



Genotypic variation and relationships among nitrogen-use efficiency and agronomic traits in tropical maize inbred lines

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Genet. Mol. Res. 16 (3): gmr16039757

Received June 21, 2017

Accepted August 11, 2017

Published September 21, 2017

DOI <http://dx.doi.org/10.4238/gmr16039757>

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ABSTRACT. Improvement in nitrogen-use efficiency (NUE) on maize is among the best strategies to mitigate the problems of poor soil fertility in tropical conditions. The objectives of this study were: i) to quantify the genetic variability for NUE-components and agronomic traits in a set of tropical maize inbred lines; ii) to study the genetic divergence among tropical maize inbred lines under contrasting nitrogen (N) levels; iii) to identify the secondary traits associated with NUE in tropical maize inbred lines; and iv) to identify maize inbred lines efficient in NUE and its components. Sixty-four tropical maize inbred lines were evaluated in the field under low- and high-N conditions for NUE-components and agronomic traits. Genetic variability for NUE-components and agronomic traits was found; lines in eight different groups for each N condition were allocated, and N-efficient inbred lines were identified in different groups. Furthermore, we suggest flowering time traits and kernel number as great secondary traits for selecting tropical maize inbred lines for NUE under both N conditions, and chlorophyll content for selecting for NUE under N stress.

Key words: Nitrogen uptake efficiency; Nitrogen utilization efficiency; Genetic variability; Abiotic stress; Secondary traits; Genetic diversity

INTRODUCTION

Maize is one of the most globally important cereals because it is used for food and feed (Timmer, 2017) and biofuels, and it is the most produced cereal in the world (USDA, 2017). Tropical regions are significant for food security (Powlson et al., 2016), but poor soils with mineral deficiency, especially in phosphorus and nitrogen (N), are often prevalent. The availability of N, in nutrient form, is a limiting factor for crop growth and yield (Edmeades et al., 1998). N-use efficiency (NUE) for cereal production is approximately 33%, and, consequently, 50-70% of the applied N is lost from the plant-soil system (Fageria, 2002). Besides, there are many concerns about the negative impacts of excess N on the environment (Han et al., 2015).

For maize, NUE has already been defined by Moll et al. (1982) as the grain yield (GY) per unit of N available in the soil. The two primary components of NUE are N-uptake efficiency (NUpE) and N-utilization efficiency (NUtE). The identification of maize cultivars with higher NUE is very significant for sustainable agriculture. Some studies have demonstrated that there is large genetic variability for NUE and its components in maize (Ferro et al., 2007; Worku et al., 2007). According to Hirel et al. (2001), maize cultivars with increased NUE can be obtained by genetic improvement of its components (NUpE and NUtE). Differences in the NUpE may arise from differences in the root system, assimilation of N in the soil and their regulation (Schmidt et al., 2002), while differences in the NUtE may arise from differences in ability for translocation, distribution and mobilization of absorbed N to and from various organs, capacity of N import-export; and the efficiency of converting CO₂ into carbohydrates (Ladha and Reddy, 2003).

There are few studies that have evaluated tropical maize inbred lines for NUE under field conditions. The challenges have been the degree of phenotypic variation in this complex trait and the difficulty in obtaining reliable data from field trial studies (Han et al., 2015). Hence, genetic gains from the direct selection of NUE and its components are small because it has low heritability and takes time (Banziger et al., 2004). Early selection is very significant to accelerate the breeding process and obtain greater genetic gains. Secondary traits related to NUE can be used to select cultivars that have greater NUE in less time (Yadav et al., 2017). Abdel-Ghani et al. (2013) found a high correlation between the traits of plant architecture and GY in maize inbred lines evaluated under high N (HN) and low N (LN). Miranda et al. (2005) used ear leaf chlorophyll content (SPAD) to discard maize inbred lines that were inefficient in NUpE. In another study, Badu-Apraku et al. (2012) found that plant (PH) and ear height (EH) were great traits for indirect selection of maize inbred lines for NUE under LN. These results demonstrate that it is possible to evaluate traits correlated with NUE and optimize the breeding process.

The main purpose of this investigation was to characterize genetic variation and to study the relationships among traits in a set of tropical maize inbred lines. Our specific aims were: i) to quantify the genetic variability for components of NUE and agronomic traits in a set of tropical maize inbred lines; ii) to study the genetic divergence among tropical maize inbred lines under LN and HN; iii) to identify the secondary traits associated with NUE in tropical maize inbred lines; and iv) to identify maize inbred lines efficient in NUtE, NUpE, and NUE.

MATERIAL AND METHODS

Plant material

In this study, 64 tropical maize inbred lines that represent a set of diverse germplasm

from the maize breeding program of the Universidade Federal de Viçosa (UFV), Viçosa, Minas Gerais State, Brazil, were used. They were obtained from different sources of tropical maize germplasm: commercial maize hybrids, maize populations, and open-pollinated maize varieties.

Field experiments

The 64 tropical maize inbred lines were evaluated in 2014 at UFV Experimental Station in Coimbra (latitude 20°51'24''S; longitude 42°48'10''W; altitude of 720 m), located in southwest Minas Gerais State, Brazil. Maize inbred lines were evaluated in two independent experiments under different levels of N: LN and HN. The design of each experiment was an 8 x 8-lattice square with two replications and two-row plots. The plot size was 6.4 m² (4 m long with 0.8 m row spacing and 0.2 m plant spacing). Plant density was 62,500 plants/ha. For the LN experiment, 30 kg/ha N was applied, while for the HN experiment, 180 kg/ha was applied. Trail management was the same for both experiments (LN and HN) employing standard agricultural practices.

Measurements

Days to pollen shedding (DTP) and days to silking (DTS) were recorded as the number of days from sowing to the day when 50% of anthers extruded outside the glumes and when silk became visible, respectively. SPAD was measured 15 days after silking on five competitive plants in the middle of the upper ear leaf, using the SPAD-502 chlorophyll meter (Minolta Camera Co., Osaka, Japan). Kernel density (KD, g/L) was recorded as the weight of a liter of kernels. PH and EH were measured in centimeters on five competitive plants as the distance from the ground level to the collar of the upper most leaf and upper ear leaf, respectively. Above (AENN) and below (BENN) ear node number was recorded as the number of nodes above and below ear leaf on five competitive plants, respectively. Ear diameter (ED, cm) and ear length (EL, cm) were measured on five competitive ears with a paquimeter and a rule, respectively. The number of kernel rows (NKR) was recorded as the number of rows measured on five competitive ears. Ear kernel number (EKN) was recorded as the number of kernels on five competitive ears after shelling. One thousand kernel weights (TKW) were measured in grams as the weight of 1000 kernels adjusted to 145 g/kg moisture. GY was recorded from all ears on the plot at physiological maturity. Ears were shelled, the grain weight and grain moisture percentage were recorded, and GY (kg/ha) was calculated at 145 g/kg moisture. At physiological maturity, five competitive plants were harvested from each plot by cutting them close to the soil surface. All plant stover, together with cobs (with kernels removed) were chopped at maturity and oven-dried to a constant weight at 70°C for 72 h. The harvest ears were also oven-dried at 70°C for 72 h. Grain and stover samples were milled using a Wiley-type mill and analyzed for N according to the Kjeldahl method (Bremner and Mulvaney, 1982). The components of NUE were calculated according to Moll et al. (1982): NUpE was calculated as the ratio of the total N (kg/ha) in the aboveground biomass to total N in the soil (kg/ha); and NUtE was calculated as the ratio of GY (kg/ha) to total N (kg/ha) in the aboveground biomass. Besides, the NUE was calculated as the ratio of GY (kg/ha) to total N in the soil (kg/ha) or $NUE = NUpE \times NUtE$. Also, the contribution of variation in NUpE and NUtE to inbred line sum of squares for NUE was calculated for each N level based on Moll et al. (1982) methodology.

Statistical analysis

Lattice analyses were performed for each N level and were combined across N levels on a plot basis for all traits using the R package “agricolae”. The genotypes, N levels, and block effects were considered as random effects. Estimates of variance components were obtained based on the expected mean squares (Searle et al., 1992). For each N level, the broad sense heritability ($\hat{h}_{\bar{x}}^2$) was estimated on a plot basis (Hallauer et al., 2010) as

$$\hat{h}_{\bar{x}}^2 = \frac{\hat{\sigma}_G^2}{(\hat{\sigma}_G^2 + \hat{\sigma}^2 / r)}$$

where $\hat{\sigma}_G^2$ is the estimate of genotypic variance, and $\hat{\sigma}^2$ is the estimate of error variance of replication, and r is the number of replications.

Regarding NUE, to identify the efficient and inefficient inbred lines, a 99% confidence interval (CI) for the adjusted means of the inbred lines for each N level was calculated according to the following formula:

$$CI(\mu)_{99\%} = \mu \pm t_{(1\%, 49)} \sqrt{\left(\frac{\hat{\sigma}^2}{r}\right)}$$

Where μ is the adjusted mean of each inbred line for NUE, and $\hat{\sigma}^2$ is the estimate of error variance of replication. Inbred lines with adjusted means higher than the upper limit of the confidence interval were classified as efficient; those with adjusted means lower than the confidence interval were classified as inefficient, similar to Mundim et al. (2013).

The genetic diversity assessment among the inbred lines at LN and HN was performed using the R packages “ade4” and “adegenet”. We generated the distance matrix by Mahalanobis generalized distance, which accounted for residual correlations among traits (Mahalanobis, 1936). Then, a Mahalanobis distance matrix was used as input data for cluster analysis based on unweighted pair-group method of arithmetic average (UPGMA). The UPGMA dendrogram was created based on Mahalanobis generalized distance to estimate the level of relatedness among inbred lines. The Mojena (1977) method was then used to allocate the inbred lines into clusters, where the dendrogram must be cut in function of the mean value of the genetic distance of fusion levels and the standard deviation of the distance values.

At LN and HN, Pearson correlation coefficients among pairs of traits were estimated using adjusted means of inbred lines. Spearman correlation coefficients were estimated between pairs of the same traits under LN and HN conditions. Finally, to partition the phenotypic correlations into causal and residual effects, a path analysis for NUE was performed at LN and HN (Wright, 1921). The path analysis and correlations were calculated using the R package “agricolae”.

RESULTS

Means, variance components, and broad-sense heritability estimates

Phenotypic variation among inbred lines for each trait was confirmed by the mean and accuracy (Table 1). When we observed the performance of inbred lines under different N

levels, the mean value of GY (-25.5%), EL (-7.5%), EKN (-9.5%), TKW (-17.0%), and SPAD (-25.6%) was substantially decreased under LN conditions, compared with traits under HN. The other traits did not change in their mean value as functions of N levels.

Table 1. Variance components and broad sense heritability estimates (\hat{h}_x^2), means and accuracy for traits measured in 64 tropical maize inbred lines under contrasting N levels.

Traits	Combined		High N				Low N			
	Line	Lines x N	Line	\hat{h}_x^2	Mean	Accuracy	Lines	\hat{h}_x^2	Mean	Accuracy
DTP	28.36***	0.12	15.74***	0.91	68.55	95.37	12.53***	0.93	68.02	96.25
DTS	33.13**	0.55**	17.46***	0.92	69.87	95.85	16.90***	0.89	69.38	94.31
KD	2261.60***	51.23	1215.50***	0.52	800.97	72.22	1160.50***	0.61	806.11	78.35
SPAD	36.75***	2.05*	14.51***	0.67	47.67	81.58	18.97***	0.64	35.44	80.10
PH	664.64***	5.41*	347.85***	0.92	153.17	96.10	293.25***	0.91	150.66	95.56
EH	332.36***	3.02*	164.09***	0.91	76.50	95.43	152.48***	0.90	76.01	94.98
BENN	1.20***	0.0122	0.5422***	0.76	7.30	86.99	0.5877***	0.87	7.20	93.20
AENN	0.77***	0.00	0.2730***	0.80	6.13	89.33	0.2618***	0.83	6.31	91.19
ED	0.21***	0.00	0.1090***	0.84	3.81	91.58	0.0899***	0.76	3.67	86.99
EL	2.43***	0.1653**	1.48***	0.79	12.59	88.98	1.1384***	0.66	11.65	81.44
NKR	4.58***	0.1210***	2.55***	0.89	13.60	94.11	2.1642***	0.89	13.52	94.11
EKN	3475.00***	0.00	1944***	0.70	258.77	83.48	1544.65***	0.62	234.30	78.54
TKW	0.0033***	0.0001***	0.00234***	0.86	0.273	92.55	0.0012***	0.87	0.214	93.04
GY	523384***	17943*	355500***	0.84	2505.01	91.51	178291***	0.72	1867.74	84.85
NUpE	0.0630***	0.0290***	0.00288***	0.48	0.495	69.02	0.0988***	0.57	2.11	75.55
NUtE	67.49***	0.00	42.76***	0.76	28.84	87.09	31.03**	0.47	30.36	68.62
NUE	165.00***	37.50***	10.98***	0.84	13.92	91.50	198.12***	0.72	62.26	84.85

DTP: days to pollen (days); DTS: days to silk (days); KD: kernel density (g/L); SPAD: ear leaf chlorophyll concentration; PH: plant height (cm); EH: ear height (cm); BENN: below ear node number; AENN: above ear node number; ED: ear diameter (cm); EL: ear length (cm); NKR: number of kernel rows; EKN: ear kernel number; TKW: one thousand kernel weight (g); GY: grain yield (kg/ha); NUpE: N-uptake efficiency; NUtE: N-utilization efficiency; NUE: N-use efficiency. *, **, ***Significant at P = 0.10, P = 0.05, and P = 0.01, respectively.

There were significant differences among maize inbred lines for all traits between the two N levels (Table 1), revealing genetic variability for agronomic traits and NUE-components under LN and HN. Variance component estimates due to lines x N levels interaction were significant for NKR, TKW, NUpE, and NUE at a level of P = 0.01, for DTS and EL at a level of P = 0.05, and for SPAD, PH, EH, and GY at a level of P = 0.10. The estimates of heritability (\hat{h}_x^2) were intermediate to high and ranged from 0.48 to 0.92, under LN, and from 0.47 to 0.93, under HN. The \hat{h}_x^2 values were consistently greater than 0.60 under both N levels, with two exceptions, KD and NupE at HN, and NupE and NUtE at LN. For the traits, EL, GY, NUtE, and NUE, the \hat{h}_x^2 values were consistently higher at HN, while for BENN and NupE the \hat{h}_x^2 values were higher at LN than HN.

Selection of tropical maize inbred lines

Means of 64 inbred lines for NUtE and NUpE under LN and HN are shown in Figures 1 and 2, respectively. The 95% CI for the means of the inbred lines for NUpE, NUtE, and NUE under each N level allowed us to identify the most efficient inbred lines under each N level. The inbred lines L27, L23, L22, L2, L26, and L28 under LN (Figure 1) had the highest mean for NUpE and were classified as efficient in N-uptake. In contrast, the inbred lines L38, L46, L5, and L10 under LN had the highest means for NUtE and were classified as efficient in N-utilization. At HN, the inbred lines L11, L23, L18, L10, and L49 (Figure 2) and the inbred lines L50, L27, L39, L28, L60, L38, and L21 had the highest means for NUpE and NUtE,

respectively, and were classified as efficient in N-uptake and N-utilization. The inbred lines L27 and L10 were selected as efficient in N-uptake and N-utilization, respectively, under LN and HN. For NUE, the inbred lines L28, L21, L38, L27, L63, and L43 were classified as efficient in N-use under LN, and the inbred lines L21, L60, L28, L27, L17, L10, L50, L6, and L38 were identified as efficient under HN. The inbred line L28 was identified as efficient in N-use and N-uptake under LN, and the inbred lines L28, L38, L21, and L27 were identified as efficient in N-use and N-utilization under HN.

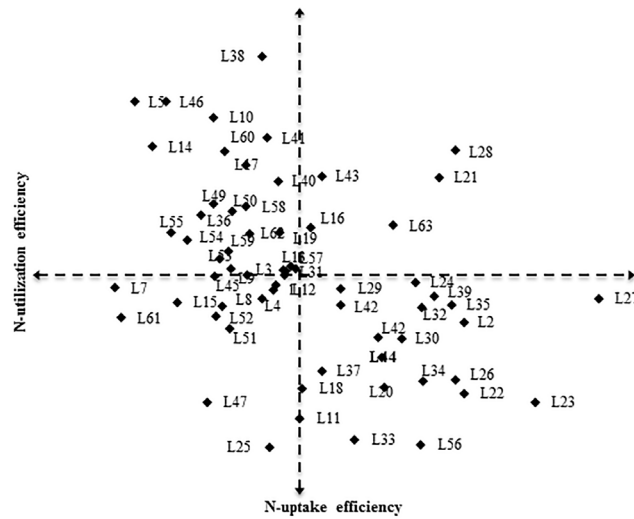


Figure 1. Mean values for N-uptake (NUpE, kg dry grain/kg) and N-utilization (NUtE, kg dry grain/kg N uptake) efficiencies measured in 64 inbred lines under LN.

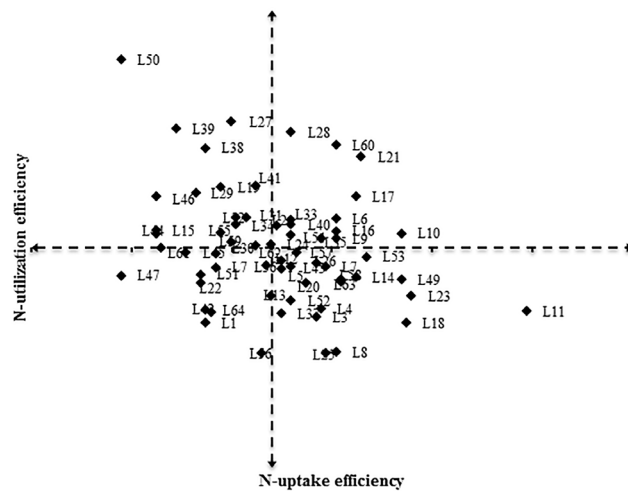


Figure 2. Mean values for N-uptake (NUpE, kg dry grain/kg) and N-utilization (NUtE, kg dry grain/kg N uptake) efficiencies measured in 64 inbred lines under HN.

Genetic diversity

Mahalanobis distance coefficients were estimated for all tropical maize inbred lines based on 16 agronomic and NUE traits under both N conditions (Figures 3 and 4). Based on the Mojena (1977), the genetic diversity assessment grouped the 64 inbred lines into eight clusters at LN (cut-off point = 114.47) and HN (cut-off point = 121.82). Under LN, all eight clusters had more than one inbred line, but clusters three, five, and six, with nine, 26, and 12 inbred lines, respectively, were the largest (Figure 3). The inbred lines classified as efficient in N-uptake were grouped in clusters two (one line), three (one line), and five (four lines), while the four lines classified as efficient in N-utilization were grouped in clusters four, five, six, and seven. Cluster one and eight did not group any N-efficient inbred lines. For NUE, inbred lines were grouped in clusters two and seven. Under LN, the mean value of genetic distance was 136.83. The highest genetic distance (423.99) was between the inbred lines L58 and L61, while the most similar inbred lines were L10 and L17 (distance = 20.92).

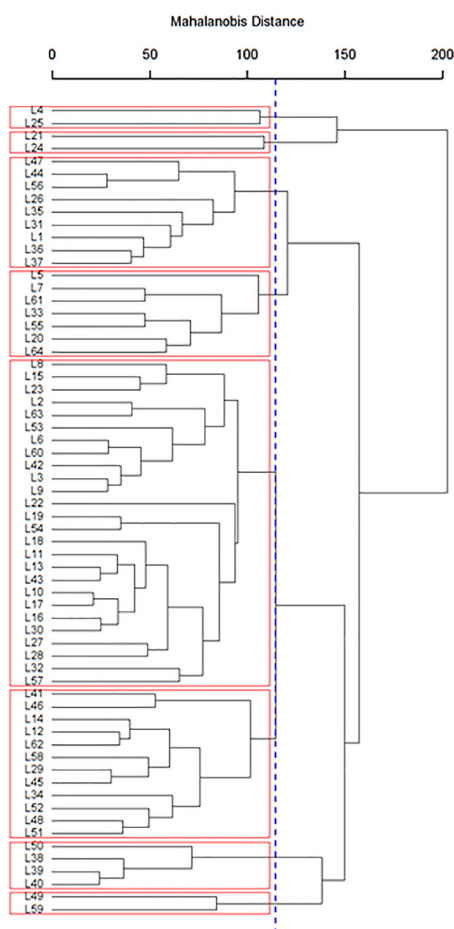


Figure 3. Dendrogram of the unweighted pair-group method of arithmetic clustering for 64 tropical maize inbred lines using Mahalanobis distance under LN.

Under HN, there were three clusters with just one inbred line, and clusters four and seven were highlighted as the largest clusters, with 10 and 41 lines, respectively (Figure 4). Concerning N-efficiency, most of the inbred lines classified as efficient in NUE, and its components were allocated to clusters five and seven. The inbred lines L21 and L50, classified as efficient in NUpE, and L18, classified as NUtE, were grouped in clusters two, eight, and six, respectively. Under HN, the mean value of genetic distance was 130.29. The highest distance (475.10) was obtained from the inbred lines L7 and L41, while the shortest distance (15.40) was between the inbred lines L36 and L37.

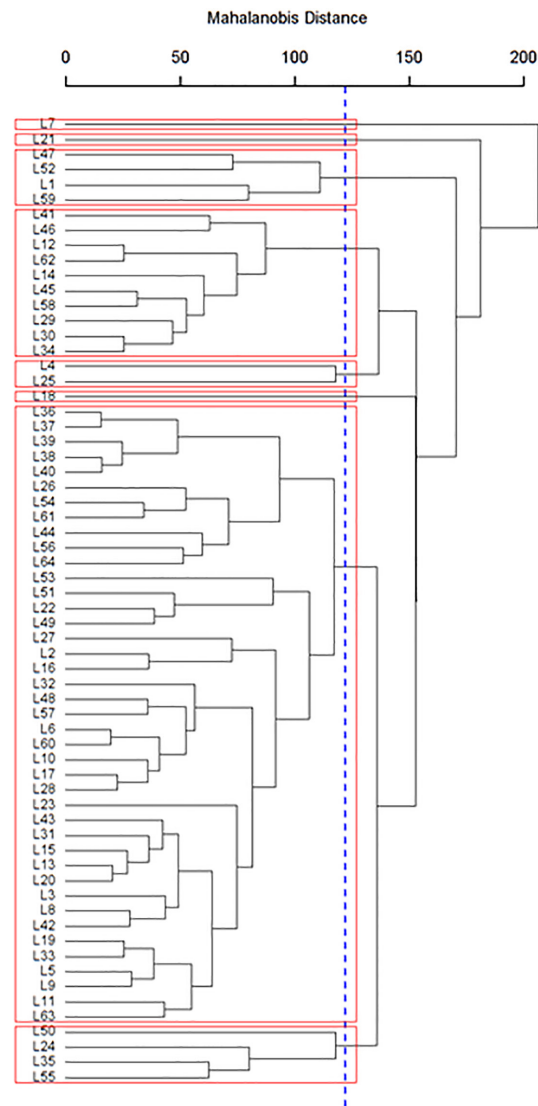


Figure 4. Dendrogram of the unweighted pair-group method of arithmetic clustering for 64 tropical maize inbred lines using Mahalanobis distance under HN.

Phenotypic correlations and path analyses

Spearman correlations between the same traits evaluated under LN and HN were significant and positive ($P < 0.01$) for almost all traits, except for NUtE (Table 2, diagonal). The significant correlations were from intermediate to strong, except for KD (0.51) and NUtE (0.49). Most of the phenotypic correlations between pairs of traits under LN (Table 2, upper diagonal) or HN (Table 2, lower diagonal) were not significant ($P > 0.10$), and most of the significant correlations exhibited low magnitude values ranging from weak to average correlations.

Table 2. Estimates of phenotypic correlation coefficients among pairs of traits across 64 maize inbred lines evaluated under low N (lower diagonal) and high N (upper diagonal) conditions; and estimates of Spearman correlation coefficients between pairs of the same traits under low and high N conditions (diagonal).

Traits	DTP	DTS	KD	SPAD	PH	EH	BENN	AENN	ED	EL	NKR	EKN	TKW	NUpE	NUtE	NUE
DTP	0.91***	0.90***	-0.17**	-0.09	-0.01	0.13	0.21*	-0.13	-0.25**	0.02	-0.02	-0.21*	-0.12	-0.14	-0.36***	-0.43***
DTS	0.90***	0.84***	-0.19	-0.16	-0.06	0.05	0.18	-0.09	-0.20	0.08	0.06	-0.25**	-0.10	-0.11	-0.50***	-0.54***
KD	-0.13	-0.14	0.51***	-0.02	-0.02	-0.03	-0.15	0.02	-0.14	-0.17	-0.17	-0.20	-0.05	0.06	0.04	0.06
SPAD	0.01	0.04	-0.01	0.62***	0.18	0.18	0.13	0.07	0.11	-0.06	0.02	0.18	-0.06	0.16	0.13	0.22*
PH	-0.03	-0.01	0.03	-0.09	0.89***	0.78***	0.43***	0.27**	0.21*	0.35***	0.00	0.27**	0.12	0.31**	0.22*	0.38***
EH	0.14	0.09	0.14	-0.11	0.76***	0.88***	0.76***	-0.08	0.08	0.18	0.08	0.29**	-0.06	0.18	0.21	0.29**
BENN	0.20	0.18	0.09	-0.19	0.38***	0.71***	0.79***	-0.06	0.15	0.16	0.20	0.24*	-0.02	0.14	0.17	0.23***
AENN	-0.29**	-0.14	-0.01	-0.04	0.32**	0.01	0.03	0.87***	0.16	0.06	0.14	0.16	-0.06	0.10	0.08	0.12
ED	-0.26**	-0.18	-0.14	0.23*	0.01	-0.17	-0.12	0.15	0.81***	0.20	0.42***	0.24*	0.57***	0.50***	-0.13	0.18
EL	-0.03	0.01	-0.16	0.21*	0.22*	0.03	-0.16	-0.17	0.12	0.59***	-0.01	0.29**	0.18	0.42***	0.05	0.35***
NKR	-0.22*	-0.14	0.16	0.07	-0.06	0.06	0.25**	0.19	0.46***	-0.16	0.80***	0.49***	-0.04	0.06	0.00	0.03
EKN	-0.40***	-0.37***	0.17	0.18	0.29**	0.28**	0.18	0.18	0.30**	0.32**	0.47***	0.60***	-0.34***	0.32***	0.28**	0.47***
TKW	-0.11	-0.04	-0.07	0.14	-0.01	-0.70**	-0.25**	0.00	0.55***	0.21*	-0.14	-0.15	0.78***	0.24*	-0.05	0.14
NUpE	-0.05	0.00	-0.11	0.45***	0.10	0.07	-0.15	-0.17	0.20	0.48***	-0.10	0.22*	0.33***	0.08	-0.31**	0.32**
NUtE	-0.37***	-0.43***	0.34***	-0.03	0.15	0.12	0.15	0.19	0.05	-0.12	0.27**	0.42***	-0.11	-0.40**	0.49***	0.78***
NUE	-0.42***	-0.45***	0.25**	0.33***	0.26**	0.16	0.01	0.08	0.21*	0.32***	0.20	0.60***	0.15	0.34***	0.70***	0.70***

DTP: days to pollen; DTS: days to silk; KD: kernel density; SPAD: ear leaf chlorophyll concentration; PH: plant height; EH: ear height; BENN: below ear node number; AENN: above ear node number; ED: ear diameter; EL: ear length; NKR: number of kernel rows; EKN: ear kernels number; TKW: one thousand kernel weight; NUpE: N-uptake efficiency; NUtE: N-utilization efficiency; NUE: N-use efficiency. *, **, ***Significant at $P = 0.10$, $P = 0.05$, and $P = 0.01$, respectively.

The N supply influenced some estimates of phenotypic correlations, and some pairs of traits showed significant correlations under HN but did not exhibit correlations under LN and vice versa. For example, SPAD was significant and positively correlated with NUpE (0.45) under LN, but it did not exhibit a correlation with NUpE under HN. TKW exhibited a negative correlation with EH under HN, but this pair of traits showed no correlation under LN. Under both N levels, NUE showed significant and negative correlations with flowering time traits, and positive correlations with SPAD, PH, EL, EKN, NUpE, and NUtE. Besides, NUE correlated significantly and positively with KD and ED, under LN, and with EL and BENN, under HN. For NUE-components, we estimated the relative importance of the two NUE-components to NUE: based on Moll et al. (1982) methodology, we found that NUtE accounted for 75.87 and 78.35% of the genetic variability observed in the inbred lines for NUE, under LN and HN, respectively (Table 3); and the NUpE accounted for 21.79 and 18.82% of this variability for NUE, under LN and HN, respectively. In contrast, we found significant and negative correlations between NUtE and NUpE under LN and HN (-0.40 and -0.31, respectively). Among the agronomic traits, we found that EKN was significantly correlated with almost all traits: it was positively correlated with NUE (0.60 and 0.47), NUtE (0.42 and 0.28), and NUpE (0.22 and 0.24) under LN and HN, respectively.

Path analysis involving NUE and agronomic traits revealed that the 13 agronomic traits accounted for almost 60 and 65% of total variation in NUE, under LN and HN, respectively

(Tables 4 and 5). Under LN, among the 13 traits, EKN (0.418), TKW (0.300), SPAD (0.271), PH (0.212), and DTP (201) had the highest direct effects on NUE, and the lowest was DTS (-0.484) followed by ED (-0.236). However, under HN, EKN (0.448), TKW (0.381), BENN (0.309), and DTP (0.203) had the highest direct effects on NUE, and DTS (-0.649), ED (-0.320) and EH (-0.200) had the least direct effects on NUE. Despite the negative correlation between DTP and NUE (-0.42 and -0.43, under LN and HN, respectively), it contributed directly and positively to NUE (0.201 and 0.203). However, under both N levels, DTP contributed indirectly and negatively to NUE through DTS, and this contribution was lower under LN (-0.548) than HN (-0.436). The other indirect effects of agronomic traits on NUE were low, and most were close to zero, except for EH, which contributed indirectly (0.235) to NUE through BENN, under HN.

Table 3. Contribution of variation in efficiency components to maize inbred lines sum of squares for NUE.

Traits	Logarithm	N levels	r_{xy}	S_{x_i}/S_y	Contribution (%)
N-use efficiency	Y	-	-	-	-
N-uptake efficiency	X_1	HN	0.795	0.986	78.35
		LN	0.742	1.023	75.88
N-utilization efficiency	X_2	HN	0.322	0.584	18.82
		LN	0.304	0.717	21.79

r_{xy} : correlation coefficient between x_i and y ; S_{x_i} , S_y : standard deviation for x_i and y , respectively.

Table 4. Direct and indirect effect estimates involving dependent trait N-use efficiency (NUE) and the independent traits: days to pollen (DTP); days to silk (DTS); kernel density (KD); ear leaf chlorophyll concentration (SPAD); plant (PH) and ear height (EH); below (BENN) and above ear node number (AENN); ear diameter (ED, cm); ear length (EL, cm); number of kernel rows (NKR); ear kernel number (EKN); and one thousand kernel weight (TKW, g) across 64 maize inbred lines evaluated under LN.

Trait	Association effect	Independent traits													
		DTP	DTS	KD	SPAD	PH	EH	BENN	AENN	ED	EL	NKR	EKN	TKW	
NUE	Direct	0.201	-0.484	0.127	0.271	0.212	-0.084	0.034	-0.031	-0.236	0.101	0.123	0.418	0.300	
	Indirect through DTP		0.180	-0.026	0.002	-0.006	0.028	0.040	-0.058	-0.052	-0.006	-0.044	-0.080	-0.022	
	Indirect through DTS	-0.436		0.068	-0.019	0.005	-0.044	-0.087	0.068	0.087	-0.005	0.068	0.179	0.019	
	Indirect through KD	-0.017	-0.018		-0.001	0.004	0.018	0.011	-0.001	-0.018	-0.020	0.020	0.022	-0.009	
	Indirect through SPAD	0.003	0.011	-0.003			-0.024	-0.030	-0.052	-0.011	0.062	0.057	0.019	0.049	
	Indirect through PH	-0.007	-0.002	0.006	-0.019			0.161	0.080	0.068	0.002	0.047	-0.013	0.061	
	Indirect through EH	-0.012	-0.008	-0.012	0.009	-0.064		-0.059	-0.001	0.014	-0.003	-0.005	-0.023	0.023	
	Indirect through BENN	0.007	0.007	0.003	-0.007	0.013	0.024		0.001	-0.004	-0.006	0.009	0.006	-0.009	
	Indirect through AENN	0.010	0.004	0.000	0.001	-0.010	0.000	-0.001		-0.005	0.005	-0.006	-0.006	0.000	
	Indirect through ED	0.062	0.043	0.033	-0.054	-0.002	0.040	0.028	-0.035		-0.028	-0.109	-0.071	-0.130	
	Indirect through EL	-0.003	0.001	-0.016	0.021	0.022	0.003	-0.016	-0.017	0.012		-0.016	0.033	0.021	
	Indirect through NKR	-0.027	-0.017	0.020	0.009	-0.007	0.007	0.031	0.023	0.056	-0.020		0.058	-0.017	
	Indirect through EKN	-0.167	-0.155	0.071	0.075	0.121	0.117	0.075	0.075	0.125	0.134	0.197		-0.063	
	Indirect through TKW	-0.033	-0.012	-0.021	0.042	-0.003	-0.081	-0.075	0.000	0.165	0.063	-0.042	-0.045		
	Total	-0.420	-0.450	0.250	0.330	0.260	0.160	0.010	0.080	0.210	0.320	0.200	0.600	0.150	
	Determination coefficient (R^2)														0.597
	Residual effect														0.635

DISCUSSION

Breeding for enhanced NUE is the most economical way of reducing the use of N fertilizers and increasing maize yield in tropical areas where poor soils are prevalent. Evaluating tropical maize inbred lines under contrasting N conditions is an important step in a maize breeding programs focused on increasing N-use efficiency. However, due to the difficulty in determining N concentrations in plants and kernels, studies with large numbers

Table 5. Direct and indirect effect estimates involving dependent trait N-use efficiency (NUE) and the independent traits: days to pollen (DTP); days to silk (DTS); kernel density (KD); ear leaf chlorophyll concentration (SPAD); plant (PH) and ear height (EH); below (BENN) and above ear node number (AENN); ear diameter (ED, cm); ear length (EL, cm); number of kernel rows (NKR); ear kernel number (EKN); and one thousand kernel weight (TKW, g) across 64 maize inbred lines evaluated under HN.

Trait	Association effect	Independent traits													
		DTP	DTS	KD	SPAD	PH	EH	BENN	AENN	ED	EL	NKR	EKN	TKW	
NUE	Direct	0.203	-0.649	0.111	0.090	0.170	-0.200	0.309	0.030	-0.320	0.213	-0.028	0.448	0.381	
	Indirect through DTP		0.183	-0.035	-0.018	-0.002	0.026	0.043	-0.026	-0.051	0.004	-0.004	-0.043	-0.024	
	Indirect through DTS	-0.584		0.123	0.104	0.039	-0.033	-0.117	0.058	0.130	-0.052	-0.039	0.162	0.065	
	Indirect through KD	-0.019	-0.021		-0.002	-0.002	-0.003	-0.017	0.002	-0.016	-0.019	-0.019	-0.022	-0.006	
	Indirect through SPAD	-0.008	-0.015	-0.002		0.016	0.016	0.012	0.006	0.010	-0.005	0.002	0.0163	-0.005	
	Indirect through PH	-0.002	-0.010	-0.003	0.031		0.132	0.073	0.046	0.036	0.059	0.000	0.046	0.020	
	Indirect through EH	-0.026	-0.010	0.006	-0.036	-0.156		-0.152	0.016	-0.016	-0.036	-0.016	-0.058	0.012	
	Indirect through BENN	0.065	0.056	-0.046	0.040	0.133	0.235		-0.019	0.046	0.050	0.062	0.074	-0.006	
	Indirect through AENN	-0.004	-0.003	0.001	0.002	0.008	-0.002	-0.002		0.005	0.002	0.004	0.005	-0.002	
	Indirect through ED	0.080	0.064	0.045	-0.035	-0.067	-0.026	-0.048	-0.051		-0.064	-0.134	-0.077	-0.182	
	Indirect through EL	0.004	0.017	-0.036	-0.013	0.074	0.038	0.034	0.013	0.043		-0.002	0.062	0.038	
	Indirect through NKR	0.001	-0.002	0.005	-0.001	0.000	-0.002	-0.006	-0.004	-0.012	0.000		-0.014	0.001	
	Indirect through EKN	-0.094	-0.112	-0.090	0.081	0.121	0.130	0.107	0.072	0.107	0.130	0.219		-0.152	
	Indirect through TKW	-0.046	-0.038	-0.019	-0.023	0.046	-0.023	-0.008	-0.023	0.217	0.069	-0.015	-0.130		
	Total	-0.430	-0.540	0.060	0.220	0.380	0.290	0.230	0.120	0.180	0.350	0.030	0.470	0.140	
	Determination coefficient (R ²)														0.651
	Residual effect														0.591

of genotypes for NUE-components in field conditions under contrasting N levels are scarce. Moreover, to our knowledge, no previous study has investigated NUE-components under contrasting N levels in tropical maize inbred lines. In our study, we evaluated 64 tropical maize inbred lines for agronomic traits and NUE-components; substantial genetic variability was observed for all traits under both N conditions, indicating that good progress can be made in selecting for NUE and agronomic traits. Genetic variability for agronomic traits and SPAD was reported in temperate maize inbred lines (Wu et al., 2011; Abdel-Ghani et al., 2013), in tropical maize hybrids (Worku et al., 2007; Abe et al., 2013; Ertiro et al., 2017), and in open-pollinated tropical maize varieties (Badu-Apraku et al., 2011) under contrasting N levels. Li et al. (2015) evaluated a recombinant inbred line population for NUE-related traits under LN and HN, and they also found genetic variability for all traits.

Some studies have shown that genetic variability for GY and NUE-related traits seems to be differently expressed according to the N-level in maize (Presterl et al., 2003; Wu et al., 2011; Li et al., 2015; Ertiro et al., 2017). To detect differentially expressed alleles and significant genotypes x N levels interaction in field experiments, the severity of N stress under LN must decrease GY by around 40% compared to HN (Presterl et al., 2002; Banziger et al., 2004). In our study, the observed 25.6% reduction in GY under LN was lower than the results of Abe et al. (2013) and Wu et al. (2011) who reported 35 and 40% of the reduction in maize GY under LN, respectively. However, we found lines x N levels interaction for NUE, NUpE, GY, and some secondary traits, indicating that, for those traits, the inbred lines responded differently to N supply, and that the selection must be made for two different N levels. This is in agreement with Presterl et al. (2003), who reported that direct selection under N conditions is more efficient at improving NUE than indirect selection under HN. According to Presterl et al. (2002), maize inbred line development at LN resulted in hybrids being better adapted to LN, but less adapted to HN. Coque and Gallais (2006) highlighted in their studies that N stress could reduce genetic variability, and consequently, heritability. Under LN, we found that the $\hat{h}_{\bar{x}}^2$ value of NUtE was 0.46, which was much lower than under HN (0.76). However, the other $\hat{h}_{\bar{x}}^2$ values did not range so much across N levels, and most values were intermediate to

high, implying good perspectives for increasing NUE-related traits based on the phenotypic selection. Ertiro et al. (2017) evaluated 55 tropical maize hybrids under stress (LN and drought) and non-stress environments and they also found similar h_x^2 values across environments.

The main target of every maize breeding program is to develop hybrids from elite inbred lines, and every year, breeding programs develop thousands of inbred lines that must be tested in cross due to the low correlation between lines *per se* and their test cross performance (Hallauer et al., 2010). According to Reif et al. (2005), the hybrid performance was higher when parents were genetically diverse. Thus, the genetic diversity assessment among a set of inbred lines can help breeders to choose the best crosses to reduce cost and human resources. In our study, under LN, most N-efficient maize inbred lines were grouped predominantly on clusters five and seven, but there was at least one line in the opposite group for each N index. Under HN, most of the inbred lines efficient in N-uptake and N-utilization were grouped in cluster seven, except line one for NUpE and two lines for NUtE. For NUE, almost all efficient lines were grouped in cluster seven. Hence, among the N-efficient maize inbred lines, there is a choice of the most different lines to develop maize hybrid efficient in N-use and to generate breeding populations with a high frequency of favorable alleles for NUE-related traits. For instance, we can cross the inbred lines L38 with L10, and L26 with L27 for NUtE and NUpE, respectively, under LN; L10 with L23 for NUpE under HN; and, for NUE, under both N conditions, and NUtE under HN, we recommend crossing L21 with L28. In contrast, the inbred line L47, the inefficient inbred line, can be intercrossed with line L28, an efficient inbred line, to generate a segregation population for QTL mapping under both N conditions. We can also intercross a set of efficient maize inbred lines from a different cluster to generate a synthetic population that can be used as a source of alleles for the production of new N-efficient maize inbred. Mundim et al. (2013) identified popcorn maize inbred lines for NUE grouped in four different groups and recommended some specific crosses among them to generate maize breeding populations.

NUE is composed by N-utilization and N-uptake efficiency, but there is no information about the contribution of those components to NUE in tropical maize inbred lines. Moll et al. (1982) evaluated eight temperate maize hybrids under contrasting N levels and found that N utilization was more important to NUE than NUpE, under LN. Under HN, NUpE accounted for 83% of the variation in NUE. In another study, Worku et al. (2007) reported that both N-uptake and N-utilization contributed to NUE under LN. According to Presterl et al. (2002), for maize hybrids selected in LN, both NUE-components contributed to explaining NUE and were independent. In a recent review on N-use efficiency in maize, Ciampitti and Vyn (2014) also emphasized that a potential way to improve NUE in maize is to increase plant N uptake and N partitioning in grain. In our study, we found that the relative contribution of NUtE to variation in NUE among inbred lines was 3.5 and 4.2 times greater than the contribution of NUpE, under LN and HN, respectively. We also found a negative correlation between NUpE and NUtE (-0.40 and -0.31) under both N levels. Therefore, we recommend building a selection index composed by NUtE and NUpE with higher weight to NUtE to select tropical maize inbred lines more efficient in N-use.

The main limitation in characterizing a large number of maize genotypes to NUE-component related traits is associated with the cost and difficulties in determining N in kernels and adult plants in field conditions. Therefore, the identification of secondary traits associated with NUE and with high direct effects on NUE is an important step in breeding programs focused on increasing N-use efficiency. According to Edmeades et al. (1998), an

ideal secondary trait should be genetically associated with the main trait (in our case, NUE); highly heritable; genetically variable; and cheap and quick to measure. Therefore, the first step to determine whether a trait is appropriate to be used as a secondary trait is to elucidate the interrelationship between the main trait and agronomic traits. Path analysis, a straightforward extension of multiple regression analysis (Wright, 1921), has often been used to study the causal relationships between a set of traits because the correlation matrix can be partitioned into causal and spurious effects (Ullman, 1996). In this way, the aspect of ear and plant, stay-green characteristics, days to anthesis, days to silking, anthesis-silking interval, plant height, and ears per plant were identified as most reliable traits for selecting maize inbred lines and open-pollinated varieties for GY under LN conditions (Badu-Apraku et al., 2011, 2012; Talabi et al., 2017) and under *Striga*-infested environments (Badu-Apraku et al., 2014). In another study, Wu et al. (2011) evaluated a set of 189 temperate maize inbred lines under contrasting N levels and found that kernel number and kernel weight had significant and direct effects on N-use efficiency under LN conditions. Although Abe et al. (2013) did not use path analysis in their investigation, they verified that kernel number, PH, EH, and ear leaf chlorophyll (SPAD) showed high and positive genetic associations with GY for selecting tropical maize hybrids under LN and HN environments. By path analysis, we identified EKN, DTS, DTP, SPAD, ED, PH, and TKW as traits with moderate direct effects on NUE under LN and, under HN, the traits EKN, DTS, DTP, ED, EH, BENN, EL, and TKW had direct effects on NUE. Although the results of this and similar studies suggest that selection for plant architecture (PH, EH, and BENN) should improve NUE under contrasting N levels, selection for increased plant height can increase lodging, and some restrictions should be made when using those traits for selecting for NUE. DTP and DTS had positive and negative direct effects on NUE, respectively, and DTP negatively affected NUE through DTS. Thus, the selection of maize inbred lines for shortened anthesis-silking interval (increased DTP and reduced DTS) should improve NUE under both N conditions. We did not include anthesis-silking interval in our study because it did not show a normal distribution, a basic assumption for variance analysis (Searle et al., 1992).

Similar to our studies, positive associations between SPAD and NUE or GY were reported on maize evaluated under LN (Miranda et al., 2005; Wu et al., 2011; Abdel-Ghani et al., 2013; Abe et al., 2013), and between EKN and NUE (Abdel-Ghani et al., 2013; Abe et al., 2013). In a recent review, Echart et al. (2013) concluded that kernel number is the main component that accounts for GY, and it is strongly associated with NUE, under water and N limitations. Thus, EKN can be a strong secondary trait for selecting tropical maize inbred lines for NUE, and a selection index based on kernel number, flowering time traits, and ear length can be developed to increase NUE under both N conditions. Under LN, we recommend including SPAD in the selection index proposed or to use it as a great secondary trait to discard tropical maize inbred lines with lower NUE. SPAD is easy to determine in the field, is a metric rather than a subjective visual evaluation, such as stay-green characteristics, and can be measured before final harvest to discard undesirable genotypes. Furthermore, according to Fischer et al. (1989), chlorophyll content is strongly associated with stay-green characteristics in maize under drought and N stress.

In conclusion, we found high genetic variability for NUE-components and agronomy traits among a set of tropical maize inbred lines under different N conditions. We allocated the maize inbred lines in eight different groups, and N-efficient inbred lines were identified in different groups. Consequently, they could be crossed to obtain efficient hybrids in N-use.

Furthermore, we suggest flowering time traits and kernel number as great secondary traits for selecting tropical maize inbred lines for NUE under both N conditions, and chlorophyll content for selecting for NUE under N stress.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank the National Council for Scientific and Technological Development (CNPq) for financial support.

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