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Interactive effects of rice ragged stunt virus infection in rice and insect vector *Nilaparvata lugens*

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Abstract

In this study, we attempted to elucidate the interaction effects of *Rice ragged stunt virus* (RRSV) infection on the developmental and survival periods of the brown planthopper (BPH, *Nilaparvata lugens* Stål). BPH is an insect pest that is important for the transmission of rice viral diseases in irrigated rice cultivation in Thailand. This experiment was conducted in a 2 × 2 factorial in a completely randomised design (CRD) with five replications for the assessment of interaction effects between the statuses of BPH populations and rice plants. The results indicated that there were significant interactions ($p < 0.05$) among the developmental periods of the second-instar nymph BPH stage, and survival periods of adult BPH stages between the statuses of BPH vectors and rice plants. In addition, the effects of different RRSV inoculation periods on the developmental and survival periods of each life cycle stage of the nonviruliferous BPH population were investigated following CRD with five replications. Our results showed a significant difference ($p < 0.05$) in the developmental periods of the instar nymphs and in the survival periods of adults. A better understanding of the interaction between BPH vectors and rice plants is needed, a benefit of the complex of plant–virus–vector interactions, as the BPH vector is well adapted to the rice host plant, either with or without RRSV. The results can be used to provide insights into how RRSV spreads in irrigated rice fields, to discern the BPH survivorship pattern, and to improve plant protection strategies for agroecosystem management in Thailand.

Keywords: RRSV, Developmental and survival periods, Brown planthopper (BPH, *Nilaparvata lugens* Stål), Plant–virus–vector interactions, Rice plants

1. Introduction

Rice ragged stunt virus (RRSV) is one of the most destructive and widespread rice plant pathogenic viruses in the rice cultivation process of several East and Southeast Asian countries. RRSV was originally discovered in Indonesia in 1976 (called "Kerdil Hampa") and in the Philippines (called "Rice Ragged Stunt" and "infectious galls") in 1977 [1]. In Thailand, RRSV was first discovered in the Bang-Nam-Priao district, Chachoengsao province, in 1977 and was reported and described in 1978 [2]. Thai plant pathologists named the disease "Rok-Bai-Ngik," while local Thai farmers called it "Rohk-Joo" (or "Rohk-Haeng," "Mai-Ok-Look," and "Dtaai-Kaa-Gra-Bprohng"). Many important outbreaks in irrigated rice cultivation of the central plain and lower north regions of Thailand, which affected economic value, were observed in 1979, 1980–1982, 1988–1990, 1998–1999, and 2009–2010 [3]. Moreover, sporadic outbreaks have been reported from 2014 to the present.

RRSV is a member of the genus *Oryzavirus* in the family *Reoviridae*, a non-enveloped virus, 60–80 nm in diameter, with an icosahedral double-shelled particle [4]. The particle consists of 10 linear double-stranded genomic RNA (linear-dsRNA) segments (denoted as S1 to S10), with a size range of 1,162 to 3,849 base pairs (bp), a total length of 26.066 kilobases (kb) [molecular weight (MW) 16.5×10^6 daltons (Da)] [5], and

conserved nucleotide sequences, with 5'–GAUAAA— and —GUGC–3' [6]. The 10 viral segments encode 12 proteins, including seven structural proteins (SPs), P1, P2, P3, P4A(Pol), P5(Cap), P8B, and P9, and three non-structural proteins (Pns), Pns6, Pns7, and Pns10 [7]. RRSV is persistently and propagatively transmitted by an insect vector, the brown planthopper (BPH, *Nilaparvata lugens* Stål, 1854; Hemiptera: Delphacidae) [8], of which the heavy phloem sap infestation causes complete drying (yellowing) of the rice plants. This phenomenon is also known as "hopperburn," which is secondary damage to rice plants caused by the transmitting viruses. After the BPH lands on the rice plant surface area, two main phases of feeding behaviours begin, which involve (1) the stylet insertion and movement into the rice plant tissues for the purpose of continuous saliva secretion, and (2) the plant cell ingestion for nutrient feeding and honeydew excretion. However, the plant–viral–vector relationships induce physiological and phenotypic changes after initial viral infection and vector infestation, while sap-feeding behaviour is a synchronous pattern related to the biological and environmental conditions in the rice field.

Previous studies have focused on the life cycle under different conditions, such as temperatures, host plant species, and planting seasons, especially focusing on different rice plant species for testing virus and insect resistance. In this study, we hypothesised that insect carrier behaviours and viral properties depend on rice plant integrity, such as host plant and host range. In nature, BPH is a gregarious insect herbivore that migrates from tropical to subtropical and temperate areas in the central plain and lower north regions of Thailand. The migrated population has unidentified viral carrier activity, while rice host plants in large field areas might be plant sources for viral spread.

Therefore, the effect of the rice virus on the vector's reproductive potential is an important key in understanding the capability and transmissibility of viral hosts. Hence, the objective of this study was to determine the effect of RRSV infection on the developmental and survival periods of each BPH life cycle in rice plants. Thereafter, we discuss the complexity of plant–virus–vector interactions, in particular the differences in host status and viral inoculation periods on the reproduction of nonviruliferous and viruliferous BPH. The results will help to further explain the interaction between RRSV and the BPH vector to improve effective sustainable agricultural control measures.

2. Materials and methods

2.1 Virus materials

The samples of RRSV-infected rice plants were kindly provided and verified by the Division of Rice Research and Development, Rice Department, Bangkok, which were obtained from an irrigated rice field in Nong-Suea district, Pathum Thani province, Thailand, in 2018–2019. The diseased rice plant samples were multiplied for routine stock cultures in the greenhouse under conditions of 26 ± 1°C, 70–90% relative humidity (RH), and light 8 h darkness: 16 h photoperiod and used as a source of inoculum for virus infection in the vectors.

2.2 Insect vector materials

The suspected viruliferous and nonviruliferous BPH vectors (*N. lugens* Stål) were collected from the light trap in the same rice field in which viral rice plant materials were obtained. The vectors were maintained in the insect rearing cages (40 cm × 40 cm × 60 cm) on the rice plant seedlings, *Oryza sativa* L. variety 'RD-7' [7–10 cm height, 6–9 days after germination (DAG)] for routine stock cultures under greenhouse conditions. To obtain specific experimental BPH populations, mature females were generally transferred for the oviposition process. Then, after 48 h, the rice RD-7 seedlings were replaced to ensure sufficient nutrition, and the infested rice plant seedlings with the BPH eggs were cultured in insect-free cages for continuous production of the stock culture of nonviruliferous BPH populations. The various stages of BPH offspring (third generation, F_3) were used in this study.

2.3 RRSV transmission and test sample preparation

After moulting, nonviruliferous individuals of the third instar nymph BPH populations were used for RRSV inoculation, and the 50 third-instar nymphs were fed on infected rice plants for a 24 h acquisition feeding period (AFP). Then, the transmitted BPH vectors were transferred and reared on viral-free *O. sativa* L. var. RD-7 rice plant seedlings for 3 days as the latent period (LP). The standard susceptible variety 'Taichung Native 1' (TN1) rice plant seedlings were inoculated with the viruliferous BPH for a 24 h inoculation access period (IAP), and the infected rice plant seedlings were treated and grown under greenhouse conditions. The rice plant leaf test samples were collected at different viral infectious periods (10 days interval) from day 10 to day 90 after inoculation (DAI). Then, the leaf samples were cut into 5–7 cm long pieces, before being placed and stored for

at least 2 h in Petri dishes containing wet cotton to retain moisture. Subsequently, the nonviruliferous and viruliferous BPH populations of various developmental stages, which had been starved for a 2 h fasting period (FP), were placed onto the rice plant leaf test samples with a fine brush. The developmental and survival periods of the BPH population were monitored, and the following parameters were estimated: (1) period of egg fertility to hatching success, (2) individual developmental stages of instar nymphs after moulting success, and (3) longevity of adults to mortality success.

2.4 Detection of RRSV in the test samples

The dot-immunobinding assay (DIBA) was modified from the method of Hibi and Saito [9]. The infected samples were collected. The rice plant tissues (1 g) were ground in 2 mL of the plant sap extraction buffer solution [plant-EB: 0.01 M phosphate buffered saline (PBS), pH 7.4] and transferred into a 2 mL Eppendorf tube (EPP). Then, the BPH population was ground in 0.2 mL of the BPH sap extraction buffer solution [BPH-EB: 0.01 M PBS (pH 7.4), 2% polyvinylpyrrolidone (PVP)] by using a sterile pipette tip in the 2 mL EPP tube and pipetting up and down. The crude sap samples were homogenised by a vortex mixer and centrifuged at 16,532 g and 4°C for 10 min (Centurion Scientific, Ltd.). Subsequently, the supernatant was transferred into a new EPP tube and diluted with the dilution buffer solution [DB: 0.01 M PBS (pH 7.4)]. A nitrocellulose membrane (NCM) sheet (pore size 0.45 µm, Bio-Rad, Catalogue No. 1620115) was cu into a 1 × 1 cm square, immersed in DB solution, and placed on the dried Whatman® filter paper No. 1 to remove the excess solution. The paper was washed three times with the washing buffer solution [PBS-T: 0.01 M PBS (pH 7.4), 0.5% Tween-20], and then 5 µL of the test and control sap samples were dotted onto the NCM sheet and allowed to dry for 15 min at room temperature. The dotted sheet was put in the blocking buffer solution [PBS-T-SK: 0.01 M PBS (pH 7.4), 0.5% Tween-20, 5% skim milk (SK)], and shaken at 10 rpm for 30 min at room temperature. After the washing step, the anti-RRSV IgG solution (diluted 1:1,000 in PBS-T-SK) was added and incubated at 4°C overnight. Following the washing step, the dotted sheet was incubated in the goat anti-rabbit serum conjugate alkaline phosphatase (GAR-AP, ZyMax™) solution (diluted 1:5,000 in PBS-T) at 4 °C for 3–5 h. The washing step was performed before the reactions were visualised with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) solution after incubation for 1 h. A positive signal was the appearance of the blue-purple colour in the region of the blot. The reaction was terminated by putting the NCM sheet into deionised water for 10 min. The positive result was detected by observing the development of blue-purple colour on the blot in 1 h.

2.5 Experimental design and statistical analysis

The experimental scheme used in this study was a completely randomised design (CRD) in a 2 × 2 factorial pattern with five replications to investigate the interaction effects between BPH status (nonviruliferous and viruliferous BPH populations) and rice plant status (RRSV-free leaves and RRSV-diseased leaves). Simultaneously, the effects of different RRSV inoculation periods or days after inoculation (DAI; 10, 20, 30, 40, 50, 60, 70, 80, and 90) of rice plants on the developmental and survival periods of each life cycle stage of the nonviruliferous BPH population were investigated following CRD with five replications. The qualitative data were analysed using analysis of variance (ANOVA) in Statistical Package for Social Sciences (SPSS) software (version 16.0). The level of statistical significance ($p < 0.05$) and the differences between mean values were further separated using Duncan's multiple range test (DMRT), in which the results were expressed as the mean ± standard deviation (SD).

3. Results and discussion

3.1 RRSV-infected rice plant symptoms

The RRSV-infected rice plants showed different visible signs and symptoms at different growth phases and stages after infection, as shown in Table 1. The symptoms began to be expressed at 8–13 DAI. These results indicated that the increased severity and susceptibility of rice plants were closely related to the growth phases, stage of plant, latent period, and generation time. The most conspicuous signs and symptoms were noticeable during the vegetative phase (germination to panicle initiation, PI), showing stunt, abnormal leaves with short, narrow, ragged, and twisted leaf blades and leaf tips, and vein swellings or galls in the phloem tissues. For the reproductive phase (PI to heading), malformed, ragged, narrow flag leaves that roll up were observed, sometimes with the appearance of leaf yellowing. For the ripening phase (heading to maturity) delayed flowering, incomplete and rolled-up panicles in the panicle hands clenching form, nodal branch production, and unfilled grains were shown to significantly lead to between 10–100% yield loss (Figure 1).

The appearance and pattern of the plant cells and tissue arrangements of RRSV-induced galls were the same as those described in the *Southern rice black-streaked dwarf virus* (SRBSDV, *Fijivirus*: *Reoviridae*) infected rice plants transmitted by the rice white-backed planthopper (WBPH, *Sogatella furcifera* Horváth, 1899; Homoptera: Delphacidae) [10]. These results proved that the gall hyperplasia regions contained three cell types, including phloem parenchyma (PP, 49%), sieve elements (SEs, 28%), and vessels (V, 23%). The proportion of gall colouration on infected rice plants included white and pale yellow (82%), light brown (6%), and dark brown (10%); with their sizes about 1 mm to over 10 cm long, 0.2–1 mm wide, and protruding 0.1–1 mm on the outer surface of the leaf blades and leaf sheaths (21%), surface of sheaths (72%), and clum or shoot (7%) [11]. However, there was evidence of viral accumulation in the infected phloem cells, an entrance site for viral systemic infections to other cells and affecting surrounding tissues. The leaf yellowing (chlorosis) process was probably due to a delay in the formation of the intercellular spaces caused by cell enlargement and proliferation. These symptoms were similar to the infected rice plants of *Rice grassy stunt virus* (RGSV, *Tenuivirus*: *Phenuiviridae*) transmitted by the BPH vector, and rice tungro viruses of *Rice tungro bacilliform virus* (RTBV, *Tungrovirus*: *Caulimoviridae*) and *Rice tungro spherical virus* (RTSV, *Waikavirus*: *Secoviridae*) transmitted by the rice green leafhoppers (GLH, *Nephottetix virescens* Distant, 1908; Homoptera: Cicadellidae), which resulted from carbon fixation- and chlorophyll synthesis-inhibition after infection [12]. In addition, the most virulence symptoms were observed after virus infection at the vegetative and early reproductive phase (≤ 60–70 DAI) but with a low virulence rate after infection at the reproductive phase, with 1–2% of mild symptoms appearing on rice plants [13].

Table 1 Signs and symptoms of rice plants infected by RRSV.

Growth phases and stages	DAI	Rice plant external signs and symptoms	Observed symptoms*
Vegetative phase	8–13	Dark-green leaves	8/10 (80%)
	10–14	Early stunted	4/10 (40%)
	10–15	Early ragged leaves	4/10 (40%)
	12–18	Spiral shapes at the base of leaf blades	6/10 (60%)
	14–20	Leaf tip twisted	8/10 (80%)
	18–28	Leaf sheath jagged	8/10 (80%)
	25–45	Excessive tillering at the nodes, and stunted	10/10 (100%)
	15–40	Vein swellings (galls)	
		Underside of leaf blades	6/10 (60%)
		Surface of sheaths	8/10 (80%)
		Clum or shoot	4/10 (40%)
Reproductive phase	50–70	Malformed or ragged flag and shortened leaves	10/10 (100%)
		Leaf-yellowing (chlorosis pattern)	10/10 (100%)
Ripening phase	80–100	Delayed flowering	10/10 (100%)
		Incomplete panicle	10/10 (100%)
		Nodal branch production	10/10 (100%)
		Unfilled grains	10/10 (100%)

*Note: %Symptom index = (No. of symptomatic plants appeared / No. of experimented rice plants) × 100 (Experimented TN1 rice plants, n = 10); RRSV = Rice ragged stunt virus; DAI = Days after inoculation

The results of viral infection affected the biochemical and physiological changes in host plants by inducing elevated levels of plant secondary metabolites (PSMs) that attracted insect vectors for its transmission. The insect's life cycle was used as an indicator of the viral impact on the performance of insect hosts. Understanding the vector survival patterns and the effects of viral pathogenesis on disease patterns are limited by the diversity of virus–host interactions [14]. BPH is an important vector for rice virus spread in the Asian rice cultivation process. Characterisation of the rice virus was essential, especially during persistent infection throughout the developmental and survival periods, depending on the food source and population density.

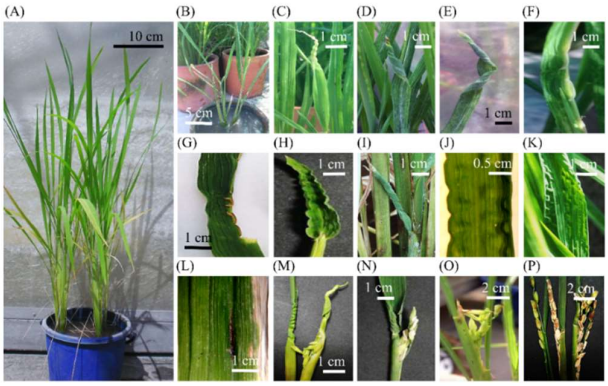

Figure 1 (A) The viral-free TN1 rice plants (45 DAG), (B) Stunted or dwarfed rice plant growth with shortened leaves,(C-E) Pale-green and dark-green twisted-leaf tip, (F-H) Ragged leaf blade with green-yellow to yellow-white discolouration, (I) Tightly wrapped-rolled dark green leaf, (J-K) Dark-green and wave-malformed leaf veins, (L) Discolouration development of vein-swelling or gall dark brown on the outer surface of the leaf blades, (M-N) Dark-green flag leaf twisted, malformed, and narrows, rolls up, and (O-P) Morphogenic abnormalities in young rice seed and incomplete panicles with yellow-brown to dark-brown unfilled grains

3.2 RRSV detection by DIBA

The samples were identified as RRSV by DIBA. The DIBA-positive reactions clearly showed blue-purple spots on the NCM blots for both the crude sap samples of viruliferous BPH vectors and infected rice plants. In contrast, the DIBA negative reactions showed pale-green colour spots for rice plant crude sap and pale-brown colour spots for BPH crude sap on the NCM blots, respectively (Figure 2). Considering these aspects, DIBA using the crude sap samples of vectors and rice plants seems to provide the most useful tool and information for preliminary RRSV screening. In addition, this assay has higher potential for viral detection, confirmation, and validation, which is simple, sensitive, specific, rapid, and easy to perform in laboratories.

(A)

BPH crude sap	Stages of instar-nymph					Wing dimorphisms of adult BPH			
	1st	2nd	3rd	4th	5th	SW-Male	SW-Female	LW-Male	LW-Female
Viruliferous BPH crude sap									
Non-viruliferous BPH crude sap									
Positive control (Viruliferous BPH crude sap)									
Negative control (BPH-extraction buffer)									

(B)

TN1 rice plant crude sap	RRSV-DAI								
	10	20	30	40	50	60	70	80	90
Infected rice plant crude sap									
Viral-free rice plant crude sap									
Positive control (Infected rice plant crude sap)									
Negative control (Plant-extraction buffer)									

Figure 2 Detection of RRSV by DIBA of both crude sap samples of (A) BPH vectors and (B) 'TN1' rice plants. Note: RRSV = Rice ragged stunt virus; DAI = Days after inoculation; SW = Short-winged brachypterous form; LW = Long-winged macropterous form.

3.3 Developmental periods of brown planthopper eggs and nymphs, and survival periods of adults

3.3.1 Developmental periods of the BPH egg stage

BPH, which was released onto RRSV-infected rice plants after various viral infection periods (10–90 DAI), exhibited typical symptoms. There was no significant (p = 0.75) interaction of the developmental periods of BPH eggs by comparing egg-laying by nonviruliferous status of adult BPH females on the viral-free (9.80 ± 0.83 days) and infected (9.800 ± 0.447 days) rice plants, and viruliferous status of adult BPH females on the viral-free (9.60 ± 0.54 days) and infected plants (9.80 ± 0.83 days) (Table 2). In addition, the effects of different RRSV inoculation periods of rice plants on the egg development periods of the nonviruliferous BPH population showed no significant (p = 0.18) difference (Table 4). This suggested that the status of rice host plants and insect vectors did not have a significant effect on a major role in the host selection, adaptation on host plant, insect fecundity, and the emergence of the instar nymphs (Figure 3).

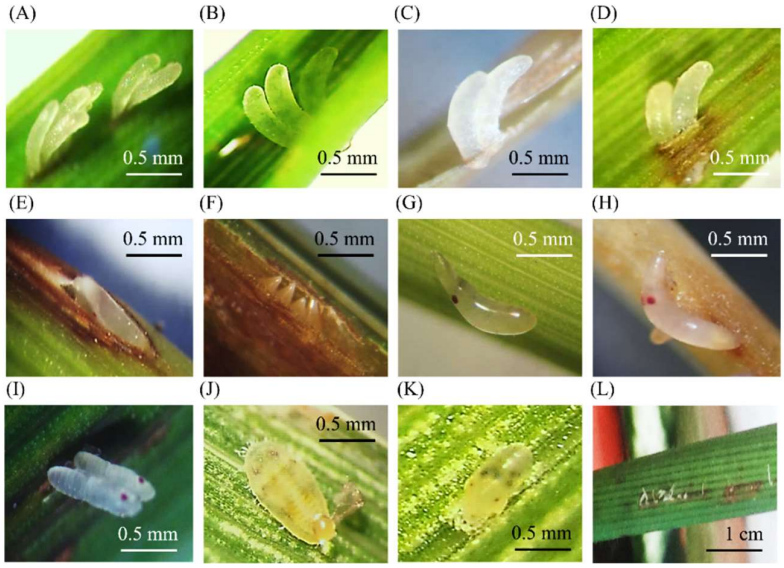

Figure 3 (A–D) Groups of inserted egg (banana shape, 2–5 eggs) in the rice plant tissue (1 day after oviposition, DAO), (E-F) BPH eggs were inside with dark brown incision on the leaf sheath tissue, (G) Eggs were covered with a dome-shaped (flat) or egg cap that protects eggs from environmental harm; and red-eye spot (3–4 DAO), (H–J) Egg stages at 5, 6–7, and 8–10 DAO, respectively, (K) Final stage of egg before hatching, and (L) Inserted eggs into the midrib of the leaf on the ventral side.

The BPH populations needed a host and food plant that were suitable for sap-feeding and living to complete the life cycle. However, the results of the egg developmental periods indicated that the eggs laid by the nonviruliferous and viruliferous status of adult BPH females on viral-free and infected rice plants had a similar trend, which was similar to the study in WBPH (*S. furcifera* Horváth, 1899) and SRBSDV [15]. However, several research studies have shown that the developmental periods of the eggs of the viruliferous vector were longer than that of the nonviruliferous vector, such as in the wheat aphid (*Sitobion avenae* Fabricius, 1775; Hemiptera: Aphididae), an insect vector of *Barley yellow dwarf virus* (BYDV; *Luteovirus*, *Luteoviridae*) [16] and the whitefly (*Bemisia tabaci* Gennadius, 1889; Homoptera: Aleyrodidae) insect vector of *Tomato yellow leaf curl China virus* (TYLCCNV) and *Tobacco curly shoot virus* (TbCSV; *Begomovirus*, *Geminiviridae*) [17].

However, RRSV did not pass through the eggs; the upregulated expression of the genes of an insect vector involved in the metabolic pathway, ubiquitin-proteasome system (UPS), cytoskeleton dynamics, and immune responses can be speculated. The egg stage of BPH developed successfully under the appropriate abiotic conditions, such as low environmental risk, and sufficient food for offspring production. Periods of pre-oviposition, oviposition, and fecundity of the LW females ranged from 2.90 to 8.40 days and averaged 421.80 eggs, while that of the SW females ranged from 3.00 to 9.20 days and averaged 485.80 eggs [18]. BPH laid eggs in 2–12 small groups or clustered inside the air cavities of the upper surface green sheath or on the base of rice plants of the young rice leaves more than on the older rice leaves, where shaded and high humidity are included, especially in the parenchymatous tissue of leaf sheaths or the midribs of leaves (11.6–86.6%), and stem sheaths (1.6%), respectively [19].

The BPH vector produced a dark brown incision, necrosis, and an ovicidal substance with a saw-like ovipositor and moved it up and down to lacerate the tissue for inserting 2–15 eggs (average egg length and width were 0.84 ± 0.06 and 0.16 ± 0.02 mm, respectively) inside the rice plant tissue. The size of egg groups did not change in response to the differences in fecundity, while the egg numbers correlated with the oviposition period and life span. In addition, phloem sap removal is direct physical damage caused by the insertional process of egg masses into rice plant tissue. The mortality of eggs on rice plant leaves is independent of egg density but dependent on the vector–virus–rice plant age interaction that induces the biosynthesis and emission of rice plant volatiles, whereas proteins, polysaccharides, and lipids were secreted from the BPH female accessory reproductive glandular (FARG) for species-specific plant responses to the developmental periods of BPH eggs [20].

3.3.2 Developmental periods of the instar nymph BPH stages

Evaluation of developmental periods of the first- to fifth-instar nymph BPH stages: We found that there were significant (p = 0.02) interactions of developmental periods of the second-instar nymph BPH between the nonviruliferous and viruliferous BPH status or between the viral-free and infected rice plants. No differences were observed in the developmental periods of the nymph BPH stages, including the first- (p = 0.62), third- (p = 0.67), fourth- (p = 0.30), and fifth-instar nymphs (p = 0.23) (Table 2). RRSV infection with typically visible symptoms in rice plants did not affect the developmental periods of the BPH instar nymphs (Figure 4). However, the developmental periods of instar nymphs of viruliferous BPH status reared and infested on viral-free and infected rice plants were markedly longer than those of the nonviruliferous BPH status. In addition, the effects of different RRSV-inoculation periods of rice plants on the developmental periods of the nymph BPH stages in the nonviruliferous BPH population were shown to be significantly different ($p < 0.05$), including the first- ($p < 0.001$), second- (p = 0.001), third- (p = 0.01), fourth- (p = 0.01), and fifth-instar nymphs (p = 0.005) (Table 4).

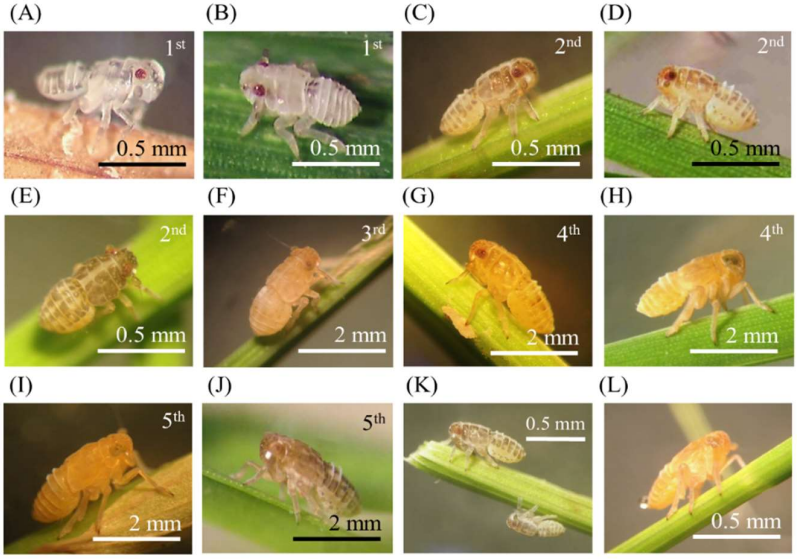

Figure 4 (A-B) First-instar nymph (1 and 3 days after hatching, DAH), (C-E) Second-instar nymph (5–7 DAH), (F) Third-instar nymph (8 DAH), (G-H) Fourth-instar nymph (11–14 DAH) with red and pale-brown eye spot, (I-J) and the fifth instar nymph (16–18 DAH) with brown and dark-brown cuticle layer, (K) Comparison of the second and third instar nymphs, and (L) honey dew after feeding on the rice plants.
Notes: The body sizes of the first to five instar nymph stages had a length of about 0.70 ± 0.07, 1.0 ± 0.07, 1.40 ± 0.14, 1.90 ± 0.12, and 2.40 ± 0.10 mm, respectively, and width of about 0.24 ± 0.03, 0.42 ± 0.03, 0.60 ± 0.05, 0.85 ± 0.06, and 0.92 ± 0.05 mm, respectively, to complete development [21].

The BPH vector has five instar nymph stages, which are distinguished by the body shapes, size, and coloured layer of the cuticle. The nymph stages are smaller, with no functional insect wings. Young nymphs are white or pale brown, but they gradually become darker brown in older instars. Moreover, the RRSV-transmission rate increased with a longer acquisition access period (AAP) [8] and the fifth-instar nymphs had the highest percentage of transmitters (28%), followed by the fourth (26%), third (18%), second (16%), and first-instar nymphs (12%) [22]. Our results indicated that the developmental periods of instar nymph BPH stages and survival periods of adult BPH stages reared and infested on infected rice plants were markedly higher than those

reared and infested on viral-free rice plants. These discontinuous trends in developmental and survival periods of both nymph and adult stages may vary depending on the status of infected rice plants and sap-feeding behaviour of BPH vectors. The RRSV transmissibility of the viruliferous BPH status regularly decreased during the insect's lifespan and was correlated with a decrease in virus acquisition, which did not disappear entirely. Although the longer developmental and survival periods of both instar nymph and adult stages benefited viral acquisition and transmission and might increase the rice infection rate, in general, RRSV infection of the BPH vectors and rice host plants was unfavourable to vector population expansion.

The longevity period of WBPH (*S. furcifera* Horváth, 1899) was slightly shorter when reared on SRBSDV-infected plants than viral-free rice plants [23]. The infection of *Rice dwarf virus* (RDV; *Fijivirus*, *Reoviridae*) showed negative impacts on the life cycle of the green rice leafhopper (GRLH, *Nephotettix cincticeps* Uhler, 1956; Hemiptera: Cicadellidae) [24], and positive impacts were shown on the fitness improvement of vectors, such as the green peach aphid (GPA, *Myzus persicae* Sulzer, 1776; Hemiptera: Aphididae) infected by *Potato leaf roll virus* (PLRV; *Polerovirus*, *Luteoviridae*) [25], western flower thrips (WFT, *Frankliniella occidentalis* Pergande, 1985; Thysanoptera: Thripidae) infected by *Tomato spotted wilt virus* (TSWV; *Tospovirus*, *Bunyaviridae*) [26], and the cotton aphid (*Aphis gossypii* Glover, 1877; Hemiptera: Aphididae) infected by *Zucchini yellow mosaic virus* (ZYMV; *Potyvirus*, *Potyviridae*) [27].

3.3.3 Survival periods of the adult BPH stages

Evaluation of the survival rates and longevity of adult BPH stages: There were significant ($p < 0.05$) interactions on the SW males ($p = 0.012$), SW females ($p = 0.03$), LW males ($p = 0.009$), and LW females ($p = 0.03$) between the nonviruliferous and viruliferous adult BPH status or between viral-free and infected rice plants (Table 3). However, the survival rates and longevity of viruliferous BPH adults reared and infested on infected rice plants was markedly longer than those reared and infested on viral-free rice plants and nonviruliferous BPH adults on rice plants (Figure 5). In addition, the effects of different RRSV inoculation periods of rice plants on the survival rates and longevity of adult BPH stages in the nonviruliferous BPH population were significantly different ($p < 0.05$), including the SW males ($p < 0.001$), SW females ($p = 0.01$), LW males ($p = 0.020$), and LW females ($p = 0.02$) (Table 5).

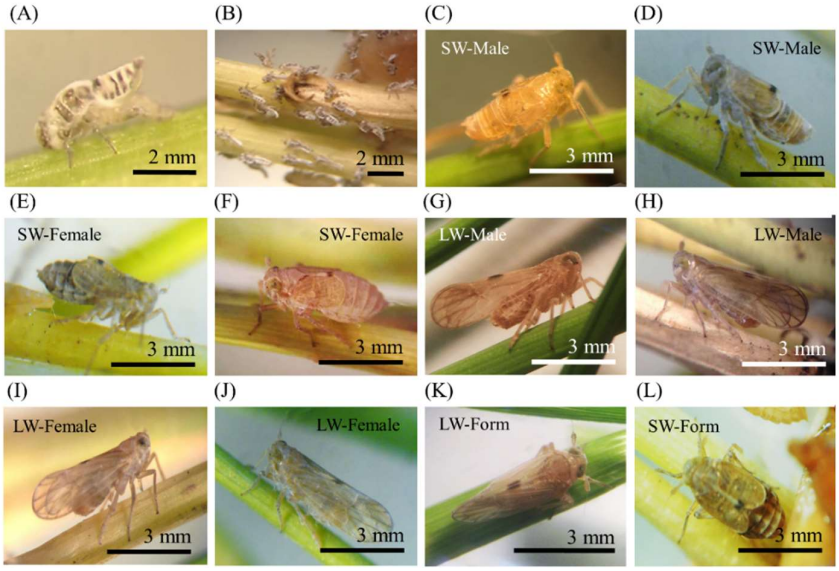

Figure 5 (A-B) BPH moulting, (C-D) Brown and dark brown short-winged (SW) brachypterous form of a male (SW-male), (E-F) Dark brown and brown SW female, (G-H) Brown and dark brown long-winged (LW) macropterous form of a male (LW male), (I-J) Dark brown and brown LW female, and (K-L) Wing dimorphism of the BPH adult stage.
Notes: The width (W) and length (L) of BPH adults, including LW female: 1.20 ± 0.05 and 3.30 ± 0.14 mm; LW male: 1.10 ± 0.07 and 2.90 ± 0.15 mm; SW female: 1.10 ± 0.04 and 3.70 ± 0.27 mm; and SW male: 1.00 ± 0.03 and 2.90 ± 0.15 mm [21], respectively.

Wing dimorphisms of adult BPH vectors were induced and triggered by environmental factors, such as nutrient content and quality of host plants, crowding, population density, temperature, and photoperiod. The LW

form has developed wings for colonising new habitats (long distances) from the site of origin by functional flight wing capable muscles, resulting in Asian rice production agricultural system damaging. The SW form has underdeveloped or less developed wings and flight muscles, which were incapable of migration but allowed reproduction. This study showed that for adult BPH stages, there were significant interactions between both the status of BPH and rice plants. However, it indicated that the viruliferous status of adult BPH fed in the phloem more frequently than the nonviruliferous status of BPH adults and thus may benefit the spread of RRSV, corresponding to the study of WBPH (*S. furcifera* Horváth, 1899), an insect vector of SRBSDV [28].

Table 2 Developmental periods of egg and instar nymph stages of the brown planthopper (BPH) reared on RRSV-free or RRSV-diseased rice plants.

BPH status (Factor A)	Rearing status (Factor B)	Developmental periods of immature BPH stages (days)					
		Egg stage	Instar nymph stage				
			First	Second	Third	Fourth	Fifth
Non viruliferous-BPH	On RRSV-free leaves	9.80±0.83^{a}	3.00±0.00^{a}	3.00±0.00^{a}	3.20±0.27^{a}	3.10±0.22^{a}	3.10±0.22^{a}
	On RRSV-diseased leaves	9.80±0.44^{a}	3.30±0.27^{ab}	3.00±0.00^{a}	3.20±0.27^{a}	3.200±0.27^{a}	3.10±0.22^{a}
Viruliferous-BPH	On RRSV-free leaves	9.60±0.54^{a}	3.20±0.27^{ab}	3.00±0.00^{a}	3.10±0.22^{a}	3.30±0.44^{ab}	3.20±0.27^{ab}
	On RRSV-diseased leaves	9.80±0.83^{a}	3.40±0.22^{b}	3.30±0.27^{b}	3.20±0.27^{a}	3.70±0.27^{b}	3.50±0.35^{b}
	BPH status (A)	0.75^{ns}	0.15^{ns}	0.026*	0.67^{ns}	0.025*	0.05^{ns}
	Rearing status (B)	0.75^{ns}	0.02*	0.026*	0.67^{ns}	0.09^{ns}	0.23^{ns}
	A × B	0.75^{ns}	0.62^{ns}	0.026*	0.67^{ns}	0.30^{ns}	0.23^{ns}

RRSV = Rice ragged stunt virus; ns = not significantly different; * = significantly different ($p< 0.05$)
Values (mean ± SD) in the same column superscripted by different lowercase letters are significantly different ($p< 0.05$).

Table 3 Survival periods of adult stages of the brown planthopper (BPH) reared on RRSV-free or RRSV-diseased rice plants.

BPH status (Factor A)	Rearing status (Factor B)	Survival periods of adult BPH stages (days)			
		Short-winged (SW) brachypterous form		Long-winged (LW) macropterous form	
		Male	Female	Male	Female
Non viruliferous-BPH	On RRSV-free leaves	13.10±0.22^{a}	14.80±0.27^{a}	13.80±0.90^{a}	14.60±0.41^{a}
	On RRSV-diseased leaves	13.10±0.22^{a}	15.00±0.35^{a}	13.80±0.57^{a}	14.70±0.44^{ab}
Viruliferous-BPH	On RRSV-free leaves	13.10±0.22^{a}	14.80±0.27^{a}	13.70±0.274^{a}	15.10±0.22^{b}
	On RRSV-diseased leaves	13.70±0.27^{b}	15.60±0.22^{b}	15.30±0.44^{b}	15.90±0.22^{c}
	BPH status (A)	0.01*	0.03*	0.01*	< 0.00*
	Rearing status (B)	0.01*	0.00*	0.00*	0.01*
	A × B	0.01*	0.03*	0.00*	0.03*

RRSV = Rice ragged stunt virus; ns = not significantly different; * = significantly different ($p< 0.05$)
Values (mean ± SD) in the same column superscripted by different lowercase letters are significantly different ($p< 0.05$).

Table 4 The developmental periods of egg and instar nymph stages of the brown planthopper (BPH) reared on rice plants at different RRSV-inoculation periods.

Days after inoculation (DAI)		Developmental periods of immature BPH stages (days)					
		Egg stage	Instar nymph stage				
			First	Second	Third	Fourth	Fifth
Vegetative growth							
10	Initial tillering stage	9.80±0.44^{a}	3.10±0.22^{a}	3.10±0.22^{abc}	3.30±0.27^{a}	3.20±0.27^{abc}	3.20±0.44^{ab}
20	Early tillering stage	10.00±0.00^{a}	3.00±0.00^{a}	2.80±0.27^{a}	3.20±0.27^{a}	3.00±0.00^{a}	3.40±0.22^{abcd}
30	Middle tillering stage	9.40±0.54^{a}	3.10±0.22^{a}	2.90±0.22^{ab}	3.10±0.22^{a}	3.30±0.27^{abc}	3.30±0.27^{abc}
40	Final tillering stage	9.80±0.44^{a}	3.20±0.44^{ab}	3.10±0.22^{abc}	3.20±0.27^{a}	3.10±0.22^{ab}	3.10±0.22^{a}
Reproductive growth							
50	Initial panicle stage	9.80±0.44^{a}	3.30±0.44^{ab}	3.20±0.44^{bcd}	3.10±0.22^{a}	3.10±0.22^{ab}	3.20±0.27^{ab}
60	Middle panicle stage	9.40±0.54^{a}	3.60±0.22^{bc}	3.40±0.22^{cd}	3.20±0.27^{a}	3.500±0.354^{bc}	3.50±0.35^{abcd}
70	Flowering stage	9.60±0.548^{a}	3.60±0.54^{bc}	3.40±0.22^{cd}	3.50±0.35^{ab}	3.60±0.41^{c}	3.80±0.27^{d}
Ripening growth							
80	Milk stage	10.00±0.00^{a}	3.90±0.22^{c}	3.40±0.22^{cd}	3.80±0.27^{b}	3.40±0.22^{abc}	3.70±0.27^{cd}
90	Initial dough stage	9.40±0.54^{a}	3.90±0.22^{c}	3.50±0.00^{d}	3.40±0.41^{a}	3.60±0.41^{c}	3.60±0.22^{bcd}
	p-Value	0.18^{ns}	<0.00*	0.00*	0.01*	0.01*	0.00*

RRSV = Rice ragged stunt virus; ns = not significantly different; * = significantly different ($p< 0.05$)
Values (mean ± SD) in the same column superscripted by different lowercase letters are significantly different ($p< 0.05$).

Table 5 Survival periods of adult stages of brown planthopper (BPH) reared on rice plants at different RRSV-inoculation periods.

Days after inoculation (DAI)		Survival periods of adult BPH stages (days)			
		Short-winged (SW) brachypterous form		Long-winged (LW) macropterous form	
		Male	Female	Male	Female
Vegetative growth					
10	Initial tillering stage	13.70±0.44^{ab}	16.10±0.96^{d}	14.30±0.27^{ab}	14.80±0.75^{a}
20	Early tillering stage	13.30±0.27^{a}	15.90±0.41^{cd}	14.20±0.27^{a}	15.30±0.57^{abc}
30	Middle tillering stage	13.40±0.54^{a}	15.10±0.65^{abc}	14.80±0.44^{bc}	14.80±0.57^{a}
40	Final tillering stage	14.20±0.44^{bc}	15.40±0.65^{abcd}	14.60±0.65^{abc}	14.90±0.41^{ab}
Reproductive growth					
50	Initial panicle stage	13.70±0.27^{ab}	15.70±0.83^{bcd}	14.70±0.27^{abc}	15.10±0.22^{abc}
60	Middle panicle stage	13.70±0.57^{ab}	14.80±0.57^{ab}	15.00±0.00^{c}	15.50±0.35^{abc}
70	Flowering stage	14.40±0.54^{c}	14.90±0.41^{ab}	14.50±0.35^{abc}	15.60±0.41^{bc}
Ripening growth					
80	Milk stage	14.30±0.27^{bc}	15.00±0.79^{abc}	14.80±0.44^{bc}	15.70±0.44^{c}
90	Initial dough stage	14.70±0.44^{c}	14.70±0.27^{a}	14.90±0.22^{c}	15.40±0.41^{abc}
	p-Value	<0.00*	0.01*	0.02*	0.02*

RRSV = Rice ragged stunt virus; ns = not significantly different; * = significantly different ($p < 0.05$)
Values (mean ± SD) in the same column superscripted by different lowercase letters are significantly different ($p < 0.05$).

Plant viruses can induce a wide variety of plant responses, such as systemic necrosis, plant pigmentation and structural change, imbalanced or excessive accumulation of photoassimilates, and inhibition of host photosynthesis. These responses might lead to changes in vector physiology, feeding, and survival behaviours; however, little is known about the viral effects on the life parameters and reproduction of BPH on host plants. The BPH vector is an economically important pest that has a significant impact on Asian rice yield loss damage. In Thailand, the preliminary BPH outbreak in irrigated rice growing areas was recorded as a serious problem in 1973, and dramatically significant and large outbreaks occurred in 1989. The BPH vectors need host and food plants that are suitable for sap-feeding and living to complete their life cycle, which is related to the complexity of plant–virus–vector interactions and biological and ecological fitness.

Our results demonstrated that there were significant interactions of the developmental periods on the second-instar nymph BPH stage and wing dimorphisms [brachypterous (SW form) and macropterous (LW form)] and sex difference (male and female) of adult BPH stages between the nonviruliferous and viruliferous BPH status or between viral-free and infected rice plants. In addition, the effects of different RRSV inoculation periods of rice plants were shown to be significantly different at the developmental periods of the first- to fifth-instar nymph BPH stages and on the survival periods of adult BPH stages. However, the external morphological characteristics of egg, instar nymph, and adult stages were not different between the statuses of nonviruliferous and viruliferous BPHs that infested viral-free and infected rice plants.

We have three hypotheses for several possible explanations. First, the infection of RRSV can continually persist in host cells, which occurs throughout the life of the insect vector [8] without changing the external morphology of the insect. This hypothesis is related to the biologically connected pathways of insects, such as multifaceted innate immune systems, RNA interference (RNAi), autophagy, and apoptosis [29], thereby affecting viruliferous BPHs as asymptomatic carriers and active transmitters. Second, RRSV did not pass to the BPH egg. This hypothesis is related to the mechanism of ovarian infection and transovarial (or transovarian) transmission (TOT) of persistent plant viruses in insect vector [30]. The RRSV coat proteins did not interact with vitellogenin (Vg), which is the precursor of the major yolk protein (MYP) of the insect ovary for developing insect embryos [31]. Therefore, RRSV cannot effectively overcome the transovarial transmission barriers and spread into the BPH ovary, resulting in the production of the status of non-infectious BPH populations after hatching. Several virus–vector systems, such as RGSV-BPH (*N. lugens* Stål, 1854) [32] and SRBSDV-WBPH (*S. furcifera* Horváth, 1899) [15], similarly operate. Third, after infection, plant viruses change plant compounds and morphologies, resulting in the plant feeding behaviour of insects [33]. All of these may cause plant–virus–vector interactions in discontinuous trends for the developmental and survival periods of each life cycle stage of BPH. However, the confirmation and validation of these explanations will require additional research in rice fields.

Viruliferous BPHs were longer, and they fed on (sucked out) phloem sap more than nonviruliferous BPHs. This phenomenon is important for RRSV persistent transmission by spending more time on stylet salivation during plant–virus–vector interactions. Infected rice plants have a higher potential to produce a viruliferous-vector status and serve as viral sources for longer periods than viral-free rice plants. The phenotypic changes of host plants involved alteration of vector orientation, quality, palatability, and transmission. In addition, the infected plants were determined by the concentration of plant photoassimilates in source rice plant leaves. They may not markedly decrease in the RRSV-diseased leaves because of the positive correlation between increased RRSV accumulation and symptom expression in the growth phases and stages of the rice plants.

Viral-infected plants can alter the plant sap–nutrient content and subsequently change the vector feeding parameters of those plants. This phenomenon has revealed that after infection on the host plants, vector suitability, the population trend index, and reduction of plant nutrition value were affected by leaf nitrogen concentration (LNC) and plant morphology [34]. Rice-tillering vegetative stages have a high nitrogen (N) nutritional value. N is the most important plant nutrient and a major component of chlorophyll (Chl) and protein, which plays a key role in the process of photosynthesis. However, the considerable variation of Chl and N content regularly leads to the degree of rice leaf greenness alteration, size, shape, growth phases and development, yield, and quality [35]. Rice host plant quality defines host plant components, which is clearly a potential threat to insect behaviour, causing a positive or negative performance in the survival rate and developmental period pattern and depends on both environmental characteristics and internal physiological processes. Changes in host plant quality are induced by damage from insect feeding behaviours and viral infections, which resulted in the reduction of pre-survival periods and an increase in developmental periods.

The differences in the lifespan of BPH populations affected the vector and the propagation of RRSV. However, the most important concept of normal practice to remove RRSV-diseased plants from the rice field is to remove not only the potential transmission sources of viruses but also a source of enhancing vector population growth. In our view, RRSV infection was caused by the inconstancy of developmental and survival periods of each BPH life cycle stage. The plant–virus–vector interactions, as revealed by this study, might offer some advantages to the BPH in its competitive displacement of RRSV and can be used as a guideline hypothesis for BPH survival on viral rice plant variation. Therefore, further careful studies are needed under irrigated rice field conditions, and environmental approaches will be developed for the successful management of RRSV and insect vectors.

4. Conclusion

Persistently transmitted RRSV can enhance the rice host plant quality to attract the BPH vector and increase viral-ability and transmissibility. We primarily attempted to evaluate the interaction effects between the BPH status of nonviruliferous and viruliferous populations and the rice plant status of the viral-free leaves and RRSV-diseased leaves on the developmental and survival periods of each life cycle stage. The results showed that the second-instar nymph BPH stage and adult BPH stages showed significant interactions between the statuses of BPH and rice plants. The effects of different RRSV inoculation periods of rice plants in the nonviruliferous BPH population on the developmental periods of the instar nymph BPH stages and on the survival periods of adult BPH stages were shown to be significantly different. Thus, a better understanding of the developmental and survival periods of BPH on virus–plant interactions would improve insect control capability and agroecosystem management policies and strategies in Thailand.

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