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Changes of Phenolic Contents and Antioxidant Activity in Soybean Seeds Harvested from *Phakopsora pachyrhizi* Infected Crops

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Abstract

Asian soybean rust caused by *Phakopsora pachyrhizi* is one of the most destructive foliar diseases on soybean. Severe infection of this disease causes early defoliation and reduces the yield. To determine the response of soybean genotypes to this disease and the changes of metabolites in seeds, a greenhouse study was conducted using eight Indonesian soybean cultivars, i.e. Malabar, Wilis, Ringgit, Pangrango, Argomulyo, Grobogan, Dena 1, and Dena 2. The experiment was arranged in a randomized completely block design and repeated three times. The soybean crops were inoculated with the pathogen and another set was not inoculated. Infection of *P. pachyrhizi* reduced fresh biomass, seed weight per plant, and weight of 100 seeds. However, total flavonoid contents in seeds increased from 12 to 50% in all infected genotypes. The increase of daidzein from 27 to 67% in seeds was observed, except for Malabar and Argomulyo . The increase of genistein was genotypic dependence. The increase of total phenolic contents as well as antioxidant activity was also depending on the genotypes. *P. pachyrhizi* could be one of the biotic elicitors to increase total flavonoid contents in soybean seeds. Dena 1 less suffered from the rust infection as represented by the least pustule number, less reduction in seed weight and weight of 100 seeds. Secondary metabolites particularly phenolics and isoflavones in seeds of this cultivar increased significantly after the rust infection. This cultivar could be considered as an alternative tolerant genotype where cultivated area is favorable for soybean rust infection.

How to Cite

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INTRODUCTION

Asian soybean rust (ASR) caused by an obligate pathogen *Phakopsora pachyrhizi* is foliar disease which infects soybean (*Glycine max* (L.) Merr.) crops. Early infection may cause premature defoliation, therefore affecting the number of intact pods, reducing pod numbers, yield and seed sizes (Kumudini *et al.*, 2008; Ribeiro *et al.*, 2008, 2009). Significant yield loss up to 80% occurs in heavily infected crops especially in susceptible genotypes (Twizeyimana *et al.*, 2009). This disease, therefore, is considered the most destructive foliar disease on soybean (Li and Young, 2009).

Initial symptom of infected plants appears on the older leaves as lesions. These lesions consist of pustules which contain uredospores. Generally, the first symptom appears on the leaves at the lower canopy after flowering in sub tropical regions and appears earlier in tropical regions (Inayati and Yusnawan, 2016b). Three lesion reaction types may appear on the infected soybeans. The three lesions are without visible lesions as immune (IM) reaction, reddish-brown (RB) lesions as resistant reaction that can be sporulating and non sporulating, and tan (TAN) lesions as susceptible reaction which is associated with uredinia and high levels of sporulation (Walker *et al.*, 2014a, b; Harris *et al.*, 2015). Since environmental factors such as temperature and humidity may influence the crop reaction; therefore categorizing susceptibility of soybean genotypes is not easily conducted.

Studies on ASR have been extensively conducted in gene levels, especially genes responsible for the resistant reactions. These approaches are believed to provide more in depth resistant mechanism of ASR in soybean compared to those of investigating the lesion types only. *Rpp* genes have been reported responsible for soybean crop resistance, which are located in six resistance loci to *P. pachyrhizi* (*Rpp*) (King *et al.*, 2016). So far, the six resistance loci*, Rpp1* to *Rpp6*, consist of at least 10 described resistance alleles (Chakraborty *et al.*, 2009; King *et al.*, 2016). *Rpp1* was reported responsible for immunity response, whereas resistance with little or no sporulation was controlled by *Rpp1b*, *Rpp2*, *Rpp3*, *Rpp4*, and *Rpp5* (Garcia *et al.*, 2008).

Commercial cultivars resistant to ASR have not been available yet (Goellner *et al.*, 2010), even though several efforts both conventional and molecular approaches have been conducted to address this issue (Monteros *et al.*, 2010). Several factors affecting the susceptibility of resistant cultivars which are easily broken are pathogen variability, gene complexity which controls resistance to ASR and environmental factors (Garcia *et al.*, 2008). The use of tolerant or partially resistant cultivars could be one of the alternative approaches to manage ASR in endemic areas.

Since resistant cultivars have not been available yet, cultivation of tolerant cultivars may prolong the existence of ASR to widespread in the field. Soybean crops will interact with the pathogen during infection and its lifecycle of ASR, considering the ASR is an obligate pathogen. Secondary metabolites such as phenolic acids, flavonoids, saponins, and triterpenoids are produced in large amount to protect crops from further infection. *Macrophomina phaseolina*, *Phytophthora sojae, Sclerotinia sclerotiorum, Rhizoctonia solani* and *Phakopsora pachyrhizi* are inhibited by these secondary metabolites (Bellaloui *et al.*, 2012; Lygin *et al.*, 2009, 2013, 2014).

Research on the production of secondary metabolites of phenolic compounds particularly on leaves after pathogen infection has been conducted (Lygin *et al.*, 2009, 2010, 2013). However, reports of secondary metabolite contents in seeds harvested from crops infected by *P. pachyrhizi* have not been available yet. This study therefore, aimed to determine the changes of phenolic, flavonoid contents including two main isoflavones, daidzein and genistein as well as antioxidant activity in seeds of infected crops. The study also aimed to assess the susceptibility of Indonesian cultivars against ASR disease.

METHODS

Plant materials and experimental design

Eight Indonesian soybean cultivars i.e. Malabar, Wilis, Ringgit, Pangrango, Argomulyo, Grobogan, Dena 1 and Dena 2 were sowed in 5 kg plastic pots. Two sets of experiments, i.e. one set was soybean for rust inoculation treatment and the other set was soybean for uninoculated treatment, were grown in a separate greenhouse. This experiment was arranged in a randomized completely block design and repeated three times. Each pot consisted of two plants and every soybean genotype was planted on three pots.

Source of inoculum and inoculation

Uredospores of *P. pachryhizi* were propagated on a susceptible cultivar, Ringgit. Inoculum for artificial inoculation was prepared by suspending the uredospores in 1L of sterile water containing 200 µL Tween 20. The set of soybean plants for inoculation study was treated as follows. Artificial inoculation with uredospores at

concentration of 10^4 spore/mL was conducted twice at 21 and 28 DAP (days after planting). The uredospores were sprayed on the leaves in the evening (Inayati and Yusnawan, 2016b). For the set of uninoculation study, the artificial inoculation was not conducted. Temperature and relative humidity were daily monitored.

Response of soybean against rust disease

Responseof soybean and the resistant level against the rust disease was rated using the modified three digits of International Working Group of Soybean Rust (IWGSR), and categorized into five groups, i.e. immune (I), resistant (R), moderately resistant (MR), moderately susceptible (MS), and susceptible (S) (Shanmugasundaram *et al.*, 2004).

Agronomic performance and yield components

Agronomic performance and yield components per plant at harvesting including plant height, fresh plant weight, number of filled pods, number of empty pods, and dry seed weight were recorded from both inoculated and uninoculated treatments.

Sample preparation and extraction for secondary metabolite analyses

Intact soybean seeds (50 g) were ground with a sample mill (Cyclotec sample mill, Sweden). Extraction was conducted using 80% methanol (1:10 w/v) for measuring total flavonoid and phenolic contents as well as antioxidant activity. Extraction was carried out twice and the supernatant was combined (Yusnawan, 2016).

Extraction of soybean flour for isoflavone analyses was conducted using 50% methanol (Cho *et al.*, 2011). Soybean flour was extracted in the extraction solvent (1:20 w/v) and macerated for 24 hours on an orbital shaker at 100 rpm. The supernatant was filtered and followed by 0.45 µm PVDF syringe filter. The filtered supernatant was stored in amber vials at 4 ^{o}C for 24 h prior to injection into HPLC system.

Determination of total phenolic content

Phenolic content was estimated using Folin-Ciocalteu's reagent according to Singleton *et al.* (1999); Yusnawan (2016). The absorbance values were recorded using a spectrophotometer (Genesys 10uv, US) at 765 nm and the phenolic contents were expressed as gallic acid equivalents per gram of sample (mg GAE/g sample).

Determination of total flavonoid content

Total flavonoid content was measured with $AlCl_3$ (Heimler *et al.*, 2005). Total flavonoid content was expressed as catechin equivalents per gram of sample (mg CE/g sample) based on dry basis using the calibration curve of the catechin.

Determination of two isoflavone aglycones

Daidzein and genistein in aglycone forms were measured using high performance liquid chromatography with modification (Achouri *et al.*, 2005). Optimization was performed on Elite LaChrom HPLC (Hitachi, Japan) using a reversed phase Cosmosil C-18 column (250 mm x 4.6 mm, 5 µm, Hitachi Japan) and equipped with uv-vis detector (L-2420 Hitachi, Japan). Soybean samples (20 µL) were injected into the system. Mobile phases were 0.1% acetic acid (solvent A) and 0.1% acetic acid in acetonitrile (solvent B). Absorbance value was monitored at 280 nm.

DPPH free radical scavenging activity

Antioxidant capacity of soybean extract was measured according to Xu and Chang (2008a, b) using 0.1 mM ethanolic DPPH solution. The radical scavenging activity of each sample was expressed as micromoles of Trolox equivalent per gram of sample (µmol TE/g sample).

RESULTS AND DISCUSSION

Response of soybean against rust disease

Eight soybean genotypes were screened against rust disease. Rust pustules appeared on the lower surface of the leaves (Fig. 1). Three genotypes, i.e. Malabar, Grobogan and Dena 2 were categorized moderately resistant at 49 DAP (Table 1). The number of pustules was from 5 to 8 pustules per cm^2. Infection rate of the rust disease was not only determined by total number of pustules, but also position of the pustules and sporulation incidence. The pustules appeared on the middle third of the leaf canopy as observed in all genotypes except Malabar and Grobogan in which their upper third canopy had been infected. As the plant grew older, the number of pustules also increased. Only one genotype, Malabar was categorized susceptible with number of pustules up to 17 pustules per cm^2 at 56 DAP. Sporulation occurred on all genotypes.

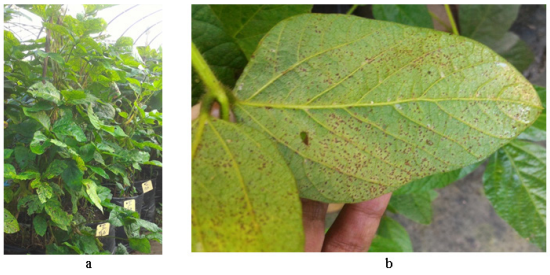


Figure 1. Soybean crops infected by *P. pachyrhizi* (a) and pustules appeared on the leaves (b)

Classification of the susceptibility of genotypes against rust disease was influenced by the intensity of uredospore sporulation and assessment of periodical severity (Araujo and Vello, 2010), as conducted in the present study. Apart from genotypes, other factors influenced the disease progress were environmental factors such as relative humidity of leaves (85-95%) and canopy temperature (21-24 °C) as investigated by Del Ponte and Esker (2008). Environmental humidity at 80-90% and temperature at 20-30°C recorded during this study were favorable for pathogen infection and rust development, therefore this condition was also favorable for soybean genotype screening against the ASR. Color lesions may also correlate with the susceptibility of the genotypes. According to Kato and Yorinori (2008), reddish brown lesion was found in resistant genotypes and tan lesion or mixture of reddish brown and tan appeared in susceptible genotypes. However, these lesion colors were easily influenced by the environmental factors (Yamanaka *et al.*, 2010), therefore this parameter was not investigated in the present study.

Agronomic performance and yield components

Soybean rust infection reduced plant weight, number of intact pods, seed weight and 100-seed weight of all genotypes (Fig 2, 3, 4). The reduction of plant weight at harvesting could be used as one of the signs of early defoliation since this pathogen caused premature leaf loss (Kumudini *et al.*, 2008; Ribeiro *et al.*, 2008, 2009). Severe leaf loss occurred in Wilis (Fig 2), even though this genotype was classified moderately susceptible cultivar (Table 1). Nevertheless, the seed weight loss per plant of Wilis was less than that of Grobogan (Fig 4a). Grobogan genotype also had the least number of intact pods (Fig 3a), suggesting the most suffered genotype to the rust pathogen. The seed size of all genotypes as represented by 100-seed weight was smaller than seeds harvested from uninoculated crops (Fig 4b).

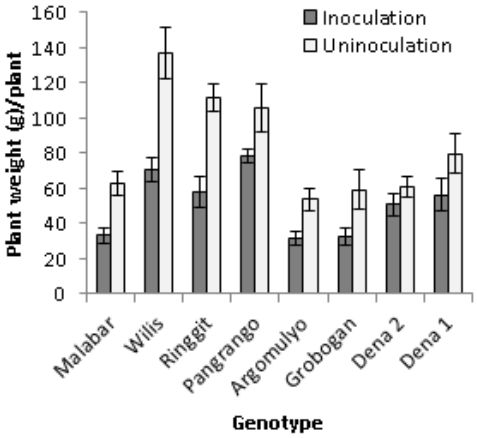


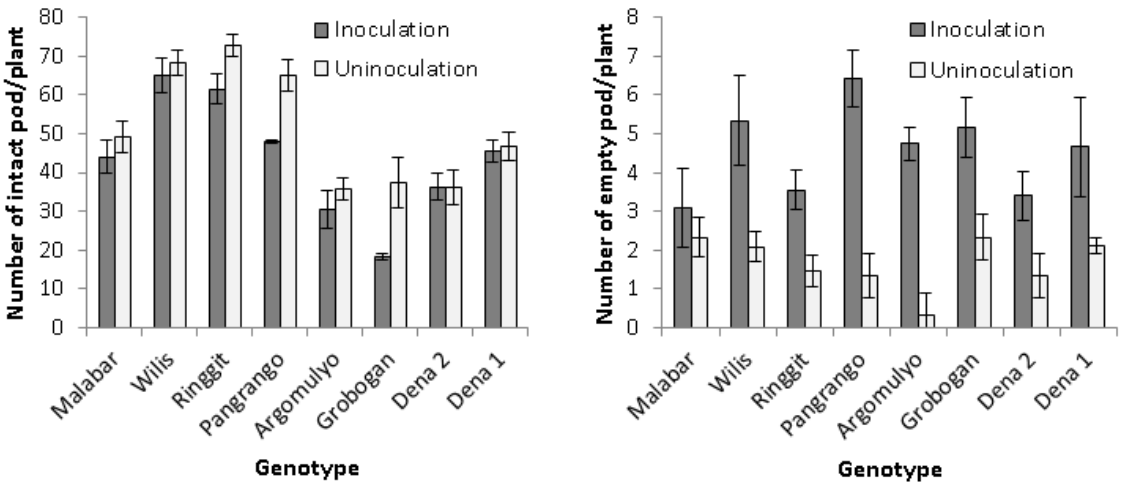
Figure 2. Plant weight of eight soybean genotypes of *P. pachyrhizi* infected and uninfected crops

The reduction of yield and yield components including number of intact pods, empty pods, and seed weight per plant was also reported

Table 1. Resistant criteria of eight soybean genotypes to soybean rust disease

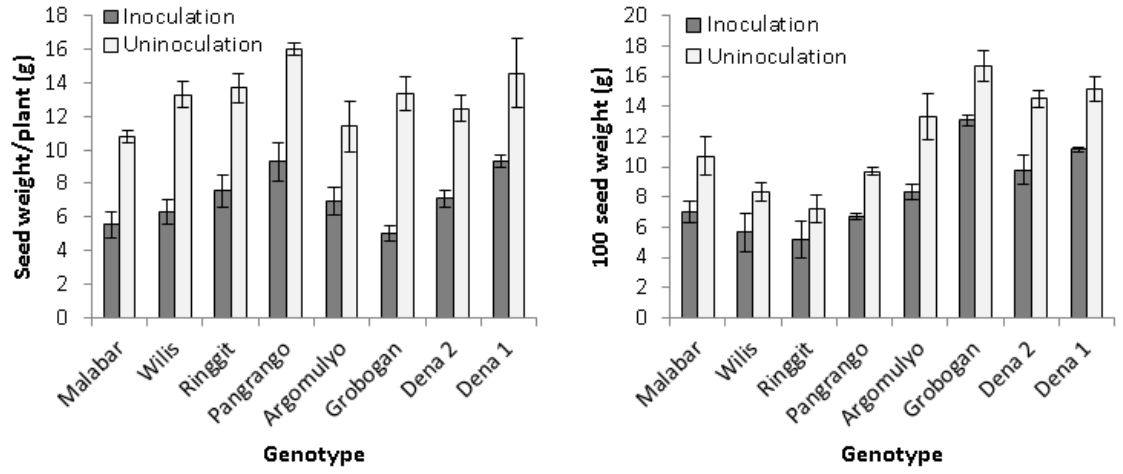
	49 DAP			56 DAP		
	Score	Σ pustule	Criteria	Score	Σ pustule	Criteria
Malabar	322	6	MR	343	17	S
Wilis	222	6	R	333	13	MS
Ringgit	222	6	R	333	12	MS
Pangrango	222	6	R	333	12	MS
Argomulyo	222	8	R	333	13	MS
Grobogan	322	5	MR	333	14	MS
Dena 2	232	8	MR	333	13	MS
Dena 1	222	8	R	333	11	MS

DAP = day after planting, R = resistant, MR = moderately resistant, S = susceptible, MS = moderately susceptible (scoring and resistant criteria based on a method by Shanmugasundaram *et al.* (2004))



a b

Figure 3. Number of intact pods (a) and empty pods (b) per plant of infected and uninfected crops of eight soybean genotypes



a b

Figure 4. Seed weight per plant (a) and weight of 100 seeds (b) of infected and uninfected crops of eight soybean genotypes.

by other studies. Inayati and Yusnawan (2016a, b) reported that susceptible genotypes had fewer intact pods than those of resistant genotypes. The pod filling was not optimal in crops infected by *P. pachyrhizi* due to early defoliation (Kumudini *et al.*, 2008; Ribeiro *et al.*, 2009). This foliar pathogen was not only influencing the healthy green leaves but also disturbing the photosynthetic process of the green parts of the leaves (Kumudini *et al.*, 2008), therefore may reduce the number of pods and seed size.

Total phenolic contents and isoflavones

Total phenolic contents in seeds of infected crops increased in all genotypes, except Wilis (Fig. 5a). The increase of total phenolic contents (11.0 to 35.4%) was genotypic dependence. Dena 1 was the highest increase (35.4 %) among other genotypes. Although phenolic contents are produced in excess amount especially in area around infection, the increase of these chemicals was also observed in seeds as observed in this study. The increase of phenolic contents in plants was in line with the function of these compounds to combat plant pathogens. Several phenolic compounds in leaves, i.e. glyceollin, formononetin, and kaempferol have been reported to inhibit rust infection, with glyceollin was the most prominent

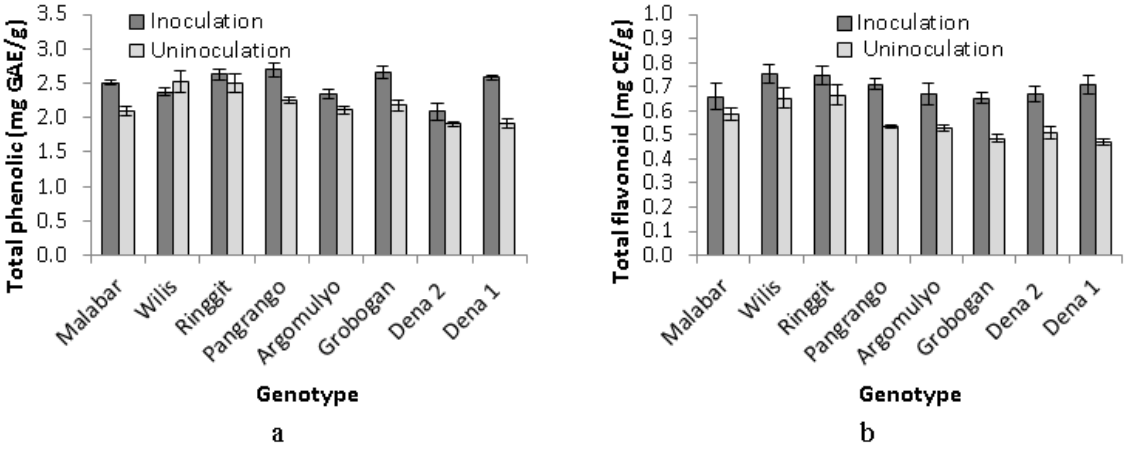


Figure 5. Total phenolic (a) and flavonoid contents (b) in seeds of eight soybean genotypes on infected and uninfected crops

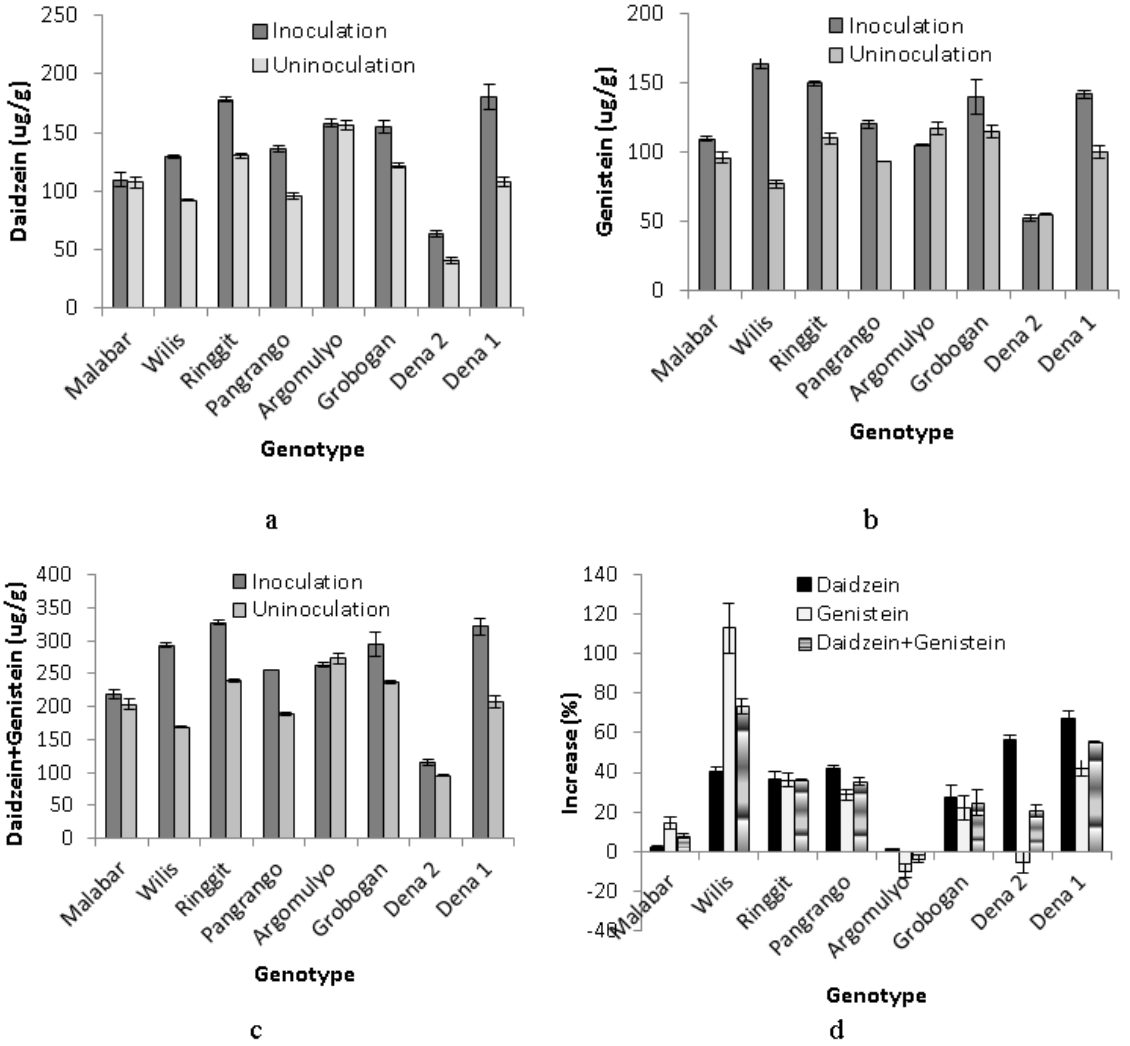


Figure 6. Daidzein (a), genistein (b), sum of daidzein and genistein (c) contents as well as the increase of isoflavone aglycones (d) in seeds of eight soybean genotypes on infected and uninfected crops

inhibition (Lygin *et al.*, 2009).

Total flavonoid contents increased in seeds of all soybean genotypes after *P. pachyrhizi* infection ($P > 0.05$) (Fig 5b). Malabar was the soybean genotype which had the lowest increase in this secondary metabolite (12.7%). This genotype also contained the lowest flavonoid content (0.66 mg CE/g) similar to that of Grobogan (0.65 mg CE/g) after pathogen infection. Dena 1 which showed the highest increase in total phenolic content also had the highest increase in total flavonoid content (50.7%). Accumulation of total flavonoid increase in seeds was noted in our study, even though in gen levels, the expression of genes involved in flavonoid biosynthesis increased at 12 hours after inoculation and returned to basal expression levels in *P. pachyrhizi* infected crops (Schneider *et al.*, 2011).

Isoflavones as one of the flavonoid groups were also investigated both in inoculated and uninoculated treatments. The concentration of daidzein in Malabar and Argomulyo only slightly increased, whereas the remarkable increase of this isoflavone aglycone was found in other six genotypes in the inoculated treatment (Fig 6a). In line with the highest increase of phenolic and flavonoid contents in Dena 1 seeds, this genotype also had the highest daidzein (180.2 ± 10.4 μg/g) similar to that in Ringgit (178.0 ± 1.9 μg/g) in infected crops. Dena 1 also had the highest increase of this isoflavone (67.5 ± 3.6 %) compared to other genotypes (Fig 6d).

The increase of genistein was genotypic dependence. Argomulyo was the only genotype which decreased in genistein content, whereas Dena 2 remained unchanged after *P. pachyrhizi* infection (Fig 6b). The increase of genistein in seeds of Wilis was remarkable, reaching up to 112.9 ± 12.7 % (Fig 6d), from 77,2 ± 2.7 μg/g to 164.2 ± 4.0 μg/g after the pathogen infection. Dena 1 also increased in genistein content (42.3 ± 4.2 %), however the increase was less than a half of that of Wilis. Daidzein and genistein were calculated together to investigate the changes of main isoflavone aglycones in crops infected by soybean rust disease. In general, the isoflavones increased in seeds of all infected genotypes, except Argomulyo which was slightly decreased (-3.7 ± 1.6 %) (Fig 6d). Dena 1 had consistently high total isoflavones (321.9 ± 12.8 μg/g), which was not different to those in Ringgit (327.7 ± 3.8 μg/g) (Fig 6c).

Research on isoflavone contents in soybean genotypes has been extensively conducted from preharvest to postharvest. Factors affecting the changes of content and composition of isoflavones have been well understood during preharvest. Isoflavone variations were noted among genotypes and soybean parts including the seed, cotyledon, embryo and seed coat (Kim *et al.*, 2007; Lee and Cho, 2012). However, reports of these compounds in seeds of infected crops were still limited. Lee *et al*. (2015) investigated the changes of isoflavones in seed diseased by *Phomopsis longicolla* and *Cercospora kikuchii*, two fungal pathogens caused deterioration of the seeds. In their study, even though total isoflavones (malonylglucoside, glucoside, aglycone, acetylglucoside forms) were remarkably decreased 5 folds and 2 folds in seeds infected by *P. longicolla* and *C.kikuchii* than those in healthy seeds, aglycone forms of daidzein, genistein, and glycitein slightly increased. This report was in agreement with our study.

DPPH scavenging activity

Many studies have investigated the antioxidant capacity in soybean seeds because of the functional properties. However, changes of the antioxidant activity in seeds harvested from the diseased crops by *P. pachyrhizi* have not been conducted in the previous studies. Therefore, this study investigated the antioxidant activity using DPPH in the seeds of the soybean crops infected by this disease. A study conducted by Lee *et al*. (2015) on diseased seeds by *P. longicolla* and *C. kikuchii* showed that inhibition percentages of potent free radical scavengers were higher in healthy seeds. This finding suggested the contribution of isoflavones for the major portion of antioxidant activity.

In our study, however, DPPH scavenging activity of eight soybean genotype seeds varied depending on the genotypes (Fig 7). Antioxidant activity in seeds of Malabar slightly decreased, whereas that of Ringgit and Argomulyo remained unchanged after infection of rust disease. Even though the increase of antioxidant activity of Dena 1 (15.7 %) was not as high as Grobogan (19.2 %), Dena 1 had the highest antioxidant activity in inoculated crops. Isoflavones may be responsible for the major portion of antioxidant activity as described in the previous study (Devi *et al.*, 2009).

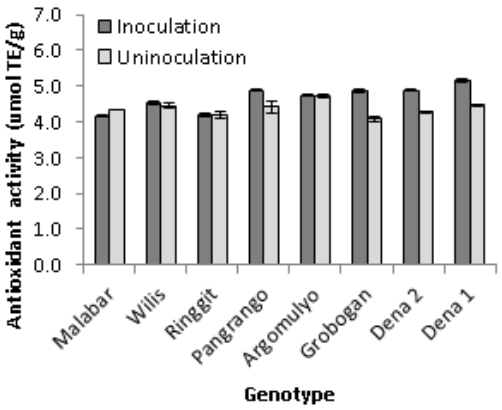


Figure 7. DPPH scavenging activity in seeds of eight soybean genotypes on infected and uninfected crops

This study has investigated for the first time the changes of total phenolic, flavonoid contents, two main isoflavones of daidzein and genistein as well as antioxidant activity in seeds of healthy and diseased crops by *P. pachyrhizi*. The soybean rust pathogen could be one of the biotic elicitors to increase total flavonoid contents in soybean seeds. Dena 1 showed a tolerant cultivar since this genotype had the least pustule number, less reduction in seed weight and weight of 100 seeds. In addition, the considerable increase of phenolics and the two main isoflavones were observed in this cultivar, therefore, could be considered as tolerant cultivar against soybean rust disease.

CONCLUSIONS

All eight soybean cultivars were reacting moderately susceptible to susceptible to soybean rust disease at the observation of 56 days after planting. This disease reduced plant weight, number of intact pods, seed weight and 100-seed weight. The increase of total flavonoid contents from 13 to 51% and daidzein from 1 to 66% were observed in all soybean genotypes. DPPH scavenging activity changes were genotypic dependence. Further studies are needed to observe other phytochemical as well as primary metabolite changes that may be affected by *P. pachyrhizi*.

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