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The Effects of Fungal Stress on the Antioxidant Contents of Black Soybeans under Germination

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Black soybeans were germinated with or without *Rhizopus oligosporus* for 3 days. The samples collected from each day were freeze-dried and extracted with chloroform−methanol−water mixture to simultaneously obtain organic phase (lipophilic extract) and aqueous phase (hydrophilic extract). In the lipophilic extract, α-, γ-, and δ-tocopherol were drastically decreased by 51%, 97%, and 88%, respectively, after 3 day germination under stress. On the other hand, the lipid peroxide concentration was increased in the fungi stressed, germinating beans from day 2 onward, while it was continually decreased in the nonstressed germinating counterpart. The change of antioxidant capacity ($ORAC_{oil}$ value) was correlated to the changes of tocopherols and lipid peroxide concentrations. In nonstressed germinatng beans, $ORAC_{oil}$ value dropped by 38% after 3 days, while in the fungi-stressed germinating beans, $ORAC_{oil}$ was decreased by 80%. In hydrophilic extract, the antioxidant activity, measured by $ORAC_{hydro}$ assay, is about 80 times higher than the $ORAC_{oil}$ values, but there is no significant change before and after germination (either with or without stress). The total phenolic content in the hydrophilic extract increased only slightly by 9% in germinated beans and 15% in fungus-stress germinated samples, respectively. The advantage of stress germination of soybeans in enriching phytoalexins is traded off by loss of tocopherols.

KEYWORDS: Black soybean; fungal stress; antioxidants; tocopherols

INTRODUCTION

Phytoalexin-enriched functional foods are becoming an emerging frontier on functional food research field (*1*, *2*). Using food grade microbes to stress living soybeans has been shown to be an effective way of introducing phytoalexins and improving digestibility of the soybeans (*3*, *4*). Nutritional values of the soybean-based foods made from the treated soybean seeds may be ideal because of lowered amount of antinutrient (oligosaccharides) and enriched glyceollins, which have been shown to possess strong antiestrogenic effects (*5*), cancer prevention activity in cell line models (*6*), and insulin-stimulating activity in 3T3-L1 adipocytes (*7*). While using food grade fungi to induce biosynthesis of glyceollins from soybean was an attractive option to develop functional foods containing glyceollins, the effects of fungal stress on other nutritional quality of the processed soybeans such as the antioxidant contents are still unclear. Fungal stress will result in increased oxidative stress in the hosts, and thus it may have negative impact on the antioxidant nutritients. Soybeans are one of the most abundant sources of vitamin E (particularly γ-tocopherol) (*8*, *9*). Chow demonstrated that vitamin E acted as a defense system to scavenge free radicals and singlet oxygen to protect cellular components from the deleterious effects of fungi-caused oxidative stress and further prevent the oxidation reaction from continuing (*10*).

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Accumulating evidence suggests that overproduction of free radicals by mitochondria is the causative factor for chronic diseases such as cancer and cardiovascular diseases (*11*); therefore, dietary antioxidant continues to be an hotly pursued research topic in relation to chronic disease prevention and health promotion. Soybean is a good source of dietary antioxidants due to its high content of vitamin E family compounds, isoflavones, and other phenolic compounds (*12*, *13*). Therefore, as a part of our efforts in developing functional foods derived from black soybeans by bioprocessing, the objectives of this study are to investigate the changes of antioxidants in the fungus stress and germination process and to evaluate the effects of germination and fungus stress on the antioxidant capacity of the black soybeans.

MATERIALS AND METHODS

Reagents. The tempeh starter culture, *Rhizopus oligosporus*, was bought from PT. Aneka Fermentasi Industri (Bandung, Indonesia). Tocopherol standards, 13-hydroperoxy-9*Z*,11*E*-octadecadienoic acid ($C_{18}H_{32}O_4$) (13-HpODE) (1 mg/mL in ethanol), and 2,2-azobis(2,4-dimethylvaleronitrile) (AMVN) were purchased from Cayman Chemical (Michigan, USA). Methyl linoleate (ML, 97%) was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (97%), 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH), and fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1[3*H*],9′[9*H*]-xanthen]-3-one) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Folin−Ciocalteu (FC) reagent and gallic acid were purchased from Sigma Aldrich (USA).

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Isoflavone standards were purchased from Acros (USA). The oxygen biosensor system (OBS), a 96-well, round-bottom microplate coated with an oxygen-sensitive ruthenium dye, was purchased from BD Biosciences Discovery Labware (Mississauga, Canada). The 96-well polystyrene microplate with flat bottom was purchased from VWR International Inc. (Bridgeport, USA). *n*-Hexane, decane, methyl alcohol, and other solvents were of spectroscopic grade or HPLC grade from commercial sources.

Instruments. A Synergy HT fluorescent microplate reader (Biotek Instruments Inc., Winooski, USA) was used to monitor lipid $ORAC_{oil}$ value in OBS plate and hydro $ORAC_{hydro}$ value in transparent polystyrene microplate. Total phenolic analysis was also performed in the transparent polystyrene microplate using the same system. Sample dilution and reaction solution mixing were performed manually or by the Precision 2000 automatic liquid handling systems (Biotek Instruments Inc.). Vitamin E analysis was carried out on a Waters HPLC system with a Waters 2475 fluorescence detector (Milford, USA). Isoflavone analysis was also carried out on the same HPLC system with a Waters 2996 UV detector. Lipid peroxide analysis was performed using a Perkin-Elmer LS55 luminescence spectrometer (Waltham, MA, USA).

Black Soybean Germination and Fungal Inoculations. The black soybean germination and fungal inoculation methods followed our previous report (*3*). Briefly, black soybean seeds (100 g) were first surface-sterilized in 200 mL of 70% ethanol for 3 min and then rinsed with sterile water to wash away the ethanol thoroughly. The treated seeds were soaked in sterile water for 10 h. After draining the water, each bean was peeled without spoiling the radicals. *R. oligosporus* culture powder (1.0 g) was dissolved in sterile deionized water (15.0 mL). The suspension was mixed well with the soybeans (15.0 mL of each fungal solution inoculated 200 g of black soybeans). The inoculated beans were laid on a sterile container (30 × 50 cm) lined with two autoclaved filter papers moistened with 30 mL of sterile water. The containers were sealed with parafilm and incubated at 25 °C in the dark. Germinated black soybeans were also prepared with identical procedures except that no fungal was inoculated onto the beans. The soaked beans without any further treatment were prepared as control. To be more concise, in this study, the abbreviation codes used were UG for control (ungerminated sample), G for germinated bean sample, and GS for germinated bean sample under *R. oligosporus* stress.

Sample Preparation Procedures. Soybean samples were homogenized with distilled water (approximately 1 mL/g of dry bean seeds) and then lyophilized. The freeze-dried powder was stocked at −20 °C for future use. Ice-cold chloroform−methanol mixture (1:2, v/v, 0.75 mL) and 50 μL of ice-cold water were added to an Eppendorf tube and mixed with 50 mg of dry bean seeds. The mixtures were votexed for 30 s and then incubated on ice bath for 3 min. The shaking and incubation step were repeated for another three times. Ice-cold water (0.3 mL) and 0.25 mL of ice-cold chloroform were subsequently added into the tube and votexed for 30 s. After that the tube was incubated on ice for 1 min. Subsequently, the shaking and incubating step were repeated for three times. The mixtures were centrifuged at 11000 rpm for 3 min. The lower organic phase was collected and dried under nitrogen stream and kept at −20 °C before analysis. The upper hydro phase was also collected (0.6 mL). After filtration through a 0.45 μm polytetrafluoroethylene (PTFE) filter membrane, it was kept at −20 °C before subsequent analysis.

Vitamin E Analysis. Vitamin E analysis was performed according to the American Oil Chemists' Society (AOCS) method with some modifications (*14*). Separation was carried out on the Waters HPLC with a PDA detector and a Spherex 5 silica column (250 × 2.3 mm, 5 μm particle size, column temperature 25 °C) (Phenomenex Inc., Torrance, USA). The mobile phase was a mixture of 99.1% hexane and 0.9% 2-propanol with an isocratic flow rate of 1 mL/min. The emission wavelength is 330 nm.

Quantification of Lipid Hydroperoxides. Hay and co-workers established a high-throughput luminescent method using the luminescent semiconductor nanocrystals (NCs) as the fluorescent probe to quantify hydroperoxides (hydrogen peroxide and lipid hydroperoxide) (*15*). In this method, the oleic acid coated CdSe NCs (i.d. ≈2.5 nm) was used as the fluorescent probe. 13-Hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13(*S*)-HpODE) was used as the standard hydroperoxide. The dose-dependent manner of fluorescence quenching with the standard concentrations can give a correlation between the probe and the lipid hydroperoxides. The CdSe nanocrystal (NC) stock solution was synthesized in our laboratory. A diluted CdSe NC solution (25 nM) was prepared in hexane and activated under room light for 1 h. Serial concentrations of 13(*S*)-HpODE, including 64, 32, 16, 8, and 4 μM, were prepared in decane as standards. Samples were also diluted to desired concentrations with decane. Two milliliters of CdSe NC solution was first placed in a glass cuvette and read in the Perkin-Elmer LS55 luminescence spectrometer. The fluorescence spectra were measured with an excitation wavelength of 400 nm and emission wavelength between 500 and 700 nm. The fluorescence intensity (I_0) at the maximum peak (near 560 nm) was recorded. Twenty microliters of 13(*S*)-HpODE standard solution was immediately added to the CdSe NC solution. The mixture was vortexed thoroughly and kept in the dark at room temperature for 40 min before another reading (I) under the same instrumental conditions. The same measuring procedures were carried out for samples and blank (decane). After subtracting the blank reading, a standard curve of $\ln(I_0/I)$ against the concentration of 13(*S*)-HpODE in the cuvette was plotted. The lipid peroxide concentration of the sample was calculated from the standard curve and expressed as micromoles of 13(*S*)-HpODE equivalents per gram of dry bean seeds. All of the samples were run in triplicate unless otherwise stated.

Measurement of Antioxidant Capacity in the Lipophilic Extracts of Soybean. $ORAC_{oil}$ assay method, standing for oxygen radical absorbance capacity in bulk oil, is used to calculate the radical scavenging capacity in the lipids and fats of the beans (*16*). The assay was carried out with an excitation wavelength of 485 ± 20 nm and an emission wavelength of 590 ± 20 nm. The temperature of the incubator was set to 37 °C. AMVN was used as the peroxyl generator and Trolox as the standard with concentration ranging from 400 to 25 μM. This method measures the oxygen consumption of autoxidation reaction in the oxygen biosensor system (OBS), a 96-well, round-bottom microplate coated with an oxygen-sensitive ruthenium dye as the fluorescence probe. Methyl linoleate and decane mixture (8:2, v/v, 150 μL), 25 μL of AMVN (50 mg/mL), and 25 μL of sample were mixed in each well, fluorescence readings were taken every 2 min interval for 2 h, and the area under the curve was calculated. All dilutions were carried out using methyl linoleate and decane mixture (8:2, v/v). Data were expressed as micromoles of Trolox equivalents per gram of dry weight.

Measurement of Antioxidant Capacity in the Hydrophilic Extract. $ORAC_{hydro}$ assay was applied in the hydrophilic extract study. Fluorescein is used as fluorescence probe, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) is used as a peroxyl radical generator, and Trolox is used as a standard antioxidant for calibration. The assay was carried out in the Biotek Synergy HT fluorescent microplate reader according to the reported method (*17*). The results were expressed as micromoles Trolox equivalents per gram of dry weight.

Determination of Total Phenolic Content. Total phenolic content was measured using the Folin−Ciocalteu method (*18*). The assay was carried out on a microplate reader in the spectrophotometric mode. The samples were diluted appropriately with water prior to analysis. Gallic acid (200, 100, 50, 25, and 12.5 mg/L) was used to establish the standard curve. Folin−Ciocalteu reagent (100 μL) was diluted ten times from the original reagent and mixed with 80 μL of 30 mg/mL sodium carbonate and 20 μL of sample in each well. Absorbance was measured at 765 nm after standing for 30 min at 37 °C. Data were expressed as milligrams of gallic acid equivalents per gram of dry weight.

Isoflavone Analysis. Isoflavone analysis was carried out in Waters HPLC with a Shimadzu ODS-VP (4.6 × 250 mm, 5 μm particle size) column and a PDA detector. The mobile phases were water (A) and acetonitrile (B). The gradient was as follows: 0−1 min, A 97.5%; 1−17 min, A from 97.5% to 55%; 17−27 min, A from 55% to 10%; 27−33 min, A 10%; 33−35 min, A from 10% to 97.5%; 35−40 min, A 97.5%.

Statistical Analysis. All of the samples were tested in triplicate. The analysis of variance (ANOVA) and multiple mean comparisons were carried out by Statistic Analysis System (SAS 9.0). Differences among mean values were determined using Duncan's multiple range test at $p \leq 0.05$ when ANOVA indicated model and treatment significances.

RESULTS

Our initial research found a novel process of fermenting black soybean yogurt with dramatically reduced flatulence-causing oligosaccharides but enriched soy phytoalexins, and fungal-stressed

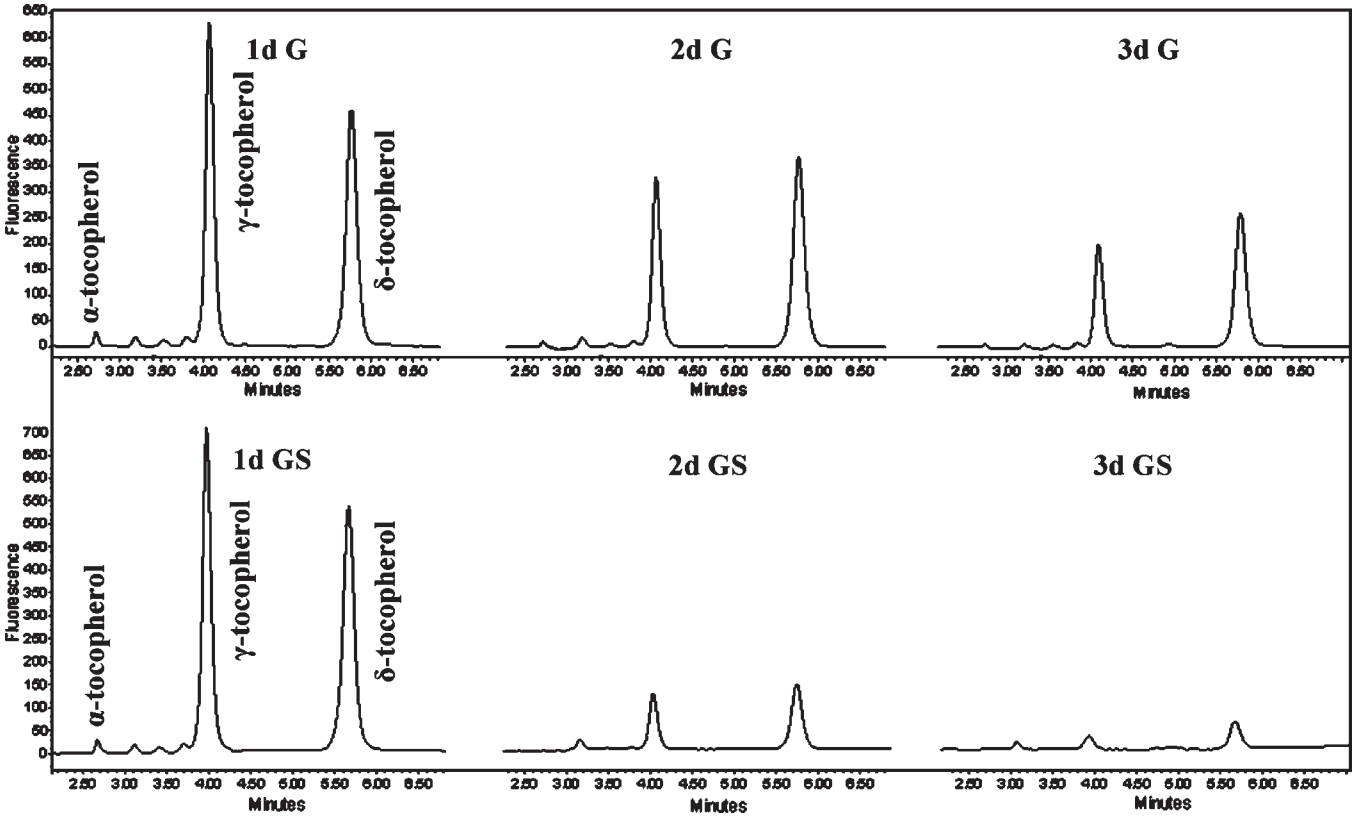

Figure 1. HPLC chromatogram of tocopherols in nonstressed germinating black soybeans (G) and *R. oligosporus* stressed germinating black soybeans (GS).

germination of black soybeans leads to generation of oxooctadecadienoic acids in addition to glyceollins (*3*, *4*). We were concerned that the excessive oxidation of the fatty acid may have profound impact on the antioxidant contents of soybean and this will have negative impact on its nutritional values. Therefore, we investigate the effects of fungal stress on the antioxidant status of germinating black soybeans by quantifing the vitamin E contents, lipid hydroperoxide values, total (both water and lipid phases) antioxidant capacity, total phenolic content, and isoflavone analysis.

Vitamin E Content. Three major tocopherols including α-, δ-, and γ-tocopherols were dominant in black soybeans but nondetectble amount of tocotrienols (**Figure 1**). About 74.75 μg/g (dry matter) δ-tocopherol and 92.00 μg/g γ-tocopherol were found in the ungerminated black soybeans, whereas α-tocopherol content was more than ten times less at 6.66 μg/g. As the germination occurs from day 1 onward, all three tocopherols declined gradually in both GS and G beans (**Figure 2**); about half of α-tocopherol was consumed in both samples after 3 days. Similarly, 65% of δ-tocopherol was depleted in G beans on day 3, while 89% was lost in GS counterpart. After 3 days, 83% and 97% of δ-tocopherol were decomposed in the two samples, respectively. Overall, tocopherols in the GS beans declined significantly more than that in G beans ($p < 0.05$).

Lipid Peroxides. Different trends were observed between GS and G beans (**Figure 3**). For both samples, lipid peroxides decreased from 1.75 μM to 0.84 and 1.35 μM, respectively, on the first day. But from day 1 onward, the concentration of lipid peroxide in GS beans was drastically increased and reached 3.70 μM on day 3. On the contrary, the concentration of lipid peroxide in nonstressed germinating sample was continually decreased and finally reached a concentration ten times less (0.35 μM).

Antioxidant Capacity and Total Phenolic Contents. The radical scavenging capacity of the beans was evaluated using oxygen radical absorbance capacity assay for both lipophilic and hydrophilic fractions of the extract. The initial $ORAC_{oil}$ value in black soybeans was 0.25 μmol of TE/g of dry bean seeds (**Table 1**). After 1 day germination, the $ORAC_{oil}$ values remained stable in both samples ($p > 0.05$). But on day 2 and day 3, the $ORAC_{oil}$ value in nonstressed sample was significantly decreased by 22% and 38%, respectively ($p < 0.05$). In addition, the $ORAC_{oil}$ value decreased by 68% in GS sample and 80% on day 2 and day 3 ($p < 0.05$). The initial $ORAC_{hydro}$ value was 19.93 μmol of TE/g of dry bean seeds, which was 80-fold higher than in lipophilic extract (0.25 μmol of TE/g of dry bean seeds, **Table 1**). The $ORAC_{hydro}$ value in sample G was slightly but not significantly increased ($p > 0.05$) to 20.78 μmol of TE/g, while in the GS germinating sample, it was gradually changed to 21.13 μmol of TE/g along the 3 days ($p < 0.05$). The total phenolic concentration in hydrophilic extract was 0.632 mg of GAE/g of dry bean seeds (**Table 1**). In germinated beans (G), the total phenolics were increased by 12% on day 1 ($p < 0.05$), 4% on day 2 ($p > 0.05$), and 9% on day 3 ($p < 0.05$) compared with the control (UG). While under stress (GS) it increased by 8% after 1 day ($p < 0.05$), 7% after 2 days ($p < 0.05$), and 15% after 3 days ($p < 0.05$) compared with the control (UG).

Residual Isoflavones in Hydrophilic Extract (Aqueous Phase). Since Isoflavones are the main polyphenolic compounds that normally contribute to free radical scavenging activity, we decided to quantify them in the aqueous phase, and the results were shown in **Table 2**. Only very small smounts of isoflavone were detected in the ungerminated beans. Similarly, the level of isoflavones in aqueous phase of the germinated beans (G) and stress-germinated beans are also very low. There are some changes upon germination under stress over the time, but overall the amount is so low that it does not contribute significantly to the antioxidant activity of the aqueous phase. The $ORAC_{hydro}$ contributing compounds are likely from total phenolic compounds and soy proteins.

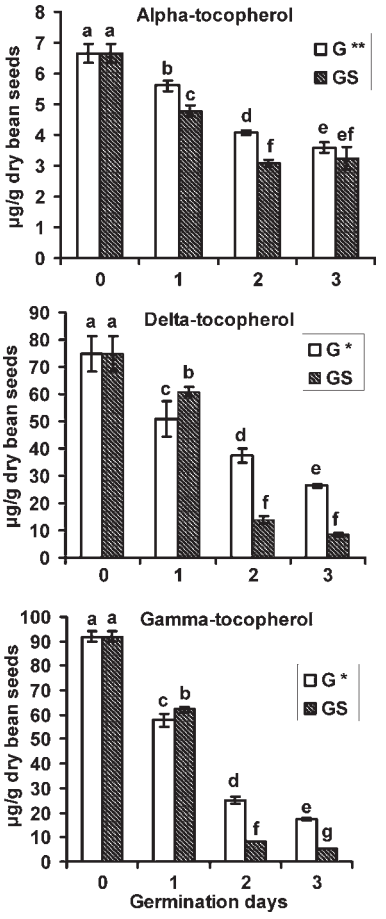

Figure 2. Comparison of α-, γ-, and δ-tocopherol changes between germinated black soybeans without stress and germinated black soybeans under *R. oligosporus* stress. ** = significant ($p < 0.01$), * = significant ($p < 0.05$). Data are expressed as means ± standard deviations ($n = 3$) on a dry weight basis. Values marked above the bar with different letters are significantly different ($p < 0.01$).

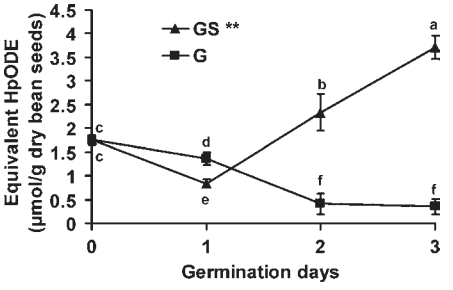

Figure 3. Comparison of lipid peroxide concentration between germinated black soybeans without stress and germinated black soybeans under *R. oligosporus* stress. ** = significant ($p < 0.01$). Data are expressed as means ± standard deviations ($n = 3$) on a dry weight basis. Values marked above the bar with different letters are significantly different ($p < 0.01$).

DISCUSSION

It was demonstrated that, during fungal stress, oxidative stress occurred accompanying the chemical or metabolic generation of large amount of reactive oxygen species (ROS) such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxy radicals, singlet oxygen, or related species (*19*), which will trigger a rapid lipid peroxidation (*20*). Although a number of naturally contained soybean peroxide-scavenging enzymes, including peroxidase, catalase, ascorbate peroxidase, and superoxide dismutase, can prevent PUFA from oxidation, it was reported that the external lipoxygenase from fungi may promote lipid peroxidation (*19*). It was thus possible that the drastic increase of lipid peroxides in fungus-stressed germinating beans (GS) was caused by both external enzymatic and free radical chain reactions, which was consistent with the observed lipid hydroperoxide accumulation during fungal stress on black soybeans. The increase of lipid peroxides in *R. oligosporus* stressed germinating beans (GS) correlates the decrease of tocopherol concentrations nicely. During seed storage, germination, and early seedling development, vitamin E plays a pivotal role to limit nonenzymatic lipid oxidation (*8*). In the present study, when *R. oligosporus* was grown as a pathogen infection to the soybeans, vitamin E is certainly in high demand due to the increased oxidative stress, and thus the vitamin E level decreased as a sacrificial antioxidant. This significantly decreases the nutritional value of the treated beans in terms of vitamin E content.

In addition to effects of fungi-caused oxidative stress, several researchers had investigated the effect of germination on the tocopherol content of legumes but obtained controversial results. Frias and co-workers investigated the correlations between seedling condition and vitamin E changes in lupins (*21*). Their results showed that germination brought about an increase in the content of α-tocopherol and a decrease in the content of γ-tocopherol and did not affect the content of δ-tocopherol. Fordham and co-workers, on the other hand, found an overall decrease of tocopherol content in kidney beans (*22*). Marero and co-workers studied the germination of mung beans and cowpeas (*23*). Their results showed an increase of α-tocopherol while a sharp decrease of γ-tocopherol. Our present study showed that all three tocopherols had significantly decreased (**Figure 2**). The discrepancy among these studies was possibly due to different legume varieties and germinating conditions such as temperature, time, moisture, and light, which had been demonstrated to have critical correlations with tocopherol synthesis (*22*,*24*). In line with the changes of tocopherol content and lipid peroxide concentration, $ORAC_{oil}$ value of the lipophilic extract was decreased accordingly in both fungus-stressed (GS) and nonstressed germinating (G) beans. However, $ORAC_{oil}$ values in GS beans declined more rapidly than in nonstressed germinating beans from day 2 onward (**Table 1**), indicating that fungal stress-induced free radical production had significantly affected its antioxidant activity.

However, the total isoflavones were significantly increased in both GS and G black soybeans (**Table 2**), but the changes of $ORAC_{hydro}$ value are not in this case. The ORAC values of individual isoflavones were measured by Rufer and Kulling (*25*) and this study; the ORAC values of total isoflavones were calculated as shown in **Table 3**. Total isoflavones in the water phase contribute only ~2% of antioxidant capacity to the total $ORAC_{hydro}$ values in the aqueous phase, suggesting that the high antioxidant ability of aqueous phase in black soybeans was mainly contributed by other antioxidants including water-soluble soy proteins.

Although the isoflavone content in the aqueous phase represents only a small fraction of the total isoflavones (majority of them are in the lipid phase), the amount extracted in the aqueous phase can still give overall trend of changes. Isoflavones in both G and GS beans are significantly increased ($p < 0.05$) by 103.8% and 72.5%, respectively, indicating the stimulatory effects of germination and fungal stress on biosynthesis of the isoflavones. It was reported that germination is able to initiate isoflavone biosynthesis in soybean seeds (*26*). Zhu and co-workers also indicated that a controlled germination can dramatically increase the total isoflavones particularly the mannoyl glucoside forms of

Table 1. Comparison of $ORAC_{oil}$ and $ORAC_{hydro}$ Values and Total Phenolic Content[a]

	UG	G				GS			
analysis	0d	1d	2d	3d	mean	1d	2d	3d	mean
$ORAC_{oil}$	0.25a	0.24a	0.19b	0.15c	0.20**	0.25a	0.08d	0.05e	0.13
$ORAC_{hydro}$	19.93	21.03	20.68	20.78	20.83	20.69	21.63	21.20	21.17
TPC	0.63e	0.71ab	0.66d	0.69bc	0.70	0.68cd	0.68cd	0.73a	0.70

[a] ** = significant ($p < 0.01$). Data are expressed as means ± standard deviations ($n = 3$) on a dry weight basis. Values marked with different letters are significantly different ($p < 0.01$). ORAC values were expressed as μmol of Trolox equivalents/g of dry bean seeds (μmol of TE/g); total phenolic contents were expressed as mg of gallic acid equivalents/g of dry bean seeds (mg of GAE/g); $ORAC_{hydro}$ and TPC were measured for the aqueous phase.

Table 2. Isoflavone Contents in the Aqueous Phases of the Soybean Samples[a]

	UG	G				GS			
isoflavone	0d	1d	2d	3d	mean	1d	2d	3d	mean
daidzein	0.039e	0.057c	0.058b	0.068a	0.061*	0.058b	0.058b	0.041d	0.052
glycitein	0.008d	0.010bc	0.010b	0.011a	0.010*	0.010b	0.010c	0.008e	0.009
genistein	0.032e	0.042d	0.044c	0.056a	0.047	0.046c	0.048b	0.044c	0.046
genistin	0f	0.022e	0.026c	0.026c	0.0244	0.024d	0.033b	0.043a	0.034**
total isoflavones	0.079f	0.130e	0.138c	0.161a	0.143	0.139c	0.150b	0.136d	0.142

[a] ** = significant ($p < 0.01$). Data are expressed as means ± standard deviations ($n = 3$) on a dry weight basis. Values marked with different letters are significantly different ($p < 0.01$). Isoflavone contents were expressed as mg/g of dry bean seeds.

Table 3. Contribution of $ORAC_{hydro}$ Values from the Isoflavones in the Aqueous Phase[a]

	UG	G				GS			
$ORAC_{hydro}$	0d	1d	2d	3d	mean	1d	2d	3d	mean
daidzein	0.163e	0.238c	0.244b	0.284a	0.256*	0.244b	0.245b	0.171d	0.220
glycitein	0.010d	0.012bc	0.012b	0.013a	0.012*	0.012b	0.012c	0.009e	0.011
genistein	0.153e	0.200d	0.212c	0.270a	0.227	0.221c	0.232b	0.211c	0.222
genistin	0f	0.086e	0.102c	0.104c	0.098	0.097d	0.134b	0.174a	0.135**
total $ORAC_{hydro}$	0.326f	0.537e	0.571cd	0.671a	0.593	0.575c	0.623b	0.566d	0.588

[a] ** = significant ($p < 0.01$). * = significant ($p < 0.05$). Data are expressed as means ± standard deviations ($n = 3$) on a dry weight basis. Values marked with different letters are significantly different ($p < 0.01$). ORAC values were expressed as μmol of Trolox equivalents/g of dry bean seeds (μmol of TE/g).

daidzein and genistein (*27*). Glycitein and its derivatives remained almost the same during germination, which were in agreement with the present study (**Table 2**). With a synchronized fungal stress and germination, the increase of genistin was higher than in the nonstressed germinating counterpart, while genistein was dropped by 8% after 3 days, which is possibly due to the isoflavone transformation among genistein derivatives (*28*). Such hypothesis can be demonstrated from the inproportional changes between glyceollins and their precursor daidzein. The observed differences may also be due to the biosynthesis of daidzein from phenylalanine (*29*, *30*).

In summary, it was found that germination and fungal stress on black soybeans had caused significant loss of antioxidant activity of lipophilic matters as measured by the $ORAC_{oil}$ value. Inconsistent with this observation, tocopherol contents were drastically reduced while lipid peroxide content increased significantly after 3 days of fungal stress and germination. In sharp contrast, there is insignificant change of antioxidant capacity in the aqueous phase of the extract. The advantage of stress germination of soybeans in enriching phytoalexins is traded off by loss of vitamin E family compounds.

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