

# Potential Physiological Frameworks for Mid-Season Field Phenotyping of Final Plant Nitrogen Uptake, Nitrogen Use Efficiency, and Grain Yield in Maize

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## ABSTRACT

Improved phenotyping tools for simultaneously characterizing maize (*Zea mays* L.) genotypes with superior grain yield (GY) and N use efficiency (NUE) would be beneficial for breeding progress. Possible phenotypic predictors of the crowding intensity and N availability effects on maize plant N uptake, GY, and NUE were evaluated for different genotypes in two environments. Our objectives were to develop phenotyping framework tools to predict plant N uptake, GY, and NUE via (i) identification of important mid-season morpho-physiological traits (from a total of 80 parameters), (ii) assessment of correlations between predictive traits (principal component analyses identified 21 traits), and (iii) arrangement of key traits into sequential pathways of mechanistic functions (3 traits). Plant phenotyping measurements taken during vegetative stages were poor predictors of GY and NUE. Plant N status at silk emergence was strongly associated with grain components. At silking, the chlorophyll contents (Soil Plant Analysis Development [SPAD] readings) were highly correlated to leaf N concentration, and the latter with the N nutrition index (NNI). As expected, NNI fairly reflected plant N uptake at silking and correlated well to relative GY. Maize plant biomass and N uptake at maturity were predicted via stem volume estimation at silking. The latter predictive model accurately simulated both GY and NUE in other field experiments. Physiologically based frameworks for mid-season prediction of maize GY and NUE require further testing but hold promise.

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**Abbreviations:** %N, N concentration; %N<sub>c</sub>, critical N concentration; 0N, 0 kg N ha<sup>-1</sup>; 90N, 90 kg N ha<sup>-1</sup>; 112N, 112 kg N ha<sup>-1</sup>; 146N, 146 kg N ha<sup>-1</sup>; 165N, 165 kg N ha<sup>-1</sup>; 202N, 202 kg N ha<sup>-1</sup>; 224N, 224 kg N ha<sup>-1</sup>; 330N, 330 kg N ha<sup>-1</sup>; ACRE, Purdue University Agronomy Center for Research and Education; B<sub>E</sub>, ear biomass; BM, biomass; CRM, comparative relative maturity; E, environment; G, genotype; Gl, number of green leaves; GY, grain yield; GY<sub>A</sub>, grain yield per unit area; HI, grain harvest index; Kn, kernel number; Kw, kernel weight; LAI, leaf area index; LAI<sub>d</sub>, reduction in leaf area index expressed as a proportion of the maximum attained at silking time; N<sub>E</sub>, ear N uptake; NIE, N internal efficiency; NLAI, N content per unit of leaf area index; NNI, N nutrition index; NRE, N recovery efficiency; NUE, N use efficiency; PCA, principal component analysis; PH, plant height; RGY, relative grain yield; RSDR, robust standard deviation of the residuals; STD, stem diameter; Sink:Source, relationship between the final kernel number (sink) achieved at R6 stage relative to the leaf area index (source) at R1 stage; SPAD, Soil Plant Analysis Development.

THE three-way interaction of genotypes, environment, and management practices (genotype [G] × environment [E] × management practice [M]) (Messina et al., 2009) is highlighted in the diverse tolerances and yield responses of modern maize genotypes to specific abiotic and biotic stresses under varying management practices and environments. During the last century,

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productivity improvements in maize have been attributed to farmer adoption of changes in genetics (from double- to single-cross hybrids), overall management practices (irrigation, nutrient management, conservation tillage, planting dates, soil testing, and integrated pest control), and transgenic pest resistance and herbicide tolerance technologies (CAST, 2006). The conjunction of all these changes allowed maize productivity in the United States to rise from approximately 4 Mg ha<sup>-1</sup> in the 1960s to approximately 9 Mg ha<sup>-1</sup> in 2011 (USDA, 2012).

Breeding progress for maize grain yield (GY) was achieved, in part, through an increase in the kernel number (Kn) per unit area (Tollenaar et al., 1992; Edmeades et al., 2000; Duvick et al., 2004; Duvick, 2005), but the kernel weight (Kw) influence cannot be overlooked (Borrás and Gambín, 2010). In addition, direct breeding progress in GY has also indirectly impacted the N use efficiency (NUE) (Moose and Below, 2008; Ciampitti and Vyn, 2012). Future improvement in maize GY and NUE might also benefit from direct selection for “phenotypic traits” that govern physiological processes (Donald, 1968) but only if sufficiently predictive physiological assessment tools are made available. In that regard, combined source–sink evaluations are important for future maize progress, and incremental gains in resource capture and efficiency (“source”) should be coupled with a larger sink capacity to allocate additional plant-acquired resources (Tollenaar and Lee, 2011).

Total maize N uptake over the entire growing season is dependent on management practices such as plant density and N fertilization rate (Lemcoff and Loomis, 1994; Ciampitti and Vyn, 2011). At low N supply, reduced plant growth rate and partitioning to reproductive structures during the period bracketing silking were accompanied by a low N uptake rate (Ciampitti and Vyn, 2011). Low Kn was observed under the combined stresses of crowding intensity and N deficiency (Lemcoff and Loomis, 1994) but for apparently dissimilar reasons. When the crowding intensity was intensified, yields were limited more by the failure of grains to establish, yet when the primary stress was low N supply, yields were limited by delays or failure of silk emergence (Lemcoff and Loomis, 1994). Low N and C levels around the period bracketing silking (e.g., due to abiotic stresses) can exert a substantial impact on GY and its components (Jacobs and Pearson, 1991; Lemcoff and Loomis, 1994; Ciampitti and Vyn, 2011).

Advances in the physiological understanding of the interacting effects of hybrids, plant densities, and N rates are possible through improved awareness of the morpho-physiological parameters most relevant to achieving gains in maize GY and NUE. In this context, a morpho-physiological trait selected to construct a physiological framework should be sufficiently predictive as to be used as a “physiological marker” for the selection process in maize breeding programs. Some common morpho-physiological traits used previously

when phenotyping for hybrids and inbreds were stem diameter (STD), chlorophyll content (Soil Plant Analysis Development [SPAD] measurements), plant height (PH), biomass (BM), and N accumulation, partitioning indices, leaf area index (LAI), number of green leaves (Gl), and GY and its components among others (Greef, 1994; Bänziger and Lafitte, 1997; D’Andrea et al., 2006; Cirilo et al., 2009). The incorporation of a measurement for the efficient use of N, such as that based on GY per unit of N fertilizer, is needed (Moll et al., 1982). Despite numerous reports of individual or multiple plant phenotype relationships to final maize GY or NUE outcomes, few studies are published showing a functional and comprehensive approach in using those tools at mid season (before or at silk emergence) for predicting future plant behavior related to GY, plant N uptake, and NUE. Earlier and more accurate predictions can speed up the phenotyping process (discarding bad phenotypes and selecting for superior materials) for testing hybrids as well as inbreds.

The primary objective of this work was to develop conceptual frameworks for the mid-season estimation of the final plant N uptake, GY, and NUE. Two different phenotyping frameworks were investigated following the determination of the associations of multiple phenotyping parameters during vegetative and reproductive stages to final GY and NUE responses to a wide range of plant density and N rate treatments in multiple hybrids. One framework related to the estimation of plant BM through the determination of the stem diameter and plant height by silk emergence, and the second one related to the estimation of the leaf N concentration (leaf N concentration [%N]) at canopy level with the determination of the SPAD measurement at leaf level. The statistical approach proceeded logically in a step-like fashion from a more complex analysis to a final simple validation. Briefly, those steps were to (i) evaluate the morpho-physiological traits associated with maize GY and plant N uptake differing in environments, plant density, and N supply (from a total of 80 parameters), (ii) assess correlations among predictive traits (identified through principal component analysis [PCA]) and (iii) arrange key physiological traits into a sequential pathway of mechanistic functions for the estimation of N uptake and GY and then, by autocorrelation, to quantify the NUE parameter.

## MATERIALS AND METHODS

### Management Practices, Experimental Design, and Treatments

The approach is novel in that it builds a physiological framework from previously published information such as plant BM, N uptake, and GY (Ciampitti and Vyn, 2011) and then validates the framework with additional datasets from studies conducted at the same locations.

A brief description of the experiment is presented in this section; further details of site characterization (soils, slope, and

nutrient content), management practices (planting, harvest, and phenological measurement dates, etc.), and experimental conditions at the two sites evaluated can be reviewed at Ciampitti and Vyn (2011). One site was located at the Purdue University Agronomy Center for Research and Education (ACRE) (soil type, Typic Endoaquoll) near West Lafayette, IN, and the second site was located at the Pinney-Purdue Agricultural Center (soil type, Typic Argiaquoll) near Wanatah, IN. In each site, the study was arranged as a split-split plot design with six blocks. The 18 treatments evaluated in these experiments come from the combination of two hybrids (the main plot), three plant densities (54,000, 79,000, and 104,000 plants ha<sup>-1</sup>) (the sub-plot), and three side-dress N rates (0, 165, and 330 kg N ha<sup>-1</sup>) (the sub-sub-plot). All three N rates included a starter N application (25 kg N ha<sup>-1</sup>) applied at planting.

## Morpho-Pheno-Physiological and Agronomic Measurements

Individual plants were tagged (30 plants per plot for approximately 3240 plants in total for both sites) in nondestructive areas for each treatment combination. Maize phenology was tracked from V5 to R6 for tagged plants in each plot. Various morpho-physiological measurements were taken primarily at V10 and V14 stages (vegetative period) and at R1, R3, and R6 stages (reproductive period). The PH parameter was recorded at V10, V14 (measured from the stem base to the uppermost developed leaf tip), R1, R3, and R6 (measured from the stem base to the collar of the uppermost leaf) stages. The STD variable was measured using a Mitutoyo ABSOLUTE Digimatic caliper (Mitutoyo America Corporation) at V14, R1, R3, and R6 stages (i.e., by recording maximum diameter at the middle of the sixth internode). The SPAD measurements were determined using the Konica Minolta SPAD-502 Chlorophyll Meter (Konica Minolta Sensing Americas, Inc.) at V10, V14, R1, R3, and R6 phenological stages; three readings were obtained on each uppermost developed leaf (vegetative stages) or earleaf (reproductive stages). The LAI estimates at R1 stage (via leaf area meter, Model LI-3100, Li-Cor, Inc.) were derived from Ciampitti and Vyn (2011). Declination of LAI during the postsilking period was estimated from the Gl measurements (>50% of the leaf area was green) from tagged plants five times during the grain filling period (Ciampitti and Vyn, 2011). In addition, the anthesis-silking interval was measured in all plots, but due to inconsistencies in the proportion of developed tassels actually shedding pollen, this parameter is not reported.

Data for plant BM and N uptake at different phenological stages (V14, R1, R3, and R6 stages) and GY and its components at maturity were incorporated into the statistical analysis and used to enhance the conceptual physiological framework. Details about plant BM, N uptake, and GY and its components for these field studies are found in Ciampitti and Vyn (2011).

The NUE was calculated from the multiplication of its main components, the N internal efficiency (NIE) and N recovery efficiency (NRE). As a result, NUE was determined as the ratio of GY to N applied (note that, in the 0 kg N ha<sup>-1</sup> [0N] plot, starter N fertilizer equaled 25 kg N ha<sup>-1</sup>). For this reason, the NIE and NRE were calculated as

$$\text{NUE (g g}^{-1}\text{)} = [\text{NIE} = \text{GY}_A / \text{Nupt}] \times [\text{NRE} = (\text{Nupt} / \text{Nfert})],$$

in which GY<sub>A</sub> is the GY per unit area (g m<sup>-2</sup>), Nupt is the plant N uptake (g m<sup>-2</sup>) at R6 stage, and the Nfert is the sum of the starter N and the N side-dress applications (g m<sup>-2</sup>). As a precondition for derivation of biologically meaningful NUE, a treatment-specific boundary was set such that the maximum NRE never exceeded the N rate applied.

Furthermore, the reduction in leaf area index expressed as a proportion of the maximum attained at silking time (LAId) (from R1 to R3) (%), during the postsilking period, was calculated with the following ratio:

$$\text{LAId (\%)} = [(\text{LAI at R1} - \text{LAI at R3}) / \text{LAI at R1}] \times 100,$$

in which LAI is the leaf area index (m<sup>-2</sup> m<sup>-2</sup>) measured at silking (R1) and milk stage (R3).

Following a similar reasoning, the proportional increase in ear N content [N<sub>E</sub>(%)] from R1 to R3 with respect to the total ear N uptake (N<sub>E</sub>) achieved at (R6) was determined as

$$\text{N}_E(\%) = [(\text{N}_E \text{ at R3} - \text{N}_E \text{ at R1}) / \text{N}_E \text{ at R6}] \times 100,$$

in which N<sub>E</sub> is the proportion of N accumulated in the ear (%) calculated at silking (R1), milk (R3), and at physiological maturity (R6) stages.

To investigate the relative proportions of the plant BM and N uptake partitioned to the ear organ, the ratio ΔB<sub>E</sub>:ΔN<sub>E</sub> at R1 stage was determined as

$$\Delta B_E : \Delta N_E \text{ (dimensionless)} = [(B_E / \text{BM}) / (N_E / N_t)],$$

in which B<sub>E</sub> is the ear biomass, BM is the plant BM (aboveground), N<sub>E</sub> is the N accumulated in the ear, and N<sub>t</sub> is the N taken up by the plant (g m<sup>-2</sup>) at the R1 stage.

The N nutrition index (NNI) was calculated to evaluate the N status within the plant at V14, R1, R3, and R6 stages. The NNI was determined as the ratio of the actual N concentration to the critical N concentration (%Nc), and the latter was calculated as

$$\%Nc \text{ (g g}^{-1}\text{)} = 3.4 \times \text{BM}^{-0.37},$$

in which BM is the plant BM ranging from 1 to 22 Mg ha<sup>-1</sup> (Plénet and Lemaire, 2000). The NNI for maize crop was first proposed by Lemaire et al. (1996) and, more recently, by Ziadi et al. (2008a, 2008b, 2009) as a reliable index of the N stress level.

Information regarding the plant N uptake to the green LAI ratio (g N m<sup>-2</sup> LAI) at silk emergence, understood as the capacity of the plant to store N per unit of green LAI, was used from Ciampitti and Vyn (2011) and included in the statistical analyses. Lastly, an indicator of the sink strength relative to source supply during silking was quantified as the ratio of Kn at R6 to the green LAI at R1.

## Physiological Frameworks

Two conceptual physiological frameworks were developed to capture the functionality of plant N uptake and GY formation processes. Both physiological frameworks estimated plant N uptake at silk emergence with the intent of correlating N status with final GY. The latter relationship was evident in a recent large-scale review by Ciampitti and Vyn (2012). For the first physiological framework, data from three growing seasons were used to develop and calibrate the model. The SPAD values collected at R1 stage for the 2009, 2010, and 2011 seasons

(Ciampitti and Vyn, 2011; Ciampitti, 2012) were correlated with the leaf %N (at canopy level) at R1 stage (for 2010 and 2011) or at V14 stage (in 2009, when no leaf %N at R1 stage was available). Except for 2009, all the experiments (four site-years in the 2010/2011) involved the same hybrids, densities, and N rate levels (further details are presented in this section). The framework relied on NNI estimation from the knowledge of the leaf %N at the canopy level. Next, plant N uptake was estimated by autocorrelation with the NNI because both parameters shared the same component (plant %N). Lastly, NNI was associated with the relative GY (RGY), calculated as the ratio of  $GY_A$  for a given treatment with the maximum  $GY_A$ .

The development of the second physiological framework was based on PH and STD measurements at silk emergence. Model development, based on the 2009 data, for predicting both GY and NUE was tested with other experimental data involving maize response to plant density and N rate interactions in the same crop sequence (maize-soybean [*Glycine max* (L.) Merr.]) from the same location or locations in 2007, 2010, and 2011. Data of PH and STD at the silk emergence stage (required for the calibration of model) from the 2007 growing season was cordially provided by Dr. Boomsma (Boomsma et al., 2009). The latter 2007 data involved identical plant densities and N rate treatments (but different hybrids Pioneer 31G68 [2830 growing degree days to R6 and comparative relative maturity {CRM} of 118] and Pioneer 31N28 [2910 growing degree days to R6 and CRM of 119] [Pioneer Hi-Bred International, Inc.]) as those in 2009. Calibration data from 2010 and 2011 experiments conducted at the same two locations by Ciampitti (2012) involved different N rates but equivalent plant densities levels as for the 2009 experiment. Data from the latter four site-years (2010 and 2011 seasons) involved N rates of 0, 112, and 224 kg ha<sup>-1</sup> and the same two hybrids at each site-year (Mycogen 2T789 and Mycogen 2M750, both with similar CRM at 114 d [Dow AgroSciences, Inc., Indianapolis, IN]). Calibration data from a third 2010 field experiment conducted at the West Lafayette location (courtesy of P. Kovacs and T. Vyn, personal communication, 2012) involved N rates of 0, 90, 146, and 202 kg N ha<sup>-1</sup> at one plant density (equivalent to the medium density) and another hybrid (Pioneer 1395XR, a hybrid with CRM of 113).

Except for the 0N treatment, calculations were simply based on interpolation of the slopes and intercepts for each specific N rate. For the 90 kg N ha<sup>-1</sup> (90N), 112 kg N ha<sup>-1</sup> (112N), and 146 kg N ha<sup>-1</sup> (146N) rates, interpolations were calculated using the 2009 season equations for 0N and 165 kg N ha<sup>-1</sup> (165N) as reference whereas, for estimation of the 202 kg N ha<sup>-1</sup> (202N) and 224 kg N ha<sup>-1</sup> (224N) rate formulas, the reference equations were those for the 165N and 330 kg N ha<sup>-1</sup> (330N) treatments from the same year (2009). The resulting equations were the following:

$$NUE = 0.050 \times GY_A + 11, \text{ for } 90N;$$

$$NUE = 0.046 \times GY_A + 12, \text{ for } 112N;$$

$$NUE = 0.040 \times GY_A + 13, \text{ for } 146N;$$

$$NUE = 0.036 \times GY_A + 12, \text{ for } 202N; \text{ and}$$

$$NUE = 0.035 \times GY_A + 12, \text{ for } 224N.$$

For the 2007 calibration data (Boomsma et al., 2009) NUE simulation was limited to the N fertilized treatments (165N–330N), but the lack of plant N uptake values from physiological maturity prevented calculation of the NIE component ( $n = 94$  from 418 data calibration points). For a portion of the 2010 (P. Kovacs, personal communication, 2012) data, the NUE simulation was evaluated for 90N and 146N, which are both medium N rates, and 202N, which is a high N rate. For the 2010 and 2011 dataset ( $n = 216$ ; Ciampitti, 2012), the calculation of NUE at all N levels was similar to that used for 2009.

## Statistical Analyses

A PCA was performed to identify patterns or correlations among traits and to select those having the largest impact on plant response. Variables (measured at V14 or R1 stage) with a predictive value for N uptake and grain yield were emphasized due to our goal of finding morpho-physiological traits for predicting phenotyping purposes. A biplot graph was constructed by plotting the symmetrically scaled components (components 1 and 2) obtained via the PCA (Fig. 1) and the most responsive trait(s) for that specific combination were identified. Analyses were conducted using the R program (R Development Core Team, 2009).

Models were fitted with GraphPad Prism 4 software (Motulsky and Christopoulos, 2003) using the equation  $y1 = I1 + B1x + B2x^2$  (Fig. 2). Models were selected by comparing independent fits with a global fit. All parameters were selected to test whether one curve fitted the entire data. Similar procedures guided development of the frameworks. In addition, quantile regression (the R program [R Development Core Team, 2009]) was used to estimate quantiles and interquantile ranges (Koenker, 2005) for the RGY and the NNI relationship (Fig. 3D).

For the validation procedure, the observed versus simulated data points for the maize  $GY_A$  and NUE relationships were fitted to a 1:1 line and lines within  $\pm 20\%$  of the measured values define a “boundary region.” For the outlier determination and detection, the robust standard deviation of the residuals (RSDR) was calculated (Motulsky and Brown, 2006).

## RESULTS AND DISCUSSION

### Treatment Factors and Morpho-Physiological Traits

The PCA (dimensionality reduction) suggested that there were 21 significant variables (predictive and physiologically meaningful) from the 80 traits measured. A synthesis of the morpho-physiological traits selected (Table 1) are presented as means across hybrids and sites. The PCA method explained approximately 60% of the total variation using just two components (Fig. 1).

The biplot graph confirmed that GYs in 2009 were more influenced by plant density and N rate than by hybrids and environments (Fig. 1). Use of more contrasting hybrids (e.g., divergent grain %N, grain harvest index [HI], GY at low N, and N responsiveness) or environments (soils and climate) would naturally change the relative factor influences on GY. In our case,  $GY_A$  improved as both plant density and N rate increased (Table 1). According to the biplot,

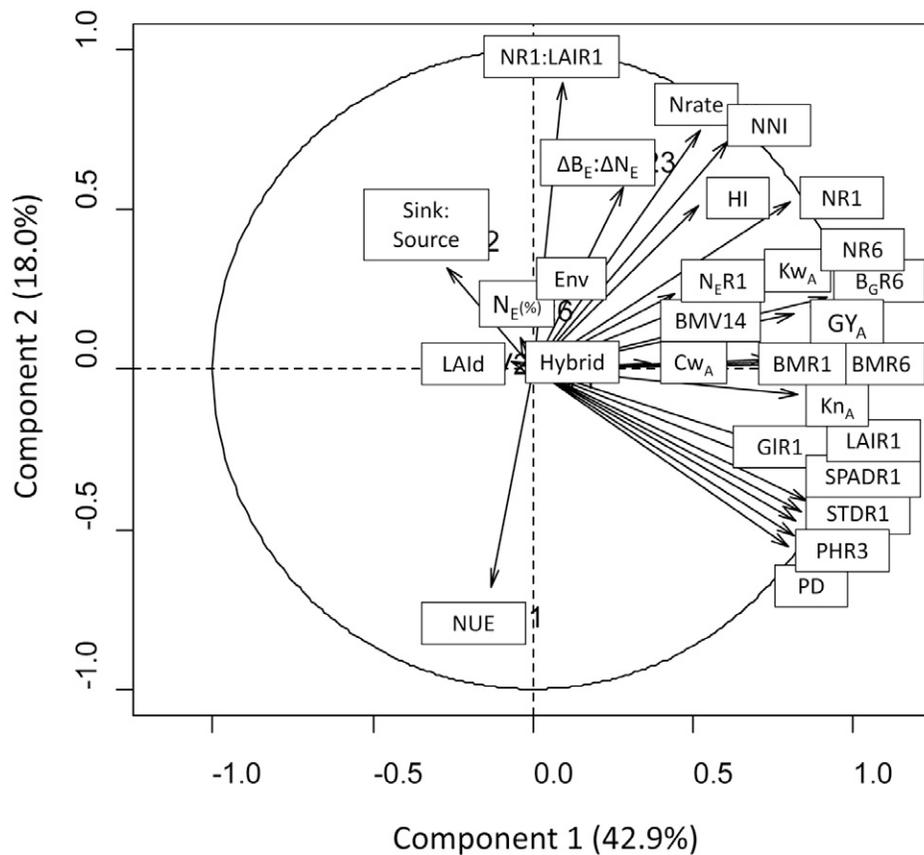


Figure 1. Response variable by morpho-physiological trait biplot derived from principal component analysis of grain yield per unit area ( $GY_A$ ) basis, N use efficiency (NUE) ( $GY_A$  per unit N applied), and plant N uptake (at R1 [NR1] and at R6 [NR6] stages) at two environments (Env) (Purdue University Agronomy Center for Research and Education and Pinney-Purdue Agricultural Center sites), four hybrids (Hybrid) (Mycogen 2M749, 2M750, 2T780, and 2T787), three densities (PD) (low, medium, and high plant density: 54,000, 79,000, and 104,000 plants  $ha^{-1}$ , respectively), and three N rates (Nrate) (0, 165, and 330 kg N  $ha^{-1}$ ) for maize during the 2009 season.  $N_{E(R1)}$ , ear N uptake at R1 stage;  $GIR1$ , number of green leaves per plant at R1 stage; SPADR1, Soil Plant Analysis Development units at R1; SDR1, stem diameter at R1 stage; PHR3, plant height at R3 stage; BMV14, BMR1, and BMR6, plant biomass at V14, R1, and R6 stages, respectively, expressed per unit area; Sink:Source, relationship between the final kernel number (sink) achieved at R6 stage relative to the leaf area index (source) at R1 stage, expressed per unit area; LAId, reduction in leaf area index expressed as a proportion of the maximum attained at silking time (from R1 to R3 stages); HI, (grain) harvest index;  $Cw_A$ , per unit area cob weight;  $Kn_A$ , per unit area kernel number;  $Kw_A$ , 1000 kernel weight adjusted by the plant density (per unit area);  $BGR6$ , grain biomass at R6 stage, expressed per unit area;  $N_E(\%)$ , proportional increase in ear N content (from R1 to R3 stages);  $\Delta B_E:\Delta N_E$ , [(ear biomass/plant BM)/(ear N uptake/plant N uptake)]; LAIR1, leaf area index at R1 stage; NNI, N nutrition index.

hybrids responded to treatments similarly regardless of the environments, and each environment resulted in similar GY range regardless of hybrids evaluated (no changes in ranks in the  $G \times E$  plot). Therefore, the emphasis in the discussion that follows is on the plant density and N rate interactions (the two important management practice factors).

From the PCA, one of the most striking results was the strong association documented between plant N uptake at silking and the GY at maturity (Fig. 1). Additionally, the evaluation of morpho-physiological plant traits before silk emergence showed either poor GY or NUE prediction power (confirming the challenge and futility of early-stage phenotyping). Plant BM, N uptake, and GY and its components ( $Kn$ ,  $Kw$ , and cob weight) were all reduced as crowding intensity and N deficiency intensified (Table 1). Similar effects of plant density and N rate on GY were

previously documented by Lemcoff and Loomis (1986, 1994), Camberato (1987), and Boomsma et al. (2009). The HI response followed a similar trend (Table 1). Nitrogen partitioning to the grain (N harvest index) did not present any evident trend. The ratio of N uptake presilking versus cumulative postsilking was lowest at low plant density and with 0N level (0.56), suggesting lower N uptake during the reproductive period (Table 1). Further details for the abovementioned traits at different stages, sites, and hybrids can be reviewed at Ciampitti and Vyn (2011).

Little association seemed apparent between the  $GY_A$  and NUE (Fig. 1), confirming that highest NUE is not automatically related to high  $GY_A$ . Additionally, the NUE showed weak correlation with the other parameters (Fig. 1). Under severe N deficiency, NUE was higher but the N content per unit of LAI (NLAI) was reduced (Table 1).

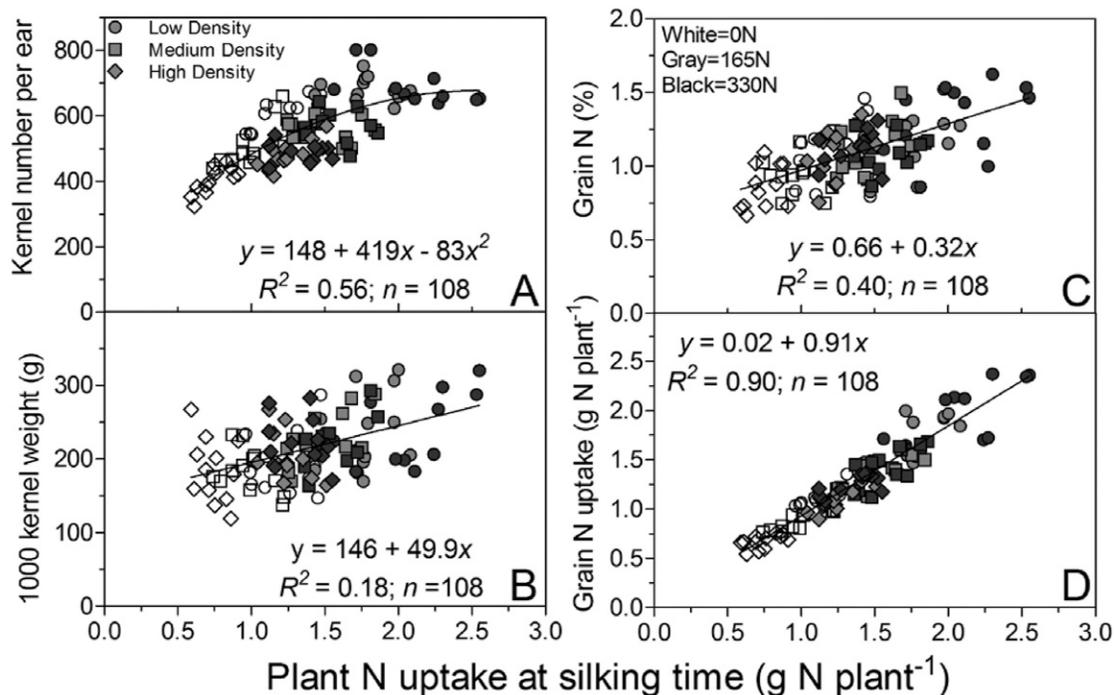


Figure 2. Relationship between the number of kernels per single ear (A), 1000 kernel weight (g) (B), the grain N concentration (C), and the per-plant grain N uptake (g N per plant) (D), all measured at the physiological maturity stage, all relative to per-plant N uptake at the silking time (g N per plant). White refers to the 0 kg N ha<sup>-1</sup> (0N) plots (low N), gray to the 165 kg N ha<sup>-1</sup> (165N) (medium N) rate, and black to the 330 kg N ha<sup>-1</sup> (330N) (high N) rate. Circles refer to 54,000 plants ha<sup>-1</sup> (low plant density), squares to 79,000 plants ha<sup>-1</sup> (medium plant density), and diamonds to 104,000 plants ha<sup>-1</sup> (high plant density).

The NLAI was similar within an N rate across plant density levels, but the highest N rate showed superior NLAI ratio, presumably via (luxury N uptake at similar LAI levels; Table 1). Variation in the NLAI ratio at equivalent LAI was well documented by Lemaire et al. (2008b); these authors showed contrasting scenarios (two different slopes) with lower plant N content in N limiting versus nonlimiting N environments. Nonetheless, in our research the leaf area ratio (LAI to plant BM ratio) attained at silk emergence clearly changes with plant density. Due to lack of data for leaf and stem fractions during silking, it is not certain if plant leaf area ratio changes result from modifications in the specific leaf weight (leaf BM per LAI) or in the leaf:stem BM ratio.

The relationship between the final kernel number (sink) achieved at R6 stage relative to the leaf area index (source) at R1 stage (Sink:Source) and proportional LAI reduction (LAId) were neither associated with GY nor with treatment factors. In accordance with our results, only minor changes in the postflowering Sink:Source were documented by Borrás et al. (2003) for three plant density levels (3, 9, and 12 plants m<sup>-2</sup>). The onset of LAId and the leaf senescence processes are programmed by the genetic component (Noodén et al., 1997; Dangl et al., 2000). Leaf senescence rate is known to be affected by both N deficiency and crowding stresses (Eik and Hanway, 1965; Pearson and Jacobs, 1987; Borrás et al., 2003). Although minor differences were evident in the LAId, in absolute terms, greater LAId occurred at highest plant density (Table 1) presumably due to lower assimilate

supply (Tollenaar and Daynard, 1982). Thus, in agreement with Borrás et al. (2003), differences in the Sink:Source were unrelated to the absolute LAI changes observed during the early postsilking period.

Lastly, ear N uptake during early-grain filling period (mg N per kernel) exhibited only small differences among plant densities but substantial increases from approximately 0.21 to 0.31 mg N per kernel in response to N rates between 0N and 330N (Table 1). Little or no variation in N uptake rate responses to varying N supply levels during early grain filling was also observed in sorghum [*Sorghum bicolor* (L.) Moench] (van Oosterom et al., 2010) and wheat (*Triticum aestivum* L.) (Martre et al., 2006). In our research, the ear N uptake represented the cumulative uptake by the grains, husk, and cob. Therefore, higher N reservoir in husk and cobs at higher N levels might have masked the uniqueness of the grain N uptake rate. Both Crawford et al. (1982) and Cliquet et al. (1990) documented that cob, husk, and shank acted as a sink of N, at least until the kernel dough stage, and then became a N source.

Parameters including GI, LAI, SPAD units, PH, and STD are only presented at the R1 stage for the purposes of predicting plant N uptake, GY<sub>A</sub>, and NUE responses at maturity. However, the seasonal trend can be synthesized as a constant decrease (except for the PH) in all these morpho-physiological traits during the postsilking period. Throughout the reproductive period, the GI, LAI, and SPAD units were proportionally more affected by plant density but still showed the N effect (data not shown). Faster decline rates

**Table 1. Summary of descriptive statistics—mean and standard error (within parenthesis)—for plant biomass accumulation (at V14, R1, R3, and R6) expressed in g m<sup>-2</sup>, harvest index, grain yield per unit area and its components expressed on a per plant basis (kernel number, kernel weight, and cob weight), total N uptake accumulation (at V14, R1, R3, and R6) expressed in g m<sup>-2</sup>, dry matter and N harvest indexes, and morpho-physiological traits (number of green leaves, leaf area index [m<sup>-2</sup> m<sup>-2</sup>], chlorophyll content [Soil Plant Analysis Development {SPAD}] units, plant height [cm], and stem diameter [mm]; all these parameters measured at R1 stage) for the combinations of three densities (54,000, 79,000, and 104,000 plants ha<sup>-1</sup>) and three N rates (0, 165, and 330 kg N ha<sup>-1</sup>) calculated as an average of four hybrids at two different locations (2 hybrids within each site) for the 2009 growing season. LAId (%), reduction in leaf area index expressed as a proportion of the maximum attained at silking time (from R1 to R3); N<sub>E</sub>(%), proportional increase in ear N content (from R1 to R3, expressed as a percentage of the maximum achieved at R6); ΔB<sub>E</sub>:ΔN<sub>E</sub>, proportional ratio of ear biomass relative to plant biomass (ΔB<sub>E</sub>) vs. ear N uptake relative to plant N uptake (ΔN<sub>E</sub>) at R1; Nupt:LAI, ratio of the plant N uptake to green LAI at R1 (g m<sup>-2</sup>); Sink:Source, relationship between the final kernel number (sink) achieved at R6 stage relative to the leaf area index (source) at R1 stage; NNI, N nutrition index (actual N concentration:critical N concentration) at R1 stage; NUE, N use efficiency.**

Traits	Low density (54,000 plants ha <sup>-1</sup> )			Medium density (79,000 plants ha <sup>-1</sup> )			High density (104,000 plants ha <sup>-1</sup> )		
	0N <sup>†</sup>	165N	330N	0N	165N	330N	0N	165N	330N
Biomass at V14	562.3 (10.3)	606.5 (12.9)	649.0 (17.6)	558.7 (12.3)	752.2 (24.3)	712.8 (25.1)	688.5 (34.7)	811.0 (24.2)	782.5 (25.6)
Biomass at R1	815.5 (26.5)	910.5 (34.4)	908.2 (52.4)	910.0 (44.5)	1075.0 (22.9)	1080.8 (23.5)	1040.7 (35.9)	1212.5 (21.0)	1252.5 (43.0)
Biomass at R3	1137.0 (32.5)	1333.3 (21.7)	1301.2 (24.3)	1313.2 (39.7)	1505.5 (27.1)	1474.0 (21.3)	1406.2 (38.5)	1707.7 (49.6)	1596.5 (45.2)
Biomass at R6	1417.8 (18.7)	1644.8 (15.5)	1672.7 (15.3)	1659.7 (31.4)	1920.7 (25.3)	2003.8 (38.5)	1822.8 (34.8)	2188.7 (36.9)	2226.2 (13.5)
Harvest index	0.44 (0.01)	0.48 (0.01)	0.48 (0.01)	0.45 (0.01)	0.50 (0.01)	0.50 (0.01)	0.44 (0.01)	0.49 (0.01)	0.51 (0.01)
Grain yield	650.5 (38.0)	866.9 (36.4)	839.3 (40.5)	797.0 (39.3)	1005.0 (38.9)	1066.4 (41.8)	851.5 (47.3)	1133.3 (36.1)	1187.3 (47.6)
Kernel number	605.8 (6.6)	683.0 (10.8)	694.0 (18.6)	504.8 (7.6)	557.3 (9.5)	567.0 (8.7)	402.0 (6.2)	500.8 (8.3)	485.0 (7.3)
1000 kernel weight	193.0 (4.3)	216.5 (4.7)	217.3 (4.3)	189.0 (3.5)	216.5 (3.7)	225.5 (4.1)	188.3 (4.9)	205.5 (4.6)	223.5 (4.1)
Cob weight	21.8 (0.9)	26.3 (0.9)	26.3 (0.8)	14.3 (0.7)	20.8 (0.6)	19.7 (0.8)	15.4 (0.8)	18.3 (0.7)	19.9 (0.9)
Nitrogen uptake at V14	5.8 (0.2)	8.8 (0.2)	9.6 (0.3)	5.9 (0.2)	10.2 (0.4)	10.3 (0.3)	6.3 (0.3)	10.4 (0.3)	11.1 (0.4)
Nitrogen uptake at R1	6.9 (0.2)	10.4 (0.3)	11.5 (0.4)	8.2 (0.3)	12.4 (0.4)	13.1 (0.4)	8.3 (0.3)	13.7 (0.4)	14.6 (0.5)
Nitrogen uptake at R3	8.7 (0.3)	13.1 (0.2)	14.4 (0.2)	10.1 (0.5)	15.2 (0.2)	17.4 (0.4)	9.9 (0.4)	17.4 (0.5)	19.1 (0.6)
Nitrogen uptake at R6	12.4 (0.2)	17.5 (0.1)	19.5 (0.2)	13.1 (0.2)	19.5 (0.2)	21.1 (0.2)	13.6 (0.2)	21.7 (0.3)	23.5 (0.2)
Green leaves at R1	12.7 (0.3)	14.3 (0.1)	13.7 (0.4)	12.7 (0.3)	13.4 (0.4)	13.3 (0.3)	12.3 (0.3)	12.8 (0.3)	13.6 (0.3)
Nitrogen harvest index	0.56 (0.02)	0.57 (0.03)	0.56 (0.03)	0.57 (0.03)	0.58 (0.02)	0.57 (0.02)	0.55 (0.03)	0.56 (0.02)	0.58 (0.03)
Leaf area index at R1	3.2 (0.1)	3.4 (0.1)	3.5 (0.1)	4.1 (0.1)	4.6 (0.1)	4.7 (0.1)	4.8 (0.2)	5.5 (0.1)	5.5 (0.1)
SPAD at R1	52.3 (0.8)	56.1 (0.7)	56.0 (0.7)	49.5 (1.0)	52.4 (0.9)	53.8 (1.0)	45.6 (0.9)	50.3 (1.1)	50.4 (1.3)
Plant height at R1	198.9 (3.9)	209.2 (8.2)	207.7 (5.1)	202.8 (6.8)	203.7 (5.3)	205.9 (5.1)	198.5 (7.2)	203.5 (4.0)	203.3 (4.5)
Stem diameter at R1	28.1 (0.6)	29.1 (0.7)	30.1 (0.5)	24.6 (0.5)	25.8 (0.4)	26.0 (0.4)	22.3 (0.3)	23.1 (0.3)	23.3 (0.3)
LAId (%)	14.1 (0.9)	11.9 (0.8)	11.6 (0.8)	14.1 (0.7)	9.8 (0.7)	10.0 (0.7)	13.3 (0.6)	11.0 (0.7)	11.6 (0.8)
N <sub>E</sub> (%)	0.42 (0.01)	0.43 (0.01)	0.40 (0.02)	0.44 (0.01)	0.39 (0.01)	0.41 (0.01)	0.52 (0.01)	0.44 (0.01)	0.39 (0.01)
ΔB <sub>E</sub> :ΔN <sub>E</sub>	0.57 (0.01)	0.70 (0.01)	0.73 (0.01)	0.52 (0.02)	0.69 (0.02)	0.69 (0.01)	0.49 (0.02)	0.70 (0.02)	0.68 (0.02)
Nupt:LAI at R1	2.2 (0.03)	3.1 (0.04)	3.3 (0.05)	2.0 (0.03)	2.7 (0.03)	2.8 (0.05)	1.8 (0.02)	2.5 (0.02)	2.7 (0.03)
Sink:Source	1072.2 (11.1)	1176.8 (11.3)	1104.8 (18.1)	1029.1 (12.6)	1014.6 (14.2)	1013.3 (14.4)	954.4 (12.8)	1020.2 (19.7)	967.6 (11.4)
NNI	0.55 (0.01)	0.77 (0.01)	0.85 (0.02)	0.60 (0.01)	0.81 (0.02)	0.86 (0.01)	0.56 (0.01)	0.83 (0.02)	0.87 (0.02)
NUE	52.4 (2.9)	49.5 (2.2)	25.4 (1.2)	60.6 (2.7)	51.6 (2.1)	32.3 (1.3)	62.6 (3.5)	52.5 (2.3)	36.0 (1.4)

<sup>†</sup>0N, 0 kg N ha<sup>-1</sup>; 165N, 165 kg N ha<sup>-1</sup>; 330N, 330 kg N ha<sup>-1</sup>.

in all three parameters were documented as the crowding intensity and N deficiency stresses intensified. Additionally, the peak value for all traits was mostly achieved at R1 stage (greatest treatment differences). Plant-level investigations for these parameters and also GY were then pursued to select the individual parameters for the physiological framework.

### Presilking N Status Effect on Maize Grain Yield and its Components

The PCA confirmed that plant N uptake at silking stage was not only highly correlated with GY but also with NNI (at R1 stage) and HI (Fig. 1). In addition, an association was observed between the N status at silking time and the grain components (Kn, Kw, and grain %N) and total N uptake at maturity (Fig. 2). Lower Kn and Kw were associated with N deficient environments, but Kw factor responded proportionately less than Kn factor. Additionally, for both Kn and

Kw, as plant N uptake increased, the relationship was curvilinear, a trend that was more prominent for the Kn parameter (plateau at 1.5 g N per plant at R1; Fig. 2A and 2B). Low levels of per-plant N uptake (<1 g N per plant) were correlated with low N supply environments (0N), resulting ultimately in low grain %N and final per-plant grain N uptake (Fig. 2C and 2D). Highest %N was reached with the 330N rate at the low plant density treatment. Such presilking N uptake influences on Kn and the grain N were previously reported (Lemcoff and Loomis, 1986; Plénet and Cruz, 1997; Uhart and Andrade, 1995). In addition, Ta and Weiland (1992) suggested a minimum ear N supply required for high maize GY.

In maize, the utility of the NNI and the consequent critical %N calculation was first proposed by Lemaire et al. (1996) and then reconfirmed by Plénet and Lemaire (2000). These studies used different site-years, genotypes, and N rates at one plant density. Other authors have also documented

NNI increments as the N rates increased in maize (Ziadi et al. (2008a, 2008b, 2009). In our research, NNI increased as N rates increased (regardless of plant density; Table 1), and it was also consistently lower under 0N for all densities during the entire season (data not shown). Overall, NNI declined with time toward silk emergence and then tended to increase slightly toward maturity (data not shown). Similar NNI trends near silking were documented by Lemaire et al. (1996) and Plénet and Cruz (1997).

Calculation of the NNI index is dependent on the critical N dilution curve determination for the %N<sub>c</sub> at different stages in different crop species (Greenwood et al., 1990; Justes et al., 1994; Lemaire et al., 1996). The question of whether the equation used to calculate %N<sub>c</sub>, and subsequently NNI, is independent of the plant density factor must still be answered. A synthesis analysis was therefore performed to attempt an answer to that question. Data for the plant density and N rate interactions from Ciampitti and Vyn (2011) and Ciampitti (2012) enabled investigation of the correlation between plant %N and plant BM as the crop aged. Treatment mean data were gathered from six site-years (288 data points during the entire maize growing season). An assumption was made that the highest N rate represented the nonlimiting N environments (330N for 2009 and 224N for 2010 and 2011). The equation proposed by Plénet and Lemaire (2000) adjusted well to all data points ( $R^2 = 0.66$ ,  $n = 288$ ) but, as expected (except for the points below 1 Mg ha<sup>-1</sup>), a better fit was obtained with the highest N rate ( $R^2 = 0.75$ ,  $n = 94$ ) regardless of the plant density evaluated (Fig. 3). As maize development progressed, the 0N and medium N treatments fell below the critical N curve, but greatest discrepancy occurred for the most limiting N treatment (0N; Fig. 3). Few treatment means (related to 224N) were above the critical N level. Discrepancies in the fitted critical N dilution curves have also been reported for wheat by Justes et al. (1994) and Greenwood et al. (1990) and to a small extent for forage maize (Herrmann and Taube, 2004). Additionally, in our study, the critical N dilution curve corresponded very well to superior plant BM levels (22–27 Mg ha<sup>-1</sup>) supporting the hypothesis offered by Herrmann and Taube (2004) that the curve proposed by Plénet and Lemaire (2000) can be extended beyond the 22 Mg ha<sup>-1</sup>. From all these considerations, we can answer the question that the N dilution curves were modified most by the N supply with only a minor influence of plant density. Proportionality between the plant %N and BM ratio was maintained within each N rate level evaluated.

## Conceptual Framework for Estimating Plant N Uptake and Relative Grain Yield

The proposed framework construction and parameter validation were based on previously proven concepts (Chapman and Barreto, 1997; Lemaire et al., 2008a; Ziadi et al., 2008a, 2008b, 2009; among others). Three steps were followed in the estimation of plant N uptake. The first

association (Fig. 4A) between the SPAD units (determined at ear-leaf level at silk emergence) and the leaf %N (measured at the canopy level) was based on previous research that conclusively confirmed high correlations for these parameters (Blackmer et al., 1994; Dwyer et al., 1995; Chapman and Barreto, 1997; Wang et al., 2011). Nonetheless, past associations were restricted to individual leaves since SPAD and leaf %N measurements typically involved the same leaf. A sufficiently positive correlation between SPAD units (at leaf level) and leaf %N (at canopy level) will permit a more reliable estimation of the overall canopy-leaf N status. Using six site-years (2009, 2010, and 2011 seasons; Ciampitti and Vyn, 2011; Ciampitti, 2012) a strong correlation was found between canopy-level leaf %N and ear-leaf SPAD units at silk emergence ( $R^2 = 0.67$ ,  $n = 108$ ). In addition, the previous correlation was independent of the site-year, hybrid, plant density, and N rate levels. The high SPAD and leaf %N correlations were plausible due to the stoichiometry relationship (close to 1:1 ratio) between ear leaf %N and canopy leaf %N calculated from different environments and plant densities (Sadras et al., 2000; Drouet and Bonhomme, 1999, 2004). This novel concept allowed the calculation of this step of the framework. However, we acknowledge that SPAD and leaf %N correlations at specific maize stages can also be affected by contrasting water supply environments and perhaps by a wider range of genotypes than those evaluated in our work to date (Schepers et al., 1992, 1996).

The second step in this estimation was to relate canopy-leaf %N with the plant %N (i.e., aboveground plant) at silk emergence. Strong associations have recently been reported between maize NNI and the leaf %N in the uppermost collared leaves (~V12) (Ziadi et al., 2009). Following the previous principle, a strong correlation was found between the NNI and canopy-leaf %N ( $R^2 = 0.76$ ,  $n = 108$ ; Fig. 4B). A similar association has been previously reported for maize, but using the leaf %N per unit of leaf area (specific leaf N) rather than leaf %N (Lemaire et al., 1997), and for perennial grasses, but for leaf %N in the upper layer leaves (Gastal et al., 2001; Farrugia et al., 2004; Gonzalez-Dugo et al., 2005). During the vegetative period, SPAD values (for the uppermost fully developed leaf) correlated as well with the specific leaf N as the leaf %N (Ciampitti, 2012). Nonetheless, as documented by Ziadi et al. (2009), the specific leaf N and NNI association was weakened (data not shown) due to the small range of variation (~1.1 to 2.1 g N m<sup>-2</sup>) as compared with the canopy-leaf %N (~14 to 34 mg g<sup>-1</sup>). Direct correlations of SPAD units with NNI were weaker (data not shown) than those between SPAD units and leaf %N (as observed by Ziadi et al., 2008b) and inconsistent (as reported by Houllès et al., 2007). Following a similar rationale postulated by Lemaire et al. (2008a), a pathway was built to indirectly estimate NNI (as a plant N status

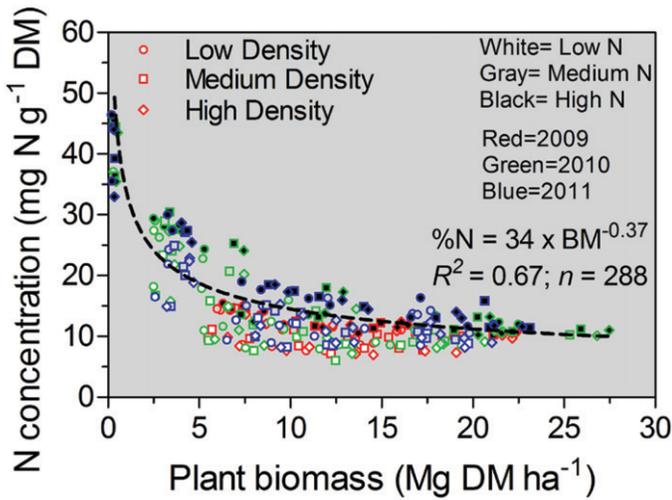


Figure 3 (left). Relationship between plant N concentration (mg N g<sup>-1</sup> dry matter [DM]) and plant biomass (BM) (Mg m<sup>-2</sup>) adjusted to the critical N dilution curve (critical N concentration) = 34 × BM<sup>-0.37</sup> (by Plénet and Lemaire, 2000) across six site-years, hybrids, three plant densities, and three N rates. Dashed line represents the N dilution curve proposed by Plénet and Lemaire (2000) adjusting for all data points presented. When only the black symbols (regardless of plant density) were adjusted to the same equation, the goodness of fit improved to R<sup>2</sup> = 0.75 (n = 94). Circles refer to 54,000 plants ha<sup>-1</sup> (low plant density), squares to 79,000 plants ha<sup>-1</sup> (medium plant density), and diamonds to 104,000 plants ha<sup>-1</sup> (high plant density). White symbols represent the 0 kg N ha<sup>-1</sup> (low N) rate, grey to the 112 or 165 kg N ha<sup>-1</sup> (medium N) rate, and black to the 224 or 330 kg N ha<sup>-1</sup> (high N) rate. Red borders correspond to the 2009, green to the 2010, and blue to the 2011 maize growing seasons. The information is summarized from Ciampitti and Vyn (2011) and Ciampitti (2012). %N, N concentration.

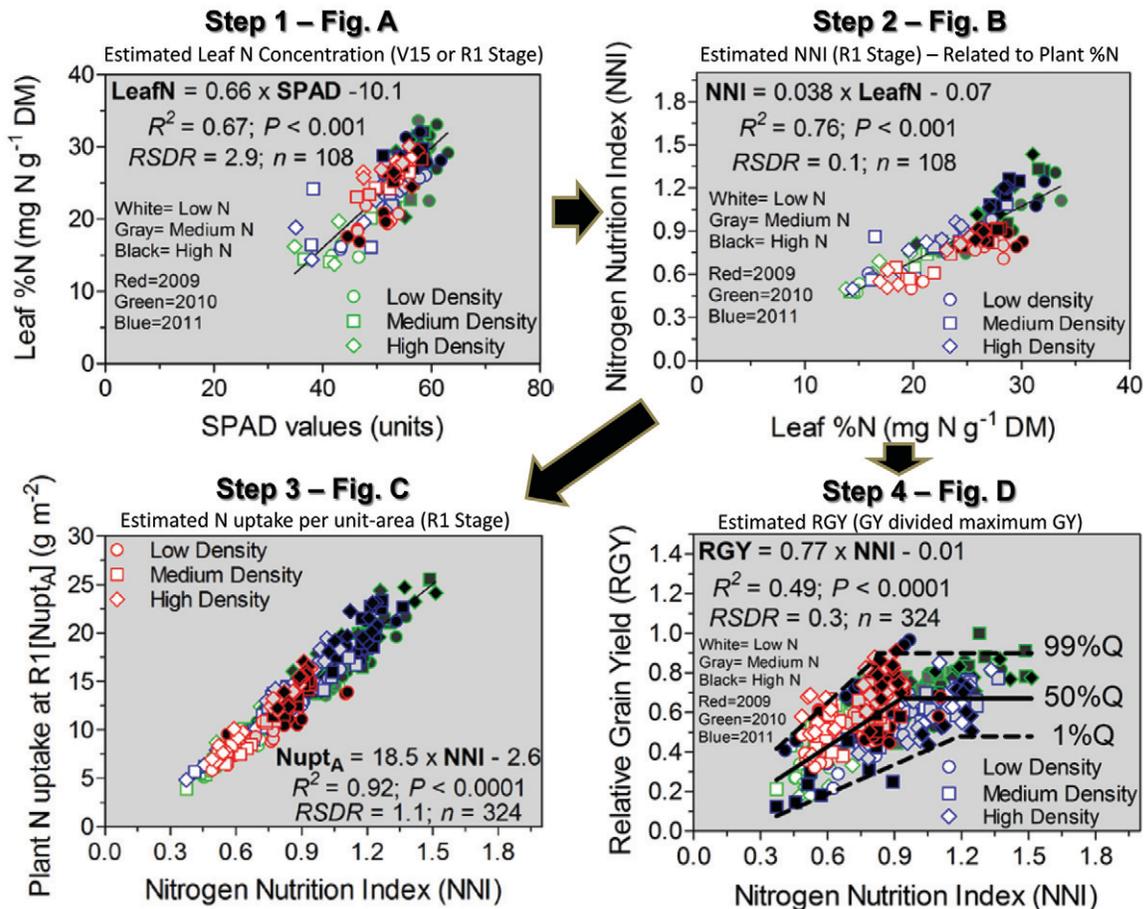


Figure 4. Conceptual framework for the estimation of relative grain yield (RGY) and plant N uptake on a per-unit area basis at R1 stage (silking time) (Nupt<sub>A</sub>) using the Soil Plant Analysis Development (SPAD) values (measured at the ear-leaf) at the silk emergence stage via estimation of leaf N concentration (%N) (Fig. A), N nutrition index (NNI) (Fig. B), Nupt<sub>A</sub> (Fig. C), and RGY (calculated as the grain yield for a given treatment combination divided by the maximum grain yield among all treatments and site-years) (Fig. D). Circles refer to 54,000 plants ha<sup>-1</sup> (low plant density), squares to 79,000 plants ha<sup>-1</sup> (medium plant density), and diamonds to 104,000 plants ha<sup>-1</sup> (high plant density). White symbols represent the 0 kg N ha<sup>-1</sup> (low N) rate, grey to the 112 or 165 kg N ha<sup>-1</sup> (medium N) rate, and black to the 224 or 330 kg N ha<sup>-1</sup> (high N) rate. For all the symbols, red borders correspond to the 2009, green to the 2010, and blue to the 2011 maize growing seasons. 1%Q, 1% quantile line; 50%Q, 50% quantile line; 99%Q, 99% quantile line; DM, dry matter; LeafN, leaf N concentration; RSDR, robust standard deviation of the residuals.

indicator) after canopy-leaf %N was estimated based on ear-leaf SPAD units.

The highest and strongest goodness of fit for the association between NNI and plant N uptake on a per-unit-area basis occurred at silk emergence (Fig. 4C). Using all individual-plot R1-stage data for the six site-years ( $n = 324$ ), the correlation between the plant N uptake (per unit-area) and the NNI was strong ( $R^2 = 0.92$ ) as well as reliable ( $P < 0.0001$ , RSDR = 1.1). The relationship demonstrated independency from the site-year, hybrid, plant density, and N rate factors. A key novel aspect of these investigations is that the proportionality between the NNI and plant N uptake did not change for either plant densities or N rates. However, the latter parameters are not independent because both share the plant %N (and plant BM) as a component. In our case, the NNI versus plant N uptake correlation is only needed to acquire the function required to use NNI as an input to estimate plant N uptake at silking. A similar interrelation between NNI and plant N uptake at silking was reported by Bertin and Gallais (2000).

The estimation of the final GY using the NNI presented a higher goodness of fit when GY was expressed as RGY (Fig. 4D). Accordingly, Ziadi et al. (2008a) also reported a very strong association between RGY and NNI, but in their report the relationship represented the average over all sampling dates (5 timings across the maize growing season). In our case, the NNI levels plateaued at 0.95 (similar to Ziadi et al. [2008a]) but at a RGY close to 0.7 (50% quantile line). The maximum  $GY_A$  value achieved at the individual plot level was close to  $1600 \text{ g m}^{-2}$  (expressed on dry weight basis for the medium plant density and highest N rate treatment combination), and this corresponded with a NNI of approximately 1.3 units. When NNI dropped below 1.0 unit, RGY also declined. It was evident that NNI clearly identified 0N versus N side-dress treatments (Fig. 4D). The lower boundary (1% quantile line) dashed line portrays conditions in which the NNI is maximum at equivalent RGY level and stresses (e.g., heat, drought, nutrient deficiencies, pest pressure, etc.) are restricting grain productivity. In contrast, the upper boundary dashed line (99% quantile line) indicates an environment in which NNI is at the lowest level (restricted by N) and therefore the conversion of N into GY is maximized. The range of NNI values reported in this paper (~0.37 to 1.51; Fig. 4D) is similar to that recorded by Lemaire et al. (1996), Plénet and Lemaire (2000), and Ziadi et al. (2008a, 2008b). Highest NNI suggests occasional luxury N uptake at the medium to high N rates.

### Conceptual Physiological Framework for Phenotyping for Actual Maize Grain Yield and N Use Efficiency

This framework construction began with estimation of plant BM at silk emergence. To pursue this goal, the allometric relationship between the per-plant stem volume (estimated via the cylindrical formula based on PH

and STD, both measured at silk emergence) and the plant BM was determined ( $R^2 = 0.83$ ; Fig. 5A). The latter is in accordance with previous findings (Miles, 1993; Vega et al., 2000; Borrás and Otegui, 2001; Maddonni and Otegui, 2004; Pagano and Maddonni, 2007; D'Andrea et al., 2008). A similar relationship was observed in our research; high correlations were observed regardless of site, hybrid, plant density, and N rate (Fig. 5A). Overall, the stem volume calculation can express potential plant BM at a given phenological stage; moreover, this parameter can facilitate genotypic selection for higher productivity.

Plant N uptake is a controlling factor in determining both LAI and plant BM, but feedback of both LAI and plant BM regulate plant N uptake at different N supplies (Lemaire et al., 2008b). Per-plant N uptake for limiting and nonlimiting N environments was estimated through the plant BM, at R1, resulting in two slopes, with (165N and 330N) and without (0N) N applied (Fig. 5B). Each slope represents a plant %N and shows relative proportional consistency. For the same dataset, a strong plant growth rate and N uptake rate relationship occurred during the period bracketing silking (Ciampitti and Vyn, 2011). Lemaire et al. (2008b) observed a similar trend, with diverse slopes for nonlimiting and limiting N environments in dissimilar maize production environments (France and Australia). Additionally, due to the well documented relationship between maize plant BM and LAI (Plénet and Lemaire, 2000), the association presented in Fig. 5B is related to the plant N uptake and LAI relationship (Lemaire et al., 2008b). Therefore, the N uptake capacity per unit of plant BM was restricted in the most limiting N environment but was only negligibly affected by plant density. Per-unit-area N uptake was calculated from the adjustment of the per-plant N uptake within each plant density level.

Our conceptual framework for simulating GY is fairly robust since its foundation is based on prior documented scientific concepts, and it follows justified steps to simulate plant N content at silk emergence. An evident connection between the present framework and the earlier one proposed for the plant N uptake estimation is that the former bases the estimation on the plant BM simulation while the latter reaches the same goal through the quantification of the NNI.

For the next step, an association was built between the plant N uptake at silk emergence with GY from concepts previously developed (Jacobs and Pearson, 1991; Lemcoff and Loomis, 1994; D'Andrea et al., 2006, 2008, 2009; Ciampitti and Vyn, 2011) but more comprehensively outlined in a recent review paper by Ciampitti and Vyn (2012). The previous research clearly identified the effect of N deficiency around the silking time over the grain components (also documented in Fig. 2). It is noteworthy that these effects were documented for inbreds as well as hybrids under contrasting N rates (D'Andrea et al., 2006, 2008, 2009). A strong correlation was also observed between the maize GY and the plant N uptake (both at 0% moisture; Fig. 5C). From this result, the effect of N status at

## Input Variables

$$\text{Stem volume (SV)} = \pi \times (\text{Stem diameter}/2) \times \text{plant height}$$

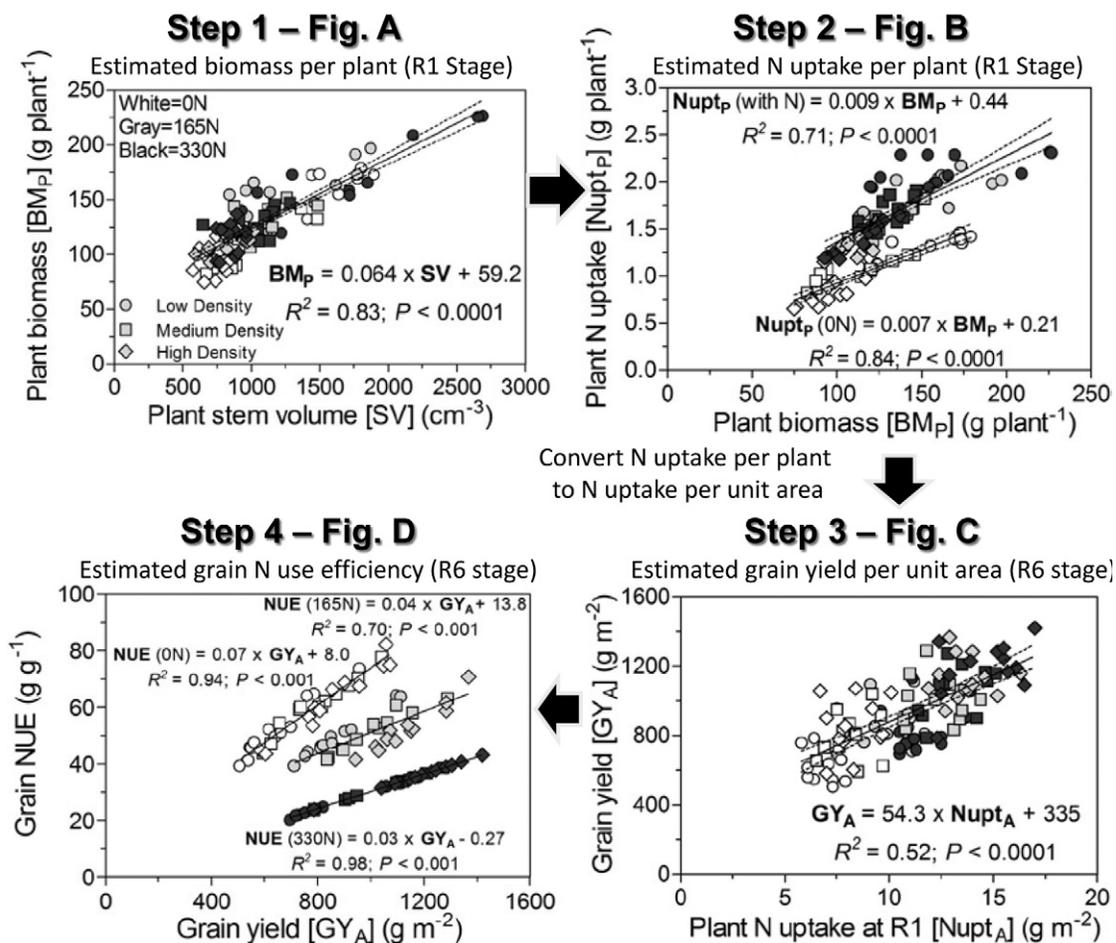


Figure 5. Conceptual framework for the estimation of maize grain yield per unit area ( $GY_A$ ) and grain N use efficiency (NUE) using the stem diameter (maximum diameter at midpoint of the sixth internode) and plant height (distance from soil surface [stem base] to the collar of the uppermost extended leaf) at the silking (R1) stage via estimation of per plant biomass in per-plant basis at R1 stage (silking time) ( $BM_p$ ) (Fig. A), plant N uptake on a per-plant basis at R1 stage (silking time) ( $Nupt_p$ ) (Fig. B),  $GY_A$  per plant N uptake on a per-unit area basis at R1 stage (silking time) ( $Nupt_A$ ) (Fig. C), and NUE for estimated  $GY_A$  at each N rate (Fig. D). White symbols refer to the 0 kg N ha<sup>-1</sup> (low N) rate, gray to the 165 kg N ha<sup>-1</sup> (165N) (medium N) rate, and black to the 330 kg N ha<sup>-1</sup> (330N) (high N) rate. Circles refer to 54,000 plants ha<sup>-1</sup> (low plant density), squares to 79,000 plants ha<sup>-1</sup> (medium plant density), and diamonds to 104,000 plants ha<sup>-1</sup> (high plant density).

silking time over grain components (Fig. 2) and, consequently, on final GY (Fig. 5C) was clearly demonstrated.

The autocorrelation between NUE and  $GY_A$  is acknowledged, but for practical purposes the relationship is just used to predict NUE with the GY parameter. In addition, the proposed NUE term is not a straightforward derivation from the equation GY divided by N applied due to the limitations imposed on the NRE (<1 unit). The framework approach permits estimation of N effectiveness for maize production at different N rates and plant densities from the context of a biologically meaningful efficiency term. It is well known that NUE declines with increasing N supply (Cassman et al., 2003; Ladha et al., 2005) even regardless of plant density (Fig. 5D).

## Simulation and Validation

For the simulation and validation steps, all equations presented in Fig. 5 were combined in a sequential fashion, based on information inputs of STD and PH parameters. A procedure similar to the latter followed using the SPAD values to estimate NNI and plant N uptake and then RGY. Both framework approaches estimate the same parameter. However, because leaf %N at the silk emergence stage was not quantified during the 2009 season, the correlation between leaf %N at V14 and SPAD units at R1 was weaker ( $R^2 = 0.51$ ,  $n = 36$ ; data not shown) as compared to that presented in Fig. 4A. Another advantage of the stem volume estimation approach is that fewer steps are required for estimating plant N uptake (Fig. 5) as compared to when the SPAD measurement is used (3 steps; Fig. 4). Although both models can be

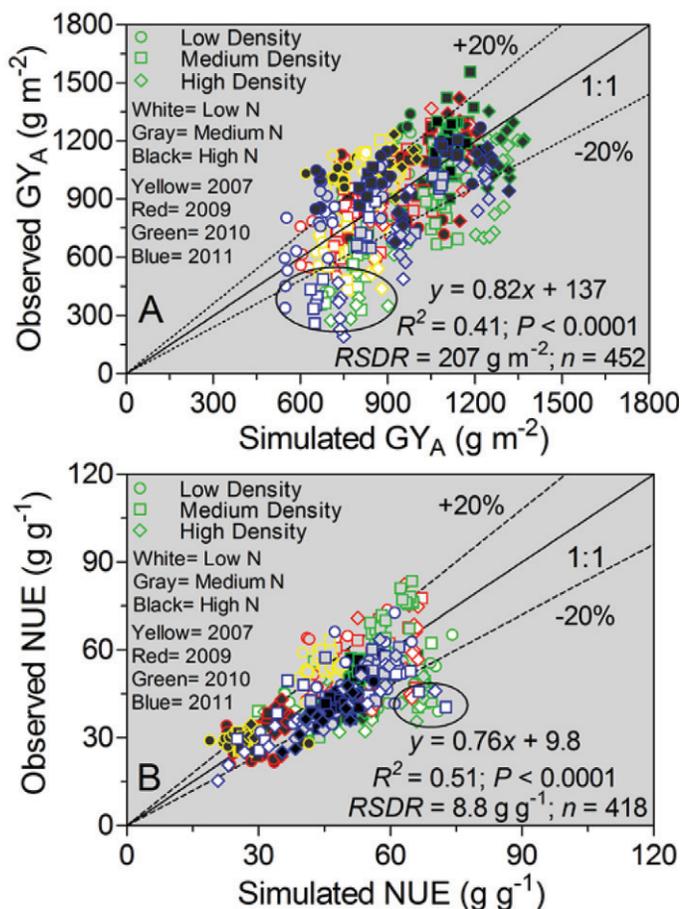


Figure 6. Observed versus simulated maize grain yields (0% moisture) (grain yield per unit area [ $GY_A$ ]) (A) and N use efficiency (NUE) (B) for a test set of different hybrids, plant densities, N rates, and years. Diagonal solid line: 1:1 ratio; dotted lines:  $\pm 20\%$  deviation from 1:1 line. Separate robust standard deviation of the residuals (RSDR) for all plant density and N rate combinations for each simulation are shown. For maize  $GY_A$  (A) and NUE (B), data points within the circle mainly correspond to non-N fertilized treatments, which were strongly affected by abiotic stresses during the postsilking period. Circles refer to 54,000 plants  $ha^{-1}$  (low plant density), squares to 79,000 plants  $ha^{-1}$  (medium plant density), and diamonds to 104,000 plants  $ha^{-1}$  (high plant density). White symbols represent the 0 kg N  $ha^{-1}$  (low N) rate, grey to the 90, 112, 146, or 165 kg N  $ha^{-1}$  (medium N) rates, and black to the 202, 224, or 330 kg N  $ha^{-1}$  (high N) rates. Yellow borders correspond to the 2007, red to the 2009, green to the 2010, and blue to the 2011 maize seasons.

useful, greater accessibility to STD and PH data from other research efforts prompted selection of the conceptual framework in Fig. 5 for validation and calibration.

All equations in Fig. 5 were estimated using the 2009 dataset. The effectiveness of these equations and overall framework to estimate  $GY_A$  and NUE were tested with other datasets (2007, 2009, 2010, and 2011) from the same locations. The latter resulted in reasonable  $R^2$  (0.41 for  $GY_A$  and 0.51 for NUE) and model simulation (62% for  $GY_A$  and 69% for NUE of the predicted data points were within  $\pm 20\%$  boundaries) for all evaluated years, based on STD and PH inputs (measured

at silk emergence; Fig. 6A and 6B). However, the  $GY_A$  was considerably overestimated with low STD and PH values arising from the non-N fertilized treatments for 2010 and 2011 growing seasons (green and blue). The greatest discrepancy in estimation occurred in both seasons at ACRE location, where low GY values for 0N treatments ( $\sim 3\text{--}4$  Mg  $ha^{-1}$ ) were documented. At the same site, the peak value ( $\sim 10\text{--}11$  Mg  $ha^{-1}$ ) corresponded to the medium density and highest N rate combination. Data points outside the  $\pm 20\%$  lines resulted in greater proportion from model overestimation (58% for  $GY_A$  and 64% for NUE; Fig. 6A and 6B).

The physiological framework developed (simply using the STD and PH inputs) was useful to accurately estimate, for different site-years, hybrids, and management practices, the final  $GY_A$  and NUE. However, it should be recognized that one of the limitations of using this simulation model—deliberately circled in Fig. 6A and 6B—corresponds to the estimation of the non-N fertilized treatments at different plant density levels. Another limitation of this simulation model is that the equations are based on maize responses from only one growing season. Nevertheless, this novel model approach was comprehensively validated with several growing seasons, hybrids, densities, and N rates.

## CONCLUSION

The initial PCA provided convincing justification that the maize phenotyping features that mattered to GY and NUE estimation were not those measured during vegetative growth stages but at the silk emergence stage. Early-vegetative-stage phenotyping of the specific parameters reported here, therefore, were less useful as morpho-physiological traits than those determined later. The most striking results that supported the physiological frameworks for the data set of hybrids and conditions used in this study were (i) the use of chlorophyll estimator (SPAD units) to predict the NNI, (ii) the stoichiometry ratio encountered in the ear leaf %N versus canopy-leaf N status before silk emergence, (iii) the indirect association (through the plant BM) between the stem volume and the plant N status before silk emergence, and (iv) the critical and fundamental relationship found between the plant N status prior silk emergence with the GY at physiological maturity (physiological foundation based on the association of the plant N content at silk emergence with grain components—Kn and Kw—and grain N uptake—grain %N). Both conceptual models were successfully related to GY but one with RGY and the other with  $GY_A$  (absolute values). Furthermore, models enabled NUE prediction from their respective GY estimates even when boundaries were imposed on maximum internal N efficiencies that could be reached in response to N fertilizer treatments.

A sensitivity analysis of the stem volume based physiological framework used other datasets from 2007, 2009, 2010, and 2011 to simulate both  $GY_A$  and NUE.

The calibration analysis showed acceptable goodness of fit, especially considering that plot-level data points (rather than treatment means) were used to validate the framework. This conceptual physiological framework appears to be a promising tool for phenotyping simultaneously for maize plant N uptake, GY, and NUE through two relatively simple variables to measure (stem diameter and plant height) at the silking stage.

Future research should focus on testing and calibration of these or other appropriately modified pathways under different environments (e.g., soil N supply and weather) and genotypes (diverse N harvest index, grain %N, and total plant N uptake). More effort should be invested in determining plant N status more directly with NNI, perhaps via an indirect association with a morpho-physiological trait, even though a tradeoff between phenotyping simplicity and quality of the NNI tool is acknowledged (Lemaire et al., 2008a).

The suggested physiological frameworks are perhaps more applicable as potential phenotyping tools to maize plant breeders than to advancing the maize physiological science itself. Physiology framework endeavors such as these should be pursued to improve efficient phenotyping for hybrids and inbred lines at diverse testing stages of the plant breeding programs for complex stress tolerance traits.

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