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Assessment of the critical period for the effect of intercepted solar radiation on sunflower oil fatty acid composition

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ABSTRACT

The fatty acid composition of sunflower (*Helianthus annuus* L.) oil closely depends on the environmental conditions during grain filling. Temperature and solar radiation are the main environmental factors driving oil fatty acid composition. Minimum night temperature and intercepted solar radiation per plant (ISR) during grain filling independently affect oleic acid percentage of traditional sunflower oil. Critical period for temperature effect on this trait has been shown to be placed between 100 and 300 °C day after flowering (°Cd af). The period of maximal sensitivity of fatty acid composition to ISR remains unknown. The aim of the present work was to identify the time window of high sensitivity (critical period) of fatty acid composition to ISR of sunflower oil. For this, ISR was modified by shading (50% or 80%) or thinning (50%) field grown sunflower hybrid DK3820 during different periods of grain filling. The timing of maximal sensitivity of fatty acid composition to source variations during post flowering periods was explored and analyzed by two widely used approaches: (i) evaluation of the relative oleic acid percentage under short shading treatments in relation to the control and (ii) window-pane analysis of the response of oleic acid percentage to ISR. The first approach generated differing estimates of the critical period depending on the level of radiation reduction. Using the second approach, a developmental interval during which oleic acid was most sensitive to ISR regardless of the radiation level was determined. The critical period began at 350 °Cd af and ended at 450 °Cd af. The critical period for radiation effect on oleic acid concentration differed from that of the radiation effect on grain weight and oil concentration and from the critical period for temperature effect on oil fatty acid composition. Different critical periods for different traits and specific environmental factors are indicative of the complexity of the interaction between environmental conditions and grain growth and oil synthesis dynamics.

1. Introduction

The quality and potential uses of vegetable oils are determined by their fatty acid composition. Sunflower oil is one of the most widely used vegetable oils because of its nutritional and industrial attributes. Sunflower oil quality is often considered in terms of oleic acid content, as this is nowadays the preferred fatty acid for both edible purposes and biodiesel production (Mensink et al., 2003; Marvey, 2008). Environmental factors have a decisive influence on sunflower oil quality (Roche et al., 2006; Izquierdo et al., 2006, 2009). It has long been known that temperature is the main factor affecting the proportion of oleic acid in the oil of traditional sunflower cultivars (Canvin, 1965). Additionally, increasing intercepted solar radiation per plant (ISR) during grain filling has been shown to increase oleic acid percentage in several crop species (Izquierdo et al., 2009; Zuil et al., 2012). In sunflower, differences in oleic acid relative concentration driven by this factor could be higher than 10 percentage points (Izquierdo et al., 2009). This effect on oleic acid percentage has been correlated with a decrease in linoleic acid concentration, with no significant changes in saturated fatty acids concentration (Izquierdo et al., 2009; Echarte et al., 2010). Intercepted solar radiation per plant and mean or minimum night temperature during grain filling independently affected oleic acid percentage of the oil of traditional sunflower (Echarte et al., 2010), soybean and maize (Zuil et al., 2012). The additive effect of temperature and radiation on fatty acid composition implies that these environmental factors affect oleic acid percentage through different mechanisms (Salisbury and Ross, 1992). In agreement with this observation, it has been shown that minimum night temperature affects oleic acid desaturation process through regulation of oleate desaturase activity (Izquierdo et al., 2006; Rolletschek et al., 2007) while intercepted solar radiation changes oleic/linoleic

Abbreviations: ISR, intercepted solar radiation per plant; PAR, photosynthetically active radiation; °Cd af, °C day after flowering.

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ratio through regulation of post flowering assimilates availability to the grains (Echarte et al., 2012).

Particular phases during plant development are more relevant for the determination of grain composition traits. Several authors have explored critical periods for the determination of grain number and grain weight in several species (Fischer, 1975; Savin and Slafer, 1991; Arisnabarreta and Miralles, 2008; Kiniry and Ritchie, 1985; Jiang and Egli, 1993; Kantolic et al., 2007).

Empirical relationships between grain composition traits and environmental conditions can be incorporated into simulation models to reflect interactions between environmental signals and physiological processes. The plant phenology, the non linearity of response of biochemical and physiological processes, as well as the erratic nature of climatic events often add to models predictions high degree of unexpected variability, which makes the output difficult to interpret. The critical period of a given trait is a modeling tool that helps to improve the accuracy of crop models predictions (Pereyra-Irujo and Aguirrezábal, 2007). Furthermore, the knowledge of many critical periods has been helpful for understanding the mechanisms underlying plant responses to environmental factors (e.g. Pleite et al., 2008).

In sunflower, critical periods for the effect of solar radiation on grain number (Cantagallo et al., 2004), grain weight and oil concentration (Aguirrezábal et al., 2003) have been found. These traits and the fatty acid composition of the oil have been shown to be more sensitive to heat stress during particular periods of grain filling (Rondanini et al., 2003, 2006). A critical period for fatty acid composition response to moderate minimum night temperature for sunflower oil has also been determined (Izquierdo et al., 2006). By contrast, the period of maximal sensitivity of oil composition to ISR throughout grain filling remains unknown. Since temperature and radiation affect fatty acid composition through different mechanisms (Echarte et al., 2012), critical periods for the effect of both factors on fatty acid composition could be different.

Different methods of experimental analysis have been used in order to elucidate these critical periods. These methods are usually based on the application of short treatments during different periods throughout the crop cycle. In many research works, the critical period is determined by evaluating the effect of these short treatments in relation to control values. The main limitation of this method of analysis is that the determined critical period depends on the treatment duration and moment of application (Cantagallo et al., 1997; Arisnabarreta and Miralles, 2008; Sandaña and Calderini, 2012). Alternatively, window-pane analysis allows determining critical periods that do not depend on the treatments. This method of analysis consists of relating a given trait to an environmental factor during time windows of different length and different starting time in order to find the period exhibiting the strongest relationship between trait response and factor (e.g. Aguirrezábal et al., 2003; Izquierdo et al., 2006).

The aim of the present work was to identify the key time window for fatty acid composition response to ISR of sunflower oil. For this, the timing and/or the sensitivity of fatty acid composition to source variations during various post flowering periods has been explored and analyzed by two widely used approaches: (i) evaluation of the dependence of the effect of short duration shading treatments on the moment in which they were imposed and (ii) window-pane analysis of the response of oleic acid percentage to ISR. The combination of both approaches allowed us to precisely assess the period during which fatty acid composition is most sensitive to solar radiation. Dynamics of grain filling and fatty acid composition empirically supports the statistically determined critical period and shed some light on the mechanisms underlying fatty acid response to environmental conditions during grain filling.

2. Materials and methods

Sunflower (*Helianthus annuus* L., hybrid DK3820) was grown in the field at the Unidad Integrada Balcarce INTA-FCA (37° S, 58° W) Balcarce-Argentina. Soil was a Typical Argiudoll. Experiments were performed during growing seasons 2008–2009 (Exp. 1) and 2010–2011 (Exp. 2). Seeds were planted on November 6th, 2008 and November 19th, 2010, for Exps. 1 and 2, respectively. Treatments were arranged in a randomized complete block design with three replicates. Experimental units were six rows six meters long spaced at 0.7 m. Plant population density at sowing was 6.5 plants m^{-2}. Crops were grown under optimal nutrient and water conditions. Soil fertility in all experiments was enough to attain maximum yields for sunflower crops grown under non-limiting water conditions (yield > 5000 kg ha^{-1}; Sosa et al., 1999; Andrade et al., 2000). Soil water content was measured every 5–7 days by the Time Domain Reflectometry method, with a moisture measuring system (Trase System, Model 6050X1, Soilmoisture Equipment Corp., Santa Barbara, CA, USA). Irrigation was applied to maintain soil water above 40% available water in the first 0.60 m of the soil profile during the entire growing season. Pests, diseases and weeds were adequately controlled. At flowering, pollination bags (Delnet, Rosario, Argentina) were used to prevent cross-pollination. Flowering of a plant was defined by the appearance of stamens in all florets from the outer whorl of the capitulum (R5.1 stage, Schneiter and Miller, 1981). Flowering of an experimental unit was considered to have occurred when 95% of the plants had reached R5.1 stage. Plants were self-pollinated manually.

Treatments meant to vary ISR during different periods of the grain filling were applied when inner flowers of 95% of the plants had been pollinated (three days after R6, Schneiter and Miller, 1981), and ended at physiological maturity. Since treatments were applied after grain set, grain number did not differ among treatments. Physiological maturity was reached at 615 °Cd af and 590 °Cd af in Exp. 1 and Exp. 2, respectively (base temperature = 6 °C). Treatments consisted of shading (Sh) and thinning (Th) whose starting and ending thermal time (°Cd af) are depicted in Table 1. Long term treatments (Th A, $Sh_{80\%}$ A and $Sh_{50\%}$ A) were applied during the whole grain filling period. Shading treatments were achieved by placing a uniform, black, synthetic, and neutral mesh cloth above the canopy of the two central rows (Dosio et al., 2000; Izquierdo et al., 2008). Shades reduced incident solar radiation by 80% (Exp. 1 and Exp. 2) or 50% (Exp. 2). Thinning treatments were performed by eliminating alternate plants in the row in order to get 50% (3.3 plant m^{-2}) of the original crop population density. Untreated sunflower plots served as control.

To estimate physiological maturity, 15 grains from rows 4 to 19 of three capitula were harvested twice a week during grain filling. To explore grain filling dynamics, 12 grains of rows 6–8 were excised in the same way. Grain removal was repeated on the same plant as long as total removal did not exceed 5% of average final capitulum grain number. Grains were oven-dried at 60 °C and weighed. Physiological maturity was determined as the time when average dry weight per grain did not further increase (Aguirrezábal et al., 2003).

2.1. Sample processing and chemical analysis

Once physiological maturity was reached, ten capitula were sampled from the two central rows of each plot and oven-dried with circulating air at 60 °C. Grains between rings 4 and 19 of the capitulum were manually separated in order to determine fatty acid composition of grains set at a similar date and therefore, exposed to similar environmental conditions (Izquierdo et al., 2002). Only non-empty grains (kernel occupying at least 20% of the internal volume of the hull) were considered in sample analyses. Oil extraction

Table 1
Treatment period and final harvest oleic acid concentration of sunflower oil corresponding to Exps. 1 and 2.

Experiment	Treatment	Period (°Cd af)	[Oleic acid] (%)
Exp. 1	Control	–	20.9 ± 2
	Th A	185–615	25.4 ± 0.6**
	Th B	250–615	24.7 ± 1.5**
	Th C	324–615	24.0 ± 0.7**
	Th D	420–615	23.4 ± 1.2*
	$Sh_{80\%}$ A	230–615	14.0 ± 0.2**
	$Sh_{80\%}$ B	230–324	21.0 ± 1.5ns
	$Sh_{80\%}$ C	324–420	16.9 ± 0.4**
	$Sh_{80\%}$ D	420–615	18.5 ± 0.6*
	MS	1.2	
	C.V. (%)	18.5	
	LSD[a]	1.90	
Exp. 2	Control	–	22.7 ± 1.9
	Th A	229–590	26.5 ± 2.4**
	Th B	307–590	26.2 ± 1.1**
	Th C	420–590	21.5 ± 2.0ns
	Th D	520–590	21.2 ± 2.2ns
	$Sh_{50\%}$ A	229–590	18.3 ± 1.6*
	$Sh_{50\%}$ B	229–295	20.3 ± 2.1ns
	$Sh_{50\%}$ C	295–437	17.5 ± 3.7*
	$Sh_{50\%}$ D	437–590	18.9 ± 1.4*
	$Sh_{80\%}$ A	229–590	12.9 ± 1.3**
	$Sh_{80\%}$ B	229–295	21.2 ± 1.9ns
	$Sh_{80\%}$ C	295–402	17.2 ± 2.8**
	$Sh_{80\%}$ D	402–590	16.8 ± 1.7**
	$Sh_{80\%}$ E	520–590	18.8 ± 0.5*
	MS	3.8	
	C.V. (%)	19.7	
	LSD[a]	3.3	

[a] Least significant difference value ($P = 0.05$). MS, mean square of the error; C.V., coefficient of variation.
* Significant effects $P < 0.05$.
** Significant effects $P < 0.01$.
ns Not significant.

from grains harvested at physiological maturity and its subsequent methylation was performed following the technique proposed by Sukhija and Palmquist (1988).

Small samples (12 grains) for dynamics determinations were processed as in Ruiz-López et al. (2003) with modifications. Lipids were extracted with hexane:isopropanol (7:2, v/v) and Na_2SO_4 (67 g l^{-1}) in the presence of 200 μl of 1, 2, 3 triheptadecanoylglycerol (50 mg ml^{-1}) as an internal standard. The lipidic phases from the extracted samples were evaporated to dryness under N_2. The residue was dissolved in 0.5 ml of hexane and incubated for 1 h at 80 °C in the presence of the methylation mixture methanol:toluene:H_2SO_4 (88:10:2) and 1 ml of heptane. After samples had cooled at room temperature, upper phase containing fatty acids methyl esters was separated.

Fatty acid composition of the extracts was determined by gas chromatography (GLC) with a Shimadzu GC-2014 equipment (Kyoto, Japan). Fatty acid content was expressed as percentage of the total fatty acids identified in the oil. Total lipids extracted were calculated with respect to the area of the internal standard. Oil content was estimated by considering that total extracted lipids represent more than 96% of the oil (Robertson et al., 1978). Oil concentration was expressed as percentage of total grain weight (oil content × 100/grain weight).

2.2. Measurements

Global daily incident radiation was measured with pyranometers (LI-200SB, LI-COR, Lincoln, NE) from weather meteorological stations located at approximately 400 m from the experiments. Mean incident radiation values for Exp. 1 and Exp. 2 were 20.8 °C and 20.5 MJ day^{-1}, respectively. Daily incident photosynthetically active radiation (PAR) was calculated as 0.48 × global daily incident radiation (Bonhomme, 1993). The proportion of photosynthetically active radiation (PAR) intercepted by the crop at noon (±1 h) was calculated according to Gallo and Daughtry (1986) as (1 − Rb/Ro), where Rb is the radiation measured below the oldest green leaf, and Ro is the radiation measured above the canopy. Rb was measured weekly with a line quantum sensor (LI-191SB, LI-COR, Lincoln, NE, USA) positioned across the rows (the length of the sensor was modified according to the distance between rows: 0.7 m). Three measurements were done per plot. In accordance with Charles-Edwards and Lawn (1984), the daily proportion of PAR intercepted was estimated as the proportion of PAR intercepted at noon × 2/(1 + proportion of PAR intercepted at noon). This correction allowed a substantial improvement on the error arising from a single measurement at noon (Trapani et al., 1992). Daily proportion of intercepted PAR between measurements was calculated by linear interpolation. Daily intercepted solar radiation per plant was calculated as the product of daily incident PAR and daily proportion of PAR intercepted divided by the plant density. Intercepted solar radiation accumulated per plant from R6 to R9 (Schneiter and Miller, 1981) was calculated as described by Dosio et al. (2000).

Temperature of the grains and the air at the capitulum level were measured using Cu constantan thermocouples (Termoquar, Buenos Aires, Argentina) and thermistors (Cavadevices, Buenos Aires, Argentina), respectively. Thermistors were protected by shields to prevent absorption of solar radiation. Measurements began after flowering and finished at physiological maturity. They were recorded by data loggers every 60 s and averaged (Cavadevices©, Buenos Aires, Argentina). Thermal time after flowering (°Cd af) was calculated from air temperature using a base temperature of 6 °C (Kiniry et al., 1992). Grain temperature did not exceed 30.5 °C for more than 3 h, making unlikely a heat stress effect such as that described by Rondanini et al. (2006).

2.3. Data analysis

Results were subjected to analysis of variance (ANOVA) and differences between the treatments and the control were tested using the Dunnett Test (Dunnett, 1955). Treatment means, standard errors, and least significant differences (LSD, $P < 0.05$) were calculated using InfoStat 1.1 Software (2002, InfoStat Group). Non linear simple regressions were fitted using Sigmaplot 8.0 software (1986–2001, SPSS Inc.). Goodness of fit was evaluated through the comparison of r^2 and root mean square error (RMSE) values.

Relationships between oleic acid concentration and ISR were described by an exponential rise up to a maximum (Eq. (1)), as performed by Echarte et al. (2010)

$$[\text{Oleic acid}] = z_0 - c\mathrm{e}^{-d\mathrm{ISR}}) \quad (1)$$

where z_0 is the oleic acid percentage attainable at maximal ISR; c represents the difference between oleic acid percentages at ISR = 0 and ISR per plant = ∞ (i.e., the maximum theoretical variation of oleic acid percentage in response to ISR), and d is the exponential curvature of oleic acid dependence on ISR per plant.

Two different approaches were used to assess the critical period for fatty acid response to ISR:

(1) Relative values of oleic acid concentration under short duration shading treatments in relation to the control were plotted as a function of the midpoint of the shading period as in Sandaña and Calderini (2012). These authors defined the average C.V. values of pea grain number and weight across treatments and experiments as the threshold for critical period estimation. According to this methodology, the threshold for the critical period

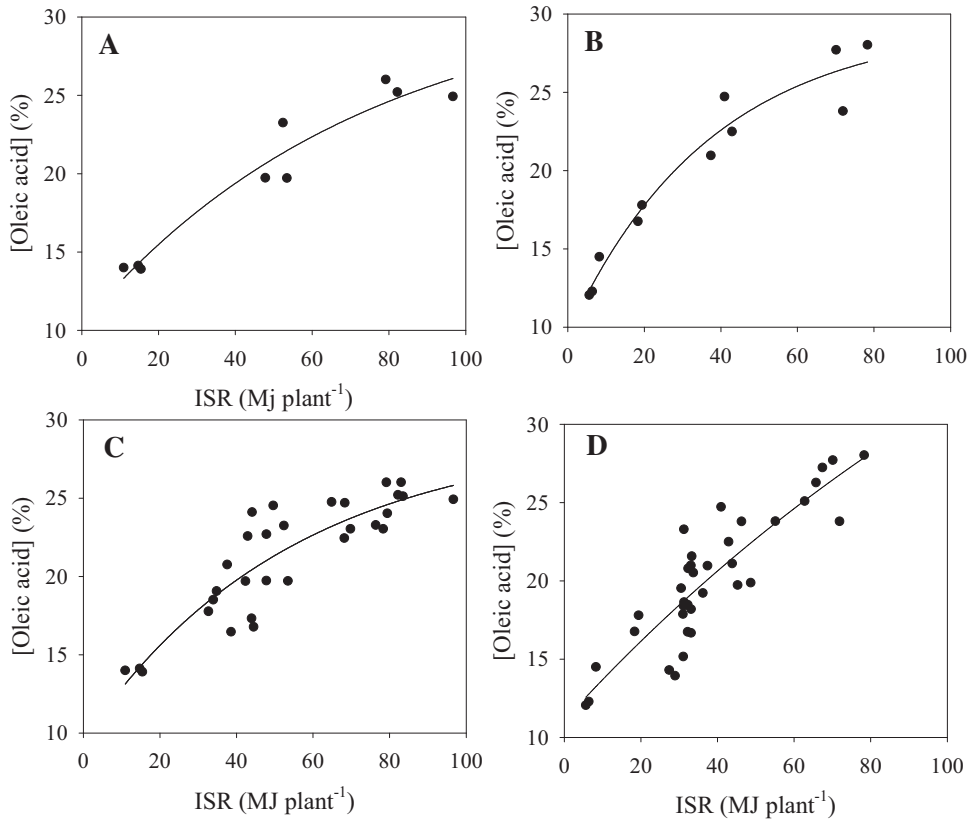

Fig. 1. Relationship between oleic acid percentage of sunflower oil and ISR accumulated from R6 to R9. Panels (A) and (B) show the results for long term treatments (R6 to R9) of Exps. 1 and 2, respectively. Panels (C) and (D) show oleic acid concentration data for long and short term treatments of Exp. 1 and Exp. 2, respectively. Each point represents a single measurement of the oleic acid percentage in the oil of sunflower plants grown under different radiative conditions during grain filling. Continuous line show functions of the form of Eq. (1) ([Oleic acid] = $z_0 - ce^{-d\mathrm{ISR}}$) fitted to the data. Values for parameters of the fitted functions are given in Table 2.

assessment was established as the average of the C.V. values for oleic acid percentages calculated in both experiments.

(2) *Window-pane analysis*: a 5-day window of time was slid in daily increments across the R6 to R9 period. The window length was arbitrarily defined in order to generate a set of data points of an adequate size (14 data sets) along the entire grain filling duration phase. Values of oleic acid percentage coming from control, shading, and thinning treatments were plotted against ISR accumulated in each different time window. Eq. (1) was fitted to experimental data and the coefficients of determination (r^2) were registered for each of the three plots. The values of r^2 for each plot were averaged and graphed against the mid-point of the thermal time window. The threshold for the critical period was established as the r^2 value obtained in each experiment for the fitting of Eq. (1) to the response of oleic acid concentration to ISR accumulated during the whole grain filling (R6–R9). Window-pane analysis results were verified by a bootstrap analysis according Izquierdo et al. (2006). For this, five hundred random samplings with replacement were obtained and Eq. (1) was fitted to each sample. Goodness of fit of the fitted function was evaluated by comparing the distribution of the 500r^2 values.

3. Results

3.1. ISR effect on fatty acid composition

Minimum night temperature during the critical period for temperature effect on oleic acid concentration (100–300 °Cd af; Izquierdo et al., 2006) did not differ between experiments (16.4 and 16.3 °C for Exps. 1 and 2, respectively). Maximum differences of oleic acid percentage among treatments were around 11 and 13 percentage points for Exp. 1 and Exp. 2, respectively (Table 1), representing the spread between long term thinning with long term

Table 2
Parameter values ± 1 standard deviation, r^2 and RMSE for the best fitting of Eq. (1) to data presented in Figs. 1 and 4.

Data source	Period of ISR accumulation	Experiment	z_0	C	d	r^2	RMSE
Long term treatments	Grain filling	Exp. 1	31.8 ± 8.0**	21.4 ± 6.8**	0.014 ± 0.001**	0.94	1.4
		Exp. 2	29.4 ± 2.8**	19.8 ± 2.2**	0.030 ± 0.010*	0.95	1.5
All treatments	Grain filling	Exp. 1	29.1 ± 3.7**	19.5 ± 2.6**	0.020 ± 0.010*	0.77	1.8
		Exp. 2	88 ± 171ns	77 ± 170ns	0.003 ± 0.008ns	0.77	2.2
All treatments	Critical period	Exp. 1	28.1 ± 2.4**	17.4 ± 1.8**	0.090 ± 0.03**	0.86	1.4
		Exp. 2	39.3 ± 10.0**	29.2 ± 11.0**	0.040 ± 0.010*	0.82	1.7

* Significant level $P < 0.05$.
** Significant level $P < 0.01$.
ns Not significant.

80% shading treatments in both experiments. Thinning treatments performed in Exp. 1 significantly increased oleic acid concentration disregarding the period during which they had been set, while in Exp. 2, late thinning treatments did not produce any significant effect on this trait ($P < 0.01$, Table 1). Oleic acid sensitivity to both 50% and 80% shading treatments depended on the moment of the grain filling period during which they have been imposed.

Oleic acid percentage was linearly and negatively related to linoleic acid percentage. The slope of this relationship was between −0.96 and −0.99, indicating that an increase in oleic acid concentration corresponded to a decrease of similar magnitude in linoleic acid concentration, with no significant change of saturated fatty acids concentration. For this reason and for the sake of clarity, in this section as well as in Sections 3.2 and 3.3, variations in fatty acid composition will be discussed in terms of variations on oleic acid concentration.

The relationship between oleic acid concentration and ISR accumulated during the entire grain filling was well described by an exponential rise to max model (Eq. (1)) fitted to experimental data collected from long term treatments (Fig. 1A and B, Table 2).

When oleic acid concentration values from short term treatments were added to the pool of data (Fig. 1C and D), the fitting of Eq. (1) resulted in lower r^2 and higher RMSE values than those obtained when considering only data from long term treatments (Table 2). Moreover, in Exp. 2, although the general trend of the response was consistent with those shown in the remaining panels, equation parameters became non-significant and the shape of the response to ISR changed slightly (Fig. 1D). These observations strongly suggest that the oleic acid concentration depends not only on the level of radiation variation but also on the timing at which ISR changes occur, and therefore, a critical period for ISR effect on oleic acid concentration could exist.

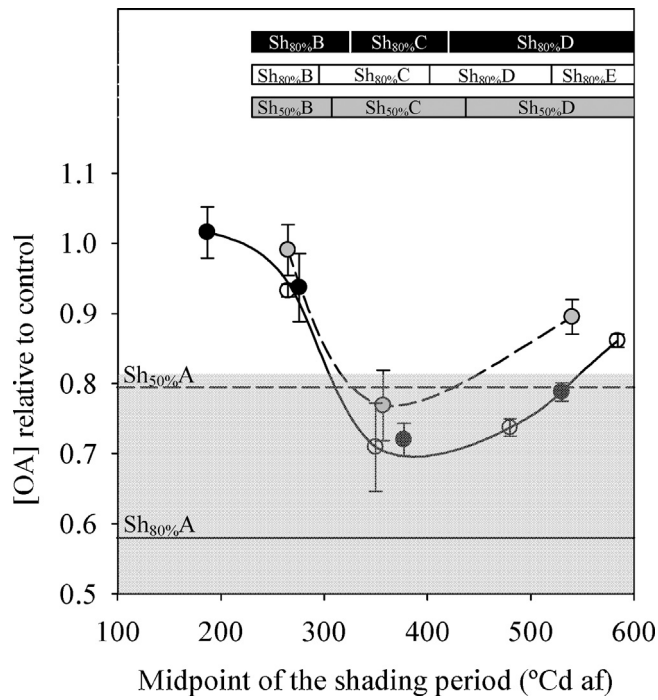

Fig. 2. Oleic acid proportion ([OA]) for short term 50% and 80% shading treatments relative to the control as a function of the thermal time elapsed at the midpoint of the shading period. Horizontal bars show the timing of application of the treatments. Black closed circles and black horizontal bars: short term 80% shading in Exp. 1, open circles and white horizontal bars: short term 80% shading in Exp. 2, gray symbols and gray horizontal bars: short term 50% shading in Exp. 2. Continuous horizontal line: [OA] relative to the control for long term 80% shading treatments ($Sh_{80\%}$ A). Dashed horizontal line: [OA] relative to control for long term 50% shading treatment ($Sh_{50\%}$ A). Gray zone: [OA] relative values lower than the threshold for the critical period (19% of [OA] reduction), as defined by C.V. across treatments and experiments (see Sections 3 and 3.2). Vertical bars show the standard error of the mean ($n = 3$).

3.2. Dependence of fatty acid composition on the timing and intensity of ISR variation

As a first approach to determine the time window of maximal sensitivity of oil fatty acid composition to radiation effect, oleic acid concentration values relative to control were plotted against the thermal time elapsed at the midpoint of the shading period for the short term treatments (Fig. 2). Since this kind of analysis requires that short treatments were all of similar duration, thinning treatments were not included. Shading of 80% of incident solar radiation during the whole grain filling period reduced oleic acid concentration to a level relative to control of 0.57 (continuous horizontal line, Fig. 2), while long term 50% shading treatment reduced oleic acid concentration to a relative value of 0.8 compared to the control (dashed horizontal line, Fig. 2). In addition, while a short term 50% shading treatment seems enough to attain maximal 50% shading effect if imposed during a certain interval of grain filling, no 80% short term shading treatment was sufficient to achieve the oleic acid reduction produced by 80% shading during the whole of grain filling.

Critical period was defined as that in which shading treatment significantly reduced oleic acid percentage ($P < 0.05$) in relation to control treatment. The threshold value to establish this critical

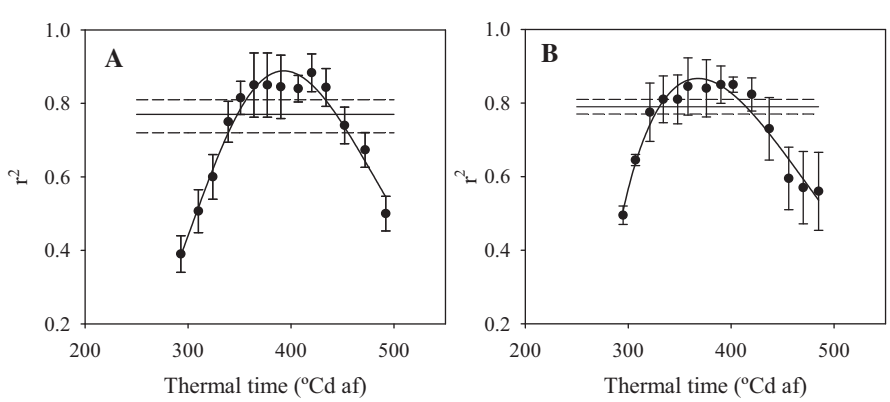

Fig. 3. Coefficients of determination (r^2) for the fitting of Eq. (1) to the response of oleic acid concentration to ISR of Exp. 1 (A) and Exp. 2. (B) as a function of the thermal time elapsed at the midpoint of the time window considered. Time windows were 5 days long. Horizontal continuous lines represent the r^2 for the fitting of Eq. (1) to oleic acid percentage vs. ISR accumulated from R6 to R9; horizontal dashed lines are the standard deviations of these r^2 values. Vertical bars show ± one standard deviation of the mean ($n = 3$) r^2 values obtained for each of the 5-day windows.

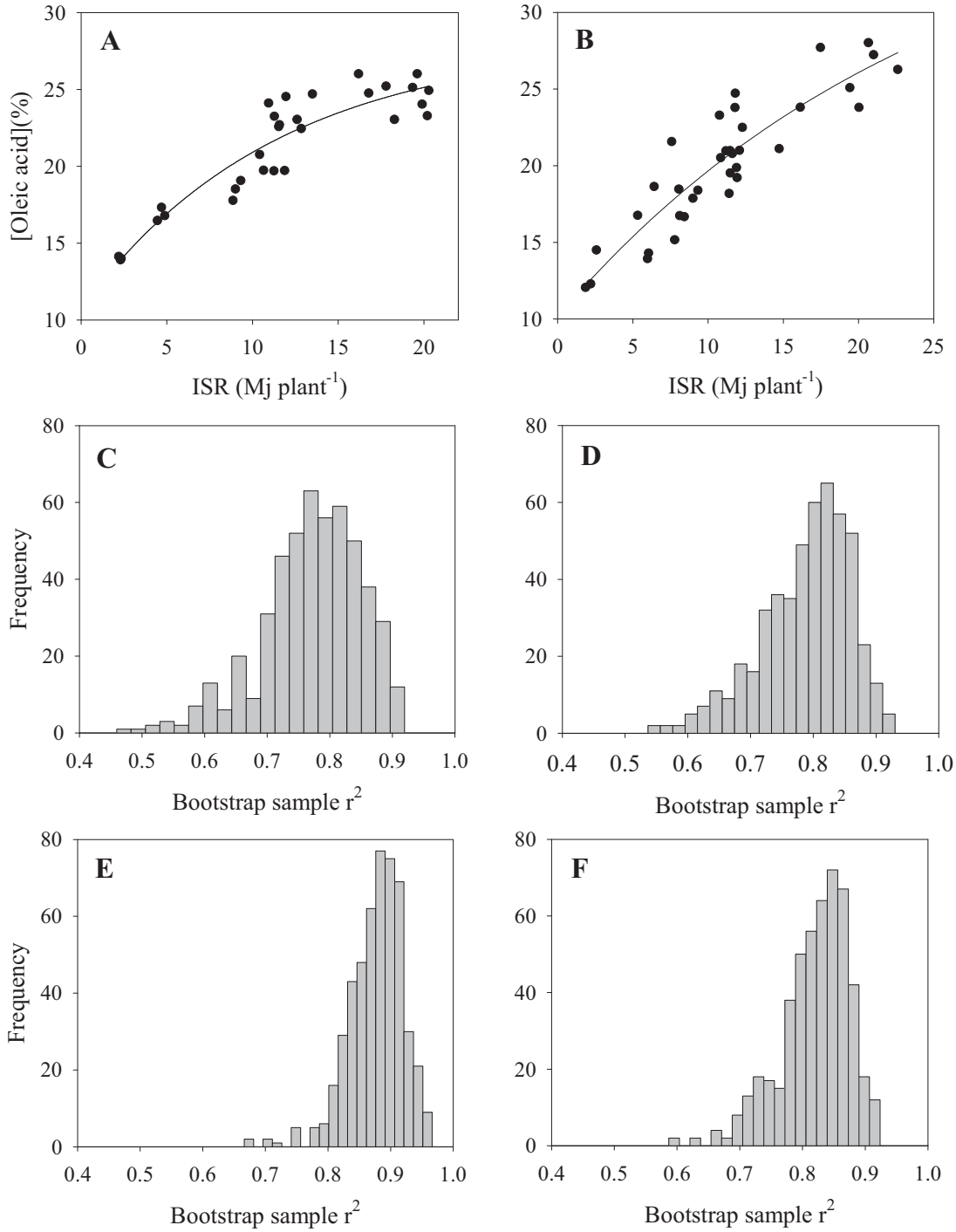

Fig. 4. (A and B) Relationship between oleic acid percentage and ISR accumulated during the critical period (350–450 °Cd af) for ISR effect. Distribution of r^2 for the relationship between oleic acid concentration and ISR accumulated during the whole grain filling (C and D) or the critical period (E and F) obtained by the bootstrap method. Left panels: Exp. 1; right panels: Exp. 2. Each point represents a single measurement of the oleic acid percentage in the oil of sunflower plants grown under different radiative conditions during grain filling.

period was defined as the average C.V. value (%) across treatments and experiments (Sandaña and Calderini, 2012). Critical period was therefore considered as the period when the estimated reduction of oleic acid concentration by shading was higher than 19% (Table 1, gray zone in Fig. 2). According to the established threshold, the critical period for the effect of the ISR on the oleic acid concentration depended on the radiation level. Critical periods from 350 to 450 °Cd af, and 300 to 550 °Cd af were defined for a reduction of 50% and 80% of the ISR during the grain filling period, respectively.

3.3. *Window-pane analysis for assessing the critical period of the effect of ISR on fatty acid composition*

To perform this analysis oleic acid concentration was related to ISR accumulated in 5-day windows from R6 to R9. As many correlations were generated as time windows used. Differences between C.V. values of ISR accumulated during R6 to R9 period and 5-day windows were not detected (data not shown). Fig. 3 shows the coefficients of determination (r^2) for the fitting of Eq. (1) to each of these relationships plotted as a function of the thermal time in

the midpoint of the time window. The critical period was defined as the period during which time windows provided relationships between oleic acid and ISR with r^2 values equal or higher than that obtained for the relationship between oleic acid concentration and ISR accumulated during the whole grain filling period (from R6 to R9).

The critical period for the effect of ISR on oleic acid concentration was placed between 350 and 450 °Cd af. By comparing Figs. 1 and 4, it can be observed that oleic acid concentration was better explained by ISR accumulated during the critical period than the ISR accumulated from R6 to R9. This result was confirmed by analyzing 500 bootstrap samples of both experiments: a higher mean r^2 value and a shorter range of r^2 distribution was achieved when considering the ISR accumulated during the critical period (Fig. 4E and F) instead of the one accumulated during the whole grain filling (Fig. 4C and D).

3.4. Grain filling and fatty acid composition dynamics under different radiative conditions

Oleic and linoleic acids dynamics were analyzed and compared with grain filling dynamics (Fig. 5). For this, grain weight, oil, oleic and linoleic acids percentages were plotted as a function of time for control, $Sh_{80\%}$ A and $Sh_{80\%}$ C treatments. Grain weight and oil percentage increased with thermal time describing a sigmoid function (Fig. 5A and B). Both $Sh_{80\%}$ A and $Sh_{80\%}$ C treatments decreased the values of these variables. The effect of the short term treatment $Sh_{80\%}$ C on grain weight and oil concentration was significantly lower than the one produced by the long term shading treatment ($P < 0.01$, $Sh_{80\%}$ A). Grain weight, in turn seemed to be more affected by $Sh_{80\%}$ C than oil percentage.

Fig. 5C shows that oleic acid percentage increased with thermal time up to a maximum value (at approx. 300 °Cd af) and after that, it slowly decreased. Linoleic acid concentration showed an opposite trend (it decreased up to a minimum and then slowly increased). Effects of shading treatments on fatty acid composition were not significant before 300 °Cd af ($P < 0.01$), regardless of when treatments were applied. Final oleic and linoleic acid percentages of both $Sh_{80\%}$ A and $Sh_{80\%}$ C were not significantly different ($P < 0.01$).

4. Discussion

The present work confirmed that oleic acid concentration exponentially increases up to a maximum value as ISR accumulated during grain filling increases (Echarte et al., 2010). Parameters describing oleic acid concentration response to ISR were very similar for both experiments when considering only long term treatments (Fig. 1A and B). Slight differences found in the response of oleic acid to ISR between experiments may be explained by differences in source/sink ratios (Izquierdo et al., 2009; Echarte et al., 2012), since minimum night temperature in both experiments were very similar. When short term treatments were included in the analysis, coefficients of goodness-of-fit significantly changed: r^2 was 20% lower and RMSE more than 20% higher than when analyzing only long term treatments. Furthermore, differences in the response parameters of oleic acid concentration to ISR between experiments appeared, and in Exp. 2, even the exponential pattern of the response was lost. The inclusion of short 50% shading treatments in Exp. 2 (not performed in Exp. 1) make the variability of ISR during certain periods higher than in Exp. 1, accounting for most of the differences between responses in both experiments.

The results reported here demonstrated that there is a short period during grain filling when sensitivity of oleic acid concentration of sunflower oil fatty acid composition to source reduction is maximal. The application of two different methodologies of analysis, widely reported in the literature, allowed determining this critical period. As a first approach, the relative values of oleic acid concentration under shading treatments in relation to the control were evaluated throughout the grain filling (threshold of oleic acid concentration reduction = 20%, Fig. 2). It was observed that the lower the radiation intensity (50% and 80% shading), the longer the critical period (350–450 °Cd af and 300–550 °Cd af, respectively). Although, it could be expected that different stress levels would present different threshold values to exert their effect, for modeling purposes, it would be necessary to define a critical period for ISR

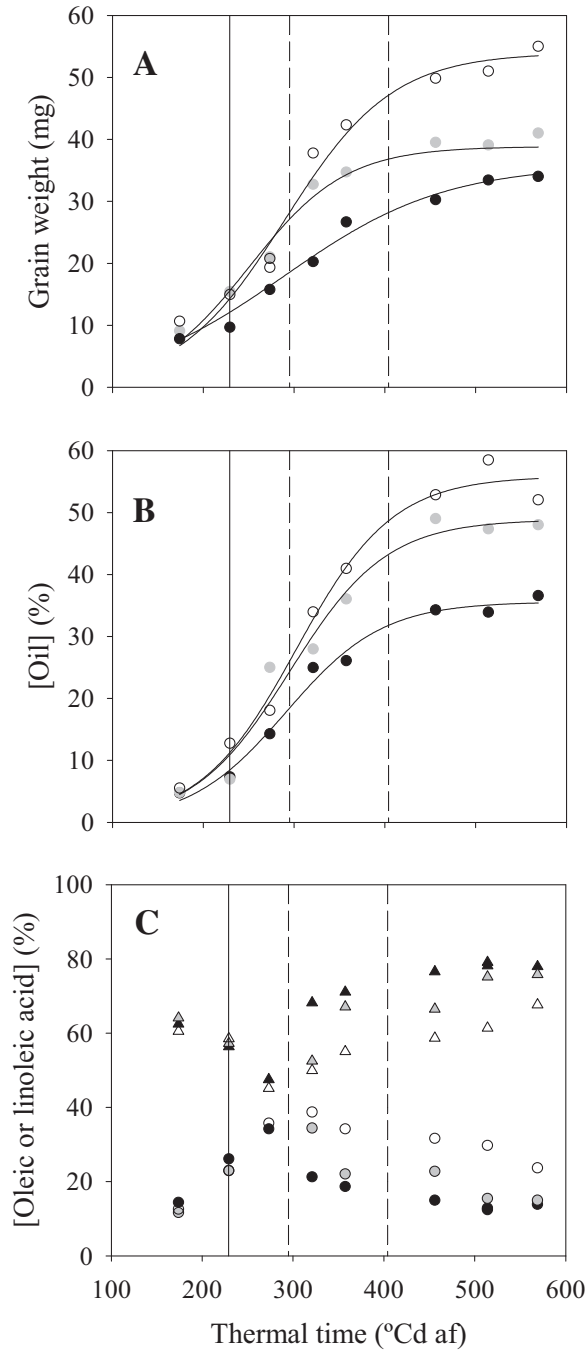

Fig. 5. Grain weight (A), oil concentration (B) and fatty acid composition (C) as a function of thermal time after flowering. Open symbols: control; closed black symbols: Sh80% A, closed gray symbols: Sh80% C. In panel C, open circles show oleic acid percentage; triangles: linoleic acid percentage. Continuous vertical line represents the moment of application of Sh80% A. Sh80% C was applied in between dashed vertical lines.

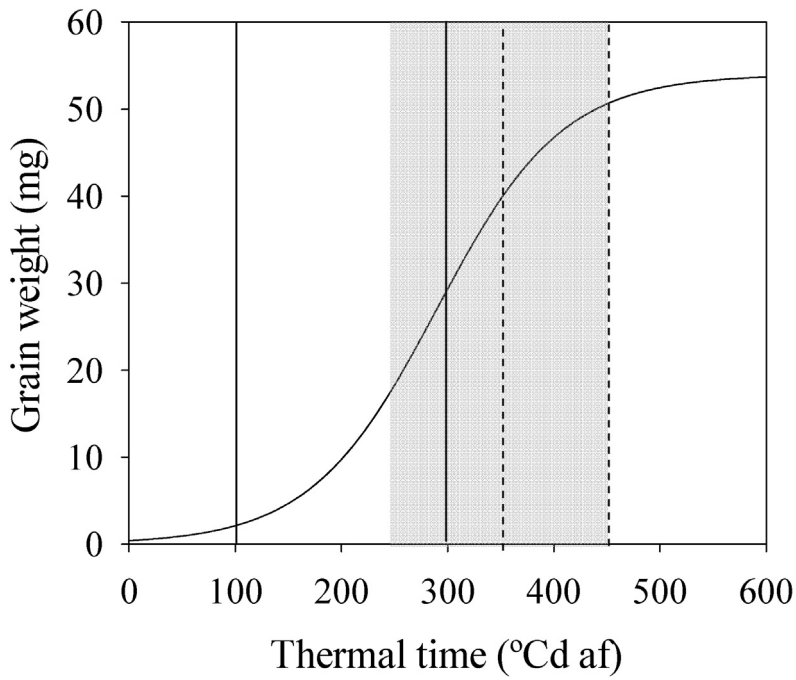

Fig. 6. Critical periods for temperature effect on fatty acid composition (in between solid vertical lines), ISR effect on fatty acid composition (in between dashed vertical lines) and ISR effect on grain weight and oil concentration (gray zone) superimposed on grain filling dynamics.

effect that is independent of the intensity of the radiation treatment applied (Pereyra-Irujo and Aguirrezábal, 2007).

Window-pane analysis allowed finding a critical period for radiation effect independent of the radiation level intercepted by the crop. This critical period was placed between 350 and 450 °Cd af. This kind of analysis has been successfully employed to assess key phases for the determination of many traits in several crop species (Aguirrezábal et al., 2003; Bertero and Ruiz, 2008; Kriss et al., 2010; Izquierdo et al., 2006). In the present work, r^2 value of the fitting of oleic acid percentage to ISR accumulated during the whole grain filling period was defined as the threshold to establish the critical period for radiation effect. The bootstrap analysis of the results (Fig. 4) confirmed the length and position of this critical period, and thus supported the threshold criteria employed.

In agreement with previous reports, grain weight and oil concentration rapidly increased from flowering (R6 + 150 °Cd af) until a few days before R9 when they stabilized (Fig. 5 this paper; Aguirrezábal et al., 2003). As has been previously reported, oleic acid percentage increased at early stages of grain filling up to a maximum and then it decreased up to R9, while linoleic acid concentration showed the opposite trend (Fig. 5; Martínez-Force et al., 1998; Santonoceto et al., 2003). Long term shading treatment immediately reduced grain weight and oil concentration, while it did not produce any effect on fatty acid composition before 300 °Cd af. The period during which the short term treatment $Sh_{80\%}$ C was imposed (295–437 °Cd af approx. for Exps. 1 and 2) almost coincided with the critical period for fatty acid response to ISR determined in this work but did not span the whole critical period for ISR effect on grain weight and oil concentration (Aguirrezábal et al., 2003). Thus, final oil fatty acid composition of plants shaded during the critical period was similar to that obtained with the long term treatment (compare $Sh_{80\%}$ A to $Sh_{80\%}$ C) but grain weight and oil concentration final values where higher than those obtained with the long term shading treatment. Taken together, these observations of grain filling dynamics empirically support the estimations of the critical periods for ISR effect on oil content, grain weight (Aguirrezábal et al., 2003) and oleic acid concentration, validating the method of analysis applied.

A conceptual model recently proposed states that ISR effect on fatty acid composition is mediated by the carbon assimilates availability for oil synthesis in sunflower grains (Echarte et al., 2012). Results obtained in the present work provide empirical evidence supporting this model. According to it, at high source levels, desaturation of oleic acid, a key step in fatty acids synthesis, becomes saturated and oleic acid accumulates with the consequent decrease of linoleic acid percentage. By contrast, at low assimilate allocation to the grains, oil synthesis is limited and most oleic acid can be converted into linoleic acid. Considering this conceptual model, results showed in Fig. 5 could be interpreted as follows: at very early stages of grain filling (before 150 °Cd af) most of the oil synthesis proceeds to linoleic acid. As grain filling progresses, oleate desturase activity increases (Gray and Kekwick, 1996), but carbon accumulates faster in the grain than the increment of oleate desaturase catalytic activity. Therefore, between 150 and 300 °Cd af, oleic acid begins to accumulate. Between 300 and 350 °Cd af, a high desaturation activity produces a decrease in oleic acid percentage with a concomitant increase in linoleic acid, being more evident when carbon is more scarce. Later on, during the critical period for ISR effect on fatty acid composition, desaturation activity is stabilized and oleic acid depends on carbon accumulation; low carbon levels can be mostly turned into linoleic acid while high carbon levels are accumulated as oleic acid (Echarte et al., 2012). To confirm this model, future experiments designed to measure the effect of the carbon flux from the maternal plant to the growing embryo on the fatty acid composition of the oil should be performed. This could be achieved, for instance, by in vivo labeling as shown by Alonso et al. (2007).

By using a similar experimental approach, Izquierdo et al. (2006) found that the critical period for temperature effect on fatty acid composition of sunflower oil was between 100 and 300 °Cd af. This critical period is different from the one for ISR effect on fatty acid composition (350–450 °Cd af) determined in the present work (Fig. 6). These results agree with previous reports showing that no interaction between the effects of these environmental factors

exists (Echarte et al., 2012; Zuil et al., 2012). Results reported in the present work supports the proposal that the mechanisms underlying temperature and radiation effects on fatty acid composition are independent (Echarte et al., 2012).

Temperature affects total activity of oleate desaturase, the enzyme that catalyzes the conversion of oleic to linoleic acid. Total activity of this enzyme is greatest at early grain filling (Garcés and Mancha, 1991; Gray and Kekwick, 1996; Kabbaj et al., 1996). Considering that the effect of temperature on fatty acid composition is more important during early stages of grain filling, when oil accumulated per grain is low, Izquierdo et al. (2002) suggested a "memory effect" of an early temperature treatment on the fatty acid desaturation mechanism. Results found in this work show that once oleate desaturase activity has been defined by temperature conditions (between 100 and 300 °Cd af; Izquierdo et al., 2006), fatty acid composition could still vary depending on the carbon allocated to the grains between 350 and 450 °Cd af (Figs. 5 and 6).

The critical period found here for the effect of radiation on oleic acid concentration was shorter and placed later in the grain filling than the one described for grain weight an oil concentration per seed (250–450 °Cd af; Aguirrezábal et al., 2003; Fig. 6). The varying degree of temporal correspondences between different traits and specific environmental factors are indicative of the complexity of the interaction between environmental conditions and growth dynamics. Crop models are useful tools for evaluating interactions between grain yield and oil quality, and may help to take management decisions (like sowing date) in order to avoid unfavorable conditions during the most critical stages (Pereyra-Irujo and Aguirrezábal, 2007). The critical period reported in the present work, should be considered in crop growth models (e.g. Pereyra-Irujo and Aguirrezábal, 2007) to explore yield and oil quality relationships.

In summary, we have demonstrated the existence of a critical period for the effect of intercepted radiation on oleic acid concentration. This critical period was different from the one described for temperature effect, and supports the idea that mechanisms underlying radiation and temperature effects are different. In order to predict fatty acid composition before crop harvest, it is necessary to know the minimum night temperature between 100 and 300 °Cd af and the photosynthetically active radiation intercepted by the crop during 350-450 °Cd af period as input variables in a crop growth model. In this sense, a model combining critical periods in an additive manner (as shown by Echarte et al., 2010) would improve oil fatty acid composition prediction and or simulation.

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