Isoflavonoid Accumulation Pattern as Affected by Shading from Maize in Soybean (Glycine max (L.) Merr.) in Relay Strip Intercropping System

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Abstract: Soybean is the most common resource of isoflavonoid in human food. Wide development of relay strip intercropped soybean has contributed to the soybean industry in China. Due to the shading from maize, growth and grain production of soybean is reduced. However, whether soybean isoflavonoid accumulation pattern is influenced in the relay strip intercropping system is still unclear. Here, we studied the accumulation patterns of soybean isoflavones in the relay strip intercropping system and sole cropping system at the per-harvest stage. The accumulation patterns of soybean isoflavones at the postharvest stage were also studied. The results indicated that accumulation patterns of soybean isoflavones of all varieties in leaf and seed in the two systems were similar, but the trend was delayed in the relay strip intercropping system compared with the sole cropping system. During the pod filling stage, the total content of isoflavone, M-type isoflavone content, G-type isoflavone content in seed in the relay strip intercropping system were lower than those in the sole cropping system. During the after-ripening period, seed isoflavone content, M-type isoflavone content, G-type isoflavone content in seed increased in both systems, but were higher in the relay strip intercropping system. The temperature and photosynthetically active radiation were significantly lower in relay intercropping than in sole cropping, while relative humidity showed the opposite trend. Path analysis showed that total isoflavone content in leaves and seeds showed a significantly positive correlation with temperature and photosynthetically active radiation, but significantly negative correlation with relative humidity. Correlation analysis between the highest isoflavone content in sole cropping or relay intercropping seeds and agronomic traits revealed a significant positive correlation between the number of both branches and pods with total isoflavone, M-type isoflavone and G-type isoflavone in both systems.

Key words: Accumulation, Post-ripening period, Relay strip intercropping system, Soybean isoflavones.

Soybean [Glycine max (L.) Merr.] is one of the oldest cultivated crops worldwide. Global soybean production is estimated at 251.5 million metric tons with the US as the leading producer. As consumers are becoming increasingly aware of the health benefits of soy (Barnes, 1998), attention has turned to the generation of soybeans that provide improved taste, functional characteristics, and health benefits (MacDonald et al., 2005). Soybeans are a rich source of protein, isoflavones, saponins, oils, fatty acids and fiber. Isoflavones, considered secondary metabolites in soybean, belong to a group of compounds that share a basic structure consisting of two benzyl rings joined by a three-carbon bridge, which may or may not be closed in a pyran ring (Liu, 1997; Robinson et al., 1995). Naturally occurring soy isoflavones mainly include four groups, aglycons (daidzein, genistein, and glycitein), glucosides (daidzin, glycitin, and genistin), acetylglucosides (6'-O-acetyldaidzin, 6'-O-acetylgenistin, and 6'-O-acetylglycitin) and malonylglucosides (6'-O-malonyldaidzin, 6'-O-malonylgenistin, and 6'-O-malonylglycitin) (Kim et al.,...
Isoflavone biosynthesis is affected by several factors, and genotype, environment, cultivation conditions and other conditions during seed maturation (Wang and Murphy, 2002; Hikosaka et al., 2004; Hu and Knobf, 2004; Vastag, 2007; Ma et al., 2008; Taylor et al., 2009; Kang et al., 2010). Isoflavone biosynthesis is affected by several factors, and their concentration and profile mainly depend on genotype, environment, cultivation conditions and other conditions during seed maturation (Wang and Murphy, 1994; Caldwell et al., 2005; Chennupati et al., 2011; Vamerali et al., 2012). Hoeck et al. (2000) thought genotype was the most important factor determining the individual isoflavone forms and total isoflavone contents. Eldridge (1982) and Carrão-Panizzi and Kitamura (1995) indicated that environmental conditions can cause significant variations in soybean cultivars. Figallo (2003) and Lee et al. (2003) found that variation in isoflavone content of soybean cultivars was strongly related to location and storage duration. Cultivation such as irrigation, potassium management, and nitrogen supply had a strong effect on isoflavone biosynthetic pathway and the concurrent extent of seed bulging (Vyn et al., 2002; Bennett et al., 2004; Al-Tawaha et al., 2007; Vamerali et al., 2012). Light condition was a limiting factor for isoflavone accumulation, which affected the expression of phenylalanine ammonia-lyase (PAL) (Xie et al., 1999).

Multiple cropping is a common system (Awal et al., 2006), that has contributed greatly to total grain yield all over the world. In China, the maize-soybean relay strip intercropping system (RC) is a practice particularly important in the southwestern regions (Yan et al., 2010; Zhang et al., 2011a, 2011b; Xiang et al., 2012; Yang et al., 2014), and is expected to help expand the soy isoflavone industry (Ye et al., 2010). However, RC affects the micro-environment of soybean growth differently from that of the sole cropping system (SC) (Yan et al., 2010; Liu et al., 2011; Zhang et al., 2011; Yang et al., 2014). At the soybean seeding growth stage, the spectral irradiance, light intensity, red to far red (R/FR) ratio and photosynthetically active radiation were decreased in RC compared to SC (Liu et al., 2011; Yan et al., 2011; Zhang et al., 2011; Yang et al., 2014). Temperature in RC was lower while relative humidity was higher than in SC (Wang et al., 2007). However, soybean seed is difficult to harvest and thresh under the high relative humidity condition in Sichuan province during the soybean harvest time. Soybean requires a dry condition during the post-ripening period (usually a month or even longer). Physiological and microbiological changes occur during this period (Ye et al., 2010). The objective of this research was to explore the difference in isoflavone content in leaves and seeds at the pre-harvest growth stage and isoflavone content in seeds during the post-ripening period in RC and SC. This was to provide an integrated view of all of the elements that affect isoflavone content in RC.

Materials and Methods

1. Plant materials and treatments

The experiment was performed at the farm of Sichuan Agricultural University (Ya’an, 29°50’N, 103°00’E) during the growing season of 2009 (Weather data shown in Table 1). The experiment was laid out in a two-factor randomized complete block design with four replications. Four soybean [Glycine max (L.) Merr.] cultivars that differed significantly in isoflavone content of seed were used in the experiment (Table 2). Among them, Gongxuan No.1 was a major South-western soybean cultivar. Two cropping systems, RC and SC were used. There were 32 plots in total, and the plot size was 2 m × 5 m.

Soybean was sown on June 7th in both systems. For RC, 2 m wide strips were prepared in the field. Maize (cv. Zhenghong 311) was sown in a strip with a width of 1 m on April 2nd and harvested on August 9, 2009. Two maize plants were kept in each hole with 50 cm row spacing and 38 cm hole spacing. Soybean was sown on another strip with a width of 1 m, the row spacing and hole spacing were 33 cm and 30 cm, respectively. For SC, soybean was planted with 33 cm row spacing and 30 cm hole spacing. Five seeds were sown in each hole, but only two plants were allowed to grow.

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**Table 1. Weather data for soybean growth stage.**

| Month  | Max temperature (ºC) | Min temperature (ºC) | Average temperature (ºC) | Relative humidity (%) | Precipitation (mm) | Day length per month (h) |
|--------|----------------------|----------------------|--------------------------|-----------------------|-------------------|-------------------------|
| May    | 22.2                 | 15.3                 | 20.5                     | 46.7                  | 69.0              | 172.1                   |
| June   | 29.4                 | 18.2                 | 23.6                     | 42.9                  | 143.4             | 178.3                   |
| July   | 27.5                 | 20.4                 | 25.1                     | 43.2                  | 219.6             | 202.1                   |
| August | 26.2                 | 23.2                 | 24.8                     | 39.2                  | 170.3             | 207.5                   |
| September | 25.4               | 19.5                 | 23.1                     | 42.3                  | 122.7             | 169.4                   |
| October| 20.1                 | 12.4                 | 17.4                     | 44.3                  | 59.1              | 141.2                   |

Data was from the Sichuan Province Meteorological Institute.
2. Measurement techniques

(1) Sampling technique

All the samples (leaf, pod, or seed) from each plot with four biological replicates were taken randomly. The mean value of each plot was obtained from the four biological replicates.

The top third trifoliolate leaves were collected randomly every two weeks from the third trifoliolate stage (V3 stage) to full pod stage (R4) (Table 3). The leaf samples (0.5 g) were immediately frozen in liquid nitrogen, and stored at –70°C until measurement.

Flowers that bloomed on the same day were marked. Pods from the top, middle, and under layer of a plant were taken randomly 0800 – 1000 every 7 day from 30 days after flowering (DAF) (Table 4). Seeds (3 g) were frozen in liquid nitrogen and stored in –70°C before test.

The whole plants with leaves, stems, pods and roots were hung in an airy place during the period of natural air drying. Seed samples were taken randomly every 7 days after harvest (DAH), then were frozen in liquid nitrogen immediately, and stored in ~70°C. The seed samples were shattered to a seed powder (smaller than 0.25 mm), and only 1 g was used for measurements.

(2) Isoflavone detection

Water content of fresh samples was determined from the weights obtained before and after the oven drying Primary stock standard solutions of daidzin, genistin, and glycitin were each dissolved in acetonitrile, to give a concentration of 0.2 mg / mL, filtered through a 0.45 μm membrane filter and stored at 4ºC. Working mixed standard solutions were prepared daily by mixing and diluting the stock solutions with acetonitrile. The mixed standard solutions were 0.004, 0.008, 0.012, 0.016, 0.020, 0.024, 0.028 mg mL⁻¹ for daidzin, 0.0008, 0.0016, 0.0024, 0.0032, 0.0040, 0.0048, 0.0056 mg mL⁻¹ for glycitin, and 0.004, 0.008, 0.012, 0.016, 0.020, 0.024, 0.028 mg mL⁻¹ for genistin According to the peak area at different concentrations of standard solutions, the regression equations were Y = 62057X – 20.817 (R = 0.9996**), Y = 95292X – 8.0917 (R = 0.9994**), Y = 84904X – 5.4417 (R = 0.9997**) for daidzin, glycitin and genistin, respectively.to determine quantitatively the contents of the isoflavones.

Soybeans were ground in a grinder and extracted with 80% ethanol. The mixture was extracted by ultrasonic treatment for 30 min at 4°C and then centrifuged at 5000 rpm, 4°C for 10 min. Then the supernatant was transferred to a 50-mL volumetric flask, and 80% ethanol was used to
dilute the supernatant to volume, filtered through a 0.45 μm membrane filter before injection.

All HPLC analyses were performed using an Agilent 1100 series HPLC chromatograph with a variable wavelength UV-d detector system, Hypersil Classical Packing Materials and Column-Hypersil ODS C18 (4.6 × 250 mm, 5 μm), a linear gradient of 0.3% acetic acid and 5% acetonitrile in water (A) and 0.3% acetic acid in acetonitrile (B) as follows: 0 min (90%A) → 10 min (88%A) → 15 min (85%A) → 20 min (80%A) → 30 min (30%A) → 35 min (30%A). The injection volume was 15 μL, the temperature of column was 4ºC and the flow rate was 1.0 mL min⁻¹. Spectral data were collected over the run and isoflavone elution was monitored at 254 nm. Data were obtained using ChemStation software (Agilent). Daidzin, glycitin and genistin contents were calculated according to the chromatographic peak area of the samples. Isoflavone contents were calculated on a dry weight basis. Malonyl isoflavone glucosides were obtained by alkali hydrolysis (Zhang et al., 2006; Ye et al., 2010). Optimum conditions for extraction of isoflavones with ethanol and hydrolysis of malonyl isoflavone glucosides were obtained preliminarily. The optimum condition for extraction of isoflavones with ethanol was sample to ethanol solution ratio of 1:40, and incubation for 30 min. The optimum conditions for hydrolysis to obtain malonyl isoflavone glucosides were solution pH 13, incubation for 40 min at 65ºC. Different monomer malonyl isoflavone glucosides was calculated by chromatographic peak area and the conversion factor (Zhang et al., 2006). The conversion factors were 4.875 mal⁻³, 2.676 mal⁻³, and 1.818 mal⁻³ for 6'-O-malonyldaidzin, 6'-O-malonylgenistin and 6'-O-malonylglycitin, respectively.

(3) Agronomic parameters
Ten plants selected randomly for each treatment were analyzed in four replicates. Plant height, stem diameter, node numbers, and branch numbers were determined at maturity. Yield components and grain yield were also measured. Number of pods per plant, 100-seed weight, and seed yield were recorded.

(4) Micro-climate parameters
The light environment characteristics in RC and SC were measured using AvaField (Avantes Corporation, Netherlands). Photosynthetically active radiation was estimated by integrating the spectral energy between 400 nm and 700 nm. Temperature and relative humidity were recorded.

(5) Data collection and statistical analysis
Means of four replications were calculated. Statistical analyses were performed by Excel 2003 and SPSS 13.0 software package (Chicago, IL, USA). A probability level (P) of 0.05 was used as the threshold for detection of isoflavone content.

Results
1. Leaf isoflavone contents during soybean growth stage
Isoflavone contents in leaves in both SC and RC showed the same slow increment-relative stability-sudden drop trend during the growth stage (Table 5). In SC, leaf isoflavones of all varieties peaked at 67 days after sowing except for B4, while leaf isoflavones of B3 and B4 in RC peaked at the same day of that in SC, but B1 and B2 in RC delayed the top value for one week compared with SC. Leaf isoflavones contents of all varieties in SC were higher than that in RC during the increasing period, but leaf isoflavone contents showed the opposite trend during the sudden drop period. Among all soybean varieties, leaf isoflavone content of B1 was significantly higher than in other varieties at different growth stages, and the top values in SC and RC were 0.497 and 0.476 mg g⁻¹, respectively.

Table 5. Isoflavone content in soybean leaves (mg g⁻¹).

| Varieties | Planting patterns | Days after sowing (d) |
|-----------|-------------------|-----------------------|
|           |                   | 25  | 39  | 53  | 67  | 81  | 95  | 109 |
| B1        | SC                | 0.289 a | 0.368 a | 0.454 a | 0.497 a | 0.487 a | 0.462 a | 0.194 b |
|           | RC                | 0.262 a | 0.316 b | 0.368 b | 0.462 b | 0.476 a | 0.466 a | 0.212 a |
| B2        | SC                | 0.211 a | 0.242 a | 0.281 a | 0.312 a | 0.302 a | 0.136 b | –         |
|           | RC                | 0.185 b | 0.202 b | 0.233 b | 0.285 b | 0.294 a | 0.156 a | –         |
| B3        | SC                | 0.214 a | 0.232 a | 0.275 a | 0.294 a | 0.159 a | –         | –         |
|           | RC                | 0.189 b | 0.209 a | 0.247 b | 0.266 b | 0.174 a | –         | –         |
| B4        | SC                | 0.221 a | 0.239 a | 0.264 a | 0.298 a | 0.310 a | 0.317 a | 0.134 b |
|           | RC                | 0.195 b | 0.217 b | 0.235 b | 0.267 b | 0.286 b | 0.301 a | 0.162 a |

Within columns, means followed by the same lower-case letters are not significantly at the 0.05 level of probability. SC: Sole cropping system, RC: Relay strip intercropping system. B1: Weiyuan, B2: Zhongdou No.8, B3: Nandou No.5, B4: Gongxuan No.1. The timing of maize harvest in RC was August 9, 2009, 32 days after soybean sowing.
During the seed during pod filling stage, the contents of M-type and G-type isoflavones changed in a pattern similar to that of total isoflavones (Table 7). The average rates of accumulation of M-type isoflavones in four varieties during the early accumulation period were 0.0121 and 0.0149 mg (g d)\(^{-1}\), in SC and RC, respectively, which accounted for 15.35% and 23.01%, respectively, of the total M-type isoflavones. During the period of rapid accumulation, the

\[0.0555 \text{ and } 0.0570 \text{ mg (g d)}^{-1}\], respectively, in the early accumulation period, and

\[0.0555 \text{ and } 0.0570 \text{ mg (g d)}^{-1}\], respectively, in the rapid accumulation period.

### Table 6. Total isoflavone content in soybean seeds at pod filling stage (mg g\(^{-1}\)).

| Varieties | Planting patterns | Days after flowering (d) |
|-----------|-------------------|--------------------------|
|           | 30                | 37                       | 44 | 51 | 58 | 65 | 72 |
| B1        | SC                | 0.086 b                  | 0.327 a                  | 0.480 a                  | 1.260 a                  | 1.619 b                  | 2.615 a                  | –             |
|           | RC                | 0.143 a                  | 0.328 a                  | 0.376 b                  | 0.860 b                  | 1.815 a                  | 2.303 b                  | –             |
| B2        | SC                | 0.252 a                  | 0.321 a                  | 0.419 a                  | 0.716 a                  | 0.825 b                  | 1.093 a                  | –             |
|           | RC                | 0.291 a                  | 0.301 a                  | 0.379 a                  | 0.547 a                  | 0.912 a                  | 0.991 b                  | –             |
| B3        | SC                | 0.384 a                  | 0.608 a                  | 0.914 a                  | 1.116 b                  | 1.626 a                  | –             | –             |
|           | RC                | 0.337 b                  | 0.465 b                  | 0.769 a                  | 1.258 a                  | 1.527 a                  | –             | –             |
| B4        | SC                | 0.103 a                  | 0.162 b                  | 0.285 a                  | 0.531 a                  | 0.739 b                  | 1.152 b                  | 1.513 a       |
|           | RC                | 0.124 a                  | 0.178 a                  | 0.302 a                  | 0.42 b                   | 0.865 a                  | 1.347 a                  | 1.459 a       |

Within columns, means followed by the same lower-case letters are not significantly at the 0.05 level of probability. SC: Sole cropping system, RC: Relay strip intercropping system. B1: Weiyuan, B2: Zhongdou No.8, B3: Nandou No.5, B4: Gongxuan No.1. The timing of maize harvest in RC was August 9, 2009, 32 days after soybean sowing.

### Table 7. M-type and G-type isoflavone content in soybean seeds at pod filling stage (mg g\(^{-1}\)).

| Type | Varieties | Planting patterns | Days after flowering (d) |
|------|-----------|-------------------|--------------------------|
|      |           | 30                | 37                       | 44 | 51 | 58 | 65 | 72 |
| B1   | SC        | 0.000 b           | 0.237 a                  | 0.339 a                  | 0.868 a                  | 1.149 a                  | 1.955 a                  | –             |
|      | RC        | 0.081 a           | 0.241 a                  | 0.256 b                  | 0.628 b                  | 1.238 a                  | 1.673 b                  | –             |
| M    |           | 0.197 a           | 0.261 a                  | 0.341 a                  | 0.568 a                  | 0.657 b                  | 0.907 b                  | –             |
|      |           | 0.186 a           | 0.244 a                  | 0.311 a                  | 0.449 b                  | 0.713 a                  | 0.771 b                  | –             |
| B2   | SC        | 0.384 a           | 0.608 a                  | 0.914 a                  | 1.116 b                  | 1.626 a                  | –             | –             |
|      | RC        | 0.337 b           | 0.465 b                  | 0.769 a                  | 1.258 a                  | 1.527 a                  | –             | –             |
| G    |           | 0.065 b           | 0.118 a                  | 0.210 b                  | 0.389 a                  | 0.578 b                  | 0.919 b                  | 1.230 a       |
|      | B1        | 0.083 a           | 0.131 a                  | 0.239 a                  | 0.334 b                  | 0.654 a                  | 1.100 a                  | 1.197 b       |
| B3   | SC        | 0.086 a           | 0.091 a                  | 0.141 a                  | 0.392 a                  | 0.470 b                  | 0.660 a                  | –             |
|      | RC        | 0.062 a           | 0.087 a                  | 0.120 a                  | 0.232 b                  | 0.577 a                  | 0.630 a                  | –             |
| G    |           | 0.053 a           | 0.059 a                  | 0.079 a                  | 0.142 a                  | 0.168 a                  | 0.183 b                  | –             |
|      | B2        | 0.045 a           | 0.056 a                  | 0.069 a                  | 0.101 b                  | 0.197 a                  | 0.219 a                  | –             |
| B3   | SC        | 0.106 a           | 0.141 a                  | 0.235 a                  | 0.291 a                  | 0.316 a                  | –             | –             |
|      | RC        | 0.118 a           | 0.133 a                  | 0.176 b                  | 0.251 b                  | 0.307 b                  | –             | –             |
|      | B4        | 0.038 a           | 0.044 a                  | 0.075 a                  | 0.142 a                  | 0.161 b                  | 0.233 a                  | 0.283 a       |
|      | RC        | 0.041 a           | 0.047 a                  | 0.063 a                  | 0.086 b                  | 0.209 a                  | 0.247 a                  | 0.262 a       |

Within columns, means followed by the same lower-case letters are not significantly at the 0.05 level of probability. SC: Sole cropping system, RC: Relay strip intercropping system. B1: Weiyuan, B2: Zhongdou No.8, B3: Nandou No.5, B4: Gongxuan No.1. The timing of maize harvest in RC was August 9, 2009, 32 days after soybean sowing.

2. **Seed isoflavones during pod filling stage**

At the pod filling stage, seed isoflavone contents increased with the growth stage (Table 6). Seed isoflavone contents in RC were lower than those in SC at the harvest time, the differences between RC and SC in B1 and B2 were significant. The average seed isoflavone content was significantly higher in B1 than in other varieties. According to accumulation rate, pod filling stage could be classified into two stages, the early accumulation period and rapid accumulation period. The average accumulation rates of the four varieties were 0.0197 and 0.0208 mg (g d)\(^{-1}\), in SC and RC, respectively, in the early accumulation period, and 0.0555 and 0.0570 mg (g d)\(^{-1}\), respectively, in the rapid accumulation period.

During the seed during pod filling stage, the contents of M-type and G-type isoflavones changed in a pattern similar to that of total isoflavones (Table 7). The average rates of accumulation of M-type isoflavones in four varieties during the early accumulation period were 0.0121 and 0.0149 mg (g d)\(^{-1}\), in SC and RC, respectively, which accounted for 15.35% and 23.01%, respectively, of the total M-type isoflavones. During the period of rapid accumulation, the
Fig. 1. Effect of cropping system on total isoflavone content in soybean seed during post-ripening period. Comparative graph of total isoflavone content during post-ripening period (mg g$^{-1}$). SC: Sole cropping system, RC: Relay strip intercropping system. B: Soybean varieties, B1: Weiyuan, B2: Zhongdou No.8, B3: Nandou No.5, B4: Gongxuan No.1.

Fig. 2. Effect of cropping system on M-type isoflavone content in soybean seed during post-ripening period (mg g$^{-1}$). Comparative graph of total isoflavone content during post-ripening period (mg g$^{-1}$). SC: Sole cropping system, RC: Relay strip intercropping system. B: Soybean varieties, B1: Weiyuan, B2: Zhongdou No.8, B3: Nandou No.5, B4: Gongxuan No.1.
average rates of accumulation of M-type isoflavones in the four varieties were 0.0453 and 0.0470 mg (g d\(^{-1}\)), in SC and RC, respectively, and the accumulation of total M-type isoflavones during this period was 84.18% and 63.70%, respectively. At harvest, the contents of M-type isoflavones in all varieties except B3 were significantly higher in SC than in RC, and the content was significantly higher in B1 than in other varieties.

At harvest, the content of G-type isoflavones was higher in SC than in RC, while that in B2 was lower (Table 7). The content of G-type isoflavones was significantly higher in B1 than in the other varieties. The average rate of accumulation of M-type isoflavones in four varieties at the early accumulation period was 0.0031 and 0.0041 mg (g d\(^{-1}\)), in SC and RC, respectively. During the rapid accumulation period, while those were 0.0111 and 0.0124 mg (g d\(^{-1}\)) during the rapid period. At harvest, the content of M-type isoflavones was higher than that of G-type isoflavones.

3. Postharvest isoflavone contents

As shown in Fig. 1, in SC, total seed isoflavones in B1, B2, B3 and B4 peaked at 7, 7, 21 and 14 DAH, respectively. However, in RC, the peak was for 7 days, or even 14 days. In SC, the positive accumulation rate of seed isoflavones in both B1 and B2 was from 0 to 7 DAH, while that in B3 and B4 was from 0 to 21 DAH and from 0 to 14 DAH, respectively. The content of isoflavones in seed of B1 in RC increased up to 14 DAH, while that of B2 and B4 steadily increased up to 21 DAH. Thereafter, the isoflavone content in all varieties decreased more in RC than in SC.

During the post-ripening period, the contents of M-type (Fig 2) and G-type (Fig 3) isoflavones in seeds showed a change similar to that of total isoflavones. The time of the maximum M-type and G-type isoflavone contents was coincident with that of the total seed isoflavone content. The M-type and G-type isoflavone contents showed peak values at the same time (7 to 21 days) as total seed isoflavones. The content of G-type isoflavones during post-ripening was lower than that of M-type isoflavones.

4. Seed isoflavone accumulation

The contents of total isoflavone in soybean seeds in SC and RC are shown in Table 8. The maximum isoflavone contents during the post-ripening period in soybean varieties B1 and B3 were higher in SC than in RC. Maximum isoflavone content was significantly higher in B1 than in the other varieties in both systems. At harvest, the isoflavone seed contents of B1 were higher in SC than in RC. Isoflavone accumulated before harvest occupied 64.25 – 90.33% and 61.08 – 85.27% of maximum isoflavone content in SC and RC, respectively. During the post-ripening period, isoflavone increment in B2 and B4 was
higher in RC than in SC, and isoflavone contents accumulated during the post-ripening period accounted for a higher percentage of maximum isoflavone content of B1, B2 and B4 in RC than in SC.

As shown in Table 9, the maximum M-type and G-type isoflavones was significantly higher in B1 than in the other varieties in both SC and RC. The maximum content of M-type isoflavones in B1 and B3 was higher in SC, while the maximum content of G-type isoflavones in all varieties except B1 was higher in RC. M-type isoflavones accumulated during the post-ripening period accounted for 8.15 – 43.14% of the maximum isoflavone content in all varieties, while G-type isoflavone accounted for 9.85 – 27.5%.

5. The relationship between micro-climate and isoflavones

In the strips where soybean was grown in RC, temperature and photosynthetically active radiation were much lower than those in SC, while relative humidity was higher than that in SC (Table 10). Path analysis (Table 11, 12 and 13) revealed that temperature and photosynthetically active radiation had positive direct effects on leaf isoflavone

Table 8. A comparison of total isoflavone in soybean seeds between pod filling stage and post-ripening period under SC and RC.

| Varieties | Planting patterns | Maximum content (mg g⁻¹) | Harvest time | Post-ripening period |
|-----------|-------------------|--------------------------|--------------|----------------------|
|           |                   | Content                  | Percentage (%) | Increments | Percentage (%) |
| B1        | SC                | 4.070 a                  | 2.615 a       | 64.25 a        | 1.455 a       | 35.75 b |
|           | RC                | 3.770 b                  | 2.305 b       | 61.08 b        | 1.467 a       | 38.92 a |
| B2        | SC                | 1.228 a                  | 1.090 a       | 88.73 a        | 0.138 b       | 11.27 b |
|           | RC                | 1.208 a                  | 0.990 a       | 81.99 b        | 0.218 a       | 18.01 a |
| B3        | SC                | 2.118 a                  | 1.626 a       | 76.77 a        | 0.492 a       | 23.23 a |
|           | RC                | 1.998 b                  | 1.527 a       | 76.43 a        | 0.471 a       | 23.57 a |
| B4        | SC                | 1.675 a                  | 1.513 a       | 90.33 a        | 0.162 b       | 9.67 b  |
|           | RC                | 1.711 a                  | 1.459 a       | 85.27 b        | 0.252 a       | 14.73 a |

Increments during post-ripening period = Maximum content - Content at harvest time; Percentage = Content at harvest time or Increments during post-ripening period / Maximum content.

Table 9. A comparison of M-type and G-type isoflavones in soybean seeds between pod filling stage and post-ripening period under SC and RC (mg g⁻¹).

| Type | Variety | Planting patterns | Maximum content (mg g⁻¹) | Harvest time | Post-ripening period |
|------|---------|-------------------|--------------------------|--------------|----------------------|
|      |         |                   | Content                  | Percentage (%) | Increments | Percentage (%) |
| M    | B1      | SC                | 3.160 a                  | 1.955 a       | 61.88 a        | 1.204 a       | 38.12 a |
|      |         | RC                | 2.942 b                  | 1.673 b       | 56.86 b        | 1.299 a       | 43.14 a |
|      | B2      | SC                | 0.987 a                  | 0.907 a       | 91.85 a        | 0.080 b       | 8.15 b  |
|      |         | RC                | 0.965 a                  | 0.771 b       | 79.93 b        | 0.194 a       | 20.07 a |
|      | B3      | SC                | 1.719 a                  | 1.310 a       | 76.21 a        | 0.499 a       | 23.79 a |
|      |         | RC                | 1.596 b                  | 1.220 a       | 76.45 a        | 0.376 b       | 23.55 a |
|      | B4      | SC                | 1.357 a                  | 1.230 a       | 90.64 a        | 0.127 b       | 9.36 b  |
|      |         | RC                | 1.389 a                  | 1.197 b       | 86.18 a        | 0.192 a       | 13.82 a |
| G    | B1      | SC                | 0.910 a                  | 0.660 a       | 72.50 a        | 0.250 a       | 27.50 a |
|      |         | RC                | 0.828 b                  | 0.630 a       | 76.08 a        | 0.198 b       | 23.92 b |
|      | B2      | SC                | 0.241 a                  | 0.183 b       | 75.93 b        | 0.058 a       | 24.07 a |
|      |         | RC                | 0.243 a                  | 0.219 a       | 90.15 a        | 0.024 b       | 9.85 b  |
|      | B3      | SC                | 0.399 a                  | 0.316 a       | 79.20 a        | 0.083 a       | 20.80 a |
|      |         | RC                | 0.402 a                  | 0.307 a       | 76.37 a        | 0.095 a       | 23.63 a |
|      | B4      | SC                | 0.318 a                  | 0.283 a       | 88.99 a        | 0.035 b       | 11.01 b |
|      |         | RC                | 0.322 a                  | 0.262 a       | 81.37 b        | 0.060 a       | 18.63 a |

Increments during post-ripening period = Maximum content - Content at harvest time; Percentage = Content at harvest time or Increments during post-ripening period / Maximum content.
Table 10.  Micro-climate of soybean under SC and RC at seedling stage.

| Planting patterns | Days after sowing (d) | 21     | 31     | 41     | 51     | 61     |
|-------------------|----------------------|--------|--------|--------|--------|--------|
|                   |                      | Temp (ºC) | RH (%) | PAR    |        |        |
| SC                |                      | 26.8 a  | 26.1 a | 30.2 a | 28.2 a | 29.5 a |
| RC                |                      | 25.5 b  | 24.7 b | 27.8 b | 26.5 b | 28.4 b |
| SC                |                      | 73 b    | 70 b   | 69 b   | 69 b   | 71 b   |
| RC                |                      | 79 a    | 82 a   | 83 a   | 76 a   | 80 a   |
| SC                |                      | 325 a   | 253 a  | 516 a  | 302 a  | 202 a  |
| RC                |                      | 84 b    | 66 b   | 133 b  | 77 b   | 54 b   |

Temp means Temperature, RH means Relative humidity, PAR means photosynthetically active radiation. The timing of maize harvest in RC was August 9, 2009, 32 days after soybean sowing.

Table 11.  Path analysis of Micro-climate of field and isoflavone content of soybean leaf.

| Correlation coefficient | Direct path coefficient | Indirect path coefficient | Sum of indirect path | Remaining path |
|-------------------------|------------------------|---------------------------|-----------------------|----------------|
| x1                      | 0.9664**               | 0.2342                    | 0.0133                | 0.7189         | 0.7322 |
| x2                      | -0.9564**              | -0.0138                   | -0.2258               | -0.7168        | -0.9426 |
| x3                      | 0.9808**               | 0.7399                    | 0.2275                | 0.0134         | 0.2409 |

Determine coefficient $R^2 = 0.9052$

The level of significance (*, $P < 0.05$; **, $P < 0.01$) is indicated in the table. x1 represents temperature; x2 represents RH; x3 represents PAR.

Table 12.  Path analysis of Micro-climate of field and total isoflavone content of soybean seed at harvest time.

| Correlation coefficient | Direct path coefficient | Indirect path coefficient | Sum of indirect path | Remaining path |
|-------------------------|------------------------|---------------------------|-----------------------|----------------|
| x1                      | 0.8971**               | 0.3371                    | 0.1586                | 0.4014         | 0.56 |
| x2                      | -0.8898**              | -0.1645                   | -0.325                | -0.4003        | -0.7253 |
| x3                      | 0.8999**               | 0.4131                    | 0.3275                | 0.1593         | 0.4868 |

Determine coefficient $R^2 = 0.8206$

The level of significance (*, $P < 0.05$; **, $P < 0.01$) is indicated in the table. x1 represents temperature; x2 represents RH; x3 represents PAR.

Table 13.  Path analysis of Micro-climate of field and maximum content of total isoflavone in soybean seed during post-ripening period.

| Correlation coefficient | Direct path coefficient | Indirect path coefficient | Sum of indirect path | Remaining path |
|-------------------------|------------------------|---------------------------|-----------------------|----------------|
| x1                      | 0.909**                | -0.1832                   | -0.1091               | 1.2013         | 1.0922 |
| x2                      | -0.9079**              | 0.1132                    | 0.1767                | -1.1978        | -1.0211 |
| x3                      | 0.9487**               | 1.2363                    | -0.178                | -0.1006        | -0.2876 |

Determine coefficient $R^2 = 0.9036$

The level of significance (*, $P < 0.05$; **, $P < 0.01$) is indicated in the table. x1 represents temperature; x2 represents RH; x3 represents PAR.
content, total isoflavone content in seeds at harvest, and maximum isoflavone content in seeds, while relative humidity had negative direct effects. The highest direct effect on leaf isoflavone content, total isoflavone content in seeds at harvest, and maximum isoflavone content in seeds was exhibited by photosynthetically active radiation, followed by temperature and relative humidity. Temperature had the greatest indirect effect on leaf isoflavone content, total isoflavone content in seeds at harvest, and maximum isoflavone content in seeds through photosynthetically active radiation (0.7189, 0.4014, and 1.2013), followed by relative humidity through photosynthetically active radiation.

6. The relationship between main agronomic traits and isoflavones

In SC, plant height, branch number and pod number showed significant positive correlations with total, M-type and G-type isoflavone contents (Table 14). Node number was positively correlated with total and G-type isoflavone contents in SC, and the relationship between stem diameter and G-type isoflavone content was also significant. In RC, branch number and pod number showed a significant positive correlation with total, M-type and G-type isoflavone contents.

Discussion

In our research, four soybean cultivars showed the same accumulation pattern for leaf isoflavones in SC and RC. However, the trends were delayed in RC (Table 5). This may be attributed to the decreased growth caused by shading in RC before maize was harvested. We found photosynthetically active radiation of soybean canopy was significantly lower in RC (Table 10), in agreement with the report of Liu et al. (2011) and Yang et al. (2014). Wang et al. (2007) and Song et al. (2009) found that poor light resulted in a lower specific leaf weight photosynthetic rate and lower weight of dry matter accumulation in RC. After harvest of maize, photosynthetic capacity of soybean growing in RC recovered gradually, total above-ground biomass quickly improved (Wang et al., 2007; Yan et al., 2011), accordingly improved leaf isoflavone content in our research. During the pod filling stage, flavones were transferred from leaf to seed. The higher leaf isoflavone contents in all varieties in RC during the sudden drop period may be related with the lower transfer rate compared with SC. Previous studies have shown that isoflavone concentrations are affected by various genotypic and environmental factors (Wang and Murphy, 1994; Caldwell et al., 2005; Chennupati et al., 2011; Vamerali et al., 2012). We also found that leaf isoflavone contents differed with the variety, and micro-climate (temperature and photosynthetically active radiation) had a positive direct effect on leaf isoflavone content (Table 11).

In our research, total seed isoflavone contents, and contents of M-type and G-type isoflavones in seed showed the same accumulation pattern in SC and RC (Table 6 and 7). There were significant differences in various types of isoflavones with the variety, and the content of M-type isoflavones was higher than that of G-type isoflavones in our research. The differences in the contents and types of isoflavones might be due to the light conditions since genistin, malonylgensistin and the flavone rutin increased gradually under a light condition, while daidzin and malonyldaidzin did not change markedly with time in the dark (Sun et al., 1998). We found rapid accumulation periods of total seed isoflavone contents. The accumulation of M-type and G-type isoflavones in RC was delayed for seven days or more, which might be due to the activity of PAL which was affected by light condition (Hahlbrock and Scheel, 1989). Further path analysis coefficient analysis also proved that photosynthetically active radiation had the main positive direct effect on seed isoflavone content (Table 12). Tsukamoto et al. (1995) found that among the various environmental conditions, temperature was the main limiting factor, and that the isoflavone content decreased significantly in seeds when exposed to high temperatures during pod filling. We found that temperature had a positive direct effect on seed isoflavone content as the average temperature in our
research was under 30°C.

The specific weather conditions in southwest of China, higher relative humidity, shorter sunshine hours per month, and more precipitation make it difficult for soybean to dry enough to be threshed at the harvest time. A period of over one month for natural air drying or post-ripening is needed to achieve physiological maturity and during that period, secondary activities still take place in this area (Ye et al., 2010). Lozovaya et al. (2005) indicated environmental factors can have a large effect on isoflavone concentration, but the potential for isoflavone production is largely under genetic control. Our results showed that total isoflavone content, M-type isoflavone content and G-type isoflavone content in seeds during the post-ripening period varied with the variety and Weiyuan had the highest isoflavone content. However, RC had a strong influence and delayed the peak time of isoflavone contents in all varieties for 7 – 14 days compared with SC (Figs. 1, 2 and 3). During the post-ripening period, isoflavone contents in Zhongdou No.8 and Gongxuan No.1 in RC were higher than that in SC indicating that the post-ripening period is beneficial to isoflavone accumulation in RC (Table 8 and 9), but the potential for isoflavone accumulation was different because of the genetic difference. Thus, we suggest that to produce soybean with a higher isoflavone content, late maturity varieties with a higher isoflavone content in seed should be used in RC, and should be threshed at 14 – 21 DAH during the post-ripening period.

In SC and RC, correlations between main agronomic traits with the total isoflavone content, and contents of M-type and G-type isoflavones were different (Table 14). Plant height, branch number and pod number showed a significant positive correlation with the total isoflavone content and contents of M-type and G-type isoflavones in SC. In RC, branch number and pod number showed a significant positive correlation with total isoflavone content and contents of M-type and G-type isoflavones. The main agronomic traits showing correlations with isoflavone content in previous research (Zeng et al., 2007) were different from those found in the present study.

In conclusion, our results showed that soybean isoflavone accumulation together with growth was inhibited in RC, but the suitable post-ripening period would facilitate the accumulation of isoflavone. Fast development of this system in south west China, is expected to help create a new way to improve isoflavone productivity in China.

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