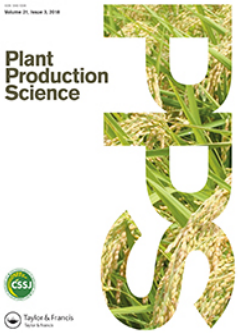

Plant Production Science

ISSN: 1343-943X (Print) 1349-1008 (Online) Journal homepage: https://www.tandfonline.com/loi/tpps20

Effect of shading on accumulation of soybean isoflavonoid under maize-soybean strip intercropping systems

Caiqiong Yang, Baoyu Hu, Nasir Iqbal, Feng Yang, Wei-guo Liu, Xiao-chun Wang, Tai-wen Yong, Jing Zhang, Wen-yu Yang & Jiang Liu

To cite this article: Caiqiong Yang, Baoyu Hu, Nasir Iqbal, Feng Yang, Wei-guo Liu, Xiao-chun Wang, Tai-wen Yong, Jing Zhang, Wen-yu Yang & Jiang Liu (2018) Effect of shading on accumulation of soybean isoflavonoid under maize-soybean strip intercropping systems, Plant Production Science, 21:3, 193-202, DOI: 10.1080/1343943X.2018.1484257

To link to this article: https://doi.org/10.1080/1343943X.2018.1484257

PLANT PRODUCTION SCIENCE
2018, VOL. 21, NO. 3, 193–202
https://doi.org/10.1080/1343943X.2018.1484257

REGULAR PAPER

Effect of shading on accumulation of soybean isoflavonoid under maize-soybean strip intercropping systems

Caiqiong Yang[†a], Baoyu Hu[†a], Nasir Iqbal[†a], Feng Yang[a], Wei-guo Liu[a,b], Xiao-chun Wang[a], Tai-wen Yong[a], Jing Zhang[a], Wen-yu Yang[a] and Jiang Liu[†a,b]

[a]Key Laboratory of Crop Ecophysiology and Farming System in Southwest, Ministry of Agriculture, Chengdu, PR China; [b]Institute of Ecological Agriculture, Sichuan Agricultural University, Chengdu, China

ABSTRACT

Maize-soybean strip intercropping system is an important ecological planting system; the wide development of intercropped soybean has contributed to the soybean industry in China. Soybean growth and seed production were affected by the shading from maize. Previous studies confirmed that relay strip intercropping shading facilitates the accumulation of isoflavone glucosides but does not take advantage of the accumulation of isoflavone aglycone. However, the regulation of isoflavones accumulation under strip intercropping system was unknown; in which soybean and maize were sown at the same time, and soybean was mainly shaded in the reproductive stage. In order to reveal the effects of intercropping shading on the isoflavones accumulation in soybean seed, two independent field experiments were conducted in eastern China. We compared the isoflavones profiles of soybean seeds, which were grown under various maize-soybean intercropping systems with different shade levels. The results showed that the intercropping shading was not benefited to the accumulation of soy-isoflavones, including aglycones and isoflavones glycosides and various forms of isoflavones were decreased with the decline of photosynthetic active radiation. Multivariate statistical analysis and significance analysis showed that acylated isoflavones may be more susceptible to intercropping shading.

Abbreviations: HCA: Hierarchical cluster analysis; PAR: photosynthetically active radiation; PCA: Principal component analysis; LC-MS: Liquid chromatography mass spectrometer.

ARTICLE HISTORY
Received 20 September 2017
Revised 14 May 2018
Accepted 19 May 2018

KEYWORDS
Soybean; intercropping; shading; isoflavone; accumulation

1. Introduction

Soybean [*Glycine max* (L.) Merrill] is one of the most important crops worldwide, and is a source of starch, dietary fiber, and protein. Soybeans contain many kinds of functional components such as isoflavone (Fehr, 2007; Kim, Ro, Kim, Kim, & Chung, 2012).

Isoflavone is a group of antioxidant composition in soybean which participate in a series of important physiological process, such as nodule formation (Sugiyama & Yazaki, 2014). Plants have formed a series of complex defense systems during the evolution process. When plants are stimulated by the environment, they will trigger defense signals such as jasmonic acid and salicylic acid, further induce the expression of defense genes, and form a variety of secondary metabolites to cope with environmental stress. Isoflavones are kinds of phytoalexins in soybean, which have important resistance to *Phytophthora sojae* and soybean root rot (Cheng, et al. 2015; Zhang et al., 2017). Soy-isoflavone biosynthesis is dual regulated by genes and environment (Chennupati, Seguin, & Liu, 2011; Li, Yao, Gong, & Li, 2010; Vamerali, Barion, Hewidy, & Mosca, 2012). The aglycone and β-glucoside concentrations of upstream constituents increased significantly, whereas the acetylglucosides and malonylglucosides of downstream constituents decreased with an increase in storage period (Jiang, et al. 2017). Geographical location is considered to affect the accumulation of soybean isoflavone (Wu et al., 2017). In addition, isoflavones in soybean leaves and grain are positively correlated with temperature and photosynthetic active radiation (PAR), and significant negative correlated with relative humidity (Yan et al., 2015) in relay intercropping system. Some researchers also found that light can promote the soybean isoflavones accumulation. For example, phenylalanine lyase (PAL) quantity expression is higher under the light condition and its influence degree relate to soybean varieties (Xie, Zhao, & Shen, 2000). In addition, cultural practices are also the important factor to regulate the plant secondary metabolites, particularly, in strip intercropping systems. Due to the influence of the environment and light distribution, the maize-soybean cultivation in the southwestern and Huang-Huai-Hai plains in

---

CONTACT Jiang Liu jiangliu@sicau.edu.cn

[†]These authors contributed equally to this work.

China has adopted different intercropping patterns. The Huang-Huai-Hai Plain is an important part of the eastern China's Great Plains, partly in the Bohai-North China Basin. Located at latitude 32° to 40°N, longitude 114° to 121°E. The light resources in Huang-Huai-Hai region are more abundant than those in southwest China. Due to the lack of light resources, the planting pattern of maize and soybean in Southwest China is dominated by relay intercropping system. Because in the relay intercropping system, the symbiotic duration of maize and soybean is shorter, soybeans can obtain sufficient light in the later stages of growth. There is a clear difference in the symbiosis between the two cropping patterns. Namely, the maize in maize–soybean relay strip intercropping systems is usually sown according to the narrow-wide row planting pattern at the end of March or the beginning of April and harvested at the end of July or the beginning of August. Soybean is sown in the wide rows between maize at the beginning of June and harvested at the end of October. The seedling phase of soybean and the reproductive phase of maize overlap over a period of approximately eight weeks between the sowing of soybean and the harvesting of maize (Moriri, Owoeye, & Mariga, 2010; Yang et al., 2014). In intercropping system, maize and soybeans are usually planted simultaneously and harvested simultaneously. Soybean plant grows under the shade of maize in intercropping systems, and the microenvironment of soybean growth varies. Especially, the change of light environment affects the photosynthetic efficiency and morphogenesis of the soybean (Moriri et al., 2010; Yang et al., 2014). However, due to the light reflection and absorption by maize leaves, the spectral irradiance, red/far-red (R/FR) ratio and PAR of the soybean canopy were decreased in maize-soybean intercropping system as compared to sole cropping (Liu et al., 2017; Yang et al., 2014). That led to increased internode lengths, plant height and specific leaf area, but reduced branching of soybean plant under intercropping. These morphological changes enabled the crop to intercept relatively more light and the shading also increased the light-use efficiency of soybean. However, due to smaller leaf size and less branching, these positive responses were not able to compensate the effect of reduced leaf area and total light interception, leading to reduced biomass and grain. The reduction in grain yield was mainly caused by the reduced number of grains produced by the intercropped soybean plants, while the grain size remained unchanged (Fan, 2017; Gong et al., 2015; Liu et al., 2017).

Appropriate cultivation methods can not only save land resources and make full use of light resources, but also change the quality of crops to a certain extent. Isoflavones are natural phytoestrogens and have many biological activities such as anti-inflammatory, anti-oxidation and anti-osteoporosis (Huang, Shen, Shen, Chiou, & Chen, 2017). If we can increase the accumulation of isoflavones in soybean grains by changing the cultivation methods, this has important implications for improving the functional value of soybeans. And our previous studies showed that the relay intercropping system can increase total isoflavone content and glycoside content in grains, reduce the content of aglycone with bitter astringency, and improve the flavor quality of soybean seeds. This indicates that the intercropping system can regulate the accumulation of isoflavones in soybean seeds. However, this is only for soybeans under relay strip intercropping system. In the strip intercropping system, maize-soybeans have a longer symbiotic period, and soybeans are exposed to shading for longer periods of time. Under such conditions, the accumulation rules of isoflavones in soybean seeds are not clear. In our current research, different experiments were conducted to reveal the isoflavones accumulation regularity of soybean grain under strip intercropping system, this study also compared the isoflavones accumulation differences between sole-cropping soybeans and intercropping soybeans. Moreover, the effect of different row spacing (or PAR) on soybean isoflavones accumulation was also conducted. From these studies, we want to clarify how isoflavones response to intercropping shading especially the photosynthetically active radiation.

2. Materials and methods

2.1. *Plant materials and experimental design*

Field experiments for maize-soybean strip intercropping system were conducted at Heze, Shandong province, China (35°26′N, 116°40′E). Two independent experiments were designed by randomized complete block design with three replications.

Experiment 1. This experiment was conducted with two treatments, each for the maize-soybean intercropping and the soybean monoculture. Maize cultivar was Jundan 20, while the soybean cultivars were Qihuang 35, Jidou 15, Hedou 18 and Zhonghuang 41. The intercropping pattern was used as wide-narrow row planting, with alternating strips of maize and soybean. The number of maize *vs*. soybean row per strip in the strip intercropping system was 2:2. The distance between the maize and soybean strips was 60 cm. The row space of the soybean monoculture was 50 cm (same as A2 treatment in Figure 1). Maize and soybean were sown on 15 June 2015.

Experiment 2. Soybean cultivar Qihuang 34 and maize cultivar Jundan 26 were used in experiment 2. The ratio of maize to soybean rows was same as experiment 1. Single-factor randomized complete block design was considered, and each block consisted of

Code	Maize narrow row spacing (cm)	Maize and soybean row spacing (cm)	soybean row spacing (cm)	Maize and soybean row spacing (cm)	PAR transmittance of soybean canopy (%)
SC	/	/	50	/	100
A1	20	70	40	70	51
A2	40	60	40	60	47
A3	60	50	40	50	42
A4	80	40	40	40	34
A5	100	30	40	30	30

Figure 1. Planting patterns and photosynthetic active radiation (PAR) transmittance. SC: sole cropping; maize–soybean intercropping patterns with different row spacings (A1: 180 + 20 cm wide-narrow row planting; A2: 160 + 40 cm wide-narrow row planting; A3: 140 + 60 cm wide-narrow row planting; A4: 120 + 80 cm wide-narrow row planting; A5: 100 + 100 cm wide-narrow row planting).

six plots treatments of cropping system (sole cropping + five intercropping patterns). The maize and soybean planting patterns under strip intercropping system are shown in Figure 1. Soybean was planted in two wide rows of maize, with 40 cm row spacing. Both the maize and soybean were sown on 14 June 2015, and harvested on 28 September 2015. The coexistence period of maize and soybean was 106 days. The PAR transmittance of the soybean canopy was measured three times in the sunny days on 10–20 August 2015 with LI-191SA quantum sensors (LI-COR Inc., Lin-coln, NE, USA), and the average PAR transmittance changed along with the row spacing gradient (Figure 1).

2.2. *Sample and chemicals*

Each treatment has three planting areas, all samples in each planting area were considered as one repeat. As the pods matured, about 4.5 kg soybean seeds were harvested from each area and were dried with natural air. Take 100 g of the seeds from each repeat then ground and stored it in tightly capped vials at −80°C until analysis. Triplicate samples of each replicate were taken separately as technical duplication, and the same extraction method was adopted to eliminate the error of the instrument and operation. Twelve isoflavone standard compounds, including daidzin (DG), glycitin (GLG), genistin (GEG), malonyldaidzin (MD), malonylglycitin (MGL), acetyldaidzin (AD), acetylglycitin (AGL), malonylgenistin (MG), daidzein (DE), acetylgenistin (AG), glycitein (GLE) and genistein (GE), were purchased from Wako Pure Chemical Industries Co., Ltd. (Japan). HPLC-grade acetonitrile was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). All aqueous solutions were prepared using ultrapure water produced using a Milli-Q system (18.2 MX; Millipore, Bedford, MA, USA). All other chemicals used in LC-MS experiments were HPLC-grade.

2.3. *Sampling and chromatographic analyses*

Extraction and chromatographic analysis procedures were based on previously published methods, with certain modifications (Wu et al., 2017). Briefly, 50.00 mg samples were subjected into 2.5 mL MeOH/H_2O (80/20 v/v) solution and ultrasonic extracted (40 kHz) for 3 h with ice-water bath. The tube samples were centrifuged at 11,000 g for 10 min, and then the supernatant fluid was filtered through a syringe filter (0.22 μm).

The isoflavones were quantified by external standardization using an Agilent 1260-series high-performance liquid chromatography (HPLC) system equipped with a mass spectrometric detector (Agilent Quadrupole LC/MS 6120). About 0.2 μL of each sample was applied to a Waters Xselect HSS T_3 (2.1 mm × 100 mm, 2.5 mm) chromatographic column, and column temperature was 30□. Mobile phase was composed of solvent A (acetonitrile) and solvent B (0.1% acetic acid aqueous solution). Gradient elution was as follows: 0–16 min starting with 15% A and ending with 25% A; 16–24 min starting with 25% A and ending with 40% A; 24–24.1 min starting with 40% A and ending with 15% A; and isocratic conditions (24.1–30 min) with 15% A and 85% B. For mass spectral conditions ESI, positive ion mode was used. Nitrogen was used as the collision gas and the desolvation gas flow rate was 10 L min^{-1}. The capillary voltage; desolvation temperature and desolvation pressure was 3.8 kV, 350□, 35 psig, respectively. In addition, selected ion monitoring (SIM) mode was used in the mass spectral acquisition. $[M + H]^+$ ions were observed at m/z 417(DG), 447(GLG), 433(GEG), 503(MD), 533(MGL), 459(AD), 489(AGL), 519(MG), 255 (DE), 475(AG), 285(GLE), and 271(GE).

2.4. *Statistical analyses*

Quantification data were subjected to SPSS (version 22.0; SPSS, Chicago, IL, USA) and the one-way ANOVA combined with Bonferroni correction multiple-range test was performed to analyze variance and significant differences among means. Z-Score was conducted before the data matrix finally subjected to principal component analysis (PCA) and hierarchical cluster analysis (HCA). The PCA and HCA were performed by SIMCA-P + 13.0 (Umetrix, Umeå, Sweden) and MetaboAnalyst version 3.0 (http://www.metaboanalyst.ca/), respectively. Then the data of isoflavones in soybean seeds were subjected to regression analyses to construct mathematical models with GraphPad Prism 7.

3. Results

3.1. *Comparative analysis of isoflavone accumulation in soybean seed in sole and intercropping systems*

This study compared the isoflavones accumulation of soybean seeds, which grown under sole-cropping and intercropping system. Liquid chromatography mass spectrometry (LC-MS) was used to detect isoflavones in soybean seeds, and the extracted ion chromatogram of mixed standard and sample is shown in Figure S1. In this study, the total isoflavones content of the five soybean cultivars were variously different. As shown in Figure 2, the highest total isoflavones content was found in Qihuang 35 (2.87 mg.g^{-1}), followed by Jidou15 as 2.80 mg.g^{-1}, while the Zhonghuang 41 with the lowest total isoflavones content of 2.20 mg.g^{-1}. In addition, Qihuang 34 is a special variety, which has higher malonylglucosides (1.80 mg.g^{-1}) and lower content of β-glucoside (0.64 mg.g^{-1}) and aglycone (0.02 mg.g^{-1}). And there were large differences of the soybean isoflavones content that grow under the two different planting patterns. Compared with the sole-cropping soybean, the total isoflavones content of intercropping soybean decreased with different degrees (0.85–8.21%) for all cultivars (Figure 2(d)). Especially to Qihuang 35, Qihuang 34 and Hedou 18, total isoflavones content of which were significantly decreased ($P < 0.05$) in intercropping system. In addition, β-glucosides and malonylglucosides were also decreased 2.22–6.37% and 5.33–8.13%, respectively, for different cultivars (Figure 2(a,b)). Although the content of β-glycoside of isoflavones was decreased not significantly, the malonylglucosides of Qihuang 35, Qihuang 34, Zhonghuang 41 and Hedou 18 was at the significant level. Distinctively, the aglycone of Zhonghuang 41 and Hedou 18 were higher in intercropping system (Figure 2(c)), although the content of Hedou 18 was not significant.

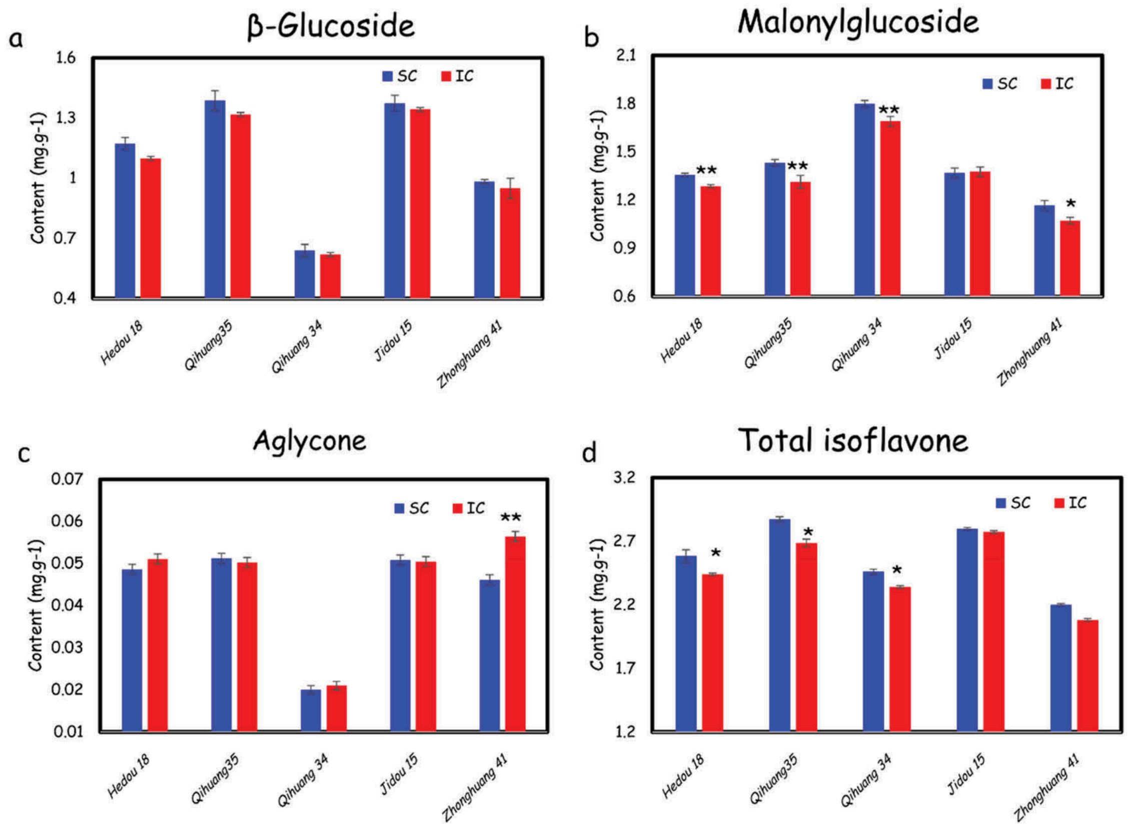

Figure 2. Differences of isoflavone constitutes between sole-cropping and intercropping system. *Represent significant difference ($P < 0.05$); **represent extremely significant difference ($P < 0.01$).

In order to investigate the effects of variety and intercropping shading on the accumulation of isoflavones in soybean seeds, two-factor analysis of variance (ANOVA) was performed on the total isoflavone, β-glucoside and malonylglucosides in soybean. as shown in the following Table 2–4, the results show that the variety and shading treatment have a significant impact on the accumulation of isoflavones, However, the interaction between the two factors has no significant effect on the accumulation of isoflavones.

3.2. *Effects of PAR transmittance on decrease level of soy isoflavones contents*

The microenvironment of intercropping soybean is changed markedly, especially the light environment of soybean growth. Because of the shading from maize leaves, the PAR (51%→30%) of soybean canopy decreased when maize to soybean row spacing decreased (70 cm→30 cm). In order to explore the relationship between the isoflavones contents of soybean seeds and the PAR transmittance, further regression analysis was conducted. Results showed that the linear regression model to the data fitting degree was optimal (R^2 > 0.78, P < 0.05). As malonylglucosides (1.80 mg.g^{-1}), GE (0.015 mg.g^{-1}), AD (0.0022 mg.g^{-1}) and total isoflavones (2.46 mg.g^{-1}) were significantly higher in sole-cropping soybean than that of heavily shaded soybean. Total isoflavones contents decreased with the PAR transmittance decreased (i.e. shade deepening), and this relationship was fit with a good linear equation with high determination coefficients (R^2 = 0.948, p = 0.005) (Figure 3 (a)). Detailed forms of isoflavones, i.e. β-glucoside (Figure 3 (b)), malonylglucoside (Figure 3(c)) and the trace isoflavones (AD+GE) in Figure 3(d) have similar trends as total isoflavones, and the determination coefficients were R^2 = 0.787 (p = 0.040), R^2 = 0.960 (p = 0.003), and R^2 = 0.893 (p = 0.015), respectively.

Table 1. Contents (mg.g^{-1}) of isoflavones in soybean with different intercropping system[a].

Isoflavone components	DG	GLG	GEG	MD	MGL	MG	AD	GE	β-Glucoside	Malonylglucosides	Total isoflavone
SC	0.47 ± 0.02a	0.03 ± 0.00a	0.14 ± 0.01ab	0.46 ± 0.02a	0.06 ± 0.00a	1.29 ± 0.04a	0.0022 ± 0.00a	0.015 ± 0.00a	0.64 ± 0.03a	1.80 ± 0.04ab	2.46 ± 0.05ab
A1	0.46 ± 0.01ab	0.02 ± 0.00a	0.17 ± 0.01a	0.45 ± 0.01a	0.05 ± 0.00ab	1.38 ± 0.07a	0.0021 ± 0.00a	0.015 ± 0.00a	0.65 ± 0.02a	1.88 ± 0.02a	2.55 ± 0.14a
A2	0.45 ± 0.01ab	0.03 ± 0.00a	0.14 ± 0.01ab	0.39 ± 0.02b	0.05 ± 0.00b	1.25 ± 0.04a	0.0024 ± 0.00a	0.015 ± 0.00a	0.62 ± 0.02ab	1.69 ± 0.03b	2.34 ± 0.107b
A3	0.42 ± 0.01c	0.03 ± 0.00a	0.13 ± 0.01b	0.34 ± 0.01c	0.05 ± 0.00c	1.09 ± 0.03b	0.0016 ± 0.00b	0.013 ± 0.00a	0.57 ± 0.01bc	1.47 ± 0.02c	2.07 ± 0.20c
A4	0.44 ± 0.01bc	0.02 ± 0.00a	0.13 ± 0.00b	0.32 ± 0.01cd	0.04 ± 0.00d	0.99 ± 0.04b	0.0015 ± 0.00b	0.009 ± 0.00b	0.59 ± 0.01b	1.34 ± 0.05c	1.95 ± 0.42c
A5	0.42 ± 0.00c	0.02 ± 0.00a	0.09 ± 0.00c	0.30 ± 0.01d	0.04 ± 0.00d	0.73 ± 0.08c	0.0014 ± 0.00b	0.010 ± 0.00b	0.54 ± 0.01c	1.07 ± 0.08d	1.63 ± 0.09d

[a] Each value represents the mean ± standard error (n = 9); values marked by the same letter within the same row are not significantly different (P□0.05). SC = sole-crop soybean samples; A1 = samples with 180 + 20 cm wide-narrow row planting; A2 = samples with 160 + 40 cm wide-narrow row planting; A3 = samples with 140 + 60 cm wide-narrow row planting; A4 = samples with 120 + 80 cm wide-narrow row planting; A5: samples with 100 + 100 cm wide-narrow row planting. DG: daidzin, GLG: glycitin, GEG: genistin, MD: malonyldaidzin, MGL: malonylglycitin, AD: acetyldaidzin, MG: malonylgenistin, GE: genistein,β-glucosides (DG + GLG + GEG);: malonylglucosides (MD+ MGL + MG); Total isoflavone (DG + GLG + GEG + MD+ MGL + MG +AD+GE)

Table 2. Two-way ANOVA of β-Glucoside.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.003	4	0.001	F (4, 20) = 0.374	P = 0.824
Variety	2.213	4	0.553	F (4, 20) = 238.1	P < 0.0001
Treatment	0.016	1	0.016	F (1, 20) = 6.835	P = 0.017
Residual	0.046	20	0.002		

Table 3. Two-way ANOVA of Malonylglucoside.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.0216	4	0.005	F (4, 20) = 2.218	P = 0.1036
Variety	0.729	4	0.182	F (4, 20) = 74.89	P < 0.0001
Treatment	0.023	1	0.024	F (1, 20) = 9.739	P = 0.0054
Residual	0.048	20	0.002		

Table 4. Two-way ANOVA of total isoflavones.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.022	4	0.005	F (4, 20) = 1.048	$P = 0.407$
Variety	1.778	4	0.445	F (4, 20) = 85.65	$P < 0.0001$
Treatment	0.109	1	0.109	F (1, 20) = 21.03	$P = 0.0002$
Residual	0.104	20	0.0052		

3.3. *Isoflavone profiles of soybean seeds grown under different row spacing of intercropping system*

To investigate the impact of intercropping row spacing on isoflavones profile of soybean seed, six field treatments, including sole cropping and five intercropping systems with different row spacing were imposed. Multivariate analysis of soybeans grown under different row spacing was conducted. The data matrix of their contents was normalized by Z score, and then a supervised multidimensional statistical method (PLS-DA) was further adopted. Two principal components were extracted using the PLS-DA model. R2X, R2Y and Q2Y of PLS-DA were 0.746, 0.919 and 0.852, respectively. This suggests that the PLS-DA model can make a correct interpretation of the variables. According to the PLS-DA model, two highest ranking components of PLS-DA resolved the measured composition profiles of slight shading treatments (SC, A1, and A2) from heavy shading treatments (A3, A4, and A5). This variation was attributed to all the isoflavones compounds, that means the heavy shading have lower content for all the eight isoflavones. The variables important in the projection (VIP) scores were examined to gain more insight into the isoflavones metabolic differences between the slight shading and heavy shading treatments. The variables with VIP values >1 are generally used as a criterion to identify the variables important to the model. The variables that played the greatest roles in distinguishing the different treatments were MD, MGL, MG, AD and DG (Figure 4(c)). To further explore the relationship among the six treatments and levels of the isoflavones, a heatmap was created to visualize the isoflavones metabolic differences among soybean samples (Figure 4(d)). The heat map showed a similar classification of various treatments with PLS-DA. Namely, all samples were correctly classified in the different treatments based on their isoflavones content, and samples in six treatments were separated into two parts. The green marked part was under slight shading (SS) while the red marked part was under heavy shading (HS). The most notable difference observed in these two parts was the SS parts, which showed higher levels of MD, MGL, MG, AD and GE. PLS-DA and heat map analysis showed A3 may as the demarcation point of these six intercropping treatments.

One-way ANOVA combined with Bonferroni correction multiple-range test was performed to analyze variance and significant difference. The contents of different forms of soybean isoflavones are shown in Table 1. As shown in Table 1, eight typical isoflavones belonging to four types (β-Glucoside, malonylglucoside,

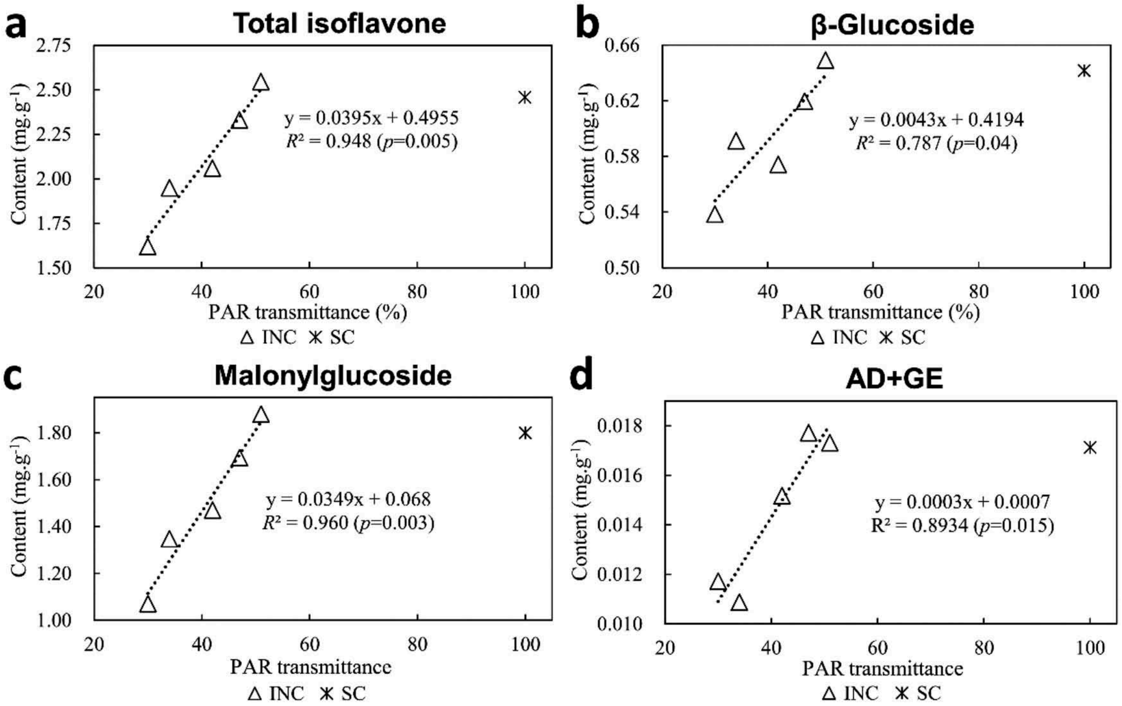

Figure 3. Effect of PAR transmittance on different forms of isoflavones in soybeans. a: total isoflavone; b: β-glucosides (DG + GLG + GEG); c: malonylglucosides (MD+ MGL + MG); d: trace isoflavones (AD+GE); INC: intercropping; SC: sole-cropping.

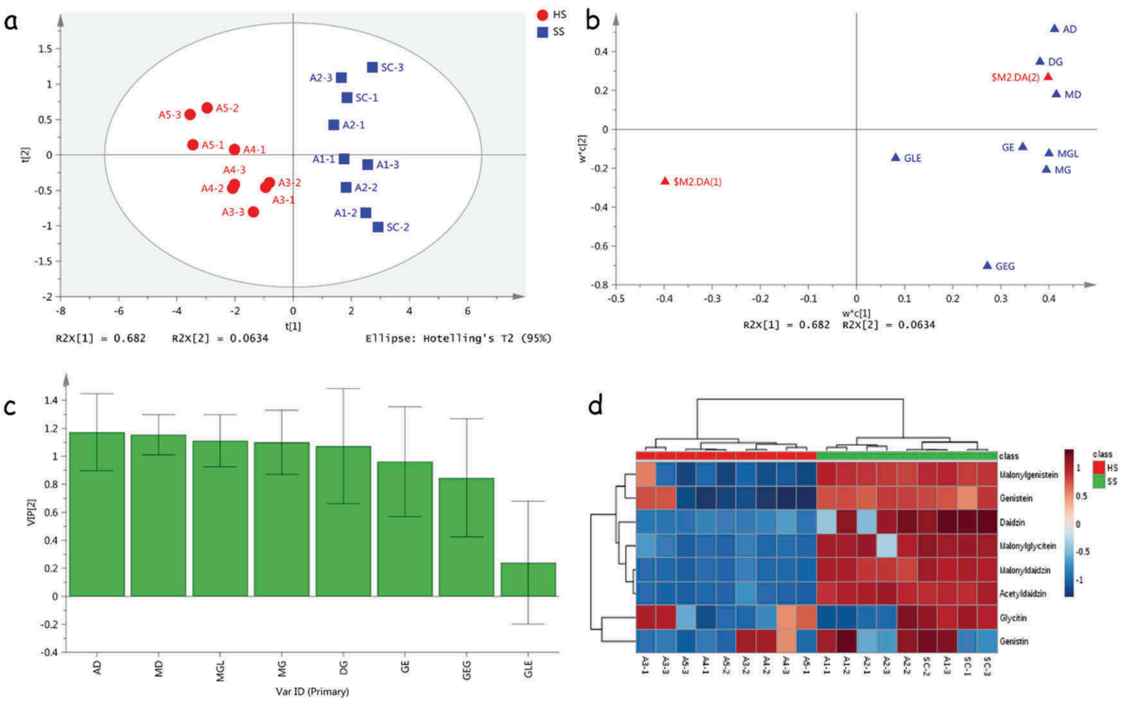

Figure 4. Multivariate analysis to separate the six treatments. (a) Complete overview of PLS-DA score plots. (b) loading plot of the PLS-DA. (c) VIP score of the isoflavones compounds. (d) Heatmap of soy-isoflavones grown with different row spacing. HS: heavy shading and SS: slight shading.

acetylglucoside, and aglycone) were detected. Both under the sole-cropping and intercropping systems, malonyl glucoside and β-glucoside were the dominant isoflavones in soybean seed, the concentrations are inconsistent with those previously detected in common soybean seeds (Kim et al., 2012). Thereinto, β-glucoside is composed of DG (0.42~0.47 mg.g^{-1}), GLG (0.02~0.03 mg.g^{-1}) and GEG (0.09~ 0.17 mg.g^{-1}); malonylglucosides is composed of MD (0.30~0.46 mg.g^{-1}), MGL (0.04~0.06 mg.g^{-1}) and MG (0.73~1.38 mg.g^{-1}). AD (0.0014~0.0024 mg.g^{-1}) and GE (0.009~0.015 mg.g^{-1}) represented acetyl glucosides and aglycones, respectively. The content of all forms of soybean isoflavones found in sole-cropping and intercropping were different. The isoflavones contents in 'A5' (most narrow row planting) were lowest (e.g. Total isoflavone 1.63 mg g^{-1}; β-glucoside 0.54 mg g^{-1}; malonylglucoside 1.07 mg g^{-1}) and significantly different from the soy-isoflavones content of sole-cropping. These observations are generally consistent with those obtained in experiment 1, but inconsistent with the research results in relay intercropping (Liu et al., 2016)

4. Discussion

Isoflavone is a group of antioxidant composition in soybean which participate in a series of important physiological process. Here, we designed experiments to explore the effect of intercropping conditions on soybean isoflavone metabolism. We plant soybean under the normal intercropping system and the distance between the maize and soybean strips was 60 cm. We found that the total isoflavones and malonylglucoside were decreased in intercropping soybeans compared to sole-cropping soybeans, particularly, in Qihuang 35, Qihuang 34 and Hedou 18. Previous studies indicated that dark environment was unfavorable to the isoflavone accumulation in soybean seeds (Phommalth, Jeong, Kim, Dhakal, & Hwang, 2008), sufficient light condition can trigger the biosynthesis of isoflavones. This might be related to the activity of PAL enzymes. The light energy obtained by intercropping soybean in the symbiotic period of maize-soybean is lower than that of sole-cropping (Yang et al., 2014), and the accumulation of organics in the leaves, stems and other organs may be also less than sole-cropping. Therefore, less isoflavone can be transferred from other organs to seeds during the development period. Additionally, there were response differences to intercropping among the tested five varieties. Isoflavones contents of Jidou 15 were not different between the two planting patterns, while the Qihuang 35 and Hedou 18 were decreased markedly. It is indicated that the sensitivity of different cultivars to intercropping shading may differ. Although total isoflavones contents in soybean seed were reduced in the intercropping system, the accumulation regularity of different type of isoflavone components was different; such as the significant increase of free aglycone content in Zhonghuang 41 grown under the intercropping condition. All above information confirmed that isoflavones accumulation is not only affected by environmental factors, but also regulated by genotype-specific traits. Furthermore, our previous studies indicated that aglycone in soybean seeds was associated with shade-resistant (Jiang, et al. 2017). Coincidently, Zhonghuang41 was evaluated as a shade sensitive variety in our previous studies; the increase of aglycone in intercropping soybean seed may be related to the specific shade response.

In fact, the photosynthetic radiation of soybean canopy was significantly affected in the intercropping system. We designed different maize-soybean row spacing to see how the PAR transmittance influence the isoflavones accumulation. And conducted regression analysis to study the relationship between the isoflavone and PAR transmittance. The results indicated that the quadratic model was fit for the experimental data well and the different type isoflavone presented the same tendency as the PAR transmittance decreased. Generally, when the PAR transmittance decreased, the isoflavone contents decreased. The PCA analysis (Figure 3) also confirmed the result and the six treatments were clearly divided into two groups. The first group contained A3, A4 and A5, whereas SC, A1 and A2 were included in the second group. Corresponding loading and HCA heatmap showed that higher content of MG, MGL, MD, DG, GE and AD was found in SC, A1 and A2 treatment. And the VIP plot showed that the intercropping environment mainly affects the accumulation of acylation isoflavones. Isoflavonoid accumulation in soybean seed is a result of both synthesis within the seed and transport from maternal tissues (Dastmalchi & Dhaubhadel, 2014). Previous studies have shown that light stimulates the synthesis of isoflavones like daidzin, genistin and their malonyl conjugates while darkness reduces the accumulation of isoflavones in cotyledons (Sun, 1998). And the activity of phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) required for isoflavones synthesis were stimulated by sunlight (Saengnil, 2011; Zoratti, Karppinen, Escobar, Häggman, & Jaakola, 2014). It indicated that the shading of maize leaves under intercropping conditions led to a decrease in light levels in the canopy of the soybean, which may inhibit expression of PAL and CHS to affect the synthesis of isoflavones in soybean seeds. In addition, some studies have shown that isoflavones can accumulate at stress sites (Gutierrezgonzalez et al., 2010; Malik, Pence, Calvert, & Bauer, 1984), For example, shaded signals increase the accumulation of soy-isoflavones directly at leaves, and drought stress increases the accumulation of soy-isoflavones directly at roots (Qin et al., 2017). Under intercropping conditions, the leaves are the direct light sensing sites. In order to cope with the weak light stress, the isoflavones synthesized in the soybean may be mainly accumulated in leaves, thereby reducing the accumulation in soybean seeds (Qin et al., 2017).

Interestingly, the observations described above are generally inconsistent with those obtained in our previous studies on the relay intercropping system, especially for the total isoflavones content (Liu et al., 2016). That may be related to light condition in these two different planting patterns. The Symbiotic period of the two crops under the relay intercropping system (42 days) is shorter than intercropping (106 days). And the relay intercropping mainly affected the vegetative growth stage (VE-R1) of soybean while the intercropping mainly affected the reproductive growth stage (R1-R8). Thus, intercropping soybeans were grown in weak light till harvest but relay intercropping soybeans undergo a light recovery process (Wu et al., 2016; Yang et al., 2017). When the maize was harvested, the soybean was suddenly exposed to strong light, the efficiency of photosynthesis can be significantly reduced. This effect is known as ‘photoinhibition’ and the primary target for light damage is photosystem II (Tyystjärvi, 2008). Previous studies have shown that phenolics, especially flavonoids, which accumulate in the epidermis can act as a darkening filter and shield the mesophyll from the excessive radiation (Solecka, 1997; Tattini et al., 2005). In addition, it is widely accepted that the enrichment of light can directly influence the basis material of quality formation and distribution of photosynthetic assimilation in the source and sink (Liu et al., 2006; Su et al., 2014). Previous studies have shown that after maize harvest in relay intercropping system, leaf area and leaf mass increased rapidly, contributing to compensation growth in intercropped soybean. Meanwhile, physiological and anatomical traits of leaf went back to similar levels as grown in sole cropping (Wu et al., 2016). Thus, the grains and leaves are capable of synthesis of more isoflavones and that more energy is available to help the accumulation of isoflavones into the grain. This may explain why relay intercropping increases the amount of isoflavones in soybean grains and intercropping decreases isoflavones.

Author Contributions

All authors contributed to this study. Wenyu Yang and Jiang Liu conceived and designed the experiments; Caiqiong Yang, Xiaochun Wang and Taiwen Yong performed the experiments; Caiqiong Yang, Baoyu Hu and Nasir Iqbal analyzed the data; Caiqiong Yang, Baoyu Hu and Nasir Iqbal wrote the paper. Jing Zhang, Weiguo Liu and Feng Yang provided research facilities.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was financially supported by the National Key Research and Development Program of China [Grant No. 2016YFD0300209]; the National Natural Science Foundation of China [Grant No. 31401329]; and the China Postdoctoral Science Foundation [Grant Nos. 2014 M560724 & 2017T100707];

ORCID

Caiqiong Yang http://orcid.org/0000-0003-3847-4167
Feng Yang http://orcid.org/0000-0003-3847-4167
Wen-yu Yang http://orcid.org/0000-0003-3847-4167

References

Cheng, Q., Li, N., Dong, L., Zhang, D., Fan, S., Jiang, L., … Zhang, S. (2015). Overexpression of soybean isoflavone reductase (GmIFR) enhances resistance to phytophthora sojae in Soybean. *Frontiers in Plant Science*, 6, 1024. doi:10.3389/fpls.2015.01024

Chennupati, P., Seguin, P., & Liu, W. (2011). Effects of high temperature stress at different development stages on soybean isoflavone and tocopherol concentrations. *Journal of Agricultural & Food Chemistry*, *59*, 13081–13088.

Dastmalchi, M., & Dhaubhadel, S. (2014). *Soybean seed isoflavonoids: Biosynthesis and regulation*. In: Jetter, R. (Eds.), *Phytochemicals – Biosynthesis, Function and Application. Recent Advances in Phytochemistry* (vol 44; pp. 1–21). Cham: Springer. doi:10.1007/978-3-319-04045-5_1

Fan, Y. F., Liu, Q. L., & Wang, R. (2017). Effects of shading on growth, photosynthetic fluorescence characteristics and yield of soybean in maize-soybean intercropping systems. *Journal of Nuclear Agricultural Sciences*, (5), 972–978. doi:10.11869/j.issn.100-8551.2017.05.0972

Fehr, W. R. (2007). Breeding for modified fatty acid composition in soybean. *Crop Science*, *47*, S-72-S-87.

Gutierrezgonzalez, J. J., Guttikonda, S. K., Tran, L. S. P., Aldrich, D. L., Zhong, R., Yu, O., … Sleper, D. A. (2010). Differential expression of isoflavone biosynthetic genes in soybean during water deficits. *Plant & Cell Physiology*, *51*, 936–948.

Huang, Y. L., Shen, C. C., Shen, Y. C., Chiou, W. F., & Chen, C. C. (2017). Anti-inflammatory and antiosteoporosis flavonoids from the rhizomes of helminthostachys zeylanica. *Journal of Natural Products*, *80*, 246–253.

Kim, E.-H., Ro, H.-M., Kim, S.-L., Kim, H.-S., & Chung, I.-M. (2012). Analysis of isoflavone, phenolic, soyasapogenol, and tocopherol compounds in soybean [Glycine max (L.) Merrill] germplasms of different seed weights and origins. *Journal of Agricultural & Food Chemistry*, *60*, 6045–6055.

Li, J., Yao, W., Gong, S., & Li, X. (2010). The research progress of the circumstance factors on the effect of soybean isoflavones content. *Chinese Agricultural Science Bulletin*, (9), 167–170.

Liu, J., Hu, B., Liu, W., Qin, W., Wu, H., Zhang, J., … Du, J. (2017). Metabolomic tool to identify soybean [Glycine max (L.) Merrill] germplasms with a high level of shade tolerance at the seedling stage. *Scientific Reports*, *7*, 42478.

Liu, J., Qin, W.-T., Wu, H.-J., Yang, C.-Q., Deng, J.-C., Iqbal, N., … Yang, W.-Y. (2017). Metabolism variation and better storability of dark-versus light-coloured soybean (Glycine max L. Merr.) seeds. *Food Chemistry*, *223*, 104–113. doi:10.1016/j.foodchem.2016.12.036

Liu, J., Yang, C. Q., Zhang, Q., Lou, Y., Wu, H. J., Deng, J. C., … Yang, W. Y. (2016). Partial improvements in the flavor quality of soybean seeds using intercropping systems with appropriate shading. *Food Chemistry*, *207*, 107–114.

Liu, X., Herbert, S. J., Hashemi, A. M., Litchfield, G. V., Zhang, Q., & Barzegar, A. R. (2006). Yield and yield components responses of old and new soybean cultivars to source-sink manipulation under light enrichment. *Plant Soil & Environment*, *52*, 150–158.

Liu, X., Rahman, T., Song, C., Su, B., Yang, F., Yong, T., … Yang, W. (2017). Changes in light environment, morphology, growth and yield of soybean in maize-soybean intercropping systems. *Field Crops Research*, *200*, 38–46.

Malik, N. S., Pence, M. K., Calvert, H. E., & Bauer, W. D. (1984). Rhizobium infection and nodule development in soybean are affected by exposure of the cotyledons to light. *Plant Physiology*, *75*, 90–94.

Moriri, S., Owoeye, L. G., & Mariga, I. K. (2010). Influence of component crop densities and planting patterns on maize production in dry land maize/cowpea intercropping systems. *African Journal of Agricultural Research*, *5*, 1200–1207.

Phommalth, S., Jeong, Y. S., Kim, Y. H., Dhakal, K. H., & Hwang, Y. H. (2008). Effects of light treatment on isoflavone content of germinated soybean seeds. *Journal of Agricultural & Food Chemistry*, *56*(21), 10123–10128.

Qin, W. T., Feng, Y. R., Lei, Z., Yang, C. Q., Hai-Jun, W. U., Iqbal, N., … Liu, J. (2017). Effect of shading signal on isoflavone biosynthesis of soybean seedling. *Natural Product Research & Development*, *9*, 1470–1474.

Saengnil, K. (2011). Sunlight-stimulated phenylalanine ammonia-lyase (PAL) activity and anthocyanin accumulation in exocarp of 'Mahajanaka' mango. *Maejo International Journal of Science & Technology*, *5*, 365–373.

Solecka, D. (1997). Role of phenylpropanoid compounds in plant responses to different stress factors. *Acta Physiologiae Plantarum*, *19*, 257–268.

Su, B. Y., Song, Y. X., Song, C., Cui, L., Yong, T. W., & Yang, W. Y. (2014). Growth and photosynthetic responses of soybean seedlings to maize shading in relay intercropping system in Southwest China. *Photosynthetica*, *52*, 332–340.

Sugiyama, A., & Yazaki, K. (2014). Flavonoids in plant rhizospheres: Secretion, fate and their effects on biological communication. *Plant Biotechnology*, *31*, 431–443.

Sun, J. (1998). Light effect on the content and distribution of isoflavones in the developing seedling tissues of soybean. *Journal of Integrative Plant Biology*, *40*, 1015–1021.

Tattini, M., Guidi, L., Morassi-Bonzi, L., Pinelli, P., Remorini, D., Degl'Innocenti, E., … Agati, G. (2005). On the role of flavonoids in the integrated mechanisms of response of Ligustrum vulgare and Phillyrea latifolia to high solar radiation. *The New Phytologist*, *167*, 457–470.

Tyystjärvi, E. (2008). Photoinhibition of photosystem II and photodamage of the oxygen evolving manganese cluster. *Coordination Chemistry Reviews*, *252*, 361–376.

Vamerali, T., Barion, G., Hewidy, M., & Mosca, G. (2012). Soybean isoflavone patterns in main stem and branches as affected by water and nitrogen supply. *European Journal of Agronomy*, *41*, 1–10.

Wu, H.-J., Deng, J.-C., Yang, C.-Q., Zhang, J., Zhang, Q., Wang, X.-C., … Liu, J. (2017). Metabolite profiling of isoflavones and anthocyanins in black soybean [Glycine max (L.) Merr.] seeds by HPLC-MS and geographical differentiation analysis in Southwest China. *Anal. Methods*, *9*, 792–802.

Wu, Y., Gong, W., Yang, F., Wang, X., Yong, T., & Yang, W. (2016). Responses to shade and subsequent recovery of soya bean in maize-soya bean relay strip intercropping. *Plant Production Science*, *15*, 1–9.

Xie, L., Zhao, W., & Shen, L. (2000). Light regulation of the expression of PAL gene in soybean leaves and isoflavone synthesis. *Bulletin of Botany,* (5), 443. doi:10.3969/j.issn.1674-3466.2000.05.011

Yan, W., Yan, Y. H., Xiang, D. B., Ye, M. Y., Yang, W. Y., & Liu, J. (2015). Isoflavonoid accumulation pattern as affected by shading from maize in soybean (Glycine max (L.) Merr.) in relay strip intercropping system. *Plant Production Science*, *18*, 302–313.

Yang, C.-Q., Iqbal, N., Hu, B.-Y., Zhang, Q.-H., Wu, H.-J., Liu, X., … Liu, J. (2017). Targeted metabolomics analysis of fatty acids in soybean seeds using GC-MS to reveal the metabolic manipulation of shading in the intercropping system. *Anal. Methods*, *9*, 2144–2152.

Yang, F., Huang, S., Gao, R., Liu, W., Yong, T., Wang, X., … Yang, W. (2014). Growth of soybean seedlings in relay strip intercropping systems in relation to light quantity and red: Far-redratio. *Field Crops Research*, *155*, 245–253.

Yang, W. Y., & Agronomy, C. O. (2015). Effects of shade and lighting recovery on growth and yield of soybean in maize-soybean relay strip intercropping. *Chinese Journal of Oil Crop Sciences*, (4), 475–480. doi:10.7505/j.issn.1007-9084.2015.04.007

Zhang, C., Wang, X., Zhang, F., Dong, L., Wu, J., Cheng, Q., … Fan, S. (2017). Phenylalanine ammonia-lyase2.1 contributes to the soybean response towards Phytophthora sojae infection. *Scientific Reports*, *7*, 7242.

Zoratti, L., Karppinen, K., Escobar, A. L., Häggman, H., & Jaakola, L. (2014). Light-controlled flavonoid biosynthesis in fruits. *Frontiers in Plant Science*, *5*, 534.