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The Infectivity Survival and Transmissibility of *Rice ragged stunt virus* from the Frozen-Infected Rice Leaves by the Brown Planthopper, *Nilaparvata lugens* Stål

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Abstract

Rice ragged stunt virus, which was transmitted by the brown planthopper (*Nilaparvata lugens* Stål), was an economically important rice plant pathogen, and caused the yield losses of 10 - 100 % in Thailand and Asian rice cultivation areas. The purpose of this research was to assess the infectivity survival and the transmissibility of *Rice ragged stunt virus* from the frozen infected rice leaf tissues to the hosts, and the efficiency detection by the indirect enzyme-linked immunosorbent assay on nitrocellulose membranes. The result was shown that rice virus was successfully acquired from the infected rice leaf frozen samples at – 20 °C (7 days and 10 months) by the insect vectors, which the reactions can be clearly detected on the nitrocellulose membranes. The efficiencies of the insect vector inoculated, and the rice plant transmitted were 60 - 100 % and 20 - 100 %, and the direct variations with increasing of the viral-minimal latent period at the 3 and 10 days after inoculation, respectively. The maximum efficiency detection of crude sap sample dilutions of both viral hosts in the insect vector and the rice plant were the 1:16 to 1:64, and 1:64 to 1:256, respectively. These new idea transmission methods can be applied for the long-term stability, viability and infectivity of the infected samples, the laboratory protocol development of the cryopreservation techniques for the future study purpose of a plant virus, the viral-resistance breeding process, and the impose rice plant protection policies and strategies in Thailand.

Keywords: *Rice ragged stunt virus*, Brown planthopper, Infectivity survival, Indirect NCM-ELISA, Minimal latent period

Introduction

Rice ragged stunt virus (RRSV, *Oryzavirus*: *Reoviridae*) was one of the most seriously re-emerging rice plant pathogenic virus in the irrigated rice-cultivation system of the central plain and lower northern regions of Thailand and caused about the 10 - 100 % yield losses, which was transmitted by a sap-sucking insect vector, the brown planthopper (BPH), *Nilaparvata lugens* Stål (order Homoptera: Family Delphacidae) in the persistent-propagative manner [1,2]. RRSV was originally discovered in Indonesia in 1976 [3] and in the Philippines in 1977 [4], respectively, and spread in various Asian rice cultivation areas, such as Malaysia in 1977 [5]; India, Sri Lanka [6], and Taiwan [7] in 1978; China [8], and Japan [9] in 1979; and Vietnam in 2005 - 2007 [10], respectively. In Thailand, RRSV caused the 1st yield loss about 32 ha [ha (hectare), 1 ha = 6.25 Rai)] in the rice (var. RD7) of Bang-Nam-Priao district, Chachoengsao province, in 1978 [1,11], which was known as Rok-Bai-Ngik by Thai plant pathologists and Rohk-Joo (or Rohk-Haeng, Mai-Ok-Look, and Dtaai-Kaa-Gra-Bprohng) by local Thai farmers. The high damage levels were observed in 1979, 1980 - 1982, 1988 - 1990, 1998 - 1999 and 2009 - 2010 [12], and they have been the sporadic outbreaks from 2014 to present, respectively.

RRSV is the non-enveloped, icosahedral double-layered capsid protein shells of complete and core viral particle approximately 75 - 80 and 50 - 60 nm in diameter, respectively, and the regular surface structure consists with A and B spike is that a critical determinant of major inducer for viral-host interactions [3,13]. The genome of RRSV is about 26.164 Kb [14] with molecular weight (MW) ranging from 0.76×10^6 to 2.46×10^6 Daltons and containing multiple segments of 10 linear-dsRNA and conserves nucleotide sequences with 5' GAUAAA- and -GUGC 3' [15], which encode at least 8 structural proteins (SPs), P1, P2, P3, P4a, P5, P8a, P8b and P9, and 3 non-structural proteins (Pns), Pns6, Pns7 and Pns10, respectively [16]. Many researchers reported that plant viruses transmitted successfully to both hosts of plants (symptomatic host), and insect vectors (asymptomatic host) by direct injection [17] of vectors feeding through the membranes on liquid viral solution preparations [18], and of vectors feeding on the fresh viral-infected plant samples. All these methods relied on the fresh samples, which were required for exploration, collection and preparation before using in a research.

However, only vector transmitted from one host plant to another by the BPH vector as soon as possible, which limited its application in viral genetic researches, viral biological activities, crop improvements and breeding strategies for rice disease resistance. A simple, rapid, less cost, high sensitivity, strong specificity and reliable detection methods were developed, through which the non-viruliferous BPH status acquired RRSV from the frozen infected rice plant leaves and transmitted to the viral-free rice plants, respectively. Here, our research presented in the main topic for conducting by the indirect enzyme-linked immunosorbent assay on nitrocellulose membranes (Indirect NCM-ELISA) and included; (1) Assessing the minimal latent period of RRSV in both hosts, and (2) Infectivity survival and transmissibility from the different storage conditions of frozen and non-frozen infected rice plant leaves, respectively. These finding were provided the useful information for rice plant protections and research applications, which were important and met the requirements of the agricultural policy and strategy, and managing rice plant disease caused by RRSV in the outbreak areas of Thailand.

Materials and methods

The viral resource

RRSV was collected from rice plants showing specific morphological symptoms from the irrigated rice field in Nong-Suea district, Pathum Thani province, Thailand, which were obtained by the courtesy from the Division of Rice Research and Development, Rice Department, and identified as RRSV by the Indirect NCM-ELISA. Next, the infected rice plant leaves were cut into the long pieces (5 - 7 cm), pre-cleaned and rinsed by the tap water and deionized water, respectively and then, stored in the different conditions of the frozen (–20 °C) and refrigerator (4 to 8 °C) before experiments. The others were planted in a greenhouse for symptom observation.

The vector populations

During 2015 and 2016, the researchers at the Division of Rice Research and Development, Rice Department collected the expected viruliferous and non-viruliferous BPH populations from the light trap in the rice field in Nong-Suea district, Pathum Thani province, Thailand. Next, the BPH populations were maintained in the insect raring cages (16×16×24 inches) under the greenhouse conditions [26 ± 1 °C, 70 - 90 % relative humidity (RH), and L8 h:D16 h photoperiod] on the rice plant seedlings, *Oryza sativa* L. variety RD-7 (7 - 10 cm height, 6 - 9 days after germination, DAG). To obtain the experimental BPH populations, the mature females were transferred for an oviposition process. Next, after 48 h, the infested rice RD-7 seedlings were replaced and the rice seedlings with the BPH-eggs were cultured in the insect-free cages for the continuous producing non-viruliferous BPH populations of a stock culture before the experimental use, which the presence of non-viruliferous BPH stock culture was pre-tested by the Indirect NCM-ELISA before a transmission, respectively.

The infected rice plant and BPH sample preparation

The preserved samples of the infected rice plant leaves were placed and thawed in the Petri dishes containing the wet-absorbent cotton that retained moisture for at least 1 h, and spread out completely. Then the pieces of infected rice leaves were transferred into the beaker containing wet-absorbent cotton bedding. About the 250 non-viruliferous BPH populations of the 3rd instar-nymphs, which had been starved (fasting period) for 1 h, and inhaled the anesthetic by CO_2 for 3 - 5 min, were placed onto the infected rice leaves in a beaker with a fine brush. And then, the beaker was sealed with filter cloth and placed under a greenhouse condition. Finally, after 48 h of the acquisition feeding period (AFP), the surviving BPH populations were transferred from the infected rice leaves in the beaker with a fine brush and were calculated.

The transmission experiments

The surviving BPH populations after infestation were transferred and reared on the viral-free *Oryza sativa* L. variety RD-7 rice seedlings in the insect raring cages under greenhouse condition for the latent period (LP), and the rice seedlings were changed after 50 % leaf wilting. Next, the 5 infected BPH populations were removed with a fine brush every day in order to pass the virus detection through a LP in a vector. The negative controls (NC) of both frozen and non-frozen infected rice leaves were the unfed BPH populations by placing on the wet-cotton and fresh viral-free rice leaves. After the LP, the BPH populations were transferred to the viral-free Taichung Native 1 (TN1) rice plant seedlings (7 - 10 cm height, 6 - 9 DAG) for the viral inoculations. A single rice plant seedling sample was planted, and each sample was inoculated with 1 BPH vector. Then the BPH was removed after 48 h of inoculation access period (IAP), and the rice plant seedlings were treated routinely with insecticide and grown under the greenhouse conditions. Next, the 5 infected rice plant seedlings were removed every day in order to pass the viral detection through the LP in the rice plants. Negative controls were transmitted rice plants of the BPH fed on wet-cotton and non-viruliferous BPH populations, and the non-frozen RRSV infected rice leaves were done the same as the frozen one.

The detection of RRSV in the viruliferous BPH vector and rice plants

The indirect NCM-ELISA was modified from the method of Hibi and Saito [19], and Na Phatthalung [20], which the reagents and solutions were shown on **Table 1**. The infected samples were collected. The rice plant tissues (1 g) were ground in the 2 mL of the Plant sap extraction buffer (Plant-EB) solution and transferred into the 2 mL eppendorf tube (EPP). Then the BPH population was ground in 0.2 mL of the BPH sap extraction buffer (BPH-EB) solution by using a sterile pipette tip in the 2 mL EPP tube and pipetting up and down. Next, the crude sap samples were homogenized by a vortex mixer, and centrifuged at 16,532×g and 4 °C for 10 min (Centurion Scientific, Ltd.). After that, the supernatant was transferred into a new EPP tube, and diluted with the Dilution buffer (DB) solution at 2-fold serial dilutions (1:2 to 1:4,096), respectively. A NCM sheet (pore size 0.45 µm, Bio-Rad, Catalog No. 1620115) was prepared into a square of 1×1 cm^2, and then, immersed in the DB solution, and placed on the dried Whatman® filter paper No.1 to reduce the excessive solution. The washing step was washed 3 times with the Washing buffer (PBS-T) solution, and then, the 5 µL of the tested and the 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 (PBS-T-SK) solution, and shaken 10 rpm at room temperature for 30 min. After washing step, the anti-RRSV IgG solution was added and incubated at 4 °C for overnight. Following by the washing step, the dotted-sheet was incubated in the goat anti-rabbit serum conjugate alkaline phosphatase (GAR-AP) solution at 4 °C for 3 - 5 h. The washing step was performed before the reactions were visualized with the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) solution after an 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 the deionized water for 10 min. The positive result was detected by observing the development of blue-purple colour on the blot in 1 h, respectively.

Table 1 The reagents and solutions for RRSV detection by the indirect NCM-ELISA.

Ingredients	
Plant sap extraction buffer (Plant-EB)	0.01 M phosphate buffered saline (PBS), pH 7.4
BPH sap extraction buffer (BPH-EB)	0.01 M PBS (pH 7.4), 2 % polyvinylpyrrolidone (PVP)
Dilution buffer (DB)	0.01 M PBS (pH 7.4)
Blocking buffer (PBS-T-SK)	0.01 M PBS (pH 7.4), 0.5 % Tween-20, 5 % skimmed milk (SK)
Washing buffer (PBS-T)	0.01 M PBS (pH 7.4), 0.5 % Tween-20
Antiserum buffer (Anti-RRSV IgG)	Anti-RRSV IgG diluted 1:1,000 in PBS-T-SK
Enzyme buffer	Goat anti-rabbit serum conjugate alkaline phosphatase (GAR-AP, ZyMax™) diluted 1:5,000 in PBS-T
Substrate solution	BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) alkaline phosphatase substrate (SIGMA*FAST*™) (pH 9.5)
Stop solution	Deionized water

Results and discussion

The pathogenicity of RRSV from its viruliferous hosts of BPH vector and rice plant

The pathogenicity associated with a persistent propagative way of RRSV infection on its susceptible hosts, which can be established and multiplied in the BPH vector of instar nymph and adult developmental stages after an infestation, but not transmitted through the eggs of the viruliferous adult BPH females. However, that are virtually external asymptomatic (**Figure 1**), whereas in the rice host plants showed that continuity symptomatic developed after the inoculation (**Figure 2**) based on the varieties of rice, dates of infections, stages of growths and rice field managements.

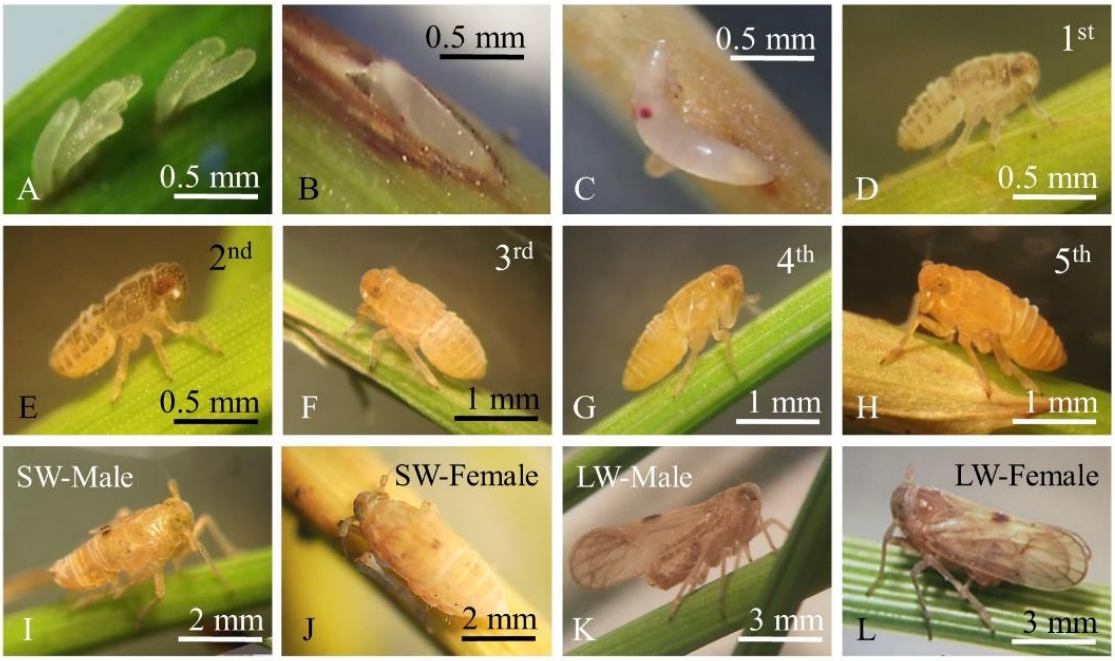

Figure 1 The BPH vector developmental stages.

Notes: (A) The groups of inserted eggs were in the rice plant tissues (1 days after oviposition, DAO). (B) The eggs were inside the leaf sheath tissues with dark brown incision area and modified further as follows: (C) The egg-banana shape with red eye spots, (D - H) developmental stages (day after hatching, DAH) of the 1st to 5th instar nymph at 3, 5, 8, 11 and 15 DAH respectively, (I and J) developmental stages [day after molting (DAM) and developing through 5 instars] of the adult stages of short-winged (SW) brachypterous forms of male and female and (K and L) the adult stages of long-winged (LW) macropterous forms of male and female at 7 DAM, respectively.

The natural host plants of RRSV were weeds or cereals, as the BPH vector survived and reproduced mainly in rice plants (*Oryza sativa* L.). The rice host plants infested with RRSV viruliferous BPH vector, fed previously on either frozen, non-frozen or fresh infected rice plants for 48 h of the IP and began to express the typical symptoms after the 8 - 13 days after inoculation (DAI), and clear symptoms after the 25 DAI, respectively. The experimentally infected rice plants were similar to those occurring in naturally infected rice plants. The researchers reported that the rice resistant varieties were resistant to viral-infection and did not multiply [21]. In addition, the heavy BPH vectors fed directly on the rice plant and caused yellowing and complete drying (withering) of plants as shown by symptoms referred to as hopper burn.

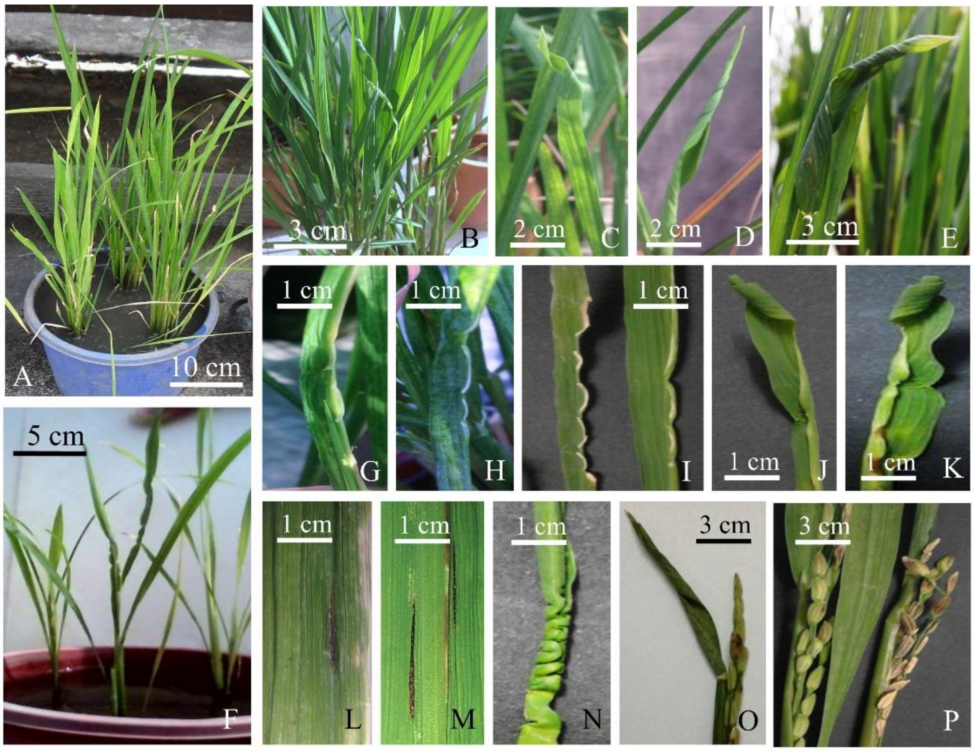

Figure 2 The symptoms of RRSV-infected cultivar TN1 rice plants.
Notes: (A and B) Rice tillering morphology after RRSV infection at 50 DAI with excessive branching, (C - E) Dark-green twisted and serrated rice leaf tip, (F - K) Dark-green ragged and jagged rice leaf blades, (L and M) Vein-swelling or galls on leaf blade which caused by hyperplasia and hypertrophy (proliferation) of the phloem tissues, (N) Malformed and narrow-roll up leaf blade and (O and P) Incomplete panicles and dark-brown unfilled grains.

The specific symptoms on rice plant caused by RRSV infection that showed the rice plant stunt and excessive tillering at the nodes (**Figures 2A** and **2B**), dark-green twisted on leaf tips (**Figures 2C** to **2E**) and ragged leaf blades (**Figures 2F** to **2K**), and vein-swelling or galls on leaf blade (**Figure 2L** and **2M**). In addition, previous researchers demonstrated that the galls occurred variously of width, lengths, and vein colour on the leaf blades (21 %), surface of sheaths (72 %), and clum or shoot (7 %), which found during the late tillering and after [4,22]. Moreover, RRSV-induced galls on infected rice plants, were the same as *Rice black streaked dwarf virus* (RBSDV, *Fijivirus*: *Reoviridae*) transmitted by the small brown planthopper (SBPH: *Laodelphax striatellus*, Hemiptera: Delphacidae), *Southern rice black-streaked dwarf virus* (SRBSDV) transmitted by the white-backed planthopper (WBPH: *Sogatella furcifera*), and *Rice gall dwarf virus* (RGDV, *Phytoreovirus*: *Reoviridae*) transmitted by the rice green leafhopper (RGLH: *Nephotettix nigropictus*, Hemiptera: Cicadellidae) [23]. The reproduction stages were showed malformed or ragged and shortened flag leaves, and appeared the leaf yellowing (chlorosis or yellowing syndrome) which were the same as Rice grassy stunt disease (RGSD) caused by *Rice grassy stunt virus* (RGSV, *Tenuivirus*: *Phenuiviridae*) transmitted by the BPH vector [24], and Rice tungro disease (RTD) caused by the combination of rice tungro viruses (RTVs) of *Rice tungro bacilliform virus* (RTBV, *Tungrovirus*: *Caulimoviridae*) and *Rice tungro spherical virus* [RTSV (helper virus), *Waikavirus*: *Secoviridae*) transmitted by the rice green leafhoppers (GLH: *Nephottetix virescens*, Homoptera: Cicadellidae) infected rice plants [25]. In addition, the ripening stage was shown the delayed flowering with the incomplete panicle, nodal branch production, and unfilled grains (**Figures 2O** and **2P**), respectively.

The detection of RRSV in a single BPH vector fed on the rice leaf samples

The number of surviving-BPH after 48 h of the AFP that fed on rice leaf samples, including the frozen infected rice leaf samples (–20 °C: 7 days, 74.40 % and 10 months, 78.4 %) were less than those fed on the non-frozen infected (4 to 8 °C, 7 days: 92 %), fresh infected (97.2 %), and viral-free rice leaf samples (96.4 %), when compared with the wet-cotton (NC, no food: 12.4 %), respectively (**Table 2**).

The results showed that the BPH vector could feed successfully on the frozen rice leaf samples, due to the soft and moist rice leaf tissues after thawing under the experimental conditions. The BPH can be fed and suck up-out the sap from rice leaf tissues. After the LP experiments, the BPH survivors that had fed on rice leaf treatments were tested by the Indirect NCM-ELISA and shown that the 3-day LP to pass RRSV through a circulative period, the minimal latent period of RRSV (RRSV-MLP) in the BPH vector. **Table 2** shows that the preliminary insect vector efficiency (BPH-preliminary infection appeared, BPH-PIA) when fed on the frozen (–20 °C, 7 days and 10 months) and non-frozen (4 to 8 °C, 7 days) infected rice leaf samples. Then, they were compared with the BPH fed on the fresh infected rice leaf samples, the BPH-PIA rates were 60 and 100 %, respectively. However, no the BPH-PIA rates that had fed on the fresh viral-free rice leaf and wet-cotton (NC, no food) samples, respectively. In addition, the BPH-PIA percentage which were fed on the rice leaf treatment testing are increased between the 60 - 100 % after the BPH-LP increasing from 5 - 20 days testing. These results suggested that RRSV was acquired from the frozen infected rice leaf treatments and multiplied in the BPH vector for the survival and the biological inoculum potential (IP) to a viral transmission. The viruliferous-BPH efficiency was direct variation with increasing of the viral-LP and the relative virus infectivity titer in the BPH vector, respectively.

Table 2 The percentages of the viruliferous BPH vector, that had acquired RRSV by feeding on the rice leaf samples.

Treatments	**Total No. of BPH**	**No. of surviving BPH**	**Proportion and percentages of RRSV-viruliferous BPH vector (No. of positive/total) (N = 5)**					
Days of total latent period (LP) in BPH			**1**	**3*****	**5**	**10**	**15**	**20**
BPH fed on rice leaf samples:								
Frozen rice leaf samples (–20 °C, 7 days)	250	186 (74.40 %)	0/5 (0 %)	3/5 (60 %)	5/5 (100 %)	5/5 (100 %)	5/5 (100 %)	5/5 (100 %)
Frozen rice leaf samples (–20 °C, 10 months)	250	196 (78.40 %)	0/5 (0 %)	3/5 (60 %)	3/5 (60 %)	4/5 (80 %)	5/5 (100 %)	5/5 (100 %)
Non-frozen rice leaf samples (4 to 8 °C, 7 days)	250	230 (92.00 %)	0/5 (0 %)	3/5 (60 %)	5/5 (100 %)	5/5 (100 %)	5/5 (100 %)	5/5 (100 %)
Fresh infected rice leaf samples	250	243 (97.20 %)	0/5 (0 %)	5/5 (100 %)	5/5 (100 %)	5/5 (100 %)	5/5 (100 %)	5/5 (100 %)
Fresh viral-free rice leaf samples (NC)	250	241 (96.40 %)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)
Wet-cotton (NC)	250	31 (12.40 %)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)
Extraction buffer (NC)	-	-	-	-	-	-	-	-

Note: ***The minimal latent period (MLP) for detection of RRSV-viruliferous BPH crude sap by Indirect NCM-ELISA; Negative control (NC).

The efficiency detection of RRSV-MLP by the indirect NCM-ELISA

The positive reaction (blue-purple spot on the blot) could be clearly detected on a NCM by the Indirect NCM-ELISA and could be detected the RRSV-MLP in the crude sap of BPH vector at 3-days (**Figure 3**). There were acquired RRSV by either feeding on frozen and non-frozen rice leaf samples. The maximum dilutions of the viruliferous BPH crude sap, that were fed on the rice plant treatments, including the frozen (–20 °C, 7 days and 10 months) and the non-frozen infected rice plant (4 to 8 °C, 7 days), were at 1:64, 1:16 and 1:8, respectively. By comparing with the BPH fed on the fresh infected rice plant samples, the maximum dilution was at 1:64, whereas the negative reaction (brown spot on the blot) of treatments that had fed on the fresh viral-free rice plants and wet cotton (NC, no food) could not be detected on the NCM, respectively (**Figure 4**).

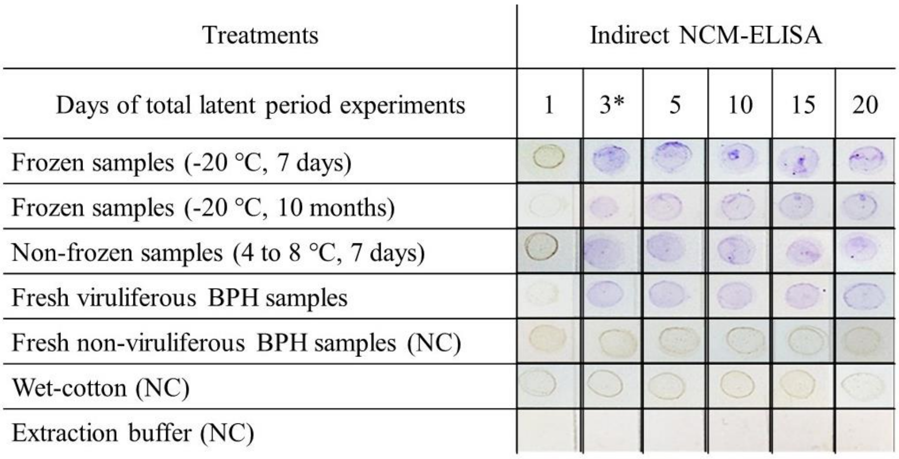

Figure 3 The detection of RRSV-viruliferous BPH vector by the Indirect NCM-ELISA.
Notes: Detection of RRSV-viruliferous BPH crude sap was fed on the infected rice leaf samples by the indirect NCM-ELISA, which the positive and negative reactions were shown the blue-purple and yellow-brown colour spots on the blots, respectively. *The RRSV-MLP in BPH crude sap samples at 3 DAI.

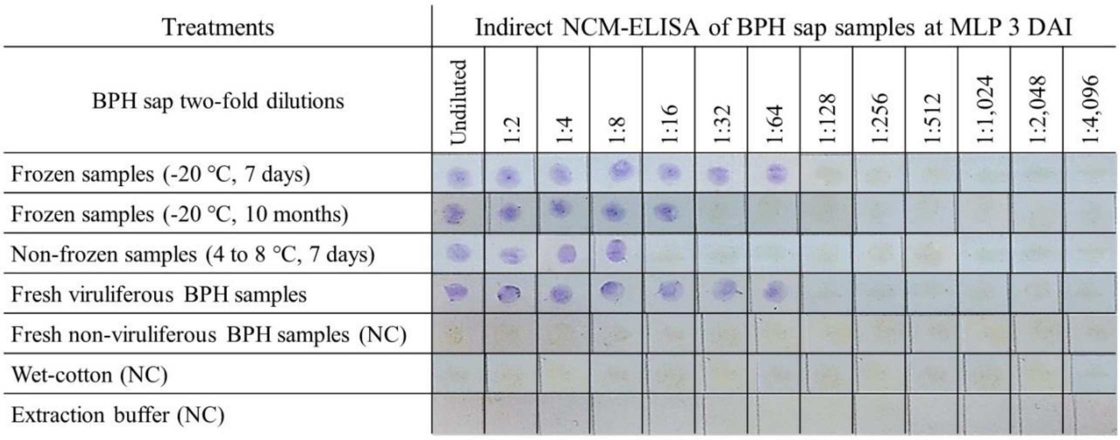

Figure 4 The efficiency detection of the RRSV-viruliferous BPH crude sap by the indirect NCM-ELISA.

The transmission experiment and RRSV detection in the rice plants

The RRSV-infected rice plants testing before asymptomatic, which were inoculated by a single BPH vector at the 3-days of RRSV-MLP and 48 h of the IP. These results were shown that, the efficiency transmitted from the BPH fed on the frozen (–20 °C, 7 days and 10 months) and the non-frozen (4 to 8 °C, 7 days) infected rice leaf samples, and the fresh infected rice leaf samples to the rice plants testing were 60, 20, 40 and 60 % at the 10-days test LP to pass RRSV through a replication in the rice plant cells, the RRSV-MLP in the rice plants (rice plant-preliminary infection appeared, RPIA), when the efficiencies transmitted from the BPH fed on the wet cotton (NC, no food) were compared with 0 %, respectively (**Table 3**).

The RRSV-infected percentages which were transmitted and increased between 60 - 100 % after the RRSV-LP increasing in the rice plants from 15 - 20 days testing. These results were demonstrated that the BPH acquired the RRSV virions from frozen (–20 °C, 7 days and 10 months) and non-frozen infected (4 to 8 °C, 7 days) rice plant treatments and transmitted to viral-free rice plants, replicated in the rice plant cells for the survival and biological inoculum potential (IP) to a viral transmission, and the host plants efficiency were direct variation with the increasing of viral-LP and the relative virus infectivity titer in the rice plants after 10-days.

The efficiency detection method of rice plant crude sap and the coloration effected on a NCM by the indirect NCM-ELISA. The positive reactions can be clearly detected on the NCM, which detected RRSV in the rice plant crude sap at the MLP (10 days) (**Figure 5**). The maximum dilutions of rice plant crude sap that transmitted by the RRSV-MLP at 3 DAI of the BPH fed on the rice plant treatments, including the frozen (–20 °C, 7 days and 10 months) and the non-frozen (4 to 8 °C, 7 days) infected rice leaf samples were at the 1:256, 1:64 and 1:32, respectively. By comparing with the fresh infected rice plant leaf samples, the maximum dilution was at the 1:256, whereas the negative reaction of treatments that fed on fresh viral-free rice leaf samples and wet cotton (NC, no food) and transmitted to rice plant testing could not be detected on the NCM, respectively (**Figure 6**).

Table 3 The percentages of RRSV-infected rice leaf samples, which were transmitted by the viruliferous BPH vector.

Treatments	**Proportion and percentages of RRSV-infected rice plants (No. of positive/total) (*N* = 5)**					
Days of total latent period in rice plants	**1**	**3**	**5**	**10*****	**15**	**20**
Frozen samples (–20 °C, 7 days)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	3/5 (60 %)	5/5 (100 %)	5/5 (100 %)
Frozen samples (–20 °C, 10 months)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	1/5 (20 %)	3/5 (60 %)	5/5 (100 %)
Non-frozen samples (4 to 8 °C, 7 days)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	2/5 (40 %)	3/5 (60 %)	5/5 (100 %)
Fresh infected plant samples	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	3/5 (60 %)	5/5 (100 %)	5/5 (100 %)
Fresh viral-free plant samples (NC)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)
Wet-cotton (NC)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)	0/5 (0 %)
Extraction buffer (NC)	-	-	-	-	-	-

Notes: ***Rice plants were transmitted by RRSV-viruliferous BPH vector (RRSV-MLP 3 DAI) after fed on rice leaf samples; Negative control (NC).

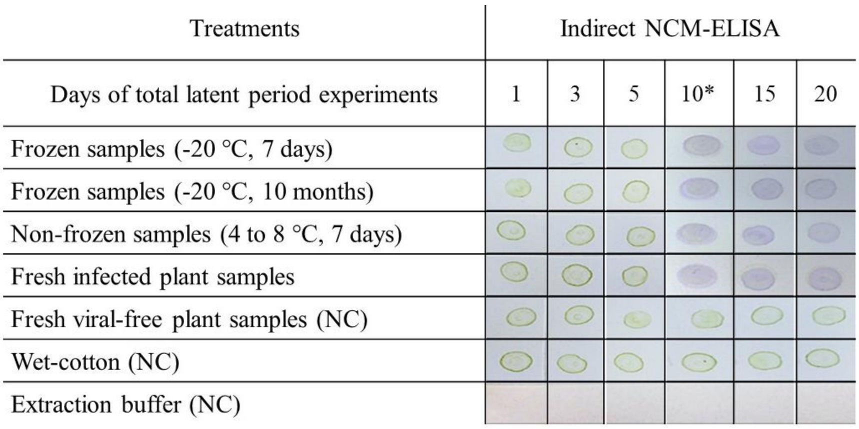

Figure 5 The detection of RRSV-infected rice plant crude sap by the indirect NCM-ELISA.
Notes: Detection of RRSV-infected rice plant crude sap, which were transmitted by the viruliferous BPH vector (RRSV-MLP 3 DAI) and detected rice plant crude sap by the indirect NCM-ELISA. The positive and negative reactions were shown the blue-purple and pale-green colour spots on the blots, respectively. *The RRSV-MLP in rice plant crude sap samples at the 10 DAI.

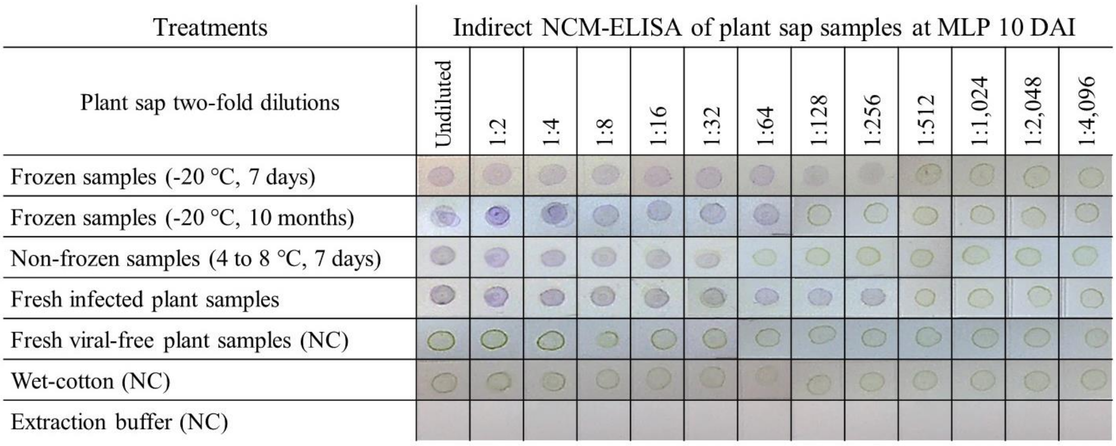

Figure 6 The efficiency detection of an infected rice plant crude sap by the indirect NCM-ELISA.

Many plant viruses depended on the piercing-sucking insect vector for their transmissions, which transmitted by different and specific feeding behaviours [26]. Like all other insect vector-transmitted plant viral diseases, the vector behaviour, spread and severity of the RRSV infection resulted from a complex interaction among numerous biotic factors or plant pathogen-vector-host interactions, which the living organisms were the pathogen-RRSV, BPH population density and specific hosts, for virus to colonize a multicellular host. In addition, the abiotic factors such as climate: e.g. temperature, wind, and rainfall, and human activity enhanced the virulence on host plants. These were the significant factors of the advance researches for the empirical data and knowledge management about the RRSV and developing insect-virus-resistant rice varieties. For our research, the main topics were identification, discussion, and summary as follows.

1) The duration of the RRSV-MLP in the susceptible both hosts of the BPH vector and rice plant, this phenomenon was very important for the successfully viral-transmission. Our results were shown that the RRSV-MLP in the BPH vector and range of crude sap dilution testing could be detected clearly at the 3 DAI (1:8 to 1:64). These results might depend on the RRSV-BPH interactions in a persistent-circulative (-propagative) manner, which can be replicated in the internal BPH organs, and infection route, and began during feeding on diseased plants [27,28]. RRSV must enter the epithelial cells of the BPH alimentary canal (AC) that consisted of oesophagus (Oe), midgut epithelium (MgE), midgut (Mg), hindgut (Hg), haemolymph (Ha), and salivary glands (Sg) at 1, 3, 3 to 4, 6 and 9 DAI, in 50, 30, 28, 26 and 40 % respectively. Next, the RRSV-virions were inoculated back to the plant hosts during feeding due to the BPH salivary glands that were principal secretory function for a biological success to transmission. In addition, the RRSV-MLP in the rice host plants and the range of crude sap dilution testing could be detected clearly at the 10 DAI (1:32 to 1:256). Many previous reviews and researches have been explained that the pathogenesis and transmission dynamics of plant viruses caused by the viral-host movement proteins (MPs) interactions for the intra- and intercellular viral movement through the plasmodesmata (PD) pathways for infection, long-distance transport *via* the phloem, and depended on the viral accumulation within host distribution.

2) The infectivity survival assessment of RRSV transmission were able to transmit virus from the frozen and non-frozen infected rice leaf samples to the BPH vector and rice host plant successfully. The survival of most plant viruses required the repeated infection of the host plants in terms of the continuous infection chains, which viruses transmitted from infected rice plants to another plants of the same species via a vector (alternate host). The icosahedron viruses had uniquely the stable structural characteristics and genome properties. This hypothesis supported the RRSV due to the non-enveloped icosahedral virus which has been double-viral capsid protein layers encode dsRNA genome. In fact, the non-enveloped virus was more virulent and survived in some disinfection processes as compared to the enveloped viruses [29].

Previously researchers reported that *Rice yellow mottle virus* (RYMV, *Sobemovirus*: *Solemoviridae*) could mechanically transmit from the frozen-infected leaves (–20 °C) [30]. In the same way, *Rice stripe virus* (RSV, *Tenuivirus*: *Phenuiviridae*) were acquired from the 2-year frozen infected rice leaves (–20 °C) and multiplied in the SBPH vector (*L. striatellus*) which in the 2 DAI and 7 LP, and transmitted to viral-free rice plants approximately 30 and 16.67 %, respectively, when compared with fed on the fresh infected rice plants and transmitted to viral-free rice plants approximately 36.67 and 23.33 % [31], respectively. In addition, the vector (*L. striatellus*)-RBSDV transmitted efficiency from the 10 months-frozen infected rice leaves (–70 °C) in the 2 DAI, 15 and 22 LP, approximately 20 and 71.43 % and could be transmitted to rice and maize host plants at the 22 LP approximately 37.5 and 50 %, respectively, when compared with the 28.57 and 71.43 % of the vector-LP treatment tests fed on the fresh infected rice plants, and the 50 and 66.67 % of viral-free rice and maize plants-transmitted efficiency [32,33], respectively. Moreover, RBSDV were acquired from the 232-days frozen infected plants and transmitted to the vectors [34], and the frozen extract of the SRBSDV-infected rice plants at the –80 °C and 220 days were successfully transmitted to the WBPH (*S. furcifera*) by a microinjection and caused the viruliferous ability about 63.3 % [35]. Although the transmission efficiency of RSV- and RBSDV-frozen infected samples are less than the fresh infected samples, but there are preliminary accepted which were indicated and supported that can act the ability for the long term survivability namely, months and years, infectivity an transmissibility in hosts.

The process of the RRSV transmission and RRSV-MLP experimental evidence were clearly detected in the BPH vector and rice plant at the 3 and 10 DAI, respectively. In addition, no difference between the BPH-egg and instar-nymph stage rearing on the infected and viral-free rice plants, but the BPH-adult stage rearing on the infected rice plants were about 5 days-shorter than the viral-free rice plants. These suggested that RRSV could be replicated in both hosts, which the minimum acquisition feeding period (MAFP) was 3 h (11 - 22 %), and the 2 and 6 DAI as RRSV antigenicity and multiplied in the BPH vector [36], respectively. The transmitted BPH as active transmitters, namely carrier or viruliferous insect after the 3 to average 8 MLP-DAI [11], which the BPH vector had ability about 40 % (range 14 - 76 %) and were also no difference observed in both nymphs (19 %, range 0 - 100 %) and adults, female (46 %, range 15 - 100 %) and male (42 %, range 6 - 91 %) adults, brachypterous (42%, range 16 - 100 %) and macropterous (48 %, range 0 - 100 %) forms [13]. However, the 5th (28 %) and 4th (26 %) instar nymphs showed the highest percentages of active transmitters followed by the 3rd (18 %), the 2nd (16 %), and the 1st (12 %) instar nymphs [37], respectively. A study was depicted that no significant differences of ability RRSV transmitted by the different BPH-biotypes [4]. The active BPH vector was sucked sap on the viral-free host plants for 1 h of the minimum inoculation (MI) or minimum transmission feeding period (MTFP) [11], which often failed to infect host plants although became old, and the non-active period averaging 7 days before an insect’s death [2], respectively.

Therefore, this result was the 1st report and novel method on the preservation and storage conditions of RRSV-infected host samples, which this paved the possibility for the long-term stability, viability and infectivity of the samples; developing of the cryopreservation techniques and protocols in a laboratory; maintaining the culture collection of plant viruses for a future study purpose; applying for the identification and facilitating the breeding process of rice plant variety resistance to RRSV and other rice viruses; and improving the pest-control and green modern rice technology strategy, respectively.

Conclusions

Our experimental results were showed successfully that the BPH vector could obtain RRSV from frozen infected rice leaves at –20 °C for 7 days to 10 months, and the pathogenicity pattern in both insect vector and rice plant host were the direct variation with increasing of the latent periods. According to the proportions of the inoculation tests, the BPH vector fed on the frozen infected rice leaves acquired about 60 % and up to 100 % after latent periods at the 3 and 5 DAI, which maximum dilution at the 1:64, respectively, whereas for the transmission tests with a single viruliferous BPH vector, fed previously on the frozen rice plant leaves about 20 % and up to 100 % after the latent periods at the 10 and 15 DAI, which maximum dilution at the 1:256, respectively. The new transmission method from the frozen RRSV-infected rice leaves could be applied to solve the problem under the intention and goals of the Thailand rice plant protection, and the research policy and strategy such as the rice plant variety resistant identification of RRSV, a breeding and the rice plant viral genetics research.

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