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Effects of Light Orientation and Mechanical Damage to Leaves on Isoflavone Accumulation in Soybean Seeds

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Abstract: Soybean is largely cultivated worldwide providing high amounts of proteins and oil for food and feed, and isoflavones for nutraceutical uses. The increasing interest in agroforestry practices for improving carbon sequestration and mitigating climate changes suggests the need to assess soybean response to variations of light availability and direction. A two-year pot trial was carried out at Legnaro (NE Italy) in order to mimic the response of the soybean var. Sponsor to contrasting light orientation (east or west) by artificial shading, associated or not with mechanical leaf damage, in terms of protein accumulation, total cotyledon isoflavone concentration (TCIC) and isoflavone profile. Here, we demonstrate that a different intensity of the isoflavone metabolism exists in response to lighting conditions, with higher TCIC and slightly increased seed crude proteins in plants lighted from the east (morning time) and after mechanical leaf damage. The isoflavone profile was not changed, but low temperatures and high rainfall during seed filling (1st year) were associated with increased accumulation of medium-high molecular weight (MW) forms (i.e., glycosyls and malonyls), while high temperatures and low rainfall (2nd year) with increased accumulation of medium-low MW forms (i.e., glycosyls and aglycones). It is concluded that within agroforestry systems, there is possibly a large scope for maximizing isoflavone accumulation by selecting the harvesting area in the neighboring of the east side of the tree alleys, with further improvements if a mild shoot stripping is applied before flowering.

Keywords: agroforestry; light orientation; soybean; isoflavones; mechanical stress

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1. Introduction

Soybean is a staple crop for human nutrition and animal feed worldwide and for nutraceutical/cosmetic uses, being one of the largest sources of proteins and isoflavones, respectively. Isoflavones production has received increasing attention in the last decades in nutraceutics, as epidemiological and clinical studies have highlighted many health benefits, such as anti-aging activity, cholesterol reduction and prevention of cardiovascular diseases, after long-term consumption of soyfoods [1,2]. Isoflavones are synthetized in the green tissues and seeds of the plant, through the secondary metabolic pathway of the phenylalanine and shikimic acid (phenyl propanoids pathway), mainly during the pod-filling stage during July–August in northern Italy. In the seeds, isoflavones are accumulated in the cell vacuoles of the embryo (hypocotyl) and cotyledons, the latter representing the largest part of the seed (50 times the hypocotyl mass). Most of the isoflavones (~90%) accumulate in the cotyledons, although the concentration in cotyledons is on average 5–8 times lower than that of the hypocotyl (1–3 mg g^{-1} vs. 20 mg g^{-1}) [3].

Isoflavones are generally produced as conjugated forms; among these, malonyls are those with highest molecular weight, followed by the acetyl-glycosyl and the glycosyl forms. The glycosyl forms have the highest antioxidant activity [4], but cannot be absorbed by the intestinal mucosa. Instead, the aglycone forms, which result from the separation of the glycosyl group, can efficiently enter into the blood circulation [5]. Twelve different isoflavones are synthesized in soybean seeds, classified into three families, which are distinguished by the different aglycone groups daidzein, genistein and glycitein, the latter found only in the embryo. Isoflavones synthesis is affected by several factors, and their concentration and profile highly depend on genotype and environmental conditions during seed maturation, such as water availability and temperature [6,7]. The effects of the environmental light on isoflavone synthesis are poorly studied, although solar radiation may have an impact due to its essential role in anthocyanin production [8]. Indeed, the metabolic pathway of isoflavones synthesis shares naringenin as an intermediate compound with the biosynthesis of anthocyanins. In this way, it is hypothesized that light might have an effect on isoflavones synthesis as well.

Soybean cultivation in light reduced environments, such as in agroforestry systems under the shade of trees, could, therefore, affect seed isoflavone content and profile. Agroforestry is an integrated land use management that combines a woody component with a lower story agricultural production [9], which is receiving increasing attention for improving resilience to climate change in temperate regions, through carbon sequestration, microclimate buffering and regulation of water flow [10,11]. Soybean is potentially a relevant intercrop in silvoarable agroforestry systems. In widely spaced tree lines inter-cropped with annual crops (i.e., alley cropping systems), soybean can effectively contribute in nutrients cycling and improve soil fertility through nitrogen fixation. As trees modify the microclimate parameters, we assume that in alley agroforestry systems the metabolic pathways of seed proteins and isoflavones accumulation might be changed compared to full sun conditions. In these farming systems with generally a north–south tree line orientation, a significant role could also be played by the direction of the light (east or west), a factor that might further affect isoflavones synthesis, depending on the side of the tree line considered. In this way, two possible farming conditions can be created, with soybean plants mainly lighted from the east (morning time) or west (afternoon time). As a summer crop, soybean can be easily affected by hailstorm events, an occurrence that might affect isoflavone accumulation through leaf mechanical damage. Additionally, the effects of leaf mechanical damage to isoflavone accumulation has not been studied up to date.

Given this background, in this study, a two-year pot trial was carried out at the experimental farm of the University of Padova (Legnaro, NE Italy) in order to mimic the response of the soybean var. Sponsor, characterized by a high seed isoflavone content [3], to: (i) two contrasting light orientations (east or west), typically observed in alley agroforestry systems with north–south oriented tree lines; and (ii) mechanical leaf damage, potentially occurring with hailstorm events. We aimed at investigating soybean response in terms of yield, seed protein content and total cotyledon isoflavone concentration (TCIC) and profile.

2. Materials and Methods

2.1. Experimental Set-Up

The trial was conducted at the experimental farm of the University of Padova at Legnaro (NE, Italy; 45°21′ N, 11°58′ E, 6 m a.s.l.) in 2016 and 2017. The average daily temperatures and precipitations were measured by a dedicated local weather station.

The soybean var. Sponsor (Semfor, San Pietro di Morubio, Verona, Italy), characterized by elevated aptitude for isoflavone accumulation [3], was grown in 36 pots (25 cm height × 30 cm top diameter × 20 cm bottom diameter, ~6.2-L volume), with one plant per pot. The soil was silty-loam, pH = 8.04, 1.7% organic matter (on dry weight), total N = 0.1% (on dry weight), carbon-to-nitrogen ratio (C/N) = 8.88, exchangeable P = 35.2 mg kg^{-1}, exchangeable K = 60.2 mg kg^{-1}, exchangeable Mg = 191 mg kg^{-1}, exchangeable Ca 2609 mg kg^{-1}, exchangeable Na = 6.06 mg kg^{-1}, cation exchange capacity (C.E.C.) = 14.8 cmol(+) kg^{-1}, total S = 418 mg kg^{-1}.

A 3-m high, 5-m width and 3-cm thick north–south oriented wood barrier was used to mimic the presence of a tree row, this orientation being the most suitable to maximize light availability to the intercrop in agroforestry alley systems at high and medium latitudes [12]. Half of the pots were placed equally spaced on the east side of the wood barrier, and half on the west side.

The low-cloud cover and morning mist were estimated by the "Meteoblue Model" [13] for two time intervals of the same duration, i.e., morning (6:00–12:00 AM) and afternoon (2:00–8:00 PM) during the pod-filling stage of soybean in the July–August period. The choice of these times was based on the exposure of soybean plants to light coming predominantly from east or west with a north–south oriented barrier, from sunrise (6:00 AM) to sunset (8:00 PM) as average of the July–August period at Legnaro.

At each side of the barrier, half of the plants (nine pots) were subjected to mechanical damage, simulating the effects of hail (here named "D" treatment), and the remaining plants (nine pots) were used as untreated controls (named "C"). The mechanical damage to the leaves was carried out using scissors with a non-cutting blade and by applying a number of lesions on all the leaves of a plant, which varied from 6 to 10 depending on the length of the leaf. The lesions parallel to the secondary veins of the leaves were about 1–1.5 cm apart. This mechanical operation was carried before flowering. Pots were arranged following a completely randomized experimental design at the east side of the barrier (east-lighted plants; named "east") and at the west side (west-lighted plants; named "west"). In this way, soybean plants were submitted to four treatments, i.e., east-D, east-C, west-D and west-C, with nine replicates per treatment.

The pots were placed outside and covered with a shading net, supported by a metallic scaffolding, according with a 50% reduction of photosynthetic active radiation (PAR). (Figure 1). The shading net was permeable to rain and plants were watered regularly to guarantee an adequate water supply, based on evapotranspiration.

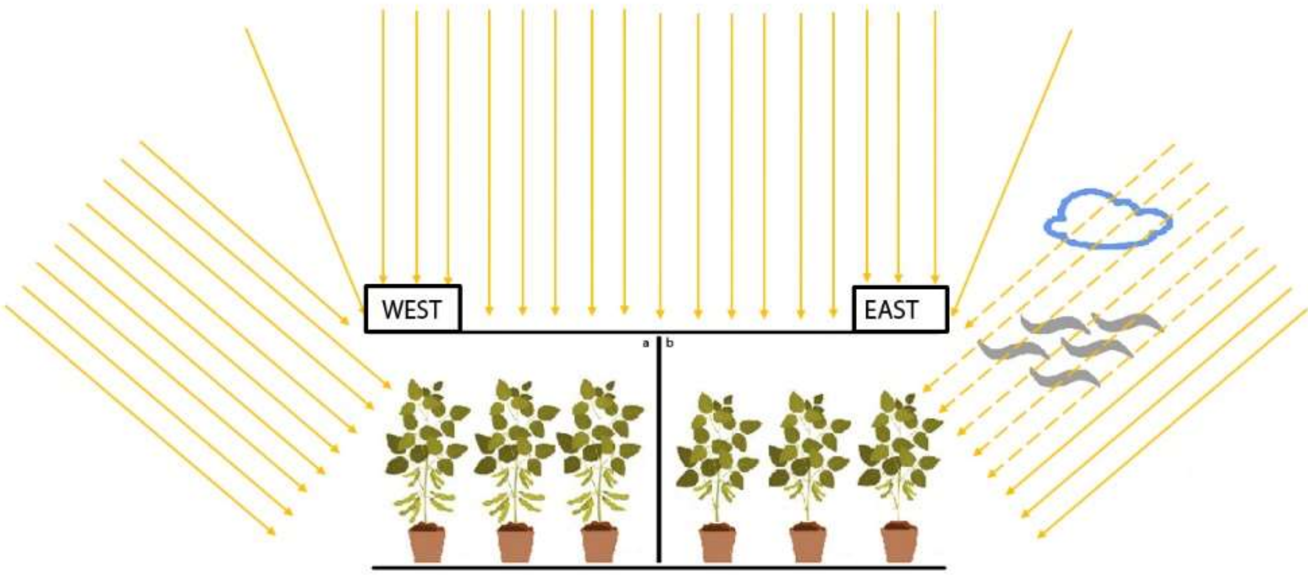


Figure 1. Sketch of the experimental trial, front section: west-lighted plants (**a**; "west" treatment) and east-lighted plants (**b**; "east" treatment).

2.2. Plant Analysis

After harvesting (134 DAS days after sowing, seeds at 12% humidity), plant biomass and grain yield (dry weights) were determined after oven drying (48 h at 60 °C), and the number of pods per plant determined by manual counting. A seed sample (60 g) from each plant/pot was lyophilized and subjected to thermal shock at 140 °C for 90 s in an oven, in order to easily remove the seed cuticle from cotyledons. Then, the seeds were pestled in a small mortar for separating the cotyledons from the embryo. Only isoflavones from the cotyledons were analyzed, according with existing literature showing significant influence of farming practices and environmental conditions on cotyledon isoflavone accumulation, but not on the germ [14]. Seed cotyledons of each sample were grinded by a laboratory mill, and the flour stored in glass tubes until further processing. About 0.1 g of cotyledons flour was suspended in 80% v/v methanol for two hours in 7 mL tubes in a rotating stirrer. After centrifugation at 10,000× g, the supernatant was filtered at 0.2-µm CA syringe-filters. The extracts were collected in plexiglass vials and stored at −20 °C until analysis. HPLC analysis of six isoflavones, such as genistein and daidzein (aglycones), and their glycosyl and malonyl forms, was carried out according to the procedure of Hubert et al. [15]. Genuine standards of aglycone- and glycosyl-conjugated forms (i.e., daidzin, genistin) were provided from a commercial source (L.C. Laboratories, Woburn, MA, USA). Due to their unstable structure, the malonyl forms were not quantified by external standards, and their response factors were derived from the corresponding glycosyl forms, corrected by molecular mass ratio, according to the procedure of Hubert et al. [15].

2.3. Statistical Analysis

Principal component analysis (PCA) and factorial discriminant analysis (Multigroup Discriminant Analysis (MDA) with Wilks' lambda and Pillai's trace tests [16] were carried out with MS Excel XLSTAT (Addinsoft, Paris, France) to describe the total cotyledon isoflavone concentration (TCIC) and the malonyls, glycosyls and aglycones concentration of isoflavones of the soybean variety Sponsor in relation to hail simulation treatment and prevailing east/west light orientation. Before analysis, multivariate data normality was verified by the Shapiro test using R 3.0.1 software [17], and data were standardized by subtracting the mean and dividing by the standard deviation for each variable. MDA was applied separately to each yearly dataset due to the contrasting weather conditions.

The Bayesian approach was also used to simulate the probability of increase in isoflavones concentration with the hail simulation treatment. In this way, the issue arising from comparing two independent binomial distributions was considered [18]. The hypotheses compared in our study were: (i) PD, as the probability (P) of maximizing the concentration of isoflavones with foliar damage (D), and (ii) PC, as the probability (P) of maximizing the concentration of isoflavones without foliar damage (C). The assumption of an independent proportion was considered following Howard's "dependent prior," which is recommended for this specific testing issue [19]. In this way, the proportions were first transformed into the real-valued logit parameters θ_1 and θ_2:

$$\theta_1 = log\frac{p_D}{1-p_D};\ \theta_2 = log\frac{p_C}{1-p_C}$$

hence, it was assumed that for each value of θ_1, the logit θ_2 was assumed to be normally distributed with mean θ_1 and standard deviation σ. Generalizing this principle, the dependent prior has the following general form:

$$g\ (pD\,;pC)\ \propto\ e^{-(1/2)\ \mu^2}\ \ ;\ pD^{\ a-1}(1-\ pD)^{\ ss-1}\ ;\ \ (1-\ pC)^{\ \delta-1}\ ,\ 0<\ pD\ ,\ pC\ <1$$

where:

$$\mu = \frac{1}{\sigma}(\theta_1 - \theta_2)$$

This class of dependent priors was indexed by the parameters:

$$(\alpha, \beta, \gamma, \delta)$$

The four parameters reflected Howard's function form and the dependence between the two proportions. The hypothesis H1: PD > PC was tested simply by computing the posterior probability of this region of the parameter space. A sample simulated by the posterior distribution of PD and PC was first produced by software, and then, the proportion of simulated pairs in which PD > PC was investigated.

3. Results

3.1. Climate Parameters

The monthly rainfall during the pod-filling period (July–August) of soybean was higher in 2016 than 2017, i.e., +6.6 mm and +37.6 mm in July and August, respectively (Figure 2c,d). The average daily temperature was inversely related to the rainfall pattern, being 1 °C lower in 2016 than in 2017 (Figure 2a,b). In 2016, the average hourly low cloud cover, estimated through the Meteoblue model, was 55% and 16% higher ($p < 0.05$) in the morning than in the afternoon, in July and August, respectively (Figure 3a,b). Similar cloud cover variations (+8% and +46% in 06:00–12:00 AM vs. 2:00–8:00 PM, respectively, for July and August) were observed in 2017 (Figure 3a,b). The average temperature measured during the pod-filling period was lower in the morning (06:00–12:00 AM) than in the afternoon (2:00–8:00 PM) times in both years: 25.2 vs. 27.4 °C in 2016 (−2.2 °C) and 26.8 vs. 28.2 °C in 2017 (−1.4 °C) ($p < 0.05$).

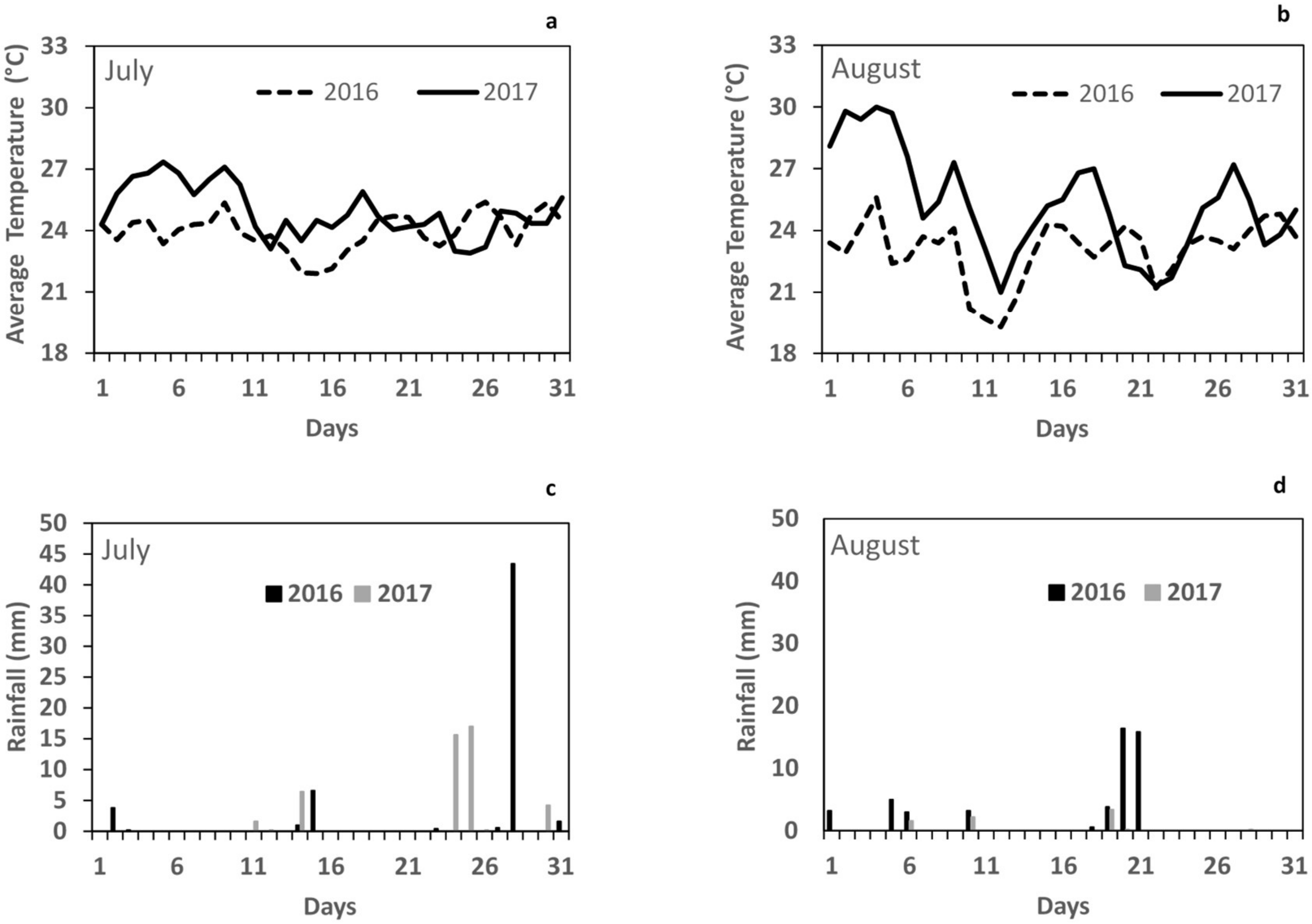


Figure 2. Average daily temperature and precipitation patterns in July (**a**,**c**) and August (**b**,**d**) during the pod-filling period of soybean in two years.

3.2. Plant Biomass and Yield

An increase in shoot biomass under mechanical foliar damaged (D) vs. control (C) plants was observed in both experimental years under the two light orientations. A trend to increase shoot biomass in west-lighted plants (afternoon time) compared to east-lighted plants (morning time) was also observed (Table 1). West-oriented damaged plants (D-west) showed +70% and +50% of biomass, respectively, in 2016 and in 2017 as compared with control plants (not damaged) oriented to the east (C-east) ($p < 0.05$; Table 1). A similar trend was also observed for grain yield, it being higher in damaged vs. control plants and in west- vs. east-oriented plants. Indeed, the D-west plants increased grain yield by 78% and 66% in 2016 and 2017, respectively, compared to the C-east plants ($p < 0.05$). As regards foliar damage, in 2017, D-west and D-east plants increased yield ($p < 0.05$) by 28% and 9%, respectively, as compared to the respective control plants, and a similar trend was observed in 2016. The same response was observed in the number of pods per plant, both in 2016 and 2017 (Table 1). The number of pods in D-west plants increased by 66% and 92%, compared to C-east plants in 2016 and 2017, respectively. Cotyledon's weight, instead, did not change significantly between years and experimental treatments (D vs. C and east vs. west).

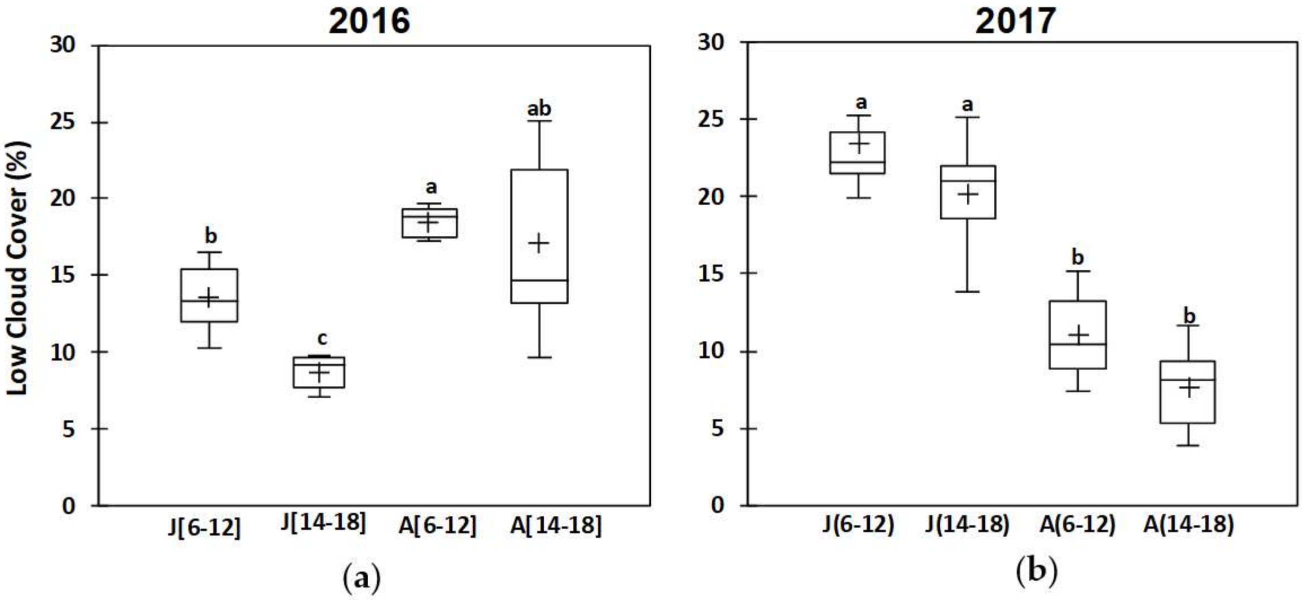


Figure 3. Low Cloud Cover (0–2 Km altitude) (by the Meteoblue model) in 2016 (**a**) and 2017 (**b**). J: July; A: August, [6–12]: values collected between 6:00 to 12:00 AM (morning time); [14–18]: values collected between 2:00 to 6:00 PM (afternoon time). Letters indicate significant differences among daytimes of July and August within each year according to Tukey-Kramer test ($p < 0.05$).

3.3. Quality Parameters

Protein concentration (% of dry weight) in soybean seeds did not change significantly between years and experimental treatments (on average 43.9%; Table 1). The Total Cotyledon Isoflavone Concentration (TCIC) was higher in 2016 (2.76 mg g^{-1}) than in 2017 (1.25 mg g^{-1}). In 2016, the foliar damage increased TCIC by 24.3% in the east-lighted plants ($p < 0.05$) compared to the respective control (C-east), while the effect was non-significant in the west-lighted plants (Table 1). In 2017, foliar damage increased TCIC, but by +7% only, again in the east-lighted plants ($p < 0.05$) compared to the respective controls (C-east) and, similarly to 2016, no effect was observed in the west-lighted plants (Table 1). In this way, in both years, the D-east plants (morning time) accumulated a higher amount of isoflavones than the D-west plants (afternoon time) and the respective controls.

3.4. PCA and MDA on Isoflavone Concentration and Profile within Treatments

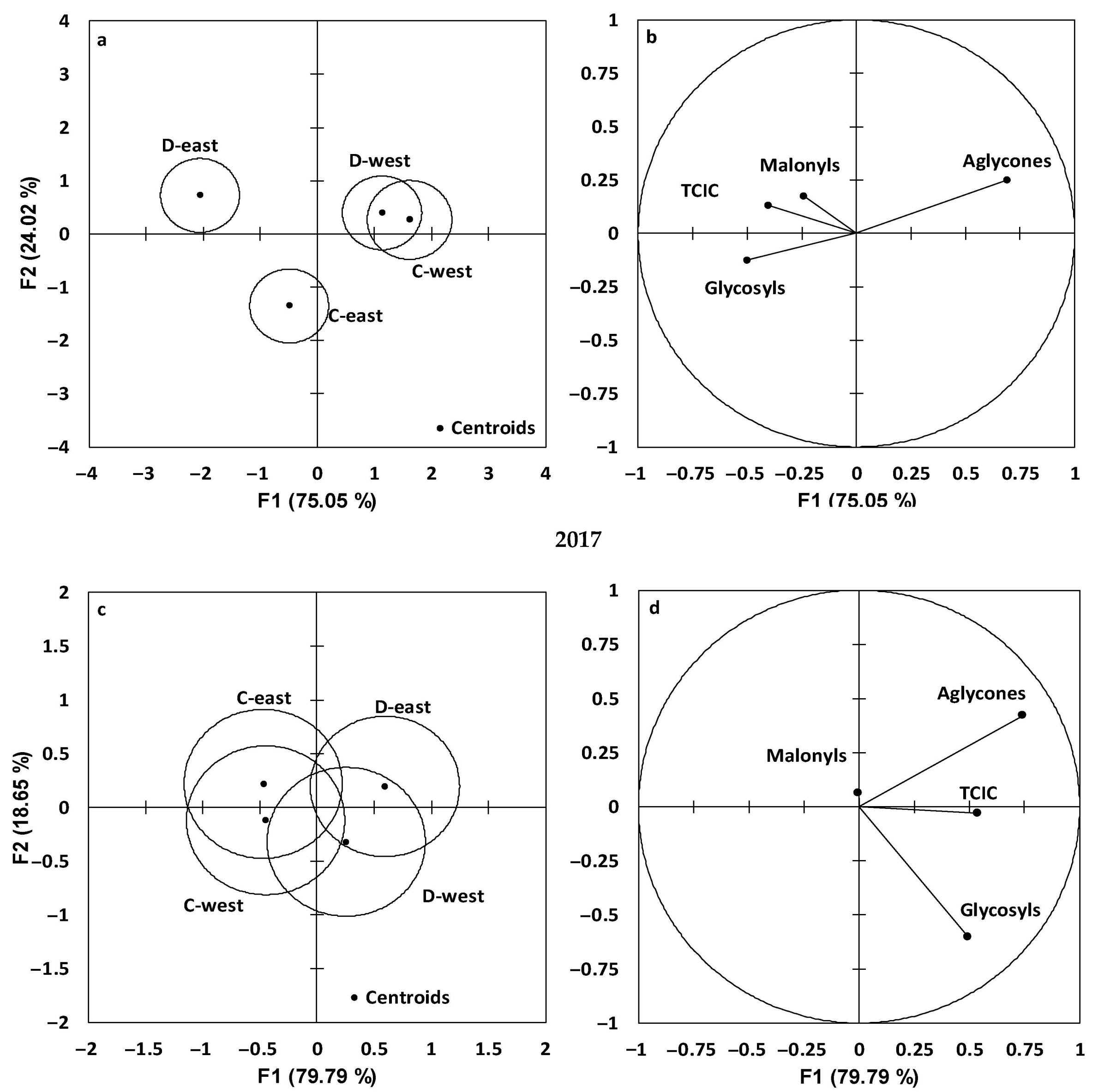
Discriminant and principal component analyses, which were carried out to summarize the effects of the experimental factors on the isoflavones profile, revealed that the increase of TCIC associated with foliar damage in 2016 was due to higher malonyl and glycosyl forms (Figure 4a,b). Instead, the increase of TCIC observed in 2017 was sustained by glycosyl and aglycone forms (Figure 4c,d). Among high informative variables (loadings > $|0.5|$), glycosyl forms of isoflavones, i.e., genistin and daidzin, were positively correlated with TCIC in both years (Figure 4; bottom table).

Table 1. Shoot biomass (g of dry matter plant^{-1}), grain yield (g of seeds plant^{-1}), number (Nb) of pods per plant, cotyledon's weight (g of dry matter), protein concentration (% of dry weight) and Total Cotyledon Isoflavone Concentration (TCIC) (mg g^{-1}) of soybean plants at harvest under foliar damage vs. controls, in east-lighted (East) and west-lighted (West) treatments. Within each parameter and year, different letters between brackets indicate significant differences among treatments according to Tukey-Kramer test ($p \leq 0.05$; $n = 9$).

Factors	2016		2017	
	West	East	West	East
Shoot biomass (g)				
Foliar damage (D)	71.7 a	58.9 ab	68.5 a	56.3 ab
Control (C)	59.6 ab	42.1 b	55.1 a	45.8 b
Grain yield (g plant^{-1})				
Foliar damage (D)	39.5 a	32.1 ab	25.6 a	19.7 a
Control (C)	32.4 ab	22.1 b	16.7 b	15.3 b
Nb of pods/plant				
Foliar damage (D)	84.7 a	69.9 a	66.2 a	55.2 a
Control (C)	57.7 ab	43.3 b	58.1 a	34.4 b
Cotyledon weight (g)				
Foliar damage (D)	0.139 a	0.136 a	0.225 a	0.279 a
Control (C)	0.138 a	0.119 a	0.223 a	0.183 a
Protein (%)				
Foliar damage (D)	43.4 a	43.6 a	43.8 a	44.0 a
Control (C)	43.6 a	43.9 a	44.1 a	44.2 a
TCIC (mg g^{-1})				
Foliar damage (D)	2.60 b	3.12 a	1.08 b	1.38 a
Control(C)	2.49 b	2.51 b	1.06 b	1.22 a

3.5. Monte Carlo Analysis on the Probability to Maximize TCIC

The Monte Carlo analysis, which was conducted with a simulation of 10,000 cases, confirmed that the probability to maximize the TCIC was higher with mechanical leaf damage in east-lighted (D-east) vs. control soybean plants (C-east), i.e., by 92% in 2016 (Figure 5a) and 80% in 2017 (Figure 5b). In west-lighted plants, the probability to increase TCIC with foliar damage was nearly 50%, with values of 45% in 2016 (Figure 5c) and 62% in 2017 (Figure 5d).



Variables	2016		2017	
	F1	F2	F1	F2
TCIC	**−0.404**	0.130	**0.539**	−0.030
Glycosyl forms	**−0.498**	−0.126	**0.496**	**−0.605**
Malonyl forms	−0.242	0.176	−0.001	0.062
Aglycone forms	**0.690**	0.249	**0.744**	**0.421**

Figure 4. Multigroup discriminant analysis (MDA; left, **a**,**c**) and principal component analysis (PCA; right, **b**,**d**) for isoflavones profile (Total Cotyledon Isoflavone Concentration (TCIC), and malonyl, aglycone and glycosyl forms) in soybean under foliar damage (D) and untreated plants (C), under a west (west; afternoon time) or east (east; morning time) light orientation, in 2016 (**a**,**b**) and 2017 (**c**,**d**). The isodensity confidence circles contain 75% of variability. In the bottom table, the highly informative variables (loadings > |0.5|) are highlighted in bold, within synthetic variables F1 and F2.

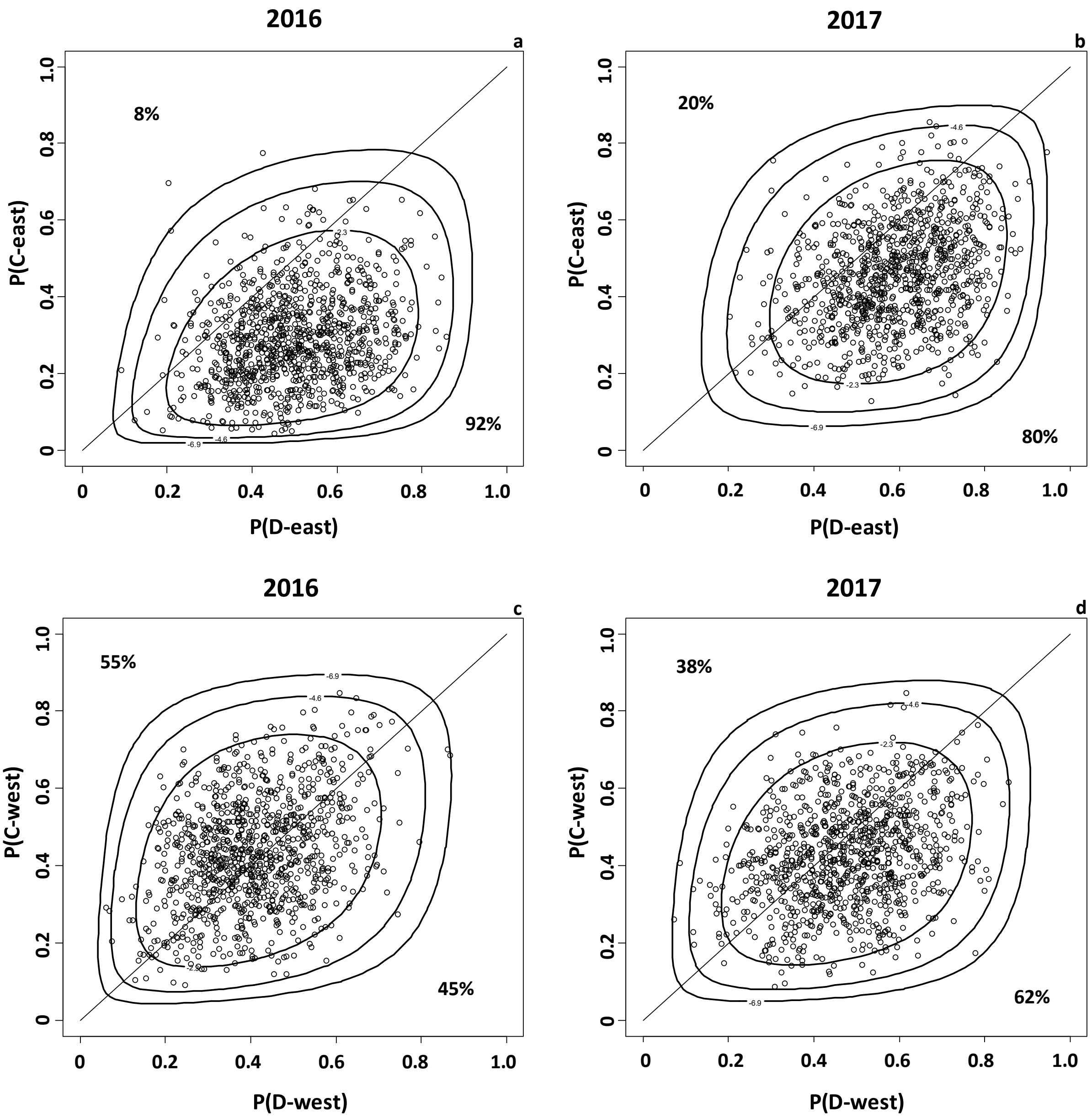


Figure 5. Monte Carlo analysis in 2016 (**a**,**c**) and 2017 (**b**,**d**): contour plot of posterior distribution with 10,000 cases (Howard function, $\delta = 0.05$) and probability of increases in TCIC (Total Cotyledon Isoflavone Concentration) under foliar damage (PD) and control treatment (PC). Contour lines are drawn at 10% (outer), 1% and 0.1% (inner) of model height. Howard-simulated random sample from this distribution overlaps with the contour plot. P(D-east): probability of maximizing TCIC under foliar damage and east light orientation; P(C-east): probability of maximizing TCIC in control treatment and east-light orientation. P(D-west): probability of maximizing TCIC under foliar damage and west light orientation, P(C-west): probability of maximizing TCIC using control treatment and west light orientation.

4. Discussion

This study provides a two-year analysis of isoflavones concentration and profile in soybean plants in response to light stress conditions with a prevailing light orientation (east or west), and after leaf mechanical damage, which can mimic hail events. The different climatic conditions during pod-filling period (July–August) between the two trial years, with higher rainfall and lower temperature in 2016 as compared to 2017, as well as the differences in light composition between morning time (plants lighted from the east) and afternoon time (plants lighted from the west), allowed better insights into the physiological mechanisms of isoflavones synthesis.

The red/far red (R/FR) ratio in the environmental light varies according to the cloud cover, and was found approximately equal to 1 during the afternoon (2.00–8.00 PM) when sunny weather conditions with fewer clouds were recorded at the experimental site during July–August in both years [20]. During morning time (6.00–12.00 AM), there was a cloud cover on average 30% higher than in the afternoon in the two years. In these conditions, the absorption coefficient due to water vapor would be expected to increase by the same percentage both for the red (R) and far red (FR) wavelength regions. However, as the water vapor absorption coefficient in far red (FR) is 43% higher than in red (R) [21], a net increase by 22% in the absorption coefficient for R/FR ratio from 1 to ~1.22 is assumed for the soybean plants mainly lighted from the east as compared to the plants mainly lighted from the west. Light with a relatively high R/FR ratio is known to stimulate the transition from the inactive phytochrome "pr" to the active localization and isomerization of the phytochromobilin in the C15 localization; this would cause a morphological modification of the phytochrome holoprotein, which exposes localization sequences (NLSs) by which the phytochrome can be transported to the nucleus, where it is dephosphorylated by an enzyme kinase and becomes stable and active (pfr form) [22,23]. Phytochrome pfr is known to prevent the expression of cell expansion genes by marking the Phytochrome Interacting Factors (PIF proteins) with ubiquitin and the consequent degradation of the latter by Proteaosome 26s [24]. In this way, it is thought that these processes might be responsible for the lower aboveground biomass of soybean plants lighted from the east (morning time) observed in our experimental conditions.

In addition, the activity of the phenylalanine ammonium-lyase (PAL) enzyme, one of the main enzymes of the flavonoids and isoflavones metabolic pathways, is reported to be proportional to the cell concentration of the phytochrome in the "pfr" form, and increase with exposure to low light intensity of the FR component [25–27]. Indeed, in our study, soybean plants lighted from the west (afternoon time), receiving light richer in the FR wavelength region than east-lighted plants, probably produced less phytochrome "pfr". As a result, PIF proteins were presumably not degraded and could regulate the expression of their target genes by promoting cell expansion and chlorophyll biosynthesis [28]. The activity of PIF proteins has also shown to be negatively regulated by "DELLA" (aspartic acid–glutamic acid–leucine–leucine–alanine) proteins, with greater abundance of red light (high R/FR ratio). On the contrary, when the FR component increases, gibberellins (GA) fix ubiquitin on the "DELLA" proteins that are destroyed by the proteosome 26s, contributing to the activity of the PIF proteins [29]. As a result, west-lighted plants, while receiving a higher level of FR light than east-lighted plants, developed a higher biomass, as previously demonstrated [30]. Previous studies have shown that with relatively low R/FR ratios, the distribution of photosynthetic compounds is directed at higher extent towards shoots rather than roots, hence reducing the root biomass [31], although we did not investigate the root system in this study. The shade avoidance response (SAR), known to stimulate the elongation of the stems and leaf petioles [32], might be responsible for morphological adaptations of west-lighted soybean plants, such as a better positioning of the photosynthetic apparatus, potentially leading to a more efficient light interception, and consequently, a higher yield than in east-lighted plants, as was observed in our study. In accordance with previous studies [14,33], higher seed yields of plants lighted from the west were associated with lower TCIC content, while the opposite was observed in east-lighted plants. As previously observed by Rasolohery et al. [7], the higher TCIC content observed in east-lighted plants might be also related to the lower air temperature measured during pod-filling in the morning hours compared to west-lighted plants. In this way, the measurement of variations in the canopy temperature may be useful to better disentangle the effects of environmental variables on isoflavones synthesis.

These findings provide relevant insights for understanding the effects of trees on yield and isoflavones concentration and profile of soybean intercrop within agroforestry cropping systems. Indeed, soybean is a relevant crop for improving nutrient cycling and soil fertility in agroforestry, while the impact of tree rows on growth and yield of the intercrop is being investigated in silvoarable systems of temperate regions [34–37]. The literature highlights a significant effect of the distance from the tree alley on soybean plant biomass and yield. However, very few studies have investigated the effect of light orientation on soybean yield and, to our knowledge, this investigation is the first attempt to study its effect on isoflavones concentration and profile. In agreement with our findings, Mantino et al. [36] found a lower number of pods per plant (−78%) and grain yield (−35%) in field soybean lighted from the east vs. west within alley cropping with poplar short rotation coppice (SRC) in central Italy. With regard to grain quality, however, we did not find a significant effect of prevailing light orientation on protein concentration, in contrast with the results of Mantino et al. [36], who recorded lower grain crude protein contents in east-lighted plants. In our study, a slight reduction of protein concentration in soybean seeds was due to mechanical damage to leaves, both in east- and west-lighted plants, which was associated to an increase of grain yield similarly to previous studies [14,33]. Regarding foliar mechanical damage vs. controls, this study also confirms previous findings on the negative relationship between seed proteins and isoflavones concentrations, likely due to the competition for phenylalanine [14,33].

With regard to hail simulation, foliar mechanical damage applied to our soybean caused the exposure of damaged tissues to air, in this way probably stimulating the production of free radicals through the NADPH synthase activity (oxidative burst). The same oxidizing species can be synthesized by the plant's metabolism after being in contact with bacterial or fungal cell elicitors [38]. A rapid production of nitric oxide through the NO-synthase activity is known to encompass an oxidative burst [39]. NO and H_2O_2 deriving from the metabolism of the OH radicals act as a signal for stimulating the biosynthetic pathway of phytoalexins, such as isoflavones. This would explain why in our study soybean plants subjected to mechanical damage accumulated higher isoflavones than control plants. Thus, a cumulative effect of both the higher R/FR ratio of east-lighted plants and foliar mechanical damage might be responsible, through a synergic action, of the higher concentration of isoflavones found in the D-east treatment. This was corroborated by the Monte Carlo analysis with a Bayesian approach [18], which confirmed the highest probability to maximize the isoflavones' concentration with east-lighted plants (under high R/FR ratio and lower air temperature) and mechanical leaf damage. In agreement with previous studies, the mechanical damage of plant tissues was also found to enhance plant biomass and seed yield, probably due to a "pruning effect" that reinvigorates the plants [3].

With regard to the isoflavone profile, the glycosyls/malonyls/aglycones content ratio has a genetic regulation, depending on the variety choice [33]. However, the effects of the experimental year in our study highlighted the role of the environment, i.e., light availability and quality, and temperature during the pod-filling stage, on the isoflavone profile. The higher concentration of isoflavones found in 2016 can be related to the higher rainfall and lower average temperatures observed during pod-filling stage as compared to 2017, these climate conditions being favorable to isoflavones' accumulation, in particular for medium–high molecular weight forms (i.e., glycosyls and malonyls). Indeed, an adequate water supply is known to be fundamental to sustain the synthesis reactions of the phenyl-propanoids metabolism. The thermal optimum for the enzymes chalcone-synthase and chalcone-isomerase, which are involved in the first part of isoflavone biosynthesis, is ~20 °C. Therefore, when temperatures increase from this value, the enzymatic activity is slowed down proportionally and the concentration of isoflavones reduced, as we observed during the hotter pod-filling period of 2017 compared to 2016 [7,33]. With regard to the isoflavones profile, the high temperatures and low rainfall of the 2nd year were associated with increased accumulation of medium-low MW forms (i.e., glycosyls and aglycones). The decreased intensity of the infrared radiation, due to low clouds and morning mist, was also likely to play a role in slightly reducing the average temperature of the plants mainly lighted from the east, favoring the isoflavones accumulation.

5. Conclusions

This trial carried out under controlled conditions provided illuminating results on the morphological and physiological response of soybean under modified lighting conditions that are typical of agroforestry alley cropping systems. It is expected that designs with north–south oriented alley trees create growing conditions that modify growth, yield and quality of the intercropped soybean, depending on the selected side of the tree alley, which is related to a prevailing light orientation. In areas where the climate is characterized by high mist and low cloud during morning time, such as in northeastern Italy, in soybean plants growing on the east side of the trees, predominantly lighted during the morning time and exposed to light with higher R/FR ratio, the secondary metabolism is expected to enhance, leading to higher isoflavones concentration. Soybean plants growing on the west side of the tree lines, instead, predominantly lighted during the afternoon, which is commonly sunny and cloudless in the area of the study and with lower R/FR ratio, are expected to enhance biomass and grain yield. Foliar mechanical lacerations applied before flowering, which may mimic the effects of hail, had cumulative positive effect with light orientation, leading to higher grain yield in west-lighted plants and higher TCIC in east-lighted plants. These findings provide relevant insights for better exploitation of soybean products in agroforestry systems, depending on the market strategies and uses. In this way, there is possibly a large scope for maximizing isoflavone production by selecting the harvesting area in the neighboring of the east side of the tree alleys, with further improvements if a mild shoot stripping is applied before flowering.

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