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Effects of Light Treatment on Isoflavone Content of Germinated Soybean Seeds

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Our research objective was to increase isoflavone content in the germinated soybean seeds of four different varieties (Pungsannamulkong, Cheongjakong, Aga4, and Aga3) by optimizing light treatments (dark, greenhouse, fluorescent, incandescent, and ultraviolet lamps). The results demonstrated that the highest isoflavone content was produced from the Aga3 variety, which was developed by an interspecific cross between Eunhakong (*Glycine max*) and KLG10084 (*G. soja*) at the Plant Genetic Laboratory, Kyunpook National University. Aga3 is known to have one of the highest isoflavone content in the world at present. Our results recommend exposure of 7-day-old Aga3 sprouts to a combined light treatment of greenhouse lamps (12 h per day) and ultraviolet light (40 min per day) for maximum isoflavone production. Aga3 produced high levels of isoflavone because, in part, it contained very high isoflavone levels within the seed as compared with the other varieties. Under stress conditions, Aga3 could produce over 1.90 times more isoflavone than its seed content and 1.53 times more isoflavone than when grown under dark conditions.

KEYWORDS: Germinated soybean seeds; light source; isoflavone

INTRODUCTION

Soybean is a popular health food in many Asian countries. Soybean is used in various forms such as soybean sprouts, soy pastes, soymilk, soybean oil, and tofu as key ingredients in cultural cuisines (*1*). Currently, there is an increasing consumption of soybean worldwide due to its nutritional properties and the beneficial characteristics of its constituent compounds like isoflavone. Consumption of isoflavone is associated with human health benefits such as decreased risk of heart disease, menopausal symptoms, cardiovascular disease, and bone resorption as well as breast, prostate, and colon cancers (*2*−*5*). The physiological function of isoflavone is mediated by a variety of mechanisms including estrogenic activity as well as inhibition of topoisomerase and protein kinases (*6, 7*).

Isoflavones are categorized chemically according to their functional groups. There are four subgroups including aglycones (genistein, daidzein, and glycitein), glycosides (genistin, daidzin, and glycitin), malonyl glycoside (malonyl genistin, malonyl daidzin, and malonyl glycitin), and acetyl glycosides (acetyl genistin, acetyl daidzin, and acetyl glycitin) (**Figure 1**) (*8*).

Isoflavone content in soybeans is generally affected by genetic factors such as cultivar as well as a number of environmental factors including cultivation year, location, temperature, storage conditions, and germination period (*9*−*11*). Wang and Murphy (*12*) reported that total isoflavone content ranged from 1176 to 3309 μg/g when grown in different years and from 1749 to 1176 μg/g when grown at different locations. Eldridge and Kwolek (*13*) measured total isoflavone contents ranging between 1160 to 3090 μg/g among four soybean cultivars. Genevese et al. (*14*) reported that isoflavone content of 14 Brazilian soybeans ranged from 570 to 1880 μg/g. Kim et al. (*15*) observed that total isoflavone of Shinpaldal-2, which showed the highest amount of isoflavone content out of three varieties tested, increased by 13.2% during initial germination and then decreased thereafter.

Light supplies energy for photosynthesis as well as signals for photomorphogenesis in plants, which in turn influences allocation and use of the products of photosynthesis. Some studies have shown that light is also directly involved in various biosynthetic pathways. Phytochromes, such as phytochrome B and crytochrome 2, have been identified as the primary photoreceptors for phenylpropanoids accumulation (*16*) and light induced carotinoid biosynthesis in ripening tomatoes (*17*). The synthesis of a flavonoid pigment was promoted by red illumination and inhibited by far-red illumination in the cuticle of tomatoes (*18*). Flavonoids and some phenylpropanoids have been thought to play roles in protecting against UV irradiation. Lee at al. (*19*) demonstrated that the content of isoflavones increased in soybean sprouts under light condition. Kirakosyan

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Table 1. Isoflavone Content in Germinated Soybean Seeds of Four Soybean Varieties Treated with Different Light Sources (μg/g)[a]

variety	light source	daidzin	genistin	acetyl daidzin	malonyl genistin	acetyl genistin	daidzein	genistein	total
dark	Pungsannamulkong	284c	403c	39a	399c	13c	429b	183b	1754c
	Aga3	2038a	2340a	52a	3206a	26a	1063a	268a	8997a
	Cheongjakong	111c	270c	39a	403c	12c	149c	109c	1097c
	Aga4	675b	1168b	57a	1622b	24b	285bc	130bc	3963b
fluorescent	Pungsannamulkong	643b	818c	54ab	987bc	11c	93c	81b	2690c
	Aga3	2536a	2405a	59a	3155a	24a	158a	89a	9329a
	Cheongjakong	248b	466c	39b	786c	11c	80c	84ab	1719c
	Aga4	1114b	1517b	48ab	1937b	16b	139b	77b	4852b
greenhouse	Pungsannamulkong	903c	739c	48b	799c	14c	163b	98c	2768c
	Aga3	3071a	2982a	91a	3941a	45a	509a	142a	10784a
	Cheongjakong	428d	655c	44b	1018c	15c	148b	131b	2441c
	Aga4	1209b	1501b	53b	1868b	18b	195b	90d	4936b
incandescent	Pungsannamulkong	197c	345c	47b	438c	12b	499b	214b	1756c
	Aga3	1498a	1846a	62a	2570a	32ab	1168a	339a	7518a
	Cheongjakong	275c	525cb	39b	970cb	16b	206c	170b	2205bc
	Aga4	492b	919b	39b	1704b	42a	443b	167b	3810b
varieties (*V*)		**	**	*	**	**	**	**	**
light source (*L*)		**	**	ns	**	ns	**	**	**
V × *L*		*	*	ns	ns	ns	**	**	*

[a] ** = Significant ($P < 0.01$), * = significant ($P < 0.05$), ns = nonsignificant. +Values with different letters in the same column within varieties in each light source are significantly different at 5% level by DMRT.

et al. (*20*) provided isoflavone levels in various genotypes of soybean that were altered by the far-red end of day light treatment.

The main objectives of this study were to evaluate the effects of environmental factors, especially light and temperature, on total isoflavone content in germinated seeds from different soybean varieties and to enhance total isoflavone content in germinated soybean seeds.

MATERIALS AND METHODS

Seed Materials and Germination Method. The soybean varieties used in this study were Pungsannamulkong (widely used for soy-sprout production in Korea), Cheongjakong (known to have the highest isoflavone content among black seed coat soybeans), and two newly developed varieties, Aga3 (green seed coat) and Aga4 (black seed coat), released from Plant Genetic Laboratory, Kyungpook National University. The isoflavone contents of seeds were 3556, 983, 7242, and 3232 μg/g, respectively. Seed germination method used was according to Lee et al. (*21*). Twenty grams of seeds of each variety were placed in a plastic container (6.0 cm W × 6.0 cm L × 15.0 cm H) with several small perforations at the bottom for drainage. The samples were first soaked in water at 20 °C for 4 h to initiate germination. After 4 h of soaking, the seeds were removed from the water and placed in the environment-controlled chamber at 20 °C with 80% humidity. A submersible pump connected to a nozzle was placed above the container and set to spray about three liters of water per minute for 4 min every 3 h.

Light Treatment. All soybean varieties, after 4 h of soaking, were treated with light from different sources such as dark (control), fluorescent lamp (40 W, FL20SD, China) 10 ± 1.00 μMol/m^2/s, greenhouse lamp (40 W, FL20 PG, Wooree Lighting co., Korea) 8 ± 1.53 μMol/m^2/s, incandescent lamp (60 W, IK04) 4 ± 1.73 μMol/m^2/s, and ultraviolet-C (Sankyo Denki, GL20, Japan) 1 ± 1.53 μMol/m^2/s (**Figure 2**).

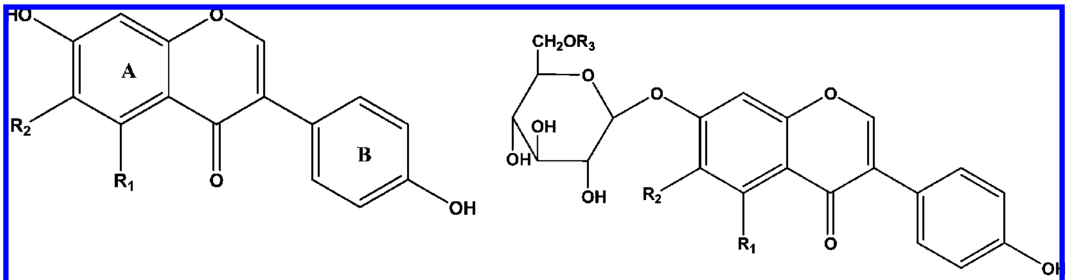
Isoflavone Extract. All isoflavone contents measured in the experiment were based on dry weight basis. The extraction of isoflavone was modified from Rostageno et al. (*22*) Dry soybean powder, 0.2 g (75 μm × 75 μm), was added to 10 mL of 80% high performance liquid chromatography (HPLC) grade EtOH and incubated in an Ultrasonic bath (Kodo Co., Korea) at 50 °C for one h. The samples were then placed in a shaking incubator (150 rpm) at 50 °C for 15 h. These were then passed through a 0.45 μm syringe filter and collected for isoflavone analysis using HPLC.

Isoflavone Analysis. HPLC analysis of isoflavone was based on the work of Wang and Murphy (*12*). The HPLC system consisted of a TOTALCHROM V6.2.0.0.1 with LC Instrument control (PerkinElmer series 200, USA) and a COL-CHOICE C18 column 4.6 mm × 150 mm (5 μm) packed. A linear HPLC gradient used acetonitrile (solvent A) and 0.1% of acetic acid in water (solvent B). After injection of a 10 μL sample volume, solvent A was increased from 0 to 45% over 10.2 min. It was further increased from 45% to 90% over 6 min, remained constant for 3.6 min, and then was reduced from 90 to 0% over 15 min. The solvent flow rate was 1.0 mL/min. The elution was monitored by UV-absorption (series 200 UV/vis detector) at 260 nm. Identification of the isoflavone was based on comparisons with retention times of genuine standards, including daidzein, genistein, and genistin (Sigma Chemical Co, USA), as well as glycitin, daidzin, 6′-0-actlygnistin, 6′-0-malonygenistin, and 6′-0-actyldaidzin (LC Laboratory, USA).

Statistical Analysis. HPLC analyses of isoflavone were conducted with two replications of each sample using completely randomized design. To identify significant treatment effects and interactions, analysis of variance (ANOVA) and multiple mean comparisons were carried out on the data comparing isoflavone content with the general linear model (GLM) by Statistic Analysis System (SAS 9.1). Differences among mean values were determined using Duncan's multiple range test at $P \leq 0.05$ when ANOVA indicated model and treatment significances.

RESULTS AND DISCUSSION

Isoflavone Content in Germinated Soybean Seeds at Different Time Intervals. Aga3 and Pungsannamulkong were treated with greenhouse lamps for 24 h per day. Samples were taken from seeds soaked for 4 h and up to 10 days of germination. Isoflavone content of germinated soybean seeds at different time intervals indicated a significant change ($P < 0.05$). Regression analysis showed a quadratic polynomial trend for both Aga3 and Pungsannamulkong (**Figure 3**). The isoflavone content of Aga3 samples at day 7 was 10788 μg/g, which was 1.49-fold greater than the content of seeds under controlled treatment (7242 μg/g). It was also observed that total isoflavone content of the Pungsannamulkong variety (3556 μg/g) after germination was 2.87-fold greater than the control treatment, which had only 1236 μg/g. These results followed a similar trend as reported by Danhua et al. (*9*), where isoflavone content



R_1	R_2	Aglycones
H	H	Daidzein
OH	H	Genistein
H	OCH_3	Glycitein

R_1	R_2	R_3	Glycosides
H	H	H	Daidzin
OH	H	H	Genistin
H	OCH_3	H	Glycitin
H	H	OCH_3	Acetyl-daidzin
OH	H	OCH_3	Acetyl-genistin
H	OCH_3	OCH_3	Acetyl-glycitin
H	H	OCH_2COOH	Malonyl-daidzin
OH	H	OCH_2COOH	Malonyl-genistin
H	OCH_3	OCH_2COOH	Malonyl-glycitin

Figure 1. Chemical structure of 12 isoflavone isomers of soybeans.

Table 2. Isoflavone Content (μg/g) of Germinated Soybean Seeds under Ultraviolet Lamps at Different Time Intervals (min per day)[a]

variety	UV (min)	daidzin	genistin	acetyl daidzin	malonyl genistin	acetyl genistin	daidzein	genistein	total
Aga3	20	3274b	4761ab	34bcd	4047ab	52b	168c	90cd	12426b
	40	3293b	4699abb	31cd	4538a	53b	342a	171a	13127a
	60	3072c	4903a	77a	3809ab	84a	175c	123b	12242b
	80	3813a	4607b	42b	3249c	88a	214b	100c	12112b
	100	3695a	4042c	37bc	3301c	61b	186c	85de	11407c
Pungsan-namulkong	20	815e	1089d	22ef	764d	18c	125d	82de	2915d
	40	1079d	1293d	28de	742d	21c	218b	118b	3499d
	60	866e	1068d	30cde	622d	16c	128d	99c	2829de
	80	684ef	811e	19f	479d	19c	132d	77de	2220ef
	100	609f	721e	16f	397d	18c	126b	75e	1963f
variety (*V*)		**	**	**	**	**	**	**	**
ultraviolet (UV)		**	**	**	**	**	**	**	**
$V \times UV$		**	*	**	ns	**	**	**	ns

[a] ** = Significant (P < 0.01), * = significant (P < 0.05), ns = nonsignificant. +Values with different letters in the same column are significantly different at 5% level by DMRT.

in seeds of 3-day-old Caviness and Hutcheson varieties incerased 21.61% and 11.51%, respectively.

The highest isoflavone content was observed in germinated seeds on day 7 for Pungsannamulkong and Aga3. After day 7, there was a decreasing trend in isoflavone content as the germination period increased. Terrence (*23*) observed that soybean primary leaf tissues underwent a programmed shift from isoflavonoid to flavonoid metabolism after 3 days of germination and became largely dominated by glycosides of kampferol, quercetin, and isorhamnetin after 5 days of germination.

Effects of Different Light Sources on Germination of Soybean Seeds. Isoflavone content in germinated soybean seeds from four different varieties, Pungsannamulkong, Cheongjakong, Aga4, and Aga3, were enhanced by exposure to different light sources such as dark (control), fluorescent, greenhouse, and incandescent lamps for 24 h per day, after 4 h of soaking, up to 7 days, as shown in **Table 1**. Statistical analysis confirmed that isoflavone content depended on seed variety and exposure to different light sources. The interaction between varieties and light sources had a significant effect ($P < 0.01$) on the total isoflavone content of samples.

The isoflavone content of seeds from different varieties under different light sources were significantly different ($P < 0.01$). Seeds exposed to greenhouse lamps had the highest total isoflavone content, which was higher than those exposed to incandescent lamps (**Figure 2**). Either significant interaction effect for daidzin, genitstin, and total isoflavone content or highly significant interaction effect for daidzein and genistain were recognized between variety and light source in isoflavone content as shown in **Table 1**. But no interaction effect was recognized in isoflavone content of acetyl daidzin, malonyl genistin, and acetyl genistin.

The isoflavone content of Aga3 germinated under greenhouse lamps was highest (10784 μg/g), while that of Cheongjakong germinated under dark conditions was the lowest (1097 μg/g) as shown in **Table 1**. According to Chi et al. (*24*), soybean

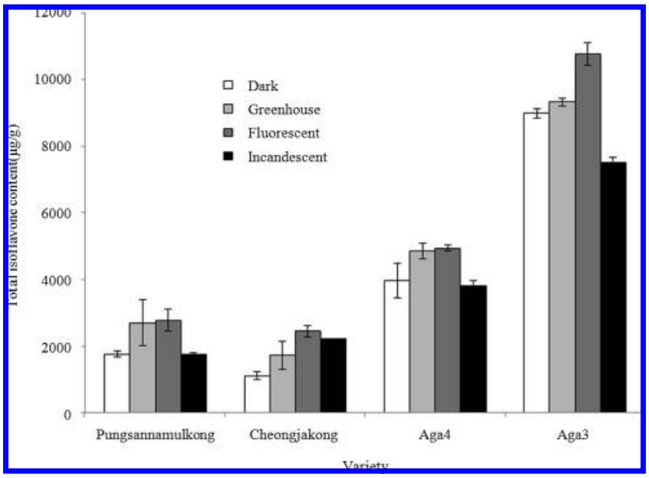


Figure 2. Distribution of isoflavone content in germinated seed of four soybean varieties under different light conditions.

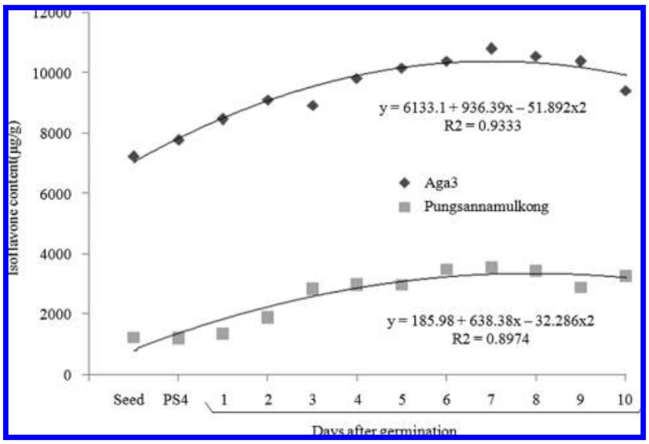


Figure 3. Variation of isoflavone content in germinated soybean seeds of Aga3 and Pungsannamulkong at different time intervals (PS4, pre-soaking for 4 hours).

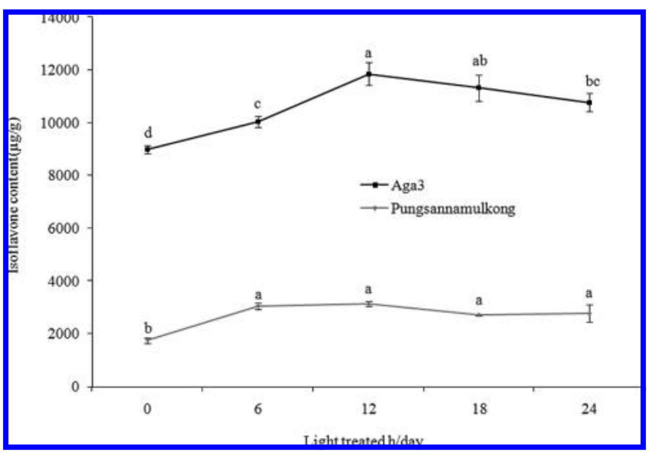


Figure 4. Changes in isoflavone content of germinated soybean seeds under greenhouse lamps at different time interval.

sprouts grown under light contained higher aspartic acid, vitamins B_1, B_2, C, and isoflavone content than those grown under normal conditions. In addition, Kim et al. (*1*) reported a total isoflavone content of 1389 μg/g in green soybean sprouts, which was 2.48-fold greater than that obtained in yellow soybean sprouts (559 μg/g), and concluded that under the two different light conditions the mean of isoflavone was higher in green than in yellow soybean sprouts. Furthermore, Maria and Daniel (*25*) conducted experiments on callus culture of *Genista* under

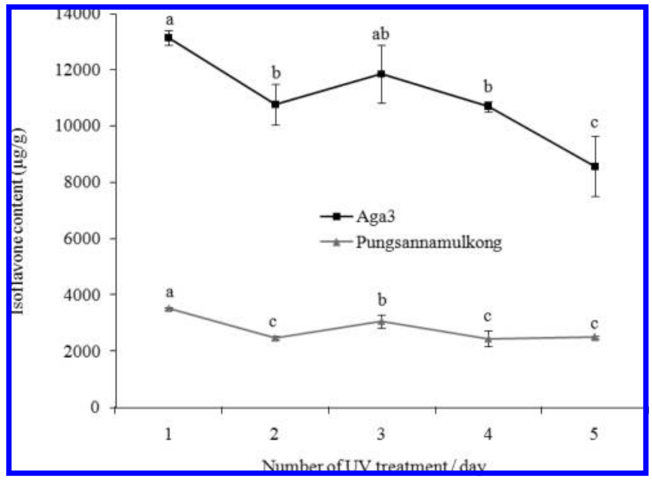


Figure 5. Changes in isoflavone content of germinated soybean seeds under ultraviolet lamps at different number of treatment (40 min per each time).

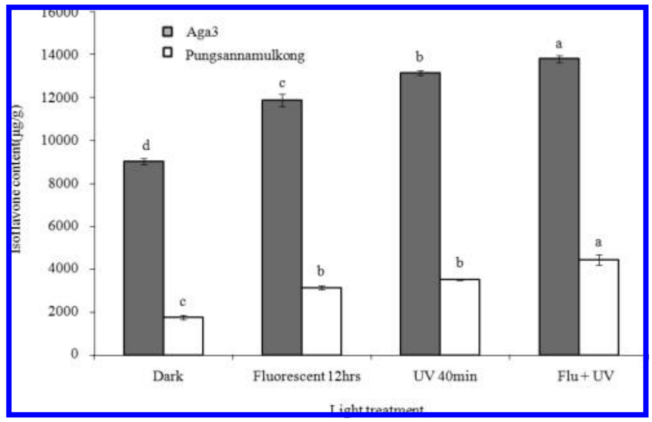


Figure 6. Verification of combined effects of greenhouse (treated 12 h/day) and ultraviolet lamps (treated 40 min/day) on isoflavone content in germinated soybean seeds (a, b, c, and d indicate the results of Duncan's multiple range test at 5% level of significant difference among varieties).

reduced and constant light (light intensity 88 μmol/m^2/s). Their results showed that the total isoflavone content was higher in samples under constant light (6587 mg/100 g DW) than in samples cultured under dark conditions (4590 mg/100 g DW). According to Heldt (*26*), light affects isoflavone content. He also reported that isoflavone is synthesized via the malonate pathway. Similarly, Maria and Daniel (*25*) predicted that light affects the enzymes that lead to the biosynthesis of isoflavone compounds.

Daidzin and genistin content in all soybean varieties increased under high light intensity including fluorescent lamps (10 $\pm$ 1.00 μMol/m^2/s) as well as greenhouse lamps (8 $\pm$ 1.52 μMol/m^2/s), whereas daidzein and genistein increased under low light intensities such as that of incandescent lamps (4 $\pm$ 1.73 μMol/m^2/s). Daidzein content in Aga3 germinated under incandescent lamps was 1168 μg/g, which was 7.36-fold greater than in those exposed to greenhouse lamps (i.e., 158 μg/g). It was also observed that genistein content in Aga3 germinated under incandescent lamps was 339 μg/g, which was 3.79-fold greater than those under greenhouse lamps (i.e., 89 μg/g). Terrence (*23*) also reported that the levels of daidzein, genistein, and their conjugates were considerably higher in dark-grown sprouts than in light-grown sprouts.

Effect of Alternate Greenhouse Light and Dark Conditions on Increasing Isoflavone Content. Greenhouse lamps were selected as the best light source based on findings of previous experiments. Aga3 and Pungsannamulkong were treated with greenhouse light at different time intervals (0, 6, 12, 18, and 24 h per day), after 4 h of soaking, up to 7 days. The effects of exposure to dark conditions and different greenhouse light durations on isoflavone content of soybean seeds germinated for 7 days are represented in **Figure 4**. Isoflavone content was significantly affected by the period of exposure to greenhouse lamps.

The isoflavone content increased from 0 h exposure to greenhouse light (1755 and 8998 μg/g for Pungsannamulkong and Aga3, respectively) with the highest values reached at 12 h (3138 and 11893 μg/g for Pungsannamulkong and Aga3, respectively). A gradual decrease was observed between 12 and 24 h of exposure to light with an isoflavone content of 2768 and 10784 μg/g for Pungsannamulkong and Aga3, respectively.

Effect of Ultraviolet Lamps on Increasing Isoflavone Content. Aga3 and Pungsannamulkong were exposed to ultraviolet lamps for the time durations of 20, 40, 60, 80, and 100 min per day, after 4 h of soaking, up to 7 days. The effects of exposure to ultraviolet lamps on the isoflavone content of germinated soybean seeds were significantly different ($P < 0.01$). The isoflavone content increased to 12426 and 2915 μg/g for Pungsannamulkong and Aga3, respectively, in 20 min of exposure to ultraviolet lamps. The highest value was reached at 40 min for both Pungsannamulkong (3499 μg/g) and Aga3 (13127 μg/g) (**Table 2**). But isoflavone content decreased at 60−100 min per day of exposure to ultraviolet light. The results agreed with observation of Zhao et al. (*27*) on callus of *Saussurea medusa* Maxim, which showed that ultraviolet light improved the content of secondary products.

Similarly, in this experiment, Aga3 and Pungsannamulkong were treated with ultraviolet lamp 1, 2, 3, 4, and 5 times per day for 40 min per treatment. According to the findings of previous experiments, 40 min was the best treatment for increasing the isoflavone content in germinated soybean seeds. Both Aga3 and Pungsannamulkong showed the highest levels in the isoflavone content in samples treated one time per day by ultraviolet lamp, 13127 and 3514 μg/g, respectively (**Figure 5**). Ryan et al. (*28*) reported that in *Petunia* and *Arabidopsis* UV light induces the synthesis of flavonols with higher hydroxylation levels. Our results indicated that ultraviolet light stimulated the isoflavone content though extended exposure stunted isoflavone synthesis, especially when exposure to ultraviolet lamp was greater than 40 min per day.

Verification of the Combined Effects of Greenhouse and Ultraviolet Lamps on Increasing Isoflavone Content. From the previous experiments, greenhouse and ultraviolet lamps were identified as efficient sources of light for increasing total isoflavone content. Aga3 and Pungsannamulkong were treated with fluorescent light 12 h per day and ultraviolet light 40 min per day, after 4 h of soaking, up to 7 days. The isoflavone content in Aga3 and Pungsannamulkong exposed to combined greenhouse and ultraviolet light was significantly different ($P < 0.01$) from other cultivation methods such as dark (24 h), only greenhouse lamp 12 h per day, and only ultraviolet light 40 min per day.

Isoflavone contents in both Aga3 and Pungsannamulkong were highly affected when exposed to combined greenhouse and ultraviolet light. The isoflavone content in both Aga3 and Pungsannamulkong were 13783 and 4428 μg/g, respectively. This was significantly higher when compared to dark treatments, which were 8998 and 1755 μg/g, respectively (**Figure 6**).

Isoflavone content of 7-day-old sprouts of Aga3, known to have one of the highest isoflavone content in the world at present, could be increased up to 13783 μg/g when a combined light treatment of green house lamps (12 h per day) and ultraviolet light (40 min per day) were applied. Soy-sprout itself and soy powder made of it would be valuable source materials for various food materials such as soy-sprout soup, soy paste, soy sauce, tofu, soy milk, and other functional foods.

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