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Characterization of banana bunchy top virus on Sumatra (Indonesia) wild banana

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Abstract. Rahayuniati RF, Hartono S, Somowiyarjo S, Subandiyah S, Thomas JE. 2021. Characterization of banana bunchy top virus on Sumatra (Indonesia) wild banana. *Biodiversitas* 22: 1243-1249. Banana bunchy top virus (BBTV) has been a major constrain on banana production worldwide. This virus infection is mostly found in banana cultivation. The study aimed to characterize the BBTV on Sumatra wild bananas. The survey to collect seeds and typical symptomatic samples had been done in 2017 and 2019 in West Sumatra, South Sumatra, and Bengkulu. The transmission test of the virus was conducted by using the viruliferous vector on four wild banana species, while the identity of BBTV confirmed by nucleotide sequences analysis using primer pairs of DNA R and DNA S. *Musa acuminata* subsp. *sumatrana* (AA) have been found naturally infected by BBTV. The transmission confirmed on *M. acuminata* subsp. *longipetiolata*, *M. acuminata* subsp. *malacensis*, *M. acuminata* subsp. *halabanensis* and *Musa* sp. with typical bunchy top symptoms. The incidence on test plants were 40%, 80%, 20%, 20% and 20%, respectively. The virus infection on Sumatra wild bananas prolonged the incubation periods in comparison to cv Mas. BBTV of *M. acuminata* subsp. *sumatrana* is similar to the BBTV MK940788 and MN073184 from Sumatra. This is the first report of BBTV on Sumatra wild banana. The result can be used as a reference to study the resistance of the banana against BBTV.

Keywords: Banana bunchy top virus, BBTV, wild banana, characterization

INTRODUCTION

Banana and plantain are valuable food crops in the world, staple food in some or cash crop for local market and export, particularly in the developing countries. Banana is considered the fourth important global food, after rice, wheat, and milk (Tchatchambe et al. 2020). Indonesian export of banana decreased by about 22.35% from 2018-2019. However, the average banana production increased by around 0.22% (BPS 2019).

Banana Bunchy Top Disease (BBTD) is one of the major diseases in banana, caused by *Banana bunchy top virus* (BBTV). The virus is transmitted by aphid *Pentalonia nigronervosa* Coquerel, in a persistent non-circulative manner. The disease might spread a long distance by its sucker, corms, and plantlet of tissue culture during the trade among islands or countries (Wickramaarachchi et al. 2016). *Banana bunchy top virus* infects most banana cultivars with stunting and chlorosis/necrosis symptoms, resulting in major economic losses of the crop. This virus is regarded as the most economically destructive of plant virus disease and contributed up to 100% decreased yield of banana and plantain (Qazi 2016).

Banana bunchy top virus consists of six components of ssDNA each size ranging from 1018 to 1111 bp. They are

DNA-R, DNA-S, DNA-M, DNA-N, DNA-C, and DNA-U3 (Stainton et al. 2015; Wickramaarachchi et al. 2016; Kumar et al. 2017; Kakathi and Nath 2018). Each DNA (Deoxyribonucleic acid) has a specific function. DNA-R encodes a master replication-associated protein (M-Rep), DNA-S encodes the coat protein (CP) (Stainton et al. 2015; Wickramaarachchi et al. 2016; Arumugam et al. 2017). DNA-N encodes nuclear shuttle protein (NSP) (Baldodiya et al. 2019; Ji et al. 2019) and DNA-M acts as movement protein (Debbarma et al. 2019). Generally, BBTV is divided into two groups; the Asian or South East Asia and the Pacific or Pacific Indian Ocean Groups (Stainton et al. 2015; Wickramaarachchi et al. 2016; Das and Banerjee 2018). Detection of Indonesian BBTV was done and reported for the isolates from Java (Lestari and Hidayat 2018) and Bali (Wirya et al. 2020). A previous study analyzed the genetic of the Sumatra population and reported low diversity compared to those from Java and Bali. The BBTV isolates in Sumatra did not show any recombination however, the infection was higher in bananas with AAB or ABB than those in bananas with AA or AAA genomes (Chiaki et al. 2015).

Wild banana (AA) is a progenitor of the cultivated one and Sumatra island is one of the origins of wild bananas, hence, vital for conservation and preservation as the

genetic resources of cultivated bananas. Information about the resistance of wild bananas to BBTB is scarce. This study is the first report of bunchy top disease on the Sumatra wild banana and it focuses on characterizing the BBTB and confirmed the susceptibility of the host through transmission test.

MATERIALS AND METHODS

Survey and sampling

The survey of BBTB was carried out in Sumatra Island, Indonesia (Figure 1) during the years 2017 and 2019. Sumatra is a renowned origin of wild banana and available abundantly on the island. Purposive random sampling was adopted and conducted across West Sumatra, South Sumatra, and Bengkulu provinces, randomly collecting young banana leaves with the symptom of bunchy top directly from the field (Figure 2). Additionally, the infected, cultivated banana around the wild ones was subjected to observation and sampled across the sampling locations. All the collected leaf samples were preserved in the silica blue gel.

Banana bunchy top virus detection and molecular characterization

The collected leaf samples were subjected to DNA extraction, conducted at the Laboratory of Plant Protection at Universitas Gadjah Mada, Yogyakarta, Indonesia. Ten grams of dried leaf sample of each material was extracted using ISOLATE II Genomic DNA Kit (Bioline Reagents Limited, London, UK). Extraction method was following the instruction provided by the kit. The virus was detected by the Polymerase Chain Reaction (PCR) using two sets of specific primers (Table 1), according to Stainton et al. (2015).

The DNA was amplified using PCR Kit of MyTaq™ HS Red Mix (Bioline). A total of 12.5 µL reaction consisted of 1 µL of DNA template, 1 µL of primer sets, 5 µL of MyTaq Red Mix, and 5.5 µL of RNase free water. Initial denaturation was performed at 94°C for 1 min, continued with 35 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 30s, extension at 72°C for 1 min, and ended with the final extension at 72°C for 3 min. The electrophoresis was carried out using 1% agarose gel in 1× TBE buffer and visualized by staining using ethidium bromide (EtBr). Then the PCR products were sent to First BASE Laboratory, Malaysia for DNA sequencing.

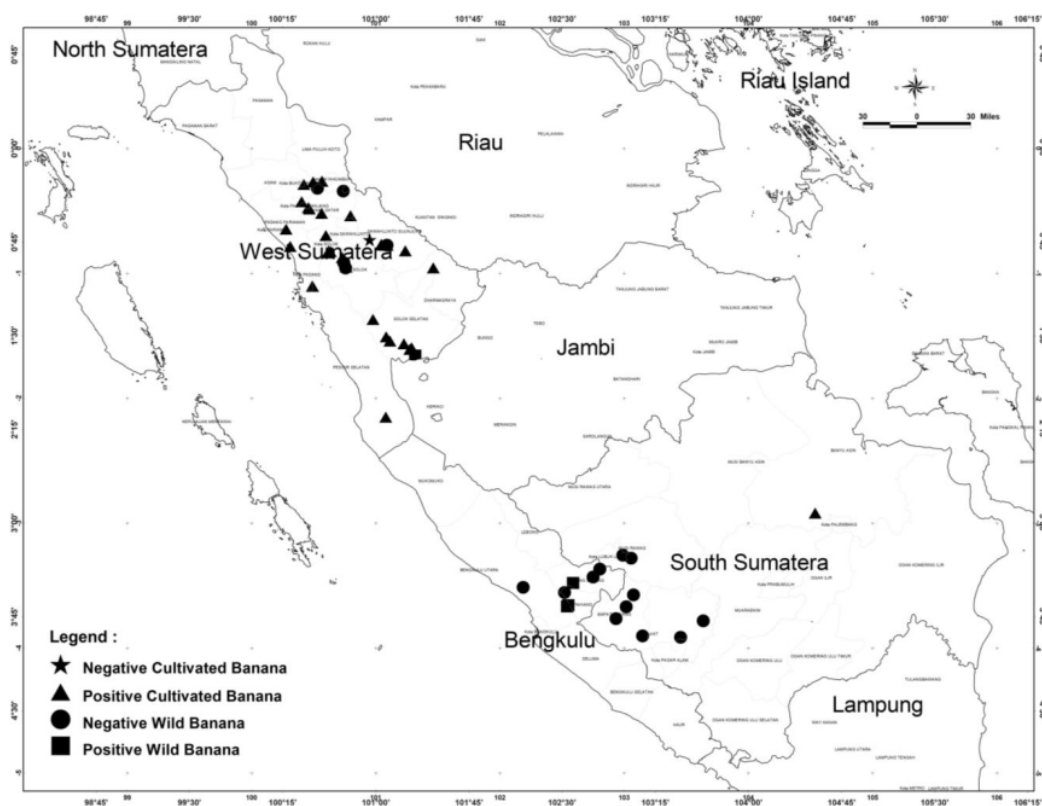


Figure 1. Sampling location of wild banana in West Sumatra, Bengkulu, and South Sumatra provinces, Indonesia



Figure 2. Disease symptom and severity on wild banana after inoculation. A: No symptom, B: Mild symptom, C: Intermediate symptom, D: Severe symptom

Table 1. Specific primer DNA R and DNA S BBTv

DNA target	Primer sequence	Amplicon size
DNA R	Forward: 5'-TTGAGAAACGAAAGGGRAGC-3' Reverse: 5'-GGTGTGCGCCTGGGAAG-3'	1111 bp
DNA S	Forward: 5'-GGTTCCTGAAAAYACCGTC-3' Reverse: 5'-AATATTGAYCCTARMGACGAG-YAGTC-3'	1075 bp

Sequence and phylogenetic analysis

Sequence contigs were assembled and aligned using Algorithm Clustal W of MEGA X program (Kumar et al. 2018). Grouping for BBTv was based on the DNA-R sequence analysis. The phylogenetic tree was constructed following the maximum likelihood method. The bootstraps of 1.000 replicates were used to verify the significance of the tree. *Banana bunchy top virus* sequence from GenBank MN073184, MK940788, AB847628, AF416468, AF416479, KM607663, KM607658, and KP876498 were used in DNA-R analysis. The DNA of *Abaca bunchy top virus* (ABTV) sequence EF546807 was used as the outer group.

Transmission test

Four species of wild banana, *M. acuminata* subsp. *malacensis*, *M. acuminata* subsp. *longipetiolata*, *M. acuminata* subsp. *halabanensis*, and *Musa* sp. were grown by seeds on the sterilized soil medium, consisted of compost and soil (1:1), and maintained in the screen house. A total of ten plants per species were under assessment. The transmission test conducted using the young plants a month after seedling. The BBTv inoculum was the isolate obtained from banana cultivar Mas and kept cultivated continuously in the greenhouse. *P. nigronevosa* mass rearing colony was used as the vector. The BBTv- infected banana cultivars Mas was used as the positive control.

According to Suparman et al. (2017) and Jebakumar et al. (2018), the transmission technique was done with a slight modification in the material usage, size, the old of the aphids, and the number of the aphids. Non-viruliferous (instar 3) aphids transferred from a healthy plant to the inoculum sources plant. After 48 hours of the acquisition period, the aphids were transferred individually using a wet paintbrush to the tested healthy seedlings. Each plant was infested with 20 viruliferous aphids, placed on the cigar leaf base. The inoculated plants were placed in a screen box and incubated in a room with a temperature range of

25-30° C for five days. On the last day of the incubation period, all the aphids were sprayed using insecticide with a 500 ppm *lambda cyhalothrin* active ingredient.

Leaf samples were collected from the infected single plant after 21-weeks post inoculation (WPI). Total DNA was isolated from the samples following the instruction on the ISOLATE II Genomic DNA Kit (Bioline Reagents Limited, London, UK). A back-to-back primer DNA-R of BBTv applied for polymerization reaction producing 1100 bp (Stainton et al. 2015). PCR products were run on 1% agarose gel, stained with ethidium bromide, and visualized under UV transilluminator.

Disease incidence

Disease incidence was assessed at the last observation and recorded based on the host's visual symptoms. The disease symptoms were inspected in all the plants, a single plant with bunchy top symptom was considered as infected (Ngatat et al. 2017). Disease incidence was calculated by the percentage of the infected plant over the total number of assessment plants.

$$\text{Disease Incidence} = \frac{\sum \text{symptomatic plant}}{\text{Total plant sample}} \times 100\%$$

Disease severity was observed weekly. A four-score infection was modified based on the visual symptom (Leiwakabessy et al. 2017; Wirya et al. 2020) (Table 2). The severity was calculated using a disease severity formula.

$$\text{Disease Severity} = \frac{\sum (n_i \times v_i)}{N \times Z} \times 100\%$$

Where;

n_i : infected sample

v_i : score at the time of observation

N : total plants

Z : highest score

Table 2. Level infection according to Leiwakabessy et al. (2017)

Score	Description of symptom
0	No symptom
1	Mild symptom. Limited vein clearing and dark green streaks on the lower part of lamina and petiole. No significant reduction of lamina width
2	Intermediate symptom. Vein clearing, upturned leaf, chlorotic, and ragged margins. Significant reduction in petiole length, distance, and lamina width
3	Severe symptom. Brittle lamina with upturned, chlorotic, and ragged margins, sometimes with necrotic symptoms. Leaves failed to emerge, giving a clear bunched appearance

Area Under Disease Progress (AUDPC)

AUDPC is measured based on the comparison between disease severity and time (Ngatat et al. 2017). It is calculated by the formula:

$$AUDPC = \sum ((y_i + y_{i+1})/2) \times (t_{i+1} - t_i)$$

Where; 'y_i' the proportion of the infected plant at the 'i' th observation, 't_i' is time in days at the 'i' th observation.

RESULTS AND DISCUSSION

Banana bunchy top virus characterization on wild banana

The wild bananas collected from West Sumatra, Bengkulu, and South Sumatra provinces were *Musa acuminata* subsp. *sumatrana*, *M. acuminata* subsp. *halabanensis*, *M. acuminata* subsp. *longipetiolata* and *M. acuminata* subsp. *malacensis*. Two *M. acuminata* subsp. *sumatrana* from West Sumatra and Bengkulu showed the specific symptoms of bunchy top disease. The initial signs were narrow leaves on the top of the plant, chlorosis on the leaves edge, and a dark green streak on the midrib and leaf stem (Figure 3). These symptoms had been reported as typical of banana bunchy top disease in Sumatra (Chiaki et al. 2015). It did not find any wild banana with typical bunchy top symptoms in South Sumatra during the survey.

Leaf samples were detected using the PCR method to ensure virus infection (Figure 4). DNA-R of BBTV on infected wild subsp. *sumatrana* from Bengkulu (W1) and West Sumatra (W2) was amplified at 1104 bp and the DNA S of W1 and W2 were amplified at 1058 bp. The infected cultivar Ambon Kuning cultivars from Bengkulu (C1) and West Sumatra (C2) was used as positive control and the healthy plant as negative control (N).

The length of DNA-R is 1104 bp and the ORF of DNA-R at the nucleotide number 103-963, encodes 287 amino acids responsible for viral putative replication initiation protein (Rep). DNA-S has a length of 1058bp and showed an ORF at nucleotide number 212-724 and encodes 170 amino acids that coding a coat protein (CP). The *sumatrana* isolates have two common CR-SL (TATTATTAC) regions at nucleotide number 13-21 and CR-M (GC rich region) in the intergenic region.

Sequence and phylogenetic analysis

According to Das and Banerjee (2018), it can be stated that BBTV of *M. acuminata* subsp. *sumatrana* from West Sumatra (GM 103047) and Bengkulu (GM 109003) is a member of the Asian/South East Asia Group. The tree shows the GM103047 share cluster with Ambon Kuning/Gros Mitchel (MN073184) from the same province. Additionally, the same BBTV is observed in two bananas from Bengkulu province, they are GM 109003 and MK 940788 (Figure 5). This phenomenon explained no differences between the isolates of wild bananas with the cultivated ones from the same area. Both *M. acuminata* subsp. *sumatrana* were group into two clusters based on the origins.

Transmission test

Virus infection on the transmission test was confirmed by a PCR using the BBTV isolate of Mas cultivar. The plant was observed after the vector was killed by insecticide. The disease incidence data are presented in Table 3, and the progression of the disease severity in Figure 6. At week 3, four plants of the subspecies *longipetiolata* showed the first symptom. The lamina margin showed chlorosis symptoms, meanwhile, dark green streak and dot emerged in the lower lamina and petiole. The new leaf decreased in size, length, and width, but the vein was clearing. The DNA R of the virus was amplified at 1104 bp. Disease severity of *longipetiolata* increased from 13.3 % on 2-3 WPI to 40% on 14-15 WPI, and the curve stabilized at 40% until 21 WPI. On week 4, cultivar Mas showed the initial symptoms of bunchy top disease. Also, mild symptoms were observed in both banana plants. Mas Cultivar showed 100% disease incidence with 93% disease severity at 20 to 21 WPI. However, the subspecies *malacensis* had a delayed first symptom for 10 weeks, followed by mild symptoms at 11 WPI with 6.67% severity. The severity increased up to 60% and stabilized on the 20 WPI; however, the disease incidence was up to 80%.

Musa sp. showed the first symptom on week 14. The disease severity increased from 6.67% on week 14 to 20% on week 18. The severity did not increase until week 21 when the disease incidence reached 20%. Subspecies *halabanensis* expressed the disease symptom in week 20 with disease severity 6.67% and incidence of the disease

about 20% in week 21. Furthermore, Figure 6 illustrates a slower response of Sumatra wild banana to the BBTv infection than that of Mas cultivar. They might have a distinct resistance response to the bunchy top virus. The response test to wild banana showed that subsp. *malacensis* is the most susceptible to BBTv. A considerably slow response to the virus infection was expressed by the subsp. *halabanensis*. Therefore, the subsp. *halabanensis* is evidently more resistant than the other wild bananas tested up to 21 weeks after inoculation. Among the wild bananas, the AUDPC indicated significant differences. Lowest AUDPC (3.33) was recorded on subsp. *halabanensis* and the highest was observed on subsp. *longipetiolata* (566.67).

Table 3. The Disease Incidence at 21-week post transmission

Banana sample	Disease incidence (%)	Disease severity (%)	AUDPC
<i>M. acuminata</i> cv Mas	100.00	100.00	1732.22
<i>M. acuminata</i> subsp. <i>longipetiolata</i>	40.00	40.00	566.67
<i>M. acuminata</i> subsp. <i>malacensis</i>	60.00	60.00	416.67
<i>Musa</i> sp	20.00	20.00	110.00
<i>M. acuminata</i> subsp. <i>halabanensis</i>	20.00	6.67	3.33



Figure 3. Banana bunchy top virus symptom on wild banana. A. Bunchy top disease symptom on *Musa acuminata* subsp. *sumatrana* in Bengkulu, B. Bunchy top disease symptom on *Musa acuminata* subsp. *sumatrana* in West Sumatra

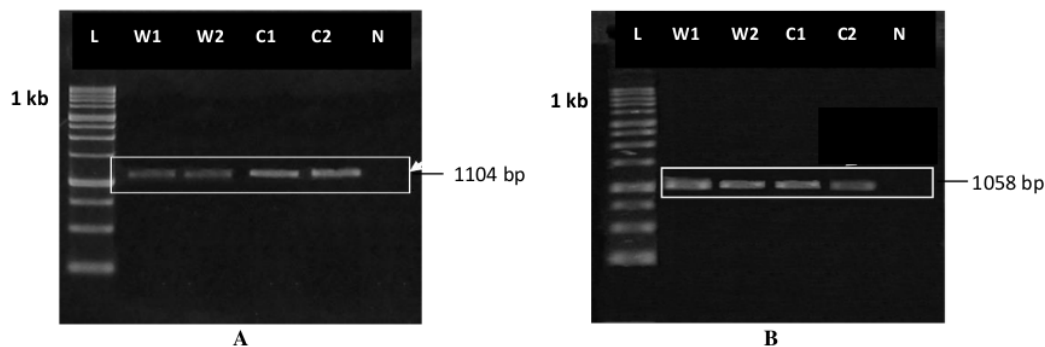


Figure 4. Visualization of DNA amplification of BBTv from wild banana and cultivated banana on 1% agarose gel. A. Amplification DNA-R of BBTv. B. Amplification DNA-S of BBTv. L= Ladder (1 Kb), W1= *M. acuminata* subsp. *sumatrana* (Bengkulu), W2= *M. acuminata* subsp. *sumatrana* (West Sumatra), C1= Banana cv. Ambon Kuning (Bengkulu), C2= Banana cv. Ambon Kuning (West Sumatra), N= Negative control (cv. Mas)

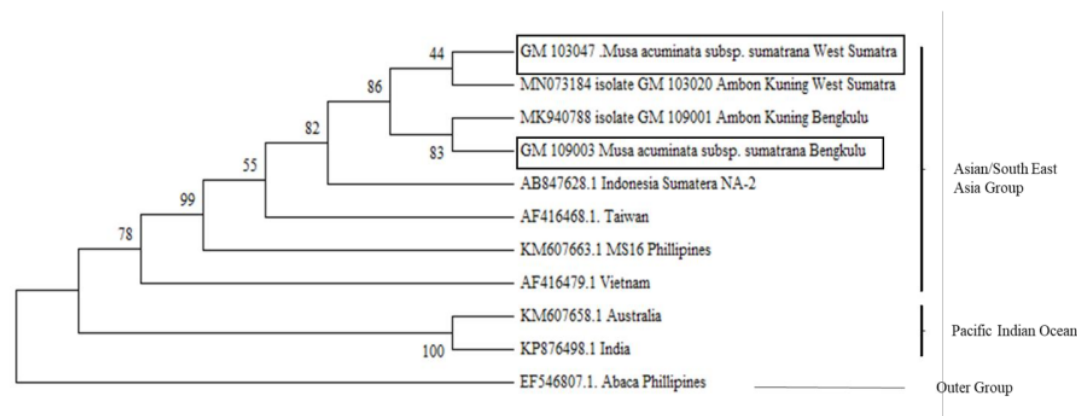


Figure 5. Phylogenetic tree showing the BBTV closely relationship of Sumatra wild banana to cultivated banana based on the DNA-R. Abaca Bunchy Top Virus on Abaca was included as an outgroup. The tree was inferred by using the maximum likelihood method with 1000-bootstrap replication

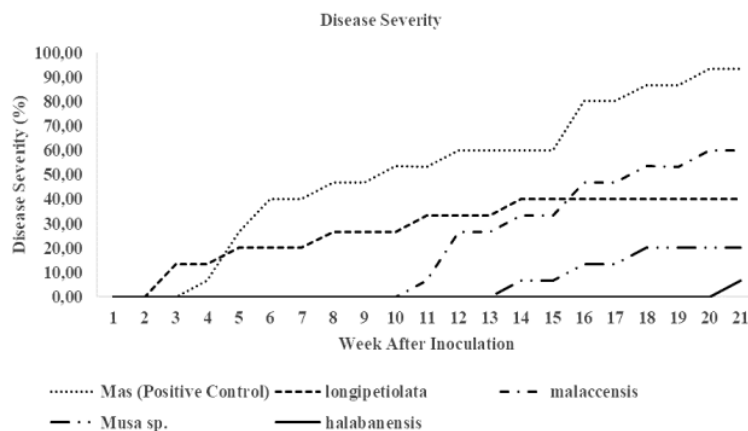


Figure 6. The development of disease severity per week

Discussion

Edible, cultivated bananas and plantains are descended from wild bananas and a hybrid of wild banana *M. acuminata* Colla (AA) and *M. balbisiana* Colla (BB). *M. acuminata* has more varieties than *M. balbisiana*. Indonesia is an area of banana origin with a high genetic diversity and known as the center of the species diversity, from the edible, seedless banana (cultivated banana) to the full-seeded banana. The diversity needs to be conserved and preserved in the natural population which is available for banana breeding (Poerba et al. 2019). Wild bananas *M. acuminata* (AA) are widely distributed in Sumatra island. The survey in West Sumatra, Bengkulu, and South Sumatra provinces identified the distribution of some subspecies of *M. acuminata* i.e. *malaccensis*, *longipetiolata*, *halabanensis*, *sumatrana*, and *Musa* sp.

The mats primarily spread and grow near rivers and in the rain forests, only a few grow near the villages and the main roads. Most of the habitats have changed to industrial estate crops. Based on the morphological symptoms, two clumps of subspecies *sumatrana* were found in West Sumatra and Bengkulu provinces. It confirmed a natural infection by BBTV and the putative replication initiation protein (Rep) encoded by DNA-R of the virus amplified at 1104bp in the subsp. *sumatrana*. This result indicated the ability of BBTV to infect and replicate their particles in the wild banana. The same genotype between wild *Musa* and the cultivated banana might affect the successful replication of the virus particles. Furthermore, the survey found that most of the infected bananas belong to the group of AA genotypes (AA, AAA, AAB).

According to the sequence of DNA-R, BBTv of the wild banana originated from West Sumatra and Bengkulu is in the same cluster with the same virus from the infected Ambon Kuning cultivar from the same locations. It is evident that *P. nigranervosa* has effectively migrated and transmitted the bunchy top virus from cultivated banana to the wild one. The weather might affect the vector survival, migration, and fecundity. Another contributing factor to the effective transmission is the titer of the virus in the aphids (Jebakumar et al. 2018). *P. nigranervosa* was also able to live in another plant such as *Colocasia* that grows wild and abundant around the banana mats. *Colocasia* and other *Zingiber* families might be the alternative host for BBTv or alternate hosts for *P. nigranervosa*. A previous study indicated the distribution and abundance of *P. nigranervosa* on *Musa* spp. are genotype-dependent (Ngatat et al. 2017). Recently, the aphid transmitted the BBTv from Ambon Kuning (AAA) to the wild *Musa* (AA) that grows in the same area. The artificial inoculation has proven that the wild bananas in the *M. acuminata* (AA) group can be infected by BBTv some of them delayed the emergence of the symptoms. The subsp. *longipetiolata* showed the fastest response to the bunchy top virus infection, however; the disease did not develop well (40% disease incidence up to 21 weeks after inoculation). This subspecies indicated a resistance response. Subspecies *halabanensis* delayed emerging symptoms until week 21st, longer than other species. *Musa* sp. showed very good resistant response to the BBTv infection. This species prevents early symptoms for 13 weeks and holds the disease incidence at 20% until week 21st. All species of wild bananas exhibited different resistant responses to BBTv infection. It may be attributed to the unique gene(s) responsible for the resistance of each species that needs to be investigated further.

The more stable DNA components in BBTv are DNA-R, DNA-N, and DNA-S. In the recent study, we analyzed DNA-R and DNA-S of the subsp. *sumatrana*. The putative Rep protein encoded by DNA-R has enzymatic activities including site-specific nicking and join, similar to other Rep proteins. *Banana bunchy top virus* of the wild banana has two conserved regions, stem-loop common region (CR-SL) and the major common region (CR-M), both in DNA-R and DNA-S. This corroborates the statement that CR-SL and CR-M are found in all BBTv's component. CR-SL is a conserved region involved in the replication process. This sequence was found in BBTv and other viruses in the Geminivirus group (Wickramaarachchi et al. 2016). This analysis indicated virus capability to replicate after transmission by the vector. When the virus can replicate in the wild banana, the plant could be a new source of the bunchy top disease and an alternative host of the virus.

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