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Anatomical Study of Pigmented Rice Leaves (*Oryza sativa* L.) Resistant to Blast (*Pyricularia grisea* Sacc.) Disease

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ABSTRACT

Pigmented Rice (*Oryza sativa* L.) is one of Indonesia's local crops with potential to be developed as a source of healthy food. A major constraint in pigmented rice cultivation is the emergence of the fungus *Pyricularia grisea* (Sacc.), which causes blast disease. This pathogen attacks the leaves, leading to growth damage and even plant death. All plants inherently possess defense mechanisms against external threats, whether biotic (insects, fungi, bacteria, viruses) or abiotic (environmental stress). These mechanisms involve anatomical response. Previous research identified a pigmented rice variety, Cempo Ireng, that exhibits resistance to blast disease caused by *P. grisea.* Pigmented rice is also known to contain high levels of phenolic compounds and flavonoids, which contribute to plant defense and coloration. This study aims to determine the total phenolic and flavonoid content in the leaves of blast-resistant and susceptible pigmented rice varieties infected by *P. grisea* (Sacc.). Two pigmented rice varieties were tested: Cempo Ireng (resistant) and Indmira Hitam (moderately resistant). Control Varieties: Asahan (blast-resistant) and Kencana Bali (susceptible). Based on the results of this study, Cempo Ireng and Asahan showed strong resistance to blast disease, Indmira Hitam exhibited moderate resistance and Kencana Bali was highly susceptible to *P. grisea* infection. Anatomical observations of leaves using leaf surface section showed differences in structural modification of epidermal thickness, mesophyll thickness and the diameter of vascular bundle, where Cempo Ireng varieties were able to form good epidermal thickness as the initial barrier of defense. The anatomical observations of leaves using SEM showed anatomical differences in trichome density in Cempo Ireng and Asahan leaves which had the highest density compared to other varieties in response to the attack of fungal pathogens.

Key words: Leave anatomy; Pigmented Rice; *Pyricularia grisea*; Resistant; Scanning Electron Microscopy

INTRODUCTION

In this modern era, public awareness about the importance of a healthy lifestyle through the consumption of functional foods has significantly increased. Consequently, a wide variety of functional food products-ranging from fermented milk and green tea to wheat-based biscuits have become readily available in the market. Functional foods are defined as foods containing active components beyond basic nutritional value that provide health benefits (Suter, 2013). This definition underscores the critical role of functional foods in promoting health due to their bioactive constituents. In Indonesia, one locally sourced ingredient used for functional food products is pigmented rice (colored rice) (Shinta, 2014). Pigmented rice is rich in polyphenols, minerals, and vitamins, and exhibits notable biological activities, including antioxidant properties. The bran of pigmented rice also contains abundant nutrients such as fiber, minerals, and vitamins (Patil and Khan, 2011), highlighting its potential for agricultural development.

Pigmented rice is a variety of *Oryza sativa* that contains anthocyanin pigments, which give its grains distinct colors ranging from purple to reddish hues. These rice varieties are commonly named based on color: Purplish-black rice is called "black rice" (*Oryza sativa* var. indica) and Reddish-brown rice is termed "red rice" (*Oryza sativa* var. japonica). (Shinta, 2014).

The color of rice is determined by its anthocyanin composition. In addition to anthocyanins, pigmented rice contains other bioactive compounds including tocopherols, tocotrienols, ferulic acid, y-oryzanol, and phenolic compounds. These phenolic compounds contribute to high antioxidant activity and free radical scavenging capacity. The antioxidant properties are often associated with other pharmacological activities such as anti-carcinogenic, anti-mutagenic, metal chelating, and antimicrobial effects (Juliano, 2003; Anil et al., 2019; Chakuton et al., 2012).

A major challenge in pigmented rice cultivation is biotic and abiotic stress. The primary biotic stress for pigmented rice is infection by *Pyricularia grisea* (Sacc.), which causes blast disease. Blast disease is significantly threat to rice crops, affecting both upland (dryland) and lowland (paddy field) cultivation systems (Sumartono et al., 1984). Damage caused by *P. grisea* can result in empty grains or complete plant death. This disease causes yield losses of 30-50% in South America and Southeast Asia, amounting to millions of dollars in economic damage (Scardaci et al., 1997). In Indonesia, blast disease affected 19,629 hectares out of 12,883,578 hectares of total rice cultivation area in 2009 (BPS, 2010).

Originally, blast disease primarily affected upland rice in dry fields, but recent reports indicate increasing occurrences in irrigated paddy fields. This shift may be due to the emergence of new *P. grisea* strains that have adapted to irrigated rice ecosystems. *P. grisea* exhibits high genetic diversity and adaptive cellular/morphological characteristics that enable infection of rice plants (Koizumi, 2009; Sudir et al., 2013). Strain 173 is particularly virulent, capable of infecting nearly all tested rice varieties except Asahan (Mogi et al., 1991).

Plants include have evolved a remarkable arsenal of defense mechanisms to protect themselves against a myriad of threats from pathogens, pests and environmental stresses. The ability of plants to resist and overcome these challenges is known as plant resistance in plants encompass a wide range of strategies that operate at different levels, from physical barriers to chemical defenses. Plants have evolved physical barriers to prevent the entry and colonization of pathogens and pests. Physical barriers in plants, such as the cell wall and cuticle, play a crucial role in defending against biotic stressors. They create a first line of defense, preventing the entry of potential attackers and reducing the susceptibility of plants to biotic stress (Naveen Kumar at al. 2023). This study focuses on the anatomical modification of pigmented rice leaves resistance to *P. grisea*. This research aimed to investigate the leaf anatomical differences between blast-resistant and susceptible pigmented rice varieties in response to *Pyricularia grisea* infection. Furthermore, to investigate the leaf anatomical differences, we formulated the following hypotheses: Leaf anatomical structures- including the epidermis, mesophyll, vascular bundle diameter, silica cells, and trichomes- exhibit statistically significant differences between resistant and susceptible pigmented rice varieties, particularly in: tissue thickness (e.g., enhanced epidermal layer) and structural dimensions (e.g., larger vascular bundles).

METHODS

Plants Preparation

The soil used in this study was sterilized and fertilized with a ratio of 5kg soil: 12.5 g urea: 0.75 g TSP: 0.6 g KCL. The soil was then transferred to seedling trays (5 kg per tray) and watered under controlled conditions at 500 mL per day for each variety. Each variety was planted in 2 sets for: Disease resistance scoring and leaf anatomical analysis (anatomical slides and SEM). Each set consisted of 2 subsets: Control subset: Asahan (blast-resistant) and Kencana Bali (blast-susceptible) varieties, and Treatment subset: Indmira and Cempo Ireng varieties. Each subset contained 3 replicates, with 5 plants per replicate per variety, resulting in 60 seedlings per subset and a total of 180 rice seedlings across all sets (Fajar, 2018). At 21 days after planting (DAP), when the rice plants reached the 5-6 leaf stage (including immature leaves), 3 uniform, healthy plants per replicate were selected for inoculation (Ginanjar, 2016).

Blast Disease Inoculation

Inoculation with *Pryricularia grisea* (blast fungus) followed the methods of Utami et al. (2006) and Hayashi et al (2009): 1) Fungal Culturing: *P. grisea* race 173 was first grown on Potato Dextrose Agar (PDA) for 7 days in a dark room. The pure isolate was then transferred to Oatmeal Agar (OMA) for 12 days to promote spore production; 2) Spore Harvesting: On day 10, fungal colonies were gently brushed with a sterile brush and sterile water containing 0,01g/L streptomycin. The isolate was then stored in an Erlenmeyer flask under 20-watt UV light for 48 hours. On day 12, the isolate was brushed again with sterile distilled water + 0,02% Tween 20 to create a conidial suspension; 3) Inoculation: Spore density was adjusted to $2x10^5$ spores/mL using a hemocytometer. The suspension was sprayed onto 21-day-old rice leaves (5-6 leaf stage). For the negative control, rice plants were treated similarly by spraying them with sterile distilled water containing 0,02% Tween 20 (without fungal spores). After inoculation, plants were incubated for 48 hours in a humid chamber (25-16^{o} C, high humidity), then transferred to a greenhouse (26-28oC) with maintained humidity using misting (Hayashi et al., 2009).

Disease Resistance Scoring and Leaf Sample Collection

Plant resistance was evaluated 7 days post-inoculation using a standardized scoring system (**Table 1**, Hayashi et al., 2009) based on lesion severity.

Table 1. Disease Severity Scoring for Rice Blast

Score	Description	Status	Image
0	No Lesions	Resistant	
1	Tiny brown specks or scattered spots	Resistant	
2	Small lesions (1 mm diameter) with: Light brown centers and distinct dark brown margins	Resistant	

Score	Description	Status	Image
3	Small spotted lesions: Smaller than 1x vein spacing, dark margins < 1,5mm diameter	Intermediate resistant	
4	Medium spotted lesions: smaller than 2x vein spacing, < 2mm diameter	Susceptible	
5	Large spotted lesions: Exceeding vein spacing, > 2mm diameter	Susceptible	

Following disease symptom observation, leaf samples from both treated and control rice plants were collected 7 days post-inoculation. From each group, 3 plants were sampled for Anatomical slide preparation and SEM analysis. Samples were immediately placed in an icebox and transferred to -80ºC storage to preserve biochemical integrity (Roessner and Bacic, 2009).

Leaf Anatomical Analysis via Paraffin Embedding

Anatomical slides were prepared using the paraffin embedding method (Johansen, 1940): 1) Fixation: Leaf sections (1 cm x 1 cm) were immersed in FAA solution for 24 hours; 2) Dehydration: Gradual alcohol-xylol substitution (3:1, 1:1, and 1:3 ratios); 4) Embedding: Infiltration with pure paraffin (24 hours), followed by block formation and 10µm slicing using a rotary microtome; 5) Staining: Sections were stained with safranin.

The parameters measured in the sectional cross anatomical analysis of rice leaves included: upper and lower epidermis thickness, mesophyll thickness and Vascular bundle diameter. These measurements were conducted on all rice varieties in the study at day 0, 3 and 7 post treatment. For each variety, epidermal thickness measurements were taken at three locations: near the midrib, left leaf lamina and right leaf lamina. The average of these three measurements was calculated for both upper and lower epidermis thickness. The same triplicate measurements protocol was applied for mesophyll thickness and vascular bundle diameter determination.

Leaf Anatomical Analysis using Scanning Electron microscopy (SEM)

Leaf samples showing significant morphological differences between blast-resistant and susceptible rice varieties were selected for SEM analysis to compare their anatomical characteristics. The SEM analysis followed a modified standard protocol adapted from Carvalho et al. (2011). Sample preparation: 1) Leaf fragments were collected 7 days post infection; 2) Fixed in 70% alcohol for 1 hour at room temperature; 3) Air-dried after fixation; 4) Coated with 20nm gold layer using a sputter coater; 5) examined under scanning electron microscope.

RESULTS

Scoring of Rice Plant Resistance to Blast Disease

Plant resistance status (resistant, moderately resistant, or susceptible) was determined using the scoring standard from Hayashi et al. (2009). The study evaluated four rice cultivars: Asahan (resistant control), Kencana Bali (Susceptible control), Cempo Ireng and Indmira Hitam (**Table 2**).

Table 2. Scoring results

Cultivar	Resistance Status	Image
Cempo Ireng (*Wildtype*)	Resistant (Score 1)	
Indmira	Moderately resistant	
Asahan (Positive resistant control)	Resistant	
Kencana Bali (Positive susceptible control)	Susceptible	

Rice Leaf Anatomy

Plant distinct defense mechanism when attacked by pathogens. These protective strategies rely on two primary systems:

- Structural Barriers
  Physical and anatomical features that prevent or limit pathogen invasion, including: cuticle thickness, cell wall lignification, trichome density and vascular bundle organization.
- Chemical Defense
- Biochemical compound that actively combat pathogens by: trapping invading organisms, direct antimicrobial activity, disrupting pathogen metabolism, halting infection progression (Agrios, 2005)

The plant’s structural anatomy plays a critical role in mechanical defense and the delivery of chemical compounds through specialized pore or glands.

Rice Leaf Anatomy (Cross-sectional Analysis)

The rice leaf epidermis consists of a single layer of oval-shaped cells. The outer epidermal wall is covered by a thick cuticle layer that serves to reduce water loss from the plant. On the adaxial (upper) surface, bulliform cells-differentiated epidermal cells are present. These cells function to roll the leaves, thereby reducing transpiration under drought stress conditions (Mulyani, 2006). In this study, further analysis was conducted on the thickness of the upper and lower epidermis (**Table 3**), mesophyll thickness, and vascular bundle diameter in transverse leaf section at day 0, 3 and 7 post inoculation, including uninoculated controls (**Fig. 1**).

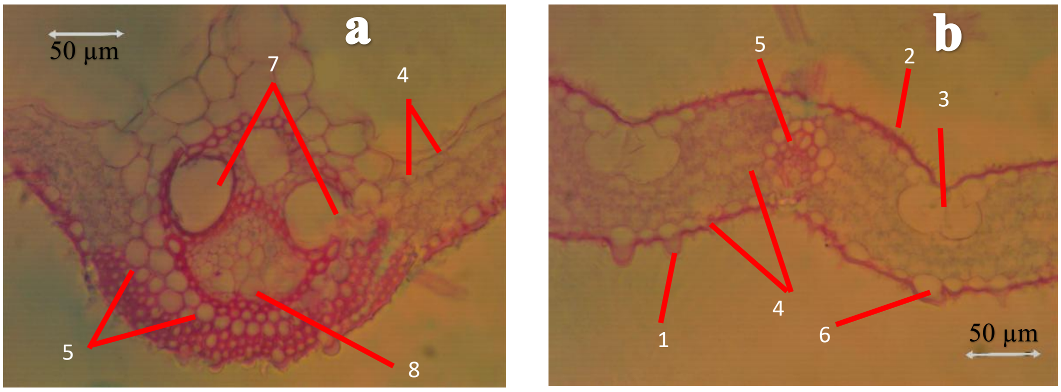

Figure 1. a. Transverse section of the Midrib of Indmira Rice Leaf on Day 3 after inoculation; b. Transverse section of the blade of Indmira rice leaf on day 3 after inoculation. Figure description: 1. Lower epidermis; 2. Upper epidermis; 3. Bulliform cells; 4. Mesophyll tissue; 5. Vascular bundle sheath; 6. Trichome; 7. Xylem; 8. Phloem.

Table 3. Analysis of the Thickness of the Upper and Lower Epidermis

Cultivar	Treatment	Thickness of Upper Epidermis	Thickness of Lower Epidermis
Cempo Ireng (*Wildtype*)	Kontrol (before-inoculation)	12,36±0,055	12,72±0,047
	0	14,006±0,188	10,85±0,075
	3	15,65±0,185	11,94±0,117
	7	16,393±0,030	12,52±0,096
Total		14,595±1.722[c]	12,012±0,966[b]
Indmira	Kontrol (before-inoculation)	9,93±0,151	11,08±0,047
	0	12,346±0,082	10,143±0,075
	3	10,597±0,062	11,117±0,023
	7	13,633±0,106	13,37±0,111
Total		11.627±1.674[a]	11,427±1,597[ab]
Asahan (Positive Resistant Control)	Kontrol (before-inoculation)	13,067±0,858	10,573±0,90001
	0	12,763±0,993	12,07±2,726
	3	12,723±1,693	11,41±1,125
	7	14.85±0,793	12,887±1,066
Total		13.351±1.336[b]	11,735±1,651[ab]
Kencana Bali (Positive Susceptible Control)	Kontrol (before-inoculation)	10,003±0,0242	9,14±0,063
	0	8,617±0,854	9,55±0,06
	3	10,653±0,079	10,072±0,117
	7	13,54±0,098	13,076±0,096
Total		10.703±2.208[a]	10.623±1.889[a]

In general, the upper epidermal thickness of Cempo Ireng leaves was the highest, followed by Asahan. Meanwhile Kencana Bali and Indmira showed relatively similar and lower thickness compared to Cempo Ireng and Asahan varieties. This trend was not observed for the lower epidermis, where the thickness did not show any significant differences. The lower epidermis, the thickest layers were found in the Indmira and Kencana Bali varieties on day 7 post-infection, which showed relatively similar results. The thinnest lower epidermal thickness was again found in Kencana Bali.

The results of mesophyll thickness measurements across all tested varieties under control treatments and at day 0, 3 and 7 after inoculation (**Table 4**) showed that he Kencana Bali variety under control (before inoculation) had the thinnest mesophyll. In contrast, the thickest mesophyll was found in the Indmira variety on day 7 post-infection.

Table 4. Analysis of the Thickness of Mesophyll in rice leaves

Cultivar	Treatment	Thickness of Mesophyll
Cempo Ireng (*Wildtype*)	Kontrol (before-inoculation)	73,343±25,523
	0	74,676±21,079
	3	69,06±22,571
	7	113,07±108,705
Indmira	Kontrol (before-inoculation)	96,417±69,301
	0	96,13±70,247
	3	119,127±90,768
	7	136,77±107,438
Asahan (Positive Resistant Control)	Kontrol (before-inoculation)	85,35±45,363
	0	86,12±41,893
	3	92,483±39,543
	7	126,84±90,029
Kencana Bali (Positive Susceptible Control)	Kontrol (before-inoculation)	47,393±22,311
	0	57,653±19,054
	3	94±48,159
	7	91,353±74,104

The vascular bundles in rice leaves found on the midrib are larger in size compared to those found on the leaf blade (**Fig. 2**).

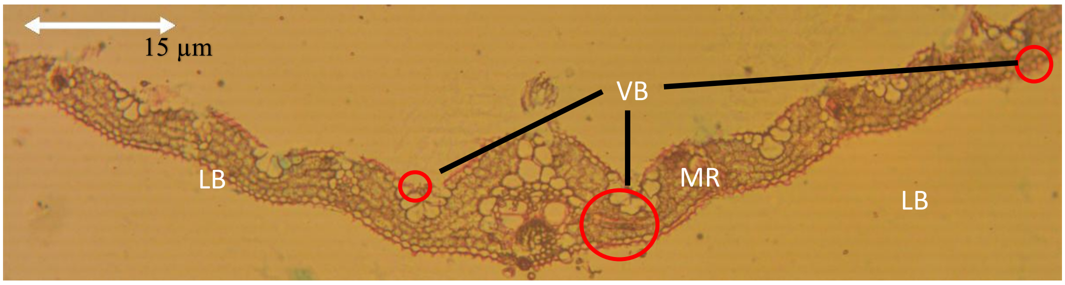

Figure 2. Cross-section of Kencana Bali rice leaf on day 0 after inoculation. Image description: MR: Midrib; LB: Leaf Blade; VB: Vascular Bundle

The analysis results of the vascular bundle diameter presented in **Table 5** show that the Cempo Ireng variety on day 7 after infection had the largest vascular bundle diameter, while the Kencana Bali variety on day 0 after infection had the smallest vascular bundle diameter.

Table 5. Analysis of the Vascular Bundle Diameter

Cultivar	Treatment	Thickness of Mesophyll
Cempo Ireng (*Wildtype*)	Kontrol (before-inoculation)	52,913±42,318

Cultivar	Treatment	Thickness of Mesophyll
	0	52,513±44,236
	3	50,537±38,585
	7	71,967±31,579
Indmira	Kontrol (before-inoculation)	54,003±41,367
	0	55,97±48,159
	3	58,873±51,267
	7	67,483±40,635
Asahan (Positive Resistant Control)	Kontrol (before-inoculation)	56,687±44,198
	0	53,887±42,570
	3	58,143±50,093
	7	58,423±28,206
Kencana Bali (Positive Susceptible Control)	Kontrol (before-inoculation)	40,87±25,980
	0	41,563±32,413
	3	60,843±52,560
	7	56,07±49,974

Rice Leaf Anatomy using Scanning Electron Microscope (SEM)

In this study, an analysis of the anatomical structure of rice leave was conducted on the 7th day after infection by the fungus *P. grisea* using SEM. The results of the observations showed differences in patterns or textures on the upper (adaxial) surface of the leaf organ (**Fig. 3**) among the four rice varieties used in this study. The Kencana Bali (**Fig. 3b**) showed a pattern or surface texture with numerous folds from the inside of the surface, which can be described as wrinkled, whereas the other three varieties- Asahan, Indmira and Cempo Ireng did not show the same pattern or folding. Although the image shows that the Indmira variety (**Fig. 3c**) also exhibited surface folds, the folding pattern it showed was different from that of the Kencana Bali variety.

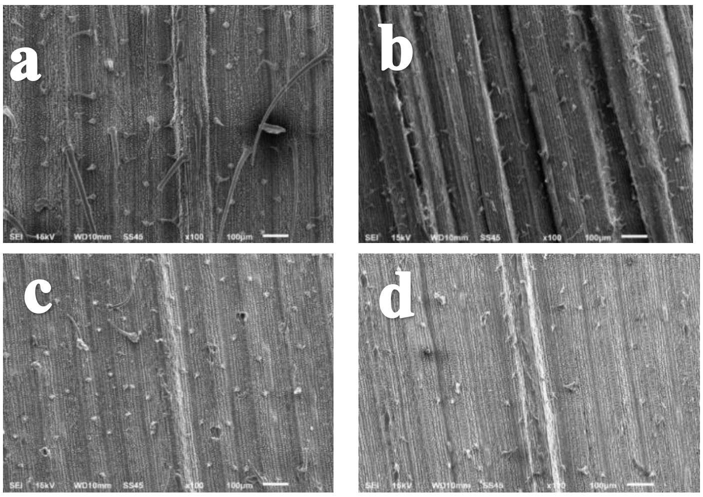

Figure 3. SEM of the Upper Surface of Rice Leaves on the 7th day after infection: a) Upper surface of rice leaves of the Asahan variety; b) Upper surface of rice leaves of the Kencana Bali variety; c) Upper surface of rice leaves of the Indmira variety; d) Upper surface of rice leaves of the Cempo Ireng variety (*Wildtype*)

This study also examined three key parameters of silica cell architecture: 1) cell diameter; 2) cell density per field of view (FOV); 3) Intercellular spacing. The comparative analysis revealed significant varietal differences (**Table 6**).

Table 6. Silica Cell Quantification

Sample	Silicon cell density (250x200 µm)	Diameter of Silicon cell (µm)	Intercellular spacing (µm)
Cempo Ireng (*Wildtype*)	913	1,93 ± 0,151[b]	2,73 ± 0,751[a]
Indmira	890	2,023 ± 0,115[b]	4,85 ± 0,750[b]
Asahan (Positive Resistant Control)	679	2,577 ± 0,168[c]	4,037 ± 0,934[ab]
Kencana Bali (Positive Susceptible Control)	899	1.443 ± 0,224[a]	4,813 ± 0,259[b]

DISCUSSION

The results of this study, presented in **Table 2**, identified the Kencana Bali variety as the most susceptible to blast disease. Observations of Kencana Bali leaves revealed lesions with spots exceeding 2mm in diameter (**Fig. 4b**). Another symptom observed in susceptible plants was yellowing and drying of the leaves. Changes in leaf coloration serve as an early physical indicator of plant health. The yellowing of leaves in susceptible rice plants is likely caused by fungal invasion of chloroplasts, leading to chloroplast damage and subsequent loss of green pigmentation. Meanwhile, the drying of leaves in susceptible plants results from fungal interference in the vascular bundles of the leaves, leading to an imbalance in water transpiration and transport.

a b

Figure 4. Leaf Morphology of: a. Cempo Ireng (Wildtype) and b. Kencana Bali, a blast susceptible rice variety, observed on the 7th day after inoculaton.

In the observation of Epidermis Thickness, both Cempo Ireng and Indmira varieties initiated structural defense mechanisms by increasing epidermal thickness immediately after inoculation (Day 0) to prevent fungal penetration into deeper leaf tissues. Only Cempo Ireng sustained this increased epidermal thickness in subsequent days, establishing an effective early barrier against *P. grisea*.

Despite an initial rapid response, Indira exhibited reduced epidermal thickness by Day 3 post-inoculation. This aligns with the life cycle of *P.grisea,* were hyphal growth peaks withi 3-5 days post inoculation (Heni & Imura, 1989) an sporulation intensifies between days 6-11 after symptom onset (Kato et al., 1970).

Rice leaves exhibit an isobilateral structure, where the mesophyll is not differentiated into palisade and spongy tissues (Maiti et al., 2012). The mesophyll contains aerenchyma and vascular bundles. According to Maiti et al. (2012), rice leaf mesophyll tissue is composed of chlorenchyma lobes. The outermost layer of chlorenchyma cells in rice contains chloroplasts. In the Kencana Bali variety, the significantly reduced mesophyll thickness indicates chloroplast loss, which explains the observed leaf yellowing (**Table 2**). This structural difference likely contributes to the varying severity of blast disease susceptibility between varieties. Anatomical modification of vascular bundle diameter represents an additional structural defense mechanism employed by the Cempo Ireng variety, following its adaptations in mesophyll and epidermal thickness.

The distinct patterns and textures of leaf surfaces observed in SEM anatomical analysis serve as key parameters differentiating rice plants defense systems against pathogen attacks. Kencana Bali variety exhibits highly grooved leaf surfaces. These grooves trap spores released by *P. grisea*, enabling spore adherence in surface folds, optimal fungal development and subsequent blast disease establishment.

In leaf anatomy, surface patterns are not the only factor contributing to mechanical defense systems. Silicon (Si) serves dual protective roles in plant defense systems, first as a mechanical barrier that forms a physical fortress against fungal penetration, impedes appressorial invasion by fungal and the second as Biochemical signaling activation acts as a defense response potentiator, a protein signaling activator, systemic acquired resistance and fungus induced resistance (Fauteux et al., 2005).

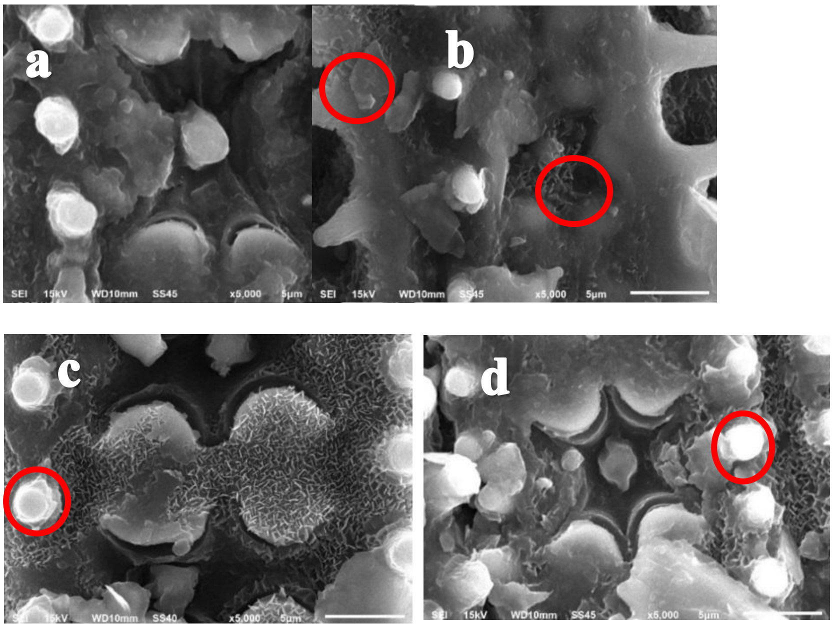

Figure 6. SEM Analysis of Silica cells in Rice leaves at 7 days post infection. a. Silica cell morphology in Asahan variety; b. Silica cell distribution in Kencana Bali variety; c. Silica cell architecture in Indmira variety; d. Silica cell formation in Cempo Ireng (Wildtype)

Agarie et al. (1996) established that higher silica cell frequency enhances rice leaf mechanical strength, our findings reveal this as only one component of pathogen defense. However, in this study it was found that high cell silica frequency was not the only factor responsible for mechanical strength in rice leaves, the distance between silica cells on the surface of rice leaves also had an impact on the formation of mechanical barriers that guard the plants from the penetration of appressorial. This is evidence by the results of the study shown in **Table 6**. The high number of silica cells followed by the distance between silica cells that are very tight in the Cempo Ireng variety makes this variety can form excellent mechanical barrier to fungal attacks. This is in accordance with previous studies conducted by Mariana (2004), where the results is that the higher the silica content, the lower the intensity of the leaf blast disease. This is also supported by the results of Hori's research (1980) in Kozaka (1995), each additional 1% the intensity of leaf blast disease drops by 23,19%.

CONCLUSION

Anatomical features of leaf organs such as epidermis, mesophyll tissue, vascular bundles, and silicon cells provide excellent anatomical responses by modifying their anatomical structures to the thickness of the epidermis, mesophyll tissue, enlargement of the diameter of the vascular bundles, silicon cells and the density of silica cells helping in the formation of an initial barrier as part of the plant's structural defense mechanism against the fungus *P. grisea* which causes blast disease.

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