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Selection of Soybean (*Glycine max)* Germplasm Against Biotrophic Fungi Disease Based on Anatomical Resistance

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Abstract. The obstacle to increasing the soybean production is an infection of rust disease caused by the biotrophic fungus, *Phakopsora pachyrhizi*. The research objectives were to determine the anatomical resistance and the level of resistance of soybean cultivars against rust disease. The embedding method for observed leaf structural anatomy. The disease severity based on the method of International Working Group on the Soybean Rust (IWGSR) rating system. The experiment was arranged as a Completely Randomized Design (CRD) with Factorial Pattern and five times repetition. The first factor was soybean cultivars, namely Gepak Kuning, Slamet, Tanggamus, and Wilis. The second factor was *P. pachyrhizi* inoculation with 0 uredospores/mL (uninoculated) and 10^4 uredospores/mL (inoculated). The results showed that the soybean cultivars that have thicker cuticle and epidermis, high trichomes and low stomatal density, and low of stomatal conductance have better anatomical resistance to leaf rust disease. Wilis and Slamet cultivars are resistant cultivars, indicated by disease intensity of 20% and 24.6%, respectively. While the Tanggamus is moderately resistant cultivar and Gepak Kuning is a susceptible cultivar, indicated by disease intensity of 56.5% and 85.3%, respectively. The novelty of selection soybean germplasm against biotrophic fungal disease are important and effectiveness in order to increase the crop productivity. These three soybean cultivars potentially serve as genetic sources to develop high yielding soybean cultivars and resistant to rust disease.

Key words: Anatomy; Biotrophic Fungi; Resistance; Selection; Soybean

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INTRODUCTION

Biotrophic fungi (*Phakopsora pachyrhizi*) is one of the most devastating foliar diseases of soybean, particularly in tropical regions of Asia where yield losses from 10 to 80% have reported (Miles et al., 2011). Domestic soybean production in Indonesia is only able to fulfill a small portion (35%) of the needs of soybean, and the remaining are obtained through imports (Direktorat Akabi, 2013).

P. pachyrhizi, an obligate biotrophic fungal pathogen is a significant threat to crop soybean production. The use of resistant soybean cultivars is one way of controlling rust disease (Murithi et al., 2016). The recommendation to develop superior cultivars of soybean is still challenging to achieve optimal results because soybean planting areas in Indonesia have different environmental conditions. The use of soybean resistant cultivars can reduce yield losses due to this disease (Sulistyo & Sumartini, 2016). Deploying resistant soybean cultivars is the preferred management method because it is more economical and impacts the environment less than other methods (Pham et al., 2010).

Anatomical characters can be used as symptoms and signs of the structural resistance of plants against pathogen attack. Therefore, it is necessary to develop superior cultivars that are site-specific. One way to get disease-resistant cultivars is by knowing the relationship between anatomical structure and disease intensity (Samiyarsih et al., 2018). Genetic factors determine plant resistance to pathogens, and some of their expressions are influenced by the environment and host interactions with the pathogen. Structural resistance has a role in the penetration of pathogens, as the opening of stomata guard cells is thought to be more important than the size and width of stomatal guard cells (Lawson & Blatt, 2014). Suriani et al. (2018) reported that each accession of maize germplasm had a significant correlation to rust severity, and every increase in stomatal density increased the rust severity to 0.73%. Leaf inoculation of the fungus of *Sphaceloma batatas* caused the decrease of stomatal length and width on ten sweet potato cultivars. Histopathological studies are essential tools to understand plant-pathogen interaction and select the germplasm resistance cultivars (Samiyarsih et al., 2018).

The primary response of plants affected by fungal infections is the presence of structural defenses, such as cell wall thickness. Besides, pathogenic infections can cause the development of plant vascular tissue structures to be disrupted (Impullitti & Malvick, 2014). Leaf anatomical structures such as stomata are predicted to influence the level of plant damages caused by a pathogen. (Grimmer et al., 2012). Djauhari (2008) showed that the density of stomata had a significant positive correlation to the intensity of rust disease on soybean leaves. Suriani et al. (2018) reported that the density of leaf hairs (trichomes), wax thickness, and the presence of cuticle has significant negative correlations to the intensity of rust disease on soybean leaves.

Research of the soybean leaf anatomy against rust disease by *P. pachyrhizi* fungus and how the intensity of rust disease in soybean germplasm has not studied before. This research's objective was to select four soybean cultivars for resistance and be susceptible to soybean rust based on structural resistance anatomy. The benefit expected from the research is to advise the community to cultivate soybean cultivars that have structural resistance to rust disease caused by biotrophic fungi.

METHODS

Plant materials and experimental design

The experiment was conducted at a greenhouse of Biology Faculty of Universitas Jenderal Soedirman, Central Java, Indonesia, from April to August 2019. Each cultivar was planted in 5kg plastic pots. This study used an experimental method with a completely randomized design (CRD) with factorial pattern (two factors). The first factor was soybean cultivar consisted of four cultivars, i.e. Gepak Kuning (K1), Slamet (K2), Tanggamus (K3), and Wilis (K4). The second factor was the inoculation of *P. pachyrhizi* as many as 0 uredospores/ml (L0), and 10^4 uredospores/ml (L1). Soybeans observation were planted two weeks after planting or when the third leaf appears—each treatment has five replications. Two sets of experiments, i.e. soybean without inoculation and soybean inoculated with *P. pachyrhizi* were grown in a separate greenhouse.

Preparation of spore suspension and inoculation of rust disease

Soybean leaves that had been infected with leaf rust were taken as a source of isolates. Rust pustules until released and then accommodated in a beaker filled with sterile aquades. Pustules were identified with the help of a microscope and identification book to ensure that the pustule was *P. pachyrhizi* fungus. Uredospore density was measured using a hemocytometer until it reached 10^4 uredospores/mL. Spore suspension obtained was used for the inoculation of all cultivars tested. Inoculation was done on a three-week-old plant or plant with three leaves by spraying the spore suspension to the surface of the leaves. Inoculation was carried out by spraying using a sprayer containing 10^4 uredospores/mL dissolved in 100 ml of water in the morning. It was repeated three times at intervals of 4 days to maximize the inoculation. The incubation period of *P. pachyrhizi* was seven days (Maman et al., 2014).

Paradermal section on stomata and trichome characters

The replica method was used for observing the stomatal and trichomes density of the leaves. The steps were as follows: (1) Cleaning the abaxial and adaxial surfaces of the leaves, (2) Applying the nail polish and leaving it for 30 minutes to be dried (3) Dried spreads were attached with transparent tape and flattened, (4) The transparent tape was peeled and removed slowly from the leaves surfaces, then attached to the object-glass, (5) Flattening and labeling with a description of the plant type, and (5) Observing the types of stomata and trichomes as well as their size and density by using a light microscope with same magnification (400x). The paradermal section was done using the quantitative (size and density) of trichomes and stomata cells. Stomata and trichomes density were obtained from the following calculation: stomata/trichomes density = total stomata/trichomes per area of the field of view (mm^2).

$$density = \frac{total\ of\ stomata/trichomata}{mm^2}$$

Embedding methods for leaf anatomy in transverse section

The observed anatomical profiles including cuticle thickness, epidermis thickness, and palisade ratio. The 5^{th} leaf from the shoot bud was taken and cut into a 1 cm^2 piece. It was then subjected to fixation in FAA solution (FAA: 10% formalin, 5% acetic acid, 50% ethyl alcohol, and 35% distilled water) for 24 hours. Preparation of leaf anatomy was based on the embedding method and the staining was done using safranin (1%) in 70% alcohol. The transversal section using to obeservations of thickness of cuticle, epidermis, and palisade ratio (cell/mm). Palisade ratio was obtained by counting the total palisade cell per area of the field of view (mm^2). Observations were conducted by using a binocular microscope, Olympus CH-20, at 400x magnification. Measurement of anatomical profiles was done using a calibrated ocular micrometer (Sass, 1951; Samiyarsih et al., 2020).

Observation of soybean respond against rust disease

The soybean response and the resistant level against the rust disease were rated using the modified three digits of the International Working Group of Soybean Rust (IWGSR). There were 5 categories used i.e., immune (I), resistant (R), moderately resistant (MR), moderately susceptible (MS), and susceptible (S) (Shanmugasundaram et al., 2004). This method used a system of the three-digit score to categorize soybean resistance against rust disease. The first digit indicated the position of diseased leaves (1= bottom third of the canopy, 2= middle third of the canopy, 3= upper third of the canopy). The second digit indicated the density of rust lesions on the most diseased leaves (1= no lesions, 2= 1-8 lesions/cm^2, 3= 9-16 lesions/cm^2, 4= more than 16 lesions/cm^2). The third digit indicated the type of infection (1= no pustule, 2= no spores in pustules, 3= pustules with spores). The categories of soybean rust disease resistance based on a three-digit scoring system are shown in Table 1.

Table 1. Relationship between disease reactions and IWGSR ratings for soybean rust

Disease reaction	IWGSR rating
Immune	111
Resistance	122, 123, 132, 133, 222, 223
Moderately resistant	142, 143, 232, 233, 242, 243, 322, 323
Moderately susceptible	332, 333
Susceptible	343

Data analysis

The data of leaf structural anatomy obtained were statistically analyzed using analysis of variance. The mean values were separated using Duncan's multiple range test at a 5% level of probability and a simple percentage error for the charts by following the standard deviation.

RESULTS AND DISCUSSION

The anatomical resistance of soybean cultivars

Quantitative leaf anatomical characters of four soybean cultivars showed a high correlation between anatomical resistance and rust disease severity. Differences in outbreak incidence rates of four soybean cultivars were due to some factors such as genetic properties, enzymatic (chemical) content, and form of leaf anatomy. Based on this research, the cuticle thickness of all cultivars increases when inoculated with rust disease. There was an increase in cuticle thickness in the Wilis cultivar by 56.6%. Based on the results, Wilis cultivar had the thickest cuticles compared to other cultivars i.e. 4.7 μm in inoculated conditions and 3.0 μm in non-inoculated condition (Figure 1 and 2). Plants have structural resistance in the form of morpho-anatomical structures such as cuticles, thick epidermis, and waxy layers that make it difficult for pathogens to enter the cells (Poerwoko et al., 2018). According to Pantilu et al. (2012), each plant cultivar has a different cuticle thickness. Plants with thick cuticle layers have relatively better structural resistance than plants that have thin cuticles. Thicker leaf cuticles can inhibit penetration or infection of a pathogen into the tissue. Samiyarsih et al. (2018) reported that the cuticle thickness is the nature of anatomical resistance to pathogen attack.

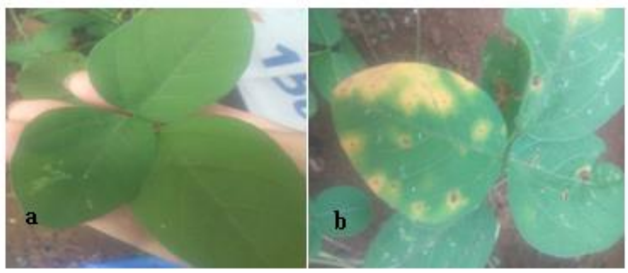

Figure 1. The comparison of biotrophic fungal infection in soybean leaf *P. pachyrhizi*. (a) Uninoculated plants (0 uredospores/mL) and (b) inoculated plant (10^4 uredospores/mL)

Four soybean cultivars have increased epidermal thickness after the inoculation of rust disease. The increases in epidermal thickness of Wilis, Tanggamus, Slamet, and Gepak Kuning cultivars were 109.6%, 55.2%, 51.35%, and 15.90%, respectively (Figure 2.). The difference in epidermal thickness is influenced by the adaptive response of an unfavorable environment due to the *P. pachyrhizi* inoculation. In the generative phase, leaf epidermis of inoculated plants is thickened. According to Rai et al. (2000), leaf epidermis will experience thickening as a result of an adaptation response from an unfavorable environment due to pest and disease attacks. Giordani et al. (2013) added that each plant cultivar has different epidermal thickness variations as environmental adaptation.

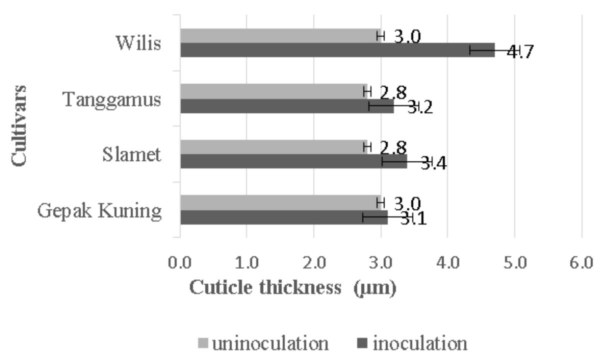

Figure 2. Average of cuticle thickness of four soybean cultivars (uninoculated and inoculated with *P. pachyrhizi*).

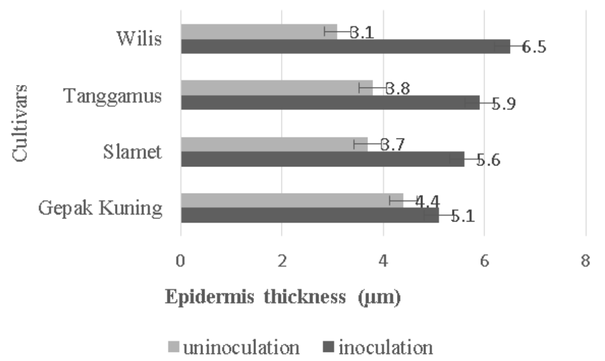

Figure 3. Average of epidermis thickness of four soybean cultivars (uninoculated and inoculated with *P. pachyrhizi*).

The palisade ratio was not related to the severity of the disease in the four soybean cultivars studied. Gepak Kuning cultivar had the most significant palisade ratio both before and after inoculation i.e. 54.4 µm, and 74.4 µm, respectively, although the cultivar was susceptible (Figure 4). Biruliova et al. (2013) stated that the palisade ratio will undergo histological changes such as hypertrophy and hyperplasia if attacked by a pathogen. Hyperplasia is a change in the shape of the palisade layer from oval to isodiametric, whereas hypertrophy is cells changing shape to become more significant attaced by pathogen.

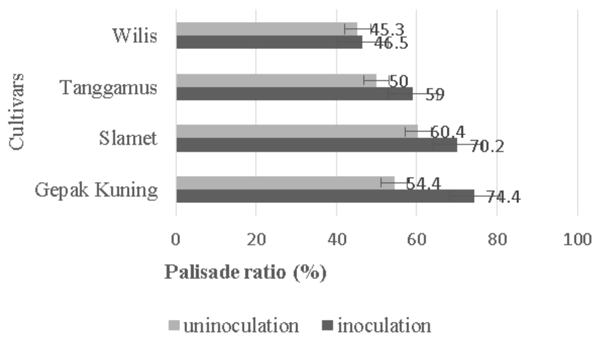

Figure 4. Average of palisade ratio of four soybean cultivars (uninoculated and inoculated with *P. pachyrhizi*).

Variance analysis results showed that soybean rust inoculation and type of cultivar affected the density of trichome on the leaf surface. Resistance cultivars, namely Wilis and Slamet, had the highest trichomata density of 6.9 and 6.7 cells / mm^2 respectively (Figure 5). After the rust inoculation, the four cultivars had increased the trichomes density. Soybean cultivars which have a high trichomes density are known to have high resistance to pathogen attack. The resistance is because trichomes can prevent pathogenic spores from attaching to the leaf epidermis surface and inhibit the infection process. Baswarsiati (2004) stated that the intensity of pathogenic attack on several varieties of superior plants is getting lower with the number of trichomes. The resistance of chili genotypes to Begomovirus that causing yellow curling leaves is associated with anatomical characters of trichomes density in leaf (Faizah et al., 2012). Trichomes density will increase if plant is infected by disease as an unfortunate adaptation response to the environment, this condition is related to trichomes, which functions as structural resistance to disease intensity (Samiyarsih et al., 2018).

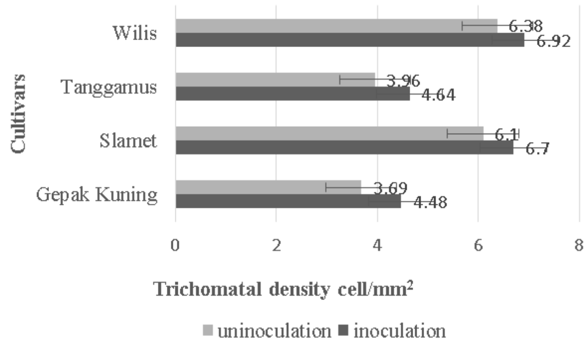

Figure 5. Average of trichomes density of four soybean cultivars (uninoculated and inoculated with *P. pachyrhizi*).

The stomatal density can be an indicator of structural resistance to the pathogen. The analysis results showed that the soybean cultivars which were inoculated with *P. pachyrhizi* in general had a higher average stomatal density than uninoculated plants. Inoculation of the rust fungus causes an increase in the stomatal density of four soybean cultivars. Gepak Kuning cultivar with the highest stomatal density has status as susceptible cultivars, with a stomatal density of 14.52 cells/mm^2. Meanwhile, Wilis cultivar is included in resistant soybean cultivars with the lowest stomatal density of 11.04 cells/mm^2 (Figure 6).

The stomatal density of soybean leaves can be an indicator of plant resistance to rust disease. The accessions of soybean germplasm with low stomatal density can be considered as materials in soybean breeding to construct new superior cultivars that are resistant to rust disease. Pradana et al. (2017) specified that the intensity of scab disease on sweet potato leaves cultivars at generative phase was extended to 85.10% due to the length of stomata on the upper leaves on susceptible cultivars, and 62.97% of the desease intensity influenced by stomata density.

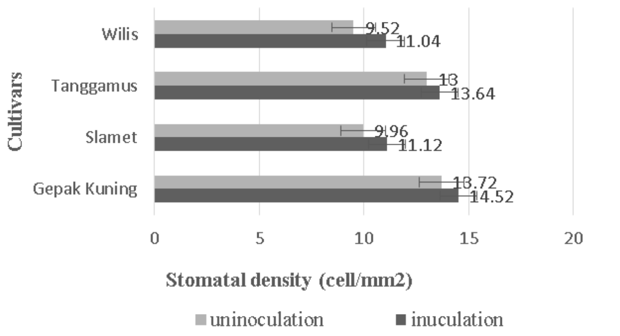

Figure 6. Average of the stomatal density of four soybean cultivars (uninoculated and inoculated with *P. pachyrhizi*).

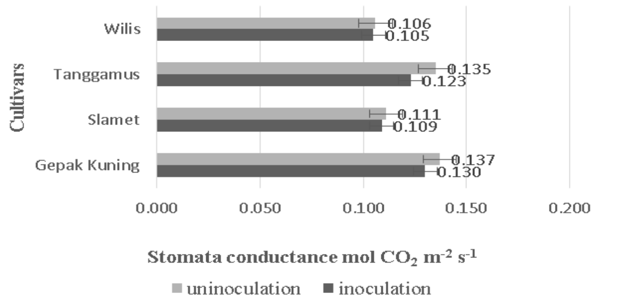

Figure 7. Average of stomata conductance of four soybean cultivars (uninoculated and inoculated with *P. pachyrhizi*).

Inoculation of the rust fungus causes a decrease in the stomatal conductance in four soybean cultivars. Variance analysis results showed that soybean rust inoculation and type of cultivar affected the conductance of stomata. Eac resistance cultivars (Wilis and Slamet) had the lowest stomatal conductance of 0.105 mol CO_2 $m^{-2}s^{-1}$ and 0.109 mol CO_2 $m^{-2}s^{-1}$ respectively (Figure 7). The conductance of stomata is supported the penetration of pathogen. It is caused by stomatal conductance roles as a path of penetration of obligate pathogens, such as rust pathogens. The higher the stomatal conductance, the higher the probability of penetration by uredospore, spore, and more infections.

Healthy soybean plants have a thick cuticle, epidermis and mesophyll layer and low stomatal density (Juwarno & Samiyarsih, 2017). Leaves natomical structure such as the adaxial epidermis, mesophyll, palisade and abaxial epidermis of soybean leaves inoculated with rust disease is damaged due to infectious diseases (Figure 8). The low stomatal density can reduce penetration and infections of pathogen into the leaf tissue. Structural resistance plays an essential role in the penetration of pathogens into host cells (Samiyarsih et al., 2018). However, the stomatal density was subjected to change due to adaptation to the local environment.

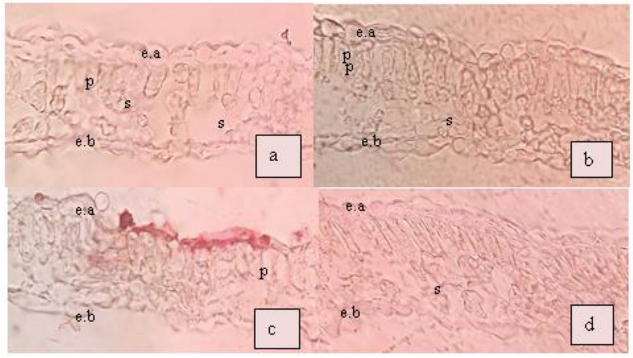

Figure 8. Anatomical structure of soybean leaves inoculated with biotrophic fungi at 400X magnification. Note: (a) Gepak Kuning cultivar, (b) Slamet cultivar, (c) Tanggamus cultivar, (d) Wilis cultivar; adaxial epidermis (e.a), palisade (p), mesophyll (s), abaxial epidermis (e.b).

Leaf surfaces and anatomical structures provide an essential substrate for the growth of a fungal pathogen widely. Thus, the physical and chemical characteristics of the leaf surface play an essential role in determining the success or failure of fungal growth both on leaf surface and inside the leaf tissue. The four soybean cultivars showed different structural anatomy responses. Cuticle and epidermal thickness, stomatal and trichomes density, and decreased stomatal conductance are the form of adaptation to the biotic stress caused by *P. Pachyrhizi* pathogenic rust disease. Anatomical resistance is the first trait to be considered in selecting resistant soybean cultivar to get superior soybean cultivars.

Rust disease severity of four soybean cultivars

Rust disease symptoms in this study started to appear from 0 to 21 days after spore inoculation. The response of soybean cultivars after the inoculation of rust diseases showed that all of the cultivars were classified as resistant, moderately resistant, and susceptible. The different resistance reaction is caused by the genetic factor of each cultivar. Kelly et al. (2015) stated that the *P. pachyrhizi* infection in soybean plants as a host begins after 24 hours of inoculation of pathogens. Uredospores will germinate and form a germ tube that produces an appressorium in the size of uredospores. Interaction and compatible of primary haustoria, is formed in mesophyll cells.

In contrast, secondary haustoria were formed within 12 days, form a dome in the epidermis, which causes rust-like spots on the leaf surface. Hyphae in mesophyll cells are not so many but are associated with mesophyll cell necrosis (Kelly et al., 2015). Qi et al. (2018) stated that the form of innouculated specialized haustoria that are hyphal structures intimately associated with host-plant cell membranes. These haustoria have roles in acquiring nutrients and secreting effector proteins that manipulate host systems of plants.

Figure 9. Presentation of rust disease severity of four soybean cultivars.

Two soybean cultivars, i.e., Wilis and Slamet, were categorized as resistant, while one cultivar namely Tanggamus cultivar was categorized as moderately resistance, and Gepak Kuning cultivar was categorized as susceptible at 21 days after planting (Table 2; Figure 9.). The presentation of rust severity is an indicator of the health of soybean cultivars. The number of pustules was from 3 to 12 pustules per cm^2. The infection rate of the rust disease was not only determined by the total number of pustules but also the position of the pustules and sporulation incidence. As the plant grew older, the number of pustules also increased. There was only Gepak Kuning cultivar that was categorized susceptible with the number of pustules up to 12 pustules per cm^2.

Table 2. Disease reaction and resistance criteria of four soybean cultivars against rust disease at 21 days after planting.

Cultivars	Status	Position of diseased leaves	The density of lesions/cm^2	Density of rust lesions	Type of infection	Score	Rust severity (%)	Reaction criteria
Gepak Kuning	Uninoculated	3	8	4	3	343	15.3	S
	Inoculated	3	12	4	3	343	85.3	
Slamet	Uninoculated	1	4	2	3	123	8.6	R
	Inoculated	1	7	3	3	133	24.6	
Tanggamus	Uninoculated	3	6	3	2	332	12.5	MR
	Inoculated	3	9	3	3	333	56.5	
Wilis	Uninoculated	2	3	2	2	222	5.8	R
	Inoculated	2	6	2	3	223	20.0	

Note: R = resistant, MR = moderately resistant, S = susceptible, MS = moderately susceptible (scoring and resistant criteria based on a method by Shanmugasundaram et al. (2004)).

The classification of the resistance and susceptibility of cultivars against rust disease was influenced by the intensity of uredospore sporulation and assessment of periodical severity (Araujo & Vello, 2010), as conducted in the present study. Based on the observation of four soybean cultivars, it can be seen that Wilis and Slamet cultivars are categorized as resistant with intensity of disease by 20 and 24.6% respectively, while Tanggamus is included in moderately resistant cultivar with disease intensity of 56.5% and Gepak Kuning is considered susceptible due to the disease intensity obtained by 85.3%. Maman et al. (2014) reported that Slamet cultivars had the lowest disease intensity, and another's cultivars had the highest-susceptible disease intensity. The higher of the disease intensity gave lower the amount of production. Sumartini and Sulistyo (2016), reported that the Tanggamus cultivar is classified as moderately resistant cultivar to rust disease because showed symptoms at almost the same time and there is a decrease in the quality and quantity of soybeans.

Environmental conditions during the study is a vital factor. Environmental conditions were relatively stable, the ambient temperatures were around 17–19.1°C in the morning, 25.3–31.7°C in the noon, and 24.1–28.7°C in the afternoon. The humidity levels ranged from 66.3 to 85.3% in the morning, 65.6 to 73.8% in the noon, and 68.5 to 75.9% in the afternoon. The soil pH value before inoculation was 6.8-7.0 and at the end of the study it was 6.6-6.9. According to Nazar et al. (2008), conditions that meet the growing requirements for soybean plants are air temperatures ranging from 23-30°C, humidity of 60-70%, and soil pH of around 5.8-7. Sumartini (2010) stated that the environmental control methods of rust disease include planting a soybean resistant cultivar, application of botanical fungicide made of clove oil, and use of antagonistic bacteria as well as antagonistic fungi.

This research has investigated for the first time the anatomical resistance and rust disease severity of four soybean cultivars inoculated *P. pachyrhizi*. Soybean cultivars that have thicker cuticle and epidermal layers as well as high trichomes density have better structural resistance to leaf rust disease. The cultivars with low severity (resistant) with typical characteristics of leaf anatomy can be selected as genetic sources used by breeders to develop new superior cultivars of rust-resistant soybean. It is advisable to obtain higher yields recommended for culturing soybean cultivars with structural resistance with cuticles and thick epidermis, high trichomata densities, low stomata density and low of stomatal conductance, in this research are Wilis, Slamet and Tanggamus cultivars. The benefit of the research is to advise the community to cultivate soybeans that have structural resistance to rust desease, that have thicker cuticle and epidermis, high trichomes and low stomatal density, and low of stomatal conductance.

CONCLUSION

Among four soybean cultivars, two cultivars were reacting resistant, while one cultivar was moderately resistant, and one was susceptible to biotrophic fungi that causes the rust. This disease increased the cuticle and epidermis thickness, palisade ratio, stomatal and trichomes density, and decreased stomatal conductance. Anatomical resistant in the form of thicker cuticle and epidermal layers, high trichomes and low stomatal density, and low stomatal conductance resulted in better structural resistance to leaf rust disease. Wilis and Slamet cultivars are resistant cultivars with disease intensity of 20% and 24.6%, respectively. While the Tanggamus and Gepak Kuning cultivars are categorized as moderately resistant and susceptible cultivar with disease intensity of 56.5% and 85.3%, respectively.

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