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DROUGHT STRESS

Changes in the Relationship Between Temperature During the Seed-Filling Period and Soya Bean Seed Isoflavones Under Water-Deficit Conditions

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Abstract

Isoflavones have been shown to have health-promoting activities in humans and are particularly abundant in soya bean. This study was conducted to determine how water deficit during seed fill affects the already known relationship between temperature and, alternately, solar radiation with soya bean seed isoflavone content. Isoflavone profile was analysed from seed samples of commercial cultivars grown in 76 environments in Argentina (29–38°S). Significant explanatory multiple linear regressions were detected for total isoflavones (TI), aglycones (AGL), glucosides (GLC), malonyl glucosides (MAL) and acetyl glucosides (ACE) regarding the following: temperature during seed fill (Tm_{R5R7}) and precipitation minus potential evapotranspiration during the reproductive period (pp-PET_{R1R7}), as well as for the combinations of these climatic variables. Cumulative solar radiation predicted isoflavone content but was less robust than Tm_{R5R7} and pp-PET_{R1R7}. To our knowledge, this is the first report of changes in the relationship between TI, as well as AGL, GLC, MAL, and ACE and Tm_{R5R7} as a function of drought in the field. When pp-PET_{R1R7} was below 70 mm (indicating drought), TI, as well as AGL, GLC, MAL, and ACE decreased linearly with rising temperatures and with increasing water deficit (decreasing values of pp-PET_{R1R7}), with both climatic variables exhibiting additive effects on isoflavones. Our results also suggest that water deficit (estimated by pp-PET_{R1R7}) would be important for modelling the relationship between temperature and soya bean seed isoflavones in rainfed crops.

Introduction

Soya beans [*Glycine max* (L.) Merr.] are unique among legumes because they are a concentrated source of isoflavones (Messina 1999). Isoflavones, a class of the more ubiquitous flavonoids, play key roles in many plant–microbe interactions and are associated with the health benefits of soya consumption (Yu and McGonigle 2005). In recent years, they have been studied for their potential role in the prevention and treatment of a number of chronic diseases, including certain forms of cancer, osteoporosis, and heart disease, as well as for their ability to relieve menopausal symptoms (Messina 1999). Twelve isoflavones from soya bean have been separated and identified and have been categorized according to their functional groups into four major subgroups: aglycones (daidzein, glycitein, genistein), glucosides (daidzin, glycitin, genistin), malonyl glucosides (malonyl daidzin, malonyl glycitin, malonyl genistin), and acetyl glucosides (acetyl daidzin, acetyl glycitin, acetyl genistin) (Lin and Lai 2006). Processed soya bean meal is typically composed of approximately 72 % MAL, 20 % GCL, 6 % ACE, and 2 % AGL (Wilson 2004).

Accumulation of isoflavones in soya bean seeds takes place 35–60 days after flowering (Medic et al. 2014); therefore, environmental factors during this period affect the final amount of isoflavones present in soya bean seeds. Temperature during seed development is one of the most important factors controlling seed isoflavone content. Temperate to cool environments promote the increase in seed isoflavone content (Carrera et al. 2011a), whereas high

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temperature during seed filling reduces isoflavone content (Tsukamoto et al. 1995, Caldwell et al. 2005, Lozovaya et al. 2005, Rasolohery et al. 2008, Chennupati et al. 2011). Soil moisture status during seed development is also an important environmental factor explaining final seed isoflavone content. Isoflavones were found to increase under irrigation treatment (Bennett et al. 2004, Rasolohery et al. 2008), and significant reductions were observed in seed isoflavone content under drought conditions (Caldwell et al. 2005, Lozovaya et al. 2005, Al-Tawaha et al. 2007, Gutierrez-Gonzalez et al. 2010). While effects of temperature during seed fill on final seed isoflavone content have been widely studied, little is known about how water deficits might affect the well-known association between soya bean seed isoflavone content and temperature. Two reports (Caldwell et al. 2005, Lozovaya et al. 2005) provided some supporting evidence of the combined effect of both environmental factors on soya bean isoflavone content. However, they may not provide a realistic approach as they were carried out in pots under controlled environments imposing the same temperature and soil moisture status treatment during the whole seed-filling period. The interaction between temperature and water deficit is a very important issue, as about 90 % of the world's soya bean production occurs under rainfed conditions, characterized by high temperatures and low or erratic rainfall (Thuzar et al. 2010). Given the current trend of rising temperatures, droughts, floods and extreme weather events (IPCC 2007), crop exposure to thermal and water stresses is expected to increase. Previous studies have not attempted to assess and quantify the effects of the interactions between temperature or solar radiation and water deficit during the soya bean seed fill period under field conditions. Accordingly, multi-environment field trials may be a useful tool to assess environmental effects on seed isoflavone content and composition because they may capture a wide range of climatic variability. They could also improve and provide ecophysiological insights to adjust and validate the management strategies in order to obtain high-quality products in terms of health-promoting activities for niche market demands. The aim of this study was to quantify changes in the relationship between temperature, and alternately, solar radiation with soya bean seed isoflavones under water deficit during the seed-filling period.

Materials and Methods

Soya bean seed samples and field experiments

A trial data set was generated from 76 multi-environment yield trials conducted during the 2001–2002 to 2003–2004 crop years by the INTA's National Soybean Network for Testing Cultivars across the Argentinean soya bean growing region. Experimental field trials were laid out as a randomized complete block design with three blocks in each environment, defined as crop season, location, and sowing date combinations. The environments used in this study are listed in Table 1. The included commercial cultivars were the following (maturity group is given between parentheses): DM 3100 RR (III), DM 3700 RR (III), DM 4400 RR (IV), DM 4600 RR (IV), DM 4800 RR (IV), A 5520 RG (V), A 6445 RG (VI), A 7636 RG (VII), and A 8000 RG (VIII). The main objective of this study was to explain the variation in isoflavone content affected by climatic variables instead of considering cultivar effects. Thus, the trial data set was constructed so as to capture the highest environmental variability possible rather than having all cultivars in all environments. Nonetheless, DM 4800 RR (one of the most frequently cropped cultivar across the Argentine soya bean region) was common to all environments. Environments were selected taking into account that the thermal ranges explored by short (III to V) and long (VI to VIII) maturity groups were similar and as wide as possible. Indeed, in the selected environments, mean daily temperature during seed filling was similar for both short and long maturity groups (21.8 ± 2.7 °C and 21.3 ± 2.3 °C, respectively). Chemical (nitrate nitrogen, phosphorus, and pH) and physical (percentage of organic matter, bulk density, and texture) analyses of soil samples from each site did not indicate any physical or nutritional constraint for crop development. Crops were grown under rainfed conditions, and following cultural practices recommended by INTA's National Soybean Network for testing cultivars. These practices include control of diseases, insects, and weeds to avoid yield reduction and/or seed chemical quality alterations. Plant density in all trials was approximately 35–40 plants m^{-2} at 0.52 m row spacing. Seeds were harvested and a 300-g grain sample from three replications of each cultivar at each location and on each sowing date was stored in walk-in refrigerators at 4 °C until analyses to avoid enzymatic degradation of the isoflavones. Temperature and solar radiation data were recorded daily for each crop season and location at INTA Meteorological Network Stations, up to 10 km away from each experimental site. Rainfall records were obtained from rain gauges placed close to each experimental plot site (less than 500 m away). Potential evapotranspiration was calculated using Penman (1948) equation. Dates of occurrence of R1 (beginning flowering), R5 (beginning seed), and R7 (physiological maturity) were assessed in the field in all trials using the scale of Fehr and Caviness (1977). By combining daily climatic data with the crop stages, we generated the following climatic variables for each environment: average daily mean air temperature during seed fill (Tm_{R5R7}, °C); cumulative solar radiation during seed fill (Sr_{R5R7},

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Table 1 Locations and sowing dates for 2001–2002 to 2003–2004 crop seasons, of 76 multi-environment field trials conducted across the soya bean Argentine crop area

Location	Coordinates (latitude, longitude)	Sowing date (day of the month)				
		Sep.	Oct.	Nov.	Dec.	Jan.
		2001/2002 crop season				
Paraná	31°44′S, 60°32′W	23	–	12	17	15
Manfredi	31°49′S, 63°46′W	–	12	27	–	–
Concepción del Uruguay	32°29′S, 58°14′W	–	–	22	7	2, 31
Marcos Juárez	32°41′S, 62°06′W	12	11	14	5	–
General Pico	35°40′S, 63°44′W	–	5	6	6	3
		2002/2003 crop season				
Reconquista	29°40′S, 59°12′W	26	23	–	12	9
Paraná	31°44′S, 60°32′W	23	10	12	26	13
Manfredi	31°49′S, 63°46′W	–	–	1, 27	27	–
Concepción del Uruguay	32°29′S, 58°14′W	23	23	22	26	–
Marcos Juárez	32°41′S, 62°06′W	23	2	5	1	5
Balcarce	37°52′S, 58°15′W	–	25	15	5, 23	–
Bellocq	38°20′S, 60°13′W	–	9, 30	13	–	–
		2003/2004 crop season				
Reconquista	29°40′S, 59°12′W	30	–	–	11	6
Rafaela	31°10′S, 61°28′W	29	–	14	19	–
Córdoba	31°25′S, 64°11′W	24	–	4	–	6
Manfredi	31°49′S, 63°46′W	–	–	–	1, 29	
Concepción del Uruguay	32°29′S, 58°14′W	29	15	14	15, 30	
Marcos Juárez	32°41′S, 62°06′W	–	–	21	–	7
General Pico	35°40′S, 63°44′W	–	14	–	3	8
Balcarce	37°52′S, 58°15′W	–	2	3	–	5
Barrow	38°19′S, 60°14′W	–	24	21	29	–
Bellocq	38°20′S, 60°13′W	–	14	19	23	–

MJ m^{-2}), calculated as the sum of daily solar radiation in that period; and precipitation minus potential evapotranspiration during the reproductive period (pp-PET_{R1R7}, mm). The variable pp-PET_{R1R7} was included because water balance from the whole reproductive period considers moisture storage in the soil profile, which could influence water availability during seed fill period (Carrera et al. 2009). The variable pp-PET was used as a simple indicator of water stress. The constructed climatic variables allowed us to summarize the environmental conditions, especially during the seed fill period, when dry mass accumulation of chemical components occurs (Wilson 2004).

Isoflavone profile analysis

Isoflavones were determined from previously defatted flour, according to the method developed by Murphy et al. (1999) and modified by Hubert et al. (2005); they are expressed as percentage of dry defatted flour (DDF, mg g^{-1}). A liquid chromatographer (Agilent 1100 high-performance resolution, Wilmington, DE, USA) equipped with a diode array detector (DAD) was used. UV absorbance was monitored at 254 nm. A ZORBAX Eclipse XDB-C18 column (4.6 × 250 mm, 5 μm) was used and maintained at 40 °C during the run. Isoflavones were separated with a mobile phase consisting of 1 % (v/v) glacial acetic acid in water and 1 % (v/v) glacial acetic acid in acetonitrile; all solvents were of HPLC purity. Injection volume was 5 μl, and flow rate was 1 ml min^{-1}. Calibration curves were determined using the following commercial standards: glycitin, acetyl daidzin, malonyl genistin, daidzein, acetyl genistin, and genistein, which were purchased from LC Laboratories (Woburn, MA, USA). Response factors of the isoflavones daidzin, malonyl daidzin, and genistin were calculated from their corresponding AGL, whereas response factors of the isoflavones glycitein, acetyl glycitin, and malonyl glycitin were calculated from glycitin and were corrected in a molecular mass ratio. Total isoflavones (TI) were calculated as the sum of the 12 forms (daidzein, glycitein, genistein, daidzin, glycitin, genistin, malonyl daidzin, malonyl glycitin, malonyl genistin, acetyl daidzin, acetyl glycitin, acetyl genistin). The variables defined as AGL, GLC, MAL, and ACE were calculated by summing the contents of their respective isomers, as follows: AGL = daidzein + glycitein + genistein, GLC = daidzin + glycitin + genistin, MAL = malonyl daidzin + malonyl glycitin + malonyl genistin, and ACE = acetyl daidzin + acetyl glycitin + acetyl genistin. All determinations

were made at the Grain Quality Laboratory, located at INTA Agricultural Experimental Station in Manfredi, Córdoba, Argentina.

Statistical analyses

A multivariate statistical analysis was performed using principal component analysis (PCA) (Johnson and Wichern 2002) to explore associations between isoflavone profile data (from nine soya bean cultivars analysed in three consecutive seasons) and the set of climatic variables characterizing the 76 multi-environment field trials. A multiple linear regression procedure was performed to model the four major isoflavone subgroups and total isoflavones (AGL, GLC, MAL, ACE, TI) as functions of Tm_{R5R7}, Sr_{R5R7}, and pp-PET_{R1R7}, as well as combinations of those climatic variables showing low correlation among them (Tm_{R5R7} with pp-PET_{R1R7}; Sr_{R5R7} with pp-PET_{R1R7}). A stepwise variable selection procedure was run to identify significant variables at 5 % significance level (used for the *t*-test on regression parameter estimates). Model selection was based on Mallows' Cp coefficients and residual analyses. All calculations were made with the statistical software Infostat (Di Rienzo et al. 2009).

Results and Discussion

A great variability of the environmental variables was observed across the soya bean region involved in this study (Table 2). Differences between maximum and minimum values were 89 % for Tm_{R5R7}, 661 % for Sr_{R5R7}, and 790 mm for pp-PET_{R1R7}. The contrasting environmental conditions to which soya bean plants were exposed during seed fill were reflected in the wide range of variation observed for all individual isoflavone isomers. Indeed, TI extreme values differed by 955 %, with MAL and ACE accounting for most of this variation (Table 3). The mean values of TI (3.42 mg g^{-1} DDF) obtained in the present study are consistent with those previously reported for non-transgenic genotypes evaluated in an area agro-ecologically similar to that involved in this work (Carrera et al. 2014a). The values reported for commercial cultivars as well as non-transgenic genotypes (3.85 mg g^{-1} DDF) across the Argentine soya bean crop area (Carrera et al. 2014a and the present study) are above the average value of 2.37 mg g^{-1} DDF observed for 233 Brazilian soya bean cultivars by Carrão-Panizzi et al. (2009). Our values were also higher than those reported in multi-environment trials testing several cultivars in Canada by Morrison et al. (2008) and Murphy et al. (2009), who observed 2.31 and 2.54 mg g^{-1} DDF, respectively; and greater than 1.82 mg g^{-1} DDF reported for American soya beans (Hoeck et al. 2000). However, Lee et al. (2003) reported average isoflavone content of 4.61 mg g^{-1} DDF for several Japanese genotypes grown at three sites during 3 years, which exceeds the values observed in other countries of the world, including Argentina.

The biplot obtained from the PCA explained 68.5 % of the variation when the first two axes were considered (Fig. 1). The cosine of the angle between two variable vectors approximates the correlation between the variables, with acute and obtuse angles indicating a positive and negative correlation, respectively. All the isoflavone isomers were positively correlated among them and with TI and responded similarly to the environmental conditions during the seed fill period (Fig. 1). One of the strongest

Table 3 Descriptive statistics for seed isoflavone composition (mg g^{-1} dry defatted flour) of soya bean grains evaluated in 76 environments from the Argentine crop region

Variable	Average	S.D.	Minimum	Maximum
Daidzein	0.0480	0.03	0.0030	0.1820
Glycitein	0.0075	0.01	0.0000	0.0220
Genistein	0.0319	0.02	0.0000	0.1149
Daidzin	0.6037	0.17	0.0990	1.1147
Glycitin	0.0975	0.03	0.0110	0.2556
Genistin	0.4219	0.24	0.0300	1.1142
Malonyl daidzin	0.8832	0.61	0.0700	3.0620
Malonyl glycitin	0.3920	0.13	0.0570	0.6700
Malonyl genistin	0.7636	0.40	0.0400	1.8127
Acetyl daidzin	0.0098	0.02	0.0000	0.0819
Acetyl glycitin	0.1467	0.08	0.0100	0.4249
Acetyl genistin	0.0170	0.01	0.0000	0.1700
Aglycones	0.087	0.06	0.000	0.305
Glucosides	1.123	0.40	0.284	2.364
Malonyl glucosides	2.039	1.03	0.260	5.194
Acetyl glucosides	0.174	0.10	0.017	0.513
Total Isoflavones	3.42	1.50	0.76	8.02

Table 2 Environmental conditions of the 76 multi-environment field trials evaluated in the Argentine crop region during 2001–2002 to 2003–2004 crop seasons

Climatic variable	Average	S.D.	Minimum	Maximum
Average daily mean air temperature during seed fill (°C)	21.6	2.6	14.1	26.7
Cumulative solar radiation during seed fill (MJ m^{-2})	697.0	277.6	220.3	1676.7
Precipitation minus potential evapotranspiration during the reproductive period (mm)	21.4	165.3	−373.0	417.0

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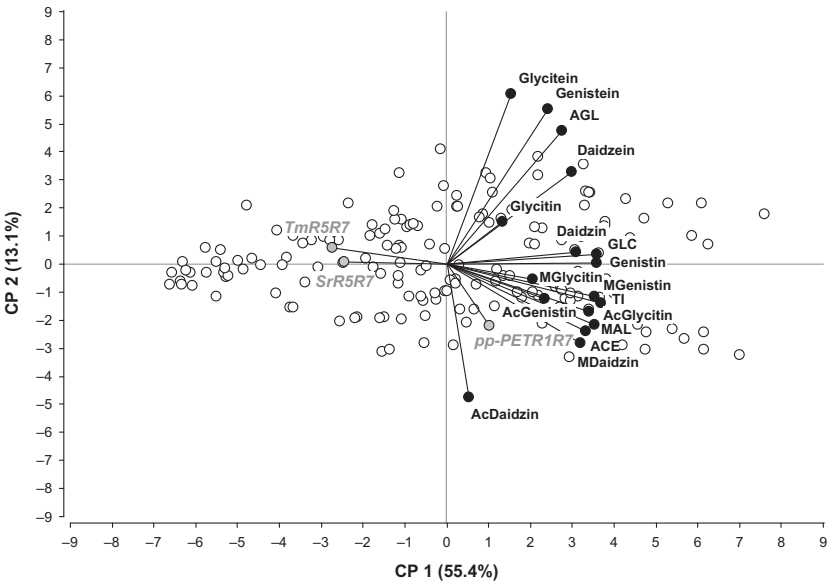

Fig. 1 Biplot of principal components 1 and 2 (PC1 and PC2) of principal components analysis (PCA) showing relations between different isoflavones (black circles) of 181 soya bean seed samples (open circles) and environmental variables (grey circles). Isoflavones (expressed as mg g^{-1} of dry defatted flour) codes: aglycones (AGL), glucosides (GLC), malonyl glucosides (MAL), acetyl glucosides (ACE), total isoflavones (TI). TI = AGL + GLC + MAL + ACE; AGL = daidzein + glycitein + genistein; GLC = daidzin + glycitin + genistin; MAL = malonyl daidzin + malonyl glycitin + malonyl genistin; ACE = acetyl daidzin + acetyl glycitin + acetyl genistin. Codes for environmental variables: average daily mean temperature during seed fill (Tm_{R5R7}), °C; mean solar radiation during seed fill (Sr_{R5R7}), MJ m^{-2}; and precipitation minus potential evapotranspiration during the whole reproductive period (pp-PET_{R1R7}), mm.

relationships between isoflavone isomers and climatic variables, as revealed in the plane determined by the two principal components of the biplot, was the negative correlation of three of the four major isoflavone subgroups (MAL, GLC, ACE) and TI to Tm_{R5R7} and Sr_{R5R7}. Another relationship observed, although less robust, was a positive association with pp-PET_{R1R7}. These associations between both sets of variables were reflected in the fitted regression model considering the whole data set ($N = 178$), where TI = 11.77 − 0.39 Tm_{R5R7} + 0.001 pp-PET_{R1R7} (adjusted $R^2 = 0.52$; $p_{TmR5R7} < 0.0001$, $p_{pp\text{-}PETR1R7} = 0.0404$). In the model including both Sr_{R5R7} and pp-PET_{R1R7}, no significant linear contribution from the latter variable was found (data not shown). Sr_{R5R7} was a significant predictor for TI (adjusted $R^2 = 0.37$; $p_{SrR5R7} < 0.0001$), but the fitted model was not as good as the model using Tm_{R5R7} and pp-PET_{R1R7}. Thus, thereafter the multiple regression including Tm_{R5R7} and pp-PET_{R1R7} as predictors was considered. Notably, although pp-PET_{R1R7} was a significant predictor of TI, the Mallows′ Cp indicated that Tm_{R5R7} effect was 27 times stronger than pp-PET_{R1R7}. This result is in agreement with findings reported by Rasolohery et al. (2008), who concluded that soil water supply had less effect than temperature on isoflavone content. When the whole data set was examined, cases from environments with and without water-stress conditions were mixed. In fact, the partial residues of this multiple regression indicated a threshold value of 70 mm for pp-PET_{R1R7}, below and above which the explanatory models elucidating TI behaviour changed. This threshold value is consistent with the one previously reported for oil, protein, and oil + protein using the same database (Carrera et al. 2009). TI content was related significantly ($P < 0.001$) only to Tm_{R5R7}, in environments without water stress (pp-PET_{R1R7} > 70 mm; range 70–417 mm) (Fig. 2). In such environments, the fitted regression indicated that the response of TI content to Tm_{R5R7} was linear and negative, showing a high value of adjusted determination coefficient (adjusted $R^2 = 0.69$) with a slope of −0.41 mg TI g^{-1} DDF $°C^{-1}$ (Fig. 2). The same tendency was observed for each of the four isoflavone subgroups considered separately, that is decreasing contents of AGL, GLC, MAL, and ACE with increasing values of temperature during seed filling (Fig. 3). The adjusted R^2 values for these equations were also high (0.54 for AGL, 0.60 for GLC, 0.76 for MAL, and 0.43 for ACE). Our results disagree with those reported by Morrison et al. (2010), who evaluated 14 cultivars across 12 years at one location and found that daily temperature was not sufficiently robust to be used as predictor of change in isoflavones concentration. Moreover, these authors did not find that high mean temperature during seed development resulted in a lower isoflavone concentration. Past studies demonstrated

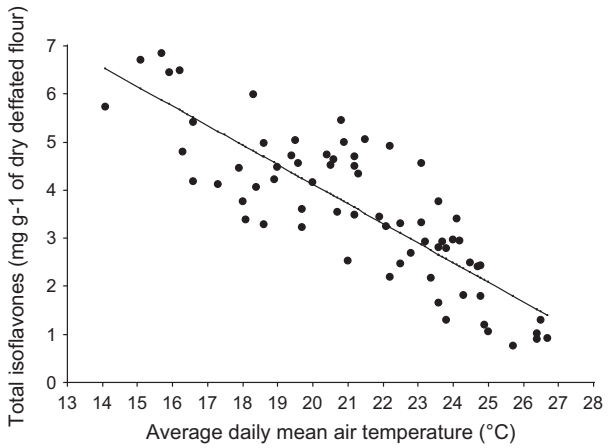

Fig. 2 Response of total isoflavones (TI) content (mg g^{-1} of dry defatted flour) in soya bean meal to average daily mean air temperature during seed fill (°C) ($Y = 12.26 - 0.41\ X$; adjusted $R^2 = 0.69$; $P < 0.0001$). The line represents fitted linear model for samples from environments (crop season, location and sowing date combinations) without water stress conditions (pp-PET_{R1R7} > 70 mm).

that, among the main environmental effects, not only year but also site was an important source of variation for isoflavones (Wang and Murphy 1994, Hoeck et al. 2000, Lee et al. 2003). The lack of relationship between isoflavone and mean temperature during seed fill observed by Morrison et al. (2010) may be attributed to the fact that those authors analysed results from soya beans grown in only one location. Thus, probably exploring a narrower range of temperature variation during the period is of interest, than the one generated in multi-environment trials involving several contrasting sites, as in the present study.

It is well known that isoflavone content is very sensitive to temperature during seed development. We found 91 % decrease in TI with rising temperatures from 14.1 to 26.7 °C in environments without water stress (pp-PET_{R1R7} > 70 mm; range 70–417 mm). Accordingly, Tsukamoto et al. (1995) observed 94 % reduction in TI content in plants grown at 33 °C with respect to plants grown at 17.5 °C during the whole seed development period in controlled environment chambers. Similarly, an

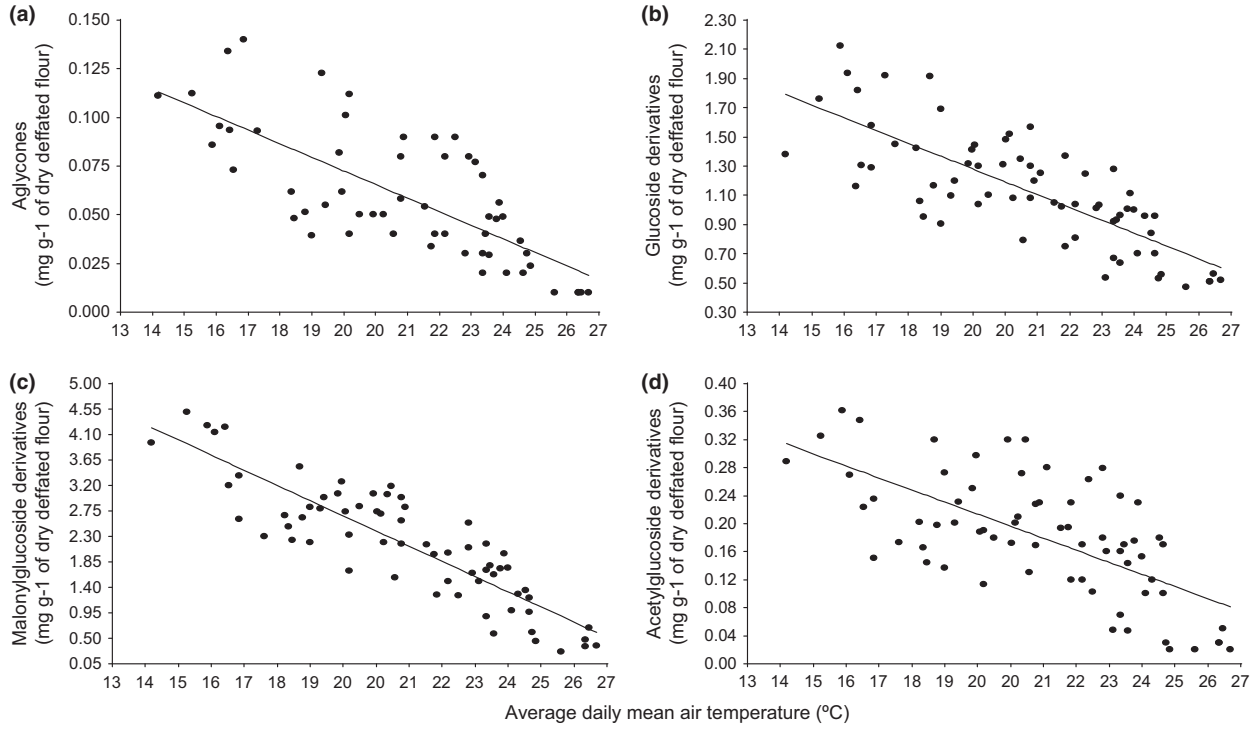

Fig. 3 Response of content (mg g^{-1} of dry defatted flour) of (a) aglycones (sum of daidzein + glycitein + genistein) (b) glucoside derivatives (sum of daidzin + glycitin + genistin); (c) malonylglucoside derivatives (sum of malonyldaidzin + malonylglycitin + malonylgenistin); (d) acetylglucoside derivatives (sum of acetyldaidzin + acetylglycitin + acetylgenistin) in soya bean meal to average daily mean air temperature during seed fill (°C). The line represents fitted linear model for samples from environments (crop season, location and sowing date combinations) without water-stress conditions (pp-PET_{R1R7} > 70 mm). Computed models were as follows: (a) $Y = 0.22 - 0.01\ X$; adjusted $R^2 = 0.54$; $P < 0.0001$; (b) $Y = 3.12 - 0.09\ X$; adjusted $R^2 = 0.60$; $P < 0.0001$; (c) $Y = 8.27 - 0.29\ X$; adjusted $R^2 = 0.76$; $P < 0.0001$; (d) $Y = 0.57 - 0.02\ X$; adjusted $R^2 = 0.43$; $P < 0.0001$.

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87 % reduction in TI under controlled conditions was reported within the 18–28 °C range during R5 to R7 (Caldwell et al. 2005). In both reports, the authors observed a decrease in all individual isoflavones, rather than changes restricted to a particular isomer. Accordingly, we found that the four subgroups of isoflavones contribute to the observed decline in TI with increasing temperatures, with a decrease of 96 % in AGL, 80 % in GLC, 95 % in MAL, and 96 % in ACE. The decrease in isoflavones with rising temperatures might be due to the effect of high temperatures on the expression of phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), and chalcone reductase (CHR). These are key metabolic entry points in the phenylpropanoid pathway for the formation of all isoflavonoids and are known to reduce their activity under elevated temperature conditions (Mori et al. 2005, Chennupati et al. 2012).

Several studies reported a negative relationship between TI and mean temperature during the seed fill period (Tsukamoto et al. 1995, Caldwell et al. 2005, Lozovaya et al. 2005, Rasolohery et al. 2008, Chennupati et al. 2011). However, no previous reports quantified the rate of isoflavone decrease as a function of mean temperature increments in seeds from environments with non-limiting water availability during the filling period. Nonetheless, we analysed the data from experiments conducted in glasshouses exploring temperatures within the range of the one in our study (14.1 and 26.7 °C) during the seed fill period to estimate the rate of isoflavone change to temperature. The calculated rates for Lozovaya et al. (2005) and Rasolohery et al. (2008) indicated 0.28 and 0.31 mg of TI g^{-1}DDF decrease per degree increase in temperature, respectively, within the 18–23 °C range. We infer that the lower rates obtained by those authors than the one obtained in our study (0.41 mg of TI g^{-1}DDF $°C^{-1}$) could be attributed in part to the shorter period of exposure of developing seeds to contrasting temperatures (R6 to R7 vs. R5 to R7 in our study). However, a primary reason behind the different values obtained is that the most active phase of isoflavone accumulation takes place during the seed growth period, between R5 and R6 (Kumar et al. 2009). Indeed, earlier rather than later (R6–R7) onset of high temperature treatments could have a greater impact on final seed isoflavone content.

Regression equations for TI content, as well as AGL, GLC, MAL, and ACE content, of samples collected from environments under water-stress conditions (pp-PET_{R1R7} < 70 mm; range −373–70 mm) showed a linearly significant contribution of both Tm_{R5R7} and pp-PET_{R1R7} (P-value, Table 4). Adjusted determination coefficients of these regressions were also high (adjusted R^2 = 0.54, 0.39, 0.46, 0.62, and 0.45 for TI, AGL, GLC, MAL, and ACE, respectively). Moreover, under water-deficit conditions, the relative contribution of pp-PET_{R1R7} in the fitted regression models increased, being approximately a third to a half of the Tm_{R5R7} magnitude, as shown by the Mallows' Cp (Table 4). These results indicate that the well-known relationship between isoflavones and temperature is influenced by water deficit during the seed-filling period. Notably, the response of all isoflavone isomers to Tm_{R5R7} and pp-PET_{R1R7} had the same direction, indicating that the effect of both climatic variables was additive. In drought environments, TI content, as well as AGL, GLC, MAL, and ACE, had a linear negative association with Tm_{R5R7}, reducing isoflavones across rising temperatures, and a linear positive association with pp-PET_{R1R7}, suggesting a decrease in isoflavones with increasing water deficit (decreasing values of pp-PET_{R1R7}) (Table 4). In field situations, elevated temperatures frequently occur under water-stress conditions (Rose 1988), and this combination results in warmer temperatures within transpiration-limited canopies in drought-stricken environments than the air temperatures from meteorological stations (Carrera et al. 2009). As a consequence of these higher temperatures sensed by the crop under water-deficit conditions, the additive effect exhibited by Tm_{R5R7} and pp-PET_{R1R7} on isoflavone variability may be expected.

Several studies have been conducted to understand the effect of water deficit on soya bean seed isoflavone content. In field experiments performed in mid-south USA (characterized by drought and high temperatures during the seed fill period), Bennett et al. (2004) indicated that irrigation enhanced isoflavone content by 92 %. Likewise, Rasolohery et al. (2008) observed a 41 % increase in final TI content with application of 30 mm of irrigation at the end of seed maturation. In glasshouse, Lozovaya et al. (2005) found 14 % of TI content reduction in plants grown at 21 % of soil water holding capacity with respect to plants maintained at 70 % of soil water holding capacity, during the R6-R7 period. Gutierrez-Gonzalez et al. (2010) observed that long-term progressive drought (spanning the whole seed developmental stage) in controlled growth chambers significantly decreased isoflavone content. Noticeable, in an experiment with two levels of irrigation (50 % and 100 % of soil water holding capacity, low and high irrigation levels, respectively), Al-Tawaha et al. (2007) observed that the low irrigation level increased TI content by 45 % compared with a non-irrigated control, whereas high irrigation level caused a 16 % reduction compared with low irrigation level. Although the authors suggest a nonlinear response of TI content to water soil availability, their approach may not be rational as 100 % of soil water holding capacity during most of the crop growing cycle might be not representative of field situations. The only study that contradicts what is known about TI response to water deficit is that of Caldwell et al. (2005), who evaluated the effects of temperature and drought on TI content in con-

Table 4 Regression coefficients for the relationship between soya bean seed content (mg g^{-1} of dry defatted flour) of total isoflavones (TI), aglycones (AGL), glucosides (GLC), malonyl glucosides (MAL), acetyl glucosides (ACE) and environmental variables1, considering the environments (crop season, location and sowing date combinations) under water-stress conditions (pp-PET_{R1R7} < 70 mm)

Dependent variable2	Explanatory variable	Regression coefficient	Standard error	P value	Mallows CP	Adjusted R2
TI	Const	11.9793	0.9823	<0.0001		0.54
	Tm_{R5R7} (Linear)	−0.3788	0.0450	<0.0001	72.08	
	pp-PET_{R1R7} (Linear)	0.0047	0.0010	<0.0001	24.71	
AGL	Const	0.3162	0.0384	<0.0001		0.39
	Tm_{R5R7} (Linear)	−0.0104	0.0018	<0.0001	36.66	
	pp-PET_{R1R7} (Linear)	0.0001	3.6E-05	0.0018	12.26	
GLC	Const	3.3893	0.2924	<0.0001		0.46
	Tm_{R5R7} (Linear)	−0.0998	0.0134	<0.0001	56.84	
	pp-PET_{R1R7} (Linear)	0.0011	0.0003	0.0004	15.54	
MAL	Const	7.3153	0.5771	<0.0001		0.62
	Tm_{R5R7} (Linear)	−0.2384	0.0264	<0.0001	82.50	
	pp-PET_{R1R7} (Linear)	0.0035	0.0006	<0.0001	37.55	
ACE	Const	0.5746	0.0706	<0.0001		0.45
	Tm_{R5R7} (Linear)	−0.0173	0.0032	<0.0001	30.18	
	pp-PET_{R1R7} (Linear)	0.0004	0.0001	<0.0001	36.23	

[1]Environmental variables taken into account for fitting regression models were average daily mean temperature during seed fill (Tm_{R5R7}), °C; and precipitation minus potential evapotranspiration during the whole reproductive period (pp-PET_{R1R7}), mm.
[2]TI = AGL + GLC + MAL + ACE; AGL = daidzein + glycitein + genistein; GLC = daidzin + glycitin + genistin; MAL = malonyl daidzin + malonyl glycitin + malonyl genistin; ACE = acetyl daidzin + acetyl glycitin + acetyl genistin.

trolled environments chambers during seed development and observed 32 % more isoflavones in water-stressed plants than in non-stressed ones at 23 °C. Of the mentioned studies, only those of Caldwell et al. (2005) and Lozovaya et al. (2005) provided some supporting evidence of the combined effect of temperature and soil moisture deficit on soya bean isoflavone content. However, as they were carried out in pots under controlled environments where the same temperature and soil moisture status treatment was imposed during the whole seed-filling period, their approaches may not be consistent with field environmental conditions. To our knowledge, the present report is the first one conducted under field conditions that demonstrates that water deficit during the seed fill period modifies the relationship between temperature and soya bean seed isoflavones.

Little is known about the mechanism underlying a decrease in soya bean seed isoflavone content under water deficit during the seed development period (Gutierrez-Gonzalez et al. 2010). Recent advances in this area include quantification of the transcripts of the most important genes for isoflavone biosynthesis in soya bean under soil moisture stress during seed filling. Indeed, Gutierrez-Gonzalez et al. (2010) found that of the two isoflavone synthase genes in soya bean (IFS1 and IFS2), IFS2 (which controls the first reaction in the pathway branch of isoflavone synthesis) was downregulated and highly correlated with isoflavone accumulation under water stress, suggesting that this gene is a main contributor to isoflavone decrease under drought. A strong induced expression of IFS2 in response to water-stress conditions during soya bean seed filling was also previously confirmed (Dhaubhadel et al. 2003). Gutierrez-Gonzalez et al. (2010) also detected that other key genes involved in isoflavone synthesis (PAL and CHS) increased their expression during severe water-stress treatment. These authors hypothesized that the phenylpropanoid pathway would direct their intermediate metabolites towards other stress-induced compounds, such as lignans, lignins, flavones, flavonols, tannins, and anthocyanins, which share this common extended metabolic route. Nevertheless, the flux of the substrate towards each pathway has not been measured (Yu and McGonigle 2005). Another important topic that needs further investigation is the quantification of the amount of TI in seed derived from translocation of maternal tissues, as it was found to be a source that substantially contributes to total seed isoflavone content (Dhaubhadel et al. 2003). These are important avenues for gaining insights into what extent the decrease in isoflavone content in response to water deficit could be the result of a decreased biosynthesis in the seeds and/or decreased isoflavones transportation from other plant tissues.

As environments were partially balanced regarding cultivars and to strengthen the results presented in Figures 2 and 3 and Table 4, we repeated the analysis considering only DM 4800 RR (N = 76) as a check cultivar, because it was common to all the environments involved in this study. The response to increasing temperature of TI content, as well as the content of the four subgroups of isoflavone isomers, is different under non-limiting water availability (Figs 4 & 5) or water-stress conditions

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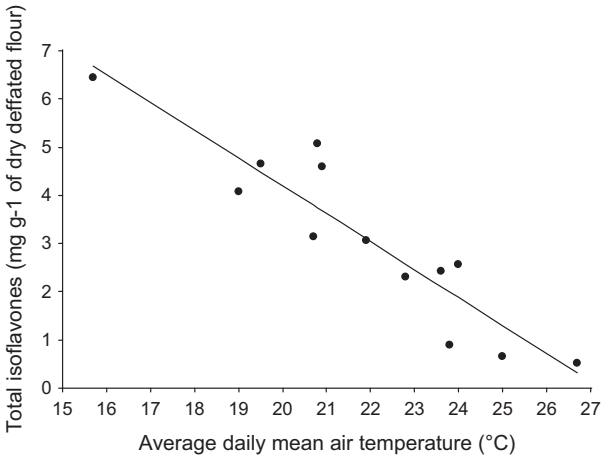

Fig. 4 Response of total isoflavone (TI) content (mg g^{-1} of dry defatted flour) in soya bean meal to average daily mean air temperature during seed fill (°C) (Y = 16.12 − 0.58 X; adjusted R^2 = 0.84; $P < 0.0001$). The line represents fitted linear model for one soya bean cultivar (DM 4800 RR) evaluated in all environments (crop season, location and sowing date combinations) without water-stress conditions (pp-PET_{R1R7} > 70 mm).

(Table 5). Figure 4 shows that under non-limiting water availability conditions, Tm_{R5R7} is still a robust predictor, being linearly and negatively related to TI content for this check cultivar (adjusted R^2 = 0.84; $P < 0.0001$). Indeed, this behaviour was supported by the four subgroups of isoflavone isomers, which also were strongly and negatively associated with Tm_{R5R7} (adjusted R^2 = 0.86, 0.83, 0.85, and 0.76 for AGL, GLC, MAL, and ACE, respectively; with a $P < 0.0001$ for all of them). On the other hand, regression equations for TI content from samples collected from environments under water-stress conditions (pp-PET_{R1R7} < 70 mm) showed linear, negative, and significant contribution of both Tm_{R5R7} and pp-PET_{R1R7} (adjusted R^2 = 0.61, Table 5). This behaviour was supported by two of the four subgroups of isoflavone isomers (GLC and MAL), which represent the predominant forms, and make up approximately 92.4 % of the TI. Both GLC and MAL were also strongly and negatively associated with Tm_{R5R7} and pp-PET_{R1R7} (adjusted R^2 = 0. 49, and 0.66 for GLC and MAL, respectively, Table 5). For the subgroups AGL and ACE, which together represent less than 8 % of the TI,

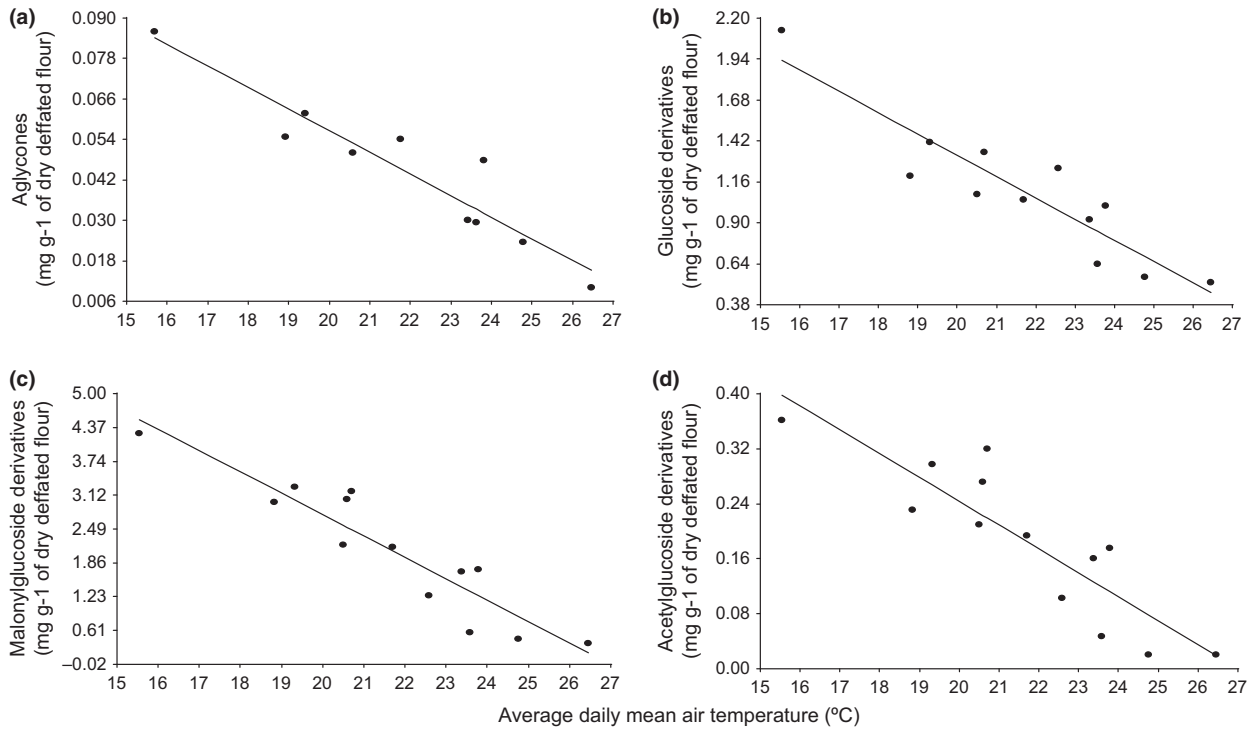

Fig. 5 Response of content (mg g^{-1} of dry defatted flour) of (a) aglycones (sum of daidzein + glycitein + genistein) (b) glucoside derivatives (sum of daidzin + glycitin + genistin); (c) malonylglucoside derivatives (sum of malonyldaidzin + malonylglycitin + malonylgenistin); (d) acetylglucoside derivatives (sum of acetyldaidzin + acetylglycitin + acetylgenistin) in soya bean meal to average daily mean air temperature during seed fill (°C). The line represents fitted linear model for one soya bean cultivar (DM 4800 RR) evaluated in all environments (crop season, location and sowing date combinations) without water-stress conditions (pp-PET_{R1R7} > 70 mm). Computed models were as follows: (A) Y = 0.183 − 0.01 X; adjusted R^2 = 0.86; $P < 0.0001$; (b) Y = 4.03 − 0.13 X; adjusted R^2 = 0.83; $P < 0.0001$; (c) Y = 10.69 − 0.39 X; adjusted R^2 = 0.85; $P < 0.0001$; (d) Y = 0.94 − 0.03 X; adjusted R^2 = 0.76; $P < 0.0001$.

Table 5 Regression coefficients for soya bean seed content (mg g^{-1} of dry defatted flour) of total isoflavones (TI), aglycones (AGL), glucosides (GLC), malonyl glucosides (MAL), acetyl glucosides (ACE) and environmental variables1, considering one soya bean cultivar (DM 4800 RR) evaluated in all environments (crop season, location and sowing date combinations) under water-stress conditions (pp-PET $_{R1R7}$ < 70 mm, N = 63)

Dependent variable2	Explanatory variable	Regression coefficient	Standard error	P value	Adjusted R2
TI	Const	12.6317	1.2301	<0.0001	0.61
	Tm_{R5R7} (Linear)	−0.4206	0.0573	<0.0001	
	pp-PET_{R1R7} (Linear)	0.0037	0.0015	0.0163	
AGL	Const	0.4291	0.0628	<0.0001	0.39
	Tm_{R5R7} (Linear)	−0.0154	0.0029	<0.0001	
	pp-PET_{R1R7} (Linear)	0.0001	0.0001	0.4797	
GLC	Const	3.4677	0.4162	<0.0001	0.49
	Tm_{R5R7} (Linear)	−0.1011	0.0195	<0.0001	
	pp-PET_{R1R7} (Linear)	0.0012	0.0005	0.0192	
MAL	Const	7.6572	0.7123	<0.0001	0.66
	Tm_{R5R7} (Linear)	−0.2610	0.0330	<0.0001	
	pp-PET_{R1R7} (Linear)	0.0024	0.0009	0.0094	
ACE	Const	0.5613	0.0858	<0.0001	0.36
	Tm_{R5R7} (Linear)	−0.0184	0.0040	<0.0001	
	pp-PET_{R1R7} (Linear)	0.0002	0.0001	0.1042	

[1]Environmental variables taken into account for fitting regression models were average daily mean temperature during seed fill (Tm_{R5R7}), °C; and precipitation minus potential evapotranspiration during the whole reproductive period (pp-PET_{R1R7}), mm.
[2]TI = AGL + GLC + MAL + ACE; AGL = daidzein + glycitein + genistein; GLC = daidzin + glycitin + genistin; MAL = malonyl daidzin + malonyl glycitin + malonyl genistin; ACE = acetyl daidzin + acetyl glycitin + acetyl genistin.

no significant linear contribution of pp-PET_{R1R7} was found (Table 5). AGL and ACE were present in very small proportions (2.5 % and 5.1 % of the TI, respectively) and exhibit a greater dispersal of the data (Table 3). Thus, we consider that the sample size for water-stress conditions using only DM 4800 RR (N = 63) may not have sufficient statistical power to capture the tendency compared with working with the whole database for samples obtained from environments under water-stress conditions (N = 108). On the basis of the similar regression models found from the check cultivar DM 4800 RR and from the whole database (N = 181), we inferred that the underlying factors contributing to the observed large isoflavones variations were due to environment rather than genotypic effects, which is common in multi-environment trials (Kang 2002). Overall, these results are in agreement with previously reported findings, in which environmental effects accounted for most of the variations in soya bean isoflavone variability (Poysa and Woodrow 2002, Lee et al. 2003, Carrera et al. 2014b). Even when the relationships between isoflavone content and climatic variables were similar for the reduced data set and for the whole database, using the latter allows us not only to explore and capture a wider range of climatic variability but also to gain power by increasing the sample sizes.

From the exploration and in-depth analysis of the associations between TI content and the set of climatic variables, we obtained fitted regressions models that explained a large proportion of the variation of this health-enhancing chemical compound. Moreover, TI variations were successfully explained in both types of environments, with and without water-stress conditions, during the seed fill period, both working with the whole database and with the reduced data set (just DM 4800 RR cultivar). Our results also demonstrate for the first time, that the overall response of TI to climatic factors in non-water-stress environments and those with water deficit was supported by the response of each subgroup of isoflavone isomers (AGL, GLC, MAL, ACE), which exhibited similar behaviour patterns to TI response in such environments. The analogous trend observed in the response of all isomers (which sum to the total isoflavone content) is not an obvious tendency, mainly for soya bean meal components, as shown by Carrera et al. (2011b) for all the amino acids (which sum to the total protein). Indeed, the authors concluded that each aminoacid behaved differently according to the environmental conditions, indicating compensatory effects among them.

In conclusion, we show here for the first time that the relationship between TI, as well as AGL, GLC, MAL, and ACE and Tm_{R5R7}, changed as function of water-deficit conditions. When pp-PET_{R1R7} was below 70 mm (environments under drought conditions) TI, as well as AGL, GLC, MAL, and ACE contents decreased linearly with rising temperatures and with increasing water deficit (decreasing values of pp-PET_{R1R7}), with both climatic variables exhibiting additive effects on isoflavones. This report also suggests that water deficit (estimated by pp-PET_{R1R7}) would be important for modelling the relationship between temperature and soya bean seed isoflavones in rainfed crops. The

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improvement of the predictive power of the models would provide useful information for implementing the adoption of management practices aimed at obtaining a specific nutraceutical quality to meet the demands of niche markets.

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