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Relationship study between the brown planthopper population and the intensity of *Rice ragged stunt virus* and *Rice grassy stunt virus,* as well as the inoculum sources

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Abstract The brown planthopper (BPH) and stunt virus are the main causes of rice crop damage in the field. Rice crops infected by viruliferous BPHs in high populations will end up with yield loss and a disease epidemic. BPH presence in the field occurred when the rice crops age was 1 week after planting (WAP). The highest population average of BPH reached 77.33 BPH/rice hills in 6 WAP, whereas the population declined on 7 to 9 WAP. Stunt disease incidence started appearing on 4 WAP and increased until 7 WAP. The increase of the BPH population was followed by increased stunt disease incidence rate in rice crops. The viruliferous BPHs found in 9 regencies in Bali carried two viruses, Rice grassy stunt virus (RGSV) and Rice ragged stunt virus (RRSV). The proportion of a BPH carrying two viruses (RGSV and RRSV) reached 20%. The proportion of BPH carrying in 1 virus for RGSV ranges around 15-44%, while the proportion of viruliferous BPHs carrying RRSV ranges around 19.04-42.85%. Other than vector insects as the cause of stunt virus spreading in the field, several weed species around rice crops are also alternative hosts of stunt virus. Weed species naturally infected by RGSV are *Axonopus compressu*s, *Eleusine indica*, *Echinochloa colona*, and *Monochoria vaginalis*. RRSV is found in several weeds around rice crops, which are *A. compressus*, *E. indica*, and *E. colona*. Meanwhile, *Paspalum distichum* is uninfected by the two viruses. The main causes of both viral infections are the vector population and the presence of weeds always found in the field, making a suitable control strategy essential to be implemented.

Keywords: RGSV, RRSV, Stunt virus, Viruliferous BPH, Weeds

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Introduction

The national rice demand continues to increase yearly, along with the population increase. This is due to rice being a primary need for Indonesian. Pest disturbance on rice crops in the field is the leading cause of rice yield decline in Indonesia. Brown planthopper (BPH) *Nilaparvata lugens* Stal is an insect that damaged rice crops (Listihani *et al.*, 2022a).

N. lugens is a prominent pest in rice crops in South Asia and South East Asia, including Indonesia (Kusuma *et al.*, 2018). BPH causes serious damage called hopperburn due to sucking fluid from the plant (Phatthalung and Tangkananond 2017). BPH makes plants turn yellow following rapid draining. BPH is a monophagous insect, its host is limited only to rice and wild rice (*Oryza parennis* dan *O. spontanea*) (Cabauatan *et al.*, 2009).

Other than as a pest, BPH is also a viral vector. Thus, its presence around plantations potentially causes larger loss (Muduli *et al.*, 2021). The BPH is the vector for rice ragged stunt disease caused by *Rice ragged stunt virus* (RRSV), and rice grassy stunt disease caused by *Rice grassy stunt virus* (RGSV) (Cabautan *et al.*, 2009).

Stunt disease is a hurdle in elevating national rice production (Dini, 2015). Rice ragged stunt disease in rice was first reported in Indonesia on 1976 (Hibino, 1979). Rice crops infected by rice ragged stunt disease suffer from stunting, leaf darkening with serrated edges or twisted tips, and swollen leaf blade or bumps at the underside of the leaf or the outer surface of the leaf sheath (Cabautan *at al.*, 2009). Rice grassy stunt disease was first reported in Indonesia on 1971 and called grassy stunt type I, and on 2006 grassy stunt type II was found. Rice grassy stunt disease symptoms are stunting, over-tillering, pale green to yellow leaves and narrow leaves with small rustic dots (IRRI, 2002). Romadhon (2007) reported that BPH endemic region in West Java are Cirebon Regency, Bekasi, Majalengka, Sukabumi, Tasikmalaya, Garut, Cianjur, Subang, Karawang and Indramayu. Moreover, stunt disease incidence has been reported in West Java (Dini *et al.*, 2015).

Almost all regions in Indonesia, including Bali, are BPH endemic areas. Nurbaeti *et al*. (2010) showed a research result on the population dynamics of BPH. During a BPH population outbreak on 2010 which caused 4,874 ha wide rice field to suffer damage, the BPH outbreak also caused the occurrence of stunt disease in the field. This shows that every insect vector has different ability to transmit and spread virus.

Rice cultivars that are resistant against BPH have been used in several countries in Asia, including Indonesia to control BPH and virus spread by BPH (Cabautan *at al.*, 2009). The incidence of RGSV in resistant plant was

originally low, but BPH can overcome plant resistance and more virulent new BPH biotype appears (Claridge and Den Hollander 1980; Ling *et al.*, 1977). This makes the resistant plant cultivars turned vulnerable.

Other than rice crops, RGSV and RRSV can also infect other Graminaceous plants through artificial inoculation by *N. lugens*. However, natural infection in weed and other graminaceous plants other than rice crops is rare because BPHs survive and breed especially on rice (Zheng *et al.*, 2014). Rice crops infected by virus becomes the viral inoculum source in the field. RGSV and RRSV outbreaks in several countries have occured when viral level on the field reached epidemic proportion due to the increase of BPH populations (Phatthalung and Tangkananond, 2021).

There has been no report about the relationship between BPH and the viruses it spreads (RGSV dan RRSV), as well as about natural infection of the two viruses among weed around rice crops, making the information crucial. This research may strengthen the understanding about the relationship between stunt disease incidence with brown planthopper population in rice cultivation. This knowledge can be referred to control pest and important disease on rice crops. Thus, the research aimed to analize the interaction between BPH and weed with the two viruses.

Materials and methods

Time and Place of Research

This research was conducted in 2022. BPH and weed samples were obtained from rice plantation in 9 regencies in Bali Province (Badung, Gianyar, Klungkung, Bangli, Karangasem, Tabanan, Denpasar City, Buleleng, and Jembrana). BPH molecular identification was conducted in plant disease laboratory, Udayana University.

Stunt Disease Insidence and Brown Planthopper Population Observation in the Field

The observation was conducted in rice fields owned by the people of Mengwi Village, Badung Regency, Bali. There were 3 observation plots, each were 300 m^2 in size, with 25 × 25 cm planting distance. Calculation of planthopper populations in every observation plot consisting of 100 plants was determined by diagonal sampling method. The disease incidence rate was calculated based on the ratio of the number of plants with symptoms against the

total number of observed plants times 100%. The observation started after the plants reached 1 week after planting (WAP) until 9 WAP with 7 days intervals.

BPH population observation was started from the initial planting of rice seedlings until the early generative phase with 7 days interval. Brown planthopper population was calculated directly from the base of the rice stalk within the sample unit.

BPH Sampling

BPH and weed were obtained from rice field in Bali Province (Badung, Gianyar, Klungkung, Bangli, Karangasem, Tabanan, Denpasar City, Buleleng, and Jembrana). BPH sample types obtained from rice plants were nymph and imago. Nymph and imago samples were used for total RNA extraction. The nymph and imago samples were stored in -20 ^{o}C upon arrival in the laboratory.

Total RNA extraction of RSSV and RGSV from BPH

One BPH stored in absolute alcohol was extracted by molecular technique to obtain total DNA using a method as described in Goodwin *et al.* (1994) with modifications. BPHs from each location were put into 1.5 mL microtube and added by 100 µL CTAB 2% extraction buffer. As much as 1 µL proteinase K was added before the sample was finely ground using a micropestle. The suspension was incubated at 65 ºC for 3 minutes. A mixture of Chloroform: isoamyl alcohol (CI) (24:1) as much as 100 µL was added into the suspension, which was then vortexed for 3 minutes. The suspension was centrifuged for 15 minutes at 10,000 rpm to produce supernatant. As much as 60 µL of the obtained supernatant was moved into a new 1.5 mL microtube. As much as 44 µL of isopropanol liquid and 6 µL of sodium acetate 3 M (pH 5.2) were added to the supernatant and the mixture was incubated at -20 ºC for 3 hours or overnight. The microtube containing incubated total RNA supernatant liquid was centrifuged at 10,000 rpm for 10 minutes. The supernatant formed was discarded, leaving total RNA containing pellet. The pellet was washed with 100 µL of ethanol 80% and recentrifuged at 8,000 rpm for 5 minutes. The supernatant formed was discarded once more. The total RNA pellet was resuspended with 20 µL of Tris-EDTA (TE).

Total RNA extraction (RRSV and RGSV) from weed

As much as 0.1 g leaf was ground by mortar with the addition of liquid nitrogen. As much as 500 µL extraction buffer containing 1% 2-β-

mercaptoethanol was added into the mortar and mixed evenly. The plant extract was put inside a 1.5 µL microtube and incubated at 65 ºC for 30 minutes. Every 10 minutes the microtube was flipped over repeatedly. As much as 500 µL mixture of Chloroform: isoamyl alcohol (24:1) was added into the microtube. The microtube was then centrifuged at 13,000 rpm for 10 minutes. The supernatant was taken and placed into a new microtube, before added by Sodium acetate 1/10 of total supernatant volume. Isopropanol was then added as much as 2/3 from supernatant total volume. The micotube was centrifuged again for 5 minutes at 13,000 rpm to form RNA pellet. The RNA pellet was washed by adding 600 µL of ethanol 70% and then centrifuged at 8,000 rpm for 5 minutes. The liquid was discarded then the RNA pellet was air dried, before then 50 µL of TE buffer 1x (10 mM Tris-HCl pH 8.0 mM EDTA) was added into the microtube.

The cDNA synthesis

Total RNA was used as the template in reverse transcription (RT) reaction to produce cDNA. The RT reaction (10 µL) consisted of 2 µL RT buffer, 1 µL dNTP 10 mM, 1 µL DTT 50 mM, 0.50 µL RNAse Inhibitor (Thermo Scientific, US), 0.50 µL M-MuLV (Thermo Scientific, US), 2 µL nuclease-free H_2O, 1 µL Oligo d(T) 10 mM, and 2 µL template RNA. First the total RNA and Oligo d(T) were incubated in water bath for 5 minutes at 60 ºC. Other ingredients were added into microtube and the microtube was then centrifuged for 1 minute at 1,000 rpm. The microtube was incubated at 42 ºC for 60 minutes, followed by incubation at 70 ºC for 5 minutes.

Target DNA amplification

After cDNA was obtained, target DNA amplification was performed. Every amplification reaction (25µL) consisted of 1 µL cDNA,1 µL primer F 10 µM, 1 µL primer R 10 µM, 12.5 µL GTG Master mix, and $2H_2O$ 9.5 µL. Primers used were specific primer RGSV and RRSV, which were RGSV primer (F1:5'-GGCTTATGATAGTCTGTGATTTG-3'/R:5'GTGTAAGATGGGGTAAAGT GCA-3') with target amplicon ±450 bp for RGSV which was designed by Nam *et al*. (2007); RRSV (F3:5'-GAC TAGGGATGTGCGTTC-3'/B3:5'-TGTAATCGAC GTTCGCTC-3') with target amplicons ±210 bp for RRSV which was designed by Le *et al.* (2010). The cDNA amplification started from pre-denaturation phase at 94 ºC for 5 minutes for 1 cycle, followed by amplification for 35 cycles with denaturation phase at 94 ºC for 1 minute, annealing at 50 ºC for 1 minute, and elongation at

72 ºC for 1 minute, and ended with 1 cycle of final elongation at 72 ºC for 7 minutes.

DNA visualization

Amplification product visualization was performed by electrophoresis on 1% agarose gel (0.5x*Tris-Borate* EDTA /TBE). The electrophoresis was conducted at 50 volts for 50 minutes, after which the agarose gel was incubated in dyeing agent containing etidium bromide (1%) for 15 minutes and then washed by H_2O for 10 minutes. Photo captures was performed by using UV transilluminator and documented by digital camera.

Results

BPH population in the field

The BPH population showed fluctuation in every observation week (Figure 1). The average highest BPH population reached 77.33 BPH/rice hills on 6 WAP, while on 7 to 9 WAP the population declined. This population fluctuation may occur due to the BPH adaptation process upon migrating from previous field. Planthoppers that managed to adapt would survive and breed while those that did not manage to adapt would die. The presence of natural predator also influenced the population of BPH. During the observation, spiders, which were the predators of nymph and imago of BPH, were found. Other than macroptera type BPH population, during the observation BPH in nymph stadia since 3 WAP and brachiptera type imago on 5 WAP were also found.

Figure 1. Average brown planthopper populations in observation plot in Badung Regency

The rice field in Badung regency was maintained conventionally and insecticide was applied on schedule, which was on the second day of every week after the second week of planting. Insecticide was applied for different pest targets according to the field's highest pest population. It showed that insecticide application every week influences the population of pests on the next week. Every time insecticide was applied, a decline in target pest population and non target pest occurred. Insecticide application was not able to kill planthopper eggs put within the rice stalk tissue and the eggs could still hatch, causing brown planthopper populations to fluctuate. The use of the pyrethroid deltamethrin in rice plants causes the plants to become susceptible to BPH.

The incidence rate of stunt disease in the field

During the observation, much of the rice crops in Badung regency suffered from stunting disease. The incidence of stunt disease started to appear on 4 WAP and increased until 7 WAP (Figure 2). The incidence rate of stunt disease is considered relatively high, probably due to the population fluctuation of its vector, the BPH. This occurred due to environmental factors influencing the vector insect population and the cultivation pattern influencing the disease's spread. Rice cultivation pattern in Badung regency is considered as monoculture with unsynchronized planting time.

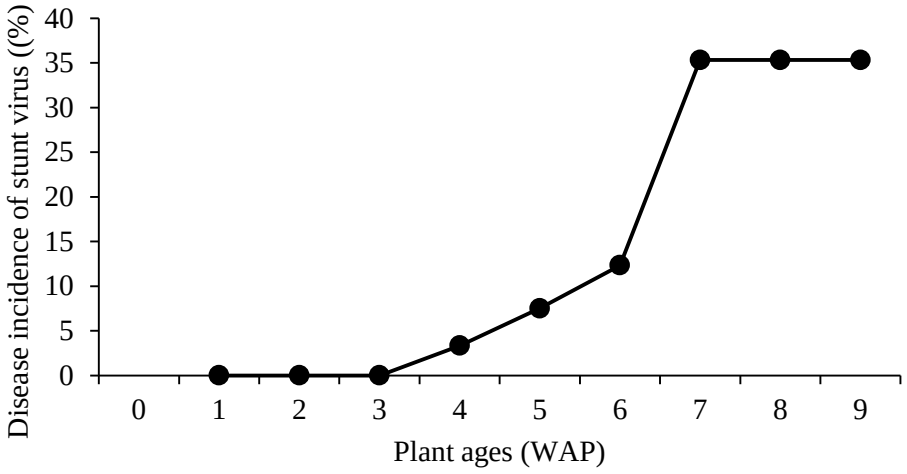

Figure 2. The average stunt disease incidence rate in observation plots in Badung Regency

The relationship between BPH population and the incidence rate of stunt disease

Although BPH population outbreak did not occur in the field, but the BPH population appeared to fluctuate and able to spread stunt viruses. The increase of BPH population was followed by the increase of rice stunt disease incidence rate (Figure 3). This showed that BPH can effectively infect stunt viruses in the field. The rice crops in Badung Regency were planted continuously without plant rotation and cultivation planting time was not synchronized, allowing planthoppers to always have rice crops as food.

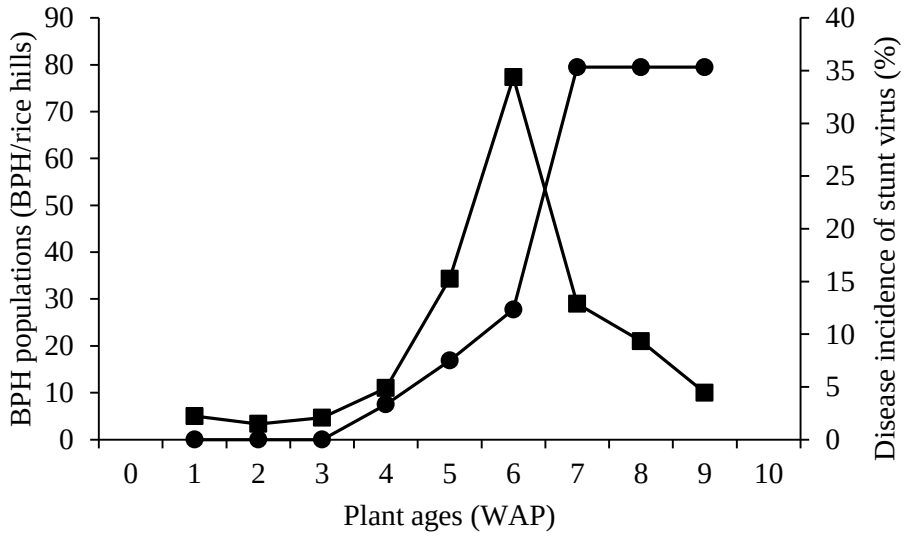

Figure 3. The relationship between brown planthopper population with disease incidence rate in Badung

The population of BPH viruliferous

The result of this research showed that BPH viruliferous RGSV and RRSV were found in 9 regencies in Bali (Figure 4). BPH viruliferous RRGV was managed to be amplified using specific primer with DNA band length 450 bp (Figure 4A), while RRSV was managed to be amplified with DNA band length 210 bp (Figure 4B). This result proved that BPH is among the influencing factor for RGSV and RRSV spread on rice crops in the field. The presence of BPH as pest and stunt disease virus on rice crops in the field remains a serious problem to this day.

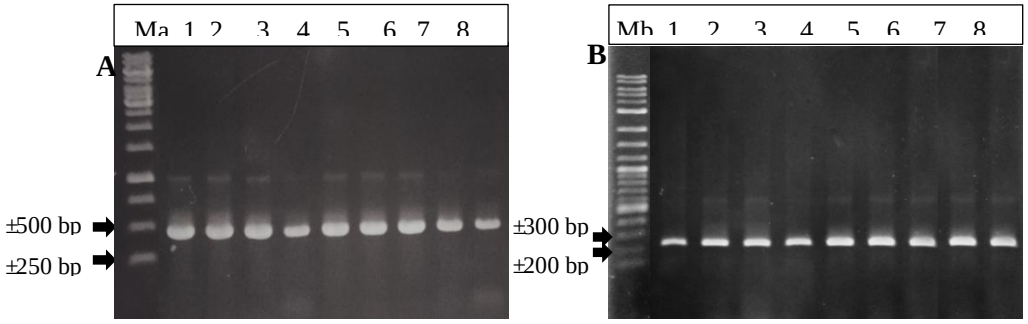

Figure 4. DNA amplification of RGSV (A) and RRSV (B) obtained from BPH Bali isolate by using specific primers. 1, Denpasar City; 2, Badung; 3, Gianyar; 4, Tabanan; 5, Buleleng; 6, Karangasem; 7, Klungkung; 8, Bangli; 9, Jembrana and; Ma,1 kb DNA marker (Thermo Scientific); Mb, 1 kb DNA marker (Fisher Scientific)

This research showed that the proportion of 1 BPH insect which carries two viruses (RGSV and RRSV) reached 20% (Table 1). The proportion of BPH which carries 1 virus for RGSV ranged between 15-44%, while the proportion of viruliferous BPHs carrying RRSV ranged between 19.04-42.85%. Between several rice cultivars, the ones most vulnerable to BPH and on which many viruliferous BPHs were found were cultivar IR-64 and Ciherang. The highest percentage of viruliferous BPH found was in Badung Regency.

Table 1. The percentage of RGSV and RRSV viruliferous BPHs based on RT-PCR detection

Location	Rice cultivars	Total of samples	Viruliferous BPH Percentage (%)		
			RGSV	RRSV	RGSV and RRSV
Denpasar City	Situbagendit	20	15.00 (3/20)	20.00 (4/20)	0.00 (0/20)
Badung	IR-64	25	44.00 (11/25)	36.00 (9/25)	20.00 (5/25)
Gianyar	Ciherang	23	43.47 (10/23)	30.43 (7/23)	13.04 (3/23)
Tabanan	Inpari 32	24	29.16 (7/24)	20.83 (5/24)	4.16 (1/24)
Buleleng	Ciherang	21	38.09 (8/21)	42.85 (9/21)	14.28 (3/21)
Karangasem	Situbagendit	20	20.00 (4/20)	25.00 (5/20)	5.00 (1/20)
Klungkung	Inpari 32	23	21.79 (5/23)	21.79 (5/23)	0.00 (0/23)
Bangli	IR-64	22	43.81(7/22)	40.90 (9/22)	18.18 (4/22)
Jembrana	Inpari 32	21	23.80 (5/21)	19.04 (4/21)	9.52 (2/21)

Weeds as inoculum sources of stunt virus in rice

Other than insect vector as the source of stunt virus spread in the field, several weed species around rice crops can also act as stunt virus alternative host. Weed species that were naturally infected by RGSV are *Axonopus compressus, Eleusine indica, Echinochloa colona,* and *Monochoria vaginalis* (Table 2). RRSV was found on several weeds around rice crops which are *A.*

compressus, *E. indica*, and *E. colona*. Meanwhile, *Paspalum distichum* was not infected by the two viruses. Weed around IR-64 cultivar rice were found to be most infected by stunt disease. This showed that the presence of weed in the field becomes the inoculum source of RGSV and RRSV, making the two viruses to always be present in the field. Thus, a good sanitation should be implemented before and during rice planting.

Table 2. Weed species infected by RGSV and RRSV via RT-PCR

Location	**Rice cultivar**	**Weed species infected by RGSV and RRSV**									
		Axonopus compressus		*Eleusine indica*		*Echinochloa colona*		*Monochoria vaginalis*		*Paspalum distichum*	
		RGSV	RRSV	RGSV	RRSV	RGSV	RRSV	RGSV	RRSV	RGSV	RRSV
Denpasar	Situbagendit	++	-	-	-	-	-	-	-	-	-
Badung	IR-64	++	-	-	++	-	-	++	-	-	-
Gianyar	Ciherang	-	-	++	-	-	++	-	-	-	-
Tabanan	Inpari 32	-	-	-	-	-	-	-	-	-	-
Buleleng	Ciherang	++	-	-	-	-	-	-	-	-	-
Karangasem	Situbagendit	-	++	-	-	-	-	-	-	-	-
Klungkung	Inpari 32	-	-	-	-	-	-	-	-	-	-
Bangli	IR-64	-	-	-	++	-	-	-	-	-	-
Jembrana	Inpari 32	-	-	-	-	++	-	-	-	-	-

Note: ++ (virus positive weed); - (virus negative weed)

Discussion

Plant stadia influences the presence of planthopper in the field. Brown planthopper appears when the plant was aged 1-9 WAP. This is supported by reports from Deng *et al*. (2022) which stated that the plant stadia vulnerable against brown planthopper infestation is from nursery until milk stage. In Indonesia brown planthopper pest outbreak had occurred on 1982 in North Sumatra, on 1998 in Subang Regency, on 2005 in Cirebon Regency, and on 2010 in Subang Regency, Karawang, and Indramayu (Baehaki, 2012). Other than causing direct damage, BPH also causes indirect damage where it acts as stunt virus vector for rice crops.

Stunt virus is persistent propagative in BPH body (Lu *et al*., 2016). After BPH acquired stunt virus, virus propagation occurs in its body which increases the viral concentration. After the vector obtained the virus, they maintain the virus throughout their lives even after molting, though they cannot pass the virus through their eggs. BPH carrying RGSV has shorter lifespan and lower fecundity compared to virus-free BPH (Phatthalung and Tangkananond, 2021). High viral concentration in insect vector will make more virus being

transmitted to plants and will further accelerate the occurrence of symptoms (Listihani *et al.*, 2022b; Temaja *et al.*, 2022).

The rise of disease incidence in the field is also influenced by the presence of planthopper in the field. Macroptera imago of brown planthopper is crucial in the spread of stunt virus as this planthopper imago has a role in long distance migration and it is unknown if the planthopper has been carrying the virus during the migration or not. Planthopper breeds rapidly as it easily adapts with the environment. Macroptera imago of immigrant brown planthopper is important as generation zero, after which nymph as the first generation from immigrant planthopper would appear later. Within one planting season, brown planthopper can produce high population from generation zero up to third generation rapidly because brown planthopper is among r-strategist breeding pests (Baehaki, 2012).

High planthopper population causes elevating incidence of stunt disease in the field, because they spread and transmit stunt virus within the field. This is because nymph actively sucks rice crops' fluid because they need enough nutrition to grow into imago. When nymphs feed on plants infected by stunt virus those nymphs can transmit the virus such as macroptera imago or brachyptera imago. Cabautan *et al.* (2009) reported that short distance dispersal of brown planthopper in plantation is done by nymph, be it brachyptera or macroptera. Short distance dispersal between planting and long distance dispersal are done by macroptera. According to Zhang *et al.* (2018), brown planthopper nymph is more efficient than imago in spreading rice ragged stunt virus. The transmitting percentage for brown planthopper nymph in first instar, second instar, third instar, fourth instar, and fifth instar in order were 19.3%, 25.8%, 29%, 27.6%, and 21.4% (Dini, 2015).

Disease incidence rate is determined by viral virulence and host plant response (Listihani *et al.*, 2019; Damayanti *et al.*, 2020; Listihani *et al.*, 2020; Listihani *et al.*, 2022c; Selangga *et al.*, 2022). Manzila *et al.* (2013) stated that disease incidence is the result of interaction between several factors, among them are viral isolate/strain, rice cultivar, and environmental condition at the cultivation site including the presence of insect vector. In addition, Ling *et al.* (1977) reported that certain rice cultivar may have the same resistance response against an infection by certain viral strain even if it has different resistance gene. Asrori *et al.* (2014) reported that IR-64 cultivar is a cultivar that is moderately resistant against tungro disease but its response against stunt disease is unknown. The result of this research provided information that cultivar IR-64 and Ciherang are very vulnerable against BPH and stunt virus infection.

Phatthalung and Tangkananond (2017) iterated that rice yield lowers along with rising planthopper infestation, earlier planthopper infestation, and higher planthopper population. Yield loss dua to BPH infestation and stunt virus infection can cause up to 90% loss (Cabautan *et al.*, 2009). This shows

that the presence of stunt virus brought by brown planthopper further inhibits panicle growth and seed growth in rice crops.

Baehaki (2012) reported that unsynchronized planting pattern will trigger the spread of stunt disease. There is a relationship between the early infection of stunt virus in the field with the population of its insect vector. The higher the intensity of early disease infection in the field, the higher the intensity of disease infection at the end of the observation. With the high number of insect vector population, the higher the probability of stunt disease to be transmitted from infected plants to healthy ones. This shows that early population of migrating planthopper determines the incidence of stunt disease during the planting season.

Other than BPH as stunt virus insect vector, the virus is always extant in the field due to the presence of alternative hosts, the weeds (Heinrichs and Muniappan 2017). However not all weeds around rice crops can become stunt virus alternative host. This is because natural infection on weeds and cereals other than rice is rare as BPH survive and breed especially on rice (Hibino 1979). This study provides new information that natural infection of stunt virus is possible on weeds present around rice crops, which are *Axonopus compressus, Eleusine indica, Echinochloa colona,* and *Monochoria vaginalis.* Stunt virus was found infecting the weed species due to the high viral stunt disease incidence and high BPH population. Moreover, nutrition competition between BPHs on rice crops cause macroptera BPHs move to other rice plantation and other brachyptera BPHs move to weeds around rice crops infected by stunt virus. Rice and weed infected by stunt virus and viruliferous BPH function as the virus source of transmission. According to Listihani (2019), the main cause of plant viral epidemic in the field is high insect vector population and proportion and the presence of weed as viral inoculum source in the field.

The implication of viruliferous BPH infestation in the field is rice production decline due to inoculum source present in the field and no appropriate control has been implemented. Thus, preventive control is required to minimize the spread of BPH infestation in the field. Considering that the main causes of the two viral infections are vector population and weed that will always be found in the field, transmitting this information to farmer is crucial to improve farmer's awareness about disease management strategy in the field.

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