all individual plants ($n = 32$) or dominant plants only, which reached the highest of the five canopy layers ($n = 12$). Results of a three-way ANOVA with genotype (G) and stand (S) as fixed factors and block as a random factor are presented. NS = not significant.

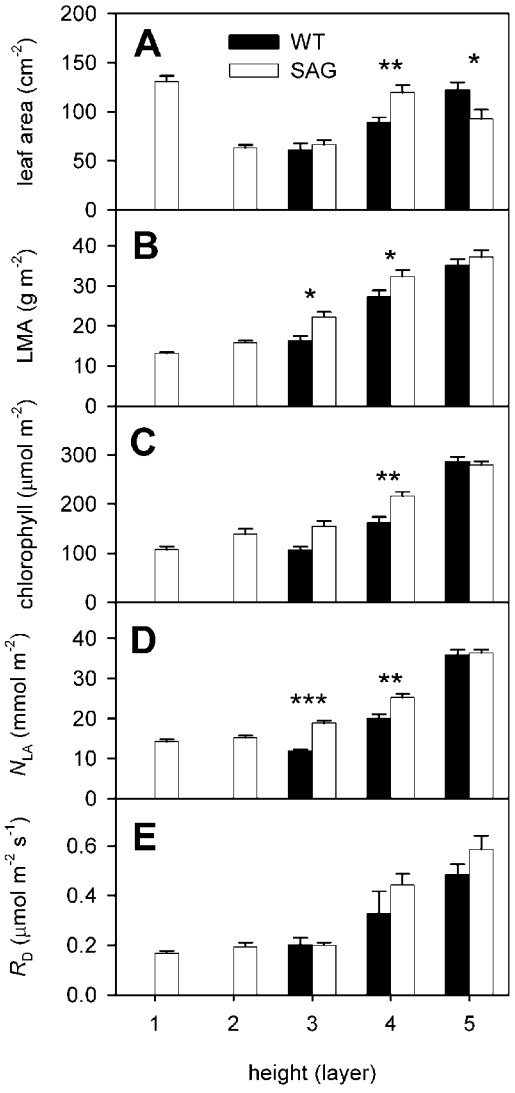

Figure 2: Characteristics of dominant wild-type (WT) and P_{SAG12}-*IPT* (SAG) tobacco plants grown for 11 weeks. *A*, Distribution of leaf area; *B*, leaf mass per unit area (LMA); *C*, chlorophyll content per unit area; *D*, organic nitrogen contents per unit area (N_{LA}); *E*, dark respiration rate (R_D). Values of R_D were measured on plants taken from the monocultures; all other data are from plants taken from the mixture. Error bars indicate the SE; $n = 8$–23. One asterisk = $P < .05$; two asterisks = $P < .01$; three asterisks = $P < .001$.

layer for the WT and equal N_{LA}, a larger amount of organic N was present there in WT plants than in SAG plants, whereas SAG plants contained more N in all the layers below that. Measurements on dominant plants taken from the monocultures showed that dark respiration did not differ significantly between genotypes in the upper three canopy layers but continued at a low level in SAG leaves in the two lower canopy layers (fig. 2*E*), where WT plants had already shed their leaves. Based on these data, daily carbon gain was calculated for dominant plants in the mixture.

Continued Respiration in Shaded Leaves Reduced SAG Whole-Plant Daily Carbon Gain

The relationships between N_{LA} and photosynthetic capacity (A_{max}; fig. 3*A*) and between chlorophyll and apparent quantum yield (fig. 3*B*) did not differ between genotypes and were used for the calculations of carbon gain. The lowest living SAG leaves in the competition experiment contained some chlorophyll (108 μmol m^{-2}) and had an N_{LA} that was lower than the *x*-intercept of the $A_{max} - N_{LA}$ relationship (14.3 and 20.2 mmol m^{-2}, respectively). For this reason, an *x*-intercept of 14.3 mmol N m^{-2} was

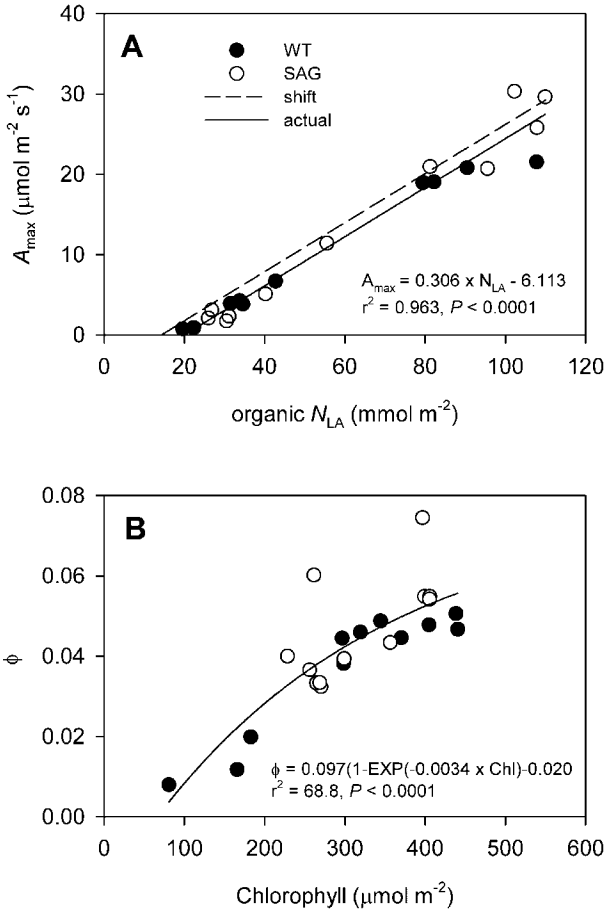

Figure 3: Regressions between organic nitrogen contents per unit area (N_{LA}) and photosynthetic capacity (A_{max}; *A*) and between chlorophyll and apparent quantum yield (Φ; *B*). Leaves were sampled at different heights from wild-type (WT) and P_{SAG12}-*IPT* (SAG) tobacco plants grown in monocultures for 12 weeks at a density of 35 plants m^{-2}.

assumed in the model, but the measured regression coefficient was used to calculate A_{max} (fig. 3*A*). This assumption did not affect the main conclusions of this study.

Irradiance strongly decreased toward the canopy bottom in the mixture, with less than 5% PPFD penetrating to the top of the third canopy layer (fig. 4*A*). Calculated daily gross photosynthesis (A_{gross}) in both genotypes was highest in the upper canopy layer, much smaller in the fourth layer, and negligible in the layers below that (fig. 4*B*). Daily respiration (R) was also highest in the upper two layers in both genotypes (fig. 4*C*). Only in the SAG plants, however, did any substantial daily respiration continue in the lower three layers (fig. 4*C*), resulting in negative daily net photosynthesis rates (A_{net} or carbon gain) in these leaves (fig. 4*D*). At the level of whole plants, this resulted in daily leaf respiration rates being 37% higher in the SAG plants compared to the WT plants (table 3). As daily gross photosynthesis rates did not differ much between genotypes, daily carbon gain was consequently lower in SAG than in WT plants. This 27% difference in carbon gain under average light conditions increased to a 46% lower rate for SAG plants under overcast conditions, when calculated photosynthesis rates consequently decreased (table 3). Note that when the originally obtained $A_{max} - N_{LA}$ relationship (fig. 3*A*) was used, generally lower but probably unrealistic calculated daily gross photosynthesis rates were found, also resulting in SAG plants having a lower daily carbon gain than the WT (data not shown).

Investment of Reallocated N in Additional Leaf Area in the Canopy Top Is Beneficial in a Competitive Situation

Since dominant WT plants in the mixture achieved a greater estimated carbon gain through shedding their lower leaves, the consequence for carbon gain of shedding lower leaves of the SAG plants and reallocating N was calculated using a sensitivity analysis (for details, see fig. 5). In scenario 1, all SAG leaves in the lower two canopy layers were shed, with all N being lost. This resulted in an increase in carbon gain of the SAG plants from 73% to 93% of the WT value under average light conditions because of reduced costs of respiration. In scenario 2, the lower leaves were shed with part of the N reallocated to increase N_{LA} of the top leaves, which further increased estimated SAG carbon gain to 4% above that of WT plants. Finally, in scenario 3, the effect of using the reallocated N to increase SAG leaf area in the upper canopy layer was simulated. This yielded an absolute SAG carbon gain similar to that found in scenario 2 but led to shading of neighboring WT plants, inhibiting their photosynthesis and resulting in a 17% higher carbon gain of SAG plants relative to that of the WT (fig. 5). Hence, the most favorable strategy for competing plants in a dense canopy appears to be the shedding of lower, shaded leaves and utilizing reallocated N to produce more leaf area in the canopy top, which is exactly what was found in WT plants.

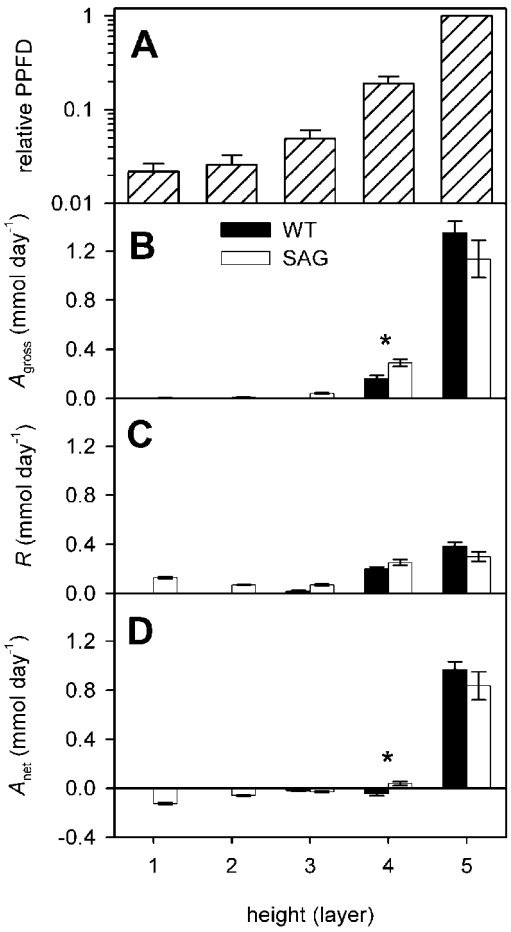

Figure 4: Distribution of irradiance and daily CO_2 exchange rates of dominant wild-type (WT) and P_{SAG12}-*IPT* (SAG) tobacco plants grown in a 1 : 1 mixture for 11 weeks. *A*, Relative photosynthetic photon flux density (PPFD); *B*, calculated daily gross photosynthesis (A_{gross}); *C*, daily respiration rate (R); *D*, calculated daily carbon gain (A_{net}). Error bars indicate the SE; $n = 8$ (*A*) and 12 (*B–D*). Asterisk = $P < .05$.

Discussion

The idea that plants form an optimal LAI and leaf N distribution at which their carbon gain is maximized in dense canopies is supported by the correspondence between model predictions and measurements on actual stands of vegetation (Grindlay 1997; Anten 2005; Hirose 2005). However, the assumption in these models of a link between optimization of these traits and plant performance has never been experimentally tested. As previously

Table 3: Daily respiration rates (R) and calculated daily gross and net photosynthesis rates of individual dominant P_{SAG12}-*IPT* (SAG) and wild-type (WT) tobacco plants in the mixture

	Daily CO_2 exchange rate (mmol day^{-1})		
	WT	SAG	Significance
R	.601 ± .028	.821 ± .032	$P < .001$
Average irradiance (16.1 mmol quanta day^{-1}):			
Calculated A_{gross}	1.509 ± .087	1.482 ± .142	NS
Calculated A_{net}	.908 ± .068	.661 ± .117	$.05 < P < .10$
Overcast (11.1 mmol quanta day^{-1}):			
Calculated A_{gross}	1.147 ± .066	1.118 ± .114	NS
Calculated A_{net}	.547 ± .048	.297 ± .090	$P < .05$

Note: Data are means ± SE; $n = 12$. NS = not significant.

reported for solitarily grown SAG plants (Gan and Amasino 1995; Wingler et al. 1998; Jordi et al. 2000), we also observed a higher LAI (table 2) and more uniform N distribution (fig. 2*D*) of SAG plants compared to WT plants in the dense leaf canopies, allowing us to test these model predictions. Indeed, estimated carbon gain of vegetative SAG plants was lower than that of the WT because of greater costs of respiration of lower, shaded leaves that were retained by SAG plants (table 3; fig. 4*C*). The somewhat greater shoot N content of SAG plants (table 2) cannot explain their lower carbon gain because higher shoot N would be expected only to increase carbon gain; rather, the cause was the differing distributions of leaf area and N. This finding suggests that shade-induced leaf senescence, and implicitly its effects on LAI and leaf N distribution, is functional for the maximization of carbon gain in a dense canopy.

Continued lower carbon gain should be a competitive disadvantage for plants in dense stands because height growth and leaf area formation, fueled by photosynthesis, may increasingly lag behind those of neighboring plants, thus further decreasing light capture and growth. Accordingly, at the time of harvest, vegetative dominant SAG plants attained a lower height (table 2), had less leaf area in the top layer of the canopy (fig. 2*A*), and achieved a 13.3% lower carbon gain than dominant WT plants in this layer (fig. 4*D*). While their predicted growth rate was higher, WT plants were not yet superior to SAG plants in terms of dry mass in this developmental stage; SAG plants actually tended to be heavier than WT plants (table 2). In the experiment that continued until the reproductive growth stage, however, a clear negative effect on SAG dry mass was observed at the higher density (fig. 1), in which the competition with surrounding WT plants was presumably more intense than at the lower density. This can be understood when the shading of lower SAG leaves is considered. Shading of lower leaves is minimal in solitary plants and early in canopy development. At this early stage, the delayed senescence phenotype of SAG plants may in fact be advantageous because plants have larger leaf areas, with which they achieve higher carbon gain and growth (Gan and Amasino 1995). As the canopy grows, light availability on the lowest leaves of SAG plants readily declines,

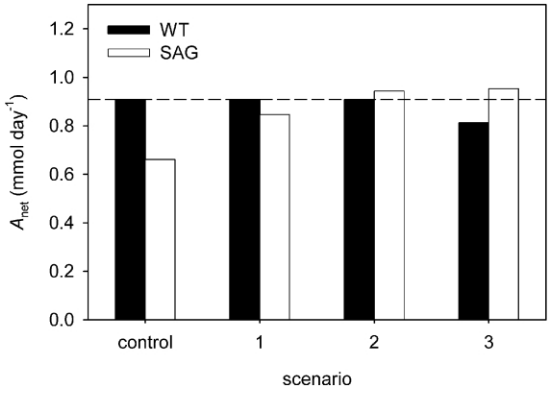

Figure 5: Sensitivity analysis of the effects of senescence and N reallocation on daily carbon gain of P_{SAG12}-*IPT* (SAG) and wild-type (WT) tobacco plants in the mixture. The control scenario is that under average light conditions in table 3. The other scenarios consider the situation where SAG plants shed all the leaves in the bottom two canopy layers, reducing respiration costs there to 0. With respect to this shedding we consider three scenarios: (1) all the N in the shed leaves is lost; (2) part of the N in the shed leaves, assumed to be equal to the N content of dead WT leaves (9.13 mmol m^{-2}), is lost, and the remainder is reallocated to the leaves in the upper canopy layer, in which N_{LA} is proportionally increased; (3) same as (2), but reallocated N is used to produce extra leaf area in the upper canopy layer, with the new leaf area having the same N_{LA} as existing leaves. In (2), photosynthetic capacity of SAG leaves in the upper canopy layer was adjusted using the N_{LA}-A_{max} relationship (fig. 3*A*), and respiration of these leaves was increased proportionally. The average leaf area increase calculated in (3) was then applied to all dominant SAG plants in the mixture, and based on this, the distribution of incident irradiance was recalculated for each block in the mixture, assuming K values were not altered. Daily A_{net} for individual dominant SAG plants in the mixture was then recalculated as described in "Material and Methods," based on these simulated changes in leaf area and N distribution. In (3), the altered light distribution was also taken into account, and the consequence for daily A_{net} of both SAG and WT plants in the mixture was calculated. Data are means; $n = 12$.

and their net photosynthesis becomes negative, which suppresses growth.

Further evidence that the negative effect of delayed leaf senescence on plant performance is density dependent comes from our observation that while solitary SAG plants can produce a greater seed mass than WT plants (Gan and Amasino 1995; T. A. Dueck, W. J. R. M. Jordi, and A. van der Werf, unpublished data), in our study seed mass was lower at both densities (table 1). These results suggest that the reproductive output of SAG plants was reduced as a result of competition with neighboring WT plants. This may have been caused by the lower carbon gain of SAG plants during the reproductive growth phase. In a dense *Xanthium canadense* stand, seed production was more strongly correlated with calculated carbon gain than with plant biomass at the time of flowering (N. P. R. Anten, unpublished data). Taken together, these data indicate that the larger LAI and more uniform N distribution caused by delayed leaf senescence negatively affect productivity and reproductive output of plants competing with neighbors in dense stands.

The strategy of a plant growing in a dense stand in relation to that of its neighbors can be framed as a game-theoretic problem. The LAI (Anten 2002, 2005) and plant height (Falster and Westoby 2003) of actual vegetations are greater than would be expected based on the optimal strategy for the stand as a whole because in a situation of stand-based optimization, taller individuals or individuals with more leaf area would have higher fitness than their neighbors. In a dense canopy, taller plants that position more leaf area in the upper parts of the canopy reduce light capture and photosynthesis of neighboring plants that have more of their leaf area in the lower parts of the canopy (Williams 1963; Barnes et al. 1990). In line with these findings, our results indicate that the shedding of lower leaves allows tobacco plants to grow taller and place more leaf area in the top of the canopy and thus, as noted, to achieve greater carbon gain than nonsenescing SAG plants.

Using sensitivity analysis, we further evaluated the strategies of WT and SAG plants in the light of the competitive interactions between plants in dense stands. First, if SAG plants shed all their leaves in the lower two canopy layers, respiration costs of these leaves would become 0 and their daily carbon gain would increase from 73% to 93% of the WT value (fig. 5). Next, the analysis showed that N reallocated from these lower SAG leaves can benefit the plant more when it is utilized to increase leaf area at the top of the canopy than when invested in existing leaves there to increase their photosynthetic capacity. This is because only the former strategy would result in the shading of neighboring WT plants, reducing their carbon gain. Since SAG plants contained more N in their shoots, the simulations in which N was reallocated from lower SAG leaves yielded a carbon gain slightly greater than that found for the WT.

The greater leaf area of WT plants in the upper canopy layer was associated with a greater total amount of leaf N there (fig. 2). Leaf area growth is generally believed to be strongly determined by the amount of N allocated to leaves (Anten et al. 1995; Lambers et al. 1998), particularly when N availability from the soil is limited, as was the case here. It can therefore be argued that leaf area growth in top layers of the SAG plants was reduced because only a limited amount of N could be reallocated from their lower leaves.

The SAG and WT plants had the same N_{LA} in their top leaves, but if N reallocation served to maximize daily carbon gain, a higher N_{LA} in upper WT leaves would be expected (Hirose and Werger 1987*a*). However, if N reallocation is more important to successfully compete with neighboring plants, more leaf area in the top layer of the canopy would be expected (see fig. 5). The latter strategy was adopted by WT plants. Our results demonstrate that senescence and the associated N reallocation are important for leaf area formation in the canopy top and height growth. Not the maximization of carbon gain per se but the increase in light capture and carbon gain relative to neighbor plants appears to dictate the distribution of leaves and N over canopy height, as predicted by individual plant-based optimality models (Anten 2005).

To conclude, we have found experimental evidence in support of model predictions that induced leaf senescence and its effects on LAI and leaf N distribution are functional for plant fitness in dense canopies. An important reason is that carbon loss through respiration is prevented by shedding shaded bottom leaves, thereby increasing whole-plant carbon gain. Moreover, N reallocation from lower leaves, which is associated with leaf senescence, contributes to the formation of leaves in the upper part of the canopy. Because carbon gain of neighbors is repressed in this manner, utilizing reallocated N for additional leaf area in the upper part of the canopy allows plants to successfully compete for light with their neighbors.

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