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Nitrogen availability effects on gas exchange measurements in field-grown maize (*Zea mays* L.) under irrigated Mediterranean conditions

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Abstract

There are limited studies about the effect of nitrogen (N) deficiency on leaf growth, N status, and photosynthetic capacity of maize grown under field conditions in a Mediterranean climate. The objective of this work was to evaluate the effect of different levels of mineral N availability on leaf gas exchange parameters of sprinkler irrigated maize. The experiment was conducted in a conventional maize field located in the central part of the Ebro valley (Spain) during two seasons. Using a portable LICOR-6400 equipment, instantaneous measurements and light response curves to gas exchange were conducted in plots with different levels of N supply ranging from deficient (no fertilized) to over-fertilized (300 kg N/ha). In addition to gas exchange measurements, mineral soil N content, chlorophyll meter readings (CMR), leaf N content, and grain yield were measured in the different plots. Results showed that grain yield reached a plateau (14.5 Mg/ha) when the mineral N available was about 179 kg/ha. CMR were linearly and highly related to total N in ear leaves. The relationship between light-saturated leaf photosynthesis measurements and CMR was significant but very weak ($R^2=0.13$) at V8 and V14 stages but increased later in the growing season ($R^2=0.52$). Plants with intermediate levels of N supply ($48 < \text{CMR} < 54$) tended to have slightly higher assimilation rates than plants with higher CMR readings. As the available N increased, the saturation point, the light compensation point and significant increases of dark respiration rate were observed. Under the conditions of the study, leaf N contents of 1.9% in the ear leaf were enough to maximize leaf assimilation rates with no need to over-fertilize the maize crop.

Additional key words: photosynthesis; dark respiration; nitrogen deficiency; stomatal conductance

Abbreviations used: An (net assimilation rate); Ci (intercellular CO₂ concentration); CMR (chlorophyll meter reading); g_{sw} (stomatal conductance to water vapour); LCP (light compensation point); LPR (linear plateau response); PAR (photosynthetic active radiation); QE (quantum efficiency); QPR (quadratic plateau response); Rd (dark respiration rate); RMSE (root mean square error); SP (saturation point); Tr (transpiration rate); WUE (water use efficiency).

Authors' contributions: Conceived and designed the experiments: RI. Performed the experiments: RI and MG. Analyzed the data and wrote the paper: RI, MG and MS.

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Introduction

Nitrogen is a major plant nutrient that is a fundamental component of leaf cell structures associated with the photosynthesis function. Nitrogen plays a key role determining plant photosynthetic capacity in natural and agricultural environments (Abrol *et al.*, 1999). It is well documented that N deficiency can lead to sig-

nificant yield reductions in maize (Di Paolo & Rinaldi, 2008; Berenguer *et al.*, 2009) as a result of reduced photosynthesis (Khamis *et al.*, 1990; Dwyer *et al.*, 1995; Zhao *et al.*, 2003; Correia *et al.*, 2005) and growth rates (Zhao *et al.*, 2003; Hammad *et al.*, 2012).

The negative effect of N deficiency in crops can be described through two mechanisms: (i) a reduced amount of radiation intercepted over the crop growth

period due to a reduction in active leaf area (limited resource capture), and/or (ii) a reduced daily rate of photosynthesis or radiation use efficiency. In maize, Vos *et al.* (2005) discussed that leaf photosynthesis is a more limiting mechanism compared to leaf area growth. In their study, the N concentration of expanding leaves from maize plants at different N supply was well related to light-saturated photosynthetic capacity with a hyperbolic function. Differences in photosynthetic assimilation rates in maize under different conditions of N supply are more likely to occur at high irradiance levels compared to low irradiance (Khamis *et al.*, 1990). Reductions in photosynthesis under conditions of N deficiency are related to a lower stomatal conductance and reduction of chlorophyll, carotenoid, and protein concentrations (Dwyer *et al.*, 1995; Correia *et al.*, 2005). On the other hand, N limited plants can show increased intercellular CO₂ concentration (C_i) (Correia *et al.*, 2005).

Under high N availability conditions, maize hybrids showed a plateau in photosynthetic assimilation rates at high leaf N concentrations, but with small differences in maximum assimilation rate among hybrids (Dwyer *et al.*, 1995). When comparing newer with older maize cultivars, the results indicate that genetic improvement can be partially attributed to higher leaf photosynthetic rates in newer cultivars, in particular during mid to late grain filling ('stay green') (Echarte *et al.*, 2008). Similarly, other studies (Ciampitty & Vyn, 2012) showed an increase in N use efficiency of new hybrids compared to old materials but as suggested by the review of Xu *et al.* (2012) the improvements in the fertilizer use management is the best option for increasing N efficiency in the agroecosystems.

The Mediterranean climate is characterized by a high evapotranspiration demand and a relatively high irradiance. Under these conditions, there is a high yield potential for maize, but maize productivity is largely dependent on irrigation supply. An adequate management of water and nitrogen fertilizer practices is therefore vital for increasing water and N productivity and reducing environmental risks. An excess of N in agroecosystems produces environmental problems associated to nitrate leaching and nitrous oxide emissions (Alvaro-Fuentes *et al.*, 2016), a powerful greenhouse effect gas. Under the conditions of high water and N demand for maize grown in a Mediterranean climate, it is well documented that N deficiency can lead to significant yield reductions (Di Paolo & Rinaldi, 2008; Berenguer *et al.*, 2009). The relationship between N supply and maize productivity has been well documented in the environments, but not at the leaf photosynthetic level. This physiological information obtained under field-grown conditions can help to optimize the

N use efficiency in agroecosystems, reducing the environmental impacts of excessive N applications.

Therefore, the objective of this study was to evaluate the effect of different levels of mineral N supply on leaf gas exchange variables of maize under irrigated Mediterranean semiarid conditions.

Material and methods

Experimental conditions

The study was conducted in a sprinkler irrigated field located in Almodévar (Ebro valley, 42°02'N/0°34'W, altitude of 390 m), a major irrigated agricultural area in Spain. The soil is a fine silt loam classified as Typic Xerofluvent. This is a calcareous soil with a total carbonate content of 38%, pH (in water) of 7.8, 2.1% of soil organic matter, 24 mg/kg of P (Olsen), and 300 mg/kg of K in the upper Ap horizon (0-35 cm). The climate is Mediterranean semiarid with high solar radiation, yearly average precipitation of 453 mm, and average air temperature of 12.4 °C. The experiment was performed during 2011 and 2012 in a 6-ha grower's field. A range in mineral N availability in the soil at pre-plant was created by applying different amounts of N fertilizer during the previous season as part of larger N fertilization trial (Isla *et al.*, 2012). A total of 15 (in 2011) and 16 (2012) plots from a larger completely randomized design fertilizer trial were selected to conduct this experiment. Soil samples were taken from 0 to 60 cm depth and analysed for nitrate concentration by colorimetry. Previous studies under similar environmental conditions have showed that ammonium content in the soil are extremely low and negligible compared to nitrate values. Soil mineral N concentration values were converted to mass values in kg/ha by considering a soil bulk density of 1.50 mg/cm³ from previous experiments in the same field. Pre-planting soil mineral N (0-60 cm) ranged from 50 to 351 kg N across years and plots. The N fertilizer treatments ranged from 0 to 300 kg N/ha which are within the normal rates used by maize growers in the area. The N fertilizer was applied as ammonium nitrate (33.5% N) and split in three applications: at pre-planting (April 12th 2011 and April 11th 2012), at V6 stage (June 9th 2011 and June 6th 2012), and at V15 stage (July 12th 2011 and July 16th 2012). Maize cv. 'PR34N43' (Pioneer Hi-Bred International) was planted on April 19th (2011) and April 26th (2012) at a planting density of 73,000 plants/ha and a 75 cm row spacing. Maize was harvested manually in October 3rd (2011) and October 1st (2012) by collecting all ears in the two central rows of each experimental plot (12

m²). The ears were threshed and grain yield was reported on a 14% moisture content. Plots were sprinkler-irrigated according to the crop evapotranspiration to avoid water stress and the total amount of irrigation water applied was similar in both growing seasons (8100 m³/ha/yr). Weed and pest control were performed according to common management practices in the area.

Instantaneous leaf gas exchange measurements

Gas exchange measurements were performed using a portable LI-6400 open gas exchange system (LI-COR, 1998) that included a CO₂ mixer and a 6400-02B led light source. These two accessories were used in our study to maintain a constant CO₂ concentration of 380 ppm and a constant incident photosynthetic active radiation (PAR) of 1850 μmol/m²/s in the leaf chamber during the instantaneous measurements. Net assimilation rate (An), stomatal conductance to water vapour (g_{sw}), transpiration rate (Tr), and intercellular CO₂ concentration (Ci) were obtained from the instantaneous measurements. The water use efficiency (WUE) at leaf level was calculated as the ratio between An and Tr.

In 2011, two sets of diurnal instantaneous light saturated leaf photosynthesis measurements were conducted in June 23th (V8) and July 7th (V14) on the last fully emerged leaf and on the ear leaf, respectively. In 2012, one set of measurements was accomplished on 31 August on the ear leaf. In both seasons, the measurements were taken in the central part of the leaf. A portable chlorophyll meter (SPAD-502, Minolta Camera Co., Ltd., Osaka, Japan) was used to select leaves with a range of values in chlorophyll meter readings (CMR). Immediately after the CMR values were recorded, the same portion of the leaf was clamped into the gas exchange chamber for measuring instantaneous photosynthesis. A total of 166 and 30 plants were measured in 2011 and 2012, respectively, across all plots within each growing season that differed in soil mineral N. A significantly lower number of plants were measured during 2012 due to a hailstorm that damaged the upper part of the canopy, reducing the number of intact leaves available to perform consistent gas exchange and CMR measurements.

Light response curves

Light response curves were obtained during the 2011 growing season at two sampling dates (27-Jul and 17-Aug) from 13 ear leaves. Similarly to instantaneous measurements, the leaves were previously selected by their CMR values to choose plants with a wide range in

N-sufficiency. The rapid light response curves were obtained according to the methodology provided in the LICOR-6400 manual. Gas exchange measurements started with a high PAR (2000 μmol) and dropped to 1500, 1000, 500, 200, 100, 50, 20, and 0 μmol. The flow rate was fixed to 400 μmol/s, and the CO₂ concentration to 380 ppm. In order to minimize diurnal fluctuations of ambient temperature, the block temperature was fixed depending on the observed ambient temperature (26 and 32°C on July 27 and August 17, respectively).

The fitted light-response models were used to determine five parameters: An_{max} as the maximum net photosynthesis rate under saturated light conditions; the saturation point (SP) as the PAR that allows to reach An_{max}; the quantum efficiency (QE) as the first derivative of An at PAR=250 μmol/m²/s; the dark respiration rate (Rd) as the assimilation rate at PAR=0 μmol/m²/s; and the light compensation point (LCP) as the PAR at which An=0.

Leaf analysis

After the light curve was obtained, the sampled leaf was collected and introduced in a plastic bag and inside a portable refrigerator. The leaves were transported to the laboratory and the leaf area, fresh weight, and dimensions (length, and maximum width) were measured. The leaf area was measured with a LI-3100C meter (LI-COR, 1998). Finally, the leaves were oven dried at 65° until constant weight, ground and analyzed for total N by combustion (TruSpec CN, LECO, St. Joseph, MI, USA). The specific leaf area (m²/kg) was calculated as the rate between leaf area and dry weight.

Statistical analysis

Statistical analyses were performed using the SAS 9.1 software. Grain yield response to N applied was fitted to a linear-plateau response (LPR) model using the NLIN procedure. The An response to PAR was fitted to a quadratic-plateau response (QPR) model using the same procedure. Analysis of variance and multiple comparison (protected LSD test) was used with a significance level of α=0.05 to compare among groups considering a completely randomized design.

Results

Maize grain yield

Maize grain yield ranged from 6-8 Mg/ha in the low-fertilized plots to 14-15 Mg/ha in the plots with high

N fertilizer applications. The total available N was estimated as the sum of preplant soil mineral N plus N applied with fertilizer. Total available N ranged from less than 100 to more than 500 kg N/ha (Fig. 1), which indicates that some plots were clearly N deficient and others had excess or non-limiting N levels. The response of grain yield to available N across the two years (31 experimental plots) was fitted to a linear-plateau model, indicating a significant crop response to the N available with a threshold value of 179 kg N/ha between N-deficient (plots with grain yield lower than the maximum) and non N-deficient plots. Plots with available N slightly over 179 kg N/ha would avoid yield penalties from N deficiencies as well as potential negative environmental effects from N excess, and thus can be considered an optimum N level from an agronomic point of view.

Instantaneous leaf gas exchange measurements and leaf chlorophyll readings

The CMR readings showed a high linear relationship ($R^2=0.85$, $n=13$, $p<0.01$) to total N concentration in the ear leaves (data not shown), indicating that CMR values can be used to estimate leaf N concentrations (RMSE=0.19%) under our environmental conditions. No saturation effect in CMR was observed at high values of total leaf N concentration. In addition, there was marginal negative relationship ($R^2=0.26$; $p=0.08$) between specific leaf area and CMR, indicating that

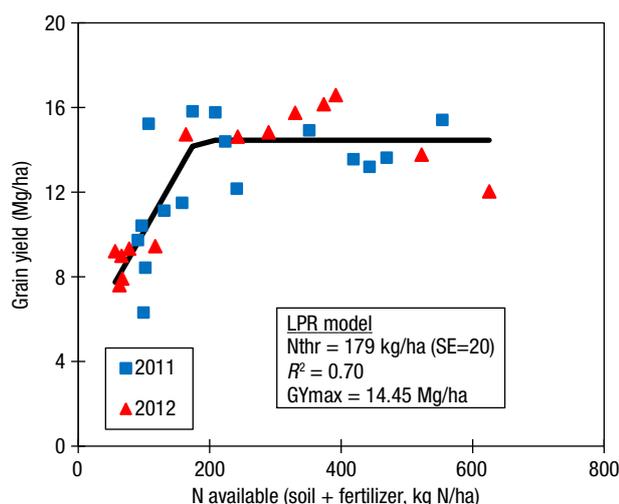


Figure 1. Relationship between grain yield and the nitrogen available to the crop. The N available is the sum of the soil mineral nitrogen (0-60 cm) before sowing and the N applied with fertilizer. The linear plateau response (LPR) model was obtained from the pooled 2011 and 2012 data. The Nthr is the value of N available that maximizes grain yield (GYmax) according to the model.

N-deficient leaves were thinner than non N-deficient leaves.

The size of the ear leaves ranged from 560 to 760 cm² depending on the N available in the soil. The area of the ear leaf was significantly related to CMR values ($R^2=0.43$, $p<0.05$) (data not shown), indicating that treatments with lower CMR values presented smaller ear leaves.

Instantaneous An rates ranged from 20 to 50 $\mu\text{mol CO}_2/\text{m}^2/\text{s}$ in 2011 and from 5 to 30 $\mu\text{mol CO}_2/\text{m}^2/\text{s}$ in 2012 (Fig. 2). Similar An rates were observed in 2011 irrespective of leaf age (last emerged leaf in V8 stage and ear leaf in V14). Figure 2a shows that during 2011, An was positively correlated to CMR at the V8-V9 stage ($R^2=0.14$, $p<0.01$), but not when measured on the ear leaf at the V14 stage. However, only a 14% of the variability in An was explained by differences in CMR, indicating that some other factors not associated with N availability might be affecting instantaneous leaf An measurements under field conditions. Since the slopes at the two sampling dates in 2011 were not significantly different ($p>0.05$), data from the two sampling dates were pooled and there was a weak positive correlation ($R^2=0.07$; $p<0.001$) between An and CMR. Leaves with high CMR showed higher variability in An compared to those with low CMR values. Similarly, in 2012 there was a weak correlation An and CMR ($R^2=0.13$, $p < 0.05$).

Data collected during the instantaneous leaf gas exchange measurements were grouped according to CMR values in three groups: high (CMR>54), medium ($48 < \text{CMR} < 54$), and low (CMR < 48). Leaves with low CMR levels showed a lower An and stomatal conductance (g_{sw}) than the Medium class ($p<0.05$) (Table 1). Surprisingly, An and g_{sw} were not higher in the High CMR class compared to the other classes. Differences in An between the Medium and Low CMR classes were relatively small (12%), although significant ($p < 0.05$). All CMR classes showed similar intercellular C_i and WUE at both sampling dates.

Light response curves and N sufficiency

The light response curves were obtained for a total of 13 leaves from plots with variable available N. The QPR model describing the relationship between An and CMR adjusted very well to the experimental data across all sampled leaves (average $R^2=99\%$). Figure 3a,b shows the obtained light response curves for the leaf with the lowest and the highest CMR value at each sampling date during 2011. The leaves with the lowest CMR (45.3 and 32.8 in the two sampling dates) presented An_{max} around 20-25 $\mu\text{mol CO}_2/\text{m}^2/\text{s}$ while the

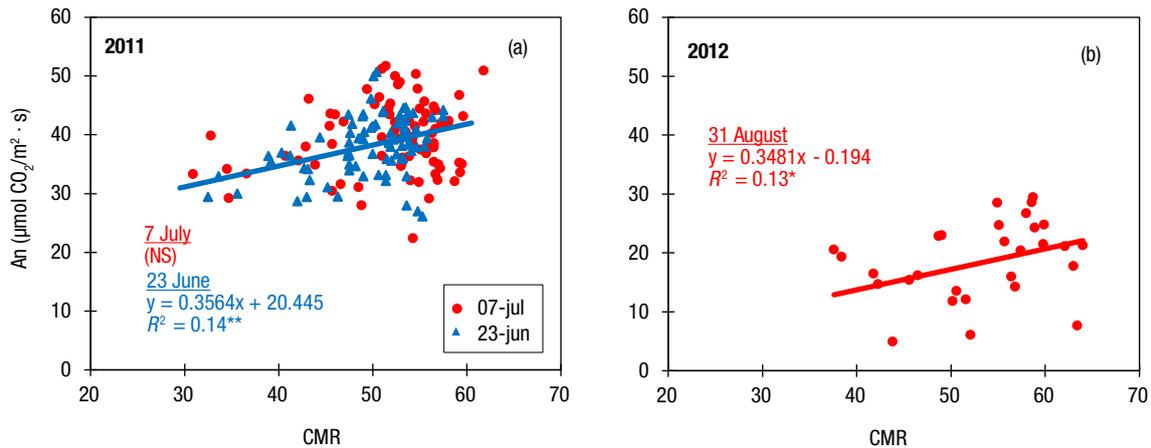


Figure 2. Relationship between light-saturated leaf net photosynthesis (A_n) measurements and portable chlorophyll meter readings (CMR) during 2011 (a) and 2012 (b) experiments. The significant linear regressions are presented. NS: no significant relationship.

leaves with higher CMR (50-60) presented $A_{n_{max}}$ values of $40 \mu\text{mol CO}_2/\text{m}^2/\text{s}$.

The maximum net assimilation rate ($A_{n_{max}}$), SP, QE, Rd, and the LCP were estimated from the thirteen fitted curves estimated at each sampling date, and their relationships with CMR values were obtained (Fig. 4). The CMR values were significantly related to $A_{n_{max}}$, the SP, the Rd, and the LCP ($p < 0.05$). However, the QE was not affected by differences in leaf N status ($p > 0.05$). The relationships between the different photosynthetic parameters and CMR readings were adjusted using a simple regression and additionally using a LPR model when a plateau effect was observed in the data. From the LPR, threshold CMRs of 52.7, 55.6, and 54.2 were obtained for the $A_{n_{max}}$, SP, and LCP, respectively. Overall, these LPR models were able to explain about a 51% of the observed variability, while the simple regression explained 43% of the variability. The Rd significantly

increased (more negative values) with CMR readings indicating a higher mitochondrial activity under optimal N conditions than under N-limiting ones.

Discussion

The observed differences in maize grain yield were highly associated to available mineral N following a typical LPR curve, agreeing with previous experiments under similar irrigated Mediterranean conditions (Berenquer *et al.*, 2009; Isla *et al.*, 2015). Low levels of available mineral N decreased leaf area, leaf N concentration, and instantaneous measurements of A_n at V8 and on the ear leaf at V14. However, the relationships between instantaneous A_n and CMR were weak in both growing seasons at these developmental stages. Therefore, the results can indicate that at low levels of

Table 1. Mean of chlorophyll meter readings (CMR), assimilation rate (A_n), stomatal conductance to water vapour (g_{sw}), intercellular CO_2 concentration (C_i), and water use efficiency (WUE) in the three N-sufficiency classes of leaves. Data from the two sampling dates from 2011 experiment are presented. For each sampling date, values followed by the same letter are not significantly different ($p > 0.05$).

N class	n	Leaf N ^[1]	CMR	A_n	g_{sw}	C_i	WUE
23 June							
High	6	2.10	55.5 a	38.3 ab	361 ab	121	6.32
Medium	41	1.90	51.7 b	39.7 a	379 a	118	6.39
Low	34	1.50	43.3 c	35.3 b	311 b	117	6.48
7 July							
High	31	2.15	56.6 a	39.1 ab	345 b	104	6.93
Medium	29	1.93	51.9 b	41.5 a	408 a	112	6.77
Low	20	1.40	41.4 c	37.2 b	306 b	97	6.91

^[1] Estimated from a linear regression between leaf total-N and CMR ($R^2=0.85$, $n=13$). Units: A_n ($\mu\text{mol CO}_2/\text{m}^2/\text{s}$); g_{sw} ($\text{mmol H}_2\text{O}/\text{m}^2/\text{s}$); C_i ($\mu\text{mol CO}_2/\text{mol}$); WUE ($\mu\text{mol CO}_2/\text{mmol H}_2\text{O}$).

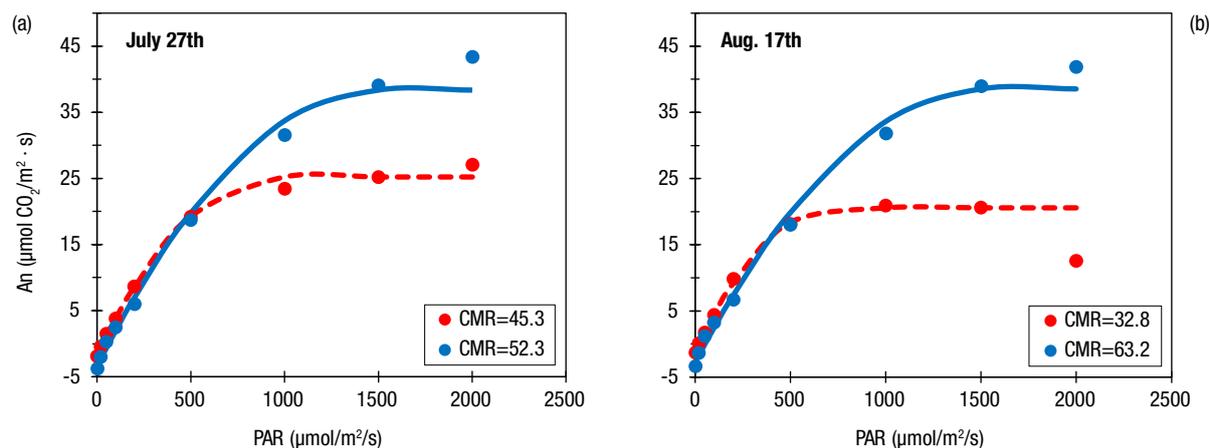


Figure 3. Light saturation curves of two maize leaves with higher differences in chlorophyll meter readings (CMR) on 27 July (a) and 17 August (b) of 2011. The quadratic plateau response (QPR) curves were adjusted to the data. The R^2 of all fitted models were higher than 0.98.

mineral by V8 to V14: (i) maize plants partially compensated for low N availability reducing leaf area, and (ii) the leaf N concentrations were able to maintain leaf photosynthetic rates only 10 to 11% lower compared with the middle or high N level classes. These results are in contrast with results by Vos *et al.* (2005) with maize grown in a glasshouse environment, where the primary strategy in maize under N limiting conditions was a reduction of leaf N content and photosynthetic capacity and not a decrease in leaf area expansion. The weak relationship obtained between instantaneous A_n and CMR readings also suggests that other environmental factors are affecting assimilation rates in addition to leaf N content.

The results also showed a tendency for higher values of A_n in the Middle CMR class or intermediate N supply, in agreement with the studies of Toth *et al.* (2002) and Hammad *et al.* (2012) that found the peak of photosynthetic rate at moderate levels of N fertilization rates and a tendency to decrease the photosynthetic rates when an excess of N fertilizer was applied. This observed tendency to decrease A_n under elevated N supply needs further research to be confirmed.

The light response curves obtained later in the growing season during 2011 indicated that there was a decrease in light-saturated photosynthesis with CMR readings except at low irradiance levels ($< 500 \mu\text{mol}/\text{m}^2/\text{s}$). This is consistent with previous studies of Khamis *et al.* (1990) in maize under controlled conditions. In their study, N concentrations in the ear leaf of 1.4-1.5% were high enough to reach similar assimilation rates to those of leaves with a higher N concentration but located at the bottom of the canopy, or at the upper part but during periods of low irradiance (early morning and late afternoon). In our study, leaves at the upper part of the canopy that received high levels of

irradiance were able to maximize photosynthesis if CMR values were higher than 53 (about 2% of total N). The increase in maximum photosynthetic rate and light saturation point with higher CMR readings or N availability found in this study were also described by Lin *et al.* (2013) in oats and by Xu *et al.* (2014) in rice. The better relationship obtained between CMR readings and light-saturated photosynthesis with measurements on July 27th and August 17th 2011 compared to the earlier instantaneous gas exchange measurements on June 23rd and July 7th can indicate that leaf photosynthesis became more limited by N availability later in the growing season. Previous studies support that differences in leaf photosynthetic rates are most likely to occur after silking (Earl & Tollenaar, 1999).

The significant decrease in the R_d observed in our experiment is coherent with the work of Osaki *et al.* (2001) and Earl *et al.* (2012) who found that N stress decreased shoot R_d rates in maize. In our study, there was no effect of N availability on C_i values, which differs from the results obtained by Correia *et al.* (2005) and Chen *et al.* (2013) where C_i increased in N-deficient maize plants compared to the control ones. Although stomatal conductance (and C_i values) tended to be lower under N-deficient conditions it is not clear that this was the main reason for lower A_n values. For a given C_i value, leaves with higher CMR presented slightly higher assimilation rates (Fig. 5), which suggests that under low N supply, light-saturated leaf photosynthesis is not stomatal limited and can be limited by a decrease in the photosynthetic enzymes and a lower rubisco activity in the mesophyll as suggested by Khamis *et al.* (1990). Similar A_n/C_i response under different N supplies was described by Chen *et al.* (2013) using CO_2 response curves corroborating that stomatal limitation is not the main reason of reduced

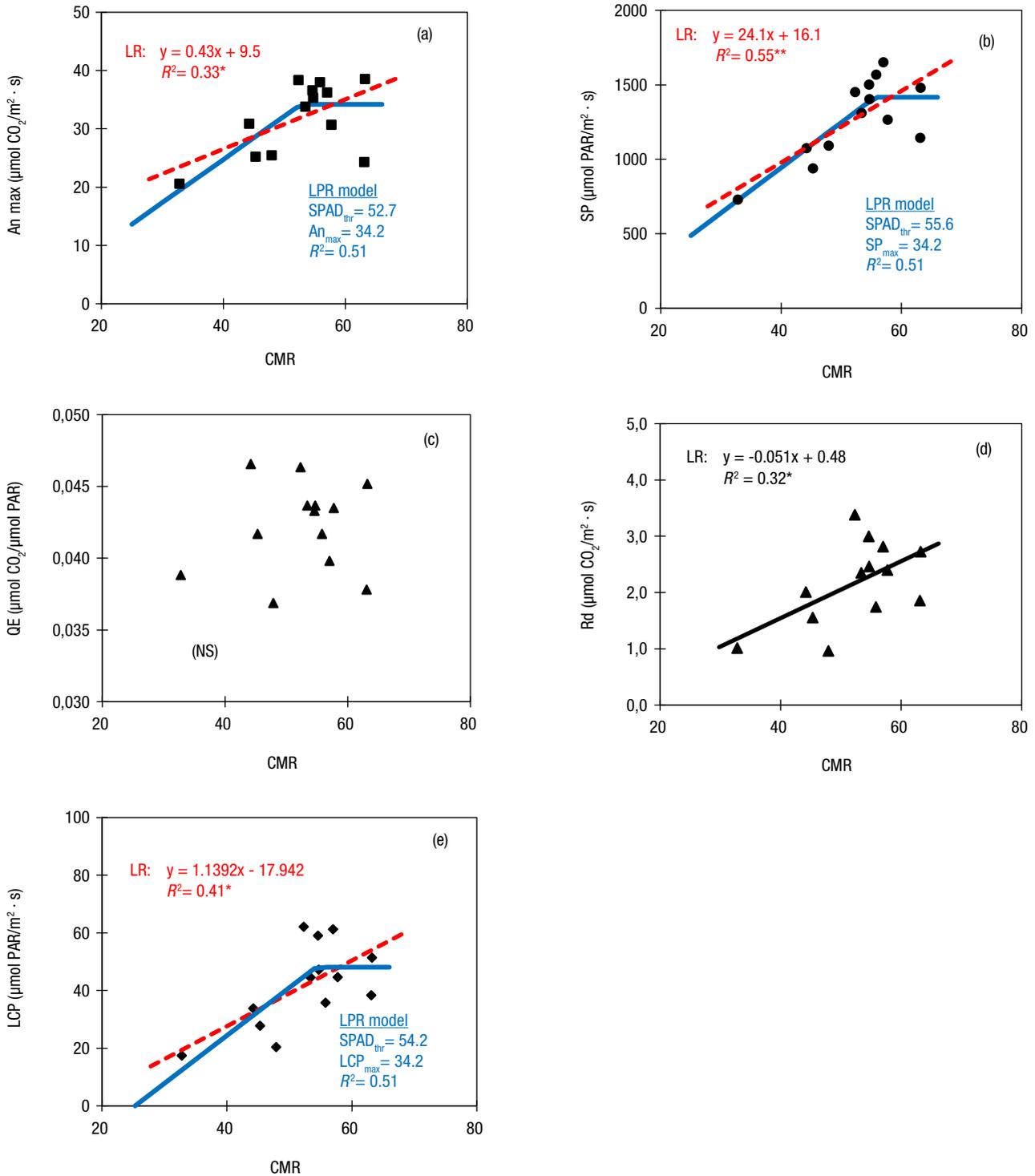


Figure 4. Effect of different levels of N sufficiency (CMR) on (a) maximum assimilation rate ($A_{n_{max}}$), (b) light saturation point (SP), (c) QE: quantum efficiency, (d) dark respiration rate (Rd), and (e) light compensation point (LCP). The linear regression (LR) and the linear plateau response model (LPR) are presented when significant. Data from 2011 experiment.

assimilation rates under N deficit. Paul & Driscoll (1997) suggested that in tobacco plants the reductions in assimilation rates in N-deficient leaves were associated from a feed-back inhibition mechanism due to lower carbohydrate content. The decrease of dark res-

piration in N-deficient plants was also described by Gonzalez-Meler (1997) and Lin *et al.* (2013) in wheat, pepper, and oats, respectively. However, the opposite effect was found in maize plants by Chen *et al.* (2013), who reported lower Rd for plants grown in high-N sup-

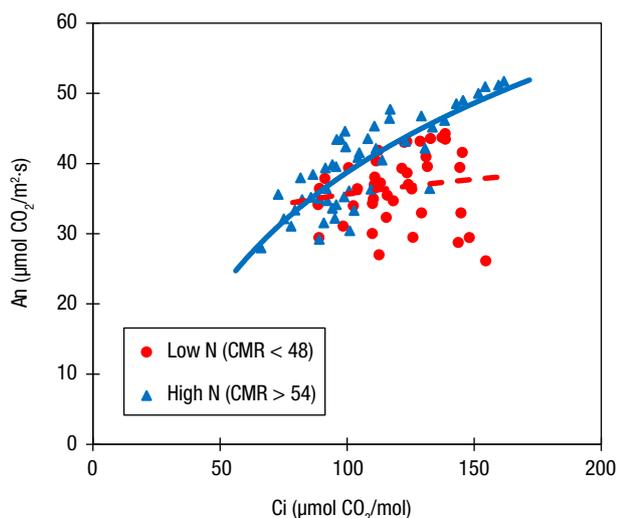


Figure 5. Relationship between light-saturated net photosynthesis (A_n) and intercellular CO_2 concentration (C_i) in maize plants under high (average leaf $\text{CMR}=55$) and low (average leaf $\text{CMR}=43$) levels of N availability. Data from 2011 experiment.

ply that can associated to the higher growing rates observed under non N-limiting conditions compared to N-limited conditions.

Our study shows that in irrigated maize, N deficit produces a significant decrease in both single leaves area and leaf light-saturated photosynthetic rates that lead to significant yield reductions. Leaves with deficient N concentration presented also reduced dark respiration rates, a lower LCP, and a lower light saturation point.

Under conditions of intensive irrigated maize with a high N demand, large N fertilizer applications and N excess are more common than conditions of N deficit, which is producing environmental impacts in water bodies (Isidoro & Aragüés, 2007) and to the atmosphere through nitrous oxide emissions (Alvaro-Fuentes *et al.*, 2016). The present study showed that low levels of N supply produced a decrease in leaf photosynthesis in maize that could be partially accounted for with SPAD readings. The results show clearly that increasing N supply above the optimum increased leaf N content but did not necessarily mean higher assimilation rates or maize yield. According our results, intermediate N ear-leaf contents of about 1.9% were enough to maximize leaf assimilation rates and yield of field-grown maize under irrigated Mediterranean conditions.

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