

# Maize Morphophysiological Responses to Intense Crowding and Low Nitrogen Availability: An Analysis and Review

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## ABSTRACT

Mounting concerns over the cost and environmental impact of N fertilizer combined with progressively higher plant densities in maize (*Zea mays* L.) production systems make progress in maize N use efficiency (NUE) and N stress tolerance essential. The primary objectives of this 3-yr field study were to (i) evaluate the N responsiveness, NUE, and N stress tolerance of multiple modern maize genotypes using suboptimal, optimal, and supraoptimal plant densities (54,000, 79,000, and 104,000 plants ha<sup>-1</sup>, respectively) with three levels of side-dress N (0, 165, and 330 kg N ha<sup>-1</sup>), (ii) identify key morphophysiological responses to the simultaneous stresses of intense crowding and low N availability, and (iii) consider our results with extensive reference to literature on maize morphophysiological responses to plant crowding and N availability. At optimal and supraoptimal plant densities, maize receiving 165 kg ha<sup>-1</sup> of side-dress N displayed strong N responsiveness, high NUE, pronounced crowding tolerance, and plant density independence. However, crowding tolerance was contingent on N application. Relative to less crowded, N-fertilized environments, the 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> treatment combination exhibited (i) reduced pre- and postanthesis plant height (PHT), stem diameter (SD), and total biomass; (ii) greater preflowering leaf senescence and lower R1 leaf areas at individual-leaf, per-plant, and canopy levels; (iii) enhanced floral protandry; (iv) lower pre- and postanthesis leaf-chlorophyll content; (v) lower per-plant kernel number (KN<sub>p</sub>), individual kernel weight (KW), grain yield per plant (GY<sub>p</sub>), andharvest index per plant (HI<sub>p</sub>); and (vi) enhanced per-plant grain yield variability (GY<sub>CV</sub>). Genetic efforts to improve high plant density tolerance should, therefore, simultaneously focus on enhancing NUE and N stress tolerance.

**N**RAIN YIELDS of modern commercial maize hybrids Greatly exceed those of their predecessors (Duvick, 2005; Lee and Tollenaar, 2007). In the United States, mean commercial grain yield increased from  $\sim 1.5$  Mg ha<sup>-1</sup> in the 1930s to  $\sim 8.5$  Mg ha<sup>-1</sup> at the end of the last century. Dramatic rates of yield improvement have also been documented in Argentina, Canada, France, and Germany within the latter half of the 20th century (Duvick, 2005). Yield increases in maize have been largely attributed to genetic gains made by public and private breeders, and to superior agronomic-management practices increasingly adopted by growers, with 50 to 70% of the observed yield improvement due to improved genetics and 30 to 50% due to superior agronomic-management practices (Cardwell, 1982; Duvick, 2005; Lee and Tollenaar, 2007). However, in actuality, nearly all prior yield advances in maize have resulted from the interaction between improved genetics and superior agronomicmanagement practices (Tollenaar and Lee, 2002).

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Environmental stress tolerance has been recognized as the physiological trait most strongly linked with the genetic improvement of maize hybrids for grain production (Tollenaar and Wu, 1999). Modern genotypes generally have a greater tolerance to insect feeding; pathogen infection; drought; low soil fertility; saturated and/or cool soils; above- and belowaverage seasonal temperatures; low night temperatures during the grain-filling period; and inter- and intraspecific competition (i.e., maize-weed and maize-maize interactions, respectively) for solar radiation, water, and soil nutrients (Dwyer and Tollenaar, 1989; Tollenaar et al., 1997; Tollenaar and Wu, 1999; Ying et al., 2000; Duvick, 2005). The tolerance of maize to intense intraspecific competition for available resources at high plant densities has improved more than many other environmental stress tolerances over the past 40 to 50 yr (Russell, 1991; Tollenaar, 1991; Tollenaar and Lee, 2002; Tokatlidis and Koutroubas, 2004). This progress has been principally driven by maize breeders selecting for grain yield

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Abbreviations: ACRE, Purdue University Agronomy Center for Research and Education; ASI<sub>p</sub>, per-plant anthesis-silking interval; *b*, a coefficient that describes the breadth/kurtosis of the distribution of green leaf area; GDD, growing degree days; GY<sub>A</sub>, per-unit-area grain yield; GY<sub>CV</sub>, per-plant grain yield variability; GY<sub>p</sub>, per-plant grain yield; GY<sub>SA</sub>, per-plant sampling area grain yield; HI, harvest index; HI<sub>p</sub>, per-plant harvest index; KN<sub>p</sub>, per-plant green leaf area; distribution; LA<sub>n</sub>, fully expanded individual leaf area; LA<sub>p</sub>, per-plant green leaf area; LA<sub>T</sub>, per-plant green leaf area of tagged, nondestructively sampled plants; LAI<sub>G</sub>, green leaf area index; NL<sub>T</sub>, total number of green leaves per plant; N<sub>R</sub>, nitrogen application rate; NUE, nitrogen use efficiency; PDA, personal digital assistant; PHT, plant height; PPAC, Pinney-Purdue Agricultural Center; SD, stem diameter; SPAD, single-photon avalanche diode; TB<sub>p</sub>, aboveground per-plant total biomass; VB<sub>p</sub>, aboveground per-plant vegetative biomass;  $x_e$ , earleaf position;  $x_H$ , position of the highest green leaf;  $x_L$ , position of the largest leaf.

and/or beneficial morphophysiological traits in environments commonly encountered in commercial maize production. This has included selection at high plant densities (Duvick and Cassman, 1999; Tollenaar and Lee, 2002; Fasoula and Tollenaar, 2005; Lee and Tollenaar, 2007). Thus, to optimize grain production and maximize grain yield potential in today's production systems, modern hybrids must be grown at higher plant densities than their predecessors (Tollenaar, 1989; Tokatlidis and Koutroubas, 2004).

Many morphophysiological traits that were either directly or indirectly selected for by maize breeders are now characteristic of today's commercial genotypes (Duvick and Cassman, 1999; Tollenaar and Lee, 2002). Traits such as greater green leaf area index (LAI<sub>G</sub>) at silking [i.e., R1 (Ritchie et al., 1996)], improved radiation use efficiency, reduced dry matter partitioning to the tassel, less temporal separation between anthesis and silking, greater postsilking dry matter accumulation, and delayed leaf senescence during the grain-filling period [i.e., visual (leaf greenness) and/or functional (photosynthetic) staygreen (Lee and Tollenaar, 2007)] are often more evident among modern hybrids in both suboptimal and optimal conditions (Tollenaar, 1991; Tollenaar and Aguilera, 1992; Rajcan and Tollenaar, 1999a; Tollenaar and Wu, 1999; Sangoi et al., 2002; Valentinuz and Tollenaar, 2004; Duvick, 2005; Tollenaar and Lee, 2006; Lee and Tollenaar, 2007). Per-plant total leaf area has not generally changed since the 1930s (Duvick, 1997), so improvements in early-, mid-, and late-season LAI<sub>G</sub> are the result of improved crowding tolerance (Tollenaar and Lee, 2002; Lee and Tollenaar, 2007). Other morphophysiological traits specifically associated with the improved crowding tolerance of modern genotypes include reduced root and/or stem lodging, shorter leaf length, increased vertical leaf angle, and greater leaf reorientation in response to intraspecific competition. Such attributes have improved light interception, canopy photosynthesis distribution, and, consequently, dry matter accumulation and grain production of modern hybrids in crowded environments (Girardin and Tollenaar, 1994; Duvick and Cassman, 1999; Sangoi et al., 2002; Tollenaar and Lee, 2006; Hammer et al., 2009). As suggested through the recent modeling efforts of Hammer et al. (2009), the enhanced high plant density tolerance of modern North American hybrids may be strongly related to changes in root system architecture, which have improved water capture at greater soil depths. Whether the harvest index (HI) of maize genotypes has improved over time is still uncertain. Duvick (2005) contends that newer commercial genotypes exhibit a higher HI than older ones when plants are subjected to biotic stresses that promote barrenness, but in general, HI has not improved when modern hybrids are grown at their respective optimal plant densities. Similarly, Tollenaar and Lee (2002, 2006) and Tollenaar et al. (2000) suggest a lack of improvement in the HI of North American hybrids, with HI generally static at 0.5 (typical range of 0.48 to 0.52) during the past 60 to 65 yr. On the contrary, Echarte and Andrade (2003) and Luque et al. (2006) report an increase in HI among Argentine hybrids. Tollenaar et al. (2000) similarly discuss an improvement in HI among tropical maize genotypes as a result of genetic improvement.

A roughly parabolic relationship exists between plant density and per-unit-area grain yield  $(GY_A)$  (Tokatlidis and

Koutroubas, 2004). A plant density therefore exists for each environment (i.e., production system) that maximizes resource capture and utilization and, consequently, grain production. At suboptimal plant densities, the addition of more plants compensates for an accompanying decline in GY<sub>p</sub> due to intraspecific competition, leading to an overall increase in GY<sub>A</sub>. Yet at supraoptimal plant densities, further crowding cannot offset reductions in GY<sub>p</sub> resulting from intense intraspecific competition, leading to a general decline in GY<sub>A</sub> (Tollenaar and Wu, 1999). Overall reductions in GY<sub>A</sub> at supraoptimal plant densities are often associated with a variety of morphophysiological responses including, but not limited to, (i) reductions in per-plant photosynthesis and shoot and (more pronouncedly) ear growth during the critical period bracketing silking [i.e., approximately 15 d before to 15 d after silking (Andrade et al., 1999; Echarte and Tollenaar, 2006)], (ii) minor and major delays in the time to anthesis and silking, respectively, (iii) declines in above ground per-plant total biomass  $(TB_p)$  and overall biomass partitioning to the ear (i.e., HI), (iv) an increase in barrenness, and (v) a rise in plant-to-plant variability for PHT, pre- and postsilking TB<sub>p</sub>, floral protandry, KW, KN<sub>p</sub>, and GY<sub>p</sub> (Glenn and Daynard, 1974; Bunce, 1990; Otegui, 1997; Tollenaar and Wu, 1999; Tollenaar et al., 2000; Maddonni and Otegui, 2004; Tokatlidis and Koutroubas, 2004; Andrade and Abbate, 2005; Hashemi et al., 2005; Maddonni and Otegui, 2006; Borrás et al., 2007).

Besides exhibiting superior tolerance to high plant densities, modern hybrids demonstrate a greater responsiveness to a variety of inputs including irrigation, pesticide application, and fertilizer use (Russell, 1991; Tokatlidis and Koutroubas, 2004). In particular, current hybrids are more responsive to N fertilizer than their predecessors in both low and high soil-N environments. For example, in a study involving 12 hybrids from three eras (1970s, early 1990s, late 1990s), O'Neill et al. (2004) found that early- and late-1990s-era hybrids exhibited larger yield responses to N application than 1970s-era hybrids. The greater responsiveness of post-1970s-era hybrids to N application has encouraged relatively high N fertilization rates among maize growers for the past roughly three decades (Cardwell, 1982; Duvick, 2001; USDA-ERS, 2008). Application of N in maize triggers a complex array of morphophysiological responses. At the per-plant and canopy levels of organization, these include, but are not restricted to, greater PHT, SD, dry matter production, leaf N concentration, LAI<sub>G</sub>, KN<sub>P</sub>, GY<sub>P</sub>, and GY<sub>A</sub> in addition to delayed leaf senescence (Ogunlela et al., 1988; Jacobs and Pearson, 1991; McCullough et al., 1994b; O'Neill et al., 2004; Ding et al., 2005; Subedi and Ma, 2005b).

In addition to improved N responsiveness, some studies suggest greater NUE and enhanced N stress tolerance among modern hybrids (e.g., McCullough et al., 1994a, 1994b; Ma and Dwyer, 1998; Ding et al., 2005; Coque and Gallais, 2007). According to Moll et al. (1982), NUE can be defined as the amount of grain produced per unit of available soil N (including fertilizer). Grain NUE can be more thoroughly expressed as the product of N-uptake efficiency (N uptake per unit available soil N) and N-utilization efficiency (grain production per unit absorbed N) (Moll et al., 1982; Coque and Gallais, 2007). Using an <sup>15</sup>N-labeling approach, Ma and Dwyer (1998) found that prior genetic improvement for NUE (when

defined as the ratio of the amount of <sup>15</sup>N recovered in grain or stover to the amount of fertilizer <sup>15</sup>N applied to the soil) was primarily associated with greater N uptake and improved dry matter production during the grain-filling period. Discussing genetic progress for NUE among European maize varieties, Coque and Gallais (2007) similarly attribute prior advances in NUE [when defined according to Moll et al. (1982)] to greater postsilking N uptake in both low N and high N environments. They also suggest potential genetic improvement for grain N-utilization efficiency. Advances in low N tolerance are evident for multiple physiological traits. For example, relative to older genotypes under N stress conditions, newer hybrids exhibit a higher C exchange rate per unit leaf N, greater N uptake capacity, improved leaf C exchange rate and higher leaf chlorophyll content during the grain-filling period, and greater KN<sub>p</sub> (McCullough et al., 1994a, 1994b; Ding et al., 2005; Coque and Gallais, 2007; Echarte et al., 2008). Despite these reported advances in NUE and N stress tolerance, recent genetic improvements for these traits have been neither consistently observed nor heavily studied. Furthermore, reductions in GY<sub>A</sub> for modern hybrids are still substantial when soil N is deficient (O'Neill et al., 2004).

Since approximately 50 to 60% of foliar N in maize is associated with chloroplasts (Hageman, 1986), a large amount of acquired N contributes to the maintenance of photosynthesis. Foliar responses to N deficiency are therefore typically pronounced and often strongly associated with GY<sub>A</sub>. Such responses commonly include reductions in total leaf area, leaf expansion and duration, leaf N and chlorophyll content, leaf stomatal conductance, and photosynthesis per unit leaf area (Wolfe et al., 1988a, 1998b; McCullough et al., 1994a, 1994b; Paponov and Engels, 2003; Monneveux et al., 2005; Echarte et al., 2008). These responses reduce radiation interception, radiation use efficiency, and, resultantly, crop growth and dry matter accumulation (McCullough et al., 1994a, 1994b; Uhart and Andrade, 1995b; Paponov and Engels, 2003, 2005; Paponov et al., 2005a; Echarte et al., 2008). With source activity diminished in low N situations, dry matter partitioning to reproductive sinks markedly decreases, silk growth commonly slows, kernel abortion often rises, and KW, KN<sub>p</sub>, GY<sub>p</sub>, and HI<sub>p</sub> consequently fall (Jacobs and Pearson, 1991; Uhart and Andrade, 1995c; Below et al., 2000; Tollenaar et al., 2000; O'Neill et al., 2004; Ding et al., 2005; Monneveux et al., 2005).

As N fertilizer costs remain relatively high, environmental concerns over excessive N application increase, and recommended plant densities move progressively higher, it is crucial that the N responsiveness, NUE, and N stress tolerance of current maize germplasm continue to be investigated and improved. Fundamental to future genetic improvement efforts is an understanding of the morphophysiological responses of modern commercial maize genotypes to deficit and adequate N availability at various plant densities. No studies to date have intensively examined the per-plant and canopy-level morphophysiological responses of modern, stress-tolerant, highly productive, North American maize hybrids to the simultaneous stresses of intense crowding and low fertilizer N availability. Thus the primary objectives of this work were to (i) evaluate the N responsiveness, NUE, and N stress tolerance of multiple modern maize genotypes using agronomic

suboptimal, optimal, and supraoptimal plant densities in conjunction with three levels of side-dress N fertilization, (ii) identify key, yield-determining morphophysiological responses to the simultaneous stresses of intense crowding and low N availability through intensive measurement of numerous perplant and canopy-level morphophysiological parameters, and (iii) provide an in-depth analysis of our experimental results through an extensive review of literature related to maize C and N physiology and metabolism. Secondary objectives for this study were to (i) investigate if the HI of certain modern, N-responsive, North American maize hybrids now potentially exceeds 0.5 when such genotypes are grown at their optimal plant densities with high N availability and (ii) develop, implement, and validate an accurate, rapid, low-cost, nondestructive technique for determining the green leaf area  $(LA_p)$  and green leaf area distribution (LA<sub>D</sub>) of individual maize plants (~4000 plants per year) at silking.

# MATERIALS AND METHODS Cultural Practices, Experimental Design, and Treatments

Research during the 2005, 2006, and 2007 growing seasons was conducted at the Purdue University Agronomy Center for Research and Education (ACRE) (40°28'07" N, 87°00'25" W) near West Lafayette, Indiana. From 1971 to 2000, the location averaged approximately 946 mm of precipitation and 1971 growing degree days (GDD) on an annual basis. The soil, which developed under prairie vegetation, was a Chalmers (fine-silty, mixed, mesic Typic Endoaquoll) silty clay loam with approximately 4.0 to 4.5% organic matter content in the top 30 cm of the soil profile and a pH of approximately 6.2. The experimental area has less than 2% slope and was systematically tile drained at 20-m intervals. In each year, maize was grown following no-till soybean [*Glycine max* (L.) Merr.]. Strip-tillage was performed in the fall of each year using a Remlinger six-row unit (Remlinger Manufacturing, Kalida, OH). Planting was done directly onto the strips the subsequent spring using a six-row John Deere 1780 planter (Deere & Company, Moline, IL) with electronic variable seeding rate controls, vacuum seed metering, and tined row cleaners ahead of each planting unit. In 2006 and 2007, both strip-tillage and planting were performed using a John Deere StarFire Real-Time Kinematic (Deere & Company, Moline, IL) automatic guidance system. Complete weed control was obtained with burndown, preemergence, and postemergence herbicides in addition to hand weeding. The insecticide Force 3G [Tefluthrin, (2,3,5,6-tetrafluoro-4-methylphenyl)methyl-(1a,3a)-(Z)-(±)-3-(2-chloro-3,3,3-trifluoro-1-propenyl)-2,2dimethylcyclopropanecarboxylate, Syngenta Crop Protection, Inc., Greensboro, NC] was applied at planting. In 2005 and 2006, the insecticide Warrior T [ $\lambda$ -cyhalothrin ([1 $\alpha$ (S\*),3 $\alpha$ (Z)]-(±)-cyano-(3-phenoxyphenyl)methyl-3-(2-chloro-3,3,3trifluoro-1-propenyl)-2,2-dimethylcyclopropanecarboxylate), Syngenta Crop Protection, Inc., Greensboro, NC] was aerialapplied at silking to limit silk-feeding by western corn rootworm (Diabrotica virgifera virgifera LeConte) and Japanese beetle (Popillia japonica Newman) populations. Postplanting insecticide application was unnecessary in 2007 and fungicide application was deemed unnecessary in all years.

The study was arranged as a split-split plot design with four blocks. Hybrid (main plot), plant density (subplot), and N application rate (sub-subplot) served as the three treatment factors. Each sub-subplot consisted of six rows (76-cm interrow spacing) and was approximately 4.5 m in width and 30.5 m (2005, 2007) or 27.5 m (2006) in length. Two hybrids were planted each year: Pioneer 33N09 [1529 GDD to R6, Comparative Relative Maturity (CRM) of 114] in 2005; Pioneer 31G68 (1585 GDD to R6, CRM of 118) in 2005, 2006, and 2007; and Pioneer 31N28 (1630 GDD to R6, CRM of 119) in 2006 and 2007 (Pioneer Hi-Bred Intl., Inc., Johnston, IA). Hybrid Pioneer 31N28 replaced Pioneer 33N09 after 2005 since the former possessed the YieldGard Corn Borer trait (Monsanto Company, St. Louis, MO) and was more similar to Pioneer 31G68 with respect to ontogeny, phenology, morphology, relative maturity, and optimal plant density than was Pioneer 33N09. In all years, planting rates were 58,000, 83,000, and 108,000 seeds ha<sup>-1</sup> to achieve the desired final plant densities of 54,000, 79,000, and 104,000 plants ha<sup>-1</sup>. Plant density measurements at V6 rarely differed from target plant densities by more than 3% (data are not shown). As suggested through multilocation, multienvironment testing by Pioneer Hi-Bred International, Inc., these final plant densities respectively corresponded to agronomic (based on maximum  $GY_A$ ) and economic (based on gross income less seed cost) suboptimal, optimal (approximately), and supraoptimal levels for each of this study's hybrids (D. Rule, personal communication, 2008). Such plant densities therefore provided highly contrasting levels of per-plant resource availability and, consequently, varying levels of intraspecific competition. For all sub-subplots in each year, starter fertilizer (10-34-0) was applied at planting 5 cm to the side and 5 cm below the seed at a rate equivalent to 25 kg N ha<sup>-1</sup>. Urea Ammonium Nitrate (UAN) (28–0–0) was applied via side-dressing at a rate equivalent to 165 kg N ha<sup>-1</sup> once (V3), twice (V3, V5), or not at all, depending on each sub-subplots' prescribed N application rate. Recent N rate trials on similar soils at ACRE in 2006, 2007, and 2008 have found the average agronomic optimal N rate (i.e., N rate that produces maximum  $GY_A$ ) for a soybean-maize rotation to be roughly 160 kg N ha<sup>-1</sup> (J.J. Camberato, personal communication, 2008). In general, the 0, 165, and 330 kg ha<sup>-1</sup> side-dress N rates were chosen for investigating this study's primary and secondary objectives because they theoretically provided (i) an environment with markedly high N stress, (ii) an N application rate similar to that used by local commercial maize growers, and (iii) an environment with little to no N limitations, respectively. Chosen side-dress N rates therefore provided highly contrasting levels of per-plant resource availability and, consequently, varying levels of intraspecific competition.

In each year, five 2-cm-diam. soil cores were taken at V4 and V8 in each sub-subplot receiving only starter fertilizer. Samples were collected from the 0- to 30-cm depth (no subdivisions) in the interrow areas on each side of the third and fourth rows (at least 25 cm from any row). All samples were air-dried and sent to A&L Great Lakes Laboratories, Inc. (Fort Wayne, IN) for determination of soil nitrate ( $NO_3^--N$ ) and ammonium ( $NH_4^+-N$ ) concentrations. In brief, soil samples were dried for 12 h at 40°C, crushed with a flail-type grinder, and sieved through a U.S. No. 10 sieve (2 mm). A portion of each sample

was extracted with 1 N KCl. Extracts were analyzed for  $NO_3^-$ -N by  $NO_3^-$  reduction and for  $NH_4^+$ -N by the phenolate method (based on the Bertholot reaction) (A&L Great Lakes Laboratories, Inc., personal communication, 2008). In all years of this study, soil  $NO_3^-$ -N levels were between 3 and 13 ppm for all sub-subplots—values well below appropriate critical  $NO_3^-$ -N levels (Brouder and Mengel, 2003). Thus side-dress N application was warranted in every year. Through appropriate fertilization, all other nutrients were kept nonlimiting.

In all years, nondestructive per-plant sampling areas (4 m in length) were established in rows three and four of each six-row sub-subplot. Within each of the designated sampling areas, individual plants (~35–70 per sampling area depending on plant density, ~4000 in total per year) were tagged with individually numbered barcodes formatted for use with a barcodescanning personal digital assistant (PDA) outfitted with MaizeMeister Phenotypic Data Collection and Seed Management System software. To accurately measure the ontogeny and phenology of each year's approximately 4000 tagged plants, orange paint was lightly applied to the tip of each plant's fourth leaf (leaves numbered from the bottom of the plant upward) at V4. When plants reached V8, bar-coded tags were stapled around the stem above the eighth leaf. As lower leaf senescence progressed, tags were moved above the 12th leaf, where they remained for the remainder of each growing season. Extensive nondestructive morphophysiological measurements were taken on these plants from seedling emergence through physiological maturity, with special attention given to limiting soil compaction from foot traffic. However, only a subset of all measured morphophysiological parameters is discussed here.

#### Weather Measurements

From 1 April to 1 October of each year, precipitation, minimum air temperature, and maximum air temperature were recorded on a daily basis at ACRE (<0.5 km from the study area) to examine the effects of these weather variables on crop phenology and physiology. The modified GDD formula was used to calculate the number of GDD accumulated during this 6-mo period for each growing season. The GDD measure of thermal time was chosen for this study since it is the most commonly used method in the U.S. Corn Belt for describing maize growth and development. Using the modified GDD formula, accumulated GDD was calculated as follows for any given date:

Accumulated GDD = 
$$\sum_{i=m}^{n} \left\{ \left[ \left( T_{max} + T_{min} \right) / 2 \right] - T_{B} \right\}$$
[1]

where  $T_{max}$  is the daily maximum air temperature (with an upper limit of 30°C),  $T_{min}$  is the daily minimum air temperature (with a lower limit of 10°C),  $T_B$  is equal to 10°C, *m* is the first day of recorded air temperature data (1 April), and *n* is the last day of available recorded air temperature data (Cross and Zuber, 1972; Bollero et al., 1996; Dwyer et al., 1999).

#### **Morphophysiological Measurements**

In each year, PHT was determined at V5, V14, and R1. At V5 and V14, PHT was measured as the distance from the soil surface to the uppermost extended leaf tip. However, PHT at R1 was measured as the distance from the soil surface to the

collar of the uppermost leaf. Internode elongation was minimal to nonexistent after R1 (c.f., Siemer et al., 1969; Fournier and Andrieu, 2000), resulting in little to no change in PHT between R1 and R6. At V14 (2005–2007), R1 (2007), R3 (2005–2007), and R6 (2005–2007), the SD of tagged plants was measured as the maximum diameter at the midpoint of the sixth internode (i.e., between Leaves 6 and 7). For a very limited number of exceptionally small and/or malformed plants without a defined sixth internode, maximum SD was determined at the internode above the stem base. All measurements were taken using a Mitutoyo ABSOLUTE Digimatic 500–171 caliper (Mitutoyo America Corporation, Aurora, IL) connected to a PDA equipped with DataGet software (Baxter Codeworks, 2004).

Single-photon avalanche diode (SPAD) measurements were taken on all tagged plants using a Konica Minolta SPAD-502 Chlorophyll Meter (Konica Minolta Sensing Americas, Inc., Ramsey, NJ). Numerous studies in maize have indicated that SPAD readings can provide (i) a rapid, accurate, indirect indication of leaf N and chlorophyll content (e.g., Dwyer et al., 1991, 1995a, 1995b) and (ii) an accurate nondestructive measure of leaf absorptance (once the relationship between SPAD readings and leaf absorptance has been determined for a particular meter) (Earl and Tollenaar, 1997). For this experiment, three SPAD measurements were taken on the 12th leaf of each plant at V14. At R1, R3, and R5, three readings were collected on each plant's uppermost earleaf. When plants did not have a fully developed ear or earshoot, SPAD readings were taken on the leaf subtending the barren earshoot or on the 12th leaf, respectively. On extremely small plants, measurements were taken on the uppermost, fully extended leaf. For all plants, SPAD measurements were taken on the middle third of the appropriate leaf (Chapman and Barreto, 1997), spaced along the midrib by 5 cm, and collected approximately 2 cm from the leaf margin. Nonrepresentative necrotic areas were intentionally avoided during data collection.

In both 2006 and 2007, the temporal separation between anthesis and silking was determined for all tagged plants [i.e., per-plant anthesis–silking interval  $(ASI_p)$ ]. Anthesis and silking were respectively defined as the beginning of pollen shed from the tassel (i.e., dehiscence of at least one anther and subsequent dispersal of pollen) and the appearance of the first pollen-receptive (stigmatic) style (i.e., silk) from the husk of the primary ear (Borrás et al., 2007). Throughout the flowering period, measurements of anthesis and silking were taken on a daily basis during mid to late afternoon. In all instances, treatment values for ASI<sub>p</sub> were calculated using the average of ASI<sub>p</sub> values for a given sampling area.

At physiological maturity in all 3 yr, the ears of plants in the per-plant sampling areas were hand-harvested and individually bagged with their respective tags. Grain was separated from each cob individually using an electric sheller. Per-plant grain samples were individually weighed, and the moisture content of each sample was determined using a Farmex MT3 portable moisture meter (Farmex, Streetsboro, OH). Per-plant grain weights were calculated to both 0% and 15.5% (GY<sub>p</sub>) moisture content. The grain yield of each sub-subplot's per-plant sampling area (GY<sub>SA</sub>) was determined as the sum of GY<sub>p</sub> values for that respective area. Plant-to-plant variability for

grain production  $(GY_{CV})$  was calculated on a sub-subplot basis as the coefficient of variation (CV) of GY<sub>P</sub> values (Fasoula and Tollenaar, 2005; Tokatlidis et al., 2005). In all 3 yr, KN<sub>p</sub> was determined for all tagged plants using an Old Mill Company Model 850-2 electronic seed counter (International Marketing and Design Company, San Antonio, TX). For each tagged plant in each growing season, KW was calculated as the quotient of GY<sub>p</sub> and KN<sub>p</sub>. In 2006 and 2007, six random representative cobs were chosen from the shelled ears of each sampling area and dried to a constant weight at 60°C to determine a common per-plant cob biomass for each subsubplot. After hand-harvesting in 2006 and 2007, six consecutive, representative plants from row three of each sub-subplot's per-plant sampling area were cut at the stem base, individually chopped to a fine consistency, and dried to a constant weight at 60°C to determine aboveground per-plant vegetative biomass (VB<sub>p</sub>). For each of these destructively sampled plants, TB<sub>p</sub> was calculated as the sum of VB<sub>p</sub>, per-plant cob biomass (common sub-subplot value), and per-plant grain weight (0%) moisture). Per-plant harvest index was subsequently calculated as the proportion of per-plant grain weight (0% moisture) to TB<sub>p</sub>. Following each year's per-plant measurements, grain was harvested from the center four rows of each six-row sub-subplot using a commercial plot harvester equipped with a four-row maize head. Grain weight and percent moisture were collected on the harvester. The  $GY_A$  (15.5% moisture content) of each sub-subplot was calculated using the sum of that sub-subplot's machine harvest grain weight and per-plant sampling area cumulative grain weight.

Within each year, NUE was determined for each subsubplot receiving N application as the incremental agronomic efficiency from applied nitrogen (AE<sub>i</sub>). According to Cassman et al. (2003), AE<sub>i</sub> is defined as the incremental grain yield increase ( $\Delta$ GY<sub>A</sub>) resulting from an incremental increase in N application rate ( $\Delta$ N<sub>R</sub>). The following function was therefore used to express NUE in this study:

$$NUE = AE_{i} = \Delta GY_{A} / \Delta N_{R} =$$

$$(GY_{A+N} - GY_{A-N}) / (N_{R+N} - N_{R-N})$$
[2]

where GY<sub>A+N</sub> is the per-unit-area grain yield (kg) of a sub-subplot receiving either 165 or 330 kg N ha<sup>-1</sup> (N<sub>R+N</sub>), and GY<sub>A-N</sub> is the per-unit-area grain yield (kg) of the corresponding subsubplot (i.e., the sub-subplot with the same block-hybrid-plant density combination) receiving no side-dress N (N<sub>R-N</sub>). For this study,  $\Delta$ GY<sub>A</sub> between the 0 and 165 kg N ha<sup>-1</sup> application rates was used as a measure of N responsiveness.

### Leaf Area Measurements

To measure the  $LA_p$  and  $LA_D$  of each tagged plant at R1, a modification of the model and techniques used by Dwyer and Stewart (1986) and Valentinuz and Tollenaar (2006) was employed. Here, we briefly describe our methodology. Readers are encouraged to consult the accompanying appendix for a more comprehensive description of this methodology's general theory, in-field measurements, and model selection, implementation, and validation components.

In both 2006 and 2007, a destructive sampling area was established in each six-row sub-subplot. In each of these areas,

three consecutive, representative plants were harvested at R1. On each of these plants, leaf length (L) and maximum leaf width (W) were measured for all green leaves. The area of each green leaf (LA<sub>n</sub>) was calculated by multiplying L × W × 0.75 (Montgomery, 1911). For all plants in the nondestructive per-plant sampling areas, earleaf position ( $x_e$ ) was determined relative to the location of each plant's tag. Values for  $x_e$  from tagged plants were used to estimate  $x_e$  for destructively sampled plants. The position of the largest leaf ( $x_o$ ) and of each green leaf ( $x_n$ ) were determined relative to  $x_e$  for each destructively sampled plant, as were the total number of green leaves (NL<sub>T</sub>) and the positions of the lowest ( $x_L$ ) and highest ( $x_H$ ) green leaves. Using estimated values for  $x_o$  from destructively sampled plants, the area of the largest green leaf of tagged plants (LÁ<sub>o</sub>) was determined at R1 as previously described (i.e., L × W × 0.75).

To describe the  $LA_D$  of destructively sampled plants at R1, we used the function:

$$LA_n = LA_o \times \exp[-b(x_n - x_o)^2]$$
[3]

where  $LA_{a}$  is the area of the largest leaf, b is a coefficient that describes the breadth/kurtosis of the distribution of green leaf area, and  $LA_n$ ,  $x_n$ , and  $x_o$  are as previously described. For the three destructively sampled plants within a sub-subplot, an average sub-subplot-level value for LA<sub>o</sub> was calculated. Using Eq. [3], sub-subplot-level LA<sub>o</sub> values, and SAS PROC NLIN (SAS Institute, 2004), a single *b* coefficient was generated for each plot based on the leaf measurements of the three plants destructively sampled within that plot. The predicted area of every green leaf  $(L\hat{A}_n)$  was resultantly determined for each destructively sampled plant. The total predicted and observed green leaf area of each of these plants ( $L\hat{A}_T$  and  $LA_T$ , respectively) was then calculated as the sum of each plant's  $LA_n$  and  $LA_n$  values, respectively. The total green leaf area at R1 of each tagged plant  $(L\dot{A}_T)$  within a sub-subplot was calculated as follows:

$$L\dot{A}_{T} = L\hat{A}_{T} \times (L\dot{A}_{o}/LA_{o})$$
[4]

where  $LA_o$  is as previously described, and  $LA_T$  and  $LA_o$  are the average total predicted leaf area and average area of the largest leaf of destructively sampled plants from that same sub-subplot, respectively. For each per-plant sampling area, the  $LAI_G$  at

R1 was calculated using the sum of all  $L\dot{A}_T$  values for that sampling area.

#### Statistical Analyses

The study was arranged and analyzed as a split-split plot design with four blocks. Hybrid (main plot), plant density (subplot), and side-dress N rate (sub-subplot) served as the three treatment factors. In some years, sub-subplot-level data was missing for a limited number of morphophysiological parameters. Years were analyzed separately due to the change in hybrids between 2005 and 2006, the enhanced opportunity to annually identify weather impacts on maize responses during the critical period, and the ability to demonstrate year-to-year consistency in responses to treatments. The majority of the subplot errors were not significant at  $P \le 0.25$  [as determined via F tests for Type III sums of squares using PROC GLM (SAS Institute, 2004)], so all subplot errors were pooled with their respective sub-subplot errors. The final ANOVA was performed using SAS PROC MIXED (SAS Institute, 2004). When treatment effects were significant at the 0.05 probability level, least-squares mean (LS mean) separation tests were performed for fixed effects (t test). Presented results/means are generally averaged across hybrids since the effect of hybrid was often not significant ( $P \le 0.05$ ), inconsistently significant across phenological stages for multistage morphophysiological parameters (e.g., SPAD), and/or of lesser importance than the plant density and N rate effects. The principal focus in the following sections is therefore placed on the fixed effects of plant density, N rate, and plant density × N rate. Readers should note that, within many of the following figures, morphophysiological responses are compared across N rates within plant density. Statistical comparisons between plant densities within N rate are discussed only in the text.

#### RESULTS

#### Weather Conditions

Total precipitation, mean maximum air temperature, and accumulated GDD for the months of April through September are indicated in Table 1 for the 2005, 2006, and 2007 growing seasons, as are accompanying 30-yr averages. Total weekly precipitation, daily maximum air temperature, and accumulated GDD for each growing season are depicted in Fig. 1. Weather conditions expectedly differed between growing seasons (Table 1, Fig. 1). Relatively dry, warm conditions in

Table I. Total precipitation, mean maximum air temperature, and accumulated growing degree days (GDD) at the Purdue University Agronomy Center for Research and Education (ACRE) for the months of April to September during 2005, 2006, 2007 and the period 1971 to 2000.

		Total pre	cipitation		Mean	maximum	air temp	erature		Accumula	ated GDE	)
				30-yr				30-yr				30-yr
Month	2005	2006	2007	mean	2005	2006	2007	mean	2005	2006	2007	mean
		m	nm ———			•	с ——					
April	51	88	108	91	19	20	15	16	152	152	109	108
May	46	131	95	111	23	21	27	23	214	204	301	228
June	51	61	72	108	30	27	28	27	368	335	354	339
July	117	156	59	102	30	30	28	29	398	445	358	401
August	51	136	148	94	30	28	31	28	402	380	416	370
September	122	72	46	76	28	24	28	25	317	234	306	271
6-mo total	438	644	528	582					1851	1749	1844	1717
6-mo avg.					27	25	26	25				



Fig. I. Total weekly precipitation, daily maximum air temperature, and accumulated growing degree days (GDD) for (A) 2005, (B) 2006, and (C) 2007 at the Purdue University Agronomy Center for Research and Education (ACRE). Timing of planting and the phenological stages V5, RI, and R6 (Ritchie et al., 1996) are indicated for each year.

2005 (Table 1, Fig. 1A) likely contributed to the lowest overall productivity being observed in this year (Fig. 2A), although rainfall events during the critical period bracketing silking likely limited reductions in kernel set and later-season kernel abortion due to water stress (Fig. 1A). Weather during the 2006 growing season was generally highly favorable for maize production (Table 1, Fig. 1B), resulting in the numerically highest GY<sub>A</sub> values in this year (Fig. 2B). During the 2007 growing season, overall precipitation was slightly below average and monthly maximum temperatures and GDD accumulation were typically above average (Table 1), generally resulting

in lower productivity in 2007 versus 2006 (Fig. 2C). As in 2005, rainfall events shortly before and after flowering in 2007 likely limited reductions in kernel set and later-season kernel abortion due to water stress (Fig. 1C). Given the precipitation patterns of each growing season, morphophysiological responses in high stress environments (i.e., supraoptimal plant crowding and/or no side-dress N fertilization) were likely principally the result of imposed treatment stresses and not water or heat stress.

## Per-Unit-Area Grain Yield and Nitrogen Use Efficiency

In all 3 yr of this study, the fixed effects of plant density, N rate, and plant density × N rate had a significant effect on GY<sub>A</sub> (Table 2). For each plant density in all years, GY<sub>A</sub> increased with an initial side-dress application of 165 kg N ha<sup>-1</sup>. When 165 kg ha<sup>-1</sup> of side-dress N was applied, values for  $\Delta GY_A$ were approximately 3.7, 5.2, and 6.1 Mg ha<sup>-1</sup> for the suboptimal, optimal, and supraoptimal plant densities in 2005, respectively; 4.9, 6.5, and 7.0 Mg ha<sup>-1</sup> for these respective densities in 2006; and 4.3, 5.8, and 5.6 Mg ha<sup>-1</sup> for these respective densities in 2007 (Fig. 2). Genotypes examined in this study therefore consistently displayed strong N responsiveness (i.e., high  $\Delta GY_A$  values) to an initial side-dress application of 165 kg N ha<sup>-1</sup>, particularly at the supraoptimal plant density. A second application of 165 kg N ha<sup>-1</sup> further improved GY<sub>A</sub> at only the optimal plant density in 2006 (Fig. 2B) and the supraoptimal plant density in 2006 and 2007 (Fig. 2B, 2C). Despite their statistical significance, these improvements were relatively small, indicating that even at the supraoptimal density further N application in marked excess of the approximate agronomic optimal N rate only marginally improved grain production. Nevertheless, side-dress N application in excess of 165 kg N ha<sup>-1</sup> was more frequently essential for maximizing GY<sub>A</sub> at the supraoptimal than suboptimal or optimal plant density (Fig. 2), suggesting a higher agronomic optimal N rate at the most intense level of crowding. When either 165 or 330 kg ha<sup>-1</sup> of side-dress N was supplied, GY<sub>A</sub> rarely increased or decreased with each incremental rise in plant density in all years. Contrarily, in the absence of sidedress N application in each growing season, GY<sub>A</sub>

declined with each incremental increase in plant density. Differences in GY<sub>A</sub> between the 54,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> and 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> treatment combinations were approximately 28, 29, and 22% in 2005, 2006, and 2007, respectively. In all 3 yr, GY<sub>A</sub> was lowest when intraspecific competition for applied N was likely most severe (i.e., 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup>) (Fig. 2). Results for GY<sub>A</sub> (Fig. 2) therefore suggest that the stress-tolerant, highly productive maize hybrids used in this study displayed limited tolerance to the simultaneous stresses of intense crowding and low fertilizer N availability.

As with GY<sub>A</sub>, plant density and N rate had a significant effect on NUE ( $\Delta GY_A / \Delta N_B$ , see Eq. [2]) in all 3 yr of this experiment. The plant density × N rate interaction was not significant in any year (Table 2). For each plant density in all years, NUE was greater for the 165 kg ha<sup>-1</sup> N rate than the 330 kg ha<sup>-1</sup> N rate, predominately owing to the very limited GY<sub>A</sub> response of the investigated hybrids to a second application of side-dress N (Fig. 2). In both 2005 and 2006, NUE rose with each increase in plant density when either 165 or 330 kg N ha<sup>-1</sup> was applied (Fig. 2A, 2B). In 2007, NUE rose for both of these N rates when plant density increased from 54,000 to 79,000 plants ha<sup>-1</sup>. However, it failed to rise for either side-dress N rate when plant density increased from 79,000 to 104,000 plants ha<sup>-1</sup> (Fig. 2C). While general increases in NUE resulting from rising plant density were due to increasing N responsiveness to 165 kg N ha<sup>-1</sup> (i.e.,  $\Delta GY_A$ , see Eq. [2]), such improvements in  $\Delta GY_A$  primarily resulted from low GY<sub>A</sub> values at the optimal and supraoptimal plant densities in the absence of side-dress N and not from exceptionally high GY<sub>A</sub> values at these same plant densities in the presence of side-dress N application (Fig. 2). In each year, GY<sub>A</sub> was only occasionally greater for the optimal than suboptimal or supraoptimal plant density when either 165 or 330 kg N ha<sup>-1</sup> was side-dress applied. Thus, when defined as kilograms of grain produced per kilogram of side-dress N  $(GY_A/N_R)$ , NUE seldom differed among the three plant densities for either the 165 or 330 kg N ha<sup>-1</sup> treatment.

As indicated in Fig. 2, values for  $GY_A$  and  $GY_{SA}$  differed slightly for each year (i.e., from 3 to 7% annually when averaged across all treatments). Nevertheless, overall grain yield responses to plant density and side-dress N rate were generally similar among machine-harvested sub-subplots and their respective hand-harvested per-plant sampling areas (Fig. 2), as were ANOVA significance levels for fixed effects ( $GY_{SA}$  data are not shown). We therefore believe that morphophysiological measurements in per-plant sampling areas were largely representative of sub-subplot-level morphophysiological behavior, and thus provided legitimate, accurate insights into observed responses for  $GY_A$  and NUE.

## Per-plant Grain Yield, Grain Yield Variability, Kernel Number, and Individual Kernel Weight

As with GY<sub>A</sub>, plant density and N rate had a significant effect on GY  $_{\rm p}$  in all 3 yr of this experiment. The plant density  $\times$  N rate interaction was significant for  $GY_p$  only in 2007 (Table 2). Similar to responses for GY<sub>A</sub>, GY<sub>P</sub> increased with an initial side-dress application of 165 kg N ha<sup>-1</sup> for each plant density in all years. However, for all plant densities in each growing season, a second application of side-dress N failed to further improve GY<sub>p</sub> (Fig. 3). Regardless of side-dress N rate, GY<sub>p</sub> decreased with each increase in plant density in all years. On a relative basis, these reductions were more pronounced when no side-dress N was applied. For example, when plant density increased from 54,000 to 104,000 plants ha<sup>-1</sup> in the absence of side-dress N,  $GY_p$  declined by approximately 50, 56, and 53% in 2005, 2006, and 2007, respectively. Yet, when either 165 or 330 kg N ha<sup>-1</sup> was applied and plant density increased from 54,000 to 104,000 plants ha<sup>-1</sup>, GY<sub>p</sub> fell by roughly 40, 45, and 46% in 2005, 2006, and 2007, respectively. In all three growing seasons, GY<sub>p</sub> was

smallest when total applied N was likely least available at the per-plant level (i.e., 104,000 plants  $ha^{-1}$ , 0 kg N  $ha^{-1}$ ) (Fig. 3). Results in Fig. 2 and 3 indicate that when plant density increased in the presence of side-dress N application, the addition of more plants per unit area compensated for accompanying declines in



Fig. 2. Plant density and N rate effects on maize per-unitarea grain yield (GY<sub>A</sub>) (15.5% moisture content) and N use efficiency (NUE) for (A) 2005, (B) 2006, and (C) 2007. For each sub-subplot, GY<sub>A</sub> was calculated using the sum of that sub-subplot's machine harvest grain weight and per-plant sampling area cumulative grain weight. Arrows approximately adjacent to each vertical bar indicate the per-unit-area grain yield (15.5% moisture content) of each treatment's per-plant sampling area (GY<sub>SA</sub>). Values for NUE are expressed as the incremental agronomic efficiency from applied N (AE<sub>i</sub>), which is defined as kilograms of additional grain (15.5% moisture content) per kilogram of additional side-dress N ( $\Delta GY_A/\Delta N_R$ , see Eq. [2]). For both GY<sub>A</sub> and NUE, means with different letters (GY<sub>A</sub>: no parentheses, NUE: parentheses) indicate statistically significant differences at the 0.05 probability level within each plant density.

grain yield (GY <sub>A</sub> ); (Ritchie et al., 1990) RI, R3, and R6 (six	nitroger 6) per-pl th-inter	h use effi lant abov node) ste	ciency ( /egroun em dian	NUE); p d vegeta neter (SI	er-plant utive (VB D); RI gr	grain yi p) and t een leaf	eld (GY otal (TE farea ind	<sub>p</sub> ); per-p ) biom dex (LA	اant gra lass; per ار); and	ain yield r-plant l I VI4 (I)	l variabi harvest 2th leaf)	ility (G) index (I ), RI, R3	( <sub>CV</sub> ); pe HI <sub>P</sub> ); pe , and R	r-plant r-plant 5 (upper	kernel nu anthesis- most eau	ımber (l -silking rleaf) sii	KN <sub>p</sub> ); in interval ngle-pho	idividua (ASI <sub>P</sub> ) oton ava	l kernel V5, VI alanche	weight 1, and RI diode (S	(KW); F PHT; V PAD).	14, 14,
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2005	¢ A		0 P	CVI	i i i		4 U P	- 60	P					Ċ				0				
Hybrid (H)	NS‡	S	SN	*	SN	*					SN	SN	SN	S		×	*		SN	SN	SN	*
Plant density (D)	*	*	*	*	×	*					SN	*	*	*		ž	*		*	\$	*	*
H×D	SN	**	SN	SN	SN	SN					SN	SN	SZ	S		SN	SN		S	SN	SN	*
N rate (N)	*	*	*	*	*	*					S	*	*	*		*	SN		*	*	*	*
Н×И	SN	S	SN	SN	SN	*					S	S	SN	*		SN	SN		S	*	SN	SN
D × N	*	S	SN	SN	SN	SN					S	SZ	SN	SN		SN	SN		*	*	**	S
H × D × N	SN	SN	SN	SN	SN	SN					S	SN	SN	SN		SN	SN		S	SN	SN	*
2006																						
т	SN	S	S	S	*	×	SN	S	SN	*	*	*	*	*		*	×	SN	S	*	ž	*
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H × Z	SN	SN	SN	**	**	**	SN	SN	*	*	SN	SN	SN	SN		SN	SN	×	SN	S	SN	*
D × N	×	SN	S	**	S	SN	SN	SN	*	**	S	S	SN	SN		S	SN	×	**	*	SN	SN
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т	SN	SN	S	*	*	SN	*	SN	S	*	S	S	*	*	*	*	*	*	*	*	*	*
D	×	**	*	**	**	*	*	*	*	*	SN	*	*	*	**	*	*	Ż	*	*	*	ž
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z	*	**	*	**	**	*	ž	*	*	*	SN	ž	*	*	**	×	ž	*	*	\$	*	*
Ч×И	SN	SN	SN	**	SN	SN	S	S	SN	×	SN	*	SN	SN	**	×	*	SN	SN	*	SN	S
D × N	*	S	*	SN	SN	SN	S	S	SN	**	SN	SN	SN	S	**	ģ	SN	*	SN	SN	SN	S
H × D × N	SN	SN	SN	SN	SN	SN	SN	ß	SN	SN	SN	SN	SN	SN	SN	SN	SN	SN	SN	SN	SN	S
* Significant at the 0.05	probability	y level.																				
** Significant at the 0.01	probabili	ty level.																				
† Per-plant grain yield v	ariability i	s expresse	d using th	e coefficiei	nt of variat	ion (CV).																
‡ NS, nonsignificant at t	the 0.05 pr	obability l	evel.																			

 $GY_p$ , therefore leading to overall increases in  $GY_A$ . Conversely, when no side-dress N was applied and plant density rose, increases in plant crowding could not offset reductions in  $GY_p$  (Fig. 3), resulting in overall declines in  $GY_A$  (Fig. 2A–2C).

Similar to trends for GY<sub>p</sub>, plant density and N rate had a significant effect on  $GY_{CV}$  in all three growing seasons of this study. The plant density × N rate interaction for  $\mathrm{GY}_{\mathrm{CV}}$  was significant in only 2006 (Table 2). In both 2006 and 2007,  $\mathrm{GY}_{\mathrm{CV}}$ decreased at the optimal and supraoptimal plant densities with an initial application of sidedress N. A second application of  $165 \text{ kg N} \text{ ha}^{-1}$  failed to further reduce  $\mathrm{GY}_{\mathrm{CV}}$  for both of these plant densities in both years (Fig. 3B, 3C). In the 2005 growing season, a total side-dress application of 330 kg N ha<sup>-1</sup> was required for reducing GY<sub>CV</sub> at both the optimal and supraoptimal plant densities (Fig. 3A). In the absence of side-dress N during each growing season, GY<sub>CV</sub> rose when plant density increased from 54,000 to 79,000 plants ha<sup>-1</sup> (Fig. 3). When plant density further rose from the optimal to supraoptimal level for the 0 kg N ha<sup>-1</sup> rate, GY<sub>CV</sub> increased in only 2006 (Fig. 3B). When either 165 or 330 kg N ha<sup>-1</sup> was applied in 2005 or 2006,  $GY_{CV}$ values increased only when plant density rose from 54,000 to 104,000 plants ha<sup>-1</sup> (Fig. 3A, 3B). However, for these same N rates in 2007, GY<sub>CV</sub> increased with each incremental rise in plant density (Fig. 3C). Overall, GY<sub>CV</sub> values were typically highest when total applied N was likely least available on a per-plant basis. For example, in 2006 the 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> environment displayed the greatest  $GY_{CV}$ , while in 2005 and 2007,  $GY_{CV}$  was highest for the 79,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> and 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> treatment combinations (Fig. 3B, 3C). The simultaneously low GY<sub>p</sub> and high GY<sub>CV</sub> values evident in this study's highly crowded, low N environments further suggest that the stress-tolerant, highly productive maize hybrids examined here displayed limited tolerance to the simultaneous stresses of supraoptimal plant density and low side-dress N availability.

As with  $GY_p$  and  $GY_{CV}$ , both plant density and N rate had a significant effect on  $KN_p$  and KW in all three growing seasons. However, the plant density × N rate interaction was not significant for either of these  $GY_p$  components in any year (Table 2). As indicated in Table 3,  $KN_p$  and KW increased with an initial side-dress application of 165 kg N ha<sup>-1</sup> for each plant density in all years. However, a second application of side-dress



Fig. 3. Plant density and N rate effects on maize per-plant grain yield  $(GY_p)$  (15.5% moisture content) and per-plant grain yield variability  $(GY_{CV})$  for (A) 2005, (B) 2006, and (C) 2007. Means with different letters  $(GY_p:$  no parentheses,  $GY_{CV}$ : parentheses) indicate statistically significant differences at the 0.05 probability level within each plant density. The parameter  $GY_{CV}$  is expressed using the coefficient of variation (CV).

N consistently failed to further improve either of these GY<sub>p</sub> components. Averaged across plant densities, KN<sub>p</sub> rose by approximately 14, 29, and 26% in response to an initial side-dress N application in 2005, 2006, and 2007, respectively. Values for KW increased by roughly 20% in each year in response to 165 kg N ha<sup>-1</sup>. Irrespective of side-dress N rate, KN<sub>p</sub> and KW decreased with each incremental increase in plant density in all years. Averaged across side-dress N rates, an overall increase in plant density from 54,000 to 104,000 plants ha<sup>-1</sup> was accompanied by an approximately 35% decline in KN<sub>p</sub> and a roughly 20% drop in KW in each year of this study. Thus both  $KN_p$ and KW were generally smallest when total applied N was likely least available at the per-plant level (i.e., 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup>). While KN<sub>P</sub> generally exhibited greater plasticity [defined here as the amount by which an individual characteristic of a particular genotype is altered by different environments influences (Bradshaw, 1965; Bonaparte and Brawn, 1975; Sadras et al., 2009)] than KW in response to both increased crowding and decreased N availability (Table 3), changes in KW were still quite pronounced and therefore accounted for a considerable portion of the observed plasticity for  $GY_{p}$  (Fig. 3).

### Per-Plant Vegetative Biomass, Total Biomass, and Harvest Index

In both 2006 and 2007, plant density and N rate had a significant effect on VBp and TBp at R6. However, the plant density × N rate interaction was not significant for either parameter in either year (Table 2). In most circumstances in both growing seasons, values for R6 VB<sub>p</sub> and TB<sub>p</sub> rose with an initial application of side-dress N (Fig. 4). While 330 kg N ha<sup>-1</sup> was necessary to improve the VBp of plants grown at 79,000 and 104,000 plants ha<sup>-1</sup> in 2006 (Fig. 4A), in most cases in both years, a second side-dress N application failed to increase VB<sub>p</sub> or  $TB_p$  (Fig. 4). For each N rate in both 2006 and 2007, R6  $VB_p$ and TB<sub>p</sub> nearly always declined with each incremental increase in plant density (Fig. 4). Although relative reductions in GY<sub>p</sub> were more pronounced for the 0 kg N ha<sup>-1</sup> rate than 165 or 330 kg N ha<sup>-1</sup> rates when plant density rose from the suboptimal to supraoptimal level (Fig. 3), this trend was not observed for VB<sub>p</sub>, as relative declines resulting from increasing plant density were similar among the three N rates for both years (Fig. 4). For example, when plant density rose from the suboptimal to supraoptimal level in 2007,  $\mathrm{VB}_\mathrm{p}$  decreased by approximately 38, 39, and 40% for the low, mid, and high side-dress N rates,

Table 3. Plant density and N rate effects on maize per-plant kernel number  $(KN_P)$  and individual kernel weight (KW) for 2005, 2006, and 2007.

<u> </u>						
		KN <sub>P</sub>			КW	
Treatment effect	2005	2006	2007	2005	2006	2007
Plant density, plants ha <sup>-1</sup>				m	ng kernel	-I
54,000	626a†	595a	513a	303a	335a	381a
79,000	494b	484b	388b	269b	298b	359b
104,000	428c	385c	341c	247c	266c	300c
N rate, kg N ha <sup>-1</sup>						
0	460a	385a	344a	233a	247a	284a
165	537b	538b	466b	288b	318b	358b
330	551b	541b	432b	297b	334b	<b>397</b> b

 $\dagger$  Within each year, means with different letters indicate statistically significant differences at the 0.05 probability level between either plant densities or N rates.



Fig. 4. Plant density and N rate effects on maize R6 (Ritchie et al., 1996) per-plant aboveground vegetative  $(VB_p)$  and total  $(TB_p)$  biomass and per-plant harvest index  $(HI_p)$  for (A) 2006 and (B) 2007. For VB<sub>p</sub>, TB<sub>p</sub>, and HI<sub>p</sub>, means with different letters  $(VB_p)$  lowercase, no parentheses; TB<sub>p</sub>: uppercase; HI<sub>p</sub>: lowercase, parentheses) indicate statistically significant differences at the 0.05 probability level within each plant density. Horizontal dashed lines indicate the commonly presumed HI<sub>p</sub> of 0.5 for modern North American maize hybrids.

respectively (Fig. 4B). As with GY<sub>p</sub> in 2006 and 2007 (Fig. 3), R6 VB<sub>p</sub> and TB<sub>p</sub> were smallest in both years when total applied N was poorly available on a per-plant basis. Thus in 2006, VB<sub>p</sub> and TB<sub>p</sub> were lowest for the 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> treatment combination, while in 2007, these parameters were smallest for the 79,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> and 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> treatment combinations (Fig. 4).

In both the 2006 and 2007 growing season, plant density and N rate had a significant effect on  $HI_p$ . The plant density  $\times$  N rate interaction for  $HI_p$  was significant in only 2006 (Table 2). Similar to responses for VBp and TBp, an initial side-dress application of 165 kg N ha<sup>-1</sup> at each plant density improved biomass partitioning to the ear in 2006 and 2007 while a second equal application of N failed to further increase HI<sub>p</sub> for all plant densities in both years (Fig. 4). When at least 165 kg N ha<sup>-1</sup> was applied at either the suboptimal, optimal, or supraoptimal plant density in either growing season, HI<sub>p</sub> values exceeded the commonly presumed 0.5 value for modern North American maize genotypes (Fig. 4), indicating strong N responsiveness for biomass partitioning to the ear among this study's hybrids. Relative differences in HIp for the suboptimal and supraoptimal plant densities in the absence of side-dress N were 22 and 8% in 2006 and 2007, respectively. However, unlike with the 0 kg N ha<sup>-1</sup> rate, increases in plant density in the presence of



Fig. 5. Plant density and N rate effects on the anthesis-silking interval of individual maize plants (ASI<sub>P</sub>) in (A) 2006 and (B) 2007. Means with different letters indicate statistically significant differences at the 0.05 probability level within each plant density.

165 or 330 kg ha<sup>-1</sup> of side-dress N resulted in no decline in  $HI_p$ . Relative differences in  $HI_p$  between the suboptimal and supraoptimal plant densities in the presence of side-dress N application were therefore small, with declines of only 5 and 3% for the 165 kg ha<sup>-1</sup> N rate and 4 and 2% for the 330 kg ha<sup>-1</sup> N rate in 2006 and 2007, respectively. Values for  $HI_p$  were lowest for the 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> treatment combination in 2006 and the 79,000 plants ha<sup>-1</sup>, 0 kg ha<sup>-1</sup> and 104,000 plants ha<sup>-1</sup>, 0 kg ha<sup>-1</sup> treatment combination in 2007 (Fig. 4). Overall, Fig. 4 indicates that while severe N stress at optimal and supraoptimal plant densities decreased VB<sub>p</sub>, biomass partitioning to the ear was more markedly reduced by intense intraspecific competition for available N at such plant densities.

#### Per-Plant Anthesis-Silking Interval

For both 2006 and 2007, the fixed effects of plant density, N rate, and plant density × N rate had a significant effect on  $ASI_p$  (Table 2). For the optimal and supraoptimal plant densities in 2006 and 2007,  $ASI_p$  decreased with an initial application of side-dress N. In both years, a second application of 165 kg N ha<sup>-1</sup> failed to further improve floral synchrony at each of these plant densities. Although the application of 330 kg N ha<sup>-1</sup> reduced  $ASI_p$  for the suboptimal plant density in 2007, side-dress N application typically had a relatively minimal effect on the floral synchrony of plants grown at this density in either 2006 or 2007 (Fig. 5). For all side-dress N rates in both

growing seasons, ASI<sub>p</sub> generally rose with each increase in plant density, with this upward trend most pronounced when no side-dress N was applied. In both 2006 and 2007, the highest floral protandry occurred when intraspecific competition for soil N was likely most severe (i.e., 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup>) (Fig. 5). This environment also exhibited the numerically lowest HI<sub>p</sub> (Fig. 4) and GY<sub>p</sub> (Fig. 3B, 3C) values in both years, suggesting that ultimately low-yielding plants subjected to the simultaneous stresses of intense crowding and low N availability already exhibited reduced biomass partitioning to the ear before the grain-filling period and, concomitantly, limited tolerance to intense intraspecific competition for available N at R1.

#### **Plant Height and Stem Diameter**

In all three growing seasons, neither plant density nor N rate had a significant effect on PHT at V5. However, in each year, both of these effects had a significant impact on V14 and R1 PHT. For all phenological stages in each year, the plant density  $\times$  N rate interaction for PHT was not significant (Table 2). At V14 and R1 for each growing season, PHT often declined with an increase in plant density from 54,000 to 79,000 or 104,000 plants ha<sup>-1</sup> regardless of side-dress N rate. Furthermore, for both of these phenological stages in each year, PHT was typically reduced by a lack of side-dress N application regardless of plant density. Values for V14 and R1 PHT were therefore often lowest for the 79,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> and 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> treatment combinations (Table 4).

In all 3 yr of this study, plant density and N rate nearly always had a significant effect on SD at V14, R3, and R6. Both of these effects also had a significant impact on R1 SD in 2007. For most phenological stages in each year, the plant density × N rate interaction for SD was not significant (Table 2). For most phenological stages within each growing season, SD (i) declined with each incremental increase in plant density regardless of side-dress N rate (Fig. 6A-6C) and (ii) was reduced by a lack of side-dress N application regardless of plant density (Fig. 6D-6F). Values for SD were therefore lowest for the 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> treatment combination for most phenological stages in each year. In all years, SD numerically declined from V14 to R6 regardless of plant density and side-dress N rate, with a majority of this decline occurring between V14 and R3 (Fig. 6). However, as indicated in Fig. 6C and 6F, measurement of R1 SD in 2007 revealed that rates of decline for SD were numerically different for the

0 kg N ha<sup>-1</sup> rate before and after silking (i.e., V14 to R1 and R1 to R3, respectively) regardless of plant density. From V14 to R1, SD numerically decreased by approximately 4.4 mm for the 0 kg N ha<sup>-1</sup> side-dress rate, but only 2.7 and 2.3 mm for the 165 and 330 kg N ha<sup>-1</sup> rates, respectively, when averaged across plant densities. However, from R1 to R3, SD numerically declined by 2.5 and 2.9 mm for the 165 and 330 kg N ha<sup>-1</sup> rates, respectively, but only 0.7 mm for the 0 kg N ha<sup>-1</sup> application rate when averaged across plant densities (Fig. 6F). Thus for these genotypes, severe N stress in highly crowded environments typically involved not only reductions in SD at each phenological stage but also early, pronounced declines in SD during the late-vegetative to presilking period (Fig. 6).

#### **Per-Plant Leaf Area Parameters**

In 2006 and 2007, both plant density and N rate had a significant effect on R1 LA<sub>T</sub>, LÂ<sub>T</sub>, and LÁ<sub>T</sub>. The plant density  $\times$  N rate interaction was significant for only LA<sub>T</sub> and LÂ<sub>T</sub> in 2007 (Table 5). For each plant density in both growing seasons, values for R1 LA<sub>T</sub>, LÂ<sub>T</sub>, and LÁ<sub>T</sub> rose with an initial application of side-dress N. In all cases, a second application of side-dress N did not further improve any of these parameters. For each N rate in both years, R1 LA<sub>T</sub>, LÂ<sub>T</sub>, and LÂ<sub>T</sub> often declined with each incremental increase in plant density. Plants therefore frequently displayed the lowest R1 LA<sub>T</sub>, LÂ<sub>T</sub>, and LA<sub>T</sub> values when intraspecific competition for soil N was likely most intense (i.e., 104,000 plants  $ha^{-1}$ , 0 kg N  $ha^{-1}$ ) (Table 5). Overall, these general responses to plant density and N rate are similar to those previously discussed for a variety of per-plant parameters [e.g., GY<sub>p</sub>, TB<sub>p</sub>, R3 SD (Fig. 3B, 3C, 5A, 5B, 7B, 7C, 7E, and 7F, respectively)].

As with R1 LA<sub>T</sub>, LÂ<sub>T</sub>, and LÁ<sub>T</sub>, both plant density and N rate had a significant effect on R1 NL<sub>T</sub> and  $x_L$  in 2006 and 2007. In the latter growing season, the plant density × N rate interaction was significant for both NL<sub>T</sub> and  $x_L$  (Table 5). For the destructively sampled plants in both years, reductions in R1 LA<sub>T</sub> and LÂ<sub>T</sub> due to increased plant density and decreased side-dress N application often corresponded with declines in R1 NL<sub>T</sub>, increases in R1  $x_L$ , and general numeric decreases in the R1 LA<sub>n</sub> of nearly all green leaves (Table 5, Fig. 7). However, these same declines in R1 LA<sub>T</sub> and LÂ<sub>T</sub> were inconsistently coupled with changes in R1 values for  $x_H$  and b. The effect of plant density and side-dress N rate was less frequently significant for each of these parameters than for NL<sub>T</sub> and  $x_L$  (Table 5). Results in Table 5 and Fig. 7 suggest that

					PHT				
-		2005			2006			2007	
Treatment effect	V5	V14	RI	V5	V14	RI	V5	V14	RI
Plant density, plants ha <sup>-1</sup>					cm				
54,000	37a†	184a	256a	<b>48</b> a	196a	258a	54a	208a	247a
79,000	38a	180ab	244b	<b>48</b> a	191b	254a	54a	200b	239b
104,000	38a	178b	242b	47a	185c	249b	53a	1 <b>97</b> b	234c
N rate, kg N ha <sup>-1</sup>									
0	38a	177a	242a	<b>48</b> a	176a	238a	54a	1 <b>89</b> a	230a
165	38a	182ab	249b	<b>48</b> a	197b	262b	54a	209b	244b
330	38a	183b	251b	<b>48</b> a	I 98b	262b	53a	208b	245b

Table 4. Plant density and N rate effects on maize V5, VI4, and RI (Ritchie et al., 1996) plant height (PHT) for 2005, 2006, and 2007.

† Within each phenological stage for a given year, means with different letters indicate statistically significant differences at the 0.05 probability level between either plant densities or N rates.



Fig. 6. Plant density (A–C) and N rate (D–F) effects on maize VI4, RI, R3, and R6 (Ritchie et al., 1996) maximum stem diameter (SD) for (A,D) 2005, (B,E) 2006, and (C,F) 2007. At each phenological stage, maximum SD was measured at the midpoint of the sixth internode. Means with different letters indicate statistically significant differences at the 0.05 probability

plants subjected to intense crowding and/or low N availability exhibited reduced R1 per-plant green leaf area (i.e.,  $LA_T$ ,  $L\hat{A}_T$ , and  $L\hat{A}_T$  values) due to premature lower leaf senescence (i.e., lower  $NL_T$  and higher  $x_L$  values) and reduced individual leaf area for most of the nonsenesced canopy (i.e., lower  $LA_n$ values for green leaves). Lower  $LA_T$ ,  $L\hat{A}_T$ , and  $L\hat{A}_T$  values were not associated with a reduction in the total number of initiated leaves (i.e., unchanged  $x_H$  values) or a modification of the general shape of the green  $LA_D$  curve (i.e., unaltered *b* values) (Table 5, Fig. 7).

level within each phenological stage.

### **Green Leaf Area Index**

In both 2006 and 2007, plant density, N rate, and plant density × N rate had a significant effect on R1 LAI<sub>G</sub> (Table 2). As generally observed for other previously discussed parameters (e.g., VB<sub>p</sub>, TB<sub>p</sub>,  $GY_{p}$ ), an initial side-dress application of 165 kg N ha<sup>-1</sup> increased R1 LAI<sub>G</sub> for each plant density in each growing season, while a second equal application of N failed to further improve R1 LAIG for all plant densities in both years (Fig. 8). When either 165 or 330 kg N ha<sup>-1</sup> was applied in 2006 or 2007, R1 LAI<sub>G</sub> increased with each incremental rise in plant density (Fig. 8), indicating that the addition of more plants more than compensated for accompanying declines in R1 LÁ<sub>T</sub> (Table 5). However, when no side-dress N was applied in each growing season, R1 LAI<sub>G</sub> was similar for the optimal and supraoptimal plant densities in 2006 and for all three plant densities in 2007 (Fig. 8), suggesting that in most circumstances increases in plant crowding barely offset pronounced reductions in R1 LÁ<sub>T</sub> (Table 5). Thus, while an initial side-dress N application increased R1 LAI<sub>G</sub> values for each plant density, these improvements were particularly pronounced at the highest plant density for which LAIG respectively rose 68 and 71% in 2006 and 2007 in response to an initial 165 kg N ha<sup>-1</sup>. In comparison, R1 LAI<sub>G</sub> respectively increased by 42 and 47% for the suboptimal and optimal plant densities in 2006, and by 30 and 56% for these same respective densities in 2007. Overall, values for LAI<sub>G</sub> at R1 were lowest for the 54,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> treatment com-

bination in 2006 and 54,000 plants  $ha^{-1}$ , 0 kg N  $ha^{-1}$ ; 79,000 plants  $ha^{-1}$ , 0 kg N  $ha^{-1}$ ; and 104,000 plants  $ha^{-1}$ , 0 kg N  $ha^{-1}$  treatment combinations in 2007 (Fig. 8).

### Single-Photon Avalanche Diode

Plant density and N rate had a significant effect on SPAD values at each sampling time in all three growing seasons of this study. Yet, for at least one phenological stage per year, the plant density  $\times$  N rate interaction was not significant (Table 2). For all phenological stages within each year, the relative

Table 5. Plant density and N rate effects on the observed  $(LA_T)$ , predicted  $(L\hat{A}_T)$ , and estimated  $(L\hat{A}_T)$  total green leaf area per plant; on the area of the largest leaf of destructively  $(LA_o)$  and nondestructively sampled  $(L\hat{A}_o)$  plants; on the total number of green leaves  $(NL_T)$  per plant; on the positions of the earleaf  $(x_e)$  and largest  $(x_o)$ , lowest  $(x_L)$ , and highest  $(x_H)$  green leaves (leaves numbered from the bottom to the top of the plant for all initiated leaves); and on the *b* coefficient of Eq. [3] for maize grown in 2006 and 2007 and measured at RI (Ritchie et al., 1996).

Voar	Plant	N rate	I A +	IÂ+	IÁ +	NI +	<b>v</b> +	<b>v</b> +	× +	<b>v</b> +	I A +	IÁ+	b+
Tear	uensity	IN TALE			<u> </u>		^LI	<u>^HI</u>	*e+	<b>^</b> 01	LA		
	plants ha <sup>-1</sup>	kg N ha⁻'		cm² plant <sup>–</sup>	·			—leaf no.—			——————————————————————————————————————	m²	
2006	54,000	0	5410a§	5445a	5203a	11.2a	11. <del>4</del> a	21.6a	14.4a	13.0a	692a	<b>66</b> 1a	0.020a
		165	7255b	7349b	7114b	I 3.7b	9.0b	21.6a	14.4a	13.3ab	756b	733b	0.022ab
		330	7417b	7456b	7229Ь	13.7b	8.7b	21.4a	14.4a	13.7b	764b	74Ib	0.023b
	79,000	0	4417a	4467a	4271a	10.5a	11.5a	21.0a	14.3a	12.9a	610a	582a	0.022a
		165	6132b	6187b	6360b	I 2.8b	9.4b	21.2a	14.3a	13.2ab	664b	684b	0.022a
		330	6059b	6093b	6377b	I 2.8b	9.4b	21.2a	14.3a	I 3.7b	657b	690b	0.024a
	104,000	0	3696a	3733a	3526a	10.1a	11.8a	20.9a	14.3a	12.8a	548a	517a	0.023a
		165	5665b	5728b	5680b	12.4b	9.6b	21.0ab	14.2a	13.2a	635b	631b	0.022a
		330	6021b	6072b	5933b	I 2.8b	9.4b	21.3b	14.2a	13.2a	663b	648b	0.023a
2007	54.000	0	5705a	5783a	5192a	11.2a	9.7a	19.9a	13.9a	12.1a	700a	628a	0.021a
	,	165	7045b	7127b	6782b	13.3b	7.6b	19.9a	13.8a	12.5a	762b	726b	0.026b
		330	7247b	7390b	6963b	I 3.4b	7.4b	19.8a	13.8a	12.4a	772b	728b	0.024ab
	79.000	0	4153a	4184a	3745a	10.0a	10.5a	19.5a	13.6a	11.9a	579a	522a	0.024a
	,	165	6765b	6913b	5893b	13.0b	7.6b	19.6a	13.7a	12.0a	752b	641b	0.026a
		330	6552b	6633b	5966b	I 3.2b	7.5b	19.7a	13.6a	12.3a	717b	645b	0.026a
	104,000	0	3698a	3724a	2906a	9.3a	11.0a	19.4a	13.5a	11.6a	578a	453a	0.024a
		165	5942b	6078b	4993b	12.5b	8.1b	19.6a	13.5a	11.8a	688b	565b	0.025a
		330	5654b	5795b	5135b	I 2.7b	8.0b	19.6a	13.5a	12.0a	<b>667</b> b	591b	0.029b
ANO	VA												
	Sources c	of variation											
2006	Hybrid	(H)	NS¶	NS	*	NS	**	**	**	NS	**	**	NS
	, Plant d	ensity (D)	**	**	**	**	**	**	**	NS	**	**	NS
	Η×D	, , ,	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	N rate	(N)	**	**	**	**	**	NS	NS	**	**	**	NS
	Η×Ν		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	D×N		NS	NS	NS	NS	NS	*	NS	NS	NS	*	NS
	Η×D	× N	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
2007	н		**	**	**	**	**	**	**	**	**	**	NS
	D		**	**	**	**	**	**	**	**	**	**	NS
	Η×D		NS	NS	NS	NS	NS	NS	NS	NS	NS	*	NS
	Ν		**	**	**	**	**	NS	NS	NS	**	**	**
	Η×Ν		NS	NS	NS	NS	*	NS	NS	NS	NS	NS	NS
	D × N		**	**	NS	**	*	NS	NS	NS	NS	NS	NS
	ΗхD	x N	NIS	NS	NIS	NIS	NIS	NIS	NIS	NIS	NIS	NS	NIS

\* Significant at the 0.05 probability level.

\*\* Significant at the 0.01 probability level.

† Determined for destructively sampled plants.

‡ Determined for tagged, nondestructively sampled plants.

§ For each column within year and plant density, means with different letters indicate statistically significant differences at the 0.05 probability level.

¶ NS, nonsignificant at the 0.05 probability level.

chlorophyll concentration (i.e., SPAD) declined with each incremental increase in plant density regardless of side-dress N rate (Fig. 9A–9C). Furthermore, for most phenological stages within each season, leaf chlorophyll content declined with each incremental decrease in N rate irrespective of plant density (Fig. 9D–9F). Values for SPAD were lowest for the 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> treatment combination for most phenological stages in 2005, 2006, and 2007 (Fig. 9). When averaged across N rates, foliar chlorophyll levels remained relatively constant between V14 and R3 for each plant density. However, leaf greenness numerically decreased from R3 to R5 in all growing seasons, with the rate of decline from R3 to R5 numerically similar among plant densities within a year (Fig. 9A–9C). When no side-dress N was applied, leaf chlorophyll concentration generally numerically declined from V14 to R5 regardless of plant density, with a majority of this reduction occurring between R3 and R5 (Fig. 9D–9F). Overall, results presented in Fig. 9 indicate that the simultaneous stresses of intense crowding and low fertilizer N availability severely reduced leaf N concentration/chlorophyll content at each phenological stage and induced premature declines in visual stay-green.

### DISCUSSION

As a result of prior genetic improvement efforts, modern maize hybrids commonly display both pronounced tolerance to intense crowding (Tollenaar and Lee, 2002; Tokatlidis and Koutroubas, 2004) and strong responsiveness to N fertilizer



Fig. 7. Plant density [(A,D) 54,000; (B,E) 79,000; (C,F) 104,000] and N rate effects on the RI (Ritchie et al., 1996) green leaf area distribution  $(LA_D)$  of destructively sampled maize plants for (A-C) 2006 and (D-F) 2007. Each curve indicates the mean predicted green  $LA_D$  for a given plant density-N rate treatment combination. The left and right limits/bounds of each curve are respectively defined by the mean positions of the lowest  $(x_L)$  and highest  $(x_H)$  green leaves of that treatment combination's destructively sampled plants (Table 5). The amplitude and point of inflection of each curve are respectively defined by the mean area  $(LA_o)$  and position  $(x_o)$  of the largest green leaf of each treatment combination's destructively sampled plants.

application (O'Neill et al., 2004; Tokatlidis and Koutroubas, 2004). As indicated by responses for  $GY_A$  (Fig. 2), the stress-tolerant, highly productive maize genotypes examined here similarly displayed both marked tolerance to high plant densities and strong responsiveness to an initial side-dress N application ( $\Delta GY_A$ , see Eq. [2]). However, in each year, the crowding tolerance of these hybrids was strongly contingent on N application rate. When side-dress N was applied, GY<sub>A</sub> rarely decreased with each incremental increase in plant density. Yet in the absence of side-dress N, GY<sub>A</sub> declined with each additional increase in plant density, indicating low crowding and (by extension of the principle of intraspecific competition) low N tolerance in this environment. Although responsiveness to an initial side-dress N application was pronounced for each plant density in all growing seasons, it was typically numerically largest at the supraoptimal level of crowding. Furthermore, while a second side-dress N application rarely led to a rise in GY<sub>A</sub> at the suboptimal and optimal plant densities, it frequently improved GY<sub>A</sub> when crowding was most severe. Overall, the observed combination at the supraoptimal plant density of (i) low crowding tolerance in the absence of side-dress N fertilization, (ii) relatively poor low N tolerance, (iii) strong N responsiveness to an initial side-dress N application, and (iv) frequent  $GY_{A}$ response to a second side-dress N application confirms that mineral N application was more essential for optimizing maize productivity at the supraoptimal than suboptimal or optimal plant density for this study's genotypes, thus lending support to our first initial hypothesis.

The general relationship between maize GY<sub>A</sub> and N supply follows the law of diminishing returns. At low soil-N levels, GY<sub>A</sub> increases dramatically with each added unit of N fertilizer since N is the primary constraint on crop growth. However, as the supply of fertilizer N rises, incremental gains in  $GY_A$  become smaller as factors other than N become more limiting. This results in diminishing NUE with increasing N supply (Cassman et al., 2003; Gallais and Coque, 2005; Barbieri et al., 2008). As indicated in Fig. 2, this trend was generally evident for each plant density in all years despite the use of only three side-dress N rates in this study. Thus in each growing season NUE

 $(\Delta GY_A/\Delta N_R$ , see Eq. [2]) consistently declined when the sidedress N rate increased from 165 to 330 kg N ha<sup>-1</sup> regardless of plant density. In most circumstances, as crowding intensified, NUE rose because of increases in plant responsiveness to 165 kg ha<sup>-1</sup> of side-dress N. However, these plant density-associated improvements in  $\Delta GY_A$  resulted more from poor N stress tolerance at the optimal and supraoptimal plant densities than from exceptionally high productivity at these same plant densities in the presence of side-dress N application. Still, genotypes examined in this study displayed relatively high NUE values in all years when grown at the optimal or supraoptimal plant density with the addition of 165 kg ha<sup>-1</sup> of side-dress N (c.f., O'Neill et al., 2004; Cirilo et al., 2009). In each year, GY<sub>A</sub> was rarely different between plant densities when either 165 or 330 kg N ha<sup>-1</sup> was side-dress applied (Fig. 2). Consequently, when broadly defined as kilograms of grain produced per kilogram of side-dress N  $(GY_A/N_R)$ , NUE seldom differed among the

three plant densities for either the 165 or 330 kg ha<sup>-1</sup> side-dress N rate. Thus higher plant densities generally failed to improve overall NUE for these N-responsive genotypes. Still, more extensive multienvironment studies on NUE and its common components (e.g., recovery efficiency, physiological efficiency) using multiple agronomic, physiological, and genetic techniques (c.f., Hirel et al., 2001; Cassman et al., 2003; Subedi and Ma, 2005a, 2007; Coque and Gallais, 2007; Barbieri et al., 2008) are clearly needed for a more complete understanding of the NUE responses of these and other modern genotypes to varying plant density and N rate.

Besides indicating little difference in overall NUE  $(GY_A/N_R)$ , similar  $GY_A$  values among the three plant densities when either 165 or 330 kg ha<sup>-1</sup> of side-dress N was applied (Fig. 2) suggest a high level of plant density independence [i.e., maximum GY<sub>A</sub> is attained over a wide range of plant densities (Tokatlidis et al., 2005)] for this study's late-maturing genotypes in this particular environment. Such plant density independence partially conflicts with the general  $GY_A$  responses observed by Pioneer Hi-Bred Intl., Inc. for both this study's and other Pioneer brand late-maturing hybrids (>113 CRM) grown in high-yielding environments (>11.9 Mg ha<sup>-1</sup>) (Paszkiewicz and Butzen, 2007; D. Rule, personal communication, 2008). In these multiyear, multilocation commercial trials,  $GY_A$  was measured at five plant densities ranging from 44,500 to 104,000 plants ha<sup>-1</sup>, with plant densities of 54,000, 79,000, and 104,000 plants ha<sup>-1</sup> corresponding to agronomic and economic suboptimal, optimal (approximately), and supraoptimal levels of crowding. For these commercial tests, increases in plant density from 79,000 to 104,000 plants ha<sup>-1</sup> led to relatively minimal reductions in  $GY_A$  ( $\approx 1\%$ ), while decreases in plant crowding from 79,000 to 54,000 plants  $ha^{-1}$  caused relatively moderate declines in GY<sub>A</sub> (≈13%) (Paszkiewicz and Butzen, 2007; D. Rule, personal communication, 2008). This latter decline in GY<sub>A</sub> clearly contrasts with the similar GY<sub>A</sub> values observed each year for this study's suboptimal and optimal plant densities when either 165 or 330 kg ha<sup>-1</sup> of side-dress N was applied (Fig. 2).

When either 165 or 330 kg N ha<sup>-1</sup> was side-dress applied, the roughly 30% decline in plant density from the optimal to suboptimal level was accompanied by an approximately 25 to 30% increase in  $GY_p$  (Fig. 3) in addition to either no increase (Fig. 3A–3B) or a decrease (Fig. 3C) in GY<sub>CV</sub>. In general, these relative gains in  $GY_p$  were associated with (i) 5 to 10% increases in KW and 15 to 25% gains in  $KN_p$  (Table 3); (ii) 25 to 30% gains in R6 VB<sub>p</sub> and TB<sub>p</sub> (Fig. 4); (iii) 10 to 15% rises in R1  $LA_T$  (Table 5); (iv) relatively small gains in V14, R3, and R6 SD (Fig. 6A–6C) and V14, R1, R3 and R5 leaf chlorophyll content (Fig. 9A–9C); (v) minor reductions in  $ASI_p$ (Fig. 5); and (vi) the maintenance of  $HI_p$  values greater than 0.5 (Fig. 4). Enhanced pre- and postanthesis LA<sub>D</sub>, VB<sub>D</sub>, and TB<sub>p</sub>; delayed postsilking leaf senescence; reduced ASI<sub>p</sub>; greater KW, KN<sub>p</sub>, and GY<sub>p</sub>; and lower GY<sub>CV</sub> are common physiological responses to the reductions in intraspecific competition provided by lower plant densities, with KN<sub>p</sub> routinely affected more by changes in plant density than KW (Tetio-Kagho and Gardner, 1988a,b; Tollenaar, 1992; Andrade, 1995; Bertin and Gallais, 2000; Edmeades et al., 2000; Sangoi et al., 2002; Bruns and Abbas, 2003; Tokatlidis and Koutroubas, 2004;



Fig. 8. Plant density and N rate effects on maize RI (Ritchie et al., 1996) green leaf area index  $(LAI_G)$  for (A) 2006 and (B) 2007. Means with different letters indicate statistically significant differences at the 0.05 probability level within each plant density.

Fasoula and Tollenaar, 2005; Hashemi et al., 2005; Monneveux et al., 2005; Sarlangue et al., 2007). In this study, the strong responsiveness of  $VB_p$ ,  $TB_p$ ,  $KN_p$ , and  $GY_p$  to reduced plant crowding and the maintenance of  $HI_p$  values above 0.5 at the suboptimal plant density suggest a relatively high level of phenotypic plasticity and reproductive partitioning among these genotypes. Such results may largely explain the plant density independence observed for these hybrids (Echarte et al., 2004; Sarlangue et al., 2007).

Despite strong plasticity for VBp, TBp, KNp, and GYp, responsiveness for R1 LÁ<sub>T</sub> to reduced plant crowding was limited for this study's genotypes, with observed plasticity for R1 LÅ<sub>T</sub> the result of not only increased LA<sub>n</sub> values for most green leaves, but also decreased lower leaf senescence (Table 5, Fig. 7A, 7B, 7D, 7E). Such findings reaffirm previous reports of maize's limited plasticity for per-plant total leaf area in response to altered resource availability (Tetio-Kagho and Gardner, 1988a; Andrade and Abbate, 2005). Restricted responsiveness for R1  $LA_T$  among this study's genotypes led to roughly 20% lower R1 LAI<sub>G</sub> values at the suboptimal versus optimal plant density when either 165 or 330 kg N ha<sup>-1</sup> was side-dress applied (Fig. 8). Despite these differences in R1 LAI<sub>G</sub>, GY<sub>A</sub> was similar among these plant densities for either side-dress N rate (Fig. 2B, 2C). Values for R1 LAI<sub>G</sub> in excess of approximately 4.0 m<sup>2</sup> m<sup>-2</sup> therefore failed to improve  $GY_A$ .



Fig. 9. (A-C) Plant density and (D-F) N rate effects on VI4, RI, R3, and R5 (Ritchie et al., 1996) leaf greenness (SPAD) for (A,D) 2005, (B,E) 2006, and (C,F) 2007. In each year, SPAD measurements were taken on the 12th leaf at VI4 and on the uppermost earleaf at RI, R3, and R5. Means with different letters indicate statistically significant differences at the 0.05 probability level within each phenological stage.

Since maize  $LAI_G$  and light interception typically reach their maximum by silking (Dwyer and Stewart, 1986; Andrade, 1995; Çakir, 2004), such results suggest that green leaf area in well-fertilized, highly crowded environments exceeded the level required for maximum light interception (c.f., Tetio-Kagho and Gardner, 1988a; Maddonni and Otegui, 1996).

Given that this study involved only a small number of late-maturing hybrids consistently grown at a single highly productive site mostly devoid of moisture and non-N nutrient limitations, care must clearly be taken in extrapolating these results to other genotypes and environments (cf, Cox, 1996). For example, the plant density independence seen in this study would likely not be observed for earlier-maturing genotypes grown in more northern latitudes since (i) shorter-season hybrids typically exhibit less biomass plasticity than later-maturing genotypes (Sarlangue et al., 2007), and (ii) high plant densities are requisite for maximizing early-season radiation interception, total biomass accumulation, and, resultantly, GY<sub>A</sub> in these environments (Westgate et al., 1997). Nevertheless, this study's results raise questions about the morphophysiological plasticity and general plant density independence of other modern North American maize genotypes, along with the environmental conditions (e.g., moisture and/or fertility regime, soil type, latitude) in which plant density independence may be most often expressed among current commercial maize hybrids.

Both supraoptimal levels of plant crowding and poor availability of N have been shown to dramatically reduce the GY<sub>A</sub> of tropical maize, with the latter having a more negative impact than the former (Monneveux et al., 2005). Similarly, in this study, GY<sub>A</sub> was more markedly reduced by severe N stress than by supraoptimal plant crowding (Fig. 2). However, it was the simultaneous imposition of both stresses that annually resulted in the lowest GY<sub>A</sub> for the 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> treatment combination. In all growing seasons, these low GY<sub>A</sub> values were accompanied by a host of per-plant and canopy-level morphophysiological responses. Relative to environments marked by lower plant densities and higher N availability,

the 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> treatment combination generally exhibited (i) reduced PHT at V14 and R1 (Table 4); (ii) lower pre- and postanthesis sixth-internode SD values, and more pronounced presilking SD declines (Fig. 6); (iii) greater preflowering lower leaf senescence (Table 5), lower R1 individual leaf area for most of the nonsenesced canopy (Fig. 7), and, resultantly, reduced R1 LÁ<sub>T</sub> (Table 5) and LAI<sub>G</sub> (Fig. 8); (iv) enhanced temporal separation between male and female floral maturity (Fig. 5); (v) lower pre- and postanthesis leaf N and chlorophyll content and decreased visual stay-green during the grain-filling period (Fig. 9); (vi) reduced VB<sub>p</sub> and TB<sub>p</sub> at R6 (Fig. 4); (vii) reduced total partitioning of biomass to the ear (Fig. 4); (viii) lower KW, KN<sub>p</sub> (Table 3), and  $GY_p$  (Fig. 3); and (ix) higher  $GY_{CV}$  (Fig. 3). As shown through the use of allometric models by Maddonni and Otegui (2004) and Pagano and Maddonni (2007), maize TB<sub>p</sub> at V14 and R1 is linearly related to stem volume when this volume is estimated via the cylinder formula using the morphometric parameters PHT and basal SD. Furthermore,  $TB_p$  at R3 is positively related to stem volume and uppermost ear diameter through a quadratic relationship (Maddonni and Otegui, 2004; Pagano and Maddonni, 2007). And rade et al. (1999) similarly determined that  $TB_p$  is related to SD through a univariate quadratic function in the preflowering period and to SD, uppermost ear diameter, and uppermost ear length through a multivariate curvilinear function in the postflowering period. Thus, although VBp and TBp were not measured at V14, R1, and R3 in this study, these general allometric relationships in conjunction with observed results for PHT at V14 and R1 (Table 4); SD at V14, R1, and R3 (Fig. 6); LÁ<sub>T</sub> at R1 (Table 5); and VB<sub>p</sub>, TB<sub>p</sub>, HI<sub>p</sub> (Fig. 4), and GY<sub>p</sub> (Fig. 3) at physiological maturity suggest that  $VB_p$  and  $TB_p$ at V14, R1, and R3 were lowest for the 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> treatment combination in each year. Overall, these observed responses (i) generally support our second initial hypothesis, (ii) strongly agree with previously reported responses to varying plant densities and/or N rates (e.g., Ogunlela et al., 1988; Tetio-Kagho and Gardner, 1988a; Wolfe et al., 1988a, 1988b; Jacobs and Pearson, 1991; Jones et al., 1995; Uhart and Andrade, 1995b, 1995c; Maddonni and Otegui, 2004; Paponov and Engels, 2003, 2005; Tokatlidis and Koutroubas, 2004; Fasoula and Tollenaar, 2005; Monneveux et al., 2005; Paponov et al., 2005a, 2005b; Subedi and Ma, 2005a, 2005b; Borrás et al., 2007; Echarte et al., 2008; Hammer et al., 2009), and (iii) provide (to the best of our knowledge) the first report on the morphophysiological responses of modern North American hybrids to the simultaneous stresses of intense crowding and low N availability. When combined with results from numerous studies in maize C and N physiology and metabolism, this experiment's per-plant and canopylevel responses offer an incomplete but improved and relatively integrated understanding of the pre- and postflowering morphophysiological behavior of these genotypes in this high stress environment.

During preflowering growth and development in maize, N deficiency commonly results in reduced leaf emergence and expansion rates along with lower LA, values for a majority of emerged leaves (Hageman, 1986; Uhart and Andrade, 1995b; Paponov and Engels, 2003). Such responses commonly limit per-plant and canopy-level leaf area, thus restricting pre- and postflowering light interception, total dry matter accumulation, and kernel production (Hageman, 1986; Uhart and Andrade, 1995b; Paponov and Engels, 2003; Subedi and Ma, 2005a, 2005b; Echarte et al., 2008). Markedly lower R1  $LA_T$ (Table 5) and  $LAI_{G}$  (Fig. 8) values in this study's 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> environment suggest impaired production and maintenance of green leaf area between seedling emergence and flowering. In fact,  $L\dot{A}_T$  was so reduced by preflowering intraspecific competition in this environment that the improvement in LAI<sub>G</sub> often conferred by high plant densities (e.g., Tetio-Kagho and Gardner, 1988a; Cox, 1996)

was largely absent (Fig. 8). As indicated by responses for  $x_{I}$ ,  $NL_{T}$  (Table 5), and  $LA_{n}$  (Fig. 7), reductions in  $LA_{T}$  were primarily the outcome of enhanced N remobilization from and subsequent senescence of lower leaves in addition to reduced individual leaf area for most of the established canopy. Measurements of SPAD at V14 suggest that preflowering productivity was further impaired in this environment by relatively low leaf chlorophyll content in the upper-canopy strata that likely lowered leaf absorptance and C exchange among these leaves (Echarte et al., 2008). When considered in conjunction with previously discussed allometric relationships (Andrade et al., 1999; Maddonni and Otegui, 2004; Pagano and Maddonni, 2007), reduced PHT and sixth-internode SD at V14 and R1 (Table 4, Fig. 6) suggest that these multiple limitations on per-plant and canopy-level photoassimilate production restricted per-plant aboveground dry matter accumulation during the preflowering period.

While N deficiency can negatively impact preflowering maize productivity, it is particularly detrimental during the critical period bracketing silking (Paponov et al., 2005b). In general, final KN<sub>p</sub> is a function of dry matter accumulation (i.e., crop growth rate) during this roughly 30-d period (Otegui and Andrade, 2000; Maddonni and Otegui, 2004; Lee and Tollenaar, 2007). Deficiency of N before and/or during this stage increases leaf N remobilization, thus decreasing leaf chlorophyll content, light absorptance, C exchange, and radiation use efficiency during the critical period (Uhart and Andrade, 1995b; Echarte et al., 2008). Crop and ear growth rates are consequently reduced, with the latter more markedly impaired than the former (Uhart and Andrade, 1995b; Otegui and Andrade, 2000; Paponov et al., 2005a). Poor dry matter partitioning to reproductive sinks commonly results in reduced rates of silk elongation, failures in fertilization, increases in kernel abortion, and, ultimately, reductions in KN<sub>p</sub>, GY<sub>p</sub>, and HI<sub>p</sub> (Jacobs and Pearson, 1991; Uhart and Andrade, 1995c; Andrade et al., 1999; Tollenaar et al., 2000; Bänziger et al., 2002; Monneveux et al., 2005; Coque and Gallais, 2007). Compared with severely N-deficient conditions, supraoptimal levels of crowding often have similar impacts on KNp and GYp. Intense crowding results in strong intraspecific competition for available radiation during the 30 d bracketing silking. As in low-N conditions, the attenuation of per-plant radiation interception, an accompanying decline in leaf C exchange, a concomitant reduction in plant growth rate, and an inflexible pattern of assimilate distribution to reproductive sinks during the critical period lead to a dramatic reduction in ear growth rate, an increase in kernel abortion, and decreases in KN<sub>p</sub>, GY<sub>p</sub>, and HI<sub>p</sub> (Edmeades and Daynard, 1979b; Edmeades et al., 1979; Otegui and Bonhomme, 1998; Otegui and Andrade, 2000; Tollenaar et al., 2000; Maddonni and Otegui, 2004; Gallais and Coque, 2005; Pagano and Maddonni, 2007). In general, the imposition of abiotic stress during this period primarily affects KN<sub>p</sub> and GY<sub>p</sub> through increased kernel abortion; it has limited effect on KW (Otegui and Andrade, 2000; Tollenaar et al., 2000; Gallais and Coque, 2005). Overall, morphophysiological responses during the critical period in this study suggest that these general physiological processes were largely responsible for the ultimately low KNp (Table 3),  $GY_p$  (Fig. 3), and  $HI_p$  (Fig. 4) values observed for the highly crowded, low N environment in each year.

Responses for R1 LÁ<sub>T</sub> (Table 5) and SPAD (Fig. 9) indicate the combination of intense crowding and low N availability markedly reduced source activity during the critical period. As previously discussed, these  $LA_T$  and SPAD reductions principally resulted from enhanced preflowering leaf senescence and reduced individual leaf area. While the general shape (i.e., *b* coefficient) of the R1 green  $LA_D$  curve (Table 5, Fig. 7) was not altered by these simultaneous stresses, both LA, values for leaves proximal to the ear (Fig. 7) and SPAD values for the earleaf itself (Fig. 9) were reduced. Because of their relative size, proximity to the developing ear, and high rates of photosynthesis, such leaves supply a substantial percentage of the C compounds utilized for plant and ear growth (Edmeades et al., 1979; Dwyer et al., 1989, 1992; Wardlaw, 1990). Observed decreases in LA, values and leaf chlorophyll content near the ear therefore suggest restricted assimilate availability for plant and ear growth during the critical period in this experiment. Still, ear growth may have been partially buffered against shortages in photosynthesis by the mobilization of stem carbohydrate reserves (Tollenaar et al., 2000). Although ear growth was not directly measured in this experiment, ASI<sub>p</sub> was used as an indirect indicator of C flux to the developing ear around the time of silking (Edmeades et al., 1993, 2000; Borrás et al., 2007; Hammer et al., 2009). Numerous studies report an increase in ASI<sub>p</sub> and a corresponding decrease in  $KN_p$ ,  $GY_p$ , and  $HI_p$  in response to high plant density or low N availability (e.g., Jacobs and Pearson, 1991; Bertin and Gallais, 2000; Edmeades et al., 2000; Gallais and Coque, 2005; Borrás et al., 2007). In every year of this study, ASI<sub>p</sub> was greatest for the 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> treatment combination (Fig. 5), suggesting that restrictions in assimilate partitioning to the ear during the critical period were at least partially responsible for the strongly depressed  $KN_p$ ,  $GY_p$ , and  $HI_p$ values of highly crowded, severely N-stressed plants.

Approximately 50% of total seasonal dry matter is accumulated during the postsilking period in maize, with a vast majority of this postsilking accumulation occurring in the grain (Bertin and Gallais, 2000; Tollenaar et al., 2004; Lee and Tollenaar, 2007). During this period, vegetative C may remobilize to developing grains in stress conditions (e.g., low N environments) or for certain genotypes (Uhart and Andrade, 1995a; He et al., 2002; Paponov and Engels, 2005). Still, grain biomass is largely the product of postsilking photosynthetic activity (Swank et al., 1982; Cliquet et al., 1990; He et al., 2002; Gallais and Coque, 2005). Kernel dry matter accumulation is therefore heavily dependent on the maintenance of photosynthesis during the grain-filling period (Bänziger et al., 2002; Luque et al., 2006; Lee and Tollenaar, 2007). Both high plant densities and N deficiency routinely accelerate leaf senescence in maize during the postsilking period. Although senescence provides kernels with requisite N via leaf N remobilization, it reduces postsilking leaf absorptance, leaf C exchange, photoassimilate production, VB<sub>p</sub>, TB<sub>p</sub>, and, consequently, KW, KN<sub>p</sub>, and GY<sub>p</sub> (Rajcan and Tollenaar, 1999a; Bänziger et al., 2002; Borrás et al., 2003; Paponov and Engels, 2003, 2005; Gallais and Coque, 2005; Coque and Gallais, 2007; Echarte et al., 2008). In this study, the simultaneous stresses of intense crowding and low N availability severely reduced R3 and R5 earleaf SPAD values (Fig. 9) and

consistently induced premature declines in visual stay-green. Leaves proximal to the ear are a major source of kernel N during senescence (Cliquet et al., 1990; Coque and Gallais, 2007). Such SPAD responses therefore indicate pronounced early degradation of earleaf chloroplasts, marked premature remobilization of earleaf N to developing kernels, and, concomitantly, considerable loss of postsilking photosynthetic activity among leaves that can supply a substantial amount of C assimilates for kernel growth (Edmeades et al., 1979; Gan and Amasino, 1997; Echarte et al., 2008). Low KW, KN<sub>P</sub>, GY<sub>P</sub>, and HI<sub>P</sub> values in this study's highly crowded, N-deficient environment (Table 3; Fig. 3, 5) were therefore likely partially the product of major reductions in postsilking C exchange (Rajcan and Tollenaar, 1999a; Paponov et al., 2005b).

Source strength during very early stages of grain-filling can largely determine kernel sink potential and KW through modifications in kernel growth rate during the effective grainfilling period (Gambín et al., 2006). Thus reductions in KW in this study's highly stressed environment (Table 3) were likely the product of source limitations both shortly after silking and during latter portions of the grain-filling period. Severe source limitations during the grain-filling period can induce C remobilization from vegetative to reproductive tissues (Uhart and Andrade, 1995a). While this process may have occurred among these hybrids in this highly competitive environment, the absence of a strong sink combined with restrictions in preflowering source production and C exchange may have limited the impact of this process. Still, this mechanism may have supplemented postsilking photosynthesis in meeting the C demands of kernel dry matter accumulation during the grain-filling period.

Although root parameters were not measured in this experiment, it is likely that pronounced constraints in postflowering source activity in the 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> environment were physiologically linked with pre- and postsilking root growth and N uptake dynamics. Deficiency of N in maize often results in greater root length, increased root dry matter, and a higher root/shoot ratio before, during, and after flowering (Eghball and Maranville, 1993; Durieux et al., 1994; McCullough et al., 1994b; Echarte et al., 2008). This increase in belowground growth is supported by enhanced translocation of C assimilates from the shoot to the roots (Mackay and Barber, 1986). However, severe senescence in low N conditions can limit carbohydrate availability to the roots, thus restricting N uptake and enhancing leaf senescence (Gallais and Coque, 2005). Lower leaves typically supply a substantial amount of carbohydrates to the lower stem and root system (Fairey and Daynard, 1978; Edmeades et al., 1979; Wardlaw, 1990). However, severe lower leaf senescence in this study's highly crowded, low N environment (Table 5) suggests this source of C assimilates may have been rather limited. As shown in Fig. 6C and 6F, early reductions in SD between V14 and R1 were evident for each plant density when no side-dress N was applied in 2007. Because of its ability to store carbohydrates and general proximity to the root system, the lower stem may have served as a C source for roots during the preflowering period to enhance N uptake and limit N remobilization (Rajcan and Tollenaar, 1999b; Coque and Gallais, 2007). Still, as suggested by low pre- and postanthesis SPAD values (Fig. 9);

pronounced senescence of lower leaves (Table 5); and high rates of decline for visual stay-green (Fig. 9); plants in the 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> environment were largely incapable of acquiring sufficient N to prevent pronounced N remobilization and subsequent reductions in GY<sub>p</sub> (Fig. 3). Though the data are not shown here, comparatively low shoot and grain N concentrations at physiological maturity in this environment confirmed limitations in N uptake.

Pronounced reductions in KW, KN<sub>p</sub>, GY<sub>p</sub>, and GY<sub>A</sub> in this study's highly crowded, N-deficient environment may not have been solely related to the production and general activity of source tissues. A number of studies suggest that poor N availability may limit kernel set through direct effects on C and N metabolism in developing grains. For example, Tsai et al. (1980) found that low N availability markedly reduced zein and glutelin deposition in developing maize kernels. This, they propose, limited sucrose transport into developing seeds, consequently restricting kernel dry matter accumulation. Singletary and Below (1990) similarly found lower endosperm concentrations of zein and glutelin in N-deficient conditions, but also discovered increased sucrose and decreased starch content in developing kernels. They therefore suggest that N deficiency limited endosperm dry weight primarily through a reduced capacity for starch synthesis. Previous experiments involving either stem infusion of C and N assimilates in the field or in vitro culture of maize kernels additionally found that N availability had a direct impact on the ability of plants to utilize sugars for ear growth, potentially through effects on the activity of enzymes directly involved in kernel C and N use (Below et al., 2000). In contrast to this collection of studies, Uhart and Andrade (1995c) found that N shortage had no direct effect on kernel set; impairment of KN<sub>p</sub> was solely the product of reduced C exchange.

Plant-to-plant variability for GY<sub>p</sub> is negatively correlated with resource use efficiency and, consequently, GY<sub>A</sub> (Tollenaar and Wu, 1999; Tokatlidis and Koutroubas, 2004; Fasoula and Tollenaar, 2005). Such variability can result from cultural (i.e., anthropogenic) practices and/or biological phenomena. Some cultural sources of variability include deviations in planting depth, seed spacing, and crop residue distribution, while biological sources of nonuniformity include variations in insect feeding, disease pressure, and soil type (Tokatlidis and Koutroubas, 2004; Andrade and Abbate, 2005). However, plant-to-plant nonuniformity is also the product of intense intraspecific competition for available resources (i.e., solar radiation, water, soil nutrients). As plant density increases in maize, greater competition between individual maize plants results in increased by-plant variability for  $GY_{p}$ , its primary components (i.e., KW, KN<sub>p</sub>), and other morphophysiological traits (e.g., plant growth rate, ASI<sub>p</sub>, pre- and postsilking VB<sub>p</sub> and TB<sub>p</sub>) (Edmeades and Daynard, 1979a; Tollenaar and Wu, 1999; Maddonni and Otegui, 2004; Tokatlidis and Koutroubas, 2004; Andrade and Abbate, 2005; Maddonni and Otegui, 2006; Borrás et al., 2007; Pagano and Maddonni, 2007). In this study,  $GY_{CV}$  generally rose with an increase in plant density regardless of N rate. However, it was the simultaneous stresses of severe crowding (i.e., 79,000 and/ or 104,000 plants ha<sup>-1</sup>) and low fertilizer N availability that resulted in the greatest GY<sub>CV</sub> values (Fig. 3). As indicated

through previous eco- and morphophysiological examinations of this study's individual plant responses (Boomsma and Vyn, 2006, 2007, 2008), these high GY<sub>CV</sub> values were primarily the product of intense intraspecific competition for available N (and potentially other limited resources). They were not principally the result of temporal (i.e., seedling emergence date) or spatial (i.e., within-row plant spacing) variability (data are not shown). Such competition encouraged the formation of plant hierarchies composed of "dominating" and "dominated" (see Maddonni and Otegui, 2004) individuals (Boomsma and Vyn, 2008), consequently leading to unequal resource sharing between plants, a reduction in overall resource use efficiency [e.g., overall NUE (GY<sub>A</sub>/N<sub>R</sub>) (Fig. 2)], and a subsequent decline in GY<sub>A</sub> in these highly crowded, low N environments (Fig. 2) (Tollenaar and Wu, 1999; Fasoula and Tollenaar, 2005).

#### CONCLUSIONS

When grown at optimal and supraoptimal plant densities with 165 kg ha<sup>-1</sup> of side-dress N, this study's stress-tolerant, highly productive, North American maize hybrids exhibited both strong N responsiveness ( $\Delta GY_A)$  and relatively high NUE  $(\Delta GY_A / \Delta N_R)$  (c.f., O'Neill et al., 2004; Cirilo et al., 2009). When N was applied roughly at or markedly above this location's approximate agronomic optimal N rate (i.e., roughly 160 kg N ha<sup>-1</sup>), GY<sub>A</sub> values were very often similar among all plant densities. Such results suggest (i) optimal and supraoptimal levels of crowding generally did not improve overall NUE  $(GY_A/N_R)$  relative to the suboptimal plant density, and (ii) this study's genotypes exhibited a degree of plant density independence in this particular highly productive environment. Regardless of plant density, this experiment's hybrids displayed HIp values in excess of the commonly presumed 0.5 value for modern North American genotypes when N was side-dress applied at 165 or 330 kg ha<sup>-1</sup>. Such results suggest that the HI of current North American genotypes may now exceed 0.5 when these hybrids are grown in highly productive environments. While this experiment's hybrids exhibited pronounced tolerance to high plant densities, this tolerance was heavily dependent on the application of side-dress N (e.g., side-dress N in excess of 165 kg ha<sup>-1</sup> was more often required at the supraoptimal than optimal plant density to maximize  $GY_{A}$ ).

In the absence of side-dress N, these hybrids displayed low crowding tolerance, poor N stress tolerance, and, consequently, low values for GY<sub>A</sub>. Markedly low grain production in the highly crowded, low N environment (i.e., 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup>) was primarily the product of intense intraspecific competition for available N. This severe competition resulted in a number of per-plant and canopy-level morphophysiological responses including, but not limited to, (i) reduced pre- and postanthesis PHT, SD,  $VB_p$ , and  $TB_p$ ; (ii) greater preflowering lower leaf senescence, lower R1 individual leaf area for most of the nonsenesced canopy, and, consequently, reduced R1 LA<sub>p</sub> and LAI<sub>C</sub>; (iii) enhanced temporal separation between male and female floral maturity; (iv) lower pre- and postanthesis leaf N and chlorophyll content and decreased visual stay-green during the grain-filling period; (v) lower KW, KN<sub>p</sub>, GY<sub>p</sub>, and  $\mathrm{HI}_{\mathrm{p}}$  and (vi) higher  $\mathrm{GY}_{\mathrm{CV}}$  Based on these responses, we suggest that poor grain production in this highly competitive environment was primarily the result of (i) reduced production

and activity of source tissues during the presilking period, (ii) decreased plant growth and markedly abated assimilate partitioning to the developing ear during the critical period bracketing silking, (iii) early remobilization of leaf N and subsequent reduction in C assimilation during the grain-filling period, and (iv) enhanced plant-to-plant variability for key morphophysiological traits (expressed ultimately in high  $GY_{CV}$ ). Overall, the observed combination at the supraoptimal plant density of (i) low crowding tolerance in the absence of side-dress N, (ii) relatively poor low N tolerance, (iii) strong N responsiveness to 165 kg ha<sup>-1</sup> of side-dress N, and (iv) frequent  $GY_A$  response to a second side-dress N application indicates that N application was more essential for optimizing maize productivity at the supraoptimal than suboptimal or optimal plant density for this study's genotypes.

While the results of this study provide an understanding of the morphophysiological behavior of modern maize genotypes in highly crowded, low-N environments, they also provide guidance for future maize advancement, particularly in the area of N stress tolerance. This experiment's responses for GY<sub>A</sub> suggest that tolerance to high plant densities is strongly contingent on adequate N application. As indicated by the poor GY<sub>A</sub> of the 104,000 plants ha<sup>-1</sup>, 0 kg N ha<sup>-1</sup> treatment combination in all 3 yr of this study, the downside risk to N deficiency is much greater in highly crowded environments. Considering the necessity for high N application rates at high plant densities, the frequently high and continually fluctuating price of N fertilizer, the unpredictable effects of weather on N availability, and environmental concerns over excessive N fertilization, losses in GY<sub>A</sub> resulting from N deficiency may increase with the progressive movement of productive North American maize systems to higher plant densities. Consequently, we propose that efforts aimed at improving a genotype's high plant density tolerance should simultaneously focus on enhancing that genotype's N stress tolerance and NUE. As evidenced by the literature reviewed and the results discussed in this publication, low N availability and intense plant crowding can generate similar morphophysiological responses in maize [e.g., attenuated per-plant radiation interception, decreased plant growth rate, and markedly reduced ear biomass accumulation during the critical period (Uhart and Andrade, 1995b; Paponov et al., 2005a; Edmeades et al., 2000; Maddonni and Otegui, 2004)]. While the genetic, metabolic, and physiological mechanisms underlying these comparable responses can differ pronouncedly by stressor, selection for some traits may improve hybrid tolerance to each of these abiotic stresses [e.g., reduced ASI<sub>p</sub> (Edmeades et al., 2000)]. Based on this study's results and much of the literature cited in this publication (e.g., Tollenaar and Wu, 1999; Edmeades et al., 2000; Otegui and Andrade, 2000; Maddonni and Otegui, 2004; Lee and Tollenaar, 2007), we suggest that genetic efforts aimed at improving maize tolerance to the simultaneous stresses of high plant density and low N availability focus initially on (i) improving crop growth and biomass partitioning to the ear during the critical period bracketing silking, (ii) maintaining photosynthetically active leaf area (i.e., functional stay-green) and kernel dry matter accumulation during the grain-filling period, and (iii) enhancing season-long plant-to-plant uniformity through improved above- and belowground per-plant

morphophysiological plasticity. Although the maize research community knows the least about mechanism (iii), its further understanding and improvement may hold considerable potential for the future advancement of maize abiotic stress tolerance. It is the aboveground aspects of this mechanism which will be intensively examined in this study's subsequent publications.

## APPENDIX

## Model Use and General Theory

Because extensive measurements were taken on the same approximately 4000 tagged plants from seedling emergence through physiological maturity each year, an accurate, rapid, low-cost, nondestructive technique was needed to measure the LA<sub>P</sub> and LA<sub>D</sub> of each tagged plant at R1. Pagano and Maddonni (2007) recently determined LA<sub>p</sub> at R1 by multiplying leaf length (L)  $\times$  maximum leaf width (W)  $\times$  0.75 (Montgomery, 1911) for all green leaves on each plant. However, this procedure was too labor-intensive, time-consuming, and costly for this study's purposes and available resources. To meet these unique needs, a modification of the model and techniques used by Dwyer and Stewart (1986) and Valentinuz and Tollenaar (2006) to describe leaf area development and the LA<sub>D</sub> of all initiated leaves (i.e., from the first seedling leaf to the uppermost leaf beneath the tassel) was employed. Here we describe this method in detail.

According to Dwyer and Stewart (1986), the  $LA_D$  for all initiated leaves on a maize plant resembles a slightly skewed bell-shaped curve. This curve is described by the model:

$$LA_n = LA_o \times \exp[-b(x_n - x_o)^2 + c(x_n - x_o)^3]$$
 [A1]

where  $LA_n$  is the fully expanded area of the *n*th leaf,  $LA_o$  is the fully expanded area of the largest leaf and dictates the amplitude of the function,  $x_n$  is the position on the stem of the *n*th leaf (leaves numbered from the bottom to the top of the plant for all initiated leaves),  $x_o$  is the position on the stem of the largest leaf and is the point of inflection of the curve, and *b* and *c* are coefficients that describe the breadth/kurtosis and skewness of the distribution of leaf area for all initiated leaves, respectively (Dwyer and Stewart, 1986; Elings, 2000; Valentinuz and Tollenaar, 2006).

## **In-Field Measurements**

To rapidly and nondestructively determine the  $LA_p$ and  $LA_D$  of tagged plants at R1 in 2006 and 2007 using a modification of this model, a destructive sampling area was established in row three of each six-row sub-subplot. In each of these areas, three consecutive, representative plants were tagged and removed (cutting at the stem base) at R1 (12 plants per hybrid-plant density-N rate treatment combination, 216 plants in total per year). In each sub-subplot, these areas were at least 3 m from the nondestructive per-plant sampling area and 4 m from the sub-subplot border (intrarow distances). To account for reductions in GY<sub>A</sub> caused by the removal of these plants, the linear distance from the point midway between plant one and its respective nonsampled neighbor to the point midway between plant three and its respective nonsampled neighbor was measured for each sub-subplot's three consecutive plants. This distance was multiplied by the interrow spacing to determine the area of plant removal. For each sub-subplot, this area of destructive sampling was subtracted from the area used for determination of GY<sub>A</sub>. In both 2006 and 2007, representative plants for destructive sampling were chosen based on overall visual similarity (i.e., size, lower leaf senescence) to tagged plants in nondestructive per-plant sampling areas. On each of these destructively sampled plants, L and W were measured for all green leaves [i.e., those leaves with greater than 50% of their area still green (Rajcan and Tollenaar, 1999a)]. As with Valentinuz and Tollenaar (2006), L was measured along the midrib as the distance from the collar to the tip. As proposed by Montgomery (1911) and employed by numerous others (e.g., Maddonni and Otegui, 1996; Stewart and Dwyer, 1999; Elings, 2000; Pagano and Maddonni, 2007), the  $LA_n$  of each green leaf was calculated by multiplying L by W by 0.75. Unlike plants in the nondestructive per-plant sampling areas, the phenology of these plants was not measured throughout the growing season.

For all plants in the nondestructive per-plant sampling areas, earleaf position  $(x_{e})$  was determined relative to the location of each plant's tag. Since the phenology of destructively sampled plants was not tracked, values for  $x_e$  from tagged plants were used to estimate  $x_{e}$  for destructively sampled plants. For tagged plants in both 2006 and 2007, neither plant density nor N rate had a substantial impact on  $x_e$  (i.e., means for  $x_e$  differed by less than 0.5 leaf positions), although the plant density effect was significant in both years (Table 5). However, the effects of year (data are not shown) and hybrid were more pronounced (i.e., means for  $x_e$  differed by greater than 0.5 leaf positions), with the hybrid effect significant in both years (Table 5). Based on these results, values for  $x_e$  were set for destructively sampled plants of each hybrid by year irrespective of plant density and N rate. These values were 15 and 14 for hybrid Pioneer 31G68 in 2006 and 2007, respectively, and 14 and 13 for hybrid Pioneer 31N28 in 2006 and 2007, respectively. For all destructively sampled plants,  $x_o$  and  $x_n$  (for green leaves only) were determined relative to these set values for  $x_e$ . For destructively sampled plants within a hybrid-plant density-N rate treatment combination,  $x_a$ , the total number of green leaves (NL<sub>T</sub>), and the positions of the lowest  $(x_{I})$  and highest  $(x_{H})$  green leaves were highly uniform (data are not shown). However, each treatment factor often significantly affected the values of these parameters in both years (Table 5). An average value for  $x_a$ was therefore calculated for each hybrid-plant density-N rate treatment combination. Since determining  $x_a$  for all tagged plants was impractical, these average values were then used to estimate x<sub>a</sub> by hybrid-plant density-N rate treatment combination for all plants within nondestructive sampling areas. To ensure that the  $x_{a}$  of a treatment combination's destructively sampled plants was an accurate estimate of  $x_a$  for tagged plants of that same treatment combination, the true  $x_a$  was verified for five consecutive, representative, tagged plants in each per-plant sampling area. Using estimated values for  $x_{\rho}$ , the area of the largest green leaf of tagged plants  $(L\dot{A}_{o})$  was determined at R1 in both 2006 and 2007 using the same method as previously described (i.e.,  $L \times W \times 0.75$ ).

## Model Selection

Whereas Eq. [A1] is a "robust predictor of the area-per-leaf profile in maize" when the area of all initiated, fully expanded leaves is measured (Valentinuz and Tollenaar, 2006), it may not be as useful an estimator of LA<sub>P</sub> and LA<sub>D</sub> at a single postanthesis phenological stage. This may be particularly true when plants are grown at a supraoptimal density or when abiotic stress (e.g., limited N or water availability) is severe. In each of these scenarios, lower leaf senescence can be pronounced and the left half of the slightly skewed bell-shaped curve described by Valentinuz and Tollenaar (2006) may be nearly or entirely missing. In such cases, the LA<sub>D</sub> would no longer resemble the slightly skewed bell-shaped curve described by Dwyer and Stewart (1986) and Valentinuz and Tollenaar (2006). Other functions that might more accurately describe the LA<sub>D</sub> at R1 were therefore considered for all destructively sampled plants. In addition to Eq. [A1], the following functions were evaluated on a by-plant basis:

$$LA_n = LA_o \times \exp[-b(x_n - x_o)^2]$$
 [A2]

$$LA_n = LA_o \times [a + d(x_n - x_o) + f(x_n - x_o)^2]$$
 [A3]

$$LA_n = LA_o \times [a + d(x_n - x_o)]$$
 [A4]

$$LA_n = LA_o \times [a + f(x_n - x_o)^2]$$
 [A5]

where  $LA_n$ ,  $LA_o$ , n,  $x_n$ ,  $x_o$ , and b are as described for Eq. [A1], and a, d, and f are model coefficients. For Eq. [A1] through [A5], the position of each green leaf was set relative to  $x_o$  (such that a describes the intercept of the curve at  $x_o$  for Eq. [A3] through [A5]). The b and c coefficients in Eq. [A1] and [A2] were estimated using the SAS NLIN procedure (SAS Institute, 2004), with starting parameter values (b = 0.03, 0.05; c = 0.00014, 0.00016) estimated from Valentinuz and Tollenaar (2006). Regression analyses for Eq. [A3] through [A5] were performed using the SAS GLM procedure (SAS Institute, 2004).

For each of the 432 plants, the best model was identified using the following criteria: (i) all model parameters were significant at the 0.05 probability level, (ii) the overall model was significant at the 0.05 probability level, and (iii) the model had the lowest P value among all significant models. Based on these selection criteria, Eq. [A2] provided the best fit for approximately 80% of the plants. For nearly all remaining plants, Eq. [A1] was the best model, but in these circumstances, its fit was only slightly better than that of Eq. [A2]. Given these results and the greater simplicity of Eq. [A2], we chose to use Eq. [A2] for all subsequent calculations of LA<sub>p</sub> and LA<sub>D</sub> at R1.

#### **Model Implementation**

For implementation of Eq. [A2] in this study, the position of each green leaf was set relative to  $x_o$  for all destructively sampled plants. For the three destructively sampled plants within a sub-subplot, an average sub-subplot-level value for LA<sub>o</sub> was calculated. Using Eq. [A2], sub-subplot-level LA<sub>o</sub> values, and SAS PROC NLIN (starting values for b = 0.03, 0.05), a single *b* coefficient was generated for each sub-subplot based on the leaf measurements of the three plants destructively sampled within that sub-subplot. The predicted area of every green leaf  $(L\hat{A}_n)$  was resultantly determined for each of these plants. The total predicted green leaf area of each of these plants  $(L\hat{A}_T)$  was then calculated as the sum of each plant's  $L\hat{A}_n$  values. The total observed/measured green leaf area of each destructively sampled plant  $(LA_T)$  was similarly calculated as the sum of green LA<sub>n</sub> values.

The total area of all initiated leaves on an individual maize plant can be related to the area of a single leaf representing a relatively large proportion of total leaf area (Pearce et al., 1975; Elings, 2000). As such, the total green leaf area at R1 of each tagged, nondestructively sampled plant ( $L\dot{A}_T$ ) within a subsubplot was calculated as follows:

$$L\hat{A}_{T} = L\hat{A}_{T} \times (L\hat{A}_{o}/LA_{o})$$
[A6]

where  $L\hat{A}_o$  is the area of the largest leaf of the tagged plant and  $L\hat{A}_T$  and  $LA_o$  are the average total predicted leaf area and average area of the largest leaf of destructively sampled plants from that same sub-subplot, respectively. The total green leaf area (m<sup>2</sup>) at R1 of each nondestructive per-plant sampling area ( $L\hat{A}_{SA}$ ) was calculated as the sum of all  $L\hat{A}_T$  values for that sampling area. For each per-plant sampling area, the LAI<sub>G</sub> at R1 was calculated as:

$$LAI_{G} = L\dot{A}_{SA}/6.08$$
 [A7]

where 6.08 is the total soil surface area  $\left(m^2\right)$  of each sampling area.

It should be noted that, while values for LA<sub>T</sub>, LÂ<sub>T</sub>, and LÂ<sub>T</sub> were numerically similar in 2006, LÁ<sub>T</sub> values were pronouncedly less than LA<sub>T</sub> and LÂ<sub>T</sub> values in 2007. Low LÂ<sub>T</sub> values in the second growing season primarily resulted from LÂ<sub>o</sub> values being consistently numerically smaller than LA<sub>o</sub> values (Table 5). Such results for 2007 suggest that plants sampled in destructive sampling areas were not perfectly representative of plants measured in nondestructive sampling areas in this growing season. This likely resulted from the inadvertent selection of slightly larger plants for destructive-sampling purposes. Still, considering patterns of lower leaf senescence were similar between destructive and nondestructive sampling areas and treatment effects had little impact on *b* values, plant sampling errors likely had minimal effect on 2007 results for all per-plant leaf area parameters.

#### **Model Validation**

The ability of Eq. [A2] to predict both the area of individual green maize leaves and total per-plant green leaf area at R1 was evaluated using two independent datasets. Data for model validation was collected in 2008 at two locations: ACRE and the Pinney-Purdue Agricultural Center (PPAC) (41°26'41" N, 86°56'41" W) in Wanatah, IN.

For the experiment at ACRE, maize was grown following soybean. Recommended practices were followed for the control of weeds and insect pests. The experiment was arranged as a split plot design with six blocks. Hybrid (main plot) and plant density (subplot) served as the two treatment factors. Each subplot consisted of four rows (76-cm interrow spacing) and was approximately 3.0 m in width and 15.0 m in length. While the experiment involved four modern hybrids and six plant densities, sampling was limited to only two hybrids [DEKALB DKC61-69 (VT3) and DEKALB DKC61-19 (VT3)] and three plant densities (49,000, 74,000, and 99,000 plants ha<sup>-1</sup>) in a single block. Both hybrids were planted at rates of 53,000, 78,000, and 103,000 seeds ha<sup>-1</sup> to achieve the desired final plant densities. For those subplots from which leaf measurements were taken, plant density measurements at V6 rarely differed from target plant densities by more than 5% (data are not shown). In all subplots, starter fertilizer [ammonium nitrate (33.5–0–0)] was applied at planting 5 cm to the side and 5 cm below the seed at a rate equivalent to 35 kg N ha<sup>-1</sup> and UAN was applied via side-dressing at V5 at a rate equivalent to 180 kg N ha<sup>-1</sup>.

At PPAC, the soil was a Runnymede (fine-loamy, mixed, mesic Typic Argiaquolls) loam with approximately 3% organic matter content in the top 30 cm of the soil profile. The experimental area had <2% slope. The modern maize hybrid Pioneer 34A20 was grown following soybean. Recommended practices were followed for the control of weeds and insect pests. The experiment was arranged as a randomized complete block design with four blocks. In addition to a no N control, treatments were a factorial combination of (i) type of starter fertilizer, (ii) rate of starter fertilizer, and (iii) timing of side-dress N application. Each plot consisted of 12 rows (76-cm interrow spacing) and was approximately 3.0 m in width and 15.0 m in length. While the experiment involved eight treatment combinations and a no-N control, sampling was limited to only the no-N control and a treatment combination involving the application of 19–17–0 starter fertilizer (at planting, 5 cm to the side and 5 cm below the seed) at a rate equivalent to 45 kg N ha<sup>-1</sup> followed by the side-dressing of UAN at V4 at a rate equivalent to 135 kg N ha<sup>-1</sup>. Leaf area measurements were further limited to only two blocks. Seeds were sown at a density of 83,000 plants ha<sup>-1</sup> to achieve a final stand density of 79,000 plant ha<sup>-1</sup>. For those plots from which leaf measurements were taken, plant density measurements at V6 rarely differed from target plant densities by more than 3% (data are not shown).

At both locations, seven consecutive, representative plants were tagged and removed (cutting at the stem base) at R1 from row three of each designated plot. In each plot, these plants were at least 2 m from the plot border (intrarow distance). As previously described, L and W were measured for all green leaves on each of these plants, and LA<sub>n</sub> was calculated by multiplying L × W × 0.75. The position of each green leaf was set relative to  $x_a$  for all plants.

Predicting the area of individual green maize leaves first involved calculating an average, plot-level value for LA<sub>o</sub> for plants one through three. Using Eq. [A2], these plot-level LA<sub>o</sub> values, and SAS PROC NLIN (starting values for b = 0.03, 0.05), a single LA<sub>D</sub> curve (i.e., *b* coefficient) was generated for each plot based on the individual leaf measurements of only these first three plants. For each plot, the *b* coefficient was significant at the 0.01 probability level. Using Eq. [A2] and appropriate model coefficients from the first three plants, values for LÂ<sub>n</sub> were calculated by plant for plants four through seven of each plot. Using SAS PROC REG (SAS Institute, 2004), LÂ<sub>n</sub> was regressed on LA<sub>n</sub> both by and across location(s) using plants four through seven from all plots. The linear relationship between LÂ<sub>n</sub> and LA<sub>n</sub> was highly significant both by



Observed total green leaf area (cm<sup>2</sup>)



(ACRE:  $R^2 = 0.88$ ,  $P \le 0.01$ ; PPAC:  $R^2 = 0.89$ ,  $P \le 0.01$ ) and across [Fig. A1(A)] location(s). Across locations, the slope was slightly less than one (P = 0.02), indicating that the use of Eq. [A2] and the techniques employed may have slightly underestimated the LA<sub>n</sub> value of green leaves. Still, across-location values for the coefficient of determination and slope [Fig. A1(A)] were only slightly lower and higher, respectively, than those reported by Valentinuz and Tollenaar (2006) in their comparison of observed individual leaf area values with estimated individual leaf area values (determined using Eq. [A1]) for all initiated leaves.

For plants four to seven of each plot, the parameters  $L\hat{A}_T$ and LA<sub>T</sub> were calculated as the sum of each plants LÂ<sub>n</sub> and  $LA_n$  values, respectively, and  $LA_T$  was estimated using Eq. [A6]. In this case,  $L\dot{A}_{\rho}$  represents the area of the largest leaf of a single plant (from plants four through seven) and  $L\hat{A}_{T}$  and  $LA_{a}$ represent the average total predicted leaf area and average area of the largest leaf of destructively sampled plants one through three from that same plot, respectively. Using SAS PROC REG,  $L\dot{A}_T$  was regressed on  $LA_T$  both by and across location(s) using plants four through seven from all plots. The linear relationship between  $L\dot{A}_T$  and  $LA_T$  was highly significant both by (ACRE:  $R^2 = 0.81$ ,  $P \le 0.01$ ; PPAC:  $R^2 = 0.84$ ,  $P \le 0.01$ ) and across [Fig. A1(B)] location(s). Across locations, the slope was not different from one (P = 0.10), indicating that Eq. [A6] and the techniques employed likely accurately estimated LA<sub>T</sub> values. Overall, given the various demands and restrictions of this experiment, this technique proved to be a relatively accurate, rapid, low-cost technique for nondestructively determining the green LA<sub>p</sub> and green LA<sub>D</sub> of a large number of maize plants at silking.

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