Effects of Genotype and Nitrogen Availability on Grain Yield and Quality in Sunflower

Natalia Diovisalvi, Nahuel Reussi Calvo,* Natalia Izquierdo,
Hernán Echeverría, Guillermo A. Divito, and Fernando García

ABSTRACT

Sunflower (*Helianthus annuus* L.) conventional (CONV) and high oleic (HO) genotypes differ in yield and quality. Nitrogen affects grain yield, quality, and by-products protein concentration. The objective was to evaluate the effect of genotype and N on grain yield, oil (O_G) and protein (P_G) concentration in grain and in by-products (P_M). The effect of genotype was evaluated in Exp. 1 with 7 CONV and 7 HO hybrids, at two planting dates (PD early and late). The effect of N (Exp. 2) was evaluated in 10 locations (3 with CONV and 7 with HO), under six N rates (0, 30, 60, 90, 120, and 150 kg N ha^{-1}). We determined yield, O_G, P_G and P_M. For the early PD of E_1, yield was higher in HO than CONV genotypes (3822 kg ha^{-1} vs. 3495 kg ha^{-1}). In Exp. 2, N increased yield in 50% of the locations (HO: 586; CONV: 597 kg ha^{-1}). In Exp. 1, genotype did not affect O_G, but P_G was higher in HO than in CONV ones (18.0 vs. 16.8%, respectively). In Exp. 2, N did not affect O_G, but increased P_G in both types of genotypes. Consequently, P_G/O_G ratio increased with N rates. The higher P_G, was also reflected in higher P_M (44.0% HO and 38.8% CONV, respectively). Increases of 2.5% points in P_G resulted in increases of 5.6 in P_M. Therefore, the application of N would allow obtaining high yields and P_G without detrimental effects on O_G, improving the quality of grains and by-products.

Core Ideas

- Positive effect of genotype and N on yield and protein in grains and meal.
- No effect of genotype and N on concentration of oil in grain.
- High oleic genotypes showed higher protein concentration of grains and by-products.
- Nitrogen increased the protein /oil ratio of the grains.
- Nitrogen improved the quality of sunflower by-products.

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Sunflower CONV and HO genotypes differ in yield and quality. In general, CONV genotypes present higher grain yield and O_G than HO ones (Del Gatto et al., 2015; Gaggioli et al., 2015). The oil produced by HO genotypes is preferred for some uses because it presents high oxidative stability. This stability is given by its high oleic acid concentration (>75%) produced by the Pervenets mutation these genotypes carry (Velasco and Fernández-Martínez, 2002; Del Gatto et al., 2015). On the other hand, CONV genotypes present lower concentrations of this fatty acid (18–56%) (Izquierdo and Aguirrezábal, 2008). Although differences in oil quality between both types of genotypes were previously described (Gaggioli et al., 2015; Gouzy et al., 2016), to our knowledge, there are no reports comparing grain (P_G) and by-products (P_M) protein concentration of both types of genotypes.

Sunflower crop needs to intercept 95% of solar radiation at flowering and maintain it high during the post-flowering period to reach high growth rates and yields (Aguirrezábal, 2010). This is important because, although grain number (N_G) is defined during the critical period around flowering, grain weight (W_G) and O_G are defined during grain filling (Dosio et al., 2000; Izquierdo et al., 2008; Alberio et al., 2015). According to Aguirrezábal et al. (2003), the interception of radiation during the period between 250 and 450°C day after flowering (base temperature: 6°C, Kiniry et al., 1992) better accounts for the variations in W_G and O_G, compared to the whole grain-filling period (Dosio et al., 2000; Izquierdo et al., 2008).

Nitrogen is the main nutrient that affects the crop growth rate and consequently, yield, O_G, P_G, and P_M (Debaeke et al., 2012; Andrianasolo et al., 2016). Sunflower crop requieres 40 to 45 kg N in plant to produce 1 Mg grain, presenting the highest absorption rates (3.5–4.0 kg ha^{-1} d^{-1}) between 25 and 70 d after emergence (Alberio et al., 2015). In general, almost 75% of N is absorbed during the 30 d pre-flowering, being post-flowering

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N. Diovisalvi and N.R. Calvo, Lab. de suelos FERTILAB, Moreno 4524, (7600) Mar del Plata, Buenos Aires, Argentina; N.R. Calvo, N. Izquierdo, and H. Echeverría, Unidad Integrada INTA-FCA Balcarce, C.C. 276, (7620) Balcarce, Buenos Aires, Argentina; N.R. Calvo and N. Izquierdo, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina); G.A. Divito, ASOCIACION ARGENTINA DE PRODUCTORES EN SIEMBRA DIRECTA (AAPRESID, Argentina); F. García, International Plant Nutrition Institute (IPNI). Received 3 Aug. 2017. Accepted 11 Apr. 2018.
*Corresponding author (nreussicalvo@laboratoriofertilab.com.ar).

Abbreviations: CONV, conventional; EP, early planting; HO, high oleic; LP, late planting; Nan, anaerobic nitrogen; N_G, number of grains; O_G, grain oil concentration; OM, soil organic matter; PAR, photosynthetically active radiation; P_G, grain protein concentration; PIR, percentage of intercepted radiation; P_M, meal protein concentration; RY, relative yield; W_G, grain weight; PD, planting date.

absorption lower. This nutrient is involved in the development and growth of leaves and florets (Alberio et al., 2015). Massignam et al. (2009) observed that N supply affected growth, development, and grain yield and that N deficiency delayed anthesis under severe N limitation. It has been reported that N deficiencies in early vegetative stages may reduce crop growth rate by reducing leaf area index, photosynthetic rate, and thus, solar radiation interception, radiation use efficiency and grain yield (Hall et al., 1995; Massignam et al., 2009). The radiation intercepted by the crop is the substrate to synthetize the oil stored in the grains and N is the main source used to synthetize proteins (Aguirrezábal et al., 2009; González-Pérez, 2015). Therefore, an adequate N availability is required to obtain grains with high O_G and P_G. However, excessive levels of this nutrient have shown to reduce O_G, which is not commercially desired as oil is the main product of these grains (Özer et al., 2004; Debaeke et al., 2012). It has been reported that N at levels of luxury consumption increased P_G/O_G ratio due to an increase of protein synthesis compared to oil synthesis. Triboi and Triboi-Blondel (2002) reported that N fertilization at planting reduced O_G, describing a lineal and inverse relationship between P_G and O_G. For some hybrids, changes in P_G and O_G would not be strictly proportional across the range of N variation, so it could be possible to increase P_G with slight or no O_G change (Alberio et al., 2015). At the present, there are no records of published works describing the P_G/O_G relationship for HO and CONV genotypes with a variable N supply in sites with different soil (e.g., organic matter, pH, nitrate, anaerobic N, P, texture) and climatic characteristics (e.g., rainfall, mean temperature, incident solar radiation).

Although O_G determines the industrial performance of sunflower grains, P_G determines the quality of by-products (González-Pérez, 2015; Dauguet et al., 2016). The latter are made from the oil industry waste after the grains have been degreased (Pedroche, 2015). In sunflower, to optimize the oil extraction process, first, the grain is dehulled leaving only 10 to 12% of residual husk (Peyronnet et al., 2012). de Figueiredo et al. (2015) reported that this process allows to reduce the content of fiber, waxes, and pigments in the oil and to increase P_M. Within the by-product market, there are "common" pellets (from not dehulled grains) with 27 to 30% protein and "low-fiber" pellets (from partially dehulled grains) with about 36% protein. The latter obtain differential prices in the market due to their higher P_M and lower fiber content (de Figueiredo et al., 2015; Dauguet et al., 2016). For this purpose, it is essential to start with grains with high P_G, since it has been observed that differences in P_G could represent differences of up to 5% in the P_M (Merrien et al., 1988). Although genetic and environmental factors affect P_G (Dauguet et al., 2016), N availability plays a fundamental role in the P_G and, consequently, in P_M (Andrianasolo et al., 2016). At the present, there is no record of studies evaluating the effect of N on P_G and, consequently, on P_M in HO vs. CONV genotypes. The objective of this work was to evaluate the effect of genotype and N availability on grain yield, oil and protein concentration in grain, and in by-products.

MATERIALS AND METHODS

The Experiments

During the 2014–2015 growing season two sets of experiments were performed (Exp. 1 and 2) covering a wide region of the southeastern Buenos Aires Province (from 37°45′ S, 58°17′ W to 38°40′ S, 60°08′ W), Argentina (Table 1). Predominant soils are Petrocalcic Argiudoll (serie fine, mixed, thermic) and Typic Argiudoll (serie fine, mixed, thermic) (Soil Taxonomy) with a slope <2%.

For both sets of experiments, we will mention "type of genotype" when we refer to HO vs. CONV and we will mention "genotype" when we refer to one specific. Within each type of genotype (HO or CONV) we have different hybrids (Table 1) but with similar characteristics: high yield potential and O_G (between 51 and 55%), resistant to lodging and good behavior toward diseases (ASAGIR, 2014). The differences between both types of genotypes (HO vs. CONV) is that HO carry the Pervenets mutation which modifies the fatty acids biosynthetic pathway and CONV genotypes do not carry such mutation, and that is why the phenotypes are different (Dorrel and Vick, 1997; Fernández-Martínez et al., 2004; Lacombe et al., 2009; Martínez-Rivas et al., 2001; Zambelli et al., 2015). In Exp. 1, we evaluated the effect of type of genotypes on grain yield, O_G and P_G at one site with seven CONV and seven HO hybrids in two planting dates: early (EP) and late (LP) (Table 1). Samples were originated from the Argentine National Trial Network of Commercial Sunflower Hybrids (INTA). The applied N rate was 120 kg ha^{-1} in the form of granulated urea (46–0–0) (50% at planting and 50% at four leaves), so that N was not limiting. The experimental design was α latice with three replications.

In Exp. 2, we evaluated the effect of N availability in 10 sites (3 with CONV and 7 with HO hybrids), all conducted under no tillage-system and with different farming history (Table 1). Evaluated N rates were: 0, 30, 60, 90, 120, and 150 kg N ha^{-1} (named as 0N, 30N, 60N, 90N, 120N, and 150N, respectively), surface-broadcasted as urea at crop emergence. Planting dates were within the recommended dates for each site. The experimental design in each site was a randomized complete block with three replications. Experimental units were 10 rows, 0.7 m apart and 12 m long.

In both sets of experiments, 20 kg ha^{-1} P (as 18–46–0) and 10 kg ha^{-1} S (as $CaSO_4$) were applied at planting to avoid deficiencies. Phenological stages were evaluated according to the scale of Schneiter and Miller (1981). Data of rainfall, mean temperature, and incident global radiation were obtained from meteorological stations of INTA from Balcarce and Barrow and from the National Weather Service (SMN, Servicio Meteorológico Nacional). The characteristics of this region are mean annual rainfall of 955 mm, potential evapotranspiration of 950 mm, and mean temperature of 13.9°C.

Soil Determinations

Before sowing, soil samples (composed by 25–30 subsamples) were taken in every block at a depth of 0 to 20, 20 to 40 and 40 to 60 cm. Soil samples were analyzed for organic matter (OM) according to the method proposed by Walkley and Black (1934) and pH was measured with an electrode in a suspension 1:2.5 (soil/water) (Thomas and Hargrove, 1984). Extractable P (Bray P) was quantified according to Bray and Kurtz (1945) with colorimetric measurement of phosphate with the method of Murphy and Riley (1962). Soil texture (0–20 cm) was determined as proposed by the technique of Bouyoucos (1962) modified by Gee and Bauder (1986). The concentration of nitrate N throughout

Table 1. Characterization of the experiments. Exp. no.: sets of experiments number (Exp. 1 or 2), PD: planting date, EP: early planting, LP: late planting, HO: high oleic, CONV: conventional.

Exp. no.	Site	Location	PD	Population	Genotype	Hybrid
				plants ha^{-1}		
Exp. 1	EP	37°45’31.72” S, 58°17’58.50” W	28 Oct.	50.000	HO	LG 5451 CL
	EP	37°45’ 31.72” S, 58°17’58.50” W	28 Oct.	50.000	HO	Aromo 105 CL
	EP	37°45’ 31.72” S, 58°17’58.50” W	28 Oct.	50.000	HO	Nusol 4500 CL
	EP	37°45’ 31.72” S, 58°17’58.50” W	28 Oct.	50.000	HO	Nusol 2500
	EP	37°45’ 31.72” S, 58°17’58.50” W	28 Oct.	50.000	HO	Mooglli CL
	EP	37°45’ 31.72” S, 58°17’58.50” W	28 Oct.	50.000	HO	ACA 868 CL
	EP	37°45’ 31.72” S, 58°17’58.50” W	28 Oct.	50.000	HO	SYN 3965 CL
	LP	37°45’ 31.72” S, 58°17’58.50” W	1 Dec.	50.000	CONV	ADW 5200
	LP	37°45’ 31.72” S, 58°17’58.50” W	1 Dec.	50.000	CONV	Vellox
	LP	37°45’ 31.72” S, 58°17’58.50” W	1 Dec.	50.000	CONV	Mobill
	LP	37°45’ 31.72” S, 58°17’58.50” W	1 Dec.	50.000	CONV	ACA 203 CL
	LP	37°45’ 31.72” S, 58°17’58.50” W	1 Dec.	50.000	CONV	MG 360 CP
	LP	37°45’ 31.72” S, 58°17’58.50” W	1 Dec.	50.000	CONV	Sungro 66 CLP
	LP	37°45’ 31.72” S, 58°17’58.50” W	1 Dec.	50.000	CONV	SYN 3970 CL
Exp. 2	1	37°05’ 20.60” S, 57°23’35.60” W	23 Sept.	60.000	HO	NTO 1.0 CL
	2	37°05’33.85” S, 57°25’ 56.87” W	29 Sept.	60.000	HO	NTO 1.0 CL
	3	38°05’18.57” S, 58°13’25.24” W	19 Oct.	66.000	HO	NTO 1.0 CL
	4	38°12’10.33” S, 57°56’16.68” W	18 Oct.	50.000	HO	NTO 1.0 CL
	5	38°40’46.87” S, 60°08’35.98” W	21 Oct.	55.000	HO	Aromo 105 CL
	6	38°30’34.07” S, 60°05’42.10” W	14 Oct.	55.000	HO	ADV 5203 CL
	7	38°29’52.53” S, 59°46’55.08” W	13 Oct.	55.000	HO	Aromo 105 CL
	8	38°12’44.66” S, 57°57’04.02” W	13 Oct.	60.000	CONV	SYN 3970 CL
	9	38°35’17.60” S, 59°08’27.00” W	16 Oct.	65.000	CONV	Paraíso 104 CL
	10	38°33’13.80” S, 58°50’36.39” W	17 Oct.	60.000	CONV	ADV CF 201

the soil profile was determined with the selective ion electrode method (Dahnke, 1971), and anaerobic incubated N (Nan) via incubation of 5 g soil during 7 d at 40°C (Keeney, 1982).

Plant Determinations

In Exp. 2 (Sites 1, 2, 4, 5, 7, 9, and 10), the percentage of intercepted radiation (PIR) from flowering to physiological maturity was measured with a lineal quantum sensor (LI-191 SB, LI-COR, Lincoln, NE) according to Dosio et al. (2000). The amount of photosynthetically active radiation (PAR) intercepted per day was calculated as the product of incident PAR and PIR. Cumulative intercepted solar radiation per plant was calculated by adding daily intercepted PAR per plant from flowering to physiological maturity.

In both sets of experiments, capitula in 10 m^2 were harvested and threshed. Grain moisture content (%) was determined and yield was expressed at a base of 11%. In E_2, the yield response to N addition was calculated as the difference in yield of the fertilized treatment and the control, and the relative yield (RY) was calculated as the ratio of the yield of each treatment to the highest yield of each site. In addition, W_G and N_G per m^2 were determined.

Nitrogen concentration in grain was determined using the method of Dumas (Jung et al., 2003), which consists of a dry combustion of the sample at high temperature (950°C) and detection via thermoconductivity with a TruSpec CN analyzer (LECO, 2010). From that value of N, P_G was calculated using a factor of 5.3 (Jones, 1941). Grain oil concentration was determined via nuclear-magnetic-resonance (NMR, Spinlock S.R.L.), using different calibrations for each type of genotype (HO and CONV).

In Exp. 2, 22 grain samples (HO: $n = 8$ and CONV: $n = 14$) were selected for P_M determination, covering a wide range of P_G (from 10.0–16.8%). Within each genotype, 50% of the samples belonged to 0N and the remaining 50% to 150N. Grains were ground in a mortar and oil extraction was performed with n-hexane (Soxhlet) at 80°C during 8 h. Deffated samples were dried at 60°C until constant weight and N concentration was determined according to the method proposed by Dumas (Jung et al., 2003). The concentration of protein in those samples was calculated using a factor of 5.3 (Jones, 1941) and corrected by the remnant hull the industry leaves after dehulling (10%) (Peyronnet et al., 2012).

Data Analysis

In both planting dates of Exp. 1, the effect of type of genotype (HO vs. CONV) on yield, O_G and P_G was evaluated through ANOVA with the statistical package R (R Core Team, 2014). Differences among treatments were evaluated with the least significant difference at the 0.05 level. The interaction between type of genotype and PD was also analyzed in E_1.

In Exp. 2, data analysis was performed by genotype (HO and CONV) taking into account that it was not possible to have both types of genotypes in each site and based on the literature related to the effect of type of genotype on yield and quality (Agüero et al., 1999; Gaggioli et al., 2015). An overall ANOVA was performed using the package R (R Core Team, 2014) for evaluating site and treatment (NR) effects on yield, N_G, W_G, O_G, and P_G. Data of P_M were analyzed with variance analysis to evaluate the effect of type of genotype and NR (0N and 150 N). Significant differences were determined at 0.05 levels using a

Table 2. Climatic and edaphic characterization of experimental sites. Pp: Total rainfall during the crop cycle, GR: global radiation during the critical period of flowering and filling of grains, OM: soil organic matter, Bray P: extractable phosphorus, Nan: nitrogen incubated in anaerobiosis, NO_3^-–N: available nitrogen content at a 0- to 60-cm depth at sowing, Exp. no.: sets of experiment number (Exp. 1 or 2), EP: early planting, LP: late planting.

Exp. no.	Site	Pp	GR	OM	pH	Bray P	Nan	NO_3^-–N	Sand	Silt	Clay	Textural class
		mm	MJ m^{-2} d^{-1}	g kg^{-1}		—— mg kg^{-1} ——		kg ha^{-1}	———— g kg^{-1} ————			
Exp.1	EP	455	24.2	50	5.7	46.4	39.9	33.2	555	238	207	Sandy clay loam
	LP	380	21.8	50	5.7	46.4	39.9	33.2	555	238	207	Sandy clay loam
Exp.2	1	500	27.0	61	5.6	8.8	42.6	76.6	561	288	151	Sandy loam
	2	500	27.0	57	5.6	6.8	56.0	69.7	597	259	144	Sandy loam
	3	450	26.3	32	6.4	7.0	55.5	54.1	593	199	209	Sandy clay loam
	4	450	26.3	67	5.9	16.5	56.4	75.5	470	336	193	Sandy clay loam
	5	550	26.4	45	6.1	13.2	57.3	70.3	644	222	134	Sandy loam
	6	550	26.4	36	6.1	10.0	56.6	91.1	650	210	141	Sandy loam
	7	550	26.4	43	5.8	17.4	53.7	65.0	579	275	146	Sandy loam
	8	450	26.3	67	5.8	17.2	51.0	39.5	427	338	235	Loam
	9	490	26.3	57	6.0	13.1	56.0	54.1	598	256	146	Sandy loam
	10	490	26.3	44	6.1	13.7	59.3	44.5	599	169	232	Loam

least significant difference test. The normality of distribution of data was confirmed using the Shapiro and Wilk (1965) procedure and while the homogeneity of variances was confirmed using the Levene (1960) test.

The relationship between PIR at flowering or RY with available N (pre-plant soil nitrate N + fertilizer N) for HO and CONV genotypes was described with quadratic model plateau as follow:

$$y = a x^2 + b x + c \text{ if } x < \text{CT}$$

$$y = \text{P if } x > \text{CT}$$

where y is PIR or RY (%), x is the level of availiable N (pre-plant soil nitrate N + fertilizer N) (kg N ha^{-1}), c is the origin, a is the quadratic coefficient and b is the slope, CT is the critical threshold of availability N that maximize the PIR or RY and P is PIR or RY value at the plateau.

Simple and multiple linear regressions were performed using the lm (linear model) procedure to explain the variation of O_G, P_G, and P_G/O_G. The stepwise selection method was used to select the best variable combination to explain P_G from pre-plant soil nitrate N, NR, pre-plant soil nitrate N + NR, clay, silt + clay, Nan, OM, rainfall from V_6 to flowering, and rainfall from flowering to physiological maturity.

RESULS AND DISCUSSION

Soils and Climate Characterization

Rainfall during the crop cycle for EP (Exp. 1 and 2) (485 mm in average) were in general enough to satisfy the demand which is 500 to 550 mm for southeastern Buenos Aires Province (Pereyra et al., 2001) (Table 2). From sowing to V_6 water excesses were 2 to 3 mm d^{-1} and during the critical periods for grain number and grain filling the water deficits were 1 to 2 mm d^{-1} depending on the experimental site. Lower rainfalls were registered in LP (Exp. 1) (380 mm), mainly during the critical periods for grain number and grain filling (100 mm in average for EP vs. 70 mm for LP, respectively), which may have affected yield and grain quality. Mean temperature during the cycle was in average 20.0 and 21.1°C for EP and E_2, and LP, respectively. These values are within the range of temperature were maximum W_G (12–22°C) can be reached (Rondanini et al., 2003). Global radiation in EP was slightly higher than the historical average for this region (25.9 vs. 24.9 MJ m^{-2} d^{-1} during grain-filling period). However, in LP, global radiation was lower (21.8 MJ m^{-2} d^{-1}) so yield and O_G may have been affected (Table 2). Nevertheless, the field sites in Exp. 1 and 2 reflect a range of optimal growing seasons for sunflower yield and grain quality.

Mean values of OM, Bray P, Nan, and pH in the first 20 cm were 51 ± 12 g kg^{-1}, 15.5 ± 11.0 mg kg^{-1}, 53.1 ± 6.3 mg kg^{-1}, and 5.9 ± 0.2, respectively (Table 2). These values agree with those typically reported for this region under long-term cropping by Sainz Rozas et al. (2011 and 2013), and Reussi Calvo et al. (2014). Sainz Rozas et al. (2011 and 2013) for agricultural soils (0–20 cm) of the Pampean and Extrapampean region reported P, OM, and pH values of 10 to 20 mg kg^{-1}, 9.5 to 65.5 g kg^{-1} and 6.0 to 7.5, respectively. While Reussi Calvo et al. (2014) reported on a regional scale in agricultural soils of the province of Buenos Aires OM and Nan values ranging from 5 to 130 g kg^{-1}, and from 12 to 260 mg kg^{-1}, respectively. The variation in OM values could be explained by the farming history and soil texture (Quiroga et al., 2006; Diovisalvi et al., 2014). No significant relationship was found between Nan and OM at sowing ($P = 0.23$). This can be a consequence of the different lability of the OM in each site, as changes in OM content not necessarily implying a proportional change in the N mineralization potential (Sharifi et al., 2007). The experimental site-years showed a narrow range of pH (5.6–6.4) which is considered optimal for the growth of crops (Sainz Rozas et al., 2011; Pagani and Mallarino, 2012) and non-limiting for nutrient cycling (Lauber et al., 2009; Barbieri et al., 2015). The concentrations of sand, silt, and clay in 0 to 20 cm were from 427 to 650, 169 to 338 and 134 to 235 g kg^{-1}, respectively.

The availability of N before planting was in average 64.0 ± 15.9 kg ha^{-1} (Table 2), being this value similar to those reported by Zamora and Massigoge (2008) for southeastern Buenos Aires Province under soils with an average content of OM of 35 g kg^{-1} and pH close to neutrality. González Montaner and Di Nápoli (2002) in studies developed in the southeastern of Buenos Aires proposed a critical initial N availability of 50 kg N ha^{-1} (0–60 cm) above which there would not be response to

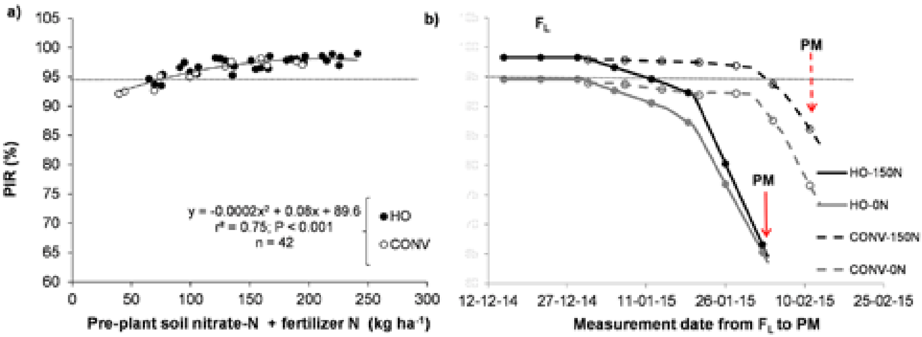

Fig. 1. Experiment number two (Exp. 2): (a) Percentage of intercepted radiation (PIR) (%) at flowering as a function of the available N (pre-plant soil nitrate N + fertilizer N) for high oleic (HO) and conventional (CONV) genotypes. *n* = number of cases. (b) Average evolution of PIR from flowering (F_L) to physiological maturity (PM) for HO and CONV genotypes under the control (0N) and 150 kg N ha^{-1} (150N) treatments (*n* = 7). PM (indicated by arrows) was the same for both treatments of the each genotype.

N fertilization, but such value is lower than the defined by other authors. For example, Zamora and Massigoge (2008), under non-limiting water conditions, obtained significant response to N fertilization in sites with initial N values between 25 and 82 kg ha^{-1}. This evidences that there are sites with low and high probability of yield responses to N fertilization.

Solar Radiation Interception and Accumulation during Grain Filling

In Exp. 2, there was an association between PIR at flowering and N availability (r^2 = 0.74). Moreover, a CT of 220 kg N ha^{-1} was defined to maximize PIR (Fig. 1a). Trápani and Hall (1996) also reported this association in an experiment in pots placed in the field at the Facultad de Agronomía, Universidad de Buenos Aires. In both types of genotypes, PIR of the 0N treatment was always less than 95%, and rapidly decreased during crop maturity, compared to PIR of the 150N treatment (Fig. 1b). The latter was over 95% and kept that value several days after flowering, thus increasing the PIR and accumulated intercepted PAR. This is reflected in better conditions for grain filling and thus, crop yield and grain quality (Tables 3 and 4, Appendix I). The benefit of presenting high levels of radiation interception on yield and grain quality were reported by other authors (Dosio et al., 2000; Aguirrezábal et al., 2003; Izquierdo et al., 2008). These authors performed their experiments under soil Typic Argiudol at the INTA Balcarce Experimental Station, Argentina.

Yield and Components

Genotype Effect

In E_1, there was significant PD × type of genotype interaction on yield and N_G ($P < 0.01$ and < 0.05, respectively). In EP, HO genotypes presented higher yields and N_G than CONV genotypes (Table 5). A similar trend between both types of genotypes was observed by Gouzy et al. (2016) in field experiments located in Toulouse (southwestern France) under conditions of rainfall and average temperature similar to those of the present study. These authors evaluated for a dry year and a wet year the changes in yield and oil fatty acid composition in different HO and CONV genotypes. However, in LP no differences in yield or N_G among genotypes were observed (Table 5). The low yields registered in LP could be explained by the low rainfalls and global radiation registered during the crop cycle (Table 2). Ross (2014) at Petrocalcic Paleudol soil with 60-cm deep, also observed reduction of potential yields in late planting dates mainly due to water stress at the beginning of the cycle and to reductions of about 28% in global radiation compared to early planting dates.

In E_2, mean yield varied from 2239 to 4149 kg ha^{-1} for HO genotypes and from 2562 to 4772 kg ha^{-1} for CONV genotypes (Table 3, Appendix I). These results are similar to those reported by Zubillaga et al. (2002) and Zamora and Massigoge (2008) for the same types of hybrids. Zubillaga et al. (2002) worked in mid-western Pampas Argentina, at Typic Hapludoll soils with pH values around 6 and P from 9 to 13 mg kg^{-1}. While Zamora and Massigoge (2008) worked in southeastern Buenos Aires Province under soils with pH close to neutrality and content of OM of 35 g kg^{-1}. These high yields would be explained by favorable meteorological conditions (rainfall, mean temperature, and global radiation) registered during the crop cycle (Table 2). Although in E_2 it was not possible to separate the effect of genotype from the effect of site, on average HO genotypes yielded 8.6% less than CONV ones (3450 and 3748 kg ha^{-1}, respectively). Agüero et al. (1999) and Gaggioli et al. (2015) also reported a similar trend between both types of genotypes, due to the obvious reason that CONV genotypes were bred mainly for productivity while HO genotypes were bred for productivity and quality. Agüero et al. (1999) conducted a network of comparative yield trials during two agricultural campaigns in seven contrasting environments covering a large area of the Argentine sunflower region. While Gaggioli et al. (2015) evaluated the productivity of sunflower hybrids differentiated in oil quality in two contrasting soils of the semiarid Pampas region (Haplustoll with frank texture with 22 g kg^{-1} of OM vs. Ustipsament of sandy texture with OM 8.6 g kg^{-1}). However, in the last years, the difference in yield between both types of genotypes has decreased (Alberio et al., 2016).

In agreement with other authors (Mercau et al., 2001; Massignam et al., 2009), in E_2, N_G explained 47% of the variation in yield (data not shown) and the effect of W_G on yield was not statistically significant ($P > 0.05$). Although N_G was the component that better account for the variations in yield, it is known that the effect of W_G in determining yield is higher than in other species as for example maize (*Zea mays* L.) (Andrade and Ferreiro, 1996; Massignam et al., 2009). As for N_G, W_G also varied among sites (Table 4, Appendix I).

This is partially explained by the accumulated intercepted PAR during the critical period for determining W_G. For

Table 3. Experiment number two (Exp. 2): Yield and components for high oleic (HO) and conventional (CONV) genotypes in the different sites and different nitrogen rates (NR) (kg ha^{-1}). N_G: number of grains, W_G: weight of 1000 grains. See detail in Appendix I.

Treatment		HO			CONV		
		Yield	N_G	W_G	Yield	N_G	W_G
		kg ha^{-1}	m^{-2}	g	kg ha^{-1}	m^{-2}	g
Site	1	3903	6074b†	57.3b			
	2	3564	5500c	58.6b			
	3	2724	4161d	59.2ab			
	4	3620	5232c	62.4a			
	5	3700	6999a	46.4d			
	6	3146	5527c	50.9c			
	7	3494	6927a	45.7d			
	8				4241	7465a	50.8b
	9				3907	6268b	55.6a
	10				3098	7288a	52.1b
NR	0N	3235	5539	53.5	3367	6608	51.9
	30N	3381	5704	53.6	3611	7002	51.0
	60N	3546	5984	54.7	3694	6923	52.7
	90N	3580	5888	55.5	3929	7050	53.7
	120N	3501	5854	54.5	4103	7526	54.0
	150N	3457	5678	54.4	3787	6932	53.8
				ANOVA			
Source of variation							
Site		**	**	**	**	**	**
NR		**	ns‡	ns	**	ns	ns
Site × NR		*	ns	ns	*	ns	ns

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

† In each Site or NR, different letters within columns indicate significant differences between treatement as determined by the LSD test ($p < 0.05$).

‡ ns: nonsignificant.

example, accumulated intercepted PAR accounted for 46% of the variability in W_G of HO genotypes. However, in CONV genotypes, the relationship between accumulated intercepted PAR and W_G was not statistically significant ($P > 0.05$) probably because these genotypes presented a narrower range of accumulated intercepted PAR (477–543 MJ m^{-2}) and W_G (49–55 g) than HO genotypes (449–632 MJ m^{-2} and 44–66 g, respectively). The relationship between both variables did not improve when intercepted PAR was accumulated during the period 250 to 450°C day after flowering by Aguirrezábal et al. (2003) as critical for determining W_G.

Nitrogen Effect

In Exp. 2, for both types of genotypes, the interaction site × N rate was statistically significant for yield (Table 4, Appendix I). This could be explained by differences in initial N availability and concentrations of silt + clay, and rainfall (Table 2), as also reported by Melgar et al. (2003). These authors developed a method of diagnosing N in sunflower, based on edaphic, crop, and environmental parameters in the Argentine sunflower region (Ustipsament, Haplustolls, Hapludolls, and Arguidolls soils with water regimes between 400 and 900 mm). Yield responded to N availability in 50% of the evaluated sites, being the magnitude of the effect similar in both types of genotypes. Yield response to N varied between 238 and 847 kg ha^{-1} in HO genotypes, and between 146 and 991 kg ha^{-1} for CONV ones.

Table 4. Experiment number two (Exp. 2): Grain oil concentration (O_G) and grain protein concentration (P_G) for high oleic (HO) and conventional (CONV) genotypes in the different sites and different nitrogen rates (NR) (kg ha^{-1}). See detail in Appendix I.

Treatment		HO		CONV	
		O_G	P_G	O_G	P_G
			%		
Site	1	56.5a†	14.8		
	2	56.1a	14.0		
	3	52.6c	15.3		
	4	51.1d	16.8		
	5	54.4b	16.3		
	6	52.3c	15.1		
	7	54.9b	13.1		
	8			57.1a	11.2
	9			54.3c	12.8
	10			55.8b	12.2
NR	0N	54.4	14.1	55.2	10.8
	30N	54.7	14.3	55.6	11.1
	60N	54.0	14.9	56.1	11.9
	90N	53.4	15.4	55.9	12.4
	120N	54.0	15.5	55.7	12.8
	150N	53.4	16.0	55.8	13.3
			ANOVA		
Source of variation					
Site		**	**	**	**
NR		ns‡	**	ns	**
Site × NR		ns	*	ns	**

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

† In each Site or NR, different letters within columns indicate significant differences between treatement as determined by the LSD test ($p < 0.05$).

‡ ns: nonsignificant.

The lack of response in the other sites (4, 5, 6, 7, and 9) could be explained by an adequate NO_3^-–N availability and the contribution of N from mineralization (Table 2). For a network of 24 experiments on the Pampas region (Argentina), Melgar et al. (2003) observed significant effect of N on yield (760 kg ha^{-1}) only in 33% of the sites, and attributed the lack of responses to the occurrence of water stress and/or high initial NO_3^-–N availability. González Montaner and Di Nápoli (2002) for southeastern Buenos Aires Province proposed a critical threshold of N availability at planting of 50 kg ha^{-1} (0–60 cm). In our work, mean availability in the sites where we observed response was 56.9 kg ha^{-1}, with values from 39.5 to 76.7 kg ha^{-1}. This indicates that it is necessary to update the threshold value since potential yield of sunflower hybrids has increased (Hall et al., 2013) and, consequently, N demand, mainly under no-tillage systems due to the low input of N from mineralization (Pereyra et al., 2001). In this sense, Fig. 2 shows that pre-plant N availability, for both types of genotypes, accounted for 44% of RY variability, being the critical threshold 125 kg N ha^{-1}. This threshold is close to the proposed by Zamora and Massigoge (2008) for the south-central Buenos Aires region for similar yields.

Table 5. Experiment number one (Exp. 1): Yield and components for different planting date (PD): early planting (EP) and late planting (LP) for high oleic (HO) and conventional (CONV) genotypes. N_G: number of grains, O_G: grain oil concentration, P_G: grain protein concentration.

PD	Genotype	Yield	N_G	O_G	P_G
		kg ha^{-1}	m^{-2}	—— %	——
EP	HO	3822a†	6175a	53.9	15.5
	CONV	3495b	5416b	53.6	14.4
LP	HO	2740a	4053a	47.5	20.4
	CONV	2878a	4359a	48.4	19.1
			ANOVA		
Source of variation					
PD		**	**	**	**
Genotype		*	**	ns‡	*
Genotype × PD		*	**	ns	ns

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

† In each PD, different letters within columns indicate significant differences between genotype as determined by the LSD test ($p < 0.05$).

‡ ns: nonsignificant.

Grain Components: Oil and Protein

Genotype Effect

In E_1, the effect of genotype and the interaction PD × genotype on O_G were not statistically significant ($P > 0.05$) in Exp. 1. However, O_G from CONV genotypes was slightly higher than that of HO genotypes (51.1 vs. 50.6%). Delaying PD reduced O_G in average from 53.7% (EP) to 47.9% (LP). As mentioned for yield, this effect is the result of better environmental conditions during the grain-filling period in EP. Delaying PD in sunflower exposes the crop to low global radiation and suboptimum mean temperature for oil synthesis (Trapani et al., 2008). In this sense, Dosio et al. (2000) under a Typic Argiudol soil at the INTA Balcarce Experimental Station, Argentina reported reductions in O_G in a late PD due to lower global radiation. Ross (2014) at Petrocalcic Paleudol soil whith average 35 g kg^{-1} OM also reported reductions of 2 to 3% points in O_G when delayed PD due to lower global radiation and mean temperature during grain filling.

The P_G was affected by genotype and PD, being genotype × PD interaction not statistically significant (Table 5). The highest values of P_G were observed in LP (19.8 vs. 14.9% for LP and EP, respectively), and HO genotypes presented higher P_G than CONV ones (18.0 vs. 16.8% for HO and CONV, respectively). Higher P_G, even between PD or types of genotypes, are explained by reductions in O_G since several authors reported a balance between both concentrations (Connor and Sadras, 1992; Alberio et al., 2015). The effect of PD on P_G can be a consequence of reductions in oil synthesis due to lower global radiation at late planting dates (Alberio et al., 2015). The difference in P_G among HO and CONV genotypes has not previously been reported and it is a novel result for these sunflower genotypes.

In Exp. 2, HO genotypes also presented, in average, lower O_G and higher P_G compared to CONV genotypes (Table 4, Appendix I). Mean values of O_G and P_G for HO genotypes were 54.2% (range 50.7–57.2%) and 15.1% (range 12.3–17.8%), respectively. In CONV genotypes, mean values were 55.7% (range 53.5–57.8%) and 12.0% (range 10.3–13.9%), for O_G and P_G, respectively. For both experiments, O_G was within the values reported by Agüero et al. (1999) and ASAGIR (2014) in their respective cultivar evaluation networks in different locations of the Argentine sunflower region, while P_G was within the values reported by Díaz Zorita (2015) for southeastern Buenos Aires Province.

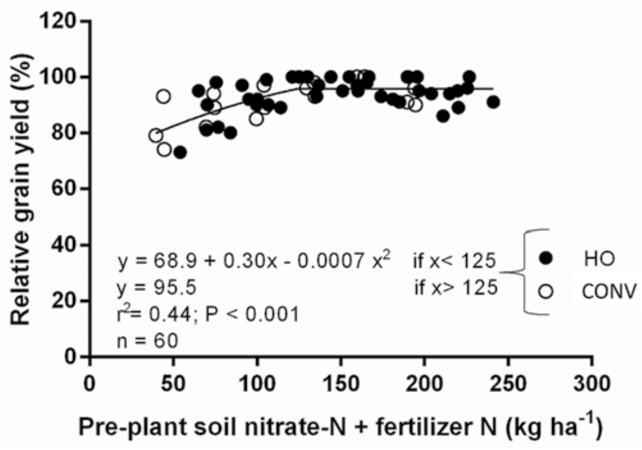

Fig. 2. Experiment number two (Exp. 2): Relative grain yield (RY) as a function of pre-plant N availability in the soil (nitrate N, 0–60 cm) before planting + fertilizer N. HO: high oleic. CONV: conventional. *n* = number of cases.

Despite the effect of PD previously mentioned in Exp. 1, the values of O_G and P_G observed in our experiments (Exp. 1 and 2) are relatively high for this species, indicating that growing conditions were in general favorable for grain filling (Table 2). Similarly to the results reported by Dosio et al. (2000), in our experiments O_G and P_G were related to accumulated intercepted PAR during grain filling (data not shown).

Nitrogen Effect

In Exp. 2, the interaction site × N rate was not statistically significant for O_G (Table 4, Appendix I). This trait was affected by site ($P < 0.001$ for HO and CONV genotypes) but not by N rate ($P > 0.05$ for HO and CONV genotypes) (Table 4, Appendix I). According to these results, there was no relationship between O_G and N availability (Fig. 3a), in agreement with Zubillaga et al. (2002) for mid-western Pampas Argentina. However, other authors reported O_G reductions when N availability increased (Scheiner et al., 2002; Triboi and Triboi-Blondel, 2002; Montemurro and De Giorgio, 2005). Li et al. (2017) in a 2-yr field experiments on the Shahaoqu Experimental Station Farm (China) under soil texture silt loam (0–20 cm) recommended not applying N fertilizer excessively at late growth stages of sunflower because they could decrease the concentration of oil in grain.

The interaction site × N rate was statistically significant for P_G in both types of genotypes (Table 4, Appendix I), which is partially explained by the different N availability at sowing. In average, P_G in HO genotypes varied from 14.1 to 16.0% for 0N and 150N, respectively. This variation for CONV genotypes was from 10.8% in 0N to 13.3% in 150N. The higher P_G could partially be explained by the higher PIR and accumulated intercepted PAR (Fig. 1). The relationship between P_G and N availability is shown in Fig. 3b. In all the range of variation, HO genotypes presented higher P_G than CONV ones. Several studies have reported a positive effect of N on grain protein concentration but have not evaluated differences between types of genotypes (Zubillaga et al., 2002; Aguirrezábal, 2010; González-Pérez, 2015). As P_G increased with N rate with nule effect

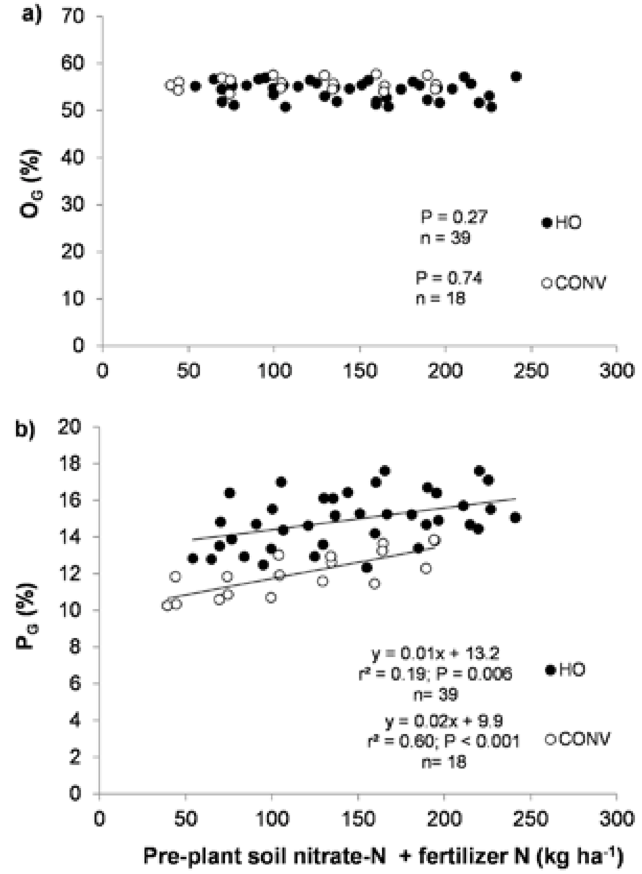

Fig. 3. Experiment number two (Exp. 2): Relationship between (a) grain oil concentration (O_G) and (b) grain protein concentration (P_G) and pre-plant soil nitrate N + fertilizer N for high oleic (HO) and conventional (CONV) genotypes. *n* = number of cases.

on O_G, this was reflected in variations in P_G/O_G ratio (HO: $P = 0.01$; CONV: $P < 0.01$). These effects are in accordance with those reported by Ruffo et al. (2003) in a 2-yr field experiment conducted in Balcarce, Buenos Aires Province, Argentina, on a soil complex of a fine, mixed, thermic Typic Argiudoll. González-Pérez (2015) reported that the variations in P_G/O_G when N varies are due to an increase in protein synthesis and a reduction in O_G. This antagonism could be explained by:

I. different relative fluxes of C and N to the grains caused by the asynchronism in the accumulation of protein and oil. The rate of protein accumulation is relatively constant while the oil accumulation rate is low at the first days after flowering but is high between 18 and 35 d after flowering, causing a dilution of stored proteins (Connor and Sadras, 1992);

Table 6. Experiment number two (Exp. 2): Parameters from a model to predict grain protein concentration from the following stepwise selected variables: preplant soil nitrate N + fertilizer N (kg ha^{-1}) (0–60 cm), silt and clay content (%), and rainfall from V_6 to flowering (mm). HO: high oleic, CONV: conventional.

Genotype	Variable	Parameter	*P* value	r^2 partial	r^2
	Intercept	3.43	<0.001		
HO	Soil + fertilizer N	0.01	<0.001	0.19	
	Silt + clay	0.15	<0.001	0.05	0.69
	Rainfall	0.03	<0.001	0.45	
	Intercept	-8.74×10^2	<0.001		
CONV	Soil + fertilizer N	1.73×10^{-2}	<0.001	0.62	
	Silt + clay	6.07	0.02	0.25	0.91
	Rainfall	4.21	0.02	0.04	

II. nitrogen remobilization to the grains, because although oil is synthetized in grains mainly with C from photosynthesis during grain filling, most part of the N used to synthetize protein is absorbed before flowering. In this context, senescence produces more translocation of N to grains but impair the photosynthetic system and, consequently, the C needed for oil synthesis (Alberio et al., 2015).

This apparent contradiction about the effect of N on O_G could be explained by variations in carbohydrates stored in the grains (Trapani et al., 2008), which was not determined in our work.

For a given value of N in Fig. 3b, the variability in P_G may be due to the effect of site, Therefore, regression models were adjusted including different variables that characterize the sites (Table 6). Nitrogen availability, surface concentration of silt + clay (%), and rainfall (mm) from V_6 to flowering accounted for 69 and 91% of the variability in P_G for HO and CONV, respectively. Soils with high silt + clay content and without water deficits from V_6 to flowering, where 75% of N is absorbed by the plant (Massignam et al., 2009), would be more efficient in N recovery.

Protein Concentration in Grains and By-Products

In Exp. 2, the concentrations of protein in grain and by-products were affected by genotype and N rate, but the interaction genotype × N rate was not statistically significant (Fig. 4). Mean P_G and P_M for CONV genotypes were 12.1 and 38.8%, respectively (Fig. 4.a), while for HO genotypes mean values were 15.0 and 44.0%, respectively (Fig. 4.a). These results indicate that the higher P_G of HO genotypes is also reflected in higher P_M compared to CONV genotypes. Dauguet et al. (2015) collected seed samples from the Terres Inovia experimental network (France)

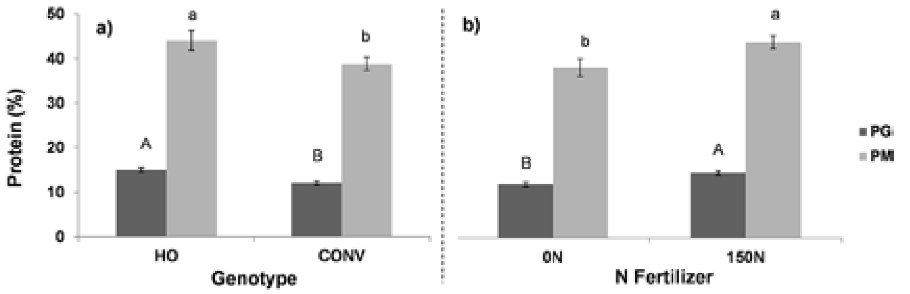

Fig. 4. Experiment number two (Exp. 2): Grain (P_G) and meal (P_M) protein concentration (average ± standard error) for (a) genotypes high oleic (HO) and conventional (CONV) and (b) control treatments (0N) and fertilized (150N). Upper and lowercase letters indicate significant differences ($P < 0.05$) in the P_G and P_M for the different treatments, respectively.

and determined that genetic improvement would increase P_G and, consequently, P_M.

Nitrogen rate increased mean P_G from 11.9% in 0N to 14.4% in 150N. For P_M, this variation was from 37.9 to 43.5% for both N rates, respectively (Fig. 4.b). Therefore, N addition allowed increasing P_G in average 2.5 (±0.9) percentage points, which were reflected in average in increases of P_M of 5.6 (±3.7) percentage points. These results are in accordance with those from Merrien et al. (1988) who reported that differences of P_G of 1 to 2% would be reflected in differences of up to 5% in P_M, assuming similar dehulling level and oil extraction.

The values of P_M observed in our work are within the ranges of values reported by Pedroche (2015) (20–60%). In addition, they were similar or higher than 30% (Fig. 4), the base value defined in the marketing regulations of sunflower by-products (INFOLEG, 2015). However, if P_M were equal or higher than 36%, it would be possible to obtain better prices during commercialization (de Figueiredo et al., 2015). For example, in 2015, the benefit for higher P_M was of EURO70 to 80 € tn^{-1}. According to Dauguet et al. (2016), with P_M of 36%, prices equivalent to 70% of the price of soybean pellets are obtained, while at 29% of P_M prices were equivalent to only 43%. In our work, for HO genotypes, values of P_M of 36% or higher were observed with either 0N or 150N. However, for CONV genotypes, only fertilized treatments overcame that limit. This reinforces the importance of the genetic effect (Dauguet et al., 2015) and N availability on P_M (Andrianasolo et al., 2016). Other studies indicate a better oil quality and stability of HO genotypes compared to CONV ones (Del Gatto et al., 2015; Alberio et al., 2016). However, this would be the first time that the higher grain quality of HO genotypes compared to CONV ones is evidenced by higher P_G and P_M. These results better position these hybrids in the sunflower market. It is of great importance for industry to have grains that allow producing by-products with high protein concentration and no reductions in yield or O_G.

CONCLUSIONS

In this work, in humid temperate agroecosystems, genotype and N availability affected yield, N_G, P_G, and P_M, with no effect on O_G. High oleic genotypes presented higher P_G and P_M than CONV genotypes, but this difference among genotypes was not reflected in reductions in yield or O_G. In addition, N fertilization allowed obtaining higher yield and P_G with no effect on O_G, increasing the P_G/O_G ratio. Therefore, the application of N would allow obtaining high yields and P_G without detrimental effects on O_G, improving the quality of grains and by-products.

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Appendix I. Experiment number two (Exp. 2): Yield, components of yield, grain oil concentration (O_G) and grain protein concentration (P_G) for high oleic (HO) and conventional (CONV) genotypes in the different sites and different nitrogen rates (NR). N_G: number of grains, W_G: weight of 1000 grains.

Genotype	Site	NR	Yield	N_G	W_G	O_G	P_G
			kg ha^{-1}	m^{-2}	g	—— %	——
OH	1	0N	3404b†	5705	53.3	56.6	13.9a
		30N	3731ab	5721	58.6	56.4	14.4a
		60N	4045ab	6138	59.0	55.4	15.2a
		90N	4130a	6281	58.8	56.1	15.2a
		120N	3961ab	6496	54.1	57.2	14.9a
		150N	4149a	6106	60.3	57.2	15.5a
	2	0N	3084b	4961	55.4	56.6	13.5a
		30N	3430ab	5419	56.4	57.0	13.4a
		60N	3793a	6245	59.2	55.8	13.6a
		90N	3686ab	5379	61.2	56.4	14.2a
		120N	3788a	5622	59.9	55.4	14.7a
		150N	3600ab	5377	59.7	55.7	14.4a
	3	0N	2239b	3621	55.0	54.5	12.8c
		30N	2477ab	3961	55.6	54.7	12.9bc
		60N	2762ab	4407	57.7	52.1	15.3ab
		90N	3086a	4590	61.1	51.3	16.4a
		120N	2884ab	4045	63.6	52.4	16.6a
		150N	2895ab	4345	62.0	50.8	17.8a
	4	0N	3641a	5063	63.6	51.1	16.4a
		30N	3690a	5167	63.6	50.8	17.0a
		60N	3457a	5048	61.1	51.9	16.1a
		90N	3654a	5240	64.3	50.8	17.6a
		120N	3716a	4999	66.2	51.6	16.4a
		150N	3562a	5875	55.7	50.7	17.1a
	5	0N	3528a	6887	45.7	55.2	14.8b
		30N	3617a	7012	46.0	55.4	15.5ab
		60N	3929a	7538	46.7	55.0	16.1ab
		90N	3724a	6902	48.6	52.7	17.0ab
		120N	3911a	7823	44.6	54.8	16.7ab
		150N	3492a	5831	47.0	53.1	17.6a
	6	0N	3259a	5558	52.5	51.9	14.7a
		30N	3363a	6069	49.3	53.3	14.6a
		60N	3182a	5216	54.4	53.0	15.3a
		90N	3110a	5819	47.9	51.9	15.2a
		120N	2892a	5267	48.9	52.3	15.7a
		150N	3068a	5235	52.2	51.7	15.1a
	7	0N	3491a	6977	49.3	55.2	12.8a
		30N	3360a	6582	45.5	55.3	12.5a
		60N	3655a	7293	44.7	55.1	12.9a
		90N	3668a	7004	46.7	54.6	12.3a
		120N	3356a	6730	44.4	54.5	13.4a

Continued

Appendix I. (cont.)

Genotype	Site	NR	Yield	N_G	W_G	O_G	P_G
			kg ha^{-1}	m^{-2}	g	%	
		150N	3431a	6975	43.8	54.6	14.7a
CONV	8	0N	3781c	7272	51.4	55.4	10.3d
		30N	3927bc	7162	49.0	56.9	10.6d
		60N	4037bc	7280	49.6	57.6	10.7cd
		90N	4574ab	7739	49.0	57.5	11.6ab
		120N	4772a	7986	53.1	57.8	11.5bc
		150N	4353abc	7350	52.7	57.5	12.3a
	9	0N	3759a	6035	55.4	54.3	11.8c
		30N	3825a	6349	53.8	53.5	11.8c
		60N	3929a	6403	54.8	54.8	13.0b
		90N	3989a	6201	57.2	54.5	13.0b
		120N	4056a	6511	55.8	54.0	13.2ab
		150N	3885a	6106	56.7	54.5	13.9a
	10	0N	2562b	6518	48.9	56.0	10.3c
		30N	3081ab	7495	50.2	56.4	10.9c
		60N	3115ab	7086	53.7	55.9	11.9b
		90N	3224ab	7209	55.0	55.7	12.6b
		120N	3481a	8079	53.0	55.3	13.6a
		150N	3122ab	7340	52.0	55.5	13.8a

† In each site, different letters within columns indicate significant differences between NR as determined by the LSD test ($p < 0.05$).

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