ASSESSMENT OF NITROGEN DIAGNOSIS METHODS IN SUNFLOWER

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DECLARATIONS

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Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest

Availability of data and material

The raw data that support the findings of this study are available from the corresponding authors, N.I.R.C and S.T.H, upon reasonable request.

Code availability

Not applicable

CORE IDEAS

Aim: evaluate nitrogen diagnosis methods for grain yield and quality of sunflower.

Nitrogen mineralized in anaerobic incubation improved the pre-plant soil NO_3^--N test.

The relative Green Seeker at V_{12} growth stage was associated with relative yield.

Grain N concentration diagnosed N availability in sunflower.

The nitrogen availability to grain yield ratio defined the N rate required.

ABSTRACT

Nitrogen (N) deficiency can severely limits sunflower (*Helianthus annuus L.*) grain yield and quality. Our objective was to evaluate N diagnosis methods based on: (i) pre-plant soil NO_3^--N test (PPSNT) and soil N mineralized in short-term anaerobic incubation (Nan), (ii) Greenness index (G_I) and the normalized difference vegetation index (NDVI) measured at six (V_6) and twelve (V_{12}) leaves, and (iii) grain N concentration (N_c). Seventeen experiments were carried out between 2010-2019 in Argentina, evaluating nine N rates (0, 30, 40, 60, 80, 90, 120, 150, and 160 kg N ha^{-1}). The G_I, NDVI, nitrogen sufficiency index and relative NDVI (NDVIr) were determined at V_6 and V_{12} growth stages. On average, yield response to N was 492 kg ha^{-1} and N_c response was 0.25% in 9 and 11 responsive experiments, respectively. The inclusion of Nan improved the PPSNT diagnosis method. The critical N availability (PPSNT + fertilizer N) threshold was 115 kg N ha^{-1} for experiments with low Nan (<60 mg kg^{-1}), and 90 kg N ha^{-1} for experiments with high Nan (>60 mg kg^{-1}). The NDVIr at V_{12} allowed monitoring the crop N status with a 0.95 critical threshold. N_c adequately diagnosed N deficiencies and the critical threshold was 2.26%. Also, N_c was predicted from the ratio between N availability and grain yield (R^2= 0.39). Our results would allow to better estimate N availability to recommend adequate N fertilizer rates for sunflower aiming to optimize grain yield and quality, and minimize the economic and environmental cost of fertilization.

Keywords: canopy indices; chlorophyll meter; mineralization; nitrogen grain concentration; soil analysis.

ABBREVIATIONS

ALCC: Arcsine logarithm calibration model.

G_I: Greenness index

G_N: Grain number.

G_W: 1000-grain weight.

NSI: N sufficiency index.

Nan: N mineralized in short-term anaerobic incubation.

NDVI: Normalized difference vegetation index.

NDVIr: Relative normalized difference vegetation index.

N_c: Nitrogen concentration.

PPSNT: Pre-plant soil NO_3^--N test

R_Y: Relative yield.

SPAD: Soil Plant Analysis Development.

1. INTRODUCTION

Nitrogen (N) is the main nutrient limiting sunflower (*Helianthus annuus L.*) grain yield and quality (Massignam et al., 2009; Ören and Çelik, 2018). Grain oil concentration defines the commercial quality, while protein concentration is important for sub products like pellets or protein powders (Merrien et al., 1988). An adequate N availability is needed to achieve high yields and grains with high protein concentration, but an excessive nitrogen level could decrease oil concentration (Ali and Ullah, 2012; Wajid et al., 2012). However, Scheiner et al. (2002), Ruffo et al. (2003), and Diovisalvi et al. (2018) have reported that oil concentration was not affected, even with a high N rate (150 kg N ha^{-1} applied at V_2-V_3). Accordingly, it is necessary to develop and calibrate accurate diagnosis methods to maximize grain yield and quality, while reducing N losses.

The most widespread N diagnosis method is based on soil nitrate (NO_3^--N) content (0-60 cm) before or at the crop sowing (PPSNT) (Diovisalvi et al., 2018; Schultz et al., 2018). Thus, different N availability thresholds (PPSNT + fertilizer N) have been proposed in sunflower to maximize grain yield (Diovisalvi et al., 2018). However, the use of high yield potential genotypes in the last years would require updating the critical PPSNT threshold due to an increase in the crop N demand (Pereyra *et al.*, 2001). Nevertheless, PPSNT determination does not consider the contribution of N from the organic matter mineralization during the growing season, which represents a significant source of N (Reussi Calvo et al., 2018). This contribution could be estimated by the N mineralized in short-term anaerobic incubation (Nan) in the soil surface layer (Keeney, 1983, Reussi Calvo et al., 2018). For example, the use of a combined index (PPSNT + Nan) improved the prediction of wheat (*Triticum aestivum L.*) yield and its grain N content, compared to the sole use of PPSNT (Reussi Calvo et al., 2013). For maize (Zea mays L.), PPSNT critical values were 75 and 90 kg N ha^{-1}, for sites with high and low Nan, respectively (Sainz Rozas et al., 2008). Therefore, it may be possible to improve sunflower N diagnosis by considering Nan combined with pre-plant N availability.

Proximal sensors allow determining the crop N status in a fast and non-destructive way throughout the crop growing season. The Soil Plant Analysis Development (SPAD) (Minolta SPAD® 502) has been widely used to quantify the leaf greenness index (G_l) which is related with both, the leaf chlorophyll content and N concentration (Waskom et al., 1996). Moreover, Green Seeker (Ntech Industries, Inc., Ukiah, CA.) determines the canopy reflectance and expresses the results as the normalized difference vegetation index (NDVI) (Qualm et al., 2010; Gutiérrez-Soto et al., 2011). Both indices are affected by factors like genotype, growth stage, diseases, water availability, and other nutrients (Blackmer and Schepers, 1995; Gandrup et al., 2004). For example, previous studies in maize performed in greenhouse (Pagani and Echeverría, 2012) and field conditions (Pagani and Echeverría, 2011; Carciochi et al., 2018) suggested that SPAD varies with sulfur status. Therefore, it is recommended to relativize the measurements to plots without N deficiency, obtaining the N sufficiency index (NSI) for the SPAD (Barker and Sawyer, 2010), and the relative NDVI (NDVIr) for the Green Seeker (Barker and Sawyer, 2010; Clay et al., 2012; Samborski et al., 2009). Both indices have been successfully evaluated in different crops like wheat and barley (*Hordeum vulgare* L.) (Reussi Calvo et al., 2020), maize (Hawkins et al., 2007; Sainz Rozas et al., 2019), and potato (*Solanum tuberosum* L.) (Giletto et al., 2010). For sunflower, the active-optical sensor readings at V_6 and V_{12} growth stages (Schneiter and Miller 1981) were related to confection yield, but not to oilseed yield (Franzen et al., 2019). However, there is a lack of information about N diagnostic for this crop.

The grain nutrient concentration analysis could be a valuable tool for recognize nutrient deficiencies post-mortem (Divito et al., 2015; Carciochi et al., 2019). Specifically, grain N concentration (N_c) has proven to be a useful tool for predicting the nutritional status of crops such as maize (Chen et al., 2010; Barbieri et al., 2013), rice (*Oriza sativa)* (Fageria, 2003), wheat (Goos et al., 1982), and cotton (*Gossypium herbaceum* L.) (Egelkraut et al., 2004). However, no attempt has been made to calibrate N_c as a N diagnosis method in sunflower.

Argentina is the fourth largest sunflower producer with 7% of the total world production and a mean grain yield of 1.9 Mg ha^{-1} (Castaño, 2018). Currently, the sunflower yield gap in Argentina is approximately 40% with an average level of 0.75 Mg ha^{-1} (Hall et al., 2013). So, N fertilization could decrease the yield gap and improve grain quality. The aim of this work was to evaluate the predictive capability of N diagnosis methods based on: 1) PPSNT and Nan, 2) canopy sensors (G_I and NDVI) at V_6 and V_{12} development stages, and 3) grain N_c, with the purpose of improving sunflower nutrition management to increase grain yield and quality.

2. MATERIALS AND METHODS

2.1. The experiments

Seventeen experiments were conducted under no-tillage since 2010 until 2019 in the southeastern Buenos Aires Province, Argentina (from 37°06’ S, 57°25’ W to 38°40’ S, 60°08’ W). In this area, the mean values of precipitation, potential evapotranspiration, and temperature are 955 mm, 950 mm, and 13.9 °C, respectively. Predominant soils are Petrocalcic Argiudoll (serie fine, mixed, thermic) and Typic Argiudoll (serie fine, mixed, thermic) (Soil Taxonomy) with a slope <2%. Additionalinformation about soil and weather characteristics is described in Table 1 and Table 2. At each experiment, treatments consisted of at least three N rates ranging from 0 to 160 kg N ha^{-1}, which were surface-broadcasted as urea (46-0-0) at the crop emergence. Sowing dates were the recommended for the area (between October and November). Plant density varied between 5 and 6 plants m^{-2} and row spacing was 0.52 or 0.70 m, depending on the site. A 60% of the experiments were sowed with conventional genotypes and 40% with high oleic hybrids, all with high yield and oil potential. At each experiment, treatments were arranged in a complete randomized block with three replicates. The size of the experimental unit was 10 rows wide by 12 m long. Phosphorus (30–40 kg ha^{-1}) and S (20–25 kg ha^{-1}) fertilizers were applied to all plots to ensure the adequate availability of these nutrients. When necessary, pesticides were applied at recommended rates to control weeds, pests, and fungal diseases. All treatments received equal pest control. Rainfall data were obtained from INTA-Balcarce (Instituto Nacional de Tecnologia Agropecuaria) and from the National Weather Service (SMN, Servicio Meteorológico Nacional). Additional information about the experiments is presented in Table 1.

(Please place Table 1 here)

2.2. Soil and plant analyses

Soil samples were taken from each site at planting at 0-20, 20-40, and 40-60 cm depth using a stainless-steel probe. Soil organic matter (SOM) (Walkey and Black, 1934), pH (1:2.5 soil-water ratio) (Thomas and Hargrove, 1984), extractable P (P-Bray) (Bray and Kurtz (1945) and Nan (Keeney, 1983) were determinate at 0-20 cm depth, while volumetric humidity and NO_3^--N (Dahnke,1971) were quantified at the three depths. Specifically, Nan method consisted of incubating 10 g soil in a stoppered tube filled with water (anaerobic condition) for 7 d at 40°C. After the incubation period, the resulting slurry was extracted with a 4 mol L^{-1} KCl solution and steam distillated. The ammonia in the distillate was trapped in a mixed boric acid and indicator solution, which was then titrated with sulfuric acid. The initial NH_4^+ content in the soil samples was subtracted from the value determined after the incubation (Keeney, 1983).

The G_I and NDVI were determined in all treatments at V_6 and V_{12} growth stages for the experiments E4 to E13. Due to operational problems, G_I and NDVI were not measured at the remaining experiments (E1 to E3 and E14 to E17). The G_I readings were performed using the Minolta SPAD® 502 chlorophyll meter (Spectrum Technologies Inc., Plainfield, IL) which measures leaf light transmittance at 650 and 940 nm. Average G_i values were obtained by taking 20 readings on the last fully expanded leaf (between 4 and 8 cm in length). NDVI measurements were made with the Green Seeker (Ntech Industries, Inc., Ukiah, CA.) which detects reflection from Red (650–670 nm) and Near Infra-Red (NIR) (755–785 nm) spectral regions. The NDVI readings were collected at 0.8-1.0 m height above canopy advancing on the plots at a constant speed (1.3 m s^{-1}). The NDVI was calculated by the sensor with an in-build software that uses the ecuation: [NDVI = (NIR - Red)/(NIR + Red)].

From G_I and NDVI readings and for each measurement time, the relative G_I (NSI) and NDVI (NDVIr) were determined as the ratio of the G_I or NDVI measured in a given N rate to the G_I or NDVI in the highest N rate, respectively.

At R9, the two middle rows from each experimental unit (10 m^2) were manually harvested and threshed with a stationary machine. Grain yield was expressed at 110 g kg^{-1} moisture content. In addition, 1000-grain weight (G_W) and grain number per m^2 (G_N) were determined. Yield response to N was calculated as the yield difference between the N-fertilized and the control (0 kg N ha^{-1}) treatment. The relative yield (RY) was calculated as the ratio between the yield of each treatment to the highest yielding treatment multiplied by 100.

The Nc was quantified using the Dumas method, which consist in a high temperature (950 °C) dry combustion of the sample and subsequent thermoconductivity detection using a TruSpec CN analyzer (LECO, 2010).

2.4 Data analysis

Due to the unbalanced database (not all experiments had the same treatments) a linear mixed model was fitted to determined the effect of N rate on the response variables (yield, G_N, G_W and N_C), in which N rate was the fixed factor and experiment was the random factor. Additionally, another model was fitted in which the hybrid was nested within the experiment as a random factor. However, this model did not differ significantly ($p>0.05$) from the previous model. Therefore, the simpler model was selected. Once the model was adjusted, a least significant difference test (LSD) ($p< 0.05$) was performed for means comparison among N rate treatments. The lme (Linear Mixed-Effects Models) function of the nlme library, the lm (linear model) function and the emmeans and multcomp libraries from the statistical software R, were used to perform the statistical analysis (R Core Team, 2014). Normality data distribution and homogeneity of variances were confirmed using the Shapiro and Wilk (1965) and Levene (1961) methods, respectively. The maximum N response was defined as the difference between the average of maximum yield, G_W, and G_N that did not differ from each other (p value > 0.05), and the control treatment. In order to improve the PPSNT diagnostic model, experiments were classified into two categories according to Nan level, low (Nan< 60 mg kg^{-1}) and high (Nan> 60 mg kg^{-1}), considering the mean value of the study-area and the classification made by Sainz Rozas et al. (2008). The ten experiments where canopy indices were tested corresponded to the low-Nan category. An arcsine logarithm calibration model

(ALCC) proposed by Dyson and Conyers (2013) and modified by Correndo et al. (2017) was used to determine the critical threshold of each diagnosis method evaluated.

3. RESULTS AND DISCUSSION

3.1. Edaphic and climatic properties

Soil pH values ranged from 5.6 to 6.2, SOM from 44 to 71 g kg^{-1}, P-Bray from 4.8 to 21.9 mg kg^{-1}, Nan from 35 to 103 mg kg^{-1}, and NO_3^--N from 40 to 88 kg ha^{-1} (Table 1). These values were within those described for soils from the Argentinean Pampas by Sainz Rozas et al. (2011) and Reussi Calvo et al. (2018). Since all experiments presented similar textural class, variations in SOM, Nan, and NO_3^--N can be mainly attributed to differences in management history and preceding crops (Bationo et al., 2007; Quiroga et al., 2006; Diovisalvi et al., 2014;). Moreover, both Nan and NO_3^--N data showed wide variability, representing different initial N availability and potential N supply through mineralization. Our study demonstrated not significant association between Nan and SOM (R^2= 0.08, p>0.05) or NO_3^--N (R^2= 0.001, p>0.05) (Table 1). This lack of correlation between Nan and NO_3^--N reinforces the validity of developing a combined index using these two variables (NO_3^--N + Nan).

Total water availability (in season rainfall + initial soil water content) at all experiments was higher than the crop demand (450-550 mm) (Berglund, 2007) (Table 1 and Table 2). As reported by Reussi Calvo and Echeverria (2006) for wheat growing in the studied area, during October to November (sunflower sowing date) there is a 25-30% probability of water excesses occurrence. An adequate water supply during these months would ensure a good crop implantation and an adequate incorporation of fertilizers. The average monthly mean temperature across experiments during the growing season was 17.8 °C (Table 2), value within the historical record for each experiment location and did not negatively affect sunflower yield and grain quality.

(Please place Table 2 here)

Table 2: Monthly rainfall and average daily mean temperature (Temp) during sunflower growing season at each experiment (Exp).

Month	**October**		**November**		**December**		**January**		**February**	
Exp	**Precipitation mm**	**Temp. °C**	**Precipitation mm**	**Temp. °C**	**Precipitation mm**	**Temp. °C**	**Precipitation mm**	**Temp. °C**	**Precipitation mm**	**Temp. °C**
E1	48	13.4	115	16.2	33	20.9	185	15.7	33	14.1
E2	56	12.5	100	14.5	81	18.6	144	21.3	88	19.5
E3	56	12.5	100	14.5	81	18.6	144	21.3	88	19.5
E4	116	13.4	128	15.5	88	19.3	123	19.1	110	20.5
E5	135	13.3	127	15.2	85	18.7	46	19.0	77	20.3
E6	135	13.3	127	15.2	85	18.7	46	19.0	77	20.3
E7	113	15.0	132	16.8	76	20.7	101	20.6	88	21.7
E8	113	15.0	132	16.8	76	20.7	101	20.6	88	21.7
E9	135	13.3	127	15.2	85	18.7	46	19.0	77	20.3
E10	53	12.1	23	15.5	56	19.3	57	20.5	140	20.7
E11	53	12.1	23	15.5	56	19.3	57	20.5	140	20.7
E12	90	12.2	43	15.8	75	20.4	115	21.1	170	21.0
E13	90	12.2	43	15.8	75	20.4	115	21.1	170	21.0
E14	66	13.5	56	16.9	192	19.2	51	21.5	74	21.1
E15	66	13.5	56	16.9	192	19.2	51	21.5	74	21.1
E16	66	13.5	56	16.9	192	19.2	51	21.5	74	21.1

E17	66	13.5	56	16.9	192	19.2	51	21.5	74	21.1

3.2. Yield response to N fertilization

The average yield was 3168 kg ha^{-1} (± 140 kg ha^{-1}) for the control treatment and 3438 kg ha^{-1} (± 162 kg ha^{-1}) for the maximum N rate treatment (Table 3, Appendix I). A significant response ($p<0.05$) to N application was observed in ~50% of the experiments with an average value of 492 kg ha^{-1} (± 290 kg ha^{-1}). The observed yield values were in the range published by Hall et al. (2013) for the studied area. The yield response to N fertilization agreed with the values reported by other authors (Scheiner et al., 2002; Ruffo et al., 2003; Wajid et al.; 2012; Schultz et al., 2018). The lack of yield response to N at some experiments could be explained by differences in: i) the yield potential of each environment (maximum attainable yield), ii) water availability, and iii) soil N supply (Table 1). However, no significant relationships were observed between the yield response to N and the maximum yield obtained ($p>0.05$; $R^2= 0.05$). Also, there was a significant relationship between total water availability and yield potential ($p<0.05$; $R^2=0.52$) (data not shown). However, water availability did not relate with yield response ($p>0,05$; $R^2=0,06$) (data not shown). Consequently, N availability was the most important factor explaining yield response to N (Figure 1). Overall, in 67% of the experiments where yield response to N was significant, the NO_3^--N values were lower than 65 kg ha^{-1}.

(Please place Table 3 here)

The mean G_N was 5944 grains m^{-2} (± 219 grains m^{-2}) and 5513 grains m^{-2} (± 320 grains m^{-2}) for the control treatment and the maximum N response rate, respectively (Table 3, Appendix I). The average G_W for the control treatment was 49 g (± 1.6 g) and for the maximum N response rate was 51 g (± 2.2 g) (Table 3, Appendix I). Significant responses ($p<0.05$) to N fertilization were observed on G_W but not G_N (Table 3). Unlike our observations, Blamey et al. (1997) reported that G_N is the most affected yield component by N availability. However, Wajid et al. (2012) suggested that, in high yield potential hybrids, N availability has also a significant effect on G_W. Although there was no significant effect of N availability on G_N, significant relationships between yield and G_N ($R^2= 0.26$) and G_W ($R^2= 0.35$) were observed. In sunflower, G_N and G_W are both important factors in determining grain yield (Marinković., 1992; Andrade., 1995; Kaya et al., 2009).

Regarding sunflower grain quality, the increase in N fertilizer rate resulted in a linear increase in grain N_c ($p<0.05$; $R^2= 0.22$). The average grain N_c value was 2.27% (± 0.07%) and 2.51% (± 0.08%) for the control and maximum N rate treatment, respectively (Table 3, Appendix I). A significant response ($p<0.05$) to N fertilization on grain N_C was observed at 65% of the experiments, with an average 0.25% response. The relationship between N_c and N addition was similar to that reported by Diovisalvi et al. (2018) for high oleic genotypes (0.33%) and lower than that reported by Ali and Ullah (2012) (0.80%). An increase in N_c has a positive effect on protein concentration (Mohammadi et al., 2013), and therefore on the by-product protein concentration (Merrien et al., 1988). Diovisalvi et al. (2018) reported that N fertilization increased grain protein concentration by 2.5% (±0.9%), which could be translated into 5.6% (±3.7%) increase in by-products protein concentration.

3.3. Nitrogen availability diagnosis based on soil analysis

The relationship between yield and N availability (PPSNT plus fertilizer N) was not significant ($p>0.05$; $R^2=0.02$). The low predictive capacity of the model was mainly due to the significant effect of the site-year ($p<0.05$) and the different N mineralization potential among experiments (Table 1). As a consequence, RY was calculated and the relationships between this variable and N availability was fitted with ALCC model, grouping experiments according to the Nan level (low or high) as Calviño and Echeverría (2003) and Sainz Rozas et al. (2008) proposed. The models were significant ($p<0.05$) and N availability was associated with the RY (r= 0.64 and 0.56 for the low and high Nan groups, respectively) (Figure 1).

(Please place Figure 1 here)

The associations observed for the two Nan groups were higher than those reported by Schultz et al. (2018) and Diovisalvi et al. (2018) who determined different N availability thresholds (PPSNT + fertilizer N) in sunflower to maximize grain yield without taking in to account different Nan groups. In part, this could be explained by i) the type of model fitted, and ii) the data grouping based on N mineralization potential. Schultz et al. (2018) used quadratic models and Diovisalvi et al. (2018) quadratic-plateau models which, according to Correndo et al. (2017), would have problems related to data normality and homogeneity of variances. Grouping experiments by Nan level would also improve the fit compare to models that do not take this variable into account.

The critical threshold was 115 and 90 kg N ha^{-1} for low and high Nan groups, respectively (Figure 1). This indicates that, due to the higher N contribution from mineralization at high-Nan experiments, less N availability at planting is needed to achieve maximum yield. As well as for maize (Orcellet et al., 2017) and wheat (Reussi Calvo et al., 2013), the addition of Nan to the conventional N diagnosis models improved yield predictive capacity in sunflower.

However, critical thresholds were lower than those reported by Schultz et al. (2018) (266 kg N ha^{-1}) in North Dakota, and slightly lower than that reported by Diovisalvi et al. (2018) (125 kg N ha^{-1}) also for the southeastern Buenos Aires Province. However, these authors did not consider the contribution of N from SOM mineralization in their diagnosis models.

3.4 Evaluation of N availability using canopy indices

ALLC models were fitted between RY and canopy indices. Only NDVIr at V_{12} allowed to predict variations in RY (r = 0.47), being the critical threshold 0.95 (Figure 2). For all the other indices (G_I, NSI and NDVI) and at both sampling times (V_6 and V_{12}), the models were not significant ($p > 0.05$, data not shown). Also, NDVIr at V_{12} was related to soil N availability (R^2= 0,19) (data not shown). In order to have an adequate predictive capacity for indices based on chlorophyll measurement (G_I or NSI), the major constraint of the crop must be the N availability, as factors such as leaf shape and roughness and disease incidence may affect its accuracy (Zillman et al., 2006). For this reason, Heege et al. (2008) suggested that sensors based on crop biomass estimation as well as N nutrition (NDVI or NDVIr) are better indicators of the crop N status than those based on chlorophyll measurement. However, both NSI and NDVIr were suggested as accurate diagnosis tools for maize (Barker and Sawyer, 2010), wheat, and barley (Reussi Calvo et al., 2020).

The lack of adjustment between RY and NDVIr at V_6 could be explained by the low N demand from the crop at the mentioned stage. Thus, NDVIr measurement at V_{12} would be a good strategy to evaluate and correct the sunflower N status. On maize, Barker et al. (2010) reported that the use of canopy indices, such as NDVIr, allowed to detect N deficiencies and correct them by applications during the growing season, improving N use efficiency by up to 15%. For sunflower, Franzen et al. (2019) observed that NDVI and CropCircle measurements at V_6, but especially at V_{12}, were related to grain yield. Considering that sunflower has a favorable response to N applications close to anthesis (Steer et al., 1984), the use of NDVIr would complement the N diagnosis based on soil analysis determined at sowing.

(Please place Figure 2 here)

3.5. Nitrogen concentration in grain

Grain N_C explained 52% of the RY variability with a 2.26% critical threshold and a 95% confidence interval from 2.19 to 2.34% (Figure 3). The fit was within the ones mentioned for maize by Cerrato and Blackmer (1990) (R^2= 0.44, p<0.01) and Barbieri et al. (2013) (R^2= 0.60; p<0.01), but the critical threshold was higher than the one reported for maize (between 1.1 and 1.3%) (Cerrato and Blackmer, 1990 and Barbieri et al., 2013). This may be explained by the higher grain protein concentration and sunflower C3 metabolism (Andrade., 1995, Andrade and Ferreiro, 1996). Our results demonstrated that grain N_C is a suitable N diagnosis method that could be used as a post-mortem tool to identify N responsive conditions in sunflower. However, the predictive capacity could be affected by situations of low water availability and temperature (Goos et al., 1982).

(Please place Figure 3 here)

The N requirement, calculated as the ratio between soil N availability and grain yield, explained 39% of the grain N_C variability (Figure 4). On the other hand, clustering by Nan level did not improve the model's fit (data not shown). As suggested by Prystupa et al. (2018), the use of N requirement would allow to define the best relationship yield-grain N_C that could be obtained with a determined N availability, considering the average yield according to the environment. As an example, to reach a grain N_C value of 2.53%, 43 kg N available (pre-plant N soil + N fertilizer) per Mg of grain was required. In other words, if the potential of the environment is 3 Mg ha^{-1}, 129 kg N ha^{-1} (N soil + N fertilizer) would be required to maximize grain yield and protein concentration, and according to Diovisalvi et al. (2018), it would not affect the grain oil concentration. In Argentina, similar N requirements have been reported (Scheiner et al., 2002; Ruffo et al., 2003). In summary, the model defined in Figure 4 represents an alternative to define the N rate when looking for an optimal grain N_C level which allows an adequate by-products quality.

(Please place Figure 4 here)

4. CONCLUSIONS

The incorporation of Nan to the traditional diagnosis method (pre-plant soil NO_3-N) improved its capacity to predict sunflower response to N fertilization. The canopy index NDVIr at V_{12} demonstrated to be a promising tool for monitoring N status during the sunflower growing season. Finally, grain N concentration adequately diagnosed N deficiencies using a 2.26% threshold. Also, we could establish that grain N concentration was determined by the ratio between N availability and grain yield. Our results will allow to better estimate N availability for sunflower and to determine the adequate N fertilizer rate to be used for optimizing the grain yield and quality, which will help minimizing the economic and environmental cost of fertilization.

Further studies should be focused on validating the prognosis and diagnosis methods we proposed under: i) different availability of other nutrients (e.g. P and S), ii) soil types, and iii) contrasting management practices.

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6. FIGURES CAPTIONS

Figure 1: Relative yield (%) as a function of pre-plant N availability (soil N plus fertilizer N) for experiments grouped by low Nan (upper panel) and high Nan (lower panel). The vertical line indicates the threshold for N available. The horizontal line represents the 95% of relative yield. Gray strips represent the 95% confidence interval for the N availability threshold calculated by the modified arcsine-logarithm method.

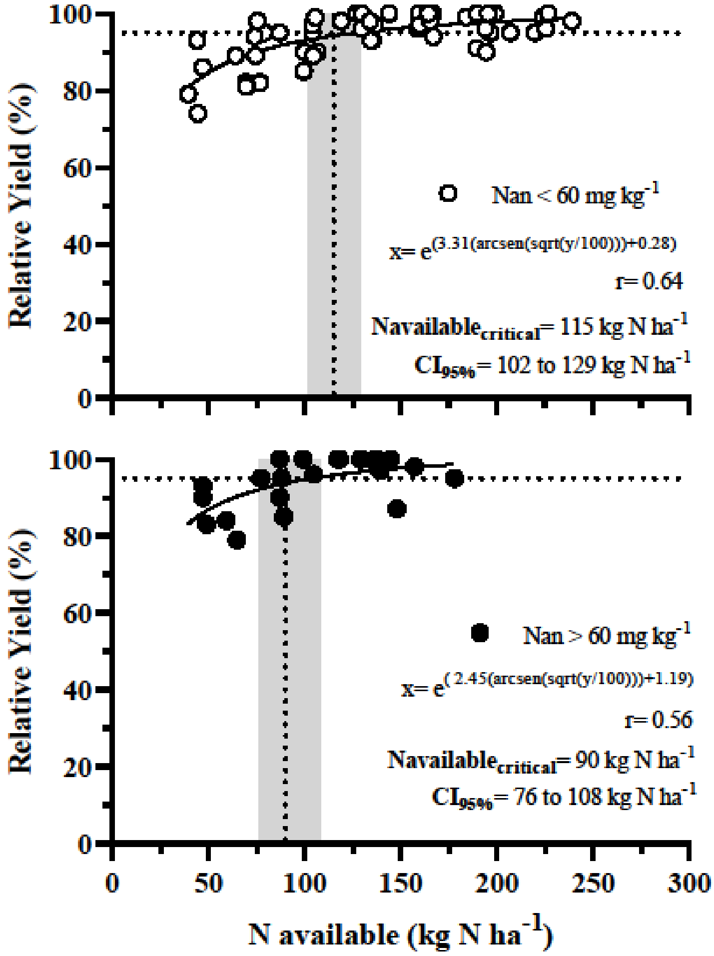


Figure 2: Relative yield as a function of relative normalized differential vegetation index (NDVIr) at V_{12} growth stage. The vertical line indicates the threshold for NDVIr. Gray strips represent the 95% confidence interval for the NDVIr threshold calculated by the modified arcsine-logarithm method.

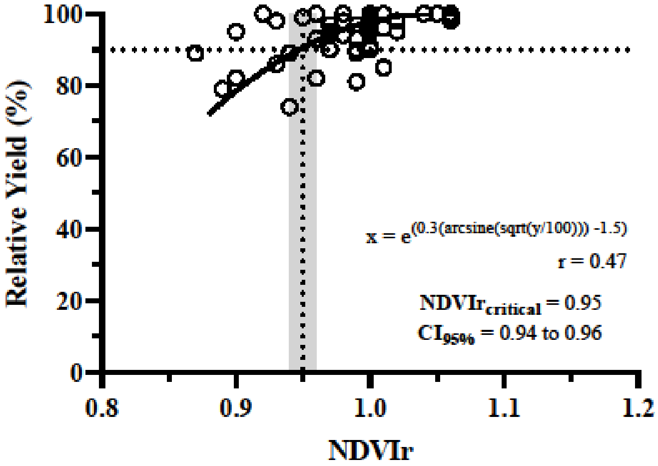


Figure 3: Relative yield (%) as a function of the grain nitrogen concentration (N_C) (%). The vertical line indicates the threshold for grain N_C. Gray strips represent the 95% confidence interval for grain Nc by the modified arcsine-logarithm method.

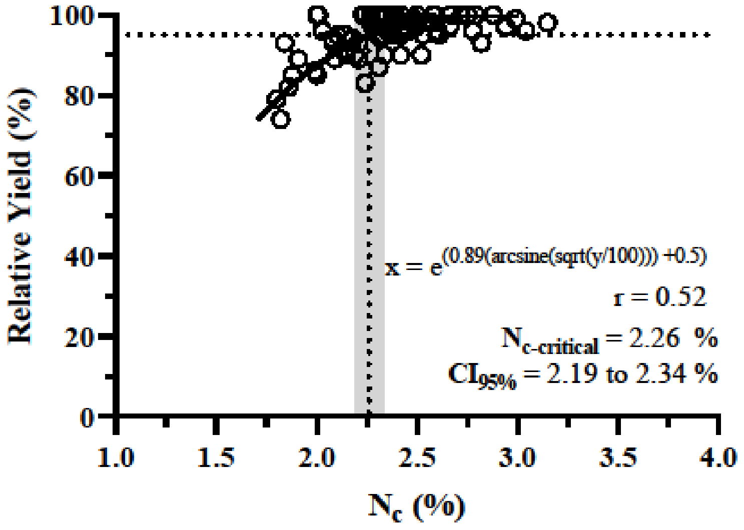
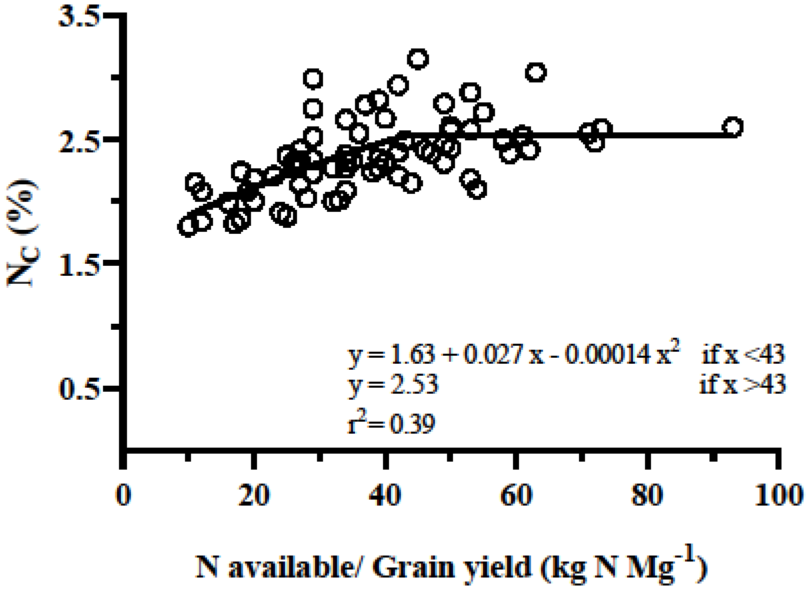


Figure 4: Grain nitrogen concentration (N_C) (%) as a function of nitrogen availability (kg N ha^{-1}) / Mg of grain produced (Mg ha^{-1}).



7. TABLES

Table 1: Experiment (Exp.), location (latitude, longitude), hybrid, soil variables [organic matter (SOM), phosphorus bray (P-Bray), nitrogen mineralized in short-term anaerobic incubation (Nan), and NO_3^--N content at 0-60cm depth], and total water availability during the crop cycle (total rainfall plus initial soil water content).

Location					Soil Variables					
Season	Exp.	Latitude	Longitude	Hybrid	pH	SOM	P-Bray	Nan	NO_3^--N	Water availability
					---------------------0-20 cm-----------------				0-60cm	
						(g kg^{-1})	-----(mg kg^{-1})------		(kg ha^{-1})	(mm)
2010/2011	E1	37.1° S	58.1°W	Paraíso 303	5.6	71	21	79	88	605
	E2	38.1° S	57.5°W	Paraíso 303	5.8	66	9	86	47	691
	E3	38.1° S	57.5°W	CF 201	5.8	61	7	79	47	691
2014/2015	E4	38.1° S	57.5°W	SYN 3970 CL	5.8	67	17	51	39	595
	E5	38.3° S	58.4°W	ADV 201	6.1	44	14	60	44	595
	E6	38.3° S	58.4°W	Paraíso 104 CL	6.0	57	13	56	44	595
	E7	37.0° S	57.1°W	NTO 1.0 CL	5.6	57	7	56	70	656
	E8	37.0° S	57.1°W	NTO 1.0 CL	5.6	61	9	43	77	656
	E9	38.1° S	57.5°W	NTO 1.0 CL	5.9	67	16	56	75	595
2016/2	E1	38.3°	58.4°W	DK 3970	6.	50	22	35	61	495

017	0	S		CL	2					
	E11	38.3° S	58.4°W	DK 3970 CL	6.1	51	15	55	47	495
	E12	38.1° S	57.5°W	SYN 4070 CL	5.9	49	7	50	64	495
	E13	38.1° S	57.5°W	SYN 4070 CL	5.8	59	9	54	79	495
2018/2019	E14	37.0° S	57.1°W	Paraíso 1500	5.8	53	5	87	49	530
	E15	37.0° S	57.1°W	Moogli	5.7	64	5	98	77	530
	E16	37.0° S	57.1°W	Aromo 105	5.7	53	10	103	65	530
	E17	37.0° S	57.1°W	Aromo 105	5.6	65	8	92	59	530

Table 2: Monthly rainfall and average daily mean temperature (Temp) during sunflower growing season at each experiment (Exp).

Month	**October**		**November**		**December**		**January**		**February**	
Exp	**Precipitation mm**	**Temp. °C**	**Precipitation mm**	**Temp. °C**	**Precipitation mm**	**Temp. °C**	**Precipitation mm**	**Temp. °C**	**Precipitation mm**	**Temp. °C**
E1	48	13.4	115	16.2	33	20.9	185	15.7	33	14.1
E2	56	12.5	100	14.5	81	18.6	144	21.3	88	19.5
E3	56	12.5	100	14.5	81	18.6	144	21.3	88	19.5
E4	116	13.4	128	15.5	88	19.3	123	19.1	110	20.5
E5	135	13.3	127	15.2	85	18.7	46	19.0	77	20.3
E6	135	13.3	127	15.2	85	18.7	46	19.0	77	20.3
E7	113	15.0	132	16.8	76	20.7	101	20.6	88	21.7

E8	113	15.0	132	16.8	76	20.7	101	20.6	88	21.7
E9	135	13.3	127	15.2	85	18.7	46	19.0	77	20.3
E10	53	12.1	23	15.5	56	19.3	57	20.5	140	20.7
E11	53	12.1	23	15.5	56	19.3	57	20.5	140	20.7
E12	90	12.2	43	15.8	75	20.4	115	21.1	170	21.0
E13	90	12.2	43	15.8	75	20.4	115	21.1	170	21.0
E14	66	13.5	56	16.9	192	19.2	51	21.5	74	21.1
E15	66	13.5	56	16.9	192	19.2	51	21.5	74	21.1
E16	66	13.5	56	16.9	192	19.2	51	21.5	74	21.1
E17	66	13.5	56	16.9	192	19.2	51	21.5	74	21.1

Table 3: Effect of N fertilization on sunflower grain yield (G_Y) (kg ha^{-1}), 1000-grain weight (G_W) (g), grain number (G_N) (grains m^{-2}) and grain nitrogen concentration (N_C) (%).

N rate (kg ha^{-1})	**G_Y**	**G_W**	**G_N**	**N_C**
0	3168**d†**	49**c**	5944**a**	2.27**e**
30	3346**c**	50**bc**	6227**a**	2.30**de**
40	3423**bc**	50**bc**	6075**a**	2.31**de**
60	3380**bc**	51**bc**	6205**a**	2.37**cd**
80	3494**ab**	53**a**	5597**a**	2.45**bc**
90	3530**ab**	52**ab**	6478**a**	2.46**bc**
120	3588**a**	50**bc**	6426**a**	2.45**bc**
150	3475**ab**	51**bc**	6359**a**	2.51**ab**
160	3438**ab**	52**ab**	5513**a**	2.55**a**

† In each column, means followed by the same letter are not significantly different according to the LSD test at 5% probability.

8. APPENDICES

Appendix I. Grain yield (G_Y) (kg ha^{-1}), 1000-grain weight (G_W) (g), grain number (G_N) (grains m^{-2}) and grain nitrogen concentration (N_C) (%) in the different experiments (Exp) and different nitrogen rates.

Exp		**Nitrogen rate (kg ha^{-1})**								
		0	**30**	**40**	**60**	**80**	**90**	**120**	**150**	**160**
E1	G_Y	3303	3485	-	3030	-	3304	-	-	-
	G_W	51	49	-	52	-	49	-	-	-
	G_N	5840	6277	-	5234	-	5979	-	-	-
	N_C	2.33	2.33	-	2.31	-	2.10	-	-	-
E2	G_Y	4239	-	4261	-	-	4387	-	-	-
	G_W	49	-	50	-	-	57	-	-	-
	G_N	7716	-	7624	-	-	7421	-	-	-
	N_C	1.84	-	2.00	-	-	2.40	-	-	-
E3	G_Y	4028	-	4310	-	-	4261	-	-	-
	G_W	52	-	57	-	-	55	-	-	-
	G_N	6911	-	6741	-	-	6852	-	-	-
	N_C	2.15	-	1.88	-	-	2.23	-	-	-
E4	G_Y	3781	3927	-	4037	-	4574	4772	4353	-
	G_W	51	49	-	50	-	49	53	53	-
	G_N	7272	7162	-	7280	-	7739	7986	7350	-
	N_C	1.81	1.85	-	1.87	-	2.03	2.01	2.15	-
E5	G_Y	2562	3081	-	3115	-	3224	3481	3122	-
	G_W	49	50	-	54	-	55	53	52	-

	G_N	6517	7495	-	7086	-	7208	8079	7339	-
	N_C	1.81	1.90	-	2.09	-	2.21	2.39	2.42	-
E6	G_Y	3759	3825	-	3929	-	3989	4056	3885	-
	G_W	55	54	-	55	-	57	56	57	-
	G_N	6034	6349	-	6403	-	6201	6511	6106	-
	N_C	2.07	2.07	-	2.28	-	2.27	2.32	2.43	-
E7	G_Y	3084	3429	-	3793	-	3685	3788	3600	-
	G_W	55	56	-	59	-	61	60	60	-
	G_N	4961	5418	-	6245	-	5378	5621	5376	-
	N_C	2.34	2.37	-	2.38	-	2.48	2.57	2.53	-
E8	G_Y	3404	3731	-	4045	-	4130	3961	4149	-
	G_W	53	59	-	59	-	59	54	60	-
	G_N	5705	5721	-	6138	-	6281	6496	6106	-
	N_C	2.43	2.52	-	2.66	-	2.67	2.60	2.71	-
E9	G_Y	3641	3690	-	3457	-	3654	3716	3562	-
	G_W	64	64	-	61	-	64	66	56	-
	G_N	5063	5167	-	5048	-	5239	4999	5875	-
	N_C	2.88	2.98	-	2.82	-	3.09	2.87	3.00	-
E10	G_Y	2320	-	2431	-	2426	-	2476		2371
	G_W	40	-	39	-	42	-	39		42
	G_N	5205	-	5641	-	5187	-	5603		5035
	N_C	2.3	-	2.40	-	2.49	-	2.59		2.6
E11	G_Y	2888	-	3168	-	3351	-	3160		3173
	G_W	41	-	53	-	53	-	47		52
	G_N	6395	-	4667	-	4730	-	4764		4756
	N_C	1.99	-	2.14	-	2.22	-	2.17		2.27

E12	G_Y	2817	-	3007	-	3152	-	3130		3110
	G_W	41	-	46	-	44	-	43		43
	G_N	6299	-	6027	-	6444	-	6448		5873
	N_C	2.21	-	2.32	-	2.42	-	2.39		2.48
E13	G_Y	3222	-	3330	-	3265	-	3409		3354
	G_W	48	-	45	-	51	-	41		47
	G_N	6103	-	6685	-	5666	-	7754		6388
	N_C	2.37	-	2.37	-	2.46	-	2.54		2.55
E14	G_Y	2540	-	2789	-	3287	-	-	-	-
	G_W	42	-	42	-	50	-	-	-	-
	G_N	5349	-	5973	-	5990	-	-	-	-
	N_C	2.29	-	2.00	-	2.43	-	-	-	-
E15	G_Y	3234	-	3028	-	2964	-	-	-	-
	G_W	54	-	54	-	58	-	-	-	-
	G_N	5293	-	4976	-	4580	-	-	-	-
	N_C	2.41	-	2.34	-	2.57	-	-	-	-
E16	G_Y	2193	-	2771	-	2978	-	-	-	-
	G_W	41	-	46	-	44	-	-	-	-
	G_N	4759	-	5345	-	6011	-	-	-	-
	N_C	2.58	-	2.77	-	2.78	-	-	-	-
E17	G_Y	2843	-	3404	-	3317	-	-	-	-
	G_W	45	-	43	-	45	-	-	-	-
	G_N	5626	-	7075	-	6579	-	-	-	-
	N_C	2.67	-	2.74	-	2.94	-	-	-	-