宮 崎 大 学 大 学 院

博 士 学 位 論 文

Fundamental breeding studies on the relationship between phenotype and antioxidant in Japanese and Indonesian soybean accessions

日本およびインドネシアにおけるダイズの表現型と抗酸化物質に関する育種学的基礎研究

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ABSTRACT

Soybean (*Glycine max* (L.) Merr.) is one of the grain legume which contains rich protein and oil, and its phytochemicals, such as flavonoids, are beneficial for human health. It is commonly used as a medicinal and traditional food in many Asian countries. In particular, in Japan and Indonesia, most soybeans are processed into soymilk, tofu, soy sauce, natto (fermented with *Bacillus subtilis*, popular in Japan), and tempeh (fermented with *Rhizopus oligosporus*, commonly consumed in Indonesia). Japan is particularly rich in soybean foods, and even in tropical Indonesia, fermented foods such as tempeh exist. Therefore, there are many genetic resources suited to each climate and culture. The soybean grade is divided based on specific seed chemical and physical properties (i.e., seed size and color). The large size of soybean seeds often makes tofu and soymilk, while the small seeds are preferable to produce natto, tempeh, and soy sauce. The seed coat color is an important parameter to assess the presence of phytochemicals (anthocyanins, flavonoids, phenol), which have antioxidant activity. For example, black soybeans have a higher anthocyanin content, while yellow soybeans have a high isoflavone content. Along with the high demand for soybean products, identifying seed phenotypic and evaluating phytochemical content has become necessary. This thesis aims to clarify the relationship between phenotypic and antioxidants by investigating the phenotypes of Japanese and Indonesian soybeans and evaluating their phytochemical content.

Twenty-six soybean accessions were first selected, of which 23 accessions are commonly consumed in Japan. These accessions were obtained from the National Agriculture and Food Research Organization (NARO) Genebank Project and the National BioResource Project (NBRP) for *Lotus*/*Glycine* in Japan. All soybean accessions used in this study were grown in a field at Saito City, Miyazaki Prefecture, Japan, in 2015. The total flavonoid, phenol, and 12 major isoflavones were determined using high-performance liquid chromatography

(HPLC). Moreover, the antioxidant activity of each soybean accession was examined using an antioxidant-responsive element (ARE) linked to a luciferase reporter in human HepG2 stable cells. As a result, the relative ARE luciferase activity rate of 26 soybean accessions varied up to 4-fold, and 22 accessions significantly increased compared to the negative control. In particular, soybean accessions, namely Williams 82, Himeshirazu, Akisengoku, Nattou kotsubu, and Akasaya, were prominent in relative ARE luciferase activity. Meanwhile, four accessions (Wase kuro daizu, Kurodaizu [Ao higuu chuu], Koito, and Oni Hadaka) showed no significant activity. In addition, 22 soybean accessions with high antioxidant activity were not necessarily high in total phenol or flavonoid content. Correlation analysis revealed that the antioxidant activity had a significant and positive correlation with the level of total isoflavone content. Moreover, quantitative data on the seed phenotype showed that the seed coat color correlated with total flavonoid and phenol contents.

The diversity of soybeans is also found in Indonesian soybean varieties. Although the amount of soybean germplasm in Indonesia is relatively small compared to other Asian countries (i.e. Japan, Korea, and China) , the variety exhibits high diversity in agronomical and morphological characteristics. A total of 20 varieties of Indonesian soybeans were obtained from the germplasm collection of the Indonesian Legumes and Tuber Crops Research Institute (ILETRI), Malang, East Java, Indonesia. These varieties are widely cultivated in Indonesia and are known for their high-quality products and resistance to pests and diseases. The phenotypic data were examined, which consisted of quantitative data (weight, seed flatness, seed index) and qualitative data (the color of the seed coat, hilum, and cotyledon). Next, whole soybean seeds were powdered and extracted to determine the biochemical components, including crude protein, oil content, total phenol, flavonoid, and antioxidant activity. The antioxidant activity was examined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method and represented by half-inhibitory concentration (IC_{50}) values. The results showed that four soybean varieties,

namely Detam 1, Detam 2, Dering 1, and Grobogan, contained high total flavonoids and total phenols. These varieties also showed low IC_{50} values, effectively inhibiting free radicals in DPPH solutions with a small amount of soybean extract. However, their biochemical components, such as protein and oil content, were not necessarily high. Correlation analysis showed that antioxidant activity correlated with total flavonoids, total phenol, and seed size (seed flatness index). Therefore, each soybean variety has advantages in its biochemical or antioxidant activity.

From these results, the relationships between the phenotypes and their antioxidants in Japanese and Indonesian soybeans have been uncovered. There was a correlation between seed coat color and total flavonoid and phenol content. In addition, isoflavone content shows a significant correlation with antioxidant activity. These findings would support the understanding of the mechanism by which varietal differences influence the antioxidant activities in soybeans. This information could be valuable in soybean breeding to generate a cultivar that contains a high number of antioxidants in the future.

CHAPTER 1

GENERAL INTRODUCTION

1. History of Soybean Breeding

The history of soybean has been recorded through phylogenetics and historical documents. According to the soybean classification, the genus *Glycine* is believed to have originated from ancient polyploidy, which is indicated by the high chromosome number of most species (n = 20), in contrast to closely related genera with lower chromosome numbers (mostly n = 10 or 11, except for one with n = 14; Goldblatt, 1981). Research on haploid *Glycine max* has shown that polyploid origin is a supported hypothesis, as demonstrated through cytogenetic studies (Crane et al. 1982). The *Glycine* genome has undergone two major rounds of duplication, the first estimated 58 million years ago and another 13 million years ago. Evolutionary events show that the recent divergence of two soybean homoeologous regions occurred 60 and 12 million years ago, respectively (Cannon and Shoemaker, 2012). The type of polyploidy was tested and discussed, with findings suggesting soybean's tetraploid nature (4 x = 40) due to the presence of chromosomes with identical morphology. Chromosome rearrangements may have occurred during the speciation of *G. max* (Clarindo et al. 2007).

The genus *Glycine* Wild. has two subgenera: *Glycine* (perennials) and *Soja* (Moench) F.J. Herm (annuals). The perennial species have a diverse range of morphology, cytology, and genome composition. They can grow in various climatic and soil conditions and have a broad geographic distribution. These species have been screened for physiological and biochemical traits and sources of resistance to economic pathogens. Some perennial *Glycine* species offer resistance to soybean cyst nematode and lack Bowman-Birk protease inhibitors, making them valuable resources (Hymowitz, 2004).

The modern cultivated soybean (*Glycine max* (L.) Merr.) was domesticated from wild soybean (*Glycine soja* Sieb. & Zucc.) in East Asia 6000-9000 years ago (Kim et al., 2010). There are several pieces of evidence that the wild soybean was domesticated by ancient people under certain agricultural conditions. The evidence shows that the cultivated soybean and the

wild soybean share the same number of chromosomes (2n=40) with a chromosome set of GG and are closely related. When cultivated and wild soybeans are crossed, the F_1 generation has normal fertility and high seed-setting percentage, similar to crosses within cultivated soybeans. This indicates that the cultivated and wild soybeans are not isolated and are closely related (Guriqbal, 2010). Another evidence is that when cultivated soybean is crossed with wild soybeans, traits such as seed size, plant height, and lodging are inherited as quantitative traits, with some intermediate types occurring. This suggests that the groups *G. max* and *G. soja* accumulated minor variants of the underlying genes. The subgenus *Soja*, which includes highly variable species, has been confirmed through various methods such as RFLP of chloroplast DNA variation, genomic DNA variation, and single-nucleotide polymorphisms of GmHs1pro-1, (Chen and Nelson, 2004; Yuan et al., 2008).

The agricultural revolution of soybean breeding started in northeastern China and became an essential crop in the Eastern Zhou Dynasty (about 2510 BP.) (Kim et al., 2010). China was the world's largest soybean producer and exporter in the first half of the 20th-century, and introducing soybeans to other regions of Asia. Landraces of soybeans have been discovered in various countries such as Japan, Indonesia, the Philippines, Vietnam, Thailand, Malaysia, Myanmar, Nepal, and north India. The dissemination of soybean from China to Japan was distributed through northern Korea about 2000 years ago. In Japan, the demand for soybeans is very high, nearly 5 million t, of which 0.2 million t is cultivated domestically (Guriqbal, 2010). Approximately one million t of soybean consumption is used to make foods such as *natto*, tofu, and soybean milk, and nearly four-fifths of soybean consumption is used for extracting oil. Most soybean cultivars grown in Japan have large seeds and are used as a vegetable soybean called *edamame*. The fresh seed weight of large seeds type has more than 70 grams per 100 seeds, while dry seeds have more than 30 grams per 100 seeds. Another variety of small-seed soybeans, used to make *natto*, weighs about 10 g per 100 seeds (Shurtleff

and Aoyagi, 2014). In Korea, most soybean varieties are medium- and small-seed with weights of 15 g per 100 seeds and are used for producing bean sprouts (Kim et al. 2022). Soybean is also introduced to other Asian countries such as Indonesia, Thailand, Vietnam, India, and Nepal. In Indonesia, fermented soybean, tempeh, is a popular soy product. India and Nepal make kinema with fermented soybean. Moreover, soybean was considered the best annual nitrogenous seed (seed that contains a lot of nitrogen) and hay-producing plant among leguminous crops. It has gained popularity as a ruminant feed and has become a primary raw feed material for swine and farmed fish (Hartman et al., 2011).

2. State of the Art of Soybean Breeding

Cultivated soybean has a protein content of approximately 40% and an oil content of around 20% (Hartman et al. 2011). This unique composition allows for various applications, including biodiesel, printing ink, feed, edible oils, and food products. Soybeans are used for three main purposes: animal feed (76%), human consumption (20%), and industrial uses (4%) (Figure 1.1). Soybeans are mainly processed into soy meals which serve as a source of protein and metabolizable energy for human and animal feed. To meet the protein requirements of animals, soybean used for animal feed should have at least 36% protein. On the other hand, food-grade soybean with 40-45% protein is ideal for making high-quality soy-based products like soy milk, tofu, soy paneer, soy sprouts, and other soy-based foods. Furthermore, soybean has recently been developed as a substitute for meat, which causes no damage to the global environment and is focused on from the perspective of the sustainable development goals (SDGs). Soybean protein is also useful for processing protein fiber, which can be combined with cotton, wool, or synthetic fibers to produce a soft, high-quality fabric (Guriqbal, 2010; Hymowitz, 2004).

Soybean has numerous applications and is associated with various health advantages, making it a supplementary addition as a functional food to the human diet. Soybeans are rich in bioactive phytochemicals such as phenolic acids, flavonoids, isoflavones, saponins, phytosterols, and sphingolipids (Lee et al. 2008). These have been found to possess various pharmacological properties. These include antioxidant, estrogenic (Hu et al. 2020), antidiabetic, antiobesity (Kanamoto et al., 2011), antihypercholesterolemic, antihypertensive, anticancer, antimutagenic, antiviral, antimicrobial, anti-inflammatory (Das et al. 2020), bifidogenic, antiosteoporosis, hepatoprotective, antihyperlipidemic, immunomodulatory, neuroprotective, wound healing, goitrogenic anti-skin aging, and anti-photoaging activities (Lim, 2012).

The discovery of the benefits of soybeans has led to improvements in breeding and crop systems. Soybean germplasms, which have valuable traits such as high nutrition content, disease resistance, high-quality yield, and adaptability to climate change, can be used to create new cultivars. These beneficial traits are preserved and recorded in the Genebank to ensure germplasm diversity (Kim et al. 2022). Moreover, it is important to understand the phenotype of soybeans and their phytochemicals to improve the production of soy-based foods. For example, the size of soybean seeds is a crucial factor in producing soy products. There are two categories of soybean products based on seed size. The first category includes products made from large seeds that weigh over 20 grams per 100 seeds, such as tofu, edamame, miso, and soy milk. The second category includes products made from smaller seeds that weigh less than 12 grams per 100 seeds, including natto, soy sauce, tempeh, and bean sprouts. The soybean phenotype, specifically the seed coat color, also correlates with health benefits, which can be valuable for pharmaceutical industries. Research on soybean seed coats has shown that they contain various essential nutrients, phytochemical compounds, and high amounts of dietary fiber. According to various studies, black soybean has a great antioxidant capacity (Takahashi et al., 2005; Xu & Chang, 2008). Extracts from black soybean may help prevent the

development of chronic diseases such as diabetes, obesity (Kanamoto et al., 2011), and thrombosis (Kim et al., 2011). Black soybean contains additional phytochemicals in its seed coat, such as anthocyanins and proanthocyanidins (Todd & Vodkin, 1993).

3. Contributions of the Thesis

This study comprised Japanese and Indonesian soybean accession materials, which serve numerous purposes, such as food and animal feed, medicinal applications, and industrial uses. These soybeans exhibit potential as antioxidants, anti-inflammatory, anti-diabetic, and anti-obesity agents, warranting further investigation. The study focused on analyzing seed phenotype and antioxidant content to provide fundamental data for breeding studies. The experiments entailed observing seed morphology, measuring flavonoid and phenol content, and evaluating antioxidant activities through luciferase reporter and DPPH assays. Comprehending genetics, phenotype, and phytochemicals associated with soybeans is of utmost importance as it plays a vital role in conducting thorough research on soybean breeding. The study has established a compelling correlation between these physical traits and their antioxidant levels. This significant correlation has enabled researchers to gain valuable insights into the impact of different soybean varieties on antioxidant levels. As a result, this finding can be instrumental in the development of new cultivars that exhibit high levels of antioxidants.

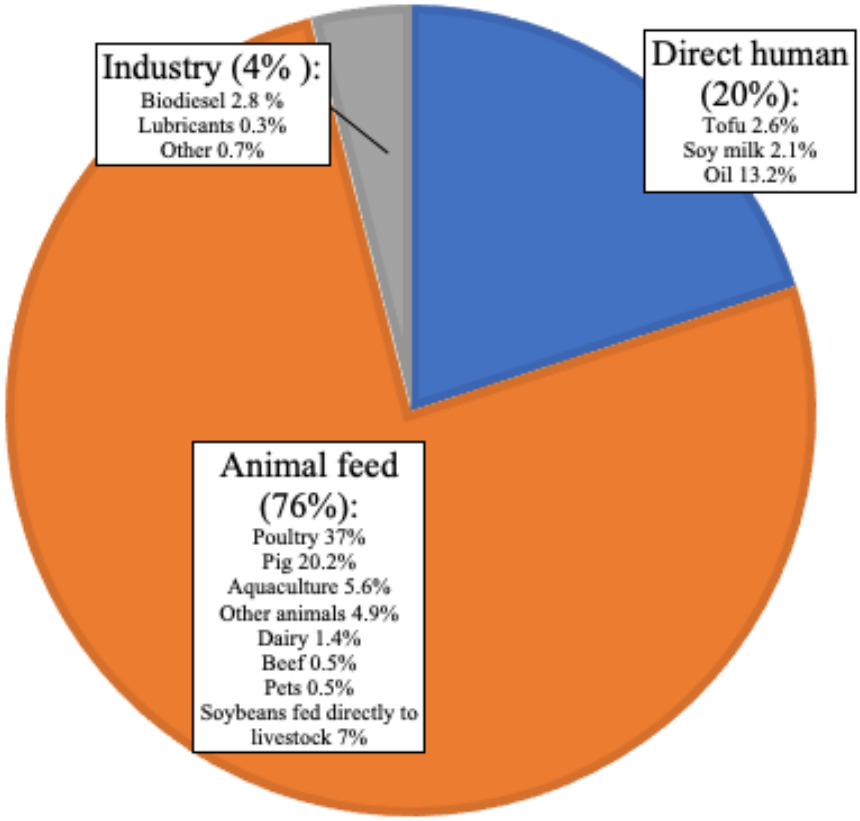

Figure 1.1. The world soybean utilization from 2017 to 2019. Source: Food and Agriculture Organization of the United Nations: https://ourworldindata.org/agricultural-production.

CHAPTER 2

VARIETAL DIFFERENCES IN FLAVONOID AND ANTIOXIDANT ACTIVITY IN JAPANESE SOYBEAN ACCESSIONS

1. INTRODUCTION

Soybean is a well-preserved plant genetic resource, with over 230k accessions in germplasm collections worldwide (Desheva et al. 2017). Its seeds are rich in phytochemical components such as isoflavones, anthocyanins, and saponins, which make soybean a prevalent choice among legumes and other vegetables (Su et al. 2000; Georgetti et al.2006; Xu et al. 2007; Yamasaki et al. 2007). Flavonoids are a group of biologically active compounds, such as flavonols, flavones, and isoflavones, composed of polyphenolic structures. They have hydroxyl groups that can fight against hydroxyl radicals and other reactive oxygen species (ROS) generated due to oxidative stress. The hydroxyl groups of flavonoids help reduce oxidative damage and act as antioxidants, preventing chronic degenerative diseases (Treml and Smejkal 2016).

Two types of antioxidants protect against oxidative stress: direct and indirect. Indirect antioxidants help maintain balance in the body by increasing the production of protective compounds and proteins such as NAD(P)H: quinone oxidoreductase 1 (NQO1) and heme oxygenase-1 (HO-1). These are activated by nuclear factor erythroid 2-related factor 2 (Nrf2)-electrophile/antioxidant responsive element (EpRE/ARE) signaling (Dinkova and Talalay 2008). In the present study, the luciferase reporter assay system was developed to detect compounds that activate ARE promoters using hepatocellular cell lines (HepG2) (Nagahama et al. 2011). While many edible plants have been examined for their health benefits, the antioxidant activity and content of flavonoids and related compounds in different Japanese soybean accessions still need to be explored. Therefore, through the luciferase reporter assay system, the antioxidant activity of Japanese soybean accessions will be detected by activating ARE promoter in HePG2 cells.

The present study focused on 26 soybean accessions, with 23 of them being commonly consumed in Japan. The total flavonoid and phenol content was measured to assess their

nutritional content, and 12 major isoflavones were used in each sample using high-performance liquid chromatography (HPLC). In addition, we evaluated the antioxidant activity of each accession through a luciferase reporter assay and examined the correlation between flavonoid content and antioxidant activity.

2. MATERIALS AND METHODS

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, geneticin, gallic acid monohydrate, quercetin dehydrate, genistein, daidzein, and glycitein were purchased from FUJIFILM Wako Pure Chemical Co., (Osaka, Japan). Daidzin, genistin, and glycitin were obtained from LC Laboratories (Woburn, MA, USA), while 6″-*O*-malonyl-daidzin, 6″-*O*-malonyl-genistin, 6″-*O*-malonyl-glycitin, 6″-*O*-acetyl-daidzin, 6″-*O*-acetyl-genistin, and 6″-*O*-acetyl-glycitin were purchased from Nagara Science Co., (Gifu, Japan). The WST-8 cell counting kit was obtained from Dojindo Laboratories (Kumamoto, Japan). Folin-Ciocalteu reagent was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

2.2. Soybean materials

Twenty-three soybean accessions from Japan were maintained at the National Agriculture and Food Research Organization (NARO) Genebank Project (https://www.gene.affrc.go.jp/). The remaining three accessions: two world accessions Moshidou Gong 503 (*G. max*), and Williams 82 (*G. max*), and one wild soybean accession B01167 (*G. soja*) were obtained from the National BioResource Project (NBRP) for Lotus/*Glycine* in Japan (https://legumebase.nbrp.jp/). The seed appearance of all soybean accessions is shown in Figure 2.2. All 26 soybean accessions were grown in 2015 in a field at Saito City (32.08°N, 131.24°E) in Miyazaki Prefecture, Japan. The field was first fertilized with 1.4 kg N, 3.2 kg P, and 3.4 kg K per 1000 m^2 and 80 kg granular magnesium lime per 1000 m^2. The field was fertilized with 1.12 kg of N, 0.7 kg of P, and 0.98 kg of K per 1000 m^2, two weeks after sowing. The harvested seeds were air-dried in the shade for two months and then kept at 4°C with a relative humidity of 35% until the sample extraction.

2.3. Sample extraction

To obtain the total isoflavones, flavonoids, and total phenols, whole soybean seeds were powdered and extracted using aqueous-acidic ethanol, including the seed coat, radicle, and plumule (Carrao-Panizzi, Fayoni, and Kikuchi 2002). 60mg of soybean seed powder was mixed with 600µL of 70% ethanol (containing 0.1% acetic acid) using a TS-100 Thermo shaker (Biosan, Riga, Latvia) for 48 hours at 25°C. After centrifugation, the supernatant was used for analysis. To conduct the luciferase reporter assay, 200 mg of seed powder was combined with 1 mL of water and agitated in a shaker for 60 min at 0.72 x *g* while heated to 90°C. After centrifuging the samples, the supernatants were lyophilized. Each soybean accession underwent extraction five times and was kept at -20°C before the assay. The detail of the experimental scheme is shown in Figure 2.1.

2.4. High Performance Liquid Chromatography (HPLC) analysis

HPLC analysis was carried out to determine the total isoflavone content using a YMC-Triart C18 column with a 4.6 x 50 mm, 3 µm particle size, and 12 nm pore size (YMC Co. Ltd., Kyoto, Japan). The mobile phase (solvent A) used a solution of acetonitrile: water: formic acid in a 10:90:0.1 ratio, while solvent B was a solution of acetonitrile: formic acid in a 100:0.1 ratio. The injection volume was 10 µL, and a programmed gradient was used, with 0 min – 2.5% (B), 18 min – 22.5% (B), and 20 min – 22.5% (B). Each soybean accession was injected three times, with five replicates for each. The flow rate was 1.5 mL min-1, and the column temperature was 30 °C. The column elute was monitored at 254 nm.

2.5. Aluminum nitrate nonahydrate colorimetric assay

The aluminum nitrate nonahydrate colorimetric assay has been carried out to determine the total flavonoid content in all soybean samples with slight modification (Mohammadzadeh

et al. 2007; Sembiring, Elya, Sauriasari 2018). The experiments were conducted in triplicates using 96-well microtiter plates, with five replicates of each soybean accession. The reaction mixture was prepared by mixing 4.3 mL of 80% ethanol, 0.1 mL of 10% aluminum nitrate nonahydrate, and 0.1 mL of 1 mol/L potassium acetate. A quercetin standard solution was added in each well, or the sample itself (20 µL), followed by 180 µL of the aluminum nitrate nonahydrate mixture. The mixture was incubated for 40 min, and the absorbance was measured at 415 nm using a microplate reader (SH-1000 Lab, Corona Electric, Ltd. Ibaraki, Japan).

2.6. Folin-Ciocalteu assay

The Folin-Ciocalteu assay has been conducted to assess the total phenol content (Magalhães et al. in 2010). In each well, 50 µL of gallic acid standard solution or sample and 50 µL of Folin-Ciocalteu reagent were added. Next, 100 µL of 0.3 mol/L NaOH was added and monitored the absorbance at 650 nm using a microplate reader. The experiment of each sample was completed in five replicates at room temperature.

2.7. Determination of antioxidant activity

The antioxidant activity was assessed by observing the induction of ARE-mediated gene expression using a reporter assay (Nagahama et al. 2011). The HepG2/ARE cells that contained the ARE luciferase reporter were preserved in DMEM with 10% FBS, penicillin-streptomycin (100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin), and 1 mg/mL of G418. The cells were incubated at 37 ºC and 5% CO_2. Five replicates of each soybean extract (1 mg mL^{-1}) or genistein (6 µmol/L) were applied to the cells for a period of 24 h. The cell survival rate was measured using the WST-8 cell counting kit (Dojindo Laboratories, Kumamoto, Japan). The calculation for cell survival rate uses the equation: cell survival rate = (absorbance of the test)/(absorbance of the solvent control) (Nagahama et al. 2011). The cells were centrifuged with the lysis buffer containing 25 mmol/L Tris-phosphate buffer (pH 7.8) and 1%

Triton X-100. The supernatant (50 µL) and luciferase assay substrate (50 µL) (Bright-Glo luciferase assay system) were mixed, and the activity was measured using a Luminescencer-PSN AB-2200 (Atto Co., Tokyo, Japan). The experiment was conducted five times using different samples.

2.8. Seed coat color analysis

Seeds were scanned without color adjustment using an Epson Perfection V700 photoscanner (Seiko Epson Corporation, Suwa, Japan). An average of 20 seeds were observed and analyzed during the scanning process. Each soybean seed coat color was accurately determined using GrainScan software (Whan et al. 2014). The color output was presented in the standardized CIELAB color space, consisting of 3 channels: L^* for lightness (ranging from 0 to 100), a^* for green (negative) or magenta (positive) values, and b^* for blue (negative) or yellow (positive).

2.9. Data analysis

The data were analyzed using analysis of variance (ANOVA) to identify significant differences between the group means with probabilities of $P \leq .01$ and .05. Correlation analysis was conducted using the Pearson product-moment correlation method (Pearson and Filon in 1898). To measure the differences between the control and treatments, post hoc multiple comparisons were used, followed by least significant difference (LSD) test (SPSS for Mac version 24 from Chicago, USA).

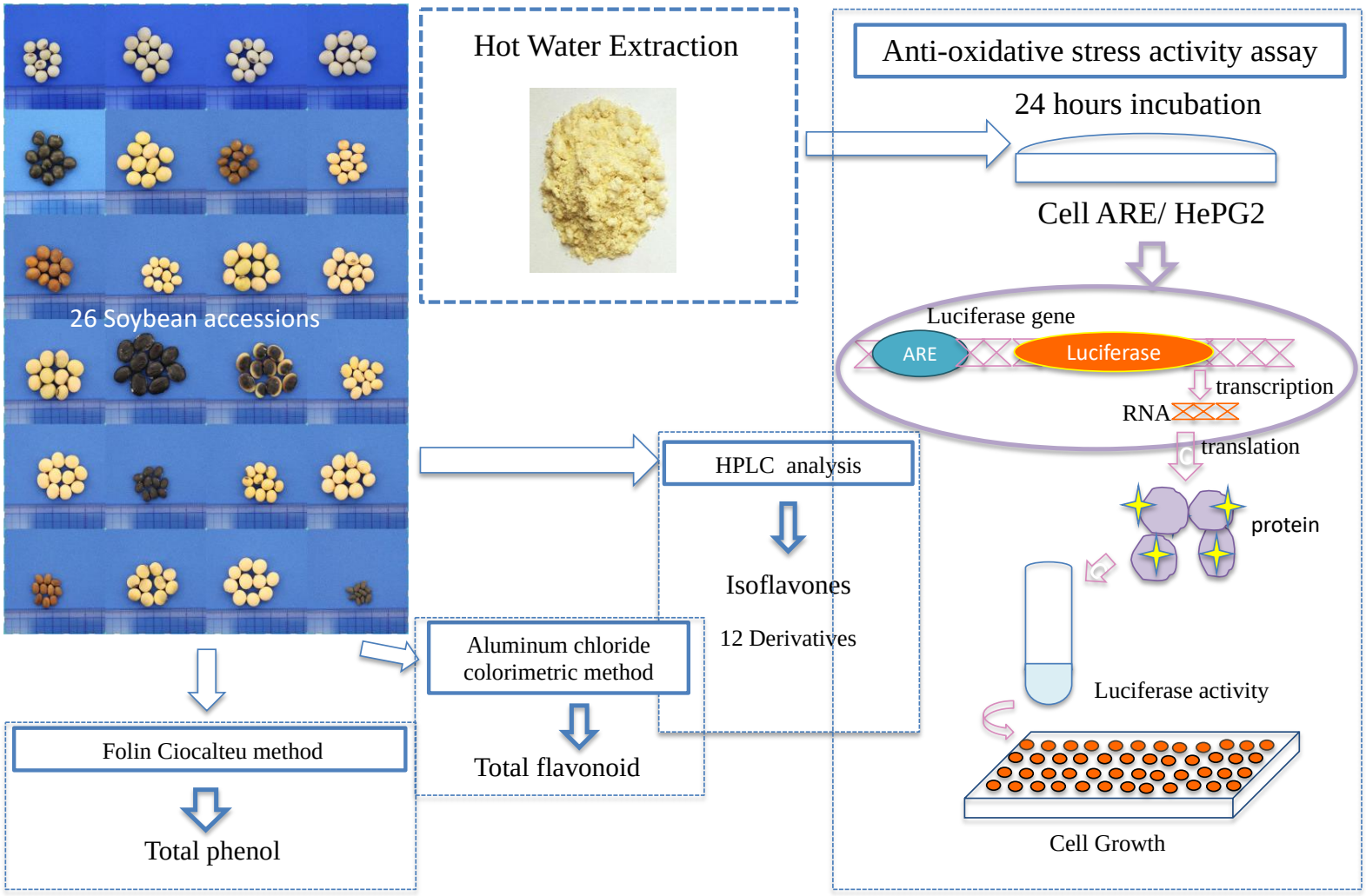

Figure 2.1. Schematic diagram of the experiment in Chapter 2

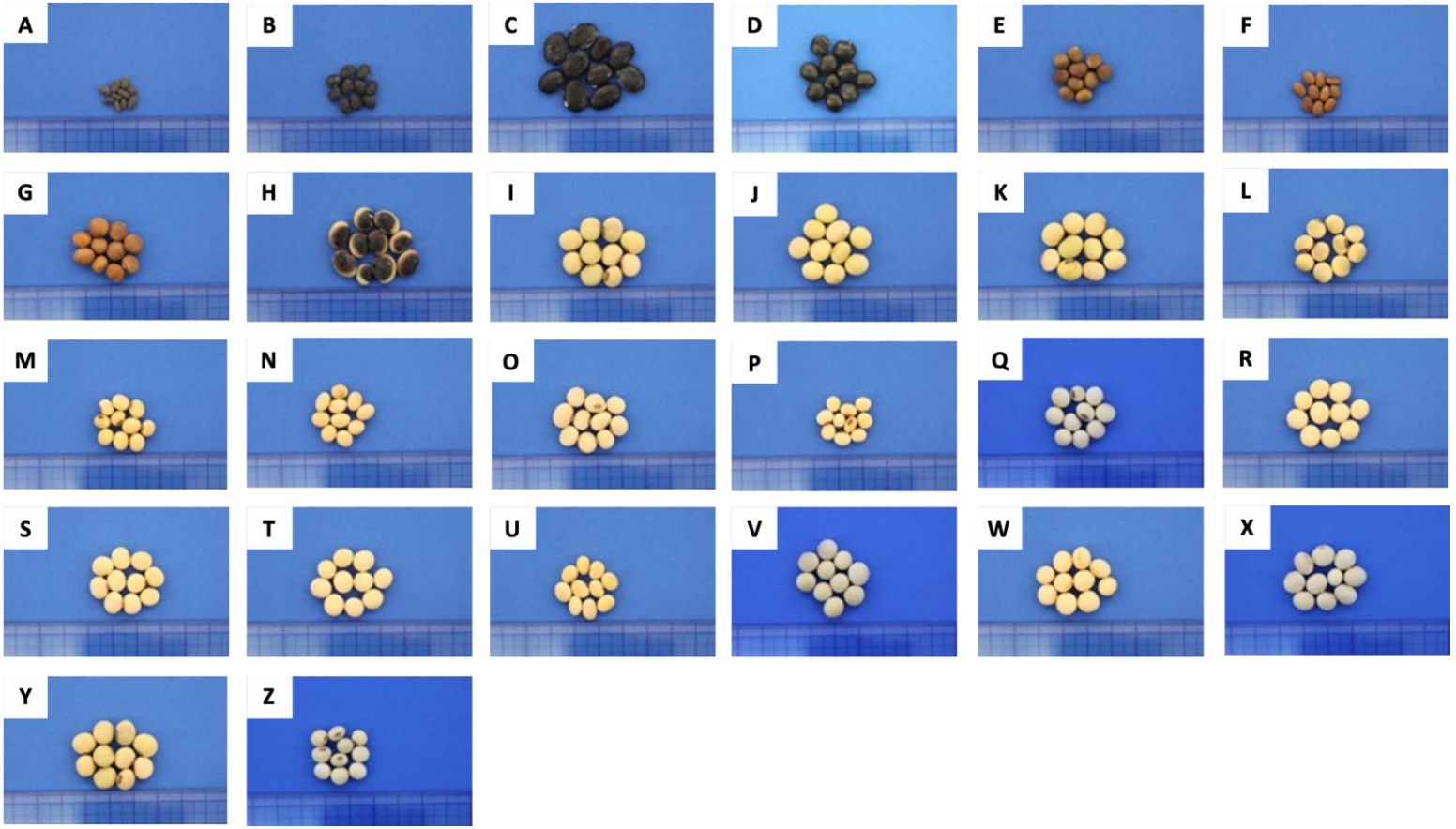

Figure 2.2. Seed appearance of 26 soybean accession used in this study. The accessions' names are according to the alphabetical order: (A) B01167 (*G. soja*), (B) Tsurusengoku, (C) Kurohira, (D) Wase kuro daizu, (E) Kurodaizu (Ao higuu chuu), (F) Moshidou Gong 503, (G) Date Cha mame, (H) Kurakake, (I) Aoakimame, (J) Koito, (K) Hitorimusume, (L) Norin 2, (M) Williams 82, (N) Nattou kotsubu, (O) Akasaya, (P) Hiku anda, (Q) Tokachi nagaha, (R) Tokei 780, (S) Akisengoku, (T) Fukuyutaka, (U) Himeshirazu, (V) Kitajiro, (W) Misizudaizu, (X) Enrei, (Y) Miyadaizu , (Z) Oni Hadaka.

3. RESULTS

3.1. Total flavonoid and total phenol content of 26 soybean accessions

This study examined 26 soybean accessions, 23 of which came from different places in Japan and have been commonly available in local food markets (Table 1). Two additional soybean types, *G. max* "Williams 82" and "Moshidou Gong 503," were selected as representatives outside Japan. One wild soybean type, *Glycine soja* "B01167", was also evaluated. All 26 accessions were extracted and subjected to aluminum nitrate nonahydrate colorimetric assay to determine the total flavonoid content in dry seeds. Table 1 shows that the average flavonoid content was 22.7 mg quercetin equivalents 100 g^{-1}, ranging from 14.7 to 40.8 mg quercetin equivalents 100 g^{-1}. After conducting the Folin-Ciocalteu assay, it was found that the total phenol content varied between 135.3 and 516.9 mg of gallic acid equivalents 100 g^{-1} with an average of 210.5 mg of gallic acid equivalents 100 g^{-1}. According to Table 2.1, *G. soja* had the highest levels of total flavonoid and total phenol content. In contrast, Akasaya and Miyadaizu had the lowest levels of total flavonoid and total phenol content, respectively.

3.2. Isoflavone content of 26 soybean accessions

Soybeans have 12 types of isoflavones, which include glycosylated, malonylated, and acetylated species (Kudou et al. 1991). The total isoflavone content of 26 soybean accessions ranged from 155.7 to 651.7 mg 100 g^{-1} with an average of 342.4 mg 100 g^{-1} (Table 2.1). In all 26 soybean accessions, malonylated isoflavone derivatives were the most prevalent form. On average, there were 264.2 mg of malonylated forms, 48.3 mg of glycosides, 16.2 mg of acetylated forms, and 13.8 mg of aglycones per 100 g. The isoflavones were divided into three forms for further classification: 1. Daidzein derivatives: daidzin, 6″-*O*-acetyl-daidzin, 6″-*O*-malonyl-daidzin, and daidzein (Figure 2.4 to 2.7); 2. Genistein derivatives: genistin, 6″-*O*-acetyl-genistin, 6″-*O*-malonyl-genistin, and genistein (Figure 2.8 to 2.11); 3. Glycitein

derivatives: glycitin, 6′′-*O*-acetyl-glycitin, 6′′-*O*-malonyl-glycitin, and glycitein (Figure 2.12 to 2.15). The range of daidzein derivatives was between 31.54% to 49.79%, while the range of genistein derivatives was between 44.70% to 60.44%. Among the three isoflavone groups, glycitein derivatives had the lowest content ranging from 2.47% to 13.21%. Hitorimusume had the highest levels of daidzein derivatives, while Fukuyutaka and Akasaya had the highest levels of genistein and glycitein derivatives.

3.3. Antioxidant activity of 26 soybean accessions

This study conducted a cell-based assay to assess the antioxidant activity of extracts from 26 soybean accessions. The test was performed on HepG2/ARE cells using an ARE-mediated luciferase reporter assay. The positive control's relative ARE luciferase activity rate (6 μmol/L genistein) was 1.74±0.30. The rate of ARE luciferase activity differed by up to four times, ranging from 1.00 to 4.02 (Figure 2.3). In 22 accessions, there was a significant increase in the activity compared to the negative control (only DMEM). Out of all the soybean accessions analyzed, Williams 82, Himeshirazu, Akisengoku, Nattou kotsubu, and Akasaya had relatively ARE luciferase activity rates of ≥3.0. However, Wase kuro daizu, Kurodaizu (Ao higuu chuu), Koito, and Oni Hadaka showed no significant activity.

3.4. Correlation analysis between flavonoid content and antioxidant activity

Correlation analysis was conducted between antioxidant activity, isoflavone content, total flavonoid content, and total phenol content (Table 2.2). There was a significant and positive correlation between the relative antioxidant activity and the total isoflavone content (r= 0.49, P < .05), including isoflavone forms: genistein (r = 0.41, p < 0.05), genistin (r = 0.43, P < .05), 6′′-*O*-malonyl-daidzin (r = 0.46, P < .05), 6′′-*O*-malonyl-genistin (r = 0.47, P < .05), and 6′′-*O*-acetyl-daidzin (r = 0.45, P < .05). Meanwhile, the total flavonoid content and total phenol content were not correlated with antioxidant activity. Out of the three-color parameters (L*,

*a**, and *b**) of soybean seed coats, only the *L** and *b** parameters showed significantly and negatively correlated with the total flavonoid content (r = –0.72, –0.75, P < .01, respectively) and the total phenol content (r = –0.63, –0.58, P <.01, respectively) (Table 2.2 and 2.3).

Table 2.1. Twenty-six soybean accessions used in this study and their isoflavone, total flavonoid and total phenol contents.

Cultivar name	JMC number*	DE	GE	GLE	D	G	GL	MD	MG	MGL	AD	AG	AGL	TI	TF	TP
		mg 100 g^{-1}													mg QE100 g^{-1}	mg GAE 100 g^{-1}
B01167 (*G. soja*)	N.A.	13.0±0.7	17.7±3.2	3.9±0.7	33.7±2.5	7.1±0.2	7.1±2.2	86.2±4.6	119.1±5.7	17.4±0.6	16.0±0.5	0.6±0.6	4.8±0.9	332.1±8.9	40.8±5.7	516.9±19.3
Tsurusengoku	GmJMC172	6.3±0.2	8.8±0.5	2.8±0.2	21.2±2.1	29.0±2.1	1.3±0.8	120.8±11.1	213.7±5.4	3.8±2.3	17.7±0.7	2.6±0.1	2.8±1.6	430.8±19.0	27.2±3.8	356.7±24.4
Kurohira	GmJMC092	4.4±0.3	4.2±0.3	0.8±0.1	16.9±1.3	16.6±0.7	2.4±0.6	86.4±4.5	111.0±1.6	5.8±1.6	7.8±0.3	0.5±0.0	1.2±0.1	257.9±10.0	28.9±8.2	219.9±9.8
Wase kuro daizu	GmJMC002	2.0±0.3	2.0±0.2	0.4±0.1	11.5±1.5	11.0±0.6	3.0±1.0	46.8±9.6	66.5±5.3	5.3±2.4	5.1±0.7	0.2±0.1	1.9±0.6	155.7±19.8	36.9±5.7	226.0±7.1
Kurodaizu (Ao higuu chuu)	GmJMC030	3.1±0.2	3.9±0.7	1.1±0.2	12.8±0.8	15.6±0.8	3.5±2.4	52.1±3.1	92.0±3.9	15.2±3.8	6.9±0.6	0.2±0.1	0.9±0.2	207.4±11.5	23.7±2.9	195.0±8.4
Moshidou Gong 503	N.A.	7.5±0.7	14.1±3.4	3.9±0.6	20.9±1.9	31.1±1.8	13.4±2.3	148.7±9.0	245.4±9.6	30.6±4.2	20.6±1.0	1.5±0.5	4.2±0.4	541.7±25.0	23.2±1.4	417.7±27.5
Date Cha mame	GmJMC041	3.7±0.5	3.4±0.4	0.9±0.4	16.2±0.5	13.6±0.3	2.5±0.7	60.6±1.7	71.7±3.7	7.1±1.2	6.9±0.6	0.4±0.3	1.5±0.4	188.6±6.9	24.5±8.9	167.0±7.1
Kurakake	GmJMC102	6.6±1.2	6.9±0.7	0.8±0.3	37.0±3.5	32.0±1.4	4.6±1.5	140.3±16.5	170.4±9.8	8.9±2.7	12.9±0.5	0.5±0.1	5.1±1.3	426.0±36.4	23.1±3.8	212.4±17.0
Aoakimame	GmJMC082	4.1±1.0	4.0±1.6	1.7±0.8	14.2±2.6	11.1±0.7	2.9±1.9	74.9±14.1	81.3±5.0	8.3±5.7	6.4±0.9	0.9±0.5	2.8±1.0	212.7±27.2	16.4±1.8	159.3±10.4
Koito	GmJMC028	6.6±1.5	6.1±0.7	1.7±0.8	29.3±2.1	23.2±1.1	3.5±0.8	121.2±7.7	134.4±5.4	6.9±1.5	10.8±0.5	1.1±0.5	5.1±0.2	349.9±19.7	17.8±2.0	181.5±13.2
Hitorimusume	GmJMC077	5.0±0.5	4.7±0.5	1.9±0.3	20.3±1.0	14.8±0.6	2.6±0.6	77.6±3.4	78.5±4.1	5.4±3.7	7.6±0.6	1.3±0.2	2.2±0.3	221.9±8.5	16.3±1.2	136.4±7.0
Norin 2	N.A.	2.3±0.5	4.0±0.5	1.0±0.4	13.1±1.4	17.0±1.0	5.9±1.9	81.2±8.7	146.9±6.9	16.1±5.8	11.2±0.7	0.6±0.1	3.2±0.5	302.4±25.2	15.7±2.4	173.9±12.1
Williams 82	N.A.	10.1±2.1	17.0±4.2	2.5±1.1	34.0±2.1	37.1±1.9	8.3±0.6	214.5±5.5	283.1±6.8	20.2±2.3	20.3±1.2	1.4±0.8	3.4±1.6	651.7±18.0	21.5±3.0	213.1±11.0
Nattou kotsubu	GmJMC032	3.5±0.4	5.8±0.4	0.8±0.2	17.3±1.4	21.2±0.8	2.7±2.2	62.1±8.1	112.8±9.6	9.7±1.8	9.9±0.5	0.2±0.1	1.6±0.2	247.7±23.8	16.3±3.2	172.5±8.5
Akasaya	GmJMC078	3.5±0.6	5.1±0.3	1.6±1.0	18.0±1.8	19.7±1.1	9.4±4.3	71.4±9.3	116.2±6.1	22.0±8.1	9.4±0.7	0.4±0.1	4.1±1.5	280.9±31.6	14.7±1.5	175.4±8.2
Hiku anda	GmJMC049	5.6±0.3	7.0±0.2	1.1±0.1	25.7±1.5	24.8±1.4	9.2±0.7	98.9±4.2	135.7±5.4	20.9±1.3	11.9±0.6	0.5±0.0	2.6±0.2	343.9±14.3	15.6±0.9	210.1±9.6
Tokachi nagaha	GmJMC007	9.4±1.7	12.1±2.8	1.3±0.9	39.7±2.4	42.4±2.4	5.1±0.7	192.6±8.5	254.5±13.5	11.7±2.4	17.6±1.9	0.9±0.6	6.9±1.3	594.3±35.4	28.2±3.2	243.7±8.0
Tokei 780	N.A.	3.8±2.7	9.0±2.3	1.0±0.7	22.3±0.7	25.8±7.8	3.2±0.6	123.3±5.5	240.1±3.4	9.8±3.0	18.0±0.6	1.1±0.6	4.1±0.4	461.4±18.6	23.8±1.9	164.9±63.4
Akisengoku	GmJMC117	6.3±0.5	6.7±0.4	0.7±0.1	28.7±2.1	25.2±1.4	2.9±0.5	149.9±6.1	178.2±4.8	8.8±2.3	13.3±1.2	0.6±0.1	6.1±0.8	427.4±15.5	16.0±2.5	172.9±7.2
Fukuyutaka	GmJMC112	2.1±0.2	4.6±0.6	0.4±0.1	11.0±1.0	15.1±1.3	1.8±0.3	69.4±3.6	135.2±6.5	5.4±0.8	10.2±0.6	0.4±0.0	1.5±0.4	257.1±12.7	21.8±1.8	159.5±7.3
Himeshirazu	GmJMC106	8.1±0.7	9.5±0.8	1.9±0.5	32.4±2.0	34.6±2.0	7.7±1.5	153.7±4.4	227.8±4.0	16.8±3.3	15.1±0.6	0.5±0.1	5.8±0.4	514.0±15.1	18.2±1.3	184.3±10.8
Kitajiro	GmJMC004	9.6±0.7	10.4±0.6	2.7±0.6	27.6±0.7	30.9±0.7	6.7±1.1	137.7±12.7	189.7±22.6	14.7±1.7	14.1±0.4	2.4±0.4	5.6±0.3	452.2±31.0	19.3±3.7	188.5±22.1
Misizudaizu	N.A.	1.8±0.5	2.8±0.6	1.2±1.0	8.4±0.9	13.4±0.8	4.6±2.4	56.8±5.4	125.3±5.7	13.9±8.5	8.4±0.7	0.5±0.2	2.1±0.4	239.2±23.9	16.6±3.6	142.3±10.6
Enrei	GmJMC025	2.1±0.8	3.4±0.6	0.6±0.2	17.3±1.2	15.6±3.7	4.3±1.6	63.5±3.4	91.5±6.4	8.2±2.8	7.0±1.0	0.3±0.1	3.4±1.0	217.2±14.4	19.1±1.9	165.0±8.2
Miyadaizu	N.A.	2.3±0.1	4.8±0.2	0.4±0.1	13.1±0.5	17.4±0.4	2.2±0.4	79.6±3.8	143.4±3.6	6.2±1.2	11.4±0.3	0.5±0.0	2.3±1.1	283.6±8.0	22.2±1.6	135.3±21.1
Oni Hadaka	GmJMC026	5.5±0.6	4.6±0.7	0.7±0.3	24.5±1.0	20.2±0.7	3.1±0.4	102.8±1.8	123.6±2.6	7.8±1.0	9.2±0.8	0.5±0.3	3.7±0.7	306.2±3.1	22.6±3.8	183.1±8.0

The total isoflavones were calculated as the sum of the 12-isoflavone forms. The data were expressed as mg 100 g^{-1} dry weight of the soybean seed. For the total flavonoid and the total phenol, the data were expressed as milligram quercetin equivalents (mg QE 100 g^{-1}) dry weight and milligrams of gallic acid equivalents per 100 grams (mg GAE 100 g^{-1}) of the soybean seed powder respectively. Data are presented as mean values with standard deviations (n=5).

Daidzein (DE), Genistein (GE), Glycitein (GLE), Daidzin (D), Genistin (G), Glycitin (GL), 6′′-*O*-malonyl-daidzin (MD), 6′′-*O*-malonyl-genistin (MG), 6′′-*O*-malonyl-glycitin (MGL), 6′′-*O*-acetyl-daidzin (AD), 6′′ -*O*-acetyl-genistin (AG), 6′′-*O*-acetyl-glycitin (AGL), Total Isoflavones (TI), Total flavonoids (TF), and Total phenols (TP).

*JMC (Japanese Mini-Core Collection) number can be searched at the NARO Genebank database (https://www.gene.affrc.go.jp/databases-core_collections_wg_en.php).

Table 2.2. Correlations between antioxidant activity, isoflavone content, total flavonoid content, total phenol content, and seed coat color.

	AA	DE	GE	GLE	D	G	GL	MD	MG	MGL	AD	AG	AGL	TI	TF	TP
AA	1.00	0.29	0.41*	0.14	0.37	0.43*	0.29	0.46*	0.47*	0.29	0.45*	0.01	0.31	0.49*	–0.29	0.03
Seed coat color:																
L*	0.32	–0.15	–0.10	–0.33	0.07	0.22	0.03	0.18	0.18	0.03	0.03	–0.35	0.27	0.16	–0.72**	–0.63**
a*	0.05	–0.04	0.11	0.08	–0.12	0.09	0.29	–0.02	0.09	0.37	0.11	–0.13	–0.12	0.06	0.09	0.17
b*	0.28	–0.04	–0.02	–0.19	0.13	0.26	0.13	0.24	0.20	0.14	0.07	–0.31	0.31	0.21	–0.75**	–0.58**

* p value < 0.05, ** p value < 0.01

Antioxidant activity (AA), Total isoflavones (TI): Daidzein (DE), Genistein (GE), Glycitein (GLE), Daidzin (D), Genistin (G), Glycitin (GL), 6′′-*O*-malonyl-daidzin (MD), 6′′-*O*-malonyl-genistin (MG), 6′′-*O*-malonyl-glycitin (MGL), 6′′-*O*-acetyl-daidzin (AD), 6′′-*O*-acetyl-genistin (AG), 6′′-*O*-acetyl-glycitin (AGL), Total flavonoids (TF), and Total phenols (TP). Seed color parameters: *L** (0 to 100 indicates lightness of the color), *a** (negative or positive values represent green or magenta, respectively) and *b** (negative or positive values represent blue or yellow, respectively).

Table 2.3. The information on seed coat, hilum, cotyledon, seed size and days to harvest of 26 soybean accessions.

Cultivar name	JMC number*	Seed coat color				Hilum	Cotyledon	Seed size		Days to harvest
		Seed coat	*L**	*a**	*b**			EI (**)	FI (***)	
B01167 (*G. soja*)	N.A.	Black	29.90±1.40	1.93±1.28	0.24±2.30	Black	Yellow	1.57	10.69	76
Tsurusengoku	GmJMC172	Black	28.11±0.69	–2.29±0.28	–4.37±0.53	Black	Yellow	1.38	22.05	96
Kurohira	GmJMC092	Black	27.87±2.05	–2.32±0.15	–4.41±0.42	Black	Yellow	1.41	62.47	115
Wase kuro daizu	GmJMC002	Black	28.95±0.91	–2.02±0.21	–5.13±0.26	Black	Yellow	1.18	51.37	74
Kurodaizu (Ao higuu chuu)	GmJMC030	Black	34.13±1.65	0.51±0.52	7.10±2.16	Black	Yellow	1.09	37.33	84
Moshidou Gong 503	N.A.	Brown	35.97±1.72	2.83±0.67	10.59±1.80	Dark brown	Yellow	1.40	17.90	84
Date Cha mame	GmJMC041	Brown	36.11±1.62	3.92±0.55	10.58±1.87	Brown	Yellow	1.05	59.02	81
Kurakake	GmJMC102	Green, black	42.16±8.36	–2.80±1.32	7.85±7.05	Black	Green	1.31	56.05	101
Aoakimame	GmJMC082	Green	57.25±2.46	–3.56±1.55	21.45±1.58	Black	Yellow	1.08	66.24	103
Koito	GmJMC028	Pale green	60.60±1.88	–3.13±3.67	23.73±1.64	Buff brown	Yellow	1.16	62.30	111
Hitorimusume	GmJMC077	Pale green	57.94±1.42	–4.40±0.81	22.68±1.46	Black	Yellow	1.12	68.24	117
Norin 2	N.A.	Yellow-green	57.70±1.91	–3.15±0.62	21.49±1.51	Black	Yellow	1.11	50.27	83
Williams 82	N.A.	Yellow	60.11±1.56	–1.20±0.59	23.86±5.65	Black	Yellow	1.08	46.12	94
Nattou kotsubu	GmJMC032	Yellow	62.17±1.65	–0.15±0.52	23.32±1.35	Light buff	Yellow	1.21	36.62	108
Akasaya	GmJMC078	Yellow	62.09±1.37	–1.09±0.27	18.64±1.22	Dark brown	Yellow	1.15	56.32	108
Hiku anda	GmJMC049	Yellow	64.17±1.36	–1.74±0.49	23.71±2.33	Dark brown	Yellow	1.10	28.91	81
Tokachi nagaha	GmJMC007	Yellow	61.68±1.05	–1.21±0.56	22.55±2.27	Black	Yellow	1.10	57.26	101
Tokei 780	N.A.	Yellow	64.10±1.22	–1.24±0.38	23.12±1.70	Light buff	Yellow	1.06	56.67	86
Akisengoku	GmJMC117	Yellow	63.92±0.87	–1.34±0.31	22.56±1.21	Brown	Yellow	1.19	46.72	108
Fukuyutaka	GmJMC112	Yellow	65.91±1.39	–1.66±0.66	22.49±2.38	Buff brown	Yellow	1.11	54.12	115
Himeshirazu	GmJMC106	Yellow	60.91±1.31	–0.98±0.44	24.35±2.07	Buff	Yellow	1.23	38.35	103
Kitajiro	GmJMC004	Yellow	57.95±1.15	–1.60±0.40	25.18±2.03	Light buff	Yellow	1.06	61.87	94
Misuzudaizu	N.A.	Yellow	62.70±0.78	–0.92±0.56	21.01±1.14	Buff	Yellow	1.08	65.48	92
Enrei	GmJMC025	Yellow	60.21±1.87	–0.34±0.58	22.97±1.43	Buff	Yellow	1.07	58.53	101
Miyadaizu	N.A.	Yellow	65.74±1.13	–1.81±0.55	21.49±1.76	Buff brown	Yellow	1.11	53.68	107
Oni Hadaka	GmJMC026	Yellow	62.58±1.07	–1.23±0.50	25.16±1.63	Dark brown	Yellow	1.04	45.50	89

*JMC (Japanese Mini-Core Collection) number can be searched at the NARO Genebank database (https://www.gene.affrc.go.jp/databases-core_collections_wg_en.php).
Seed color parameters: L* (0 to 100 indicates lightness of the color), a* (negative or positive values represent green or magenta, respectively) and b* (negative or positive values represent blue or yellow, respectively).
Seed size: (**) EI: Seed Eccentricity Index (mm), (***) FI: Seed Flatness Index (mm)

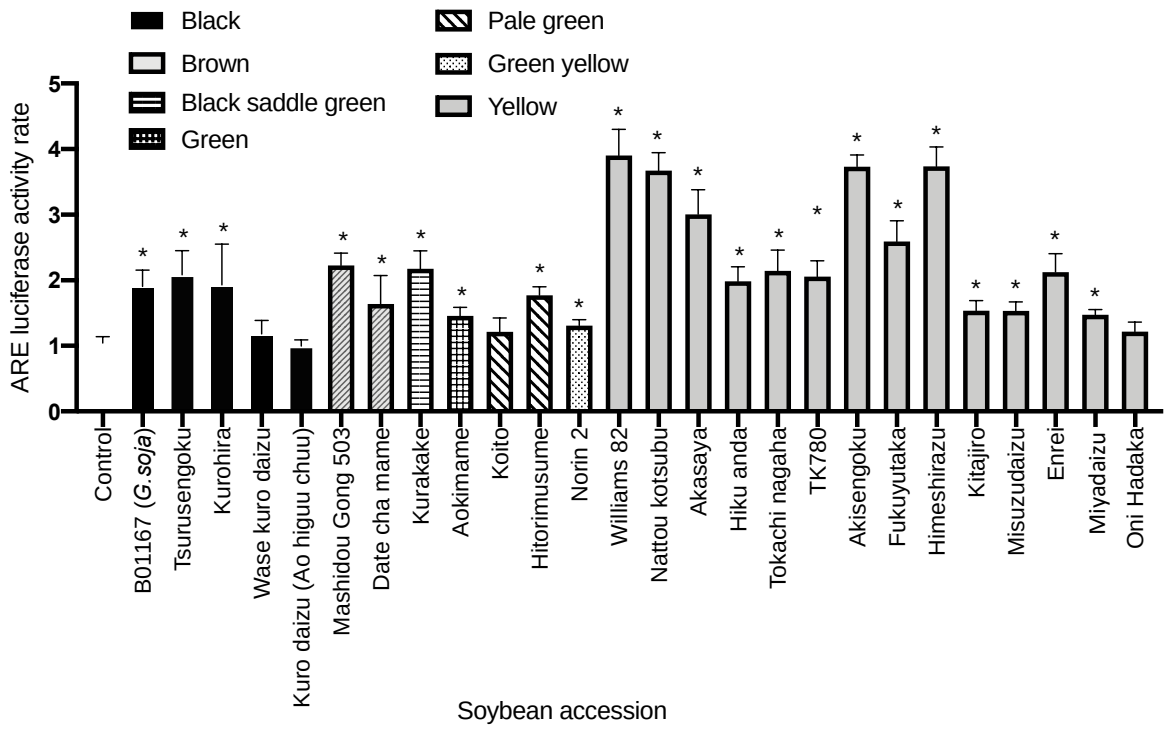

Figure 2.3. Comparison of antioxidant activities in 26 soybean accessions. Antioxidant activity was evaluated by induction of ARE-mediated gene expression using a reporter assay as described in the Materials and Methods (n=5). The level of antioxidant activity was calculated using the equation: relative ARE luciferase activity rate = (relative luciferase activity)/(cell survival rate). The data shown in the figure represent means ± SD for five experiments. Statistically significant differences were determined by the least significant difference (LSD) test for individual comparison of groups with control groups. *p <0.05.

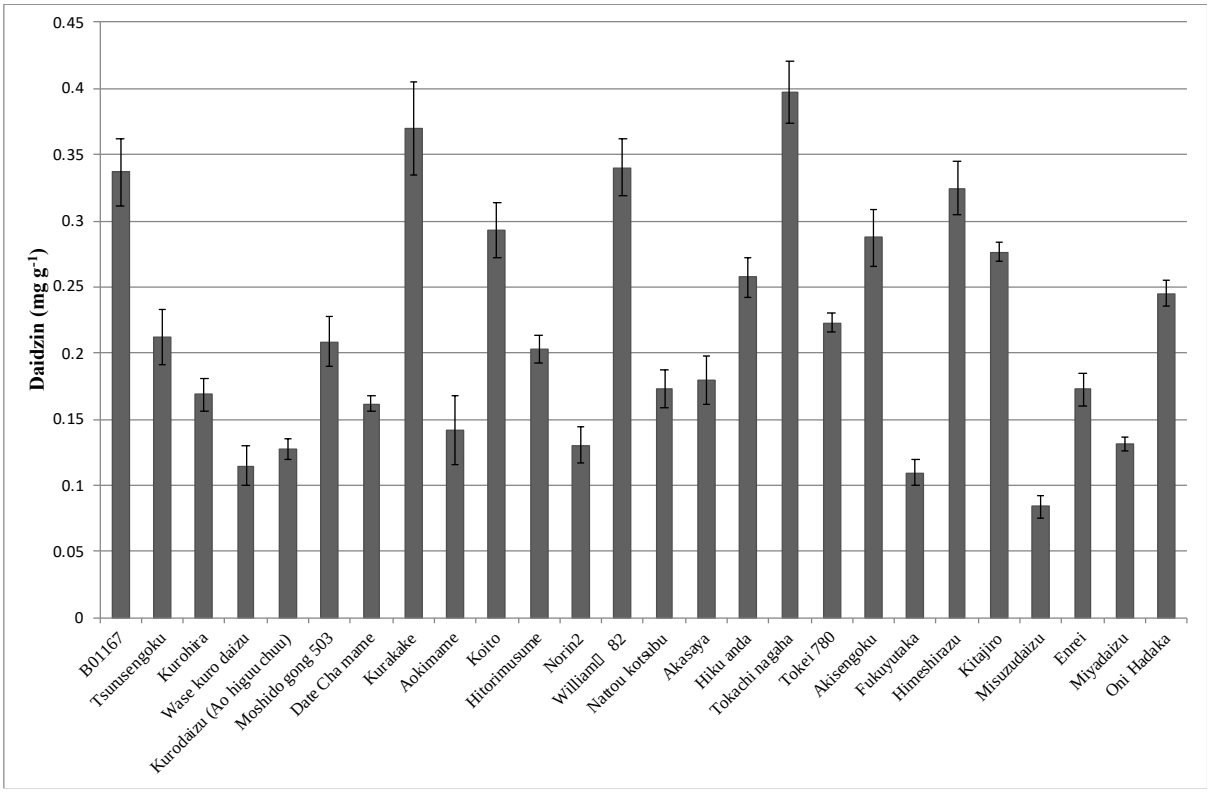

Figure 2.4. Comparison of daidzin contents in 26 soybean accessions. The isoflavones derivatives: daidzin was determined by aluminum nitrate nonahydrate colorimetric assay as described in the Materials and Methods. The experiments were performed in triplicates in 96-well microtiter plates with five replicates of each soybean accession. The data shown in the figure represent means ± SD.

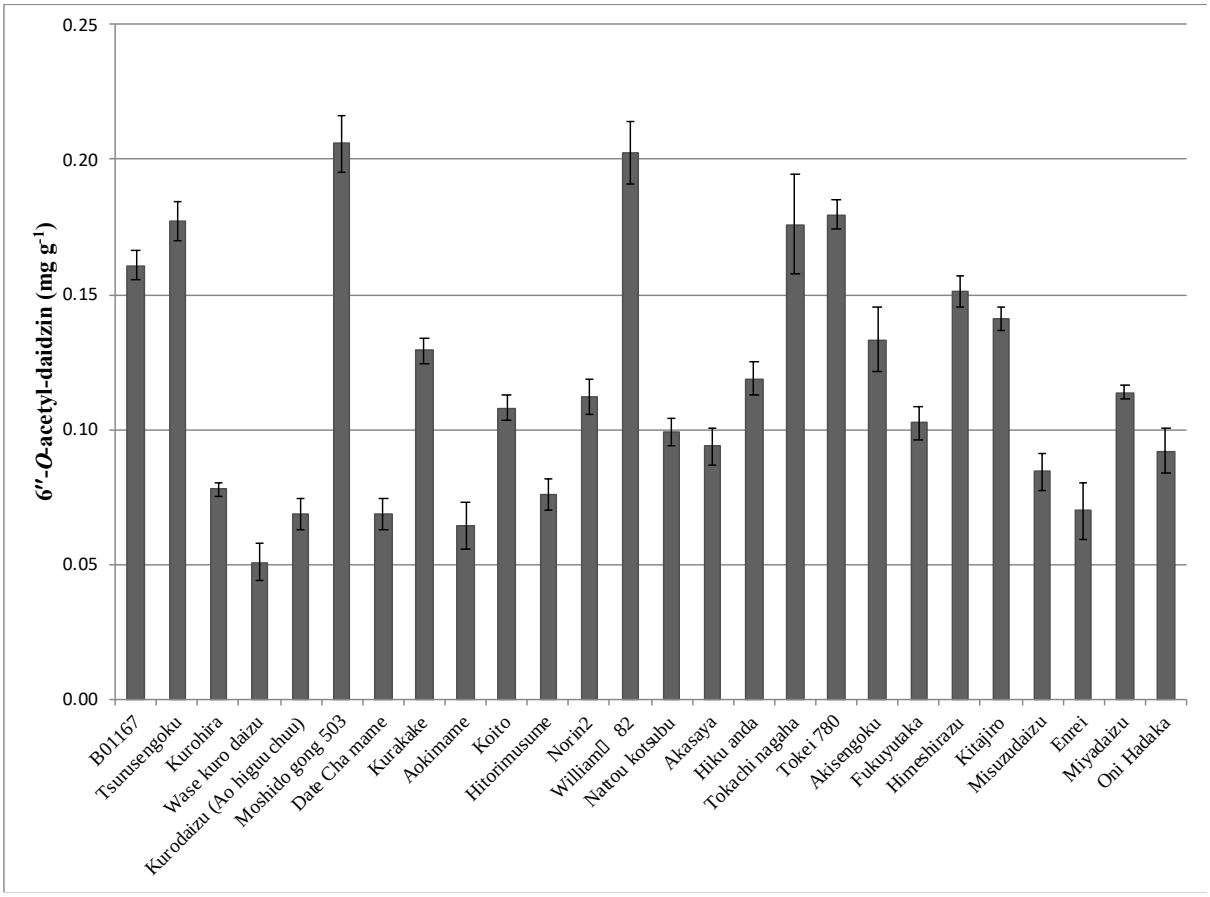

Figure 2.5. Comparison of 6′′-*O*-acetyl-daidzin contents in 26 soybean accessions. The isoflavones derivatives: 6′′-*O*-acetyl-daidzin was determined by aluminum nitrate nonahydrate colorimetric assay as described in the Materials and Methods. The experiments were performed in triplicates in 96-well microtiter plates with five replicates of each soybean accession. The data shown in the figure represent means ± SD.

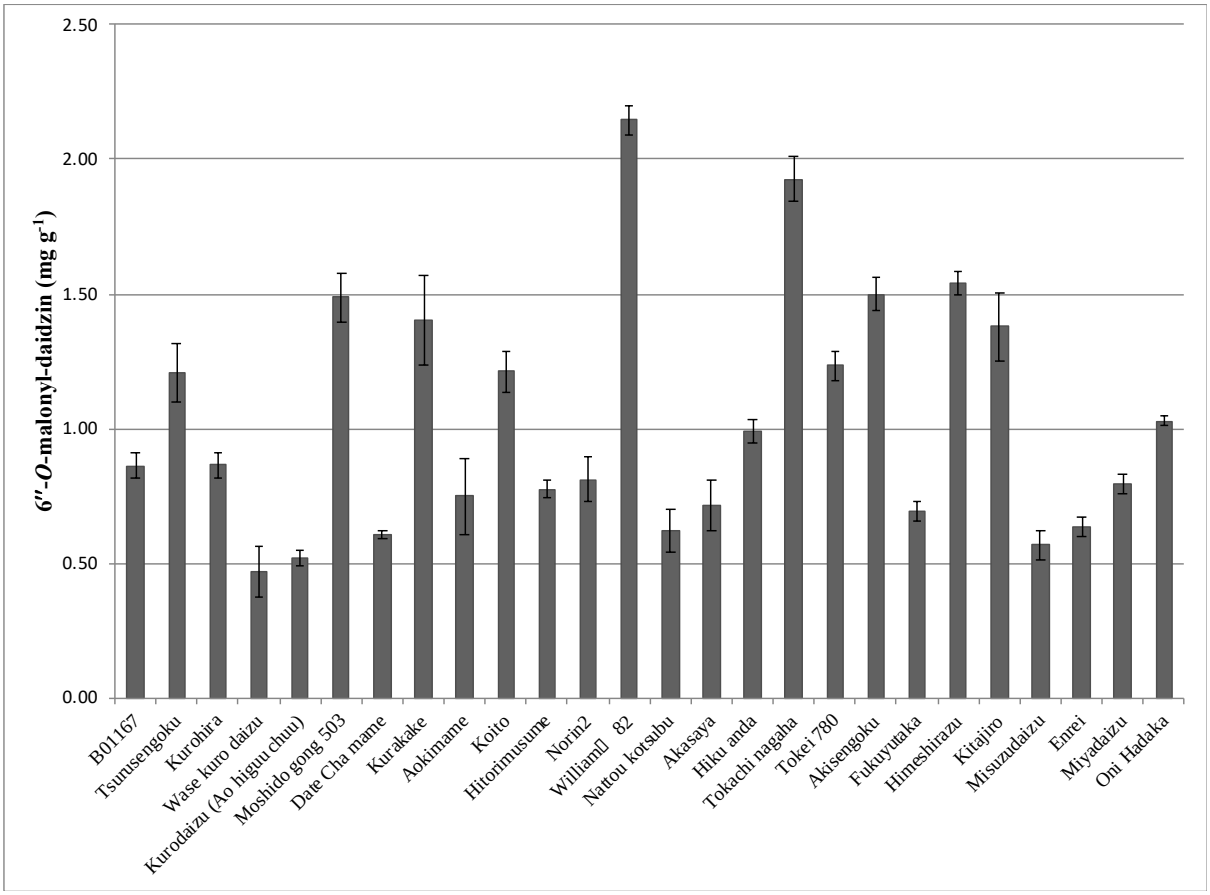

Figure 2.6. Comparison of 6′′-*O*-malonyl-daidzin contents in 26 soybean accessions. The isoflavones derivatives: 6′′-*O*-malonyl-daidzin was determined by aluminum nitrate nonahydrate colorimetric assay as described in the Materials and Methods. The experiments were performed in triplicates in 96-well microtiter plates with five replicates of each soybean accession. The data shown in the figure represent means ± SD.

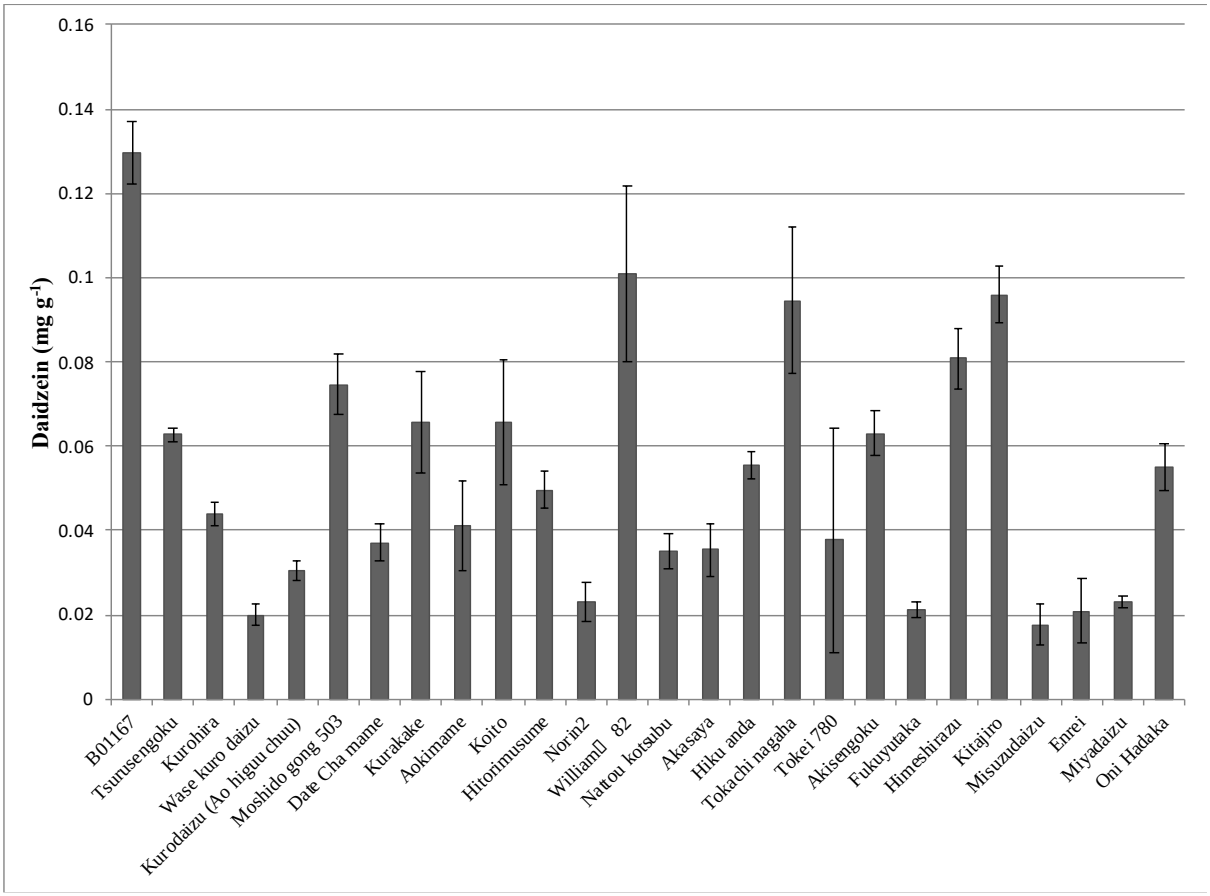

Figure 2.7. Comparison of daidzein contents in 26 soybean accessions. The isoflavones derivatives: daidzein was determined by aluminum nitrate nonahydrate colorimetric assay as described in the Materials and Methods. The experiments were performed in triplicates in 96-well microtiter plates with five replicates of each soybean accession. The data shown in the figure represent means ± SD.

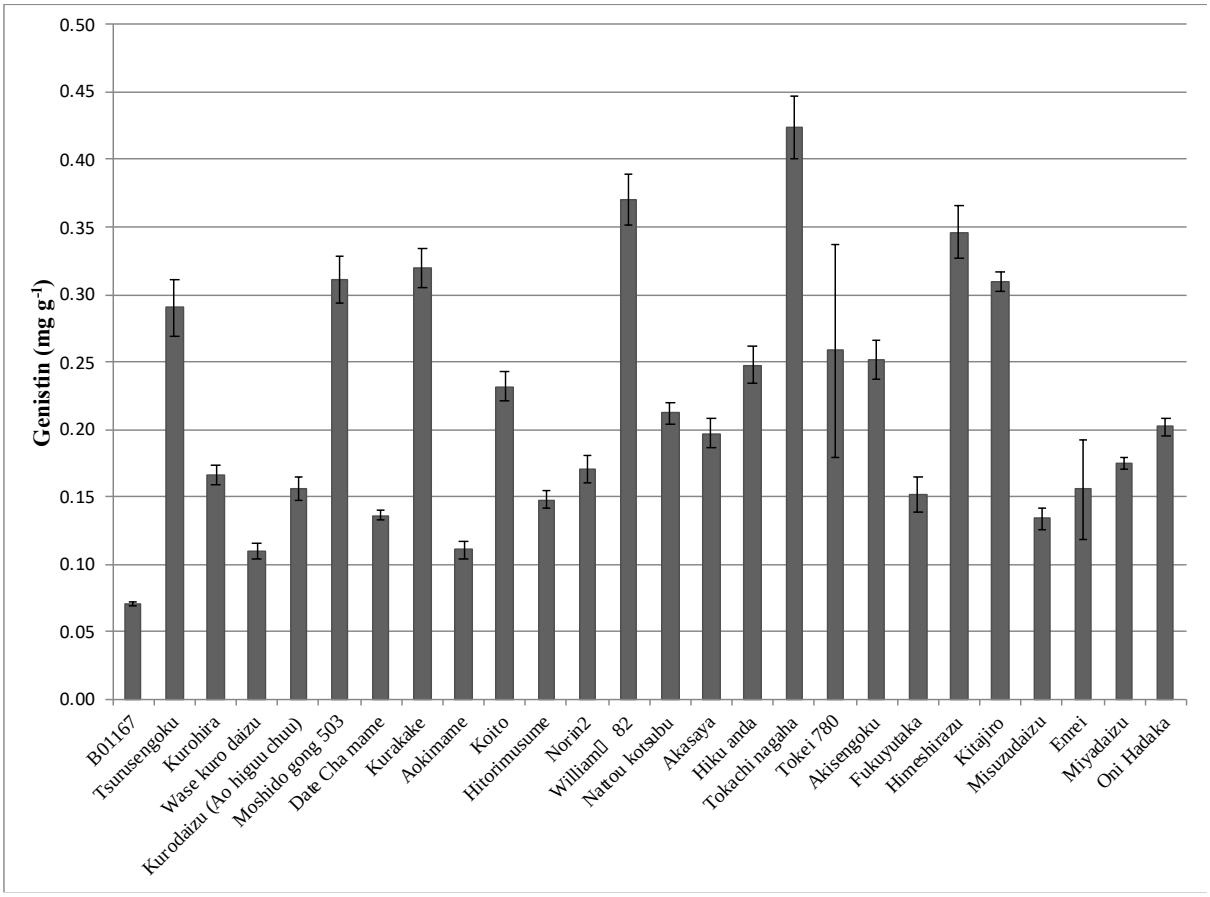

Figure 2.8. Comparison of genistin contents in 26 soybean accessions. The isoflavones derivatives: genistin was determined by aluminum nitrate nonahydrate colorimetric assay as described in the Materials and Methods. The experiments were performed in triplicates in 96-well microtiter plates with five replicates of each soybean accession. The data shown in the figure represent means ± SD.

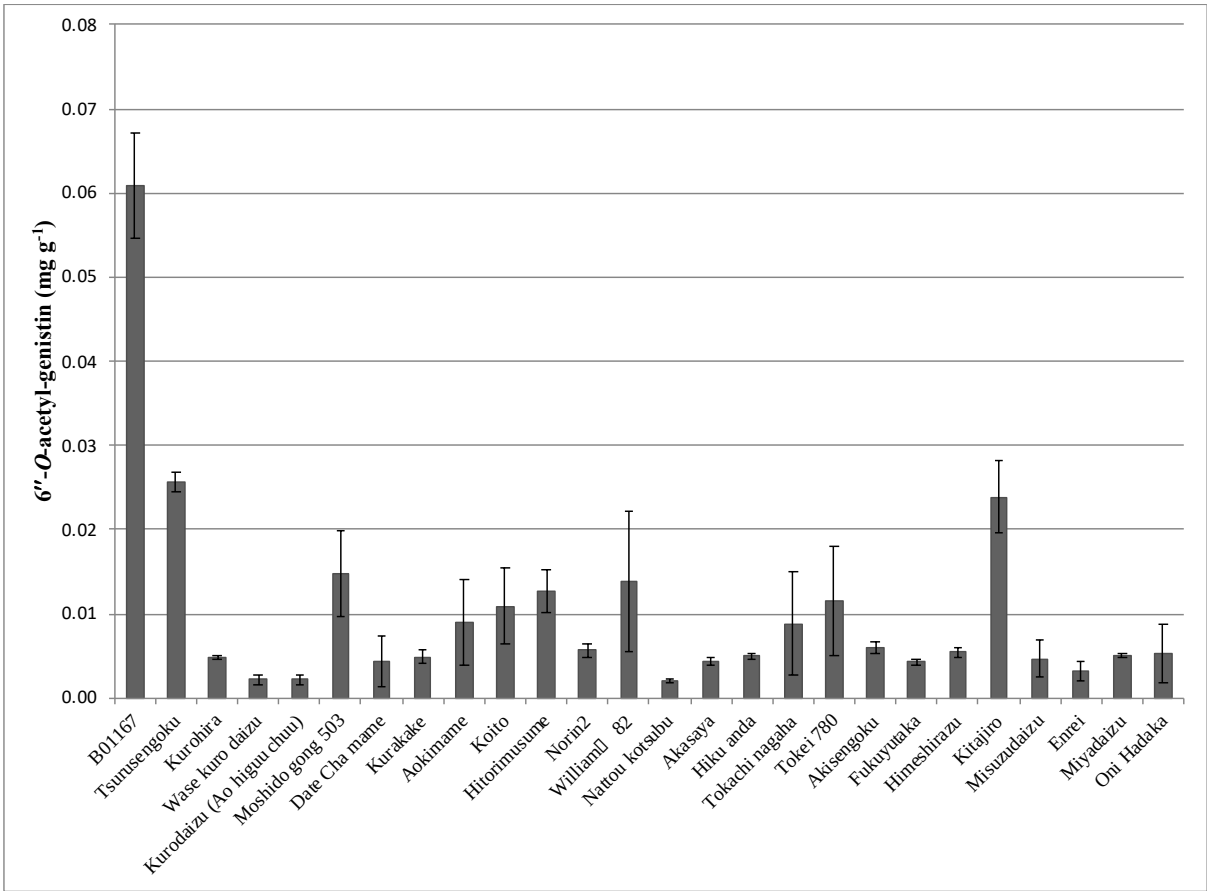

Figure 2.9. Comparison of 6′′-*O*-acetyl-genistin contents in 26 soybean accessions. The isoflavones derivatives: 6′′-*O*-acetyl-genistin was determined by aluminum nitrate nonahydrate colorimetric assay as described in the Materials and Methods. The experiments were performed in triplicates in 96-well microtiter plates with five replicates of each soybean accession. The data shown in the figure represent means ± SD.

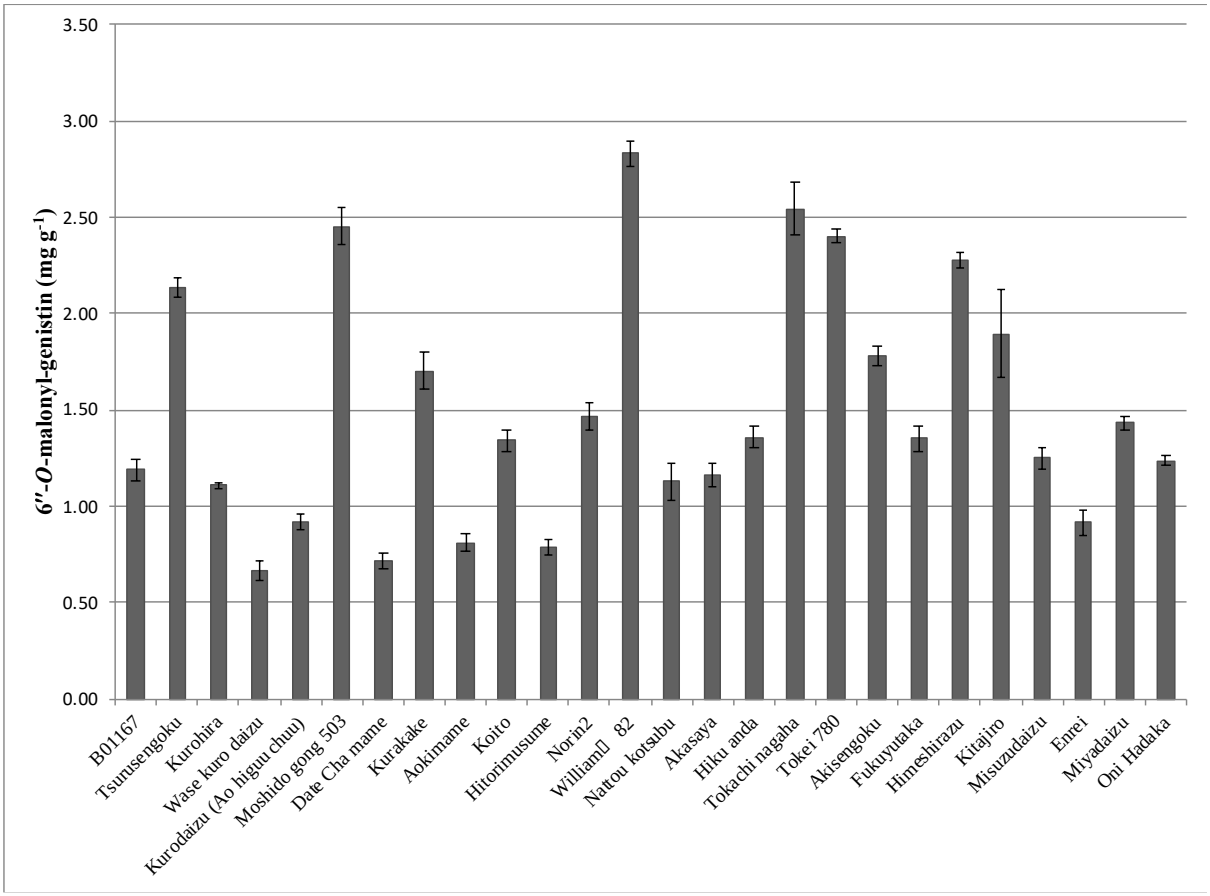

Figure 2.10. Comparison of 6′′-*O*-malonyl-genistin contents in 26 soybean accessions. The isoflavones derivatives: 6′′-*O*-malonyl-genistin was determined by aluminum nitrate nonahydrate colorimetric assay as described in the Materials and Methods. The experiments were performed in triplicates in 96-well microtiter plates with five replicates of each soybean accession. The data shown in the figure represent means ± SD.

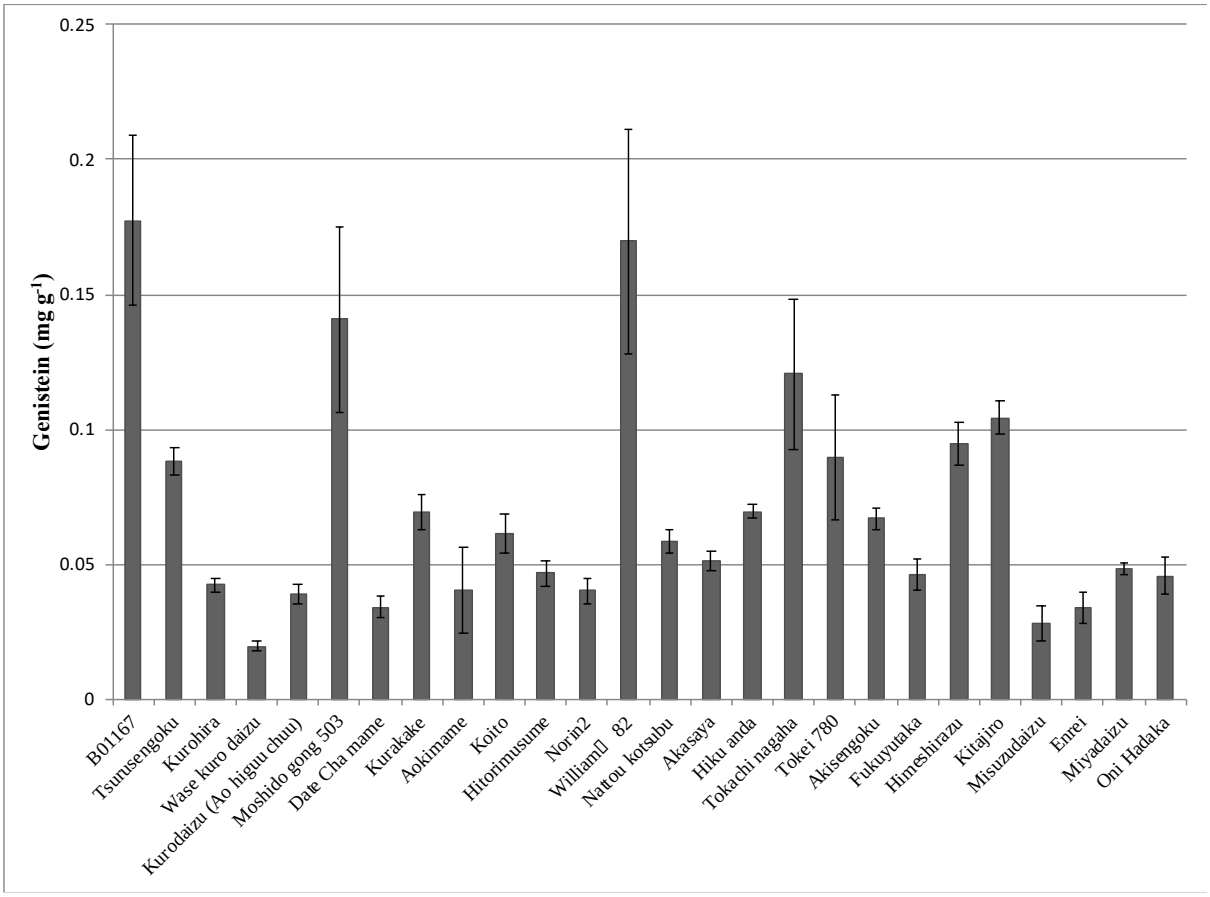

Figure 2.11. Comparison of genistein contents in 26 soybean accessions. The isoflavones derivatives: genistein was determined by aluminum nitrate nonahydrate colorimetric assay as described in the Materials and Methods. The experiments were performed in triplicates in 96-well microtiter plates with five replicates of each soybean accession. The data shown in the figure represent means ± SD.

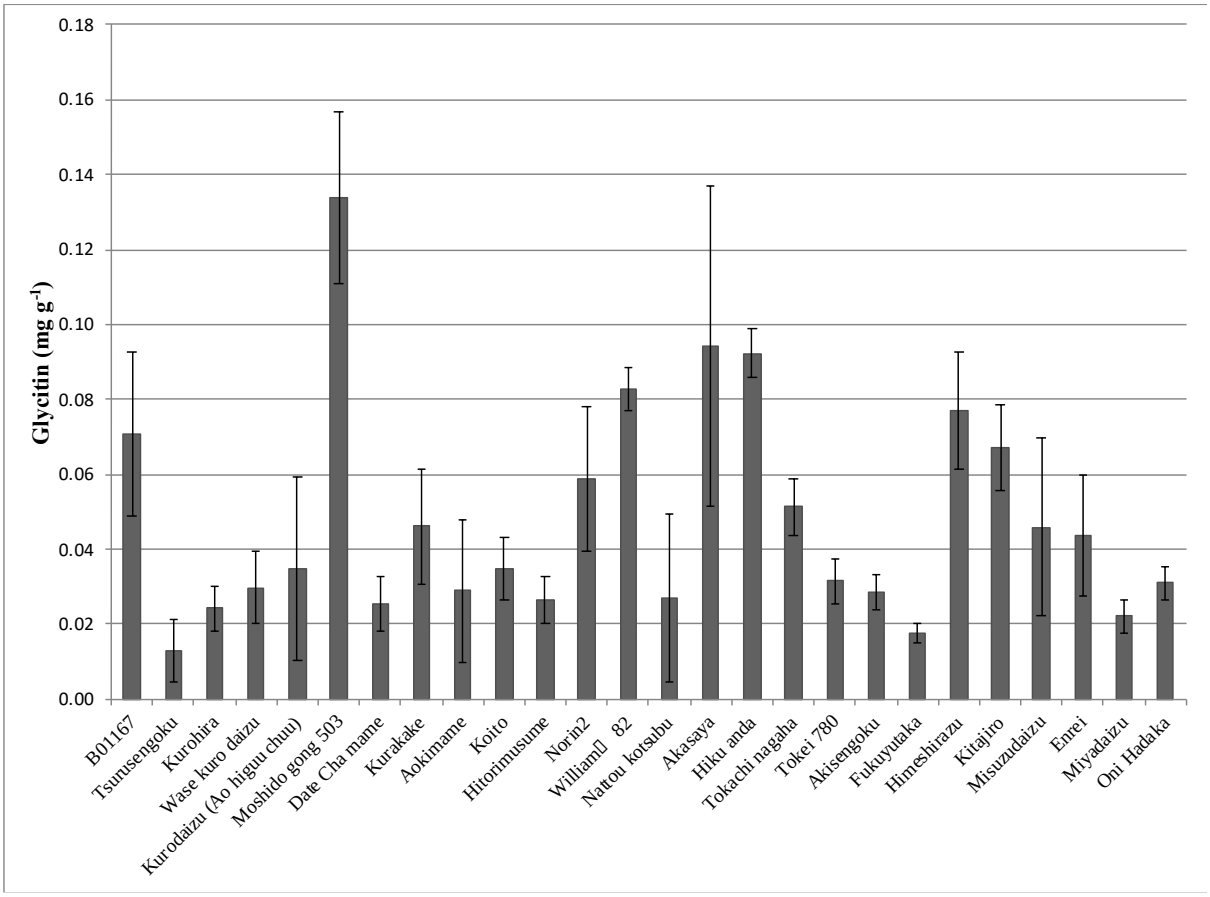

Figure 2.12. Comparison of glycitin contents in 26 soybean accessions. The isoflavones derivatives: glycitin was determined by aluminum nitrate nonahydrate colorimetric assay as described in the Materials and Methods. The experiments were performed in triplicates in 96-well microtiter plates with five replicates of each soybean accession. The data shown in the figure represent means ± SD.

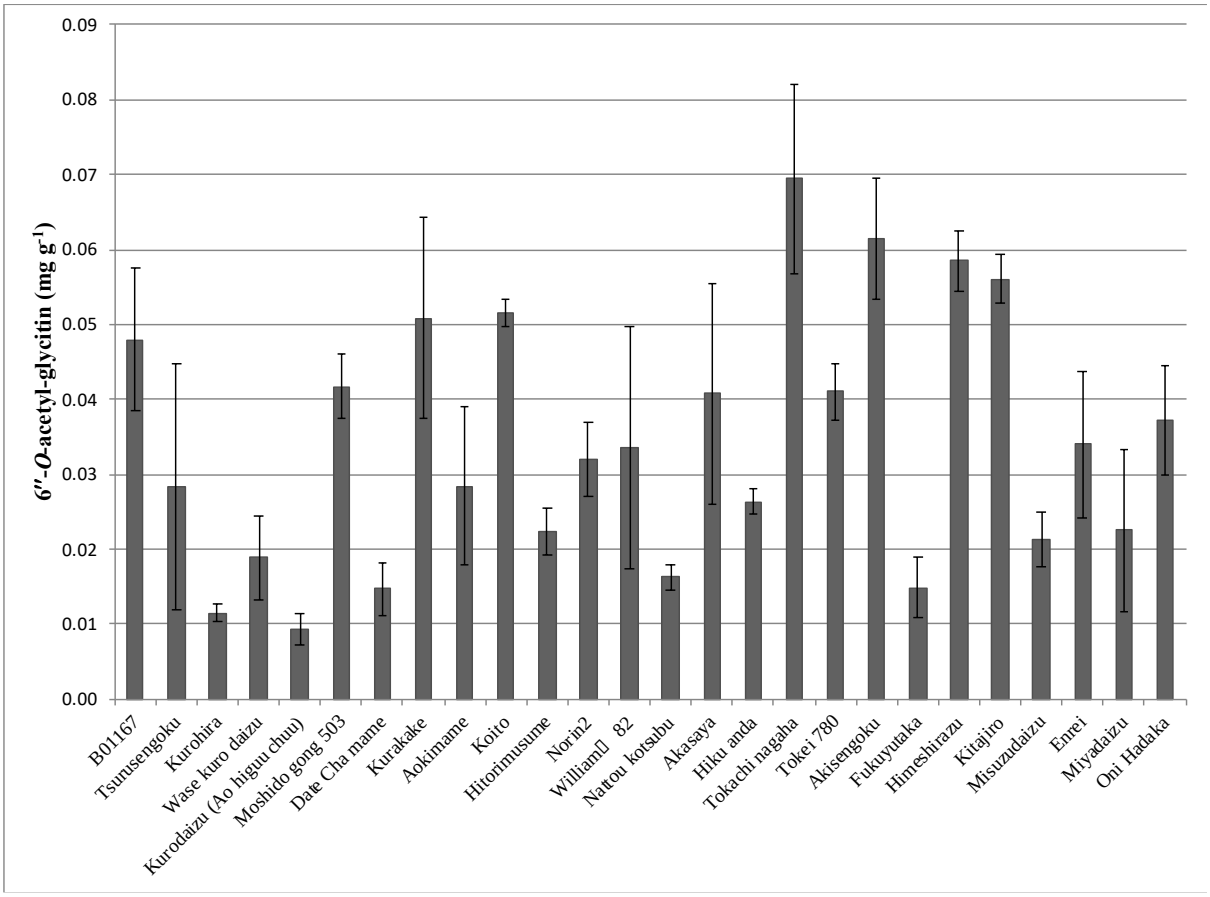

Figure 2.13. Comparison of 6′′-*O*-acetyl-glycitin contents in 26 soybean accessions. The isoflavones derivatives: 6′′-*O*-acetyl-glycitin was determined by aluminum nitrate nonahydrate colorimetric assay as described in the Materials and Methods. The experiments were performed in triplicates in 96-well microtiter plates with five replicates of each soybean accession. The data shown in the figure represent means ± SD.

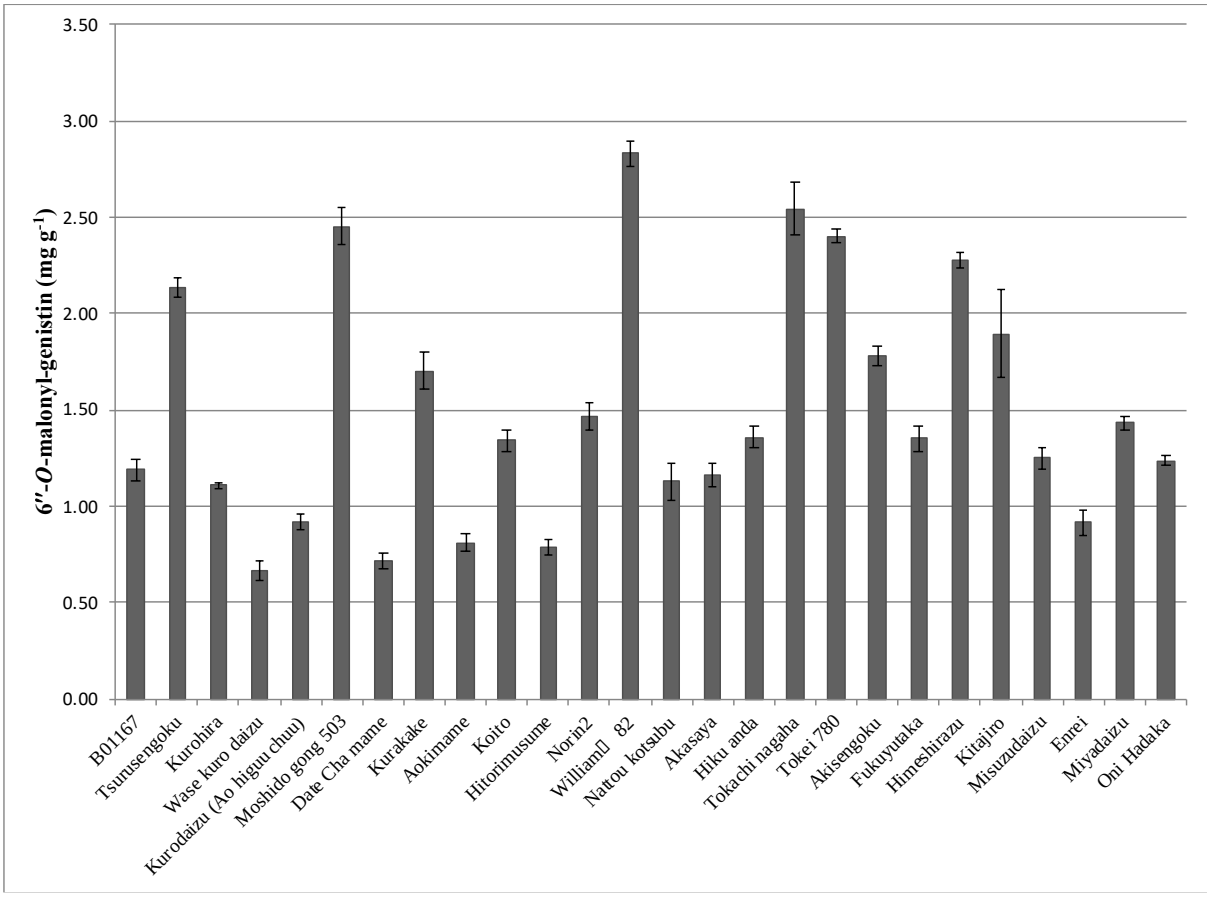

Figure 2.14. Comparison of 6′′-*O*-malonyl-glycitin contents in 26 soybean accessions. The isoflavones derivatives: 6′′-*O*-malonyl-glycitin was determined by aluminum nitrate nonahydrate colorimetric assay as described in the Materials and Methods. The experiments were performed in triplicates in 96-well microtiter plates with five replicates of each soybean accession. The data shown in the figure represent means ± SD.

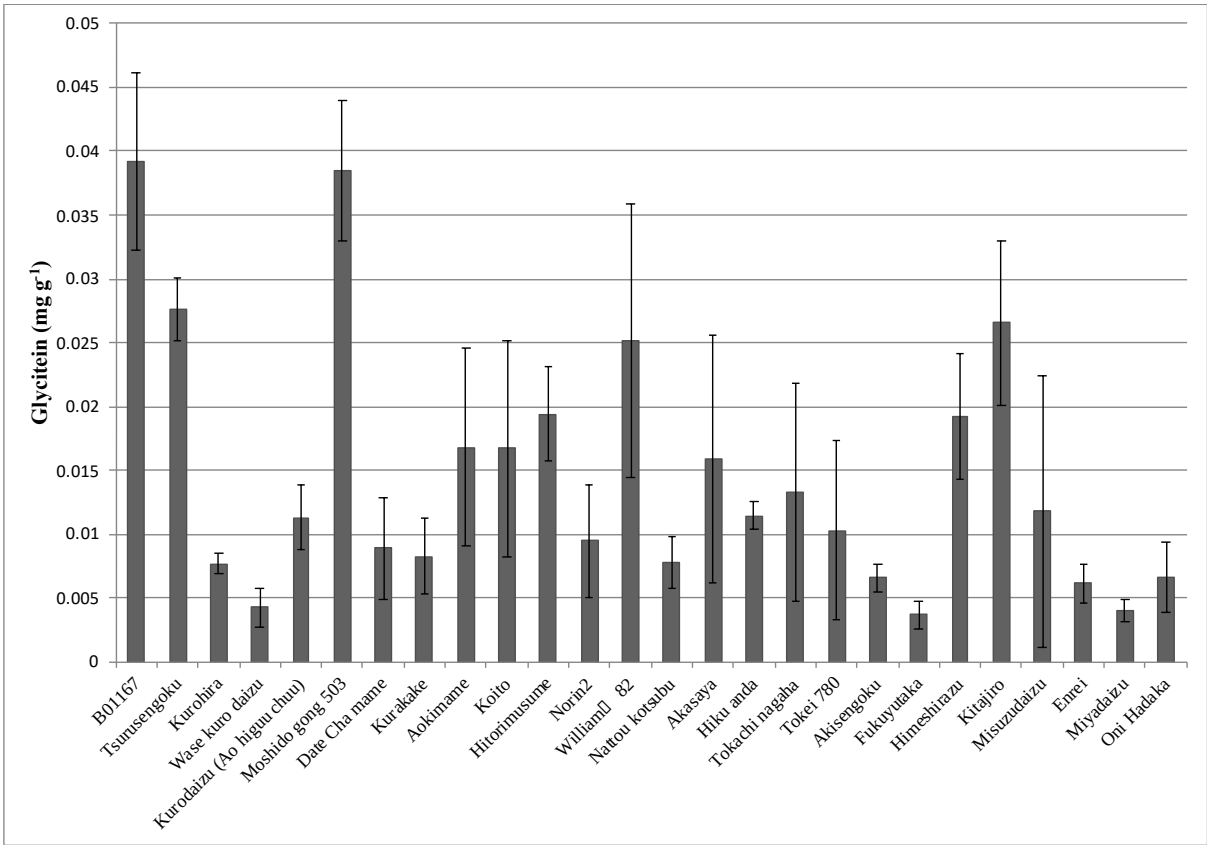

Figure 2.15. Comparison of glycitein contents in 26 soybean accessions. The isoflavones derivatives: glycitein was determined by aluminum nitrate nonahydrate colorimetric assay as described in the Materials and Methods. The experiments were performed in triplicates in 96-well microtiter plates with five replicates of each soybean accession. The data shown in the figure represent means ± SD.

4. DISCUSSION

Functional food factors are chemical substances that uniquely maintain balance and regulate the proper functioning of various bodily systems. These ingredients play an essential role in promoting and preserving overall health and well-being. The soybean crop is crucial and provides various food components as secondary metabolites. Thus far, numerous cultivars have been produced worldwide through classical breeding techniques. In the United States, cultivated soybeans were deliberately crossed to generate high-isoflavone soybean genotypes (Miladinović et al. 2019). However, the flavonoid content and antioxidant activity of Japanese soybean accessions have not been well documented. This study examined the flavonoid content and antioxidant activity of 23 Japanese soybean varieties, two world accessions, and one wild soybean accession.

Soybean seeds contain three main isoflavones: malonyl-daidzin, malonyl-glycitin, and malonyl-genistin (Ahmad et al., 2017). Malonylation plays a significant role in making the derivatives soluble, stable, and easy to transport, which is vital for their storage in the vacuole (Ahmad et al., 2017). In line with previous findings, all 26 types of soybeans analyzed were found to have high levels of malonylated isoflavone glycosides. Additionally, the total isoflavone content of the 26 accessions was higher in comparison with those from 5 Croatian soybean cultivars (Mujic et al. 2011), ranging from 80.7 to 213.6 mg 100 g^{-1}. In addition to having a high isoflavone content, this soybean's total phenol content was also higher than the 33 soybean accessions from eastern Croatia. The phenol content of those soybeans ranged from 212.1 to 316.4 mg gallic acid equivalents 100 g^{-1} (Josipovic et al. 2016). This discrepancy might be associated with genotype as they used Croatian cultivars and not Japanese cultivars, for the analysis. Nevertheless, other factors, such as environmental conditions, might affect polyphenol content (Carrao-Panizzi, 2009), and Japanese cultivars should be evaluated with Croatian cultivars in the same growth condition. This study only used the seeds harvested in

2015 at Miyazaki Prefecture, Japan. A similar trend in soybean cultivars showed that isoflavone concentrations are influenced by various factors such as cultivars, year, and site. However, genetic factors have been identified as the most significant factors in determining isoflavone accumulation (Zhang et al. 2014). Since individual and total flavonoid contents in soybeans may vary depending on genetics and/or environmental factors (Zhang et al. 2014), multi-year samples of Japanese soybean accessions are needed for future research.

The seed coat color of the 26 soybean accessions was also examined. However, there was no correlation with the antioxidant activity as shown in Table 2.2. Several studies have discussed and found that seed colors are important parameters showing a correlation with the content of phytochemicals (phenol, flavonoid, anthocyanin, proanthocyanidin, and carotene) in fruits and vegetables (Xu et al. 2007; Serafini and Peluso 2016). Also, seed color correlated with the total flavonoid and total phenol content when all accessions were analyzed in the present study.

While 22 soybean accessions showed significant antioxidant activity in a cell-based assay, it was found that those with high levels of antioxidant activity did not necessarily have high levels of total phenol or total flavonoid content. Correlation analysis showed a positive and significant connection between the amount of isoflavones and antioxidant activity. However, two specific types of isoflavones, genistein and daidzein, had only moderate effects on the ARE-mediated luciferase reporter assay, indicating that other factors may be involved in producing strong antioxidant activity (Pallauf et al. 2017). According to the previous study, biochanin A, one of the isoflavones, could directly bind to Kelch-like ECH-associated protein 1 (KEAP1), a cytoplasmic suppressor of Nrf2, and then facilitate Nrf2-ARE signaling (Liang et al. 2019). Although the biochanin A content has yet to be examined in this study, it could be found in legumes, including soybean (Mazur et al. 1998). Other food components, such as saponins (Liu et al. 2018), peptides (Yi et al. 2020), and tannins, can also potentially act as

antioxidants through an indirect mechanism by activating the ARE promoter. These components are found in the seed coats or embryos of soybeans. The wild soybean (*G. soja*), possesses genetic variability; thereby, it has been used to broaden the genetic base of soybeans. This study found that B01167 contained the highest flavonoid content but was not correlated with its antioxidant activity under this experimental condition. Additional work is warranted to clarify the mechanism by which varietal differences influence the antioxidant activities in soybeans.

CHAPTER 3

RELATIONSHIP OF PHYTOCHEMICAL AND SEED CHARACTERISTICS OF INDONESIAN SOYBEAN VARIETIES

1. INTRODUCTION

Soybean (*Glycine* max [L.] Merr.) is one of the subtropical legumes native to southeastern Asia, which is cultivated throughout the world in tropical, subtropical, and temperature climates (Alghamdi et al. 2018). Additionally, soybean has been consumed widely as a source of protein-rich foods and supplements such as tempeh (Indonesian yeast fermented soybean), soy sauce, tofu, natto (bacterially fermented soybean), miso, and also edamame (vegetable soybean). In Indonesia, in particular, the consumption of tempeh and tofu is approximately 7.61 kg and 8.23 kg/per capita/year, respectively (Harsono et al. 2021).

Due to the essential role of the soybean as one of the primary protein sources, soybean breeders continuously adapt the breeding tools and technologies to achieve the availability of soybean varieties with high quality. One of the primary soybean seed qualities is its size and color. In Indonesia, more than 90% of tempeh producers prefer large-seeded soybeans with yellow seed coats because they produce tempeh with bright colors and large volumes (Adie and Krisnawati 2018). Recently, the Indonesian soybean breeding program has released 107 soybean varieties, several of which have large seed sizes (14 – 17 g/100 seeds) (ILETRI 2021).

Generally, soybean seeds contain 40% protein, 20% oil, and are enriched in unsaturated fatty acids (FAS) and essential amino acids (AAS) (Zhang et al. 2018). They also have phytochemicals such as phenols, flavonoids, soyasaponins, and triterpenoids, which are crucial in protecting cells and preventing diseases such as cancer, diabetes, and inflammation (Yang et al. 2021). Phenols are substances in many foods such as vegetables, fruits, herbs, and tea. They come in different forms and can be grouped into categories, ranging from simple to complex (Tsimogiannis and Oreopoulou 2019). One type of phenol called isoflavones is commonly found in soybeans, while others are found in certain plants in varying amounts (Hu et al. 2020). Phenolic compounds are essential for plant growth, development, and protection against pests and predators (Zaynab et al. 2018). Flavonoids, on the other hand, are a natural

type of antioxidant found in plants. They are classified into 15 groups, and isoflavones are exclusively found in legumes, particularly soybeans (Nakayama et al. 2019). Soy isoflavones can help alleviate menopausal symptoms as they mimic estrogen (Hu et al. 2020).

It is well known that different varieties exhibit distinct properties even within the same origin. The author has shown that Japanese soybean accessions exhibited significant varieties of antioxidant activities in a cell-based assay in chapter 2 (Arifin et al. 2021). Indonesian soybean varieties, in particular, also have variations in agronomical and morphological characteristics (Kuswantoro et al. 2019; Sulistyo et al. 2019). Therefore, it is worth to investigate their phytochemicals among varieties. This study aimed to determine a relationship between phytochemicals, antioxidant activity, and morphological characteristics of soybean varieties in Indonesia.

2. MATERIALS AND METHODS

2.1. Chemicals

Quercetin dehydrates, Follin-ciocalteu reagent, gallic acid monohydrate, and 2,2-diphenyl-1-picrylhydrazyl were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol, aluminum nitrate nonahydrate, and potassium acetate were pro-analysis grades obtained from Merck (Darmstadt, Germany).

2.2. Soybean materials

This study used 20 soybean varieties, which comprised 19 Indonesian soybean varieties and 1 Korean soybean variety (Figure 3.1). The experiment was conducted in 2021 at the University of Brawijaya, Malang, East Java, Indonesia. The seeds of these soybean varieties were obtained from the germplasm collection of the Indonesian Legumes and Tuber Crops Research Institute (ILETRI), Malang, East Java, Indonesia. The information on soybean varieties used in this study is presented in Table 1.

2.3. Determination of crude proteins and oil content

The crude protein content was analyzed according to the standard by AOAC (2005) and using a Kjeldahl system (Buchi K-350 and K-426, Switzerland). The powdered soybean seeds (0.5 g) were used to detect each variety in triplicate. The protein content was calculated as a percentage of nitrogen multiplied by 6.25 (the standard Kjeldahl factor). The oil content was examined using a Soxhlet extraction apparatus. The pulverized seeds (2 g) were dried in an oven (75 °C) for 60 min. The sample was added to hexane in an extraction thimble and boiled for six h. The solvent was evaporated under a vacuum dryer (105 °C) and cooled at room temperature.

2.4. Extraction for total phenol, total flavonoid, and antioxidant activity

The soybean seeds were powdered and extracted with aqueous-acidic ethanol (Carrão-Panizzi et al. 2002). Then, the soybean seed powder (6 g) was dissolved in 60 mL of 70% ethanol (containing 0.1% acetic acid). Samples were placed on an orbital shaker at 150 rpm (Protechâ, type 722) for 48 h at room temperature. The sample was then centrifuged and the supernatant was used for analysis. All extractions for each variety were conducted in triplicate and stored at –20 °C before assay.

2.5. Determination of total phenol content

The total phenol content was analyzed using the Folin-Ciocalteau assay (Magalhães et al. 2010). In particular, 50 µL of gallic acid standard solution or sample and 50 µL of Folin-Ciocalteau reagent were placed in each well. Then, 100 µL of 0.3 mol L^{-1} NaOH was added. The absorbance was then monitored at 650 nm using microplate reader. All experiments were performed in triplicate at room temperature. The total phenol content was expressed as mg g^{-1} gallic acid equivalents (GAE).

2.6. Determination of total flavonoid content

The total flavonoids were determined using the aluminum nitrate nonahydrate colorimetric assay with a slight modification (Sembiring et al. 2018). The experiments were performed in 96-well microtiter plates with triplicates of each soybean accession. The reaction mixture was prepared by mixing 4.3 mL of 80% ethanol, 0.1 mL of 10% aluminum nitrate nonahydrate, and 0.1 mL of 1 mol L^{-1} potassium acetate. A quercetin standards solution or sample (20 µL) was placed in each well, followed by adding 180 µL of the aluminum nitrate nonahydrate mixture. The mixture was then incubated for 40 min, and the absorbance was measured at 415 nm. The flavonoid content was expressed as mg g^{-1} quercetin equivalent (QE).

2.7. 2,2-diphenyl-1-picrylhydrazyl free radical assay

The antioxidant activity of each soybean cultivar was assessed by a 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical assay according to a previously described method (Zahratunnisa et al. 2017). The analysis was conducted in 96-well microtiter plates with triplicates of each soybean variety. 20 µL of soybean extract and 180 µL of 0.147 mM DPPH solution were added to each well. After 30 min of incubation at room temperature in the darkroom, the absorbance was measured at λ 517 nm using a microplate reader. The results were plotted as absorbance value vs. sample concentration (mg/assay). The results were expressed as the half-inhibitory concentration (IC_{50}) in mg mL^{-1} reaction mixture.

2.8. Statistical analyses

The results of this study were presented as means ± standard deviation (SD). Analysis variance (ANOVA) was used to determine the significant differences between group means with probabilities of $P \leq .01$ and .05. Correlation analyses were obtained using Pearson's product-moment correlation (with normality). Statistical and clustering analysis was performed using the R software package (Version 4.1.2).

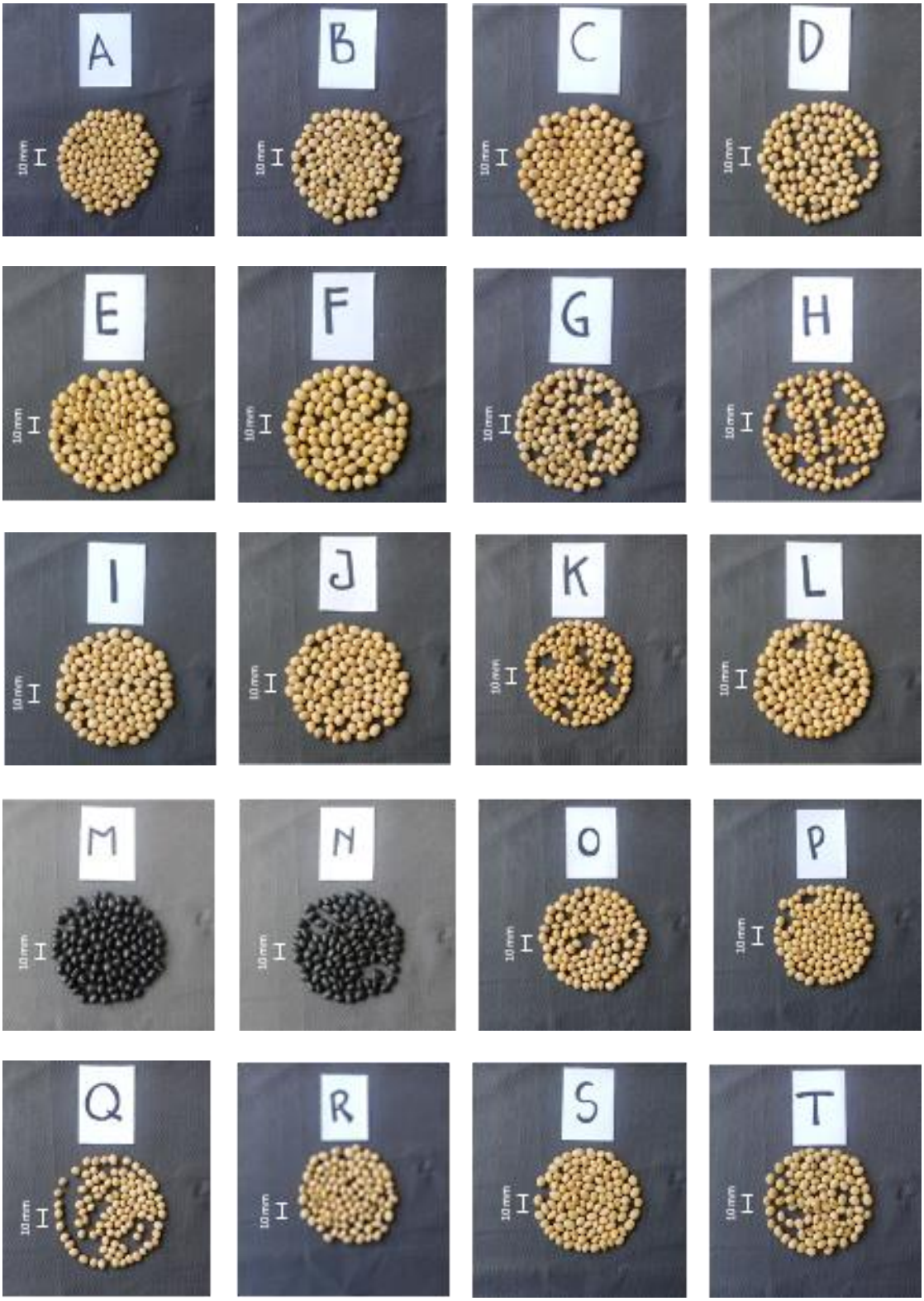

Figure 3.1. Seed appearance of 20 soybean variety used in this study. The varieties' names are according to the alphabetical order: (A) Anjasmoro, (B) Argomulyo, (C) Argopuro, (D) Baluran, (E) Daewon-Korean variety, (F) Dega 1, (G) Deja 1, (H) Demas 1, (I) Dena 1, (J) Derap 1, (K) Dering 1, (L) Detap 1, (M) Detam 1, (N) Detam 2, (O) Devon 1, (P) Devon 2, (Q) Gepak kuning, (R) Grobogan, (S) Mahameru, and (T) Rajabasa.

Table 3.1. The information on seed size, 100 seed weight, seed color, flowering time, and pod maturity of 20 soybean varieties.

Varieties	Seed size		Weight	Color									Flowering time [c]	Pod maturity
	EI (mm)[a]	FI (mm)[b]	100 seeds (g)	Seed coat	Hilum	Hypocotyl	Epicotyl	Cotyledon	Mature pod [c]	Pubescence [c]	Leaf [c]	Flower [c]	(DAP)[d]	(DAP)[d]
Anjasmoro	1.22±0.08	35.3±0.02	14.8±0.45	Yellow	Buff	Purple	Purple	Green	Light brown	Gray	Green	Purple	36	82
Argomulyo	1.20±0.04	30.0±0.01	14.8±0.45	Yellow	White	Purple	Green	Yellow	Brown	Brown	Green	Purple	35	80
Argopuro	1.15±0.09	37.8±0.03	17.4±0.55	Yellow	Buff	Green	Green	Green	Dark brown	Gray	Green	White	32	84
Baluran	1.20±0.03	28.2±0.03	10.80±0.45	Yellow	Buff	Purple	Green	White	Brown	Brown	Green	Purple	33	80
Daewon	1.25±0.12	35.4±0.03	17.80±0.45	Yellow	Yellow	Green	Green	Yellow	Yellow	Gray	Green	White	35	84
Dega 1	1.21±0.04	38.6±0.04	19.80±0.45	Yellow	Brown	Purple	Purple	Purple	Light brown	Brown	Green	Purple	29	69
Deja 1	1.27±0.06	22.2±0.04	11.20±0.45	Yellow	Buff	Purple	Purple	Yellow	Dark brown	Brown	Green	Purple	39	89
Demas 1	1.20±0.01	20.1±0.04	8.80±0.45	Yellow	Dark brown	Purple	Green	White	Light brown	Brown	Green	Purple	37	84
Dena 1	1.30±0.06	30.2±0.06	16.00±0.00	Yellow	Brown	Purple	Green	Green	Light brown	Brown	Green	Purple	33	78
Derap 1	1.22±0.10	28.9±0.04	15.60±0.89	Yellow	Buff	Purple	Green	White	Yellow	Gray	Green	Purple	34	76
Dering 1	1.26±0.11	21.8±0.03	8.80±0.45	Yellow	Dark brown	Purple	Purple	White	Dark brown	Brown	Green	Purple	35	81
Detap 1	1.22±0.07	25.7±0.07	14.80±0.45	Yellow	Yellow	Purple	Green	White	Yellow	Gray	Green	Purple	35	78
Detam 1	1.15±0.08	24.3±0.05	15.20±0.45	Black	White	Purple	Green	Yellow	Dark brown	Light brown	Dark green	Purple	35	84
Detam 2	1.32±0.11	23.4±0.02	12.20±0.45	Black	Brown	Purple	Green	Yellow	Light brown	Dark brown	Green	Purple	34	82
Devon 1	1.27±0.16	24.5±0.03	14.80±0.45	Yellow	Buff	Purple	Green	White	Light brown	Brown	Green	Purple	34	83
Devon 2	1.22±0.09	25.1±0.06	17.20±0.45	Yellow	Yellow	Purple	Green	White	Yellow	Gray	Green	Purple	33	77
Gepak kuning	1.11±0.09	17.9±0.04	7.20±0.45	Yellow-green	Brown	Purple	Green	Green	Brown	Brown	Green	Purple	28	73
Grobogan	1.25±0.07	28.7±0.06	18.00±0.71	Light yellow	Brown	Purple	Purple	Green	Brown	Brown	Dark green	Purple	30	76
Mahameru	1.31±0.04	29.1±0.03	17.40±0.89	Yellow	Buff	Purple	Purple	Green	Brown	Gray	Green	Purple	36	83
Rajabasa	1.24±0.28	29.8±0.04	14.20±0.45	Yellow	Brown	Purple	Purple	Green	Dark brown	Brown	Green	Purple	35	82

Seed size: (a): Seed eccentricity index; (b): seed flatness index; (c): data accessed from Indonesian Legumes and Tuber Crops Research Institute (2021); (d): days after planting.

3. RESULTS

3.1. Crude protein and oil content

The detailed information on 20 soybean varieties for crude protein, oil, total phenol, total flavonoid, and antioxidant activity is presented in Figures 3.2–3.6. Also, the statistical values of their phytochemicals are shown in Table 3.2. Crude protein content values ranged from 28.03% in Argopuro to 44.72% in Detam 1 varieties, with a mean value of 38.60% (Figure 3.2). The six soybean varieties containing more than 40% crude protein were Detam 1, Detam 2, Grobogan, Mahameru, Anjasmoro, and Detap 1. The results showed significant differences among varieties in crude protein content up to 2-fold, reflecting the variations in genetic background and origin. The oil content in the present study showed a significant difference among varieties from 14.50% to 33.12%, with a mean value of 18.76% (Figure 3.3). In particular, the Detam 1 variety is superior in crude protein and oil content, while Daewon has the lowest oil content.

3.2. Total phenol and total flavonoid content

Subsequent Folin-Ciocalteau assay revealed that the total phenol content ranged from 3.02 to 9.54 mg GAE g^{-1} with a mean of 5.66 mg GAE g^{-1} (Table 3.2). The highest total phenol content was present in Detam 2, while the lowest was in Anjasmoro (Figure 3.4). The total phenol content of seeds in all varieties was significantly different from each other. The total flavonoid content of 20 soybean varieties ranged from 0.21 to 2.56 mg QE mg^{-1} (Figure 3.5). Daewon variety contained the lowest total flavonoid content (0.21 mg QE mg^{-1}), while Detam 2 had the highest value of 2.56 mg QE mg^{-1}.

Table 3.2. Statistical values of phytochemical composition in 20 soybean varieties.

	Crude protein (g 100 g^{-1})	Oil (g 100 g^{-1})	Total phenol (mg GAE g^{-1})	Total flavonoid (mg QE mg^{-1})	IC_{50} (mg mL^{-1})
N	20	20	20	20	20
Min	28.03	14.50	3.02	0.21	0.11
Max	44.72	33.12	9.54	2.56	2.45
Mean	38.60	18.76	5.66	0.66	1.03
Stand. Dev.	4.08	4.15	2.04	0.57	0.55
Coeff. Var.	10.57	22.12	36.07	87.42	53.82

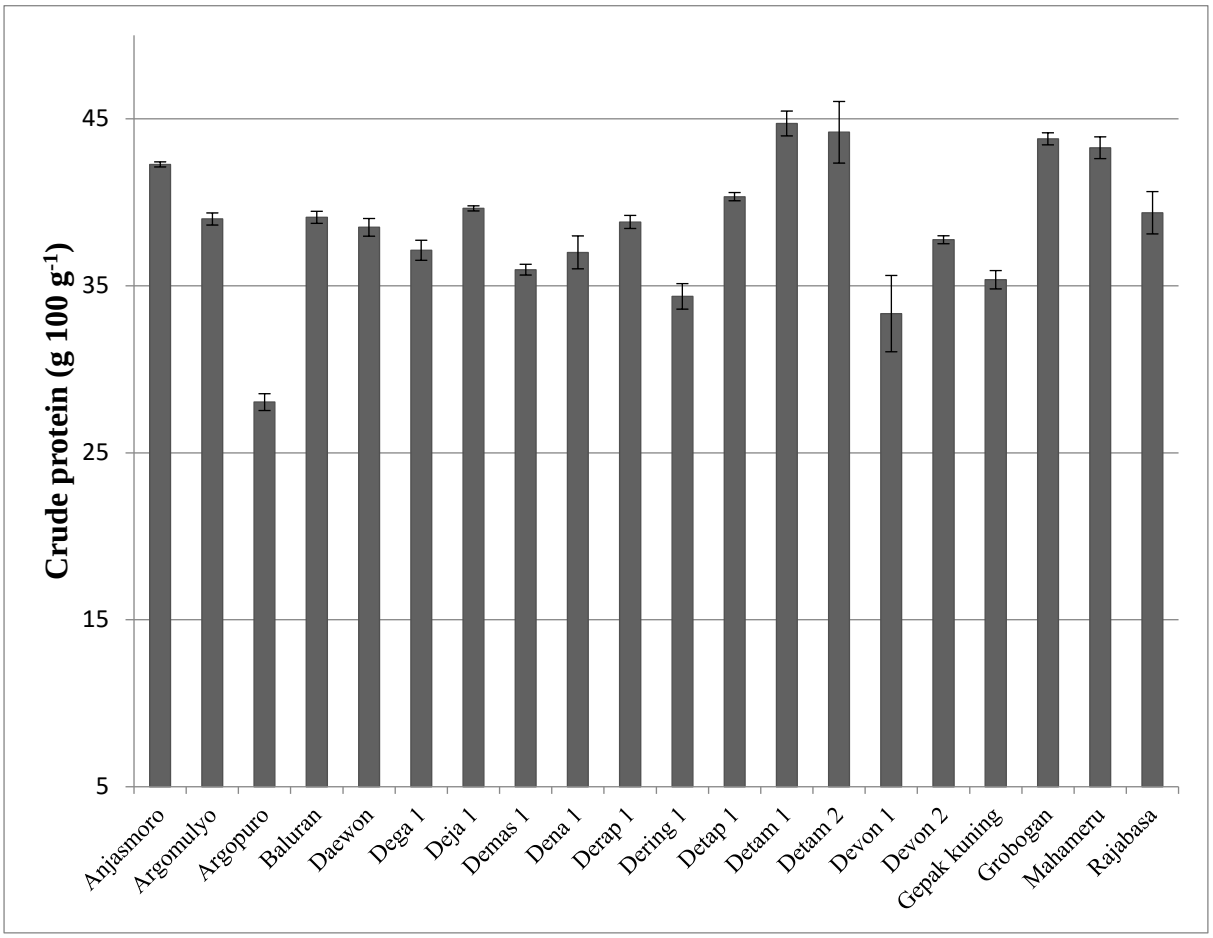

Figure 3.2. Comparison of crude protein contents in 20 soybean varieties. The crude protein content was analyzed using a Kjeldahl system. The experiments were performed in each variety for triplicates and the data shown in the figure represent means ± SD for tree replicates.

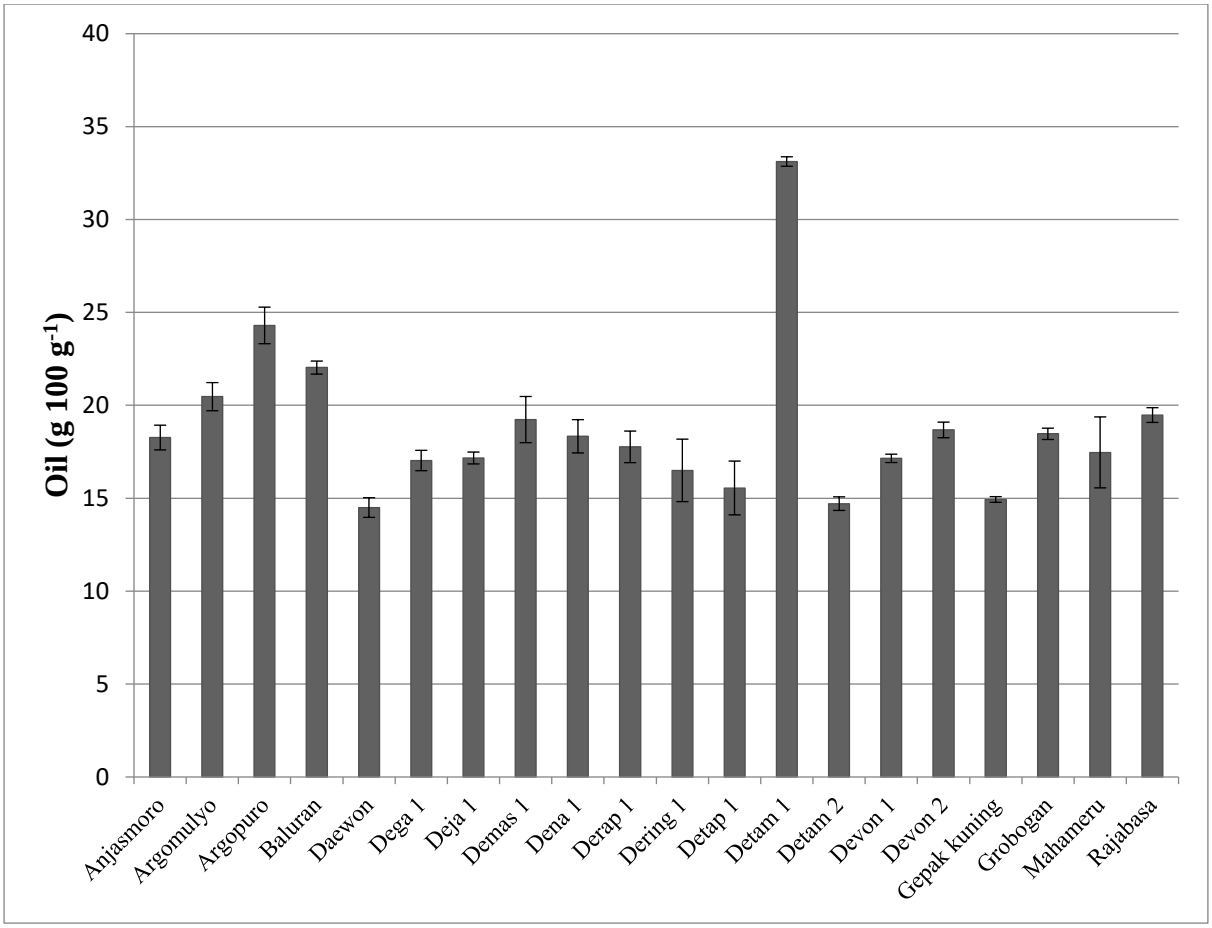

Figure 3.3. Comparison of oil contents in 20 soybean varieties. The oil content was examined by using a Soxhlet extraction apparatus. The experiments were performed in each variety for triplicates and the data shown in the figure represent means ± SD for tree replicates.

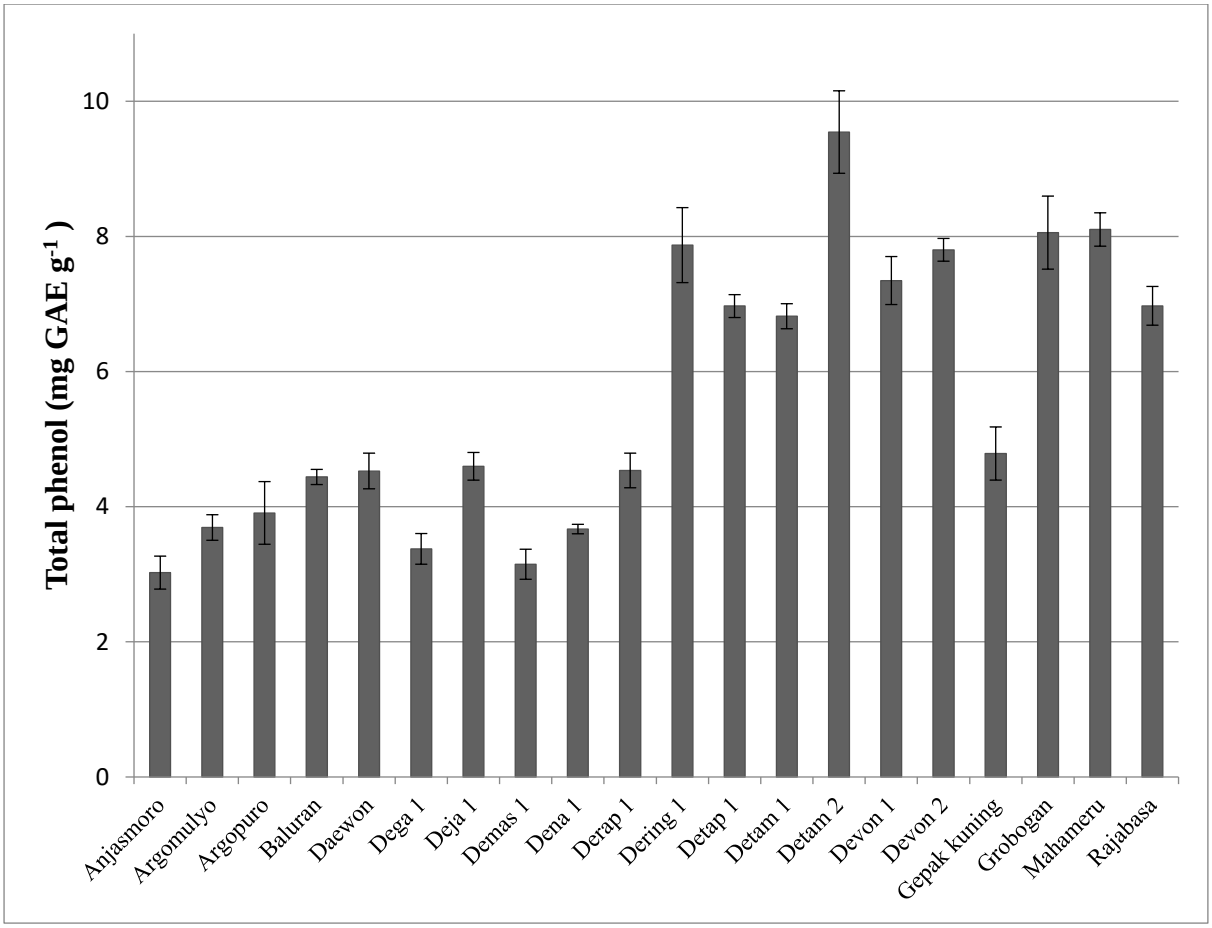

Figure 3.4. Comparison of total phenol contents in 20 soybean varieties. The phenol content was analyzed using the Folin-ciocalteau assay as described in Material and Methods. The experiments were performed in each variety for triplicates. The result was expressed as mg g^{-1} gallic acid equivalents (GAE), and the data shown in the figure represent means ± SD for tree replicates.

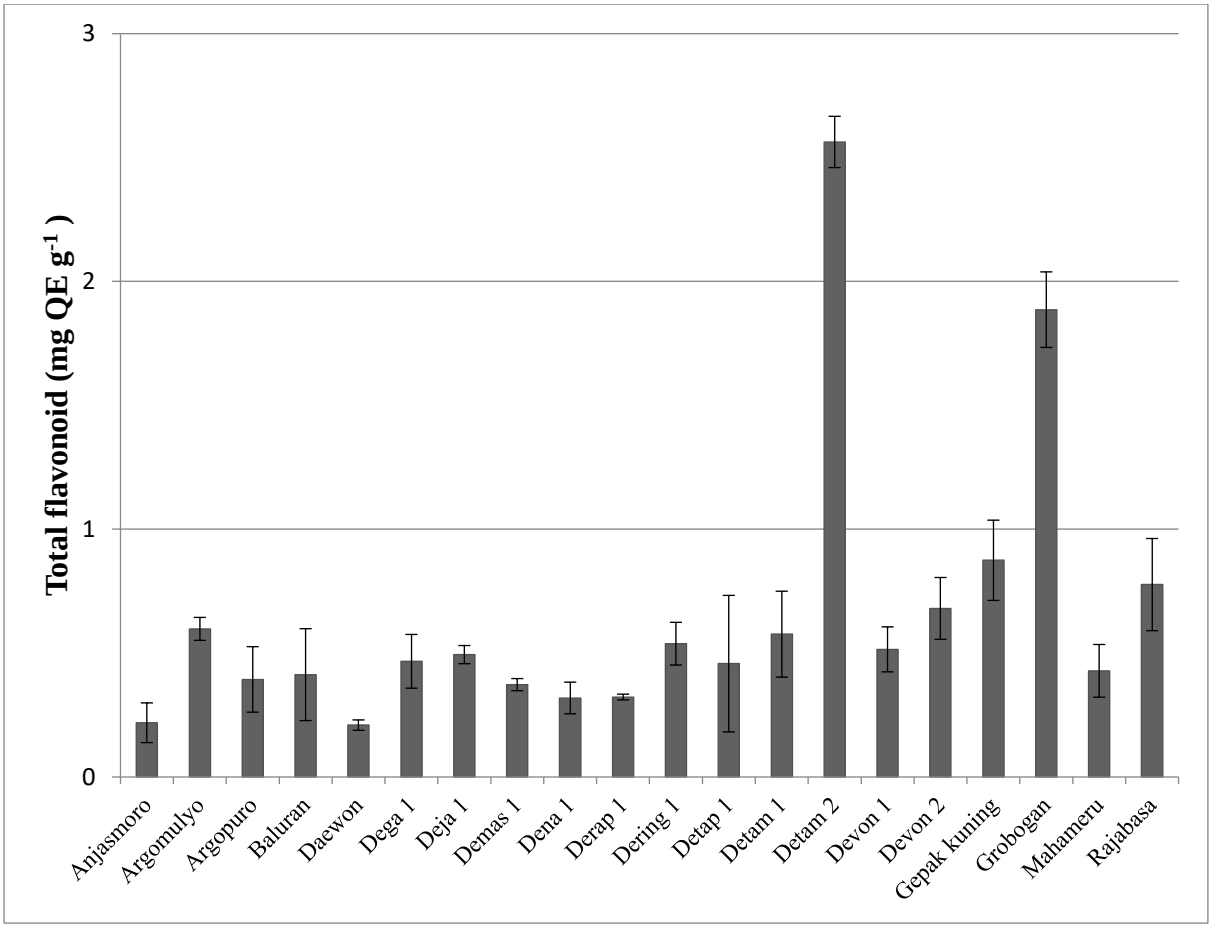

Figure 3.5. Comparison of total flavonoid contents in 20 soybean varieties. The total flavonoid content was determined using the aluminum nitrate nonahydrate colorimetric assay as described in Material and Methods. The experiments were performed in each variety for triplicates. The result was expressed as mg g^{-1} quercetin equivalents (QE), and the data shown in the figure represent means ± SD for tree replicates.

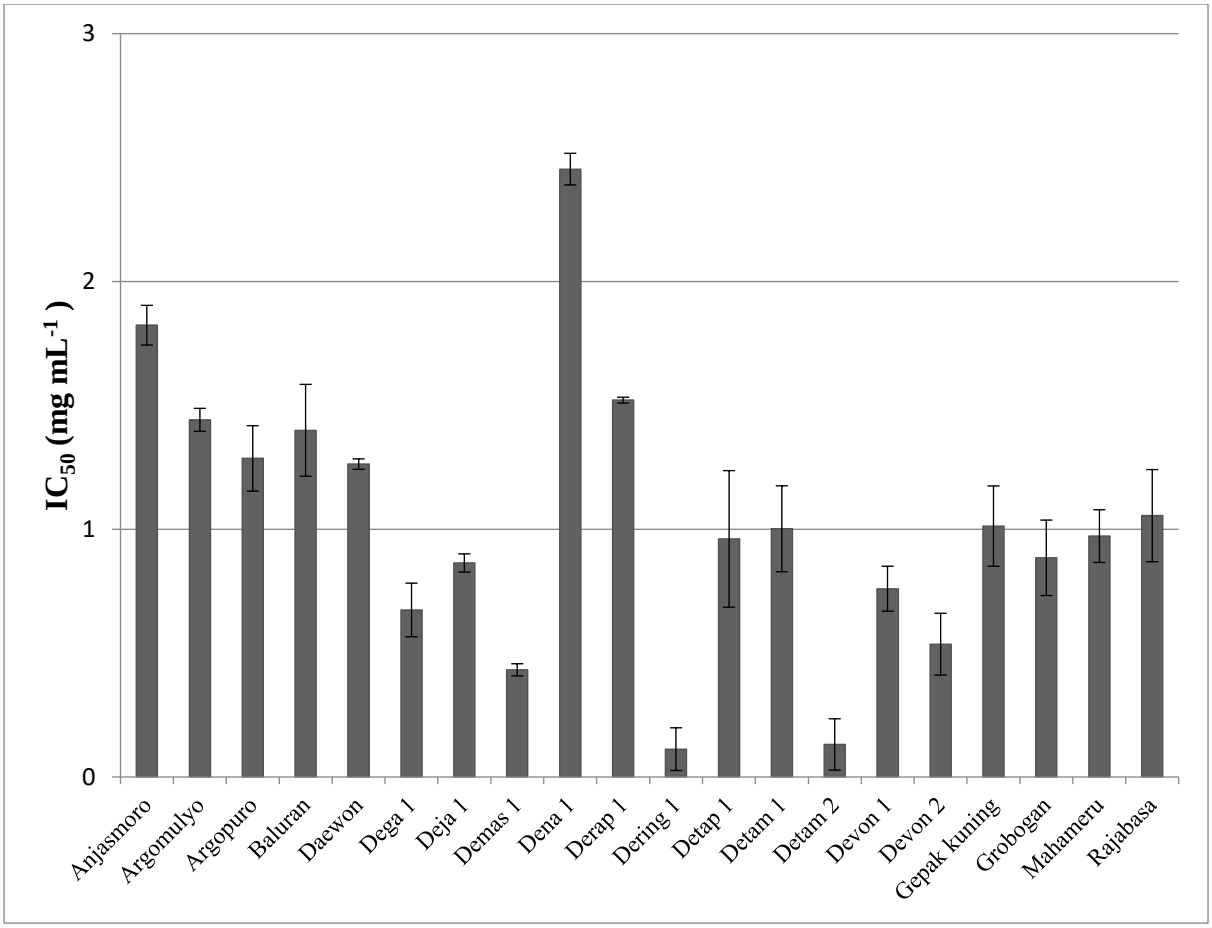

Figure 3.6. Comparison of IC_{50} value in 20 soybean varieties. Value of IC_{50} was assessed by a 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical assay as described in Material and Methods (n=3). The results were expressed as the half-inhibitory concentration (IC_{50}) in mg mL^{-1} reaction mixture. The data shown in the figure represent means ± SD for tree replicates.

3.3. Antioxidant activity

The antioxidant activity was expressed by the IC_{50} values, defined as the concentration of the sample (mg mL^{-1} reaction mixture) required to scavenge 50% of the DPPH free radicals. The lower IC_{50} value indicates that the sample has higher antioxidant activity and is more efficient in extinguishing free radicals. The result showed that IC_{50} of 20 varieties ranged from 0.11 to 2.45 mg mL^{-1} with a mean of 1.03 mg mL^{-1} (Table 3.2). The lowest value of IC_{50} was found in Dering 1, while the highest IC_{50} was in Dena 1 (Figure 3.6). Varieties that recorded lower than 0.5 mg mL^{-1} IC_{50} were Dering 1, Detam 2, Demas 1, and Devon 2.

3.4. Correlation analysis among characters observed

Correlation analyses were conducted between seed traits, crude protein, oil, total phenol, total flavonoid, and IC_{50} (Table 3.3). The seed size indicated with the seed eccentricity index was significantly and negatively correlated with oil content (r = -0.47, $P < 0.05$). The seed flatness index was significantly and positively correlated with seed weight (r = 0.77, $P < 0.01$) and IC_{50} (r = 0.47, $P < 0.05$). The total phenol content of 20 soybean varieties was significantly high and positively correlated with the total flavonoid content (r = 0.63**, $P < 0.01$). The IC_{50} value was significantly high and negatively correlated with the total phenol (r = -0.58, $P < 0.01$). Moreover, the IC_{50} value was also significantly and negatively correlated with the total flavonoid content (r = -0.46, $P < 0.05$).

3.5. Cluster and heatmap analysis

The present study performed cluster and heatmap analysis to describe the variability among soybean varieties in their seed characteristics, flowering time, pod maturity, phytochemicals (total phenol, total flavonoid, crude protein, and oil), and antioxidant activity

(IC_{50}). In particular, Figure 3.7 showed the cluster analysis according to the average linkage method.

The color represents the standardized score of the parameters. All varieties were divided into four main groups. Group 1 included only one variety (Gepak Kuning), which has the smallest seed size and fastest flowering time and pod maturity. Group 2 also contained only one variety (Dega 1) that had the highest seed weight and seed size. Group 3 consisted of 2 varieties (Grobogan and Detam 2), which are prominent in the total phenol and flavonoid content and antioxidant activity. Group 4 consisted of the remaining 16 varieties, while the Detam 1 variety was singled out as a specific group. Detam 1 showed the highest oil content with sufficient antioxidant activity, total phenol, and total flavonoid content.

Table 3.3. Profile of correlation value among the characters observed in several varieties of Indonesian soybean.

	EI [a]	FI [b]	Seed weight	Flowering time	Pod maturity	Crude protein	Oil	Total phenol	Total flavonoid	IC_{50}
EI [a]	1.00									
FI [b]	0.00	1.00								
Seed weight	0.19	0.77**	1.00							
Flowering time	0.40	-0.21	-0.19	1.00						
Pod maturity	0.26	-0.18	-0.21	0.82**	1.00					
Crude protein	0.32	-0.11	0.14	0.23	0.04	1.00				
Oil	-0.47*	0.08	0.13	0.08	0.25	0.08	1.00			
Total phenol	0.43	-0.39	0.02	0.02	0.08	0.37	-0.09	1.00		
Total flavonoid	0.28	-0.27	-0.08	-0.28	-0.12	0.41	-0.17	0.63**	1.00	
IC_{50}	-0.09	0.47*	0.29	-0.04	-0.06	-0.01	0.18	-0.58**	-0.46*	1.00

Seed size: (a): Seed eccentricity index; (b): seed flatness index. *p-value < .05, **p-value < .01

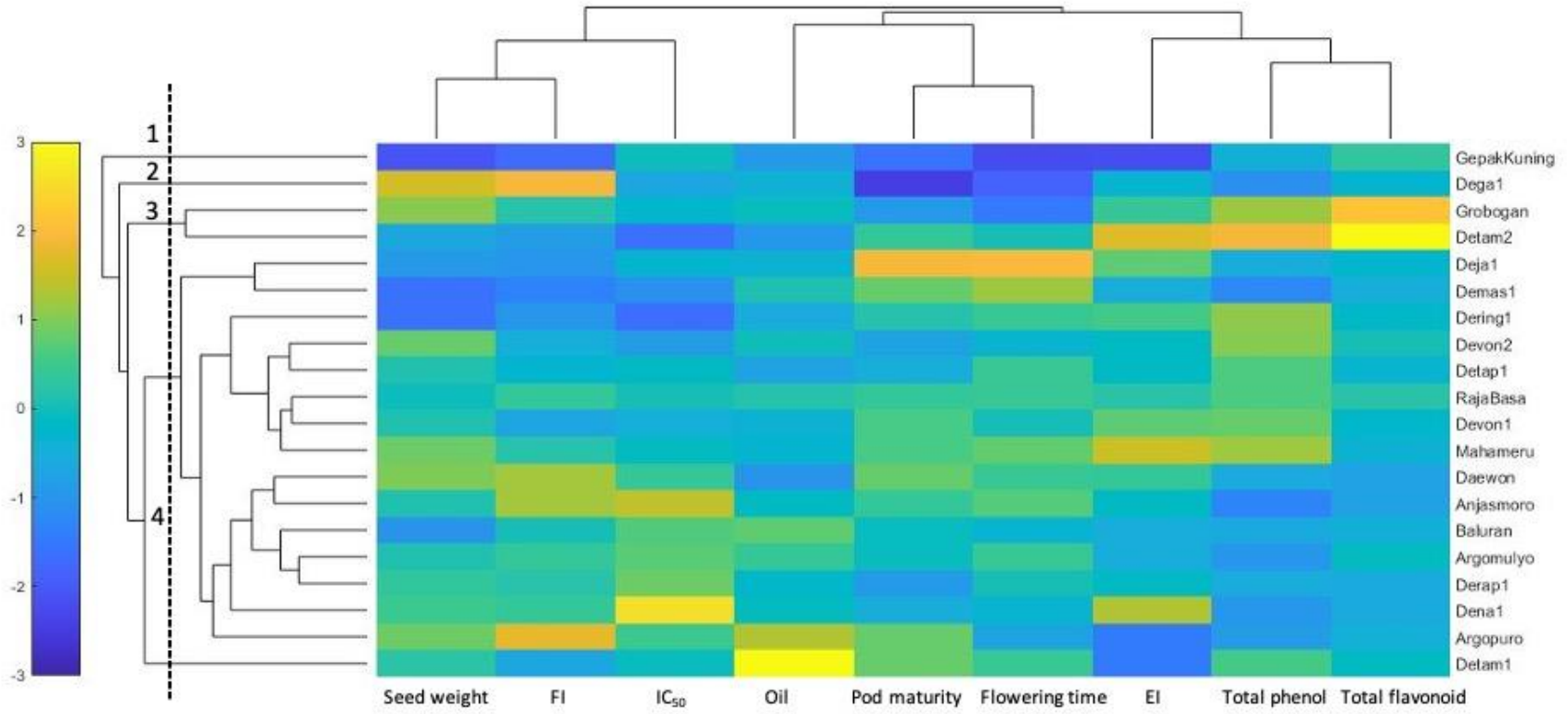

Figure 3.7. Cluster and heatmap analysis of 20 soybean varieties based on significantly correlated variables. FI: seed flatness index, IC_{50}: the 50% inhibitory concentration, EI: seed eccentricity index.

4. DISCUSSION

Research on the colors of soybean seeds' morphology has been ongoing since the early 1900s to assess the colors of the seed coat and cotyledon (Owen 1927). However, there is limited knowledge about the correlation between seed characteristics and phytochemicals in Indonesian soybeans. This study examined the relationship between seed characteristics and nutrient content, including protein, oil, and phytochemicals, such as total phenol, total flavonoid, and antioxidant activity.

The nutritional value and quality of soybean seeds are intricately tied to their oil and protein levels. These two components play a vital role in determining the worth of soybean seeds and are key factors that farmers and producers consider when assessing their crop's potential. This study showed 20 soybean varieties for crude protein values ranging from 28.03% to 44.72% (Table 3.2). In comparison to two other studies, which noted crude protein levels ranging from 35.35% to 43.13% and 39.20% to 47.90% across different soybean genotypes (Alghamdi et al. 2018, Min et al. 2015), our findings highlight a wider range of crude protein content levels. Moreover, the oil content differed significantly among varieties, from 14.50% to 33.12% (Table 3.2). In particular, the Detam 1 variety is superior in crude protein and oil content, while Daewon has the lowest oil content. Other studies found slight variations, with total oil ranging from 13.9 % to 20.4 % in the Korean soybean genotypes (Dhungana et al. 2021). The composition of soy oil and protein depends on genotype characteristics and environmental conditions (Min et al. 2015; Alghamdi et al. 2018). This result is consistent with a previous study that the Detam 1 soybean variety had a higher protein content of 45.36% compared to other Indonesian soybean varieties, making it a promising option for functional foods and medical purposes. This particular variety has also been identified as a rich source of bioactive compounds, which has gained interest in recent studies (Hidayat et al. 2015).

Phenols and flavonoids possess powerful antioxidant capabilities that allow them to counteract the effects of free radicals and reduce their generation. This suggests that these naturally occurring compounds play a significant role in protecting against oxidative stress and related health concerns. Based on Folin-Ciocalteau assay, it has been determined that the soybean seeds exhibit a total phenol content that ranges from 3.02 to 9.54 mg GAE g^{-1} (Table 3.2). The highest total phenol content was in Detam 2, while the lowest was in Anjasmoro (Figure 3.4). The total phenol content of seeds in all varieties was significantly different from each other. These findings are consistent with those described in another report (Yusnawan 2016). Detam 2 had high levels of total phenol and flavonoid content, resulting in a significant outcome overall. Detam 1 and Detam 2 varieties are black soybean seed coats (described in Figure 3.1 and Table 3.1) and have been recognized as high-antioxidant soybeans because of their phenolics, flavonoids, proanthocyanidins, and anthocyanins (ILETRI 2021). Hidayat et al. (2015) reported that Detam 1 has the potential to inhibit glucose 6-phosphate dehydrogenase (G6PD), triglyceride (TG), and cholesterol activity to prevent obesity. Moreover, Detam 1 exhibited positive results in anti-adipogenesis and antiobesity and was non-toxic to the 3T3-L1 cells. Another study revealed that flavonoids, particularly isoflavones in Detam 2, can lower blood lead levels and repair the kidney's histopathology (Christyaningsih et al. 2017).

Correlation analyses were conducted between seed traits, crude protein, oil, total phenol content, total flavonoid content, and IC_{50} (Table 3.3). The seed size indicated with the seed eccentricity index was significantly and negatively correlated with oil content (r = -0.47, P < .05). This result reflects that the lower eccentricity index values mean that seeds with more roundish shapes might contain the highest oil content. The relationship between the seed size and oil content was also reported by Liu et al. (2020). In particular, the authors claimed that essential fatty acids such as linolenic acid and oleic acid contained in soybean oil were significantly correlated with the seed width, thickness, length, and weight of 100 seeds (P

< .0001). Similar results by Kumar et al. (2006) revealed that the seed size (g/100 dried seeds) has a positive correlation with the amount of oleic acid and a negative correlation with the amount of linolenic acid.

Another parameter of seed size in the present study was the seed flatness index, which is shown to be significantly and positively correlated to seed weight ($r = 0.77$, $P < 0.01$) and IC_{50} ($r = 0.47$, $P < 0.05$). These results indicated that seeds with a small flatness index and low seed weight have low IC_{50} values which means a high antioxidant activity. The varieties in this study that show this particular trend (of having a small flatness index and seed weight) are Dering 1, Detam 2, Demas 1, and Devon 2, which indeed were reported to have high antioxidant activity (Table 3.1 and Figure 3.6). In a previous study, Choi et al. (2020) also conducted a similar investigation and found that small seeds showed the maximum ferric reducing antioxidant power (FRAP) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS) scavenging activities. They also reported that the small seeds contained higher total phenolic content than the large and medium seed sizes.

Overall, our results demonstrated the influence of seed size on antioxidant activity and oil content in soybean varieties. Thus, the information on seed size and its relationship with antioxidant activity and oil content must be considered in soybean selection to enhance the seed quality. In addition, other parameters that showed positive and significant correlations were also found in the flowering time with pod maturity ($r = 0.82$, $P < 0.01$).

The total phenol content of 20 soybean varieties was significantly high and positively correlated with total flavonoid content ($r = 0.63^{**}$, $P < 0.01$) (Table 3.3). Moreover, the IC_{50} value was significantly high and negatively correlated with the total phenol ($r = -0.58$, $P < 0.01$). In addition, the IC_{50} value was also significantly and negatively correlated with the total flavonoid content ($r = -0.46$, $P < 0.05$). This finding is consistent with a previous study that reported that the phenol content is correlated with antioxidant activity, which is expressed by

the linear relationship between IC_{50} values and the contents of total phenol content (Malenčić et al. 2012). It has been reported that phenolic compounds are responsible for the level of antioxidant activity (Zaynab et al. 2018). As represented in Figures 3.4 and 3.5, the varieties Dering 1, Detam 2, Demas 1, and Devon 2 possessed moderately high total phenol and flavonoid content, contributing to scavenging DPPH free radicals.

Seed colors are another critical parameter that correlates with phytochemicals in soybean and other legumes (Dhungana et al. 2021; Yang et al. 2021). Varieties Dering 1, Demas 1, and Devon 2 have yellow seed coats, while Detam 2 has black seed coats (Figure 3.1 and Table 3.1). The comparison between the colors of the seeds and their phytochemicals has been discussed, and it has been found that both yellow and black soybeans were predominant in certain phenolic compounds (Yang et al. 2021).

Black seed coat soybean had the highest content of total phenols, flavonoids, and anthocyanins. Anthocyanins belong to the flavonoid family and accumulate in the epidermis palisade layer of the seed coat (Choi et al. 2020), acting as pigments. On the other hand, anthocyanins have not been detected in the cotyledon and seed coats of yellow soybean (Yusnawan 2016). The reason is that the pigmentation of the seed coat is inhibited in yellow soybeans, causing low levels of anthocyanins and proanthocyanidins (Lu et al. 2021). Although yellow soybeans lack anthocyanins, they are abundant in isoflavones responsible for the quantity of flavonoid content (Zhu et al. 2018). Therefore, black and yellow soybeans have their respective roles based on their dominant content as natural ingredients that may play a crucial role in human health.

The correlation between protein and oil has become a concern in improving soybean quality. Several studies have discussed and found that protein and oil content show a strong negative correlation (Zhang et al. 2018; Wang et al. 2019; Wang et al. 2019; Zhang et al. 2018). The observed negative correlation indicates a 2 % increase in protein content for every 1 %

decrease in oil content (Zhang et al. 2018). Consequently, it is challenging to increase seed protein content without a penalty in oil content and vice versa (Wang et al. 2019). This phenomenon has become a significant bottleneck in soybean improvement. However, our results revealed no correlation between crude protein and oil content (Table 3.3). As described in Figures 3.2 and 3.3, some varieties, such as Detam 1, Grobogan, and Anjasmoro, have moderately high crude protein and oil content. Therefore, these three varieties could be used as parents to improve soybean characteristics and its phytochemicals for breeding purposes.

In summary, this study analyzed crude protein, oil, total phenol, total flavonoid, antioxidant activity, and seed characteristics of 19 Indonesian and 1 Korean soybean varieties. Moreover, 20 soybean varieties were classified using clustering and heatmap analysis. These results could contribute to the more efficient utilization of soybean varieties to enhance food and feed products, especially for Detam1, Detam2, Dering1, and Grobogan, which possess superior traits as antioxidants.

CHAPTER 4

GENERAL DISCUSSION

1. IMPORTANCE OF SEED PHENOTYPE STUDIES

Since the early 1900s, researchers have conducted inheritance studies on the morphological colors of soybean seeds (Guriqbal, 2010). These studies have focused on evaluating the colors of the seed coat and cotyledon because of the ease and simplicity of observation. In the initial stages of soybean breeding programs, assessing the plant's morphology is crucial to determine the extent of the genetic variation. This evaluation allows for the identification of different physical characteristics of the soybean, which can aid in determining the plant's antioxidant levels. The diversity of soybean genetics is a significant factor in the success of breeding efforts, and the assessment of physical traits is a valuable tool in achieving desirable outcomes. Despite this extensive research, there is still a need for more understanding of the relationship between seed characteristics and antioxidants in Japanese and Indonesian soybeans. In light of this knowledge gap, the primary objective of this study is to examine the correlation between seed characteristics and nutrient content, such as protein, oil, and antioxidant content, including total phenol, total flavonoid, and antioxidant activity. This research aims to contribute to a better understanding of the nutritional benefits of Japanese and Indonesian soybeans, which is crucial for developing sustainable agricultural practices and promoting healthy food choices.

Soybeans are widely used for medicinal and traditional food purposes in many Asian countries, particularly Japan and Indonesia. These countries mainly process soybeans into food products (Figure 4.1), animal feed (Figure 4.2), and processed soybean (Figure 4.3). In particular, soybean seed must meet specific physical and chemical requirements when producing soy food. Factors such as seed size, uniformity of seed size and shape, and a light-colored hilum and yellow seed coat without physical damage (i.e., mottling, splits, shriveling, purple stain) are vital considerations for food-grade soybeans. Seed size uniformity affects water absorption and quality of the soy product. Seeds that are shrunken or discolored are

considered undesirable due to consumer preferences (Jegadeesan and Yu, 2020). Additionally, seeds that do not absorb water properly during soaking can cause issues with the texture and consistency of soy products, especially fermented soy foods such as natto. The hardness of the seed can be estimated by its swell ratio and can affect the calcium content and water absorption. Seed composition is also important and can vary depending on the type of soy food being made. For example, soybean seeds with high protein (>45%), low oil, high sucrose, and low oligosaccharide content are ideal for making tofu. In contrast, soybean seeds with high carbohydrate content are preferable to produce soy food like natto through a short fermentation process. This allows for a faster conversion to simple sugars (Shurtleff and Aoyagi, 2014).

Moreover, phenotypes in seed coats indicates phytochemicals and their antioxidant capacity. It is crucial to note that the various colors of soybeans can lead to different health benefits. Research has shown that black soybeans contain extracts with a longer low-density lipoprotein oxidation lag time than yellow soybeans due to their high polyphenol content within the seed coat (Takahashi et al., 2005). Additionally, the brown soybean seed coat has been found to have high radical-scavenging activity because of its highly polymerized proanthocyanidin component (Takahata et al., 2001). Furthermore, a study on oncology found that a diet enriched with soybean isoflavones can enhance immune function and inhibit the growth of adult T-cell leukemia cells in vitro and in vivo (Yamasaki et al., 2007).

2. SIMILARITIES BETWEEN JAPANESE AND INDONESIAN SOYBEANS

According to our studies, there are notable similarities between Japanese and Indonesian soybeans. It is indicated that certain types of soybeans possess higher concentrations of total flavonoids and phenols, specifically those with darker seed coats. This observation was evident in various Japanese soybean samples, including *G. soja* "B01167", Tsurusengoku, and Wase kuro daizu, as well as Indonesian varieties such as Detam 1 and

Detam 2, which all possess black seed coats. The study revealed a positive correlation between seed color and total flavonoid and phenol content, with darker seed colors consistently linked to greater levels of these beneficial compounds. Notably, black and brown soybean seeds are known to have some of the highest total flavonoid and phenol content compared to other seed colors.

The study conducted has revealed that there exists a correlation between phytochemicals and antioxidant activity. Further analysis using the luciferase reporter assay has shown that isoflavones, specifically genistein and daidzein, have a moderate impact on antioxidant activity (Table 2.2). Moreover, the DPPH assays have indicated a direct relationship between the total flavonoid and phenol content and antioxidant activity, as demonstrated by the IC_{50} values (Table 3.3). These findings provide valuable insight into the role of phytochemicals in promoting antioxidant activity and their potential benefits for human health.

However, despite the similarities between Japanese and Indonesian soybean accessions, there were differences in seed phenotype and antioxidant content. The Japanese soybean accessions have heavier dry seed weight (100 seeds/ gr) than Indonesian soybean accessions (Table 2.3 and Table 3.3). Moreover, this study has observed a correlation between soybean accessions in Japan and Indonesia. The study found that in Japanese soybean accessions, there is a significant correlation between isoflavone content and antioxidant activity. However, the correlation between phenolic or flavonoid content and antioxidant activity was not clear. In contrast, Indonesian soybean accessions showed a significant correlation between phenolic, flavonoid, and antioxidant activity.

Although phenols and flavonoids are generally responsible for antioxidant activity, the study in Japanese soybean accessions found that this may not always be the case. It suggests that another mechanism could be influencing the level of antioxidant activity or that simple

content alone cannot indicate antioxidant activity. The study assessed antioxidant activities using a luciferase reporter assay. In contrast, a different method was used to evaluate the antioxidant activity in Indonesian soybean accessions, which showed a correlation between content levels (flavonoid and phenol) and antioxidant activity.

3. PROSPECTIVE FOR SOYBEAN BREEDING

The breeding of soybeans for food purposes has been focused on developing seeds with unique nutritional compositions. Some examples of such varieties are categorized by the fraction from which the desired trait originates. These food-grade soybean varieties target specific traits such as high total protein content, high β-conglycinin, low lipoxygenase, high levels of certain amino acids such as lysine, methionine, and threonine, and low levels of allergenic proteins (Min et al., 2015; Shurtleff and Aoyagi 2014). Soybeans with high protein content (over 43%) are commonly used in making tofu, soymilk, soy sauce, beverages, baked goods, pudding, cheese, and meat substitutes. There are three main categories for breeding food-grade soybeans: large-seeded, small-seeded, and unique seed compositions. Based on our study in this dissertation, a few types of seeds commonly found in the market were randomly chosen to identify the beneficial components in each variety. Even though some of these seeds are small, accessions like B01167, Tsurusengoku, and Moshidou Gong 503 have notably high levels of flavonoids and phenols (as shown in Table 2.1). Similar to Dering 1 in Indonesian soybean, it also has high total phenol and excellent antioxidant activity with smaller seed size (Figure 3.4 and Figure 3.5). On the other hand, Williams, Akisengoku, and Himeshirazu varieties have larger seeds and higher levels of isoflavones (Table 2.1).

Soybean varieties that have high levels of isoflavones are desirable because of their health benefits. High-isoflavone soybeans have more than 0.4% isoflavones, while traditional soybean varieties have levels between 0.15-0.25% (Hu et al., 2020). The amount of isoflavones

in soybeans is affected by genetic and environmental factors such as temperature and irrigation during seed maturation (Lee et al., 2008). Studies show that the growth temperature has a negative impact on the total isoflavone content of soybean seeds (Kumar et al., 2006). To obtain soybean varieties with high isoflavone levels, it is important to understand the genetic regulation of this pathway. Previous research has identified three quantitative trait loci (QTLs) that control the concentrations of daidzein, glycitein, genistein, and total isoflavones in soybean seeds (Akond et al., 2014). These QTLs were located on different linkage groups, with one controlling the daidzein content on LG A1 (Chr 5), and two controlling the glycitein content on LG K (Chr 9) and LG B2 (Chr 14). In our study, Hitorimusume had the highest levels of daidzein derivatives. Fukuyutaka had the highest levels of genistein derivatives, while Akasaya had the highest levels of glycitein derivatives. Therefore, together with the QTL information, these findings could be used to develop soybean varieties with desirable isoflavone concentrations.

Moreover, developing soybean varieties with high protein and oil content is an important objective for improving food-grade characteristics through breeding. High protein and oil content can enhance the nutritional value of soy foods. However, the negative correlation between protein and oil levels makes it challenging. The observed negative correlation indicates a 2 % increase in protein content for every 1 % decrease in oil content (Zhang et al. 2018). While higher oil content is linked to higher yields, it often comes at the cost of lower protein content. Because soybean is valued for its high protein meal and versatile vegetable oils, breeders generally aim for modest gains in oil and yield without sacrificing protein concentration. Soybean seed protein and oil content are two valuable quality traits controlled by multiple genes. This suggests the potential for genetic improvement of soybean seed protein and oil content. Therefore, to overcome the negative correlation between protein and oil content, it is necessary to identify molecular markers linked with QTLs that control

these traits. This step is crucial as it helps improve the product's overall quality. Our study has discovered some unique soybean varieties in their high protein and oil content, despite the usual correlation between the two. Figures 3.2 and 3.3 show that Detam 1, Grobogan, and Anjasmoro are among these varieties, with moderately high levels of both crude protein and oil. This information is valuable in identifying potential parent plants for breeding programs aimed at improving soybean traits.

Improving soybean seed composition traits through breeding is a complex process, but soybean breeders and researchers now have ample genomic resources and tools to make it easier. Combining conventional breeding methods and genomic approaches can help identify genomic loci, haplotypes, and improve seed composition traits. The information of soybean varieties from this study could be a standard for improving the new breeding varieties.

4. SUMMARY

This research indicates that one of the Japanese soybean accessions, identified as B01167 (*G. soja*), possesses superior traits due to its high levels of flavonoid and phenol contents, including daidzein, daidzin, genistein, acetyl-genistin, and glycitein. This makes B01167 a promising candidate for future soybean breeding programs to produce soybeans with high phenol and flavonoid contents. Additionally, among the Indonesian soybean accessions, Detam 2 showed high levels of total phenol and flavonoid content, making it a potential source of antioxidants for pharmaceutical research.

To further enhance our understanding of the antioxidant content of soybean accessions, QTL studies could be conducted using a linkage population of high and low levels for the component of interest. For instance, B01167, a promising candidate for superior traits in flavonoid and phenol content, could be crossed with Wase kuro daizu, which has a very low antioxidant content. This will help create the recombinant inbred line (RILS) to reveal the QTL.

This information allows us to develop soybean varieties with desirable flavonoid and phenol concentrations.

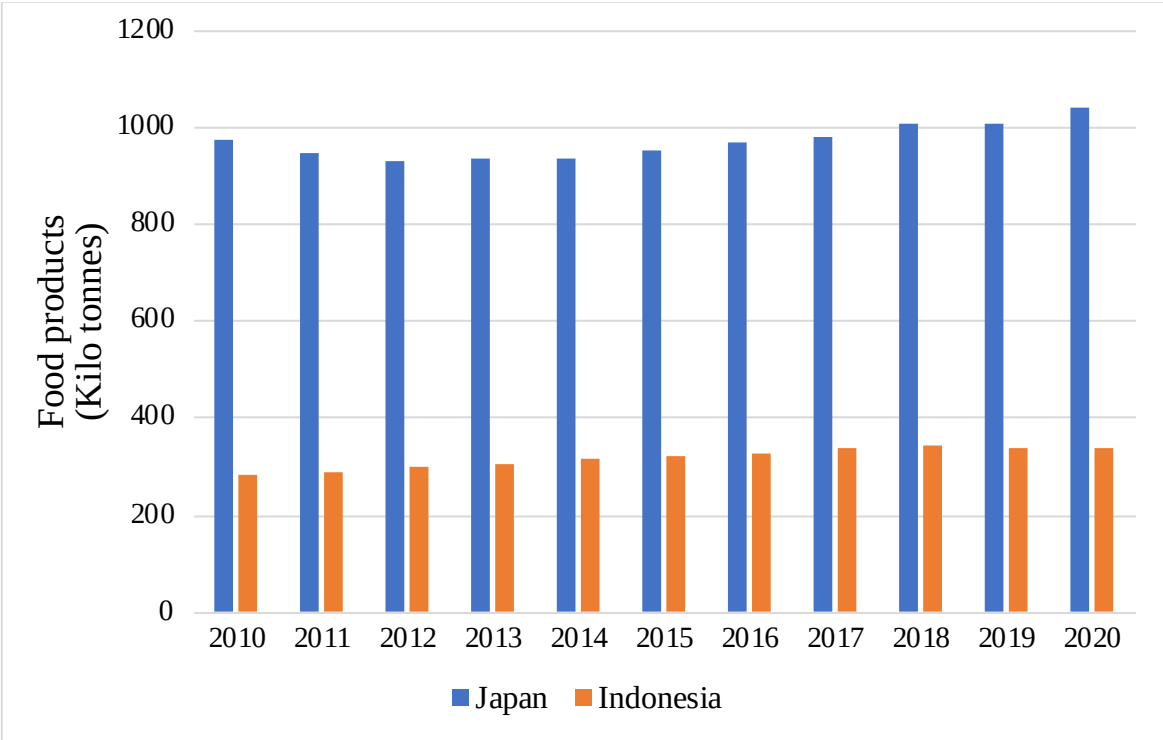

Figure 4.1. Historical trends of soybean demand in Japan and Indonesia for food products for the last ten years. The food products include tofu, soy milk, tempeh, natto, etc. Source: Food and Agriculture Organization of the United Nations: https://ourworldindata.org/agricultural-production

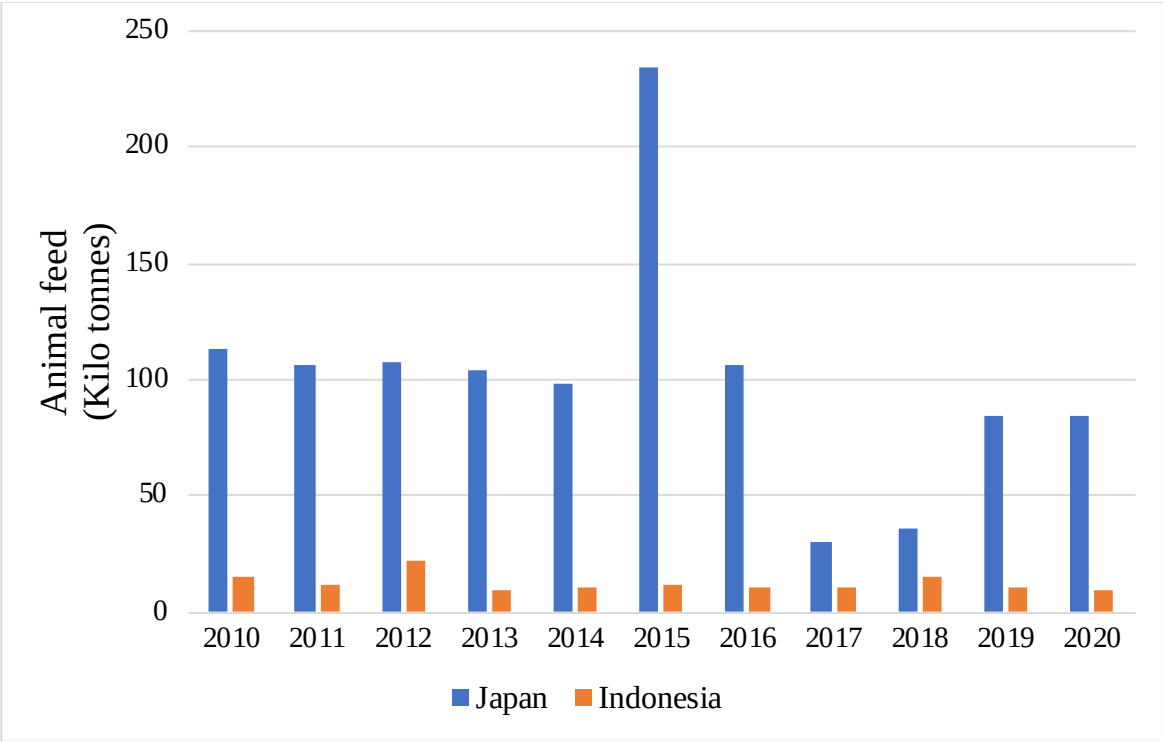

Figure 4.2. Historical trends of soybean demand in Japan and Indonesia for animal feed for the last ten years. Source: Food and Agriculture Organization of the United Nations: https://ourworldindata.org/agricultural-production

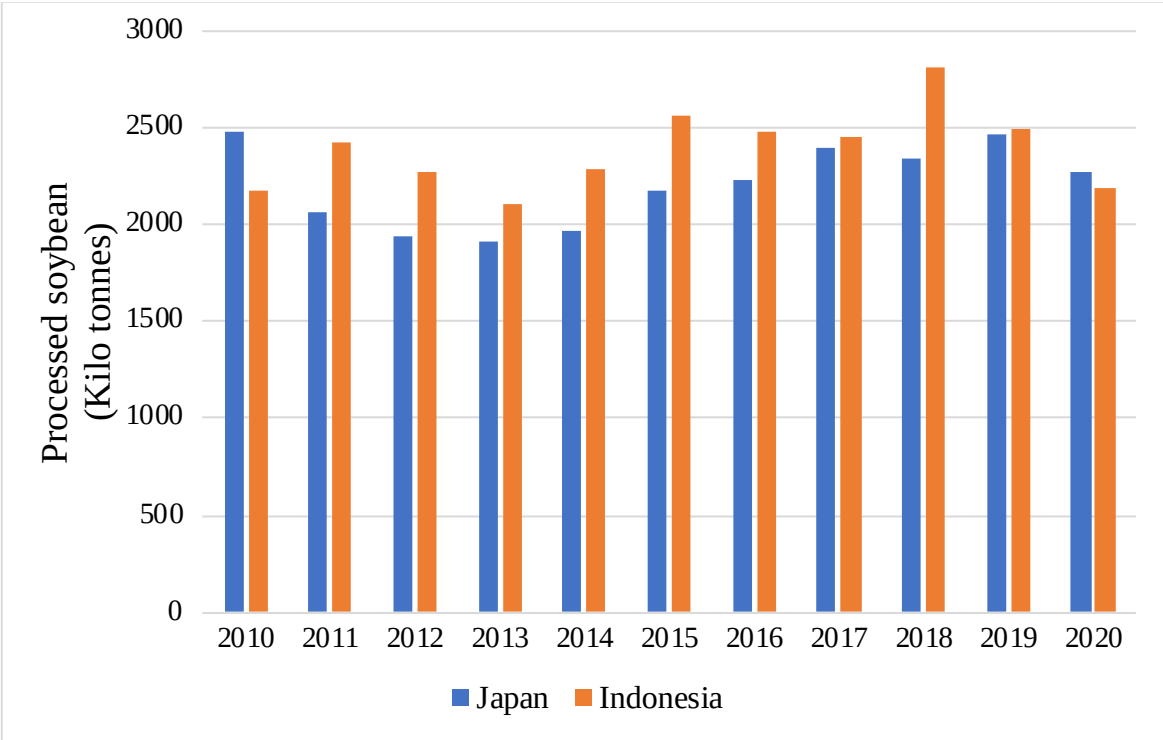

Figure 4.3. Historical trends of soybean demand in Japan and Indonesia for processed soybean for the last ten years. The processed soybean products include processed animal feed, biofuels, vegetable oil etc. Source: Food and Agriculture Organization of the United Nations: https://ourworldindata.org/agricultural-production

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