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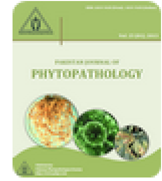

THE RESISTANCE RESPONSE OF SIX RICE VARIETIES TO *RICE RAGGED STUNT VIRUS* IN SOUTH SULAWESI, INDONESIA: A GREENHOUSE STUDY

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A B S T R A C T

Nilaparvata lugens, commonly known as the brown planthopper (BPH), transmits the *Rice ragged stunt virus* (*RRSV*) which severely affects rice plants. This study aimed to evaluate the resistance of six rice varieties to *RRSV* in South Sulawesi under greenhouse conditions. The stages of the study included the preparation of the inoculum source, seeding of rice seeds, rearing of BPH, transmission treatment, and observation of resistance response and symptom scoring based on IRRI (1996) in a greenhouse. Data analysis techniques using Duncan's 5% were also applied. The results showed that varieties Inpari 36, 37, TN1, Mekongga, Ciherang, and Tukad Unda exhibited differing resistance levels, with Tukad Unda showing the highest resistance. The disease incidence ranged from 63.33% to 100%, and disease intensity varied from 28.14% to 71.10%. These findings suggest that the selection of resistant varieties could significantly mitigate *RRSV* impact, highlighting the need for integrated pest management strategies in rice cultivation.

Keywords: Disease intensity, Plant resistance, Rice varieties, *Rice ragged stunt virus*, South Sulawesi

INTRODUCTION

Rice, as a staple food for more than half of the world's population, particularly in Asia, is under constant threat from various pests and diseases. Among these, the Brown planthopper (BPH), *Nilaparvata lugens*, is one of the most devastating due to its role as a vector for transmitting viral diseases such as *Rice ragged stunt virus* (*RRSV*) and *Rice grassy stunt virus* (*RGSV*). These viruses lead to significant agricultural losses through the stunting of growth and development of rice panicles, adversely affecting both the yield and quality of rice. The economic impact is profound, particularly in regions like South Sulawesi, Indonesia, where rice farming is a critical component of local food security and economic stability. This study focuses on the resistance of six rice varieties to *RRSV* in South Sulawesi, a region that exemplifies the challenges faced by rice-producing areas in tropical climates. The persistent threat posed by BPH and its transmitted viruses necessitates a detailed exploration of resistant rice varieties as a sustainable approach to agricultural practices in affected regions.

The Brown planthopper is capable of causing extensive damage to rice crops by directly feeding on plants and more significantly, through the transmission of viruses

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such as *RRSV* and *RGSV* (Hibino, 1996). The damage is compounded when BPH populations reach high densities, enabling the rapid spread of viruses from infected to healthy plants. Infected plants exhibit a range of symptoms including yellowing leaves, twisted growth, stunted development, and reduced tillering, each symptom varying in severity depending on the viral load transmitted by BPH (Baehaki *et al.,* 2016; Huang *et al.,* 2015b). Control strategies for BPH have traditionally included chemical insecticides, biological control agents, and the adjustment of cropping patterns to disrupt the life cycle of the pest. Additionally, creating and implementing BPH-resistant rice cultivars and the viruses it carries have been seen as a cornerstone of integrated pest management (IPM) strategies. However, the adaptability of BPH, including its ability to develop resistance to insecticides and overcome plant resistance mechanisms, poses ongoing challenges to effective control.

The first reported case of *RRSV* in Indonesia was in Pandeglang, West Java, in 1976 (Hibino *et al.,* 2017; Huang *et al.,* 2017). *RRSV* attacks were rare until an outbreak of BPH attacks on superior varieties in 2005, believed to have been caused by global climate change and the emergence of new BPH biotypes (Baehaki & Munawar, 2008; Jena & Kim, 2010). The yield loss caused by *RRSV* is considered to be significant as it can cause the rice plant's panicles to produce empty grains and decrease overall yield (Hibino *et al.,* 1977; Effendi & Munawar, 2013; Huang *et al.,* 2015a). Symptoms of *RRSV* in infected rice plants include dwarf growth, short flag leaves, and dark green leaves. The newly growing leaves may twist, have torn edges, branch off, and have swelling along the leaf bones. The discharge of panicles is also inhibited and can cause the grain to not form (Hibino *et al.,* 1977; Effendi & Munawar, 2013; Huang *et al.,* 2015a). *RGSV* is often found to infect plants separately or with other viruses (mixed infection). The cycle of spreading the dwarf disease occurs in rice plants, transmitted by the insect vector *N. Lugens* persistently propagative by multiplying in the vector's body before being transmitted to the host plant. The latent period is required for this (Chomchan *et al.,* 2002). Brown planthoppers acquire the virus during a meal acquisition period, lasting 5-10 minutes. Vectors generally acquire more viruses over a longer acquisition period. When the brown planthopper moves to other plants and feeds, the virus in its saliva is transmitted directly to rice plants (Hogenhout *et al.,* 2008). A 2006 study in Vietnam showed variations in symptoms due to mixed infections. Variations depend on the type of virus and environmental conditions (Hogenhout *et al.,* 2008). In the tropics, *RRSV* is commonly found in paddy fields with year-round cropping patterns (Cheng *et al.,* 1979; Li *et al.,* 2014). The virus is reported to be transmitted through vector insects, specifically BPH, in a trans-stadial persistence. This means the virus remains in the vector's body throughout its life cycle (Cabauatan *et al.,* 2009). Control efforts are mainly focused on suppressing the population of these insect vectors to reduce crop damage (Saenchai *et al.,* 2012). This highlights the importance of studying the transmission of the virus by BPH. According to Hibino *et al.* (1977), *RRSV* is persistently transmitted by BPH *Nilaparvata lugens* and by two other *Nilaparvata* species, *N. bakeri* and *N. muiri*

Many current control methods have been used to control *RRSV* in the field. One of these methods is by planting BPH-resistant varieties. However, leafhoppers' capacity to adapt and create new biotypes may restrict the usage of resistant cultivars, which can quickly render the released varieties ineffective (Wen *et al.,* 2009; Wang *et al.,* 2014). Varieties that have resistance to the Tungro virus can be tested and used as a source of new resistance genes that can be used to repair and create new resistant varieties (Yangon, 2011). The Central Rice Research Center (BB Padi) has produced several superior rice varieties resistant to BPH. The latest resistant varieties released are Inpari 13, Inpari 31, and Inpari 33. Inpari 13 was released in 2010 and resulted from an IRRI introduction with selection number OM1490, originating from a cross from OM606/IR18348-36-3-3 (BB Padi, 2009). The importance of studying the molecular identification of rice viruses and the interaction between insects and their hosts is crucial in understanding disease control strategies and developing resistant varieties for sustainable agriculture. The development of new genetic diversity and the utilization of genomic resources to find and integrate new genes and alleles is necessary for example, by identifying the NBS-LRR gene, which is associated with disease resistance (Wang *et al.,* 2017).

Given the significant impacts of *RRSV* on rice production, particularly in regions like South Sulawesi, this research aims to evaluate the resistance of six rice varieties to *RRSV* under controlled greenhouse conditions. By understanding the resistance profiles of these varieties, this study seeks to provide insights that could guide future breeding programs and inform integrated pest

management strategies aimed at mitigating the impacts of *RRSV* in tropical rice-growing regions.

MATERIALS AND METHODS

Research Site: The research was carried out at the Rice Tungro Disease Research Institute in Sidrap District (South Sulawesi), and the Faculty of Agriculture, Universitas Gadjah Mada. Samples were collected from Sidrap, Wajo, Luwu, and Maros between November 2018 and March 2019. A total of 120 plants per variety were examined under controlled conditions: 28°C daytime temperature, 80% relative humidity, and 12-hour photoperiods in the greenhouse.

Molecular detections for the source of viral inoculum: Samples from the suspected infected plants were taken and processed to extract the virus' DNA or RNA. Molecular detection using a PCR machine (Bio-Rad T100 TM Thermal Cycler, United States) was performed using a single set of specific primers based on the genomic sequence of *RRSV* CP gene from seqment 8 (S8) in GenBank DataBase. The forward primer (CPF 5'-ACC GTC GTT GAG CTA CCA TCC ATT-3'), corresponds to position 890 nt to 913 nt, and the reverse primer (CPR 5'GGC GGG CCA CTC AAA CCA T-3'), corresponds to position 1366 nt to 1384 nt (Kusuma *et al.,* 2018). A size amplified a conserved segment around 494 bp. (Wu *et al.,* 2014). The stages of the PCR, RT-PCR, and the reaction volume used for the detection of the slightly modified *RRSV* virus were as follows:

Table 1. Volume of the PCR reaction

Reaction Composition	Volume (µl)
RT-PCR	
RNase free H_2O 5x RT Buffer	3,5
RNase inhibitor	0,5
RT Buffer	2
Rever TraAce	0,5
Primer oligo (dt)	0,5
dNTP Mixture	1
RNA	2
PCR	
PCR mix	5
DH_2O	3
Forward Primer	0,5
Reverse Primer	0,5
cDNA	1

Table 2. Timing of the PCR Program

PCR Program		Temperature (º C)	Time	Cycle
RT-PCR	Inkubasi	42	20’	1
	Inkubasi	99	5’	1
	Inkubasi	4	∞	-
PCR	Predenaturation	95	2’	1
	Denaturation	95	30”	35
	Annealing	52	30”	35
	Ekstension	72	30”	35
	Final extension	72	7’	1
	Stop	4	∞	-

Note: The RT-PCR was carried out using the KOD Pluz Neo Kit Toyobo (*Toyobo Research Reagents*) and the PCR Mix utilizing the MyTaq HS Red Mix (*Bioline*). The primary concentration was 10 pmol, and the total volume of each reaction was 10 µl.

Electrophoresis: The PCR amplification product was then run on an electrophoresis device with an agarose gel composition of 0.30 grams dissolved with 30 ml of TBE 1x until homogeneous, then heated using a microwave for 5 minutes. 1 µl of GreenSave dye was added to the agarose gel solution. 4 µl of PCR product and 5 µl of 100 bp DNA ladder markers were loaded onto the gel. Electrophoresis was run at 50 Volts for 50 minutes. UV transilluminator was used to visualize the electrophoresis results. Digital camera images of the generated DNA bands were captured.

Test for transmission of six rice varieties to *RRSV*: This test aimed to determine the resistance of several rice varieties to disease inoculation with insect vectors.

Ciherang, Tukad Unda, Mekongga, TN1, Inpari 36, and Inpari 37 were the rice varieties that were utilised. Meanwhile, the vector insects used were brown planthoppers for the dwarf virus inoculation, obtained from rearing in the laboratory. A number of BPHs were placed into cages with sick rice plants (the source of the inoculum) for 24 hours so they might acquire the ability to feed. After that, the BPHs were transferred to several healthy test varieties for the inoculation process (inoculation feeding), then closed using a hood and using the test tube method for 24 hours. Reinoculation was carried out again after 24 hours for 3 replications per experiment.

Scoring symptoms of *RRSV* in a greenhouse: Symptom observation in a greenhouse was carried out after inoculating healthy plants with plant viruses, using vector insects (BPHs) and control plants as a comparison. Test plants were inoculated with vector insects, one plant per-1 with an acquisition and inoculation period of 24 hours. After passing the ten-day incubation period, each inoculum was used as a source of inoculum to TN1 variety rice with the same transmission stage by using the score from the standardized assessment system for rice (IRRI, 1996), the symptom presence visualisation was carried out when the plant reached 2 WAP (weeks after planting).

Table 2. Scores for the assessment of symptoms of *RRSV* on rice plants on a greenhouse scale based on scoring following *The Standard Evaluation System for Rice* (IRRI, 1996)

Criteria	Scoring
No symptoms	1
0-10% decrease in plant height, no symptoms of ragged or twisted on leaves, small or very few swelling veins at the base of leaves	3
0-10% decrease in plant height, 1-2 leaves with symptoms of ragged or twisted, slightly found vein swelling at the base of the leaf	5
11-30% decrease in plant height, 3-4 leaves with symptoms of ragged or twisted, many found swelling veins at the base of the leaves, several leaves and leaf midribs.	7
> 30% decrease in plant height, most leaves with symptoms of ragged or twisted, vein swelling at the base of leaves, leaf strands and midrib leaves are found.	9

The results of the scoring were then used to calculate the percentage of diseased plants from the total number of plants observed. The following formulas (Zadoks and Schein, 1979) were used to determine the disease intensity (DI) and incidence (I):

$$I = \frac{n}{N} \times 100\%$$

$$DI = \frac{n(1)+ n(3)+ n(5)+ n(7)+ n(9)}{tn} \times 100\%$$

Remarks: I= disease incidence, DI= disease index, n=number of plants affected by *RRSV*, N=number of plants observed, tn= total number of plants infected based on attack category

The illness incidence reflects the frequency of infected plants in a community, whereas the disease intensity reflects the severity of symptoms on infected plants. Additionally, the outcomes of the disease severity computation were used to categorise the way that plants responded to infection resistance (IRRI, 1996):

DI (%)	Reaction
0-30	resistant / tolerant
31-50	rather resistant / moderate
>51	susceptible

RESULTS AND DISCUSSION

Moleculer detections: Molecular analysis confirmed the presence of *RRSV* in four out of five locations, with varying disease incidences and intensities among the tested varieties. The study's findings are compared with existing research, indicating a consistent pattern of resistance in newer varieties such as Tukad Unda. The detection of *RRSV* using *RRSV*-CPF / CPR primers for Sidrap, Wajo, and Luwu isolates yielded a DNA band size of around 416-422 bp, while *RRSV* was not detected in Maros sample (Figure 1 and Table 3). The exact size of the amplicon will depend on the specific strain of *RRSV* and the location of the primers in the viral genome. The temperature of annealing (attachment of the primer in the target sequence that complements) has a significant impact on the success of the PCR analysis (McPherson and Moller, 2006). Determination of the virus in rice plant samples obtained from several districts proves a rice virus infection in rice plants which vector insects transmit. The results of the analysis in the laboratory indicate that it is necessary for preventive control measures to prevent the spread of rice virus disease in the field.

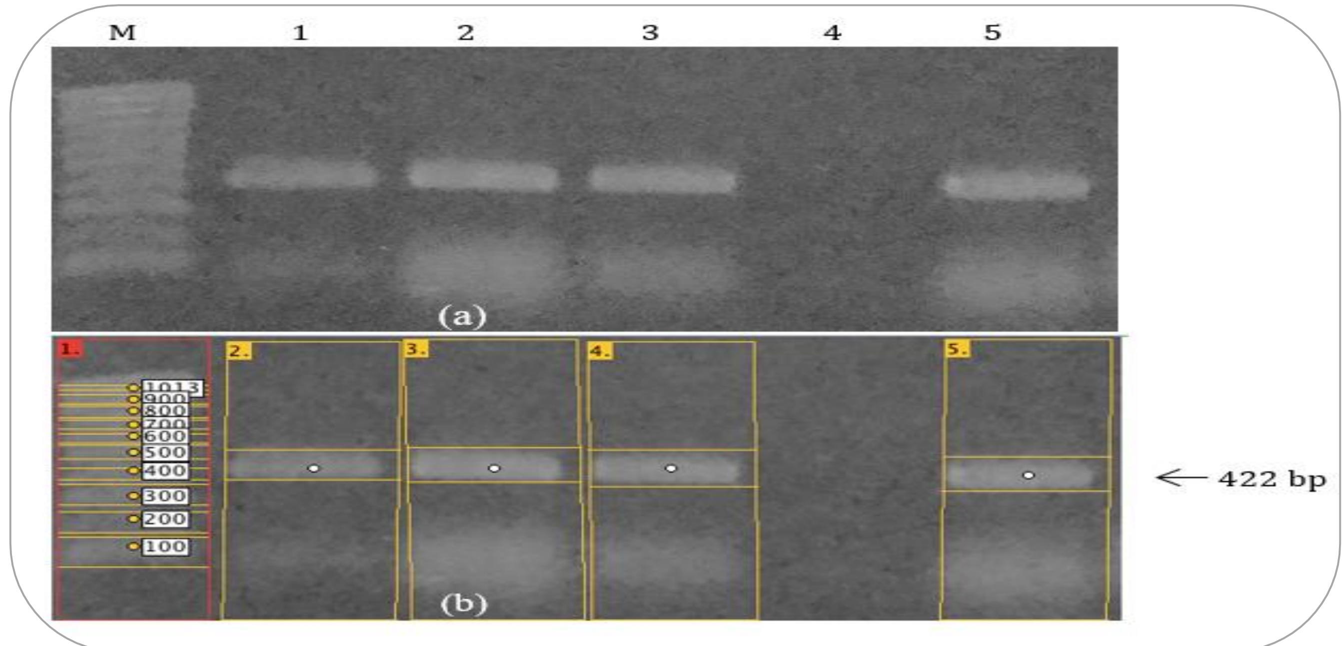
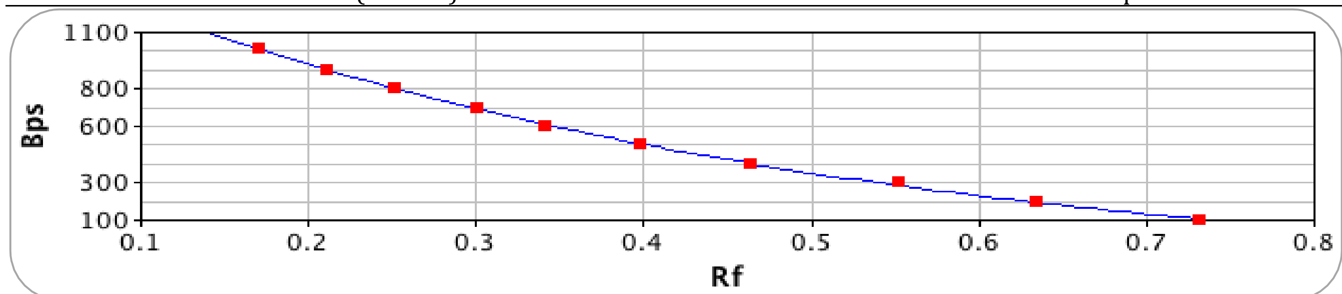


Figure 1. Results of *RRSV* detections by PCR using Gel Analizer V.19.1 Program. M. 100 bp DNA Ladder Marker. 1. Sidrap, 2. Wajo, 3. Luwu, 4. Maros, 5. Klaten (Positive control). (a). before analysis, (b). after analysis.

Molecular identification using RT-PCR method is often used to detect the presence of a virus in plants. Although the method is very sensitive, it can be difficult to distinguish between *RRSV* and other viruses, such as *Rice gall dwarf virus* (*RGDV*) and *Southern rice black streaked dwarf virus* (*SRBSDV*), because these viruses have a close genetic relationship and an RNA genome. Furthermore, samples are susceptible to cross-contamination between viruses transmitted by vector insects, leading to mixed infections and potentially false positive results. According to research by Yang *et al.* (2017), the RT-PCR multiplex method for simultaneously detecting viruses in rice plants in China found 92 samples infected with *RRSV*, 21 samples infected with both *SRBSDV* and *RRSV* and 99 samples that tested negative. The most common combination of mixed infections is *SRBSDV* and *RRSV*, possibly due to *RRSV*-carrying brown planthoppers preferring *SRBSDV*-infected plants (Wang *et al.,* 2014). It is possible that the symptoms caused by vector insect pests are similar to those caused by viruses or that a new type of virus is emerging in the field.

Table 3. RT-PCR Results and Molecular Weight from several locations

Region	Code	*RRSV* (RT-PCR)	Molecular Weight (Gel Analizer 19.1)
Sidrap	1	+	422 bp
Wajo	2	+	416 bp
Luwu	3	+	422 bp
Maros	4	-	-
Klaten (Kontrol)	5	+	380 bp
	M (Marker)		100 - 1013 bp



y= 1848.879311 *exp (-2.249997 * x) - 248.044913, R^2 = 1

Figure 2. DNA Molecular Weight Calibration Curves using Gel Analyzer V.19.1 Program

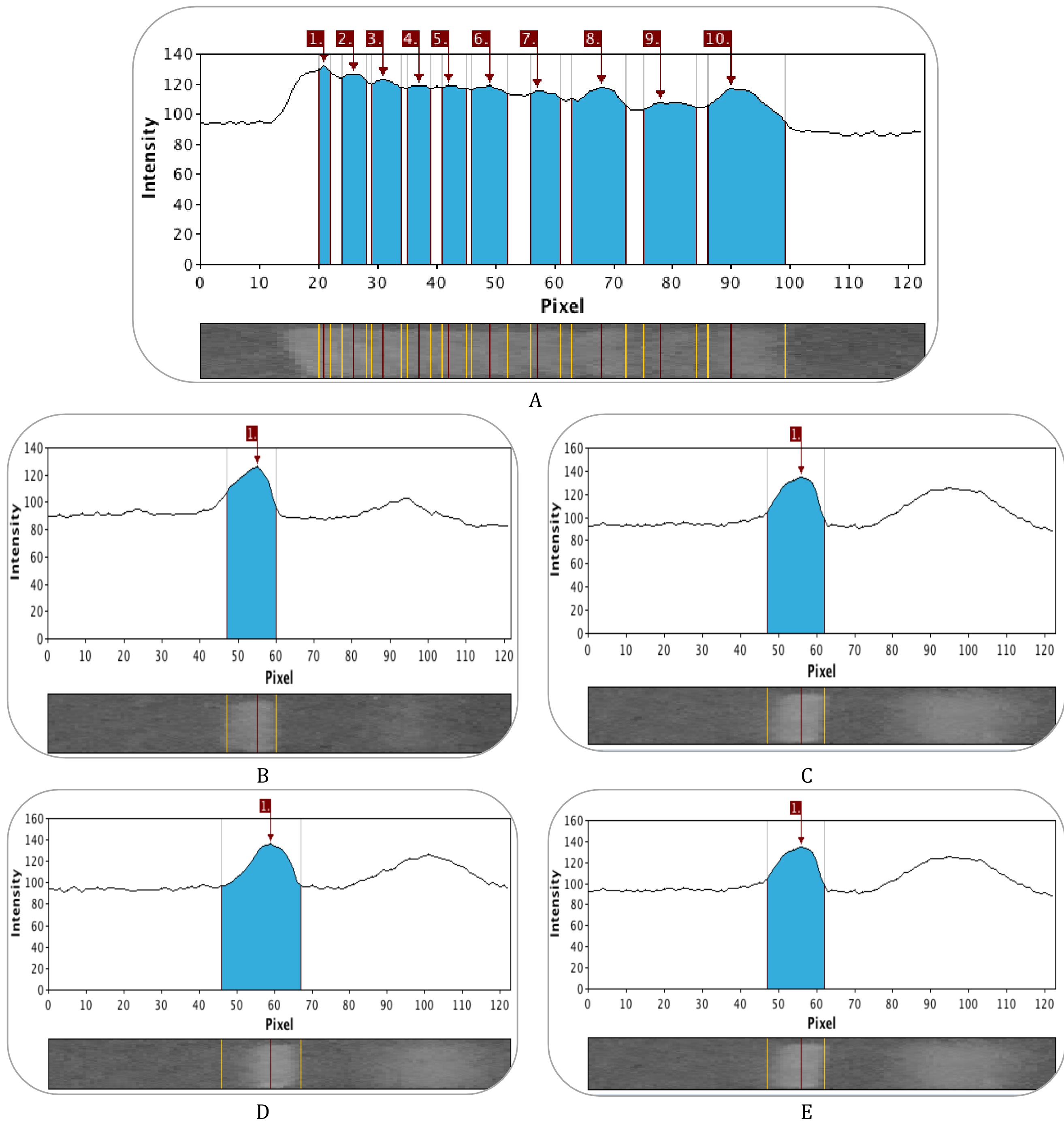


Figure 3. The intensity of DNA Molecular Pixel using Gel Analyzer V.19.1 Program. (a) Marker 100 bp DNA Ladder Marker. (b). Sidrap, (c). Wajo, (d). Luwu, (e). Klaten (Positive control)

Response of several rice varieties to the infection of *RRSV*: The results also showed differences in the resistance reactions of each variety to infection with dwarf viruses. This indicates that there is a relationship between insect vectors, viruses, and host plants, as well as symptom responses that vary between test varieties. The variety with the highest incidence of disease was TN1, with 100% incidence and a disease intensity of 68%, with an average incubation period of 11-17 days. Varieties somewhat resistant to *RRSV* infection were Mekongga, with 50% disease intensity, and Tukad Unda, with 28% disease intensity. These findings suggest that resistance to *RRSV* infection may vary among rice varieties and that further research is needed to identify the specific genetic mechanisms involved in resistance.

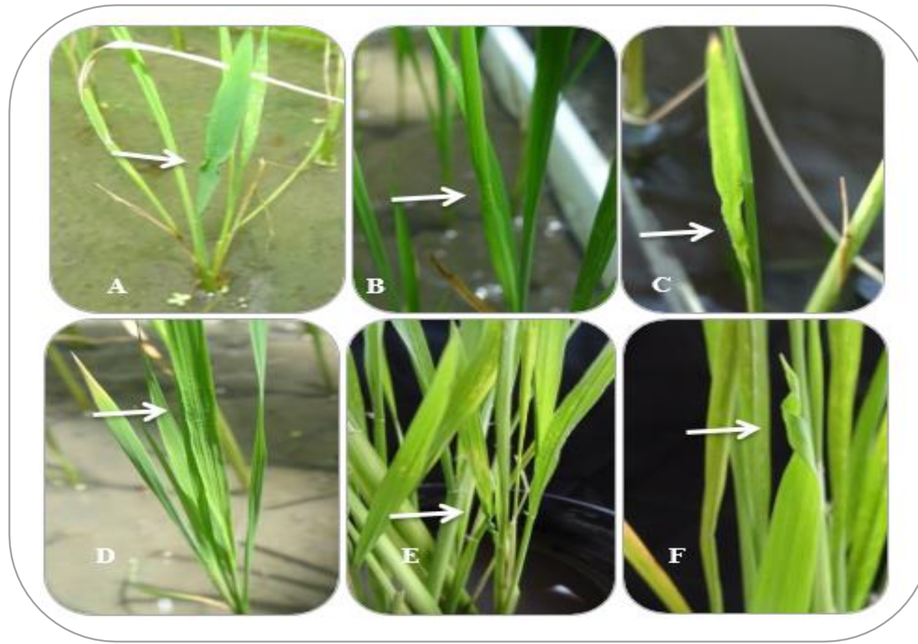


Figure 4. Variation in symptoms of *RRSV*. A.TN1, B.Ciherang, C.Mekongga, D.Tukad Unda, E.Inpari 36, F.Inpari 37

Table 4. Average incubation period, incidence and intensity of *RRSV*

No.	Varieties	Average			Reaction
		IP (Days)	I (%)	DI (%)	
1	TN1	10.26[c]	100[a]	68.88[ab]	susceptible
2	Ciherang	14.20[b]	100[a]	71.10[a]	susceptible
3	Mekongga	15.30[ab]	90[a]	50.36[d]	rather resistant
4	Tukad Unda	17.40[a]	63.33[b]	28.14[e]	resistant
5	Inpari 36	17.70[a]	96.66[a]	59.99[c]	susceptible
6	Inpari 37	15.73[ab]	93.33[a]	62.22[bc]	susceptible

Note: Numbers followed by the same lowercase letter in the column were not significantly different based on Duncan's 5% test. IP: Incubation Period, I: Disease Incidence, DI: Disease Intensity.

According to Bao and Zhang (2019), insect saliva has an impact on a host plant's adaptability because it contains bioactive substances that serve as a defense mechanism for plants and assist in the digestion of nutrients that vector insects need to consume and survive. Insect salivary gland cells have been found to undergo *RRSV*-induced apoptosis, according to other investigations (Huang *et al.,* 2015a). Virus-associated apoptosis is condition-dependent. The salivary sheath is made up of proteins called annexins and salivary envelope proteins (Huang *et al.,* 2015b, 2016). The feeding habits and survival of N. lugens in rice are significantly influenced by a number of salivary proteins, such as salivap3, carbonic anhydrase, and catalases such the Cat-1 protein (Petrova and Smith, 2015; Huang *et al.,* 2016, 2017).

The findings revealed the disease's earliest signs, which ranged from pale leaves to yellowing, a gall at the leaf's base, and twisted or rolled leaf tips. According to Cheng *et al.* (1979), *RRSV* symptoms start to show 10 days after inoculation and include pale-looking yellowing of the leaves, dwarf plants, and leaf rolling that appears 20–25 days later. The level of the sickness depends on how resilient the plants are to viral infections. Ling (1972), claimed that the type of rice and the age of the plant when infected determine the extent of plant damage. Transmission during breeding allows the rice to be more easily infected by dwarf viruses. Figure 4 shows that in each treatment variety, the brown planthopper was able to transmit the virus, even though there were differences in incubation period (IP), disease incidence (I), and disease intensity (DI) between each treatment (Table 4). The short incubation period of the virus indicates a susceptible reaction to viral infection because TN1 varieties do not have resistance genes (Sama *et al.,* 1991) and are often used as experimental plants in a study. The longer the brown planthopper acquires sick plants, the more the brown planthopper gets the virus in its body. This results in plants showing symptoms faster.

According to Hibino *et al.* (1977), the longer the vector insect acquisition and inoculation period in the plant, the higher the virus concentration found in both the vector insect and the host plant, causing the symptoms shown to be severe and faster.

The average plant height after infection with *RRSV*. Varieties with early dwarf symptoms will certainly affect plant growth. The incubation period of plant viruses starts to appear between 7-21 days after inoculation, with varying initial symptoms for each variety. The level of resistance of a variety to the dwarf virus affects the severity of symptoms caused. Surely, using resistant varieties will support efforts to control the disease.

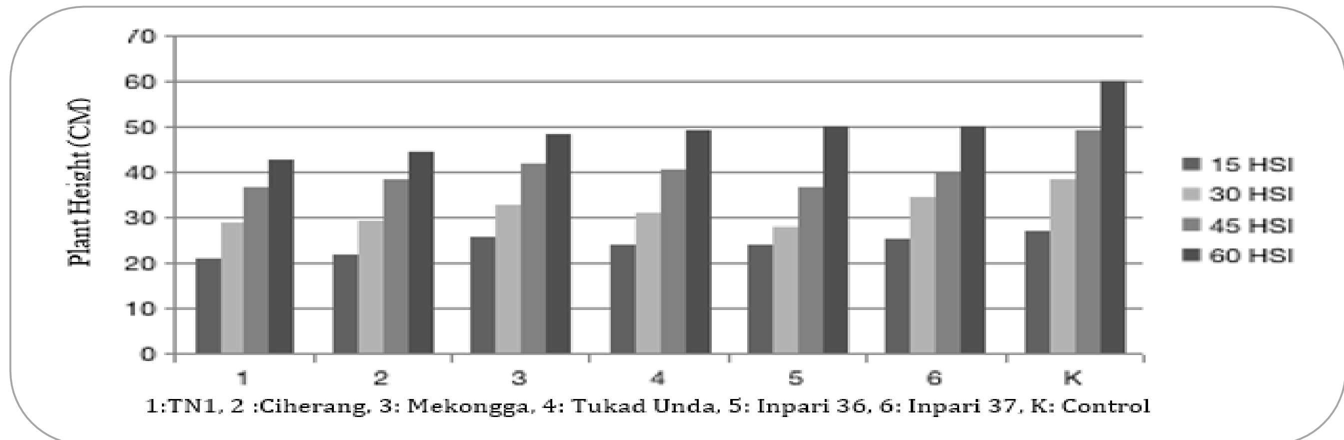


Figure 5. Plant height results of inoculation *RRSV* in several rice varieties in the Greenhouse. HSI: Days after inoculation *RRSV* (Source: Primary data source on greenhouses)

Similar research was also conducted by Helina *et al.* (2019) on the Ciherang variety 10 days after inoculation, which showed that the leaves looked yellow and pale and had growth disturbances in plants characterized by plant height and number of leaves that were different from healthy plants. *RRSV* coinfection has a high pathogenicity. This condition even causes death in rice plants at the beginning of growth (Shimizu *et al.,* 2013). The average plant height in the six test varieties differed (Figure 5). Some varieties have stunted plant growth. Based on Figure 5, shows that the TN1, Ciherang, Inpari 36, and Inpari 37 varieties have low plant growth rates compared to Mekongga and Tukad Unda varieties. Mekongga and Tukad Unda varieties do not experience only the symptoms of leaves that flutter, but there is also a gall at the base of the leaf. TN1 variety had the highest 100% DS value and the highest 68% DI value compared to other varieties, and this is because TN1 varieties are said to not have viral resistance genes (Sama *et al.,* 1991; Baehaki, 2012). While varieties that used to be resistant such as Inpari 36, Inpari 37, and Ciherang, which farmers prefer, had a susceptible reaction to *RRSV* with DI values above 51%. The breakdown of resistant varieties is due to the emergence of a new BPH biotype triggered by the introduction of resistant varieties with a single resistance or vertical resistance gene in a field that is planted continuously, thus accelerating the emergence of new biotypes (Baehaki *et al.,* 2016).

CONCLUSION

The study identified significant differences in resistance to *RRSV* among the six tested rice varieties, with specific varieties like Tukad Unda showing potential for breeding programs. Future research should focus on the genetic mechanisms conferring resistance to *RRSV*, aiming to develop rice varieties with enhanced durability against virus transmission in diverse environmental conditions.

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