



# A comprehensive study of plant density consequences on nitrogen uptake dynamics of maize plants from vegetative to reproductive stages

Ignacio A. Ciampitti, Tony J. Vyn\*

Agronomy Department, Purdue University, 915 W State Street, West Lafayette, IN 47907-2054, United States

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## ABSTRACT

Nitrogen (N) use efficiency (NUE), defined as grain produced per unit of fertilizer N applied, is difficult to predict for specific maize (*Zea mays* L.) genotypes and environments because of possible significant interactions between different management practices (e.g., plant density and N fertilization rate or timing). The main research objective of this study was to utilize a quantitative framework to better understand the physiological mechanisms that govern N dynamics in maize plants at varying plant densities and N rates. Paired near-isogenic hybrids [i.e., with/without transgenic corn rootworm (*Diabrotica* sp.) resistance] were grown at two locations to investigate the individual and interacting effects of plant density (low–54,000; medium–79,000; and high–104,000 pl ha<sup>-1</sup>) and sidedress N fertilization rate (low–0; medium–165; and high–330 kg N ha<sup>-1</sup>) on maize NUE and associated physiological responses. Total aboveground biomass (per unit area basis) was fractionated and both dry matter and N uptake were measured at four developmental stages (V14, R1, R3 and R6). Both plant density and N rate affected growth parameters and grain yield in this study, but hybrid effects were negligible. As expected, total aboveground biomass and N content were highly correlated at the V14 stage. However, biomass gain was not the only factor driving vegetative N uptake, for although N-fertilized maize exhibited higher shoot N concentrations than N-unfertilized maize, the former and latter had similar total aboveground biomass at V14. At the R1 stage, both plant density and N rate strongly impacted the ratio of total aboveground N content to green leaf area index (LAI), with the ratio declining with increases in plant density and decreases in N rate. Higher plant densities substantially increased pre-silking N uptake, but had relatively minor impact on post-silking N uptake for hybrids at both locations. Treatment differences for grain yield were more strongly associated with differences in R6 total biomass than in harvest index (HI) (for which values never exceeded 0.54). Total aboveground biomass accumulated between R1 and R6 rose with increasing plant density and N rate, a phenomenon that was positively associated with greater crop growth rate (CGR) and nitrogen uptake rate (NUR) during the critical period bracketing silking. Average NUE was similar at both locations. Higher plant densities increased NUE for both medium and high N rates, but only when plant density positively influenced both the N recovery efficiency (NRE) and N internal efficiency (NIE) of maize plants. Thus plant density-driven increases in N uptake by shoot and/or ear components were not enough, by themselves, to increase NUE.

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## 1. Introduction

Nitrogen use efficiency (NUE) in maize (*Zea mays* L.) is often defined as grain produced per unit of fertilizer N applied, and the NUE concept commonly provides a quantitative measure of the effectiveness of plants to take up and convert available N into grain yield within a cropping system. Improved maize NUE needs to be attained to (i) lessen negative environmental impacts associated

with N fertilization (e.g., N losses to surface and ground water) and (ii) to reduce N fertilizer input costs per unit of grain yield produced (Cassman et al., 2003). To achieve a better understanding of the effects of different management practices (e.g., hybrids, plant densities, and N rates) on system-level NUE, it is beneficial to examine the main components of NUE independently (Salvagiotti et al., 2009). Two important components of NUE are (i) nitrogen recovery efficiency (NRE), which reflects the ability of aboveground plant parts to recover N from the fertilizer N applied, and (ii) nitrogen internal efficiency (NIE), or the capability of plants to transform the N taken up by the crop into grain N (Moll et al., 1982; Coque and Gallais, 2007). Individual evaluation of these NUE components is useful to advance understanding of the physiological mechanisms and processes (such as N uptake, assimilation, translocation, and

\* Corresponding author at: Department of Agronomy, Purdue University, Lilly Hall of Life Sciences, 915 W. State St., West Lafayette, IN 47907, United States.  
Tel.: +1 765 496 2221; fax: +1 765 496 2221.

E-mail address: [tvyn@purdue.edu](mailto:tvyn@purdue.edu) (T.J. Vyn).

remobilization) of the N cycle within the plant that consequently affect final grain NUE (Moll et al., 1982). To achieve high grain NUE, some authors have observed that the NRE component was more important under high N supply environments; whereas the NIE component was more essential in low N availability environments (Moll et al., 1982; Ma et al., 1998).

Although the association between N uptake and crop N demand has been acknowledged, the effective N uptake potential seems to rely on the level of soluble carbohydrates supplied to the root system; under this assumption, N uptake is dependent on the uninterrupted carbohydrate mobilization from shoot to root (Tolley-Henry et al., 1988; Tolley-Henry and Raper, 1991; Rajcan and Tollenaar, 1999). During the post-silking period, the continuation of root N uptake is a critical factor in minimizing the requirement for N remobilization from vegetative to reproductive organs, thus retaining green leaf area, and concomitantly prolonging dry matter accumulation (Rajcan and Tollenaar, 1999). Continued root N uptake during grain filling should, therefore, increase canopy photosynthesis duration and final grain yield.

Nitrogen deficiency in maize is often visually apparent via reductions in leaf area, leaf chlorophyll status (especially as leaves age), and vegetative biomass. Such phenomena decrease plant light interception, photo-assimilate production, and final grain yield (Uhart and Andrade, 1995; Paponov and Engels, 2003; Monneveux et al., 2005; Echarte et al., 2008). At low N supply, dry matter allocation to reproductive structures declines, and this results in lower maize grain yield (and its components—kernel number and weight) as well as smaller harvest index (Uhart and Andrade, 1995; Below et al., 2000; O'Neill et al., 2004; Ding et al., 2005; Monneveux et al., 2005). Despite these nearly universal symptoms, there can be substantial differences among maize hybrids in their tolerance to low N, and recent results have confirmed genotypic variation in maize kernel set resulting from varying N fertilizer rates (D'Andrea et al., 2008).

When sufficient N is available to maize, extended periods of post-silking dry matter and N accumulation have been associated with higher grain yields (Swank et al., 1982; Moll et al., 1994). One characteristic response pattern is that increased N availability promotes proportionately greater absolute yield responses with high yielding hybrids (Moll et al., 1987; Osaki, 1995; Boomsma et al., 2009) than with low yielding hybrids (Anderson et al., 1985; Osaki, 1995).

Maize grain yield per unit area responds to plant density in a curvilinear fashion, with maximum yield occurring at the optimum plant density for a specific genotype and environment (Tokatlidis and Koutroubas, 2004). As plant densities decline, reduction in the number of plants per unit area is partially compensated by an accompanying increase in the productivity of each plant. Consequently, some increase in the final grain yield per unit area can be expected relative to the individual plant yields at optimum densities. However, at supra-optimum plant densities, reductions in per-plant crop growth rate, N uptake rate, and partitioning of photosynthetic products to the ear, and grain components, as well as increases in plant-to-plant variability (sometimes reflected via more barrenness) lead to grain yield reductions relative to those at optimum plant density (Andrade et al., 1999; Echarte et al., 2008; Tokatlidis and Koutroubas, 2004; Borrás et al., 2007; Boomsma et al., 2009).

Genetic selection has resulted in wide-ranging genotypic variation for NUE (Balko and Russell, 1980; Paponov and Engels, 2003; Paponov et al., 2005; Uribealarea et al., 2007, 2009). There appears to be considerable scope for further improvement in maize NUE and N stress tolerance for both temperate hybrids (Balko and Russell, 1980) and tropical maize cultivars (Bänziger et al., 1997; Lafitte and Edmeades, 1994; Muruli and Paulsen, 1981). Substantial genetic variability for adaptation to low and high soil N supply was also

observed in other research (Paponov and Engels, 2003; Paponov et al., 2005; Uribealarea et al., 2007, 2009). Both NUE itself, and N stress tolerance in general, seems to have improved with recently developed maize hybrids compared to commercial hybrids of earlier decades (e.g., Ma and Dwyer, 1998; Ding et al., 2005; Coque and Gallais, 2007). Nevertheless, when modern hybrids are grown at low N supply, grain yield losses are still considerable (O'Neill et al., 2004; Massignam et al., 2009). Furthermore, selection of hybrids with the combination of high NUE and stress tolerance to low N is even more challenging when maize plant densities are above optimum (Boomsma et al., 2009).

There are few studies to date that have intensively examined the simultaneous effects of differences in plant crowding and fertilizer N availability for multiple hybrids and locations. Enhanced knowledge of physiological relationships can be useful for developing maize management systems and hybrids that enhance system-level NUE. We will address the following questions in this manuscript: (i) Does shoot growth potential or leaf area expansion drive N uptake during vegetative stages? (ii) Does the proportionality of the ratio between N uptake and LAI remain constant when both total N uptake and LAI are themselves affected by plant density and N rate? (iii) Is there any relationship between the crop growth rate and the N uptake rate during the period bracketing silking and, does this association change with different N rates, plant densities and hybrid factors? (iv) Do plant density and N rate management practices most strongly impact aboveground biomass (BM) productivity and total N uptake, or the partitioning of dry matter and N to the grain?

Few reports in the literature have considered maize N uptake in alternate management systems using a dynamic perspective that integrates N uptake with the shifting internal N demands during vegetative stages (e.g. developing leaves and stems) versus reproductive stages (as ears and grain develop, but remain dependent on plant vegetative structures for requisite resources). The main objective of this study was to understand the physiological mechanisms that govern the N dynamics in maize plants by representing crop N dynamics as a quantitative framework of underlying component processes that fluctuate in response to plant density and N rate, thereby giving us greater ability to decipher genotype  $\times$  environment interactions (van Oosterom et al., 2010). A secondary objective was to evaluate the influence of varying plant densities (low, medium, and high densities) and N rates (three levels of side-dress N fertilization) on plant N response and grain NUE in maize hybrids with or without corn rootworm (CRW) tolerance. Advances in the knowledge of the physiological mechanisms that govern maize N dynamics will allow us to incorporate this information into simulation models (Hammer et al., 2005; Chenu et al., 2008; van Oosterom et al., 2010).

## 2. Materials and methods

### 2.1. Management practices, experimental arrangement, and treatments

During the 2009 growing season, a research study was conducted in two locations: the Purdue University Agronomy Center for Research and Education (ACRE) (40°28'07"N, 87°00'25"W) near West Lafayette, Indiana and the Pinney-Purdue Agricultural Center (PPAC) (41°26'41"N, 86°56'41"W) near Wanatah, Indiana. PPAC and ACRE have very similar average annual precipitations of 944 mm and 951 mm, respectively. Both seasonal mean minimum air temperature ( $T_{\text{air}}$ ) and mean maximum  $T_{\text{air}}$  for PPAC are 0.8 °C colder than corresponding air temperatures at ACRE site. The soil at the ACRE location was a Chalmers (fine-silty, mixed, mesic Typic Endoaquoll) silty clay loam with approximately 4.0–4.5% organic

**Table 1**

Soil analysis values at V3 maize phenological stage for non-fertilized plots [inorganic nitrogen (Nitrate-N), organic matter content (OM), soil pH, potassium content (K) and phosphorous Bray-P 1 (P)] in the uppermost 0.3 m of the soil profile, and phenological data [dates of planting, fourteen leaves stage (V14), silking (R1), milk stage (R3) and physiological maturity (R6)] and harvest date, averaged across hybrids at two locations. The value with the (+) symbol refers to the standard error of each parameter.

	Location	
	PPAC	ACRE
Soil analysis values at V3 0–0.3 m		
Nitrate-N (mg N kg <sup>-1</sup> )	13.8+0.2	18.0+0.3
OM (g 100 g <sup>-1</sup> )	2.7+0.1	4.7+0.2
pH units	5.5+0.1	6.0+0.1
K (mg N kg <sup>-1</sup> )	68.4+0.2	117.9+0.2
P (mg N kg <sup>-1</sup> )	31.2+0.3	18.9+0.4
Phenological and climatic data		
Planting date	May 7	May 14
V14 stage	July 18	July 20
Silking date (R1)	August 2	July 31
Milk stage (R3)	August 13	August 14
Physiological maturity (R6)	October 4	October 1
Harvesting date	November 19	November 15

matter content in the topsoil layer (0–30 cm soil depth) (Table 1). The soil at the other experimental site, PPAC, 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 (Table 1). Maize field experiments were established following soybean (*Glycine max* (L.) Merr.) at both locations, and conventional tillage practices at these sites involved full-width spring field cultivation before planting. Precision planting was accomplished via automatic guidance (John Deere StarFire Real-Time Kinematic) and a six-row John Deere 1770 or 1780 planter (Deere & Company, Moline, IL). At both locations, residual pre- and post-emergence herbicides achieved essentially complete weed control.

At both locations, the experimental design was a split-split plot with six blocks. The three treatment factors were hybrid (main plot), plant density (subplot), and N application rate (sub-subplot). Each sub-subplot comprised six rows (76-cm interrow spacing) and was approximately 4.5 m in width and 18.3 m in length (except for blocks 1–3 which had an extra 9.1 m in length to permit biomass sampling). Two near-isogenic hybrids with and without transgenic CRW resistance were planted in each location: at ACRE Mycogen 2T780 (without CRW resistance) and its near-isoline Mycogen 2T787 (with CRW resistance) were compared, while at the PPAC location the hybrid treatment comparison involved Mycogen 2M749 (non-CRW resistant) and its near-isoline 2M750 (with CRW resistance). All four hybrids were similar in Comparative Relative Maturity (CRM) at 114 days (Dow AgroSciences, Inc., Indianapolis, IN). At planting time, only the non-rootworm resistant hybrids were treated with the insecticide Force 3G Tefluthrin (2,3,5,6-tetrafluoro-4-methylphenyl)methyl-(1 $\alpha$ ,3 $\alpha$ )-(Z)-(±)-3-(2-chloro-3,3,3-trifluoro-1-propenyl)-2,2-dimethylcyclopropanecarboxylate, Syngenta Crop Protection, Inc., Greensboro, NC. Rootworm feeding injury symptoms on the nodal roots were systematically assessed at the R1 stage but CRW damage was either not present or very minimal in all treatment combinations (data not presented). No further insecticide or fungicide treatments were warranted or applied between planting and harvest. Final plant densities averaged 54,000, 79,000, and 104,000 pl ha<sup>-1</sup>, respectively for low, medium and high plant density sub-treatments. Plant density deviations among individual plots at the six-leaf stage (V6) (Ritchie et al., 1996) were generally <2% from the intended means (data are not shown). At planting time, all treatments received 25 kg N ha<sup>-1</sup> (10-34-0) as starter fertilizer, 5 cm below and to the side of the seed. Three side-dress N rates, namely the 0 (0N), 165 (165N), and 330 (330N) kg ha<sup>-1</sup> N

rates, were applied to investigate the physiological responses of maize hybrids at alternate densities to different N availabilities. The N fertilizer source for side-dress application was urea ammonium nitrate (UAN) (28-0-0). For the 165 kg N ha<sup>-1</sup> rate, the entire UAN application was made at the V3 stage, while for the 330 kg N ha<sup>-1</sup> rate, half the UAN was applied at V3 and the remainder at the V5 stage.

At each location, five soil cores (2-cm diameter) were taken at V3 in the sub-subplots with zero side-dress N. Soil samples were collected from the 0 to 30 cm depth near the central two rows (on each side of rows 3 and 4, and at least 25 cm away from each row). All samples were sent to A&L Great Lakes Laboratories, Inc. (Fort Wayne, IN) for determination of soil nitrate (NO<sub>3</sub><sup>-</sup>-N) concentrations via NO<sub>3</sub><sup>-</sup> reduction and ammonium (NH<sub>4</sub><sup>+</sup>-N) concentrations via the phenolate method following subsample extraction with 1 N KCL (A&L Great Lakes Laboratories, Inc., personal communication, 2010). The soil NO<sub>3</sub><sup>-</sup>-N levels for sub-subplots receiving only starter fertilizer ranged between 3 and 18 ppm—values generally below the average critical NO<sub>3</sub><sup>-</sup>-N levels for maize (Brouder and Mengel, 2003).

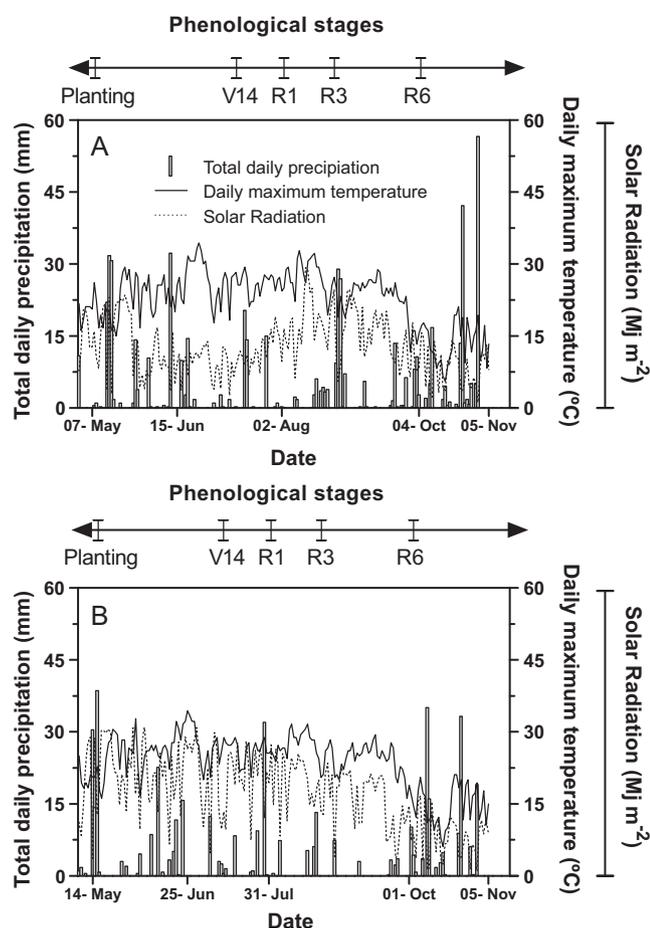
Nondestructive per-plant sampling areas (totaling 30 plants per plot in either row central two rows) were identified near the center of each six-row plot. Individual plants were tagged in these sub-subplot sampling areas to permit sequential sampling on the same plants. Orange paint was applied to the tip of each plant's fourth leaf shortly after V4 (Ritchie et al., 1996) to facilitate accurate phenology determination on approximately 1500 tagged plants at each location.

## 2.2. Weather measurements and crop phenology

At ACRE and at PPAC locations from 1 May to 1 November, precipitation, minimum and maximum  $T_{\text{air}}$  were recorded on a daily basis to study the effects of these variables during maize growing season. Fig. 1 shows the evolution of daily maximum  $T_{\text{air}}$ , precipitation and solar radiation for each location and the approximately corresponding phenological stages of V14, R1, R3 and R6 (Ritchie et al., 1996). Dates of seedling emergence (when 50% of the plants in a sub-subplot were visible above the soil surface), silking (when 50% of the plants in a sub-subplot presented visible silks), and physiological maturity (when 50% of the plants in a sub-subplot showed black layer formation in the grain from the mid-portion of the ears), were recorded for the tagged plants from each sub-subplot's per-plant sampling area (Table 1).

## 2.3. Aboveground biomass measurements

Total aboveground BM was collected and fractionated for the determination of dry matter (DM) and N uptake at V14, R1, R3, and R6 stages (Ritchie et al., 1996) from three of the six blocks at each location. Aboveground BM was determined from six consecutive plants with a treatment-representative plant density from either row three or four of each sub-subplot's per-plant sampling area. These six plants were cut at the stem base, separated into fractions, chopped, and dried to a constant weight at 60 °C. Prior to chopping, maize plants were separated into leaves plus stems at V14 (leaf sheaths were included in the stem fraction), and into vegetative BM (leaves, stem, and tassel) versus ear BM (husk, cob and kernels) at R1 and R3 stages, and into vegetative BM (now also including the husk) versus grain BM and cob BM fractions at R6 stage. The ear shanks were included with ear BM at R1, but not at R3 and R6 (when they were included with "vegetative" BM). The N content in each fraction was determined using the combustion method (AOAC International, 2000). The N content of each fraction was calculated as the product of that fraction's N concentration and biomass.



**Fig. 1.** Total daily precipitation, daily maximum air temperature, and solar radiation for (A) at the Pinney-Purdue Agronomy Center (PPAC) and (B) at the Purdue University Agronomy Center for Research and Education (ACRE). Timing of planting and the phenological stages V14, R1, R3 and R6 (Ritchie et al., 1996) are indicated for each location during the 2009 growing season.

At the end of the growing season, maize yield was measured with a small plot combine from an 18.3 m length of rows three and four (central two rows) of each sub-subplot.

At R6, all ears from the intensive per-plant sampling areas were hand-harvested and separately tagged and bagged. An electric sheller separated grain from cobs for each individual ear; the resulting grain samples were weighed to determine the individual plant grain yield (corrected to 0% moisture content after grain moisture was recorded). Kernel number per plant was determined for all tagged plants. For each ear, kernel weight was calculated as the quotient of that ear's grain weight and kernel number. The harvest index (HI) of each sub-subplot was calculated as the ratio of the grain weight (0% moisture) to the total aboveground plant BM.

#### 2.4. Nitrogen indices

For each sub-subplot receiving side-dress N application at both locations, NUE was calculated as the ratio of incremental grain yield response (N fertilized–unfertilized) to the N fertilizer applied (Cassman et al., 2003). The following equation was used for the NUE calculation:

$$\text{NUE} = \frac{\text{GY}_{\text{fert.}} - \text{GY}_{\text{unfert.}}}{\Delta \text{N applied}} \quad (1)$$

where  $\text{GY}_{\text{fert.}}$  is the per-unit-area grain yield ( $\text{kg ha}^{-1}$  at 15.5% moisture) of a treatment receiving either 165 or 330  $\text{kg N ha}^{-1}$ , and  $\text{GY}_{\text{unfert.}}$  is the per-unit-area grain yield of the ON treatment. For

this research,  $\text{GY}_{\text{fert.}} - \text{GY}_{\text{unfert.}}$  between 0 and 165  $\text{kg N ha}^{-1}$  and 0 and 330  $\text{kg N ha}^{-1}$  application rates were used as the N response measurements for the different N treatments.

Following the same reasoning, the N recovery efficiency (NRE) was calculated as

$$\text{NRE} = \frac{\text{Nupt}_{\text{fert.}} - \text{Nupt}_{\text{unfert.}}}{\Delta \text{N applied}} \quad (2)$$

where  $\text{Nupt}_{\text{fert.}}$  is N uptake in the fertilized plot (either 165 or 330  $\text{kg N ha}^{-1}$ ) and  $\text{Nupt}_{\text{unfert.}}$  is N uptake in the corresponding unfertilized sub-subplots (no side-dress N). The N internal efficiency was calculated as

$$\text{NIE} = \frac{\text{GY}_{\text{fert.}} - \text{GY}_{\text{unfert.}}}{\text{Nupt}_{\text{fert.}} - \text{Nupt}_{\text{unfert.}}} \quad (3)$$

At physiological maturity (PM), Nitrogen harvest index (NHI, %) was calculated as

$$\text{NHI} = \frac{\text{Grain N}}{(\text{Shoot N} + \text{Grain N})} \times 100 \quad (4)$$

where the shoot N fraction includes stem, leaf, cob, shank and husk components, and the grain N fraction is only composed of the grain component.

#### 2.5. Leaf area measurements

Leaf area was estimated at the R1 stage following destructive evaluations of individual leaf areas from representative plants of each hybrid. At both locations, three consecutive, representative plants were harvested at R1 stage from each sub-subplot. A traditional LAI meter (Model LI-3100, Li-Cor, Inc., Lincoln, NE) was used to estimate the total green leaf area of these plants along with the leaf areas of their respective individual leaves. In brief, this technique involved feeding individual leaves through the machine, allowing the machine to calculate the leaf area for each individual leaf, recording the individual position of these leaves, and summing individual leaf areas to determine per-plant total leaf area. From these total and individual leaf area values, a relationship was established between the largest leaf and the total leaf area using all destructively-sampled plants (data not shown). Using this relationship, and by knowing the position of the largest individual leaf area within the plant, we were able to estimate the total plant leaf area for all plants in this study's non-destructive per-plant sampling areas. This was done by measuring leaf length ( $L$ ) and maximum leaf width ( $W$ ) for the largest leaf on the individual tagged plants, and then calculating leaf area using  $L \times W \times 0.75$  (Montgomery, 1911). The previously established relationship between total plant leaf area and the area of the largest leaf was then used to determine the total leaf area of non-destructively sampled plants.

The determination of leaf area index (LAI) in the conventional per unit area basis utilized the relationship established between the length and width of the largest leaves from each tagged plant and the total leaf area observed from the harvested plants at R1, and divided this cumulative leaf area value by the respective soil surface area for designated plants in each sub-subplot.

The number of green leaves remaining per plant was measured five times from the onset of silk emergence (and at 2-week intervals thereafter). From the knowledge of the relationship between total leaf area index at R1 and the relative proportional contribution of each leaf position to the total plant leaf area, we were able to estimate the loss of total leaf area during the grain filling period.

#### 2.6. Statistical analyses

The final ANOVA was executed using SAS PROC MIXED (SAS Institute, 2004). Least-squares mean tests were performed for fixed effects only when treatment effects were significant at  $P=0.05$ . The

relationship between total aboveground N content and N concentration and biomass was fitted for the observed data points to test whether the relationship was linear and the intercept not significantly different from zero. Finally, a similar procedure was followed for testing the relationship between CGR and NUR.

For each location at R1, a response surface analysis for the ratio of the total aboveground N content ( $\text{g m}^{-2}$ ) and the green LAI (Fig. 4A and B) was performed with the R program software (R Development Core Team, 2009). Moreover, the same procedure was followed for the ratios of grain, stover biomass ( $\text{g m}^{-2}$ ) and the total aboveground N content ( $\text{g m}^{-2}$ ) (Fig. 8A and B). The rsm package for R was used for the information coding, design and fitting the response-surface model. From the analysis of variance in both locations, it was clear that the second-order terms contributed significantly to the model, so the canonical analysis was relevant (data not shown). Moreover, the stationary point was close to the experimental region, but the eigen values were of mixed sign, indicating that it was a saddle point (i.e. neither a maximum nor minimum).

### 3. Results

#### 3.1. Phenology

The mean timing of reproductive stages for maize varied (Table 1) among experiments as expected due to the combined effects of location differences in air temperatures (Fig. 1) and hybrid pair differences between locations. Average silk emergence was delayed by seven days in the lowest N treatment, relative to the same hybrid and plant density treatments with N fertilizer applied, at both locations (data not shown).

#### 3.2. Dynamics of leaf area development

Individual leaf area measurement data from the 165N treatment was used to examine genotypic effects on leaf area accumulation associated with differences in leaf length and width of the largest leaves of maize plants in each density treatment. The position of the largest leaf was associated with leaf number 14 at both intermediate and low densities (Supplementary Fig. 1). For the high plant density treatment, a greater variability was observed but the largest leaf size was associated with either leaf number 13 or 14 depending on the hybrid (Supplementary Fig. 1). Under medium N, low and intermediate plant density treatments resulted in larger leaves than the high plant density treatment, regardless of the hybrid and location factors (Supplementary Fig. 1). However, due to the number of plants per unit area, overall LAI was greater at the highest density than that at the low and medium plant densities (Supplementary Fig. 2), a difference that was particularly noticeable at the R1 stage (LAI values from 4.5 to 5.5). In treatments with side-dress N applications, the LAI remained above 4.0 units until 30–40 days after silk emergence. The overall LAI was lower at PPAC than at ACRE, and this LAI reduction might have been related to the individual or combined effects of vegetative-period air temperatures, hybrid differences and post-silking drought stress at PPAC (Supplementary Fig. 2). Moreover, in general terms, the decline in LAI after flowering was affected more by the plant density factor than by N rates, and was hybrid-independent at both locations.

#### 3.3. Grain yield and yield components

Grain yield per plant was greater at low plant density ( $54,000 \text{ pl ha}^{-1}$ ) for all hybrids, N rates and locations (Table 2). Increased stand density was accompanied by a reduction of per plant biomass (BM) and grain yield (Table 2). The 3-way interaction effects of hybrid  $\times$  plant density  $\times$  N rate were significant ( $P < 0.05$ ) for maize grain yield at both locations (Table 2). However, both

plant density and N rate factors exerted a large influence on maize grain yield. In all treatment combinations, only one ear per plant was present and almost no barren plants were detected (data not shown). At the PPAC location, the effect of the reduction of N supply (0N) was manifested in a reduction in both kernel number (KN) and weight (KW) per plant ( $\sim 10\%$  of KN and KW reduction for 0N vs. 165–330N). The plant density factor also significantly affected ( $P < 0.05$ ) the KN and KW ( $\sim 12\%$  reduction in mean KN and  $\sim 9\%$  reduction of KW reduction for high vs. medium plant density;  $\sim 29\%$  reduction in KN and  $\sim 3\%$  reduction in KW reduction for high vs. low plant density). At the ACRE site, the 3-way interaction was significant ( $P < 0.05$ ) for KN; while for the KW, only the individual plant density and N rates effects were significant ( $P < 0.05$ ; Table 2). Mean KW was approximately 14% lower with 0N than with 165–330N treatments (Table 2). Increased stand density was also accompanied by a reduction of mean cob weight (CW) at PPAC (Table 2). Cob weights were about 43% lower for high vs. low plant density, and about 21% lower for high vs. medium plant density at PPAC. Similarly, the 0N treatment resulted in  $\sim 17\%$  reduction in CW vs. 165–330N treatments (Table 2). At ACRE location, only plant density treatments, and not the N rate treatments, significantly affected CW. Increased stand density was accompanied by a reduction of mean CW values ( $\sim 5\%$  of CW reduction for high vs. medium plant density;  $\sim 29\%$  of CW reduction for high vs. low plant density).

#### 3.4. Plant biomass accumulation patterns and partitioning

Biomass accumulation patterns demonstrated significant 3-way interactions ( $P < 0.05$ ) at both locations for all plant dry matter fractions except for, at ACRE location, ear BM at R1 and shoot BM at R3 (Table 3). Moreover, ear BM at R1 was significantly affected by the 2-way hybrid  $\times$  density, and hybrid  $\times$  N rate interactions ( $P < 0.0001$ ); while the shoot BM at the R3 stage was significantly affected by the 2-way hybrid  $\times$  N rate interaction and the single effect of plant density ( $P < 0.0001$ ) (Table 3). Differences in BM among N rate and plant density treatments became apparent around 15 days before silking (first sampling time), after the onset of stem elongation (Fig. 3).

Hybrid differences in BM accumulation response to density and N rate factors were relatively small and temporal at both locations (Table 3). At PPAC location, Mycogen 2M750 (RW resistant) resulted in higher BM than Mycogen 2M749 at V14 and R1 stages ( $\sim 4\%$  and  $2\%$  for each stage, respectively), but in similar BM levels at R3 and R6 stages (Table 3). From seedling emergence to V14 stage, total shoot BM was around  $8\text{--}11 \text{ g m}^{-2} \text{ d}^{-1}$ ; the total gain between phenological stages was approximately  $27 \text{ g m}^{-2} \text{ d}^{-1}$  from V14 to R1,  $27$  to  $32 \text{ g m}^{-2} \text{ d}^{-1}$  from R1 to R3, and  $8$  to  $9 \text{ g m}^{-2} \text{ d}^{-1}$  from R3 to R6 developmental stages. Ear and shoot BM fractions at the R6 stage varied with plant density (Table 3).

At the ACRE location, Mycogen 2T787 resulted in higher total aboveground BM than Mycogen 2T780 in all sampling times ( $\sim 4\%$  overall gain in BM for 2T787 vs. 2T780, but as much as  $10\%$  at R1; Table 3). Both intermediate and high N rates promoted substantially higher shoot BM ( $\sim 23\%$ ) compared to 0N situation at all measurement times. The ear and shoot BM fractions at R6 stage increased as plant density increased (Table 3). From seedling emergence to V14 stage, rate of gain in total shoot BM ranged from  $9$  to  $13 \text{ g m}^{-2} \text{ d}^{-1}$  and gain rates between the phenological stages averaged approximately  $18\text{--}27 \text{ g m}^{-2} \text{ d}^{-1}$  from V14 to R1,  $28$  to  $32 \text{ g m}^{-2} \text{ d}^{-1}$  from R1 to R3, and  $8\text{--}10 \text{ g m}^{-2} \text{ d}^{-1}$  from R3 to R6 stages (Table 3).

The leaf/stem BM ratio at V14 was generally not affected by the different hybrid, plant density and N rate factors at either location (Table 3). However, differences were apparent between location and hybrid pair combinations since PPAC's hybrids resulted in a leaf/stem BM ratio of 0.29 (ranging from 0.23 to 0.34) while the other pair of hybrids at ACRE had a mean ratio of 0.32 (ranging from 0.29 to 0.38). Although the 2T780 and 2T787 hybrids at the

**Table 2**

Maize yields (g per plant<sup>-1</sup>), kernel number (number ear<sup>-1</sup>), 1000-kernel weight (g 1000 kernels<sup>-1</sup>) and cob weight (g ear<sup>-1</sup>) for the individual plant measurements for maize hybrids 2M749, 2M750, 2T780 and 2T787 at three different populations (54,000; 79,000 and 104,000 pl ha<sup>-1</sup>) and three N rates (0, 165 and 330 kg ha<sup>-1</sup>) at PPAC and ACRE locations in 2009 growing season. Only significant treatment or interaction effects resulting from the ANOVA analysis are presented. The standard error (SE) relates only to the comparisons between significant terms.

Treatments	PPAC location						ACRE location						
	Hybrid 2M749			Hybrid 2M750			Hybrid 2T780			Hybrid 2T787			
	PD1	PD2	PD3	PD1	PD2	PD3	PD1	PD2	PD3	PD1	PD2	PD3	
Maize yields (g per plant <sup>-1</sup> )													
ON	115.2	108.1	76.8	138.1	98.4	87.4	ON	107.6	80.8	72.6	105.7	93.8	68.5
165N	138.1	124.2	102.3	159.3	126.6	101.7	165N	145.7	122.8	100.1	147.4	108.2	104.5
330N	142.6	145.9	105.8	153.3	132.2	117.5	330N	143.9	122.0	105.9	158.0	109.9	104.2
<sup>†</sup> H × PD × N	#0.044						PD × N						
SE	11.9						#0.019						
Kernel number (number ear <sup>-1</sup> )													
ON	644	598	438	612	481	405	ON	622	456	355	545	484	410
165N	684	554	527	661	548	556	165N	687	566	459	700	561	461
330N	801	532	499	654	576	475	330N	643	597	499	678	563	467
PD/N	<0.0001/0.025						H × PD × N						
SE	21.2						SE						
1000-Kernel weight (g 1000 kernels <sup>-1</sup> )													
ON	179	181	175	226	204	216	ON	173	177	204	194	194	167
165N	202	224	194	241	231	183	165N	212	217	218	211	193	227
330N	178	274	212	234	229	247	330N	224	204	212	233	195	223
PD/N	<0.0001/<0.0001						PD × N						
SE	8.5						SE						
Cob weight (g ear <sup>-1</sup> )													
ON	25.2	16.1	15.2	23.0	16.8	12.5	ON	21.2	12.5	14.5	17.6	14.9	15.0
165N	24.8	23.9	13.9	31.1	18.9	11.8	165N	23.8	17.6	18.0	25.5	22.5	14.5
330N	26.3	22.3	25.8	31.2	18.5	13.0	330N	23.6	17.0	18.1	24.0	17.3	16.7
PD/N	£0.0001/<0.0001						PD						
SE	0.7						SE						

# P value for significant term (P < 0.05).

† Significant terms in the information ANOVA analysis for each location.

£ Significant considering a P-value < 0.01.

**Table 3**

Aboveground biomass (g m<sup>-2</sup>) for different fractions (leaves, stem and ear fractions) at V14, R1, R3 and R6 stages for maize hybrids 2M749, 2M750, 2T780 and 2T787 at three different populations (PD1 = 54,000; PD2 = 79,000 and PD3 = 104,000 pl ha<sup>-1</sup>) and three N rates (0, 165 and 330 kg ha<sup>-1</sup>) at PPAC and ACRE locations in 2009 growing season. Only significant treatment or interaction effects resulting from the ANOVA analysis are presented. The standard error (SE) relates only to comparisons between significant terms. At V14 stage, plants were fractionated between stem and leaf biomass (i.e. shoot biomass/leaf proportion), and from silking onwards plants were divided between shoot (stem + leaves) and ear biomass and thus presented as shoot/ear biomass fractions. At R6, shoot biomass fraction includes the cob biomass, while the “ear biomass” is only the grain fraction.

Hybrid	Treatments											
	V14 stage			Silking stage (R1)			Milk stage (R3)			Physiological maturity (R6)		
	PD1	PD2	PD3	PD1	PD2	PD3	PD1	PD2	PD3	PD1	PD2	PD3
PPAC location												
2M749 Aboveground biomass (g m <sup>-2</sup> )												
ON	564/0.29	540/0.33	654/0.23	717/181	949/165	803/270	764/317	998/467	938/512	763/622	953/854	1037/799
165N	613/0.31	651/0.27	784/0.25	734/174	949/189	976/308	836/514	1007/391	1047/603	830/746	906/981	1193/1064
330N	628/0.29	617/0.30	776/0.31	687/155	990/164	1102/246	869/520	1036/484	1068/621	854/770	1043/1153	1125/1100
2M750												
ON	545/0.28	597/0.29	789/0.33	749/148	766/210	873/263	880/414	972/371	1031/428	777/746	866/777	1072/909
165N	581/0.29	743/0.34	724/0.29	796/209	821/306	833/349	901/460	1110/440	1034/480	776/860	1003/1000	1008/1058
330N	631/0.29	728/0.23	701/0.28	874/210	841/269	952/359	884/470	977/490	1012/515	836/828	981/1044	1029/1222
<sup>†</sup> H × PD × N	0.0045			0.0006/NS*			£NS/<0.0001			<0.0001/<0.0001		
SE	37.6			32.5/11.5			33.3/17.7			10.3/11.5		
ACRE location												
2T780 Aboveground biomass (g m <sup>-2</sup> )												
ON	537/0.36	549/0.32	537/0.36	548/169	710/62	815/85	775/296	866/333	810/394	804/581	878/638	922/755
165N	574/0.38	833/0.33	848/0.30	589/161	873/149	1024/115	837/458	999/580	1246/473	863/787	985/970	1091/1041
330N	623/0.35	786/0.30	744/0.29	603/144	878/181	952/102	801/422	890/570	1023/387	872/777	974/964	1105/1101
2T787												
ON	603/0.29	549/0.32	774/0.34	632/118	666/112	873/181	782/320	863/383	1057/455	807/571	932/741	1085/712
165N	658/0.35	782/0.33	888/0.34	792/187	851/162	1038/207	901/426	1054/441	1305/643	921/796	983/855	1213/1087
330N	714/0.31	720/0.29	909/0.35	782/178	806/194	1097/200	831/408	1043/406	1198/562	901/853	988/868	1139/1084
<sup>†</sup> H × PD × N	0.0036			0.038/0.044			0.011/<0.0001			0.001/<0.0001		
SE	21.9			25.4/17.5			31.7/15.4			14.5/11.4		

† P values for the significant 3-way interaction (hybrid × PD × N rate) term in the information ANOVA analysis for each location.

£ Hybrid × N rate (P = 0.028) and PD effect (P < 0.0001) for shoot biomass fraction at R3 stage at the ACRE experimental site.

\* Hybrid × PD and hybrid × N rate (P < 0.0001) for ear biomass fraction at R1 stage at the PPAC experimental site.

ACRE location appeared to have higher leafiness, location was a confounding factor.

The N stress effect on BM accumulation differed among plant density treatments and locations (Fig. 2). At ACRE, the greatest differences between N rates were observed at the highest plant density treatment (Fig. 2C and F). Furthermore, strong N stress (0N) was observed to reduce BM accumulation in all treatments starting well before anthesis (Fig. 2). At PPAC, increased stand density was accompanied by incremental gains in mean BM values (Fig. 2H, I, K, L).

### 3.5. Plant N uptake timing, rates, quantities, and partitioning

The effect of N supply on the total aboveground N content became apparent before anthesis (Fig. 3). Genotypic, plant density and N rate effects were significant ( $P < 0.05$ ) at both locations (Table 4). The higher leafiness observed for the ACRE's hybrids also corresponded with a higher total shoot (leaves + stem) N content. At V14 stage, the proportion of N uptake in leaves versus total shoot ratio ranged from 49 to 64% (averaging 52%) at ACRE location; while at PPAC, the overall leaf N content ranged from 55 to 68% (averaging 62%) of the total N accumulated by maize plants (Table 4). This pattern of N allocation in leaves and stems was not significantly related with N rate, plant density and hybrid factors. As discussed earlier, increased plant density was accompanied by higher total aboveground N content as N rates increased for all hybrids evaluated (Fig. 3). In general terms, an increasing N accumulation in the shoot BM and in the ear fractions was already apparent at R1 but continued at both R3 and R6 stages (Table 4; Fig. 3).

Under high N conditions (330N), the N uptake rate (NUR) was around 0.23 and 0.28  $\text{g m}^{-2} \text{d}^{-1}$  during the period bracketing silking ( $\pm 15$  days before and after R1) across hybrids and plant density treatments at both PPAC and ACRE locations, respectively (Table 4; Fig. 3). In the 0N treatment, NUR was about 50% lower than in other N treatments from the R1 to the R3 stage (at both locations), and about 50% lower between R3 and R6 stages. Moreover, it is of interest that substantial differences in NUR were already observed from seedling emergence to V14 stage; mean NUR in the non-fertilized plants averaged 0.09  $\text{g m}^{-2} \text{d}^{-1}$ , while mean NUR in fertilized treatments (165N–330N) averaged 0.15  $\text{g m}^{-2} \text{d}^{-1}$  (~67% greater compared to the non-N treatment) (Table 4; Fig. 3).

### 3.6. Ratio of plant N quantity to LAI and biomass accumulation

At the R1 stage, the ratio between aboveground N content and green LAI was influenced by both plant density and N rate factors. Overall, the ratio declined (from 3.4 to 1.8  $\text{g N m}^{-2}$  green LAI at PPAC, and from 2.9 to 1.8  $\text{g N m}^{-2}$  green LAI) as plant density increased and N rate decreased (Fig. 4A and B). At low density (54,000  $\text{pl ha}^{-1}$ ), the ratio ranged from 2.0–2.2 to 3.0–3.4  $\text{g N m}^{-2}$  green LAI across N rates (0–330  $\text{kg ha}^{-1}$ ) (Fig. 4A and B). At high density (104,000  $\text{pl ha}^{-1}$ ), the ratio varied from 1.8 to 2.4–2.7  $\text{g N m}^{-2}$  green LAI when N rate changed from 0 to 330  $\text{kg ha}^{-1}$  (Fig. 4A and B). Less variability was observed in the ratio's changes across plant densities for each specific N rate (Fig. 4A and B). When no N was applied, lower ratio values (1.8–2.4  $\text{g N green LAI m}^{-2}$  from high to low density) were observed than with the N fertilized treatments (2.5–3.4  $\text{g N green LAI m}^{-2}$  from high to low density).

A tight linkage was observed between the total aboveground BM and N content in maize plants; moreover, it seems that this relationship is established early in the growing season. For example, a strong correlation was observed between the total aboveground BM and N content at V14 stage (Fig. 5). However, treatments with N applied achieved a higher N uptake even when the zero N treatment (empty symbols; Fig. 5) accumulated as much total

aboveground BM as the N fertilized treatments, simply because of the higher total aboveground N concentration in fertilized treatments (Fig. 5).

The crop growth rate (CGR) appeared to drive the N uptake rate (NUR) during the period bracketing silking at both locations (Fig. 6). The non-fertilized plants exhibited a lower NUR than those of fertilized treatments as the CGR increased. However, the slope of this relationship was 50% lower in the 0N compared with the adequate N treatments (165–330N) at both locations (Fig. 6). At both sites, the highest CGRs achieved (i.e. 40  $\text{g m}^{-2} \text{d}^{-1}$ ) corresponded with the highest NURs (i.e. 0.4  $\text{g m}^{-2} \text{d}^{-1}$ ). At PPAC, the incremental slope was higher than at ACRE, mainly because the low CGRs corresponded with lower NUR (at the low stand density treatment), regardless of the N rates applied (Fig. 6). Moreover, at the low density, more than 30  $\text{g m}^{-2} \text{d}^{-1}$  of CGR was required to achieve a NUR above 0.2  $\text{g m}^{-2} \text{d}^{-1}$  at PPAC; while at the ACRE location, the same NUR was attained with a lower CGR, around 20  $\text{g m}^{-2} \text{d}^{-1}$  (Fig. 6).

### 3.7. Pre- versus post-silking N uptake and consequences

The ratio of N uptake pre-silking versus cumulative post-silking was not clearly related with the hybrid and N rate factors; however, in general terms, the ratio was lower at the lowest plant density (Table 5). The complexity of this ratio was reflected in the significant 3-way interaction, hybrid  $\times$  plant density  $\times$  N rates ( $P < 0.05$ ) at both locations (Table 5). The ratio was highly variable, and ranged from 0.51 to 0.71. The total aboveground BM resulted in similar ratio values for the pre-silking period relative to BM at maturity, ranging from 0.45 to 0.65 (data not shown). There is, therefore, tight linkage between the total aboveground BM accumulation and N taken up by maize plants.

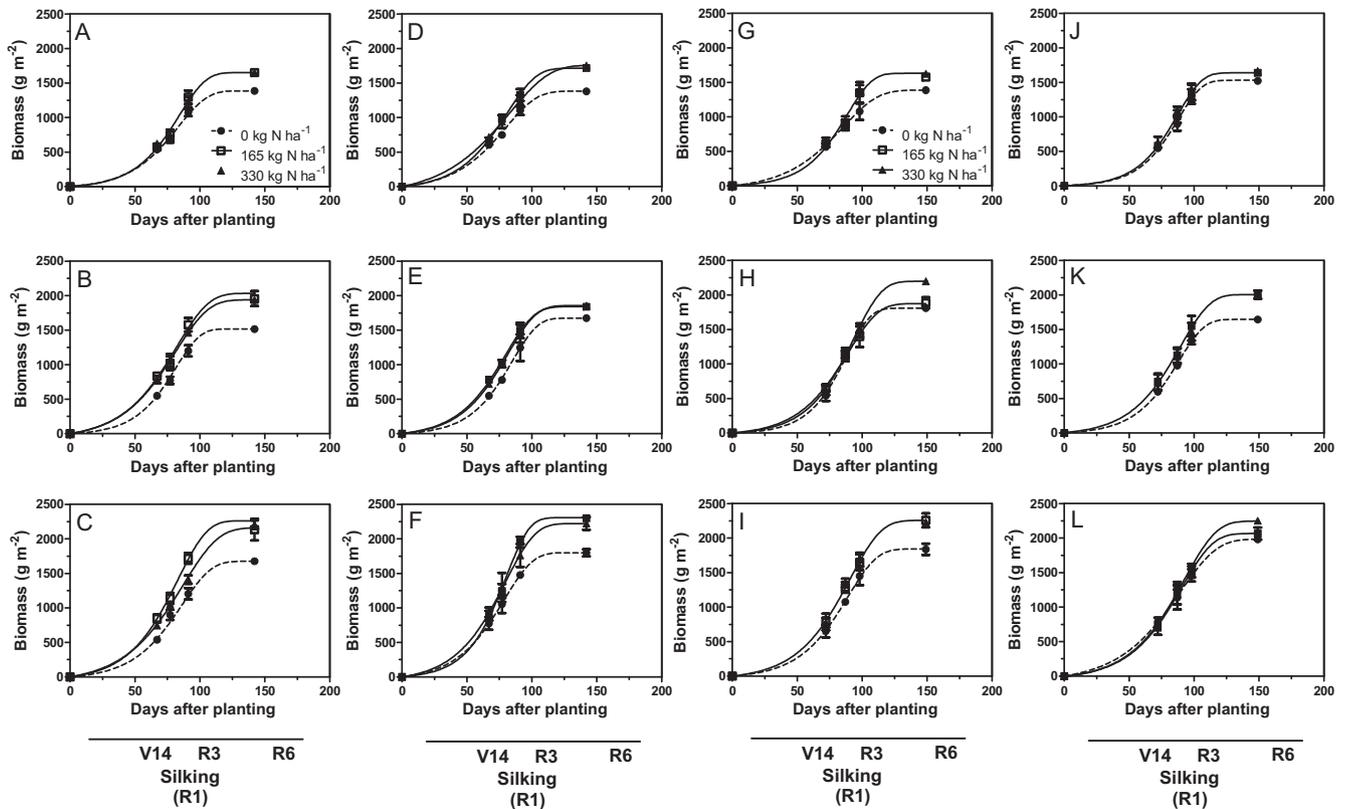
Total N uptake was very responsive to N supply. By physiological maturity, the total N uptake under low N supply was reduced by 36%, 41% and 44% than under high N supply for maize at the low, medium and high plant density treatments, respectively, across hybrids and locations (Fig. 3). At both sites, the high density treatment had greater total N uptake than the low density treatment (Table 4).

From R1 to R6, ear growth was highly dependent on total N uptake in the aboveground canopy, and this relationship was constant over time (Fig. 7). Moreover, the ratio of the ear BM to ear N uptake ranged from 82 to 98  $\text{g g}^{-1}$  (Fig. 7). Stem plus leaf BM was strongly correlated with N content in these fractions from R1 to R6; however, this relationship was not as strong as observed for the ear BM fraction (Fig. 7). Moreover, the evolution of shoot (stem + leaf) BM and N content were opposite to the ear BM and N content relationship; shoot BM achieved proportionately smaller gains in response to total plant N content within this fraction from R1 to R6 stages (Fig. 7).

### 3.8. Grain and stover biomass relationships to plant N content

The ratio of grain biomass to total aboveground N content was examined for all 3 plant densities and N rates combinations (Fig. 8A). In situations without side-dress N, this ratio was higher (52–54  $\text{g BM g}^{-1}$  N from low to high density) than with the N fertilized treatments (44–50  $\text{g BM g}^{-1}$  N from low to high density). At low density, the grain biomass and N uptake ratio varied from 44 to 52  $\text{g BM g}^{-1}$  N across N rates; while at high plant density the ratio ranged from 47 to 55  $\text{g BM g}^{-1}$  N (Fig. 8A). Regarding the plant density effect, less variability was observed across plant densities for each specific N rate (Fig. 8A and B).

The ratio of stover BM to total N uptake at equivalent N rates was also less impacted by plant density than by N rate treatments (Fig. 8B). With 0N, this ratio was higher (64–70  $\text{g BM g}^{-1}$  N from low to high density) than with the N fertilized treatments



**Fig. 2.** Biomass accumulation vs. days after planting for the different hybrids, plant densities and N rates at two different locations. For the hybrid factor: (A–C) hybrid 2T780 (non-RW), (D–F) hybrid 2T787 (RW) at ACRE location and (G–I) hybrid 2M749 (non-RW), (J–L) hybrid 2M750 (RW) at PPAC location. For the plant density factor: (A, D, G, J) low density treatment (54,000 pl ha<sup>-1</sup>), (B, E, H, K) medium density (79,000 pl ha<sup>-1</sup>) and (C, F, I, L) high density (104,000 pl ha<sup>-1</sup>). Each point is the mean of 18 individual plants per treatment. Curves are fitted using Gompertz equations.

**Table 4**

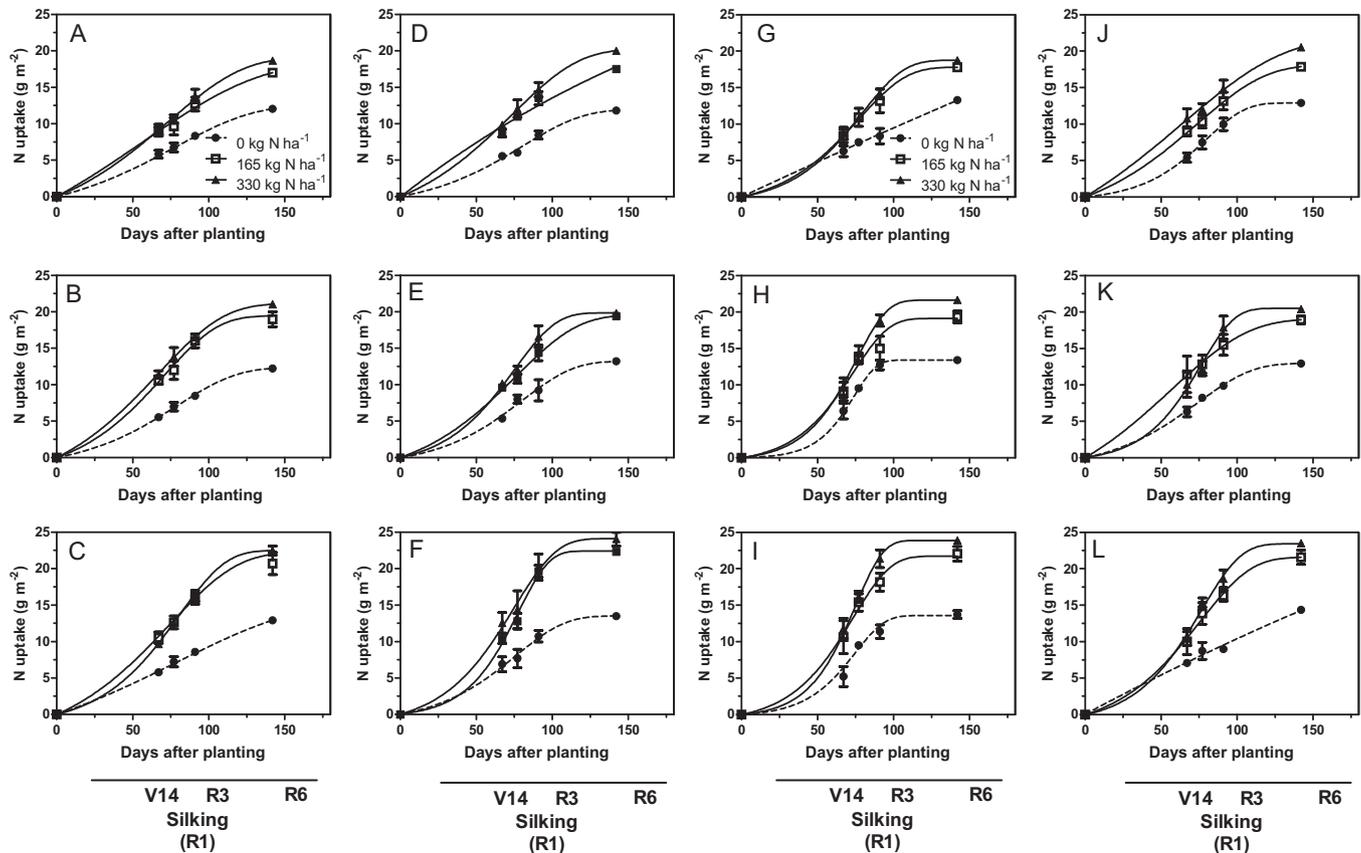
Nitrogen uptake (g m<sup>-2</sup>) for different fractions (leaves, stem and ear fractions) at V14, R1, R3 and R6 stages for maize hybrids 2M749, 2M750, 2T780 and 2T787 at three different populations (PD1 = 54,000; PD2 = 79,000 and PD3 = 104,000 pl ha<sup>-1</sup>) and three N rates (0, 165 and 330 kg ha<sup>-1</sup>) at PPAC and ACRE locations in 2009 growing season. Only significant treatment or interaction effects resulting from the ANOVA analysis are presented. The standard error (SE) relates only to comparisons between terms. At V14 stage, plants were fractionated between stem and leaf biomass (i.e. shoot biomass/leaf proportion), and from silking onwards plants were divided between shoot (stem + leaves) and ear biomass and thus presented as shoot/ear biomass fractions. At R6, shoot biomass fraction includes the cob biomass, while the “ear biomass” is only the grain fraction.

Hybrid	Treatments												
	V14 stage			Silking stage (R1)			Milk stage (R3)			Physiological maturity (R6)			
	PD1	PD2	PD3	PD1	PD2	PD3	PD1	PD2	PD3	PD1	PD2	PD3	
PPAC location													
2M749	Nitrogen uptake (g m <sup>-2</sup> )												
ON	6.3/0.54	6.4/0.61	5.2/0.51	4.6/2.9	6.6/2.9	4.9/4.6	4.5/3.8	6.8/6.0	5.0/6.3	5.6/7.4	5.6/7.8	5.5/8.2	
165N	8.4/0.64	9.1/0.51	10.6/0.49	7.5/3.4	10.1/3.6	9.8/5.6	6.3/6.9	9.5/5.5	9.7/8.4	7.2/10.6	7.3/12.1	8.8/13.3	
330N	8.7/0.63	9.7/0.53	11.8/0.53	7.8/3.0	11.1/3.3	11/5.1	6.8/7.4	11.9/6.9	11.7/9.7	7.6/11.2	8.5/13.4	9.3/14.5	
2M750	ON	5.4/0.59	6.3/0.54	7.1/0.62	5.1/2.4	4.6/3.6	4.4/4.3	4.8/5.2	5.1/4.8	3.8/5.2	5.4/7.5	5.6/7.8	6.5/7.8
	165N	9.0/0.53	11.5/0.60	10.0/0.53	6.9/3.4	7.6/5.3	7.8/6.1	7.0/6.1	9.7/5.8	9.2/7.1	7.1/10.7	8.2/11.7	9.6/12.2
	330N	10.8/0.50	10.0/0.41	10.4/0.50	8.0/4.1	7.1/5.1	8.7/6.7	8.5/6.3	10.9/7.0	11.2/7.4	8.7/11.6	9.0/12.3	9.1/14.6
	†H × PD × N	<0.0001			0.0385/NS		‡NS/ <0.0001				<0.0001		
	SE	0.30			0.27/0.21		0.28/0.24				0.11		
ACRE location													
2T780	Nitrogen uptake (g m <sup>-2</sup> )												
ON	6.0/0.64	5.5/0.62	5.8/0.62	4.6/2.1	5.9/1.1	5.8/1.4	5.1/3.2	4.8/3.6	4.2/4.4	5.5/6.5	5.4/6.8	5.3/7.6	
165N	8.9/0.65	10.6/0.63	10.5/0.59	7.0/2.6	9.7/2.3	11.0/1.7	7.6/5.0	9.5/6.5	10.8/5.0	8.0/9.1	8.7/10.3	9.6/11.1	
330N	9.2/0.68	11.4/0.58	9.7/0.59	8.1/2.6	10.8/3.0	11.0/1.6	8.8/5.0	9.2/7.3	11.5/4.8	8.8/9.9	9.5/11.5	10.2/12.3	
2T787	ON	5.5/0.63	5.3/0.61	6.9/0.67	4.0/2.0	6.1/1.9	5.0/2.7	4.8/3.6	5.0/4.2	5.8/4.9	5.4/6.4	6.5/7.0	7.2/6.3
	165N	8.7/0.68	9.6/0.63	10.4/0.67	8.2/2.8	8.4/2.4	9.9/2.9	8.2/5.3	9.3/5.1	11.6/8.0	7.9/9.6	9.4/10.3	10.9/11.4
	330N	9.9/0.61	10.2/0.55	12.6/0.65	9.5/2.9	9.0/3.0	11.4/3.0	9.8/4.9	11.5/5.1	12.9/7.3	9.6/10.5	10.4/9.8	10.9/13.1
	†H × PD × N	<0.0001			<0.0001/0.0434		0.0008/ <0.0001			<0.0001/ <0.0001			
	SE	0.28			0.26/0.27		0.28/0.18				0.13/0.12		

† P values for the significant terms in the information ANOVA analysis for each location.

‡ Hybrid × N rate (P = 0.028) and PD effect (P < 0.0001) for shoot biomass fraction at R3 stage at the PPAC experimental site.

\* Hybrid × PD and hybrid × N rate (P < 0.0001) for ear biomass fraction at R1 stage at the PPAC experimental site.

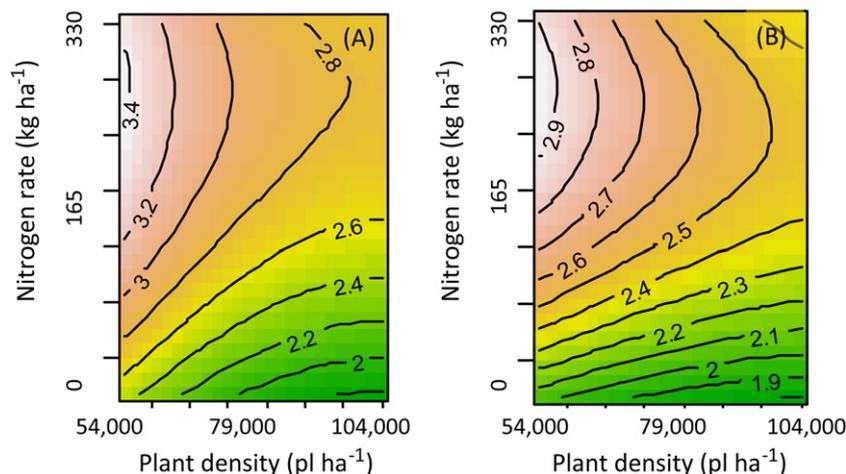


**Fig. 3.** Nitrogen uptake versus days after planting for the different hybrids, plant densities and N rates at two different locations. For the hybrid factor: (A–C) hybrid 2T780 (non-RW), (D–F) hybrid 2T787 (RW) at ACRE location and (G–I) hybrid 2M749 (non-RW), (J–L) hybrid 2M750 (RW) at PPAC location. For the plant density factor: (A, D, G, J) low density treatment (54,000 pl ha<sup>-1</sup>), (B, E, H, K) medium density (79,000 pl ha<sup>-1</sup>) and (C, F, I, L) high density (104,000 pl ha<sup>-1</sup>). Each point represents the mean of 18 individual plants per treatment. Curves are fitted using Gompertz equations.

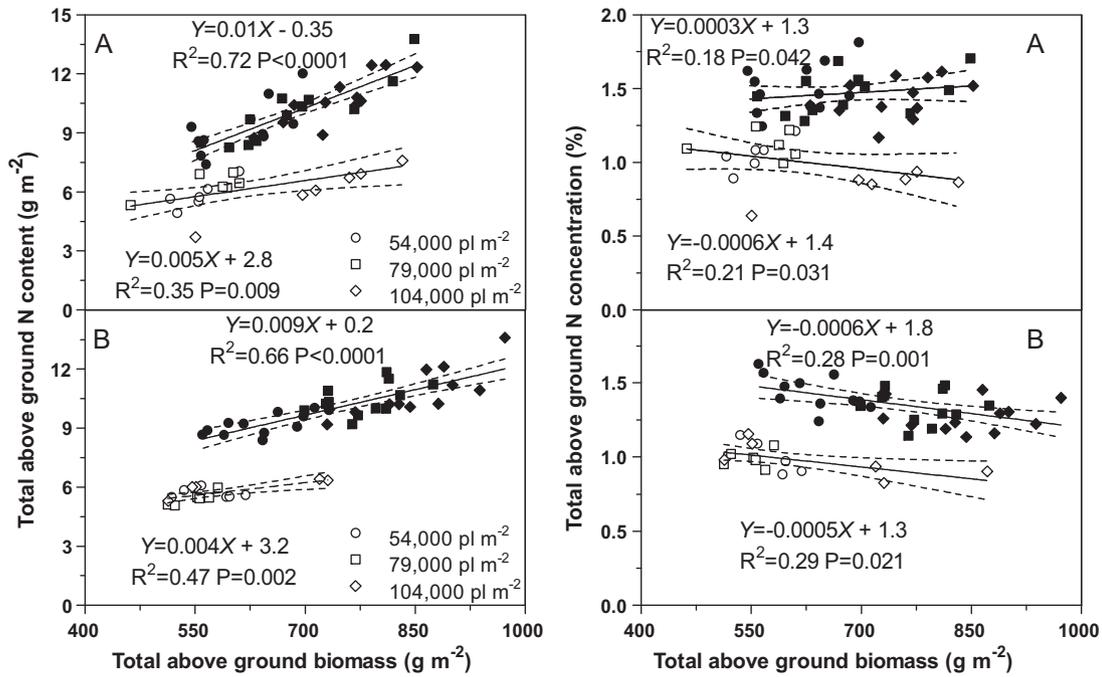
(46–58 g BM g<sup>-1</sup> from low to high density). At low density, the stover BM and N uptake ratio varied from 46 to 64 g BM g<sup>-1</sup> N across N rates; while at high plant density the same ratio ranged from 46 to 68 g BM g<sup>-1</sup> N (Fig. 8B).

The ratio of the grain BM to total N uptake was remarkably more responsive than the ratio of stover BM to total N uptake over all

plant densities and N rates combinations (Fig. 8A and B). As N rate increases, maize tended to produce much more BM per unit area for N fertilized treatments but at lower efficiency (44 g BM g<sup>-1</sup> N; low density and high N rate) than at the ON treatment (ranged from 52 to 54 g BM g<sup>-1</sup> N) (Fig. 8A). A similar trend is true for the ratio of stover BM to total N uptake; in addition, the plant density effect on



**Fig. 4.** Total aboveground N content and green leaf area index ratio at silking (R1) stage for the different N supply (zero N sidedress treatment, 0 kg ha<sup>-1</sup>; medium N, 165 kg ha<sup>-1</sup>; and high N treatment; 330 kg ha<sup>-1</sup>) and plant density levels (low plant density, 54,000 pl ha<sup>-1</sup>; medium plant population, 79,000 pl ha<sup>-1</sup>; and high plant density, 104,000 pl ha<sup>-1</sup>) across hybrids at two different locations (A–PPAC and B–ACRE sites).



**Fig. 5.** Total aboveground N content and N concentration before silking relative to total aboveground plant BM at the V14 phenological stage for the different plant densities and N rates across maize hybrids at two locations (A—PPAC and B—ACRE sites). Full symbols refer to N fertilizer rates of either N2 medium N (165 kg ha<sup>-1</sup>) or N3 = high N treatment (330 kg ha<sup>-1</sup>); empty symbols refer to the no N sidedress applied, N1 = low N (0 kg ha<sup>-1</sup>). Circle symbols correspond with low plant density (54,000 pl ha<sup>-1</sup>), squares with medium plant population (79,000 pl ha<sup>-1</sup>), and diamonds with high plant density (104,000 pl ha<sup>-1</sup>).

this ratio at the same N rate was scarcely detectable (except for the ON treatment) (Fig. 8B).

### 3.9. Harvest indices for grain and N

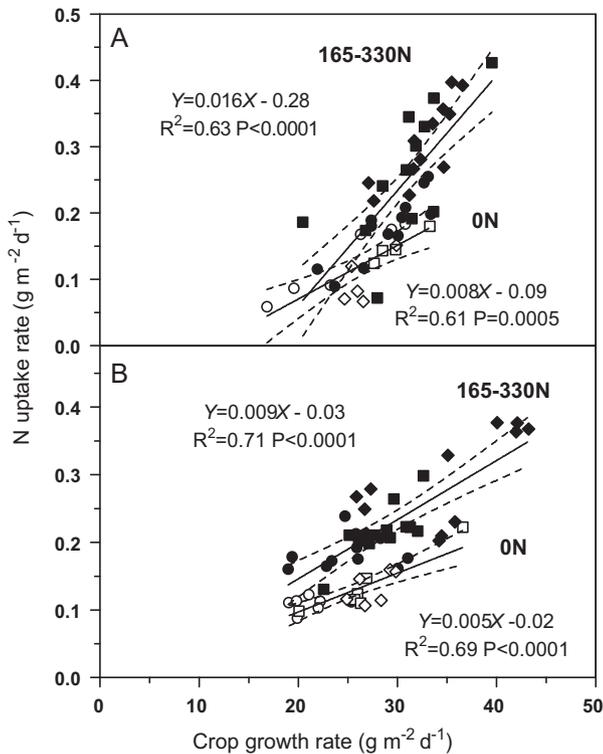
At ACRE, HI increased as N rate increased even though hybrid and plant density influences on HI were non-significant. Har-

vest index also increased significantly as N rate increased at PPAC (Fig. 9), but at this location the single factors of hybrid and plant density also significantly affected HI; the highest values were observed at the intermediate plant density in both hybrids (Fig. 9). Overall, HI significantly increased with the increase in total plant N uptake as well as with the N fertilizer rate applied (Fig. 9).

**Table 5**

Ratio of N uptake pre-silking versus cumulative post-silking (%), nitrogen internal efficiency (NIE), expressed as kg grain kg<sup>-1</sup> N uptake, nitrogen recovery efficiency (NRE), as kg N uptake kg<sup>-1</sup> N applied, and nitrogen use efficiency (NUE), expressed as kg grain kg<sup>-1</sup> N applied, for maize hybrids 2M749, 2M750, 2T780 and 2T787 at three different populations (PD1 = 54,000; PD2 = 79,000 and PD3 = 104,000 pl ha<sup>-1</sup>) and three N rates (0, 165 and 330 kg ha<sup>-1</sup>) at PPAC and ACRE locations in 2009 growing season.

Treatments	PPAC location											
	Ratio N uptake Pre/cumulative post-silking (%)			NIE (kg grain kg <sup>-1</sup> N uptake)			NRE (kg N uptake kg <sup>-1</sup> N applied)			NUE (kg grain kg <sup>-1</sup> N applied)		
Hybrid	PD1	PD2	PD3	PD1	PD2	PD3	PD1	PD2	PD3	PD1	PD2	PD3
<b>2M749</b>												
ON	0.57	0.71	0.69	–	–	–	–	–	–	–	–	–
165N	0.61	0.71	0.70	29.84	24.45	36.44	0.29	0.36	0.51	8.68	8.89	18.55
330N	0.58	0.66	0.68	29.47	40.63	34.42	0.18	0.26	0.31	5.18	10.47	10.54
<b>2M750</b>												
ON	0.58	0.64	0.61	–	–	–	–	–	–	–	–	–
165N	0.58	0.68	0.64	26.87	39.63	22.95	0.30	0.39	0.45	7.98	15.61	10.43
330N	0.59	0.60	0.65	12.80	39.04	38.46	0.22	0.24	0.28	2.87	9.35	10.96
<b>H × PD × N</b>		0.0451			0.0325			0.0036			0.0125	
<b>SE</b>		0.028			3.78			0.09			1.51	
<b>Hybrid</b>												
<b>2T780</b>												
ON	0.56	0.57	0.56	–	–	–	–	–	–	–	–	–
165N	0.57	0.64	0.61	46.65	56.39	42.35	0.31	0.41	0.47	14.42	23.24	20.02
330N	0.57	0.66	0.56	33.79	42.79	41.63	0.20	0.27	0.29	6.86	11.41	12.11
<b>2T787</b>												
ON	0.51	0.61	0.57	–	–	–	–	–	–	–	–	–
165N	0.63	0.56	0.57	45.59	21.24	49.22	0.35	0.38	0.53	15.75	7.98	26.25
330N	0.58	0.61	0.60	39.24	21.89	40.92	0.25	0.20	0.32	9.87	4.45	13.02
<b>H × PD × N</b>		0.0236			0.031			0.0042			0.0253	
<b>SE</b>		0.025			4.12			0.05			1.83	



**Fig. 6.** Total N uptake rate and crop growth rate during the period bracketing silking ( $\pm 15$  days from silking stage) for the different plant densities and N rates across hybrids at two different locations (A—PPAC and B—ACRE sites). Full symbols refer to the medium N (165 kg ha<sup>-1</sup>), and high N treatment (330 kg ha<sup>-1</sup>), and empty symbols refer to the zero N sidedress applied. Circles symbols correspond with low plant density (54,000 pl ha<sup>-1</sup>), squares with medium plant population (79,000 pl ha<sup>-1</sup>), and diamonds with high plant density (104,000 pl ha<sup>-1</sup>).

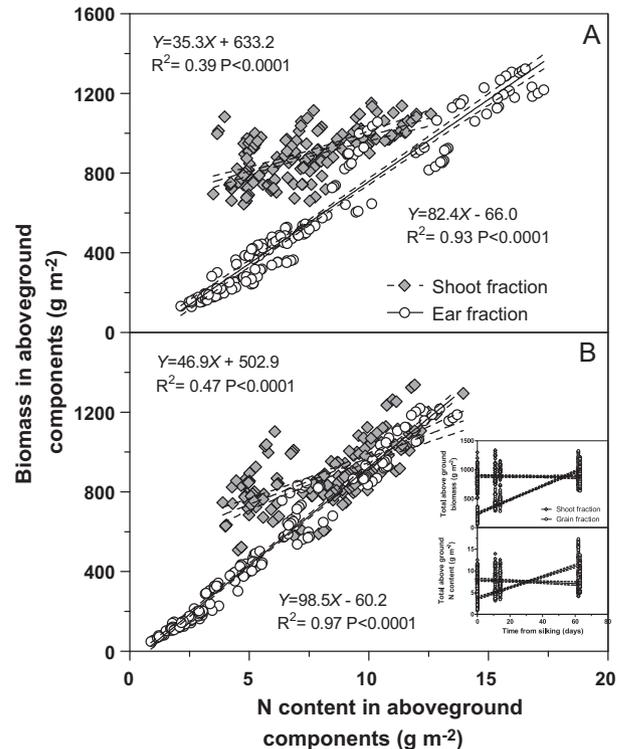
The N harvest index (NHI) response was somewhat erratic at both locations; in some treatments NHI increased while in others it did not change significantly in response to plant density and N rate factors (Fig. 9).

### 3.10. Nitrogen efficiency indices

In terms of the N indices, the N internal efficiency (NIE) was highest at the medium plant density and highest N rate at PPAC location; while at ACRE, the highest NIE was observed at the same density but at the medium N rate (Table 5). At ACRE, the grain production differential between ON and the medium N rate was greater compared with PPAC at the medium density ( $\sim 223$  g m<sup>-2</sup> for ACRE versus just 175 g m<sup>-2</sup> of grain yield at PPAC; Table 3); however, total plant N uptake differentials between N treatments were similar between locations ( $\sim 6.5$  g m<sup>-2</sup> and 6.3 g m<sup>-2</sup> of N uptake for ACRE vs. PPAC, respectively; Table 4). On the other hand, at the PPAC site, the medium plant density resulted in greater grain productivity as well as a higher N uptake differential between ON and 330N treatments (Tables 3 and 4).

Highest values for NRE (ranging from 0.45 to 0.53) were observed at the treatment combining high plant density and medium N rate (Table 5). This situation was related to the large difference in N uptake per unit area (ranging from 3.5 to 5.1 g m<sup>-2</sup>; Table 4) observed between the ON and 165N rates at the high plant density. In general terms, the highest NRE was due to a greater N uptake per unit area, but also corresponded with a moderate to high NIE (Table 5).

Both experiments clearly showed a greater N uptake when N fertilizer was added, altering the internal efficiency in a wide range from 12.8 to 56.4 kg grain kg<sup>-1</sup> N uptake (Table 5). Nitro-



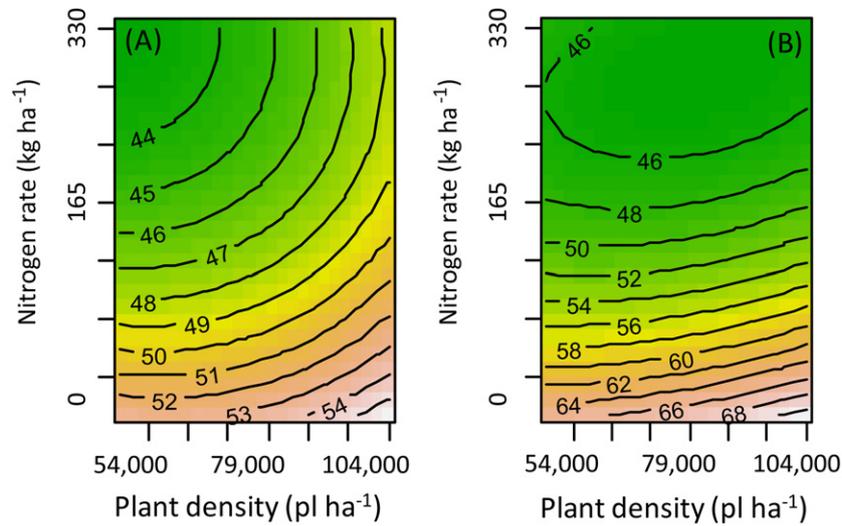
**Fig. 7.** Biomass and N content accumulated in aboveground components for two different fractions (stem + leaves and ear BM) across hybrids, plant densities and N rates factors at two different locations (A—PPAC and B—ACRE sites) as determined from leaf plus stem versus ear shoot samples taken at R1, R3 and R6 stages. Ear BM fraction includes the cob, husk and grains until R6 (at which time the husks were excluded from this fraction). Sub-figures represent the total aboveground BM and N content evolution for shoot BM versus ear BM, calculated as an average across hybrids, populations, and N rates.

gen use efficiency (NUE) increased most (ranging from 10.4 to 26.3 kg grain kg<sup>-1</sup> N applied across hybrids at both locations) when N fertilizer was added at the intermediate N rate to maize at the high density (Table 5). The highest NUE value in the entire study was observed with the high plant density, medium N rate at ACRE (26.3 kg grain increment kg<sup>-1</sup> N applied; Table 5). However, the latter response was not associated with maximum maize grain yield, which was observed at the highest N rate for that plant density treatment (Tables 2 and 3). Therefore, these results showed that variations in NUE in response to N fertilization and plant density factors were more associated with changes in N response components such as NRE and NIE than they were with grain yield *per se*.

## 4. Discussion

### 4.1. Grain yield, biomass and partitioning components

Grain yield per unit area responded positively to increasing plant density only when side-dress N was applied (rates of 165 or 330N), but not when no N was side-dress applied, over a wide range of total aboveground plant N uptake (Table 2). The difference in grain yields among N rate and plant density treatments were more associated with total plant BM rather than HI (Table 3 and Fig. 2). Moreover, stover BM at physiological maturity was also greater and more responsive to N supply for N treatments (165–330N) than the zero N treatment over a range of total N uptake (Table 3), regardless of the plant density and hybrid factors. This was largely because of higher total BM production after anthesis in situations with inter-

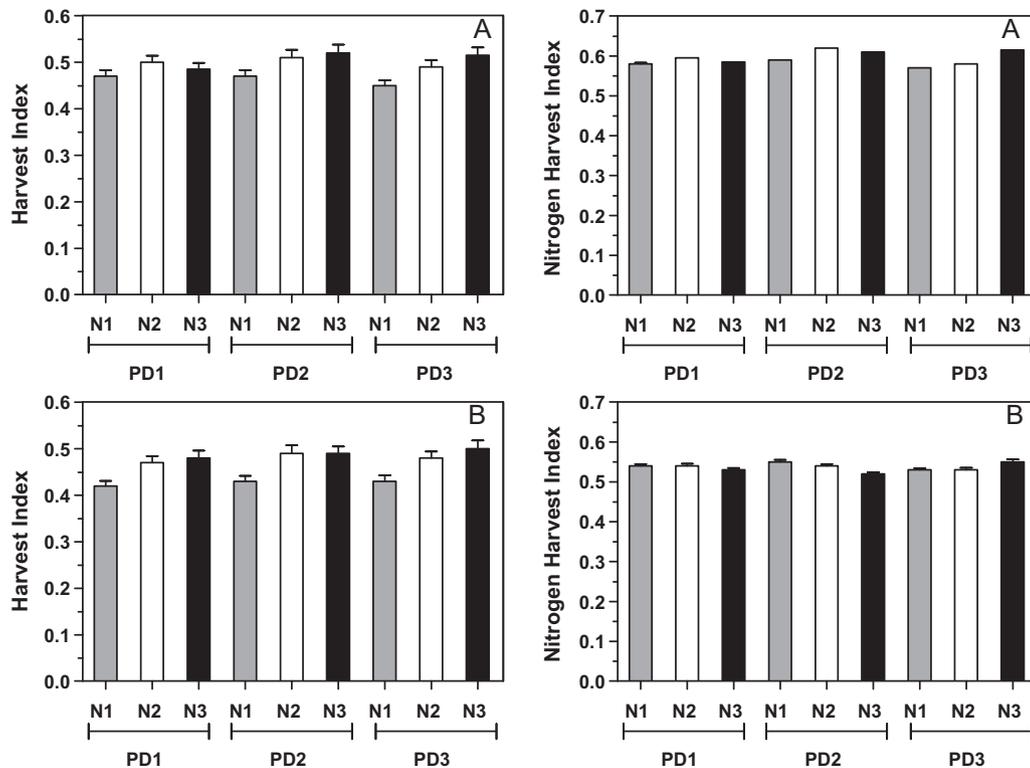


**Fig. 8.** The ratio of (A) grain BM (0% moisture), and (B) stover BM to total aboveground N content ( $\text{g BM g}^{-1} \text{N}$ ) at physiological maturity (R6) for maize experiencing different N supply (zero N sidedress treatment,  $0 \text{ kg ha}^{-1}$ ; medium N,  $165 \text{ kg ha}^{-1}$ ; and high N treatment;  $330 \text{ kg ha}^{-1}$ ) and plant density levels (low plant density,  $54,000 \text{ pl ha}^{-1}$ ; medium plant population,  $79,000 \text{ pl ha}^{-1}$ ; and high plant density,  $104,000 \text{ pl ha}^{-1}$ ).

mediate and high N fertilizer applications (Table 3; Fig. 2). However, the stover BM conversion efficiency per unit of total N taken up by maize plants (measured as the ratio of the stover BM and total above N content –  $\text{g BM g}^{-1} \text{N}$  – at R6) was lower as N rate increased and as plant density decreased (Fig. 8B). The grain HI was significantly higher in the 165–330N treatments than with zero N applied regardless of plant density and hybrid effects (Fig. 9). The decrease in HI under high plant density and without N (0.01–0.05, or approximately 10%; Fig. 9) was proportionally much less than the decline in total plant BM ( $389\text{--}529 \text{ g m}^{-2}$ , i.e. 18–25%, Fig. 2), indicating clearly that the dominant effect of plant density and N rate was on

total BM accumulation in maize. Similar effects were observed by Muchow (1994) and Massignam et al. (2009).

In terms of grain components, the most dramatic responses to N deficiency and high-density stresses were observed as a major reduction in KN and a less dramatic reduction in KW (Table 2). Deficient N environments exert an effect on C and N metabolism in developing kernels, which may constitute a constraint for the final grain set (Below et al., 2000). In addition, the effect of the N nutrition on the utilization of sugars for the ear growth regulates the kernel number response to N availability (Below et al., 2000). Contrastingly, Uhart and Andrade (1995)



**Fig. 9.** Grain harvest index (left graph) and N harvest index (right graph) for the different plant densities and N rates across hybrids at two different locations (A—PPAC and B—ACRE sites). For the plant density factor: (PD1) low density ( $54,000 \text{ pl ha}^{-1}$ ), (PD2) medium density ( $79,000 \text{ pl ha}^{-1}$ ) and (PD3) high density treatment ( $104,000 \text{ pl ha}^{-1}$ ). For the N factor: (N1) low N ( $0 \text{ kg ha}^{-1}$ ), (N2) medium N ( $165 \text{ kg ha}^{-1}$ ), and (N3) high N treatment ( $330 \text{ kg ha}^{-1}$ ).

reported that KN reduction was mainly promoted by the C exchange lessening, without presenting a direct impact of N deprivation over the final kernel set. With respect to KW, previous reports demonstrated that this component of kernel development is linked more to the duration of the effective grain-filling period than to the grain-filling rate when maize encounters water (NeSmith and Ritchie, 1992), defoliation (Echarte et al., 2006; Sala et al., 2007), and shading stresses (Tanaka and Maddoni, 2009). However, N deficient environments may affect both duration and rate of the grain-filling (Jones et al., 1996), while the additional stress of high plant density has a tendency to affect the kernel weight growth rate slightly more than the effective grain-filling period (Lemcoff and Loomis, 1994).

#### 4.2. N uptake: time course, distribution and relationship with green LAI

As expected, higher whole-plant N uptake was observed when N fertilizer rate increased. Despite significant genotypic differences, total aboveground N content was similar between hybrids compared at the same plant density and N rate (Table 4). Similar results were presented by Subedi and Ma (2005). The 0N treatment resulted in the lowest maize productivity under the high-density stress conditions (Table 3; Fig. 2). At the medium N rate (165N), maize plants did not demonstrate apparent N deficiency symptoms across all different plant density treatments, suggesting that soil plus fertilizer N may have been enough to meet the crop N demand at this N uptake level. Moreover, it is important to highlight that at the PPAC location the sharply declining trend observed for green LAI after silking (Supplementary Fig. 2) did not significantly reduce or affect the N uptake rate after that phenological stage (Fig. 3); the latter suggests the ability of maize to maintain N uptake following early cessation of photosynthetic leaf area due to an abiotic stress. However, the reduction in green LAI was most apparent 40 days after silking time (around R3–R4 stages), a phenomenon that results in lower carbohydrate remobilization from leaves to the ear (Uhart and Andrade, 1995). In that respect, Massignam et al. (2009) observed a proportional decrease in the fraction of total radiation intercepted as the green LAI declined. After silking of maize crop, the prolongation of the whole plant N uptake is a consequence of the postponement in leaf senescence because the latter extends canopy photosynthesis duration (Ma and Dwyer, 1998; Rajcan and Tollenaar, 1999). Additionally, N remobilization (mainly due to increase in leaf senescence) and post-silking N accumulation were associated in an antagonistic relationship (Coque and Gallais, 2007).

Based on reports of a linear relationship between aboveground N content and green LAI under optimum conditions for a wide range of crops (Lemaire and Gastal, 1997; Plénet and Lemaire, 2000), the prior assumption was that maize leaf area expansion (determined by the green LAI evolution during vegetative stages) seemed to be the driving factor for the aboveground N uptake. In our study, plant density and N rate interactions exerted a large influence on the ratio of the total aboveground N content to the green LAI ratio (Fig. 4A, and B); this effect can be visualized in the changes occurring from high to low density and from low N to high N (from 1.8 to 3.0–3.4 g N m<sup>-2</sup> LAI; Fig. 4A, and B). At the low plant density, no significant differences were observed in the green LAI across N treatments at both locations (Supplementary Fig. 2); in addition, significant changes were apparent in total N accumulation for the 0N vs. 165–330N treatments (Fig. 3). A similar trend is true at the intermediate density, with the exception of the hybrid 2T787 at ACRE location, which showed differences in LAI (minor) and total N uptake (Supplementary Figs. 2 and 3). With the high density treatment at ACRE location, both hybrids experienced an average reduction of 27% in green LAI from the situations with N

(165–330N) to that of zero side-dress applied (0N) (Supplementary Fig. 2) while reduction in total N uptake declined more dramatically (~46%) when no sidedress N was applied (0N) (Fig. 3). From these results, we can affirm that the capacity of the crop to accumulate N per unit of green LAI (per unit area) is reduced as N becomes more limiting as well as when plant density increases. In a similar manner, Lemaire and Gastal (2009) reported that maize plants in N deficient environments accumulated less N per unit area of LAI as the result of the plant's strategy to maximize light interception by minimizing the reduction in green LAI in response to low N availability. Two hypotheses could be formulated: (i) as plant density increases, the N crop demand per unit land area increases and, with a constant N supply, there is a shift in plant N status; or (ii) as density increases, competition for light increases. The latter would change plant morphology and leaf anatomy, and then leaf N content per unit area would change too. In support of the first hypothesis, we obtained differences between 165N and 330N treatments at both locations (Fig. 4A, and B). In general terms, from 41% to 68% of total N taken up by maize plants was partitioned to leaf blades before silking (V14 stage) (Table 3); average N partitioned to leaf blades was greatest at the 0N and high plant density combination (i.e., mean of 62%). Nevertheless, our research demonstrates that this relationship is changed substantially by both plant density and N levels (Fig. 4A and B).

If crop N uptake is determined primarily by leaf area expansion, then a positive relationship should be observed between the total aboveground N content and the total aboveground BM (leaf + stem fractions). We observed that aboveground N content was strongly correlated with total aboveground BM before the silking stage (V14) (Fig. 5), but that the increases in plant BM did not drive total N uptake for maize with 0N. However, when side-dress N was applied (165–330N) this relationship was positive and the maximum N uptake per unit of total BM was around 0.009–0.01 g N m<sup>-2</sup> g<sup>-1</sup> BM m<sup>-2</sup> (Fig. 5), which corresponded to the maximum total aboveground BM demand for N. Basically, at the V14 stage, the difference among N treatments was related to the initial N concentration, which was higher in fertilized treatments compared with the zero N treatment. Additionally, as the growing season progressed, N concentration declined while BM increased (Fig. 5). These results confirm that (1) in situations without N applied, factors other than leaf area expansion or total BM govern and limit the NUR, and (2) that the relationship between aboveground BM and N content is rather constant across the different plant densities and hybrids evaluated but changes dramatically with the N supply (with or without N applied). A pragmatic association, rather than a systematic one, was proposed for the proportionality between the aboveground N content and the green LAI, forced by the trade-off between an increase in the N partitioned to stems and a decline in N distribution to leaves as the LAI expands with time (Lemaire et al., 2007). The time course of N uptake (expressed in terms of the proportion of total N uptake reached at anthesis relative to that at maturity) varied in response to the treatment combinations (and location) even when total N uptake by the crop at maturity was similar for given N treatments in both locations. Environmental conditions during the period bracketing silking (i.e. from V14 to R3 stage) affected CGR and, consequently, the pattern of N uptake (Fig. 6A and B). Likewise, NUR per plant was dependent on N supply and plant density at both locations (Fig. 6) presumably via the effects of these treatments on CGR; because NUR increased as CGR increased there appeared to be little sink limitation to NUR during this period. Slopes between NUR and CGR ranged from 0.009 to 0.016 for 165–330N and from 0.005 to 0.008 for 0N (Fig. 6); slopes changed with the N supply regardless of the hybrid and plant density factors. Therefore, it is the CGR during the period bracketing silking that determines the NUR and, as a consequence, the amount of N that needs to

be remobilized to meet the N requirement in the ears whenever post-silking N uptake is not sufficient, by itself, to meet N demand in ears.

Measurements of N uptake and distribution showed that N translocation started before silking time (Table 4), indicating that N supply to the early ear formation from current plant N uptake during the pre-silking period did not exceed demand. In general terms, the translocation was greater in the situations without N applied (0N), where from 23% to 27% of the N accumulated before silking was translocated to the developing ears (presumably to help establish viable embryos). This pre-silking translocation is apparently responsive to the early grain requirement for structural N, which is likely temperature dependent (Lecouer and Sinclair, 2001). Moreover, N translocation to ears at this stage seems to have occurred from the stem in preference to leaves even though some N remobilization from leaves was expected in the zero N treatments. These results are consistent with observations reported by Weiland and Ta (1992).

Average post-anthesis N uptake under 0N ( $\sim 5.2 \text{ g m}^{-2}$ ) was lower than for the N fertilized treatments ( $\sim 7.4$  and  $8.3 \text{ g m}^{-2}$  for 165N and 330N, respectively; Table 4). Consequently, the 0N treatment required an increase in N translocation from vegetative plant parts to meet grain N demand even though it had fewer grains and lower kernel weights (Tables 1 and 4). Moreover, under N deficiency (0N) we observed lower apparent LAI during the grain filling period (Supplementary Fig. 2). The effect of abiotic stresses on resource use efficiency and capacity is ameliorated by the initial N remobilization from the stem organ, a situation which retains canopy photosynthesis ability and duration (Muchow and Sinclair, 1994). Differences in total post-silking N uptake among plant densities were not significant across hybrids and locations (i.e. these ranged from  $5.0$  to  $5.4 \text{ g m}^{-2}$ ); suggesting that plant density influences the pre-anthesis interval (period in which maize plants establish potential kernel number) more than the post-silking N uptake.

#### 4.3. Critical N concentration

The concept of critical N concentration (%Nc) has been proposed as the minimum %N in the aboveground BM for maize (as well as for other crops) required for production of maximum aboveground BM at a given phenological stage (Plénet and Lemaire, 2000). The model relationship between BM and %Nc variables are described through an exponential equation ( $\%Nc = 3.40 \times BM^{-0.37}$ ). In treatments without N applied, the calculation of the N critical dilution curve (%Nc) (Supplementary Fig. 3) resulted in lower values compared with treatments at moderate or high N fertilizer rates (165–330N). In agreement with other authors, both %Nc and the actual N concentration (%Na) in our experiments declined as a function of the whole plant aboveground BM accumulation (Plénet and Lemaire, 2000; Peng et al., 2010). The prior literature suggests that the equation for %Nc apparently applies to BM values from 100 to  $2200 \text{ g m}^{-2}$  (i.e. from the nine visible leaf stage to 25 days after silking). However, in our research, this relationship did not result in the best fit with different N rates and plant densities (Supplementary Fig. 3). From V14 stage to physiological maturity, almost all the points were below the %Nc; even though N treatments (165–330N) resulted in higher %Na during the growing season compared with 0N plots.

Another important observation to highlight is that medium and high plant densities resulted in a fitted curve that was closer to the %Nc. The latter suggests that differences in BM due to differences in N stress and high-density stress resulted in a weaker correlation than that observed by Plénet and Lemaire (2000). Similarly, Peng et al. (2010) observed lower values of the relationship for two different maize inbreds, which were explained by the lower

plant density ( $60,000 \text{ pl ha}^{-1}$ ) and smaller shoot dry weight. In our research, differences in whole-plant N concentration (ranging from 0.75% to 1.51% at R1 stage), were the main cause that explained the equation's lack of fit. At the same phenological stage, Plénet and Lemaire (2000) reported whole-plant N concentrations varied from 1.37% to 1.56% at a plant density of  $90,000 \text{ pl ha}^{-1}$ . These results allowed us to confirm that: (1) situations without N applied (0N) resulted in a lower %Na than other N treatments evaluated, (2) the relationship between total aboveground BM and %Na is dependent on the N supply, in which slopes change with the N supply (from insufficient to adequate N supply), (3) the relationship between total aboveground BM and %Na was independent of plant density and hybrid factors in our research (although we acknowledge the hybrid differences would be expected to be very small both because we were comparing near-isoline hybrids and because these hybrid pairs had no differential CRW feeding), and (4) a better fit between total aboveground BM and %Na was observed from R3 to R6 stages in the 165N and 330N treatments (at all plant densities), suggesting that N was a less limiting factor during this period. Additional experimentations with more contrasting hybrids, years and locations are required to validate this relationship at specific developmental stages; however, currently this association is an excellent tool to know more about the N nutrition dynamics of maize plants during the growing season, and to have a more complete idea about the deficiency or sufficiency of the N status within the plant.

#### 4.4. Grain biomass, grain N concentration, and harvest index relationships to plant N uptake and allocation

Ear BM progression during grain fill was strongly correlated with ear N content from silking until physiological maturity at both locations (Fig. 7); this relationship was relatively independent of hybrid, stand density, and N rate factors. We also investigated the dependency between grain BM (i.e. ear minus the cob) and its N content, and observed slight changes in the slope values (i.e.  $74\text{--}91 \text{ g BM m}^{-2} \text{ g}^{-1} \text{ N content m}^{-2}$ ). The latter means the effective ratio between grain BM and N content ranged from 1.1 to 1.4% (similar to the final N concentration achieved for the whole plant; Supplementary Fig. 3). The high slopes resulting from this relationship confirm the high N status required for optimal grain development. Because grain N accumulation coincided so directly with ear BM increase, we support the concept of sink (as compared to source-dominant) determination of the potential N accumulated. Below et al. (1981) observed a similar relationship (i.e. estimated slope of  $82 \text{ g BM m}^{-2} \text{ g}^{-1} \text{ N content m}^{-2}$ , for a plant density of  $46,000 \text{ pl ha}^{-1}$ ) for four different hybrids of an earlier era over a 53–72 day period after silking. Several researchers have provided data that permitted us to estimate the relationship between ear biomass and ear N content from silking until physiological maturity, resulting in slopes values from 61 to  $89 \text{ g BM m}^{-2} \text{ g}^{-1} \text{ N content m}^{-2}$  (Lemcoff and Loomis, 1986, 1994; Below et al., 1981; Swank et al., 1982; Pan et al., 1986; Camberato, 1987). These results suggest that this relationship has been relatively constant in maize of different hybrid eras, and that it might also be independent of plant densities and N rates. Nevertheless, the genetic effect exerts a large influence in the potential kernel protein concentration, given that the genetic predisposition to low grain N concentration did not change under high N supply environments (Uribelarrea et al., 2004). More research is therefore needed to better understand the relationship between N balance (supply and demand), and N dynamics, specifically during the critical period bracketing silking.

At physiological maturity, NHI displays the extent of N translocation from vegetative to reproductive structures. We observed a

large variation in NHI responses to management practices (Fig. 9). However, in general terms, greater N remobilization (higher NHI) was observed for the two hybrids at the PPAC site even though total plant BM production between locations was similar at R6 (Table 3; Fig. 2). Greater BM accumulation occurred before silking at the PPAC site; this assured a high and a constant CGR and NUR during the critical period bracketing silking (Fig. 6).

Nitrogen fertilizer treatments of either 165 or 330 N decreased NHI at both low and medium plant densities at the ACRE location, indicating that N deficient plants in the control treatment mobilized proportionately more N to the grains (Fig. 9). Therefore, the increase in BM observed in response to N (Fig. 5) can be explained in terms of a greater accumulation of N in vegetative tissues. Because change in grain N concentration was also observed (Supplementary Fig. 3), the accumulation of N in grains was not proportional to BM production. The enhanced grain N accumulation due to N fertilizer addition was explained by both increases in grain BM (~23–26%), and in grain N concentration (~15–18%) across plant densities, hybrids and locations (Table 3; Supplementary Fig. 3).

Changes in N concentration in grains were observed well beyond the N fertilization rates needed to achieve the maximum grain yield. Other reports have also shown an increase in grain N concentration when a crop is grown with non-limiting N supply (Uhart and Andrade, 1995; Gooding et al., 2007). Moreover, increases in stover BM (~7–8%) and stover N concentrations (~27–32%) at physiological maturity were also observed as N fertilization rates increased (Tables 3 and 4). For fertilized plots, in which no plant N limitation was observed, progressively greater N quantity uptake by the crop was not proportionately partitioned to the sink during the grain filling period (Tables 3 and 4). Moreover, a different response was observed in the ratios of grain BM and stover BM to the total N uptake (Fig. 8A and B); it is noticeable that for the grain BM the strong sink effect is the main driving force. In the ratio of stover BM to N uptake at a constant N rate, changes across densities were minor compared with those observed for the ratio of the grain BM to the total aboveground N content. This suggests that N remobilization is tightly linked to the final grain BM accumulation and the grain N content (%N), which is potentially genetically determined (Uribelarrea et al., 2004). Moreover, grain N concentration was changing with different N rates, plant densities and locations, which suggests a relatively modest genetic effect, but a much larger interaction effect of genotype  $\times$  environment  $\times$  management ( $G \times E \times M$ ). Continued progress in understanding relationships among N uptake dynamics (from vegetative to reproductive stages), grain yield and NUE must acknowledge the magnitude and complexity of the  $G \times E \times M$  interactions that remain a challenge to decipher and interpret (Messina et al., 2009).

#### 4.5. Overview of NUE and its components (NIE and NRE)

The use of grain NUE by itself is not enough to understand the impact of management practices on crop N dynamics because it comprises both soil and plant processes (Salvagiotti et al., 2009). Advances in the knowledge of the NIE, which is more associated with the plant N conversion or utilization efficiency to the final grain yield, and the NRE, which is more connected to the plant N uptake efficiency, are also required to improve the understanding of the grain NUE variability under the  $G \times E \times M$  framework (Salvagiotti et al., 2009). In general terms, maize grain NUE is very high at low N fertilizer rates (Ladha et al., 2005; Uribelarrea et al., 2007). The same trend was observed in this research, and NUE declined as N fertilization rate increased. In an earlier study (involving 411 data points by Ladha et al., 2005) of maize across different regions, the NUE averaged around 25 kg grain  $\text{kg}^{-1}$  N applied, with a minimum and maximum range from 0–5 to 50–60 kg grain increment  $\text{kg}^{-1}$  N applied. In our experiment, the mean NUE (around

12 kg grain  $\text{kg}^{-1}$  N applied) and range of NUE (from 3 to 26 kg  $\text{kg}^{-1}$  N applied, as reported in Table 5) was within the range previously reported by Ladha et al. (2005) but the average NUE was lower. Our average NUE was also lower than that reported by Uribelarrea et al. (2007); their mean NUE averaged 24 kg grain  $\text{kg}^{-1}$  N. However, maize NUE can be expected to decline when N fertilizer rates exceed 200 kg N  $\text{ha}^{-1}$  (NUE < 23 grain  $\text{kg}^{-1}$  N applied; Dobermann and Cassman, 2004), just as we investigated in half of the plots receiving side-dress N in our study. In addition, in prior research involving similar plant density and N rate combinations with other maize hybrids, Boomsma et al. (2009) observed that, NUE was consistently highest at the medium N rate (165N) combined with either high plant density (104,000 pl  $\text{ha}^{-1}$ ) in two of the three years, or the intermediate plant density (79,000 pl  $\text{ha}^{-1}$ ) in the third year. Average NUE in their study was approximately 25 kg grain  $\text{kg}^{-1}$  N; moreover, as it was observed in our research, highest NUE did not necessarily correspond with highest maize grain yields.

Although genotypes, N fertilizer rates, plant density, and environments all influence NUE, the primary factor affecting NUE is the proportional grain yield response to N. Our average NUE of 12 kg grain  $\text{kg}^{-1}$  N is lower than those in other studies mostly because those studies involved higher yield responses to N fertilizer. For instance, Dobermann and Cassman (2004) reported a minimum maize yield gain of approximately 400 g  $\text{m}^{-2}$  for the maximum N rate applied (close to 300 kg N  $\text{ha}^{-1}$ ) relative to the non-N fertilized treatment. Furthermore, Boomsma et al. (2009) reported maize yield gains of approximately 400–700 g  $\text{m}^{-2}$  at intermediate side-dress N rates relative to the control. In contrast, in our research the yield differential from same N treatment combinations as the latter study ranged from 114 to 375 g  $\text{m}^{-2}$ .

In our work, overall NUE was higher for the 2 hybrids at ACRE compared with those at the PPAC location (on average 13.8 and 10.0 kg grain increment  $\text{kg}^{-1}$  N for ACRE and PPAC sites, respectively). Nevertheless, they differed markedly in the strategy employed to achieve it, with ACRE's hybrids having higher NIE (~40 vs. 31 kg grain  $\text{kg}^{-1}$  N uptake for ACRE and PPAC, respectively), and both ACRE and PPAC's hybrids having similar response in NRE (0.33 and 0.32 kg N uptake  $\text{kg}^{-1}$  N applied for ACRE and PPAC, respectively; Table 5). As it was suggested by Uribelarrea et al. (2007), we anticipate that high levels of NRE would be more related with roots (size and activity), and high NIE would be more closely linked to the development of grain components (KN and KW).

When side-dress N was applied, our work showed a rise in NUR, and consequently in NRE, during the period bracketing silking that coincided with the period of increased ear N demand. Our results demonstrated that N deficiencies during this period are detrimental to both NUR and NRE. In addition, changes in grain N concentration and dry matter harvest index at varying N fertilization and plant density levels were reflected in the final NIE attained for maize crop. This result suggests that NIE varies considerably under different environmental conditions or management practices (~12.8–56.4 kg grain  $\text{kg}^{-1}$  N uptake; Table 5). Moreover, relative variability is even greater in the NRE (~0.18–0.53 kg N uptake  $\text{kg}^{-1}$  N applied; Table 5). The latter suggests that maize breeding efforts should perhaps focus on both components, NIE and NRE to achieve gains in NUE. In the study reported by Ladha et al. (2005), the average NRE was around 0.45 kg N uptake  $\text{kg}^{-1}$  N applied, with a minimum of 0.1 and a maximum of 0.7 kg N uptake  $\text{kg}^{-1}$  N applied. In our research, we observed a range from 0.18 to 0.53 kg N uptake  $\text{kg}^{-1}$  N applied (Table 5). As was the case in NUE, in general terms the highest values observed for both NRE and NIE occurred with the use of low N rates. However, if we take into account the maximum value observed by Ladha et al. (2005), there is genetic variability that warrants continued striving for improvement of both NRE and NIE components at breeding and cropping system levels.

## 5. Conclusions

Nitrogen side-dress applications (165–330N) were much more effective in increasing grain yield, relative to zero sidedress N, as plant density increased in all 4 hybrids evaluated. Total plant N uptake ranged widely, but responded positively to both N rate (as expected) and plant density. Clearly, the dominant influence of plant density and N rate was on total BM gain and N uptake achieved by maize plants in the time period from silking until physiological maturity, rather than in the partitioning components, grain HI and grain NHI. Additionally, plant density and N rate treatments impacted both important grain component factors of KN and KW, but KN proportionately more than KW.

Our tentative answer to the first question (“Does shoot growth potential or leaf area expansion drive N uptake during vegetative stages?”) is incomplete because under 0N, crop N uptake was not mainly driven by increases in LAI and crop N uptake may have been more limited by other factors that we did not measure (such as root activity or size). However, with N added (165–330N) crop N uptake was predominantly determined by the total aboveground BM accumulation up to the R1 stage.

Our tentative answer to the second question (“Does the proportionality of the ratio between N uptake and LAI remain constant when both total N uptake and LAI are themselves affected by plant density and N rate?”) is that, even when our approach was a static one (because we only evaluated this ratio at the R1 stage), the proportionality is not constant because both plant density and N rate exerted a large influence on the N uptake to green LAI ratio. From this result, one question that could be proposed is: “What will the optimum point be for this ratio (from economical, agronomical and environmental perspectives)?”. The answer to this question is being pursued in our subsequent research projects.

Regarding the third question (“Is there any relationship between the crop growth rate and the N uptake rate during the period bracketing silking and, does this association change with different N rates, plant densities and hybrid factors?”), we conclude that the highly significant relationship between NUR and CGR was most dependent on the contrasting N rates (0N vs. 165–330N) regardless of the plant density, hybrid/location factors. From this result, we suggest that NUR was driven by the potential sink capacity, which was itself modified by the CGR during the critical period bracketing silking (from V14 until R3 in this research).

Our last question (“Do plant density and N rate management practices most strongly impact aboveground biomass (BM) productivity and total N uptake, or the partitioning of dry matter and N to the grain?”) can be answered by pointing to the greater influence exerted by N rate and plant density on overall BM and N uptake, versus the smaller influence on the relative partitioning of these components to the grain.

Overall, average NUE was similar at both locations (12 kg kg<sup>-1</sup>), but its variation with different N rates and plant densities was explained by treatment influences on both internal and recovery efficiency components. Because highest NUE was not automatically associated with the highest maize yields, progress in breeding selection for improved NUE is inherently more difficult. A quantitative and comprehensive framework for N demand of vegetative and reproductive plant parts at different development stages is required to fully understand the factors governing the N dynamics within the maize plants, and to improve both NUE and maize productivity for future generations.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fcr.2010.10.009.

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