TOTAL PHENOLIC CONTENTS AND ANTIOXIDANT ACTIVITIES OF TEN SOYBEAN PROMISING LINES TOLERANT TO ACID SOIL

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Abstract
Marginal land such as acid soil has been recently used to cultivate soybean since soybean cultivated area in Java has shifted to other valuable crops. This expansion aims to fulfill national soybean demand and to reduce soybean import as well as to ensure soybean security. Ten soybean promising lines tolerant to acid soil have been bred to address this issue. Secondary metabolites in soybean, i.e. phenolic compounds vary in each genotype. These compounds function mainly as chemical defense against insects and pathogens. Also, the phenolic compounds including flavonoids which hypothetically have antioxidant activities have positive effects to human health. Phenolic contents of ten soybean promising lines ranged from 2.87 to 3.73 mg GAE/g. Five varieties, i.e. Wilis, Tanggamus, Anjasmoro, Grobogan, and Argomulyo as the checks had the phenolic contents of 3.02 to 3.93 mg GAE/g. Flavonoid contents of the ten lines varied from 0.57 to 0.76 mg CAE/g. There were no check varieties which had the flavonoid contents higher than those of the promising lines. The Tgm/Anj-784, Tgm/Anj-856, Tgm/Anj-844, and Tgm/Anj-857 had the equally high flavonoid contents, i.e. 0.76, 0.75, 0.72, and 0.72 mg CAE/g, respectively. Antioxidant activities as indicated by DPPH values of the ten soybean lines were lower than those of Wilis, Argomulyo, and Grobogan.

Keywords: *soybean promising line, acid soil, phenolic content, flavonoid content, antioxidant activity*.

Abbreviations:
CAE: Catechin Equivalent
GAE: Gallic Acid Equivalent
DPPH: 2,2-diphenyl-1-picryhydrazyl

1. Introduction

The utilization of the soybean cultivation in Java has shifted to other prospected crops; therefore, the national soybean cultivated area has decreased gradually. Efforts have been made to expand the soybean cultivated area from 700 thousand hectares to 2 million hectares through several ways [1]. One of the efforts is the expansion of cultivated area to outside Java such as Sumatera and Kalimantan. Most of soybean areas in these two islands are categorized as marginal lands, such as acidity of the lands.

The main issues of the acidic land are high phosphorus fixation; toxicity of Al, Mn, and Fe in some circumstances; lack of Ca, Mg, K, Mo, and micro elements such as Zn and Cu; low cation exchange capacity; low available mineral contents; and vulnerable to erosion [2]. Based on this fact, new soybean breeding lines should be adapted to these land properties. Several studies have reported the genetic diversity on soybeans tolerant to the soil acidity [3, 4], therefore, a chance to breed new breeding lines tolerant to such properties is promising.

The development of soybean tolerant to acid soil ideally meets several criteria such as good agronomic performance as reflected by the agronomic growth (such as determinate/ semideterminate types and early-moderate maturity), high potential yield, and good quality of seeds. Since the acid land is mostly in dry condition, the soybean breeding lines should be bred to be adaptive to this obstacle. Drought as one of the abiotic stresses will affect seed quality and yield, since flowering, pod formation, and pod filling are the critical periods of soybean to water deficit. The occurrence of the water deficit until pod formation and pod filling will affect yield loss because of the pod reduction per plant [4]. To address the acidity and drought problems in the acid land in relations to the soybean cultivation, Indonesian Legumes and Tuber Crops Research Institute has bred several breeding lines, including Tgm/Anj lines. Some of them have medium seeded soybean.

With the sub optimal agroecosystems, the breeding lines may face more biotic stresses compared to those in optimal agroecosystems. The abiotic stresses may influence the crop performance, therefore, more prone to biotic stresses (insects, pathogens, and weeds). Even though as a consequence of cultivation in the sub optimal land may decrease the yield, this condition does not always have a negative impact on soybean crops. Secondary metabolites such as flavonoid phytoalexins and phenolic compounds are synthesized in higher amounts when soybeans are stressed with biotic and abiotic elicitors [5]. Phytoalexins are antimicrobial compounds with low molecular weights synthesized *de novo* and accumulate in plants as a response to pathogen infections or abiotic stresses because of wound, UV exposure and chilling [6, 7]. The advancement in food chemistry specifically functional foods is able to proof the secondary metabolites besides having antimicrobial properties; they also have beneficial effects to human health such as reducing the risk of cancer [8-12]. This study, therefore, was conducted to determine the flavonoids, phenolic contents and antioxidant activity of soybean breeding lines tolerant to acid soil to investigate the potential antimicrobial properties as well as potential to human health.

2. Materials and methods

This research was conducted in a greenhouse and laboratory of Indonesian Legumes and Tuber Crops Research Institute (ILETRI). The experiments consisted of five activities, i.e. soybean cultivation, sample preparation, total flavonoid determination, total phenolic analysis, and antioxidant activity measurement.

2.1. Soybean cultivation

To minimize the variation of soybean materials because of different planting seasons and cultivated area, ten breeding lines tolerant to acid soil (i.e. Tgm/Anj-784, Tgm/Anj-832, Tgm/Anj-844, Tgm/Anj-847, Tgm/Anj-856, Tgm/Anj-857, Tgm/Anj-858, Tgm/Anj-862, Tgm/Anj-888, and Tgm/Anj-889) and five varieties as checks (i.e. Wilis, Tanggamus, Anjasmoro, Grobogan, and Argomulyo) were cultivated in a greenhouse. All breeding lines were cultivated in pots. Each genotype was sowed in 3 pots with 2 plants/pots and 3 replicates. Fertilizers were given 7 days after planting. Full protection from insect pests and diseases were carried out to maintain optimal growth of the genotypes. Carbofuran, sipermetrin, and sihalotrin were applied to protect the plants from bean flies, leaf feeding pests, and pod insects. Soil borne diseases were controlled with captane application. Harvest was carried based on the genotype physiological maturity.

2.2. Sample preparation for phenolic, flavonoid and antioxidant capacity measurements

Soybeans were ground to obtain 80 mesh-particles. Extraction was carried out with 50% acetone for flavonoid and phenolic analyses and 70% ethanol for an antioxidant assay [13, 14]. Briefly, the ground soybeans and 50% acetone (1:10 v/v) were placed into tubes and shaked for 30 min at 200 rpm. After centrifugation, supernatant was transferred into amber vials. The pellet was extracted with same procedure to obtain the supernatant. The first and the second supernatant were combined for analysis.

2.3. Phenolic analysis

Total phenolic contents were determined using Folin-Ciocalteu reagent with gallic acid as a standard according to [15] and [16]. The proportion of the sample extract, dH_2O, Folin-Ciocalteu reagent, $NaCO_3$ and dH_2O were 1:60:5:15:19. The sample, dH_2O, Folin-Ciocalteu reagent were incubated for 8 min. After adding dH_2O, the solution was incubated for 90 min. Absorbance values were measured using spectrophotometer at 750 nm. The total phenolic contents were expressed as gallic acid equivalents (mg of GAE/g sample) using the calibration curve of gallic acid.

2.4. Flavonoid measurement

Flavonoids were determined according to a method developed by [17]. The proportions of the sample, dH_20, 5% $NaNO_2$, 10% $AlCl_3.6H_2O$, 1 M NaOH, and dH_2O were 10:50:3:6:20:11. Sample extracts or standards were diluted in dH_2O and 5% $NaNO_2$. After 6 minutes of incubation, the solution was added with 10% $AlCl_3.6H_2O$ and incubated for another 5 min. NaOH was added and followed by adding dH_2O. Absorbance values were measured using spectrophotometer at 510 nm. The results were express as micrograms of (+)- catechin equivalents (mg of CAE/g sample) using the calibration curve of (+)- catechin.

2.5. Antioxidant activity analysis

Antioxidant capacity was determined with DPPH according to [18]. Soybean extract was diluted in 0.1 mM DPPH (1:19 v/v). The solution was homogenized and incubated at a dark room for 30 min. Absorbance values of samples, control and blank were measured at 515 nm. The percentage of inhibition was measured as an indicator of antioxidant activity.

3. Results and Discussions

3.1. Total phenolic contents

The lines of Tgm/Anj-784, Tgm/Anj-832, Tgm/Anj-844, Tgm/Anj-847, Tgm/Anj-856, Tgm/Anj-857, Tgm/Anj-858, Tgm/Anj-862, Tgm/Anj-888, and Tgm/Anj-889 were categorized as medium seeded soybeans. Their parents were Tanggamus (Tgm) and Anjasmoro (Anj). Total phenolic contents of the ten soybean lines varied between 2.87 and 3.73 mg GAE/g based on the dry weight (dwt) (Table 1). The highest phenolic content was Tgm/Anj-889 (3.73 mg GAE/g, dwt) which was not significantly different with that of Tanggamus and the check varieties. Even though the parents had the phenolic contents of 3.92 mg GAE/g for Tanggamus and 3.01 mg GAE/g for Anjasmoro, one of their lines had the phenolic content less than 3.0 mg GAE/g. The Tgm/Anj-858 had the lowest content of the phenolic content (2.87 mg GAE/g) compared to that of other soybean lines, however, similar content with Anjasmoro.

Table 1. Total phenolic contents of ten soybean promising lines tolerant to acid soil

No	Soybean Line	Total Phenolic Content (mg GAE/g)	
		Wet weight	Dry weight
1	Tgm/Anj-784	3.35 bcde	3.59 bcd
2	Tgm/Anj-832	3.07 efgh	3.28 def
3	Tgm/Anj-844	3.27 cdefg	3.49 cde
4	Tgm/Anj-847	3.30 cdef	3.51 cde
5	Tgm/Anj-856	3.12 efg	3.32 def
6	Tgm/Anj-857	3.04 fgh	3.27 ef
7	Tgm/Anj-858	2.63 i	2.87 g
8	Tgm/Anj-862	3.13 efg	3.41 de
9	Tgm/Anj-888	2.99 gh	3.28 def
10	Tgm/Anj-889	3.45 abcd	3.73 abc
11	Wilis	3.47 abc	3.76 abc
12	Tanggamus	3.67 a	3.92 a
13	Anjasmoro	2.79 hi	3.01 fg
14	Grobogan	3.16 defg	3.38 de
15	Argomulyo	3.63 ab	3.88 ab

Values with the same letters in the same columns were not significantly different based on the LSD (α = 5)

3.2. Total flavonoid contents

Unlike Tanggamus and Anjasmoro which had the flavonoid contents of 0.59 and 0.45 mg CAE/g, the lines had the average of the flavonoid contents higher, which were 0.66 mg CAE/g (Table 2). Four lines (Tgm/Anj-784, Tgm/Anj-844, Tgm-Anj-856, and Tgm/Anj-857) had higher flavonoid contents (between 0.72 and 0.76 mg CAE/g) than those of the others. Among the varieties, only Wilis had the flavonoid content (0.62 mg CAE/g) similar to those of the breeding lines.

3.3. Antioxidant activities

There were no breeding lines having % inhibition similar to that of Wilis (36.7%). The antioxidant activity of Wilis was the highest (Table 3). The % inhibition range of the breeding lines was between 18.4% and 31.0%. Two lines had the % inhibition lower than that of Anjasmoro and no breeding lines had % inhibition higher or equal to that of Tanggamus.

Table 2. Total flavonoid contents of ten soybean promising lines tolerant to acid soil

No	Soybean Line	Total Flavonoid Content (mg CAE/g)	
		Wet weight	Dry weight
1	Tgm/Anj-784	0.70 a	0.76 a
2	Tgm/Anj-832	0.56 bc	0.60 bc
3	Tgm/Anj-844	0.68 a	0.72 a
4	Tgm/Anj-847	0.58 b	0.61 bc
5	Tgm/Anj-856	0.70 a	0.75 a
6	Tgm/Anj-857	0.67 a	0.72 a
7	Tgm/Anj-858	0.51 cde	0.56 cd
8	Tgm/Anj-862	0.58 b	0.64 b
9	Tgm/Anj-888	0.53 bcd	0.58 bc
10	Tgm/Anj-889	0.59 b	0.64 b
11	Wilis	0.57 b	0.62 b
12	Tanggamus	0.56 bc	0.59 bc
13	Anjasmoro	0.42 f	0.45 e
14	Grobogan	0.47 def	0.51 de
15	Argomulyo	0.46 ef	0.49 e

Values with the same letters in the same columns were not significantly different based on the LSD (α = 5)

Among the ten soybean breeding lines tested, only the Tgm/Anj-889 had consistently high phenolic (3.73 mg GAE/g), flavonoid (0.64 mg CAE/g) contents and the antioxidant activity (30.0% inhibition) (Table 1, 2, 3). A similar result was observed in Tanggamus, however, not found in Anjasmoro. Other varieties, Wilis, also had the same trend, the antioxidant activity was the highest compared to those of Tgm/Anj-889, Tanggamus and Anjasmoro.

As observed in the ten breeding lines, the lines with high phenolic contents did not correlate with high flavonoid contents or antioxidant activities, and vice versa as shown in the correlation values (r, Pearson correlation). The correlations between flavonoid and phenolic contents, flavonoid contents and antioxidant activities, phenolic contents and antioxidant activities were 0.13,

-0.23, and 0.39, respectively. In this study, flavonoid and phenolic compounds which hypothetically as a source of antioxidant showed low correlation with the antioxidant activities. Separate experiments employing other antioxidant assays including 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant power assay (FRAP), oxygen radical absorbing capacity assay (ORAC), cupric reducing antioxidant capacity (CUPRAC) [19, 20], may explain better antioxidant activity performance than only employing DPPH in a single experiment.

The ranges of phenolic contents (2.87-3.73 mg GAE/g), flavonoid contents (0.60-0.76 mg CE/g), and antioxidant activities (18.4-31.0%) were higher than the results conducted by [13] when different solvents to extract soybean samples were used. In their study, the phenolic and flavonoid contents extracted using 50% acetone were 2.62±0.05 mg GAE/g and 0.50±0.05 mg CE/g. The slight difference in the secondary metabolite contents may be caused by different genotype used. Beside genotypic variations, the expression of the soybean secondary metabolites was also influenced by the environment, both biotic and abiotic stress [5, 21].

Genotypes with high expression of the secondary metabolites such as Tgm/Anj 889 as observed in this study may have two advantages both for plant's life cycle and potential for human health. Phenolic compounds and flavonoids play an important role in defence strategies against pathogens and insect attacks. Also, these chemicals have allelopathic properties and ultraviolet protectans [21]. Comprehensive studies of flavonoids to protect the soybean plant from pathogens were reported by [22] and [23]. Sub class of isoflavones inhibited some soil borne pathogens. Daidzein, genistein, glycitein were effective to inhibit *Fusarium culmorum* [22], *Rhizoctonia solani* and *Sclerotium rolfsii* [23].

In recent years, the role of both phenolic compounds and flavonoids as health promoting constituents has become an increasingly important area of human nutrition studies. Prolong consumption of foods containing phenolic and flavonoid compounds has been proven to prevent or reduce the risk of degenerative diseases such as an endometrial cancer, breast cancer and prostate cancer as well as other diseases related to estrogenic deficiency based on the epidemiological studies [8-12]. The line of Tgm/Anj 889 may have a potential health benefit besides the other breeding lines.

Table 3. Percentage of inhibition of ten soybean promising lines tolerant to acid soil

No	Soybean Line	% inhibition*
1	Tgm/Anj-784	19.6 ef
2	Tgm/Anj-832	18.4 f
3	Tgm/Anj-844	20.6 e
4	Tgm/Anj-847	30.3 c
5	Tgm/Anj-856	31.4 c
6	Tgm/Anj-857	25.2 d
7	Tgm/Anj-858	25.2 d
8	Tgm/Anj-862	31.0 c
9	Tgm/Anj-888	20.1 e
10	Tgm/Anj-889	30.0 c
11	Wilis	36.7 a
12	Tanggamus	31.0 c
13	Anjasmoro	23.9 d
14	Grobogan	33.7 b
15	Argomulyo	34.3 b

*% inhibition of Trolox 400 μm, 200 μm, and 100 μm = 56.5 %, 29.0%, and 14.3 %.
Values with the same letters in the same columns were not significantly different based on the LSD (α = 5)

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4. Conclusions

The Tgm/Anj-889 which had the high expression of phenolic contents, flavonoids and antioxidant activity may be considered for a genotipe adaptive to acid soil along with other agronomic characteristics and consumer preferences. This line was rich in antimicrobial compounds which may be more resistant to pre-emerging pathogen infections as well as had potential health benefits.

Acknowledgements

Authors would like to thank Asian Food and Agriculture Cooperation Initiative for providing the research funding. Thanks are also extended to Miss Etika D.S. and Mr. Suprapto for their technical assistances in the laboratory.

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