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The Mode of Transmission of *Banana streak virus* by *Paracoccus burnerae* (Homiptera; Planococcidae) Vector is Non-circulative

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SMM and LKN designed the study, wrote the protocol, gathered the initial data, interpreted, managed the literature searches and produced the initial and final copy of the manuscript. While authors FNW and LSK anchored the field study and supervised the execution of the work. All authors read and approved the final manuscript.

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ABSTRACT

The causative agent of banana streak disease is *Banana streak virus* (BSV). In tropical countries, for example Kenya, the virus causes considerable damages to banana crop as well as banana production yields. Several mealy-bug species have been reported as vectors of BSV. However, latent and retention time of the BSV in the oleander mealy-bug (*Paracoccus burnerae*) are unknown. These characteristics are important in determining the mode of transmission of viruses by their vectors. The purpose of this study was to determine the latent and retention time of the BSV in its vector, *P. burnerae*. We employed both Immuno-capture Polymerase Chain Reaction (IC-PCR) and Rolling Circle Amplification (RCA) techniques to select diseased and healthy

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plantlets for transmission trials. RCA assays were performed on the deoxyribonucleic acid samples of viruliferous mealy-bug instars of *P. burnerae* and on the deoxyribonucleic acid of virus-inoculated plantlets. The findings of the study indicated that BSV has no latent period in *P. burnerae* during transmission at ambient conditions (9-30℃). However, t he vector can retain and transmit BSV for a period of four days under ambient temperatures (9-30℃). The results revealed that vector *P. burnerae*, transmit BSV semi-persistently which is an indication of non-circulative mode of transmission of viruses. The results of this study contribute immensely to the elucidation of the mode of transmission of *Banana streak virus* by *P. burnerae,* thus enhancing development of novel control strategies of BSV transmission.

Keywords: BSV; P. burnerae; immuno-capture PCR; rolling circle amplification; latent period; retention time.

1. INTRODUCTION

The causative agent of the banana streak disease of banana (*Musa* spp.) is BSV, and the disease occurs in most banana-growing regions of the world [1]. The symptoms of the banana streak disease exhibit high heterogeneity and can include chlorotic and necrotic streaking of leaves along the leaf lamina, distortion of leaves and petioles, stem cracking, abnormal bunch development and death of the growing point [2]. In some situations, the newly emerging leaves remain non-symptomatic [2]. In addition, yield losses of 6% -15% in banana crop have been reported in regions where disease has been recorded [2,3]. However, the range of yield losses depends on various factors, such as variety of the banana, strains of the virus, number of the strains infecting the crop and the prevailing environmental factors (rain and temperature, etc.).

BSV belongs to the members of the genus *Badnavirus* in the family *Caulimoviridae*. The genome of the BSV comprises of non-covalently closed, double-stranded deoxyribonucleic acid of approximately 7.2 to 7.8 kbp. However, within the banana (*Musa* spp.) genome, two types of the integrated *Badnavirus* sequences have been reported [4]. The first type is the *Musa* spp. endogenous pararetrovirus and the incomplete virus genome which is incapable of causing infections to the crop [5]. The second type is endogenous activatable BSV. These activatable sequences are made up of the entire genome of episomal BSV sequences, which are multiple non-contiguous regions of the virus deoxyribonucleic acid combined with host-genomic sequences. However, it has been reported that under unfavourable conditions, recombination events of the integrated viral sequences occur within the banana genome, which allows the integrated viral genome to be activated, resulting in episomal infections [6-8]. The artificial factors that allow the integrated viral genome to be activated are tissue culture and hybridization conditions [6-8]. In addition to these facts, incomplete integrated viral sequences are found in both A and B genomes of the two types of domesticated banana, i.e. *Musa acuminata* and *Musa balbisiana*, respectively [9-11]. However, the activatable BSV sequences have only been reported in the B genome of different banana cultivars [9-11].

The BSV exhibits high heterogeneity at both serological and genomic levels [4,9,12-15], phenomenon that pose challenges in the application of the polymerase chain reaction (PCR) and of the antibody-based detection assays. The presence of integrants of the *Badnavirus* origin in the banana genome further complicates disease detection by PCR-based methods because of the arising false positives from integrated *Badnavirus* sequences [16-18]. To circumvent the detection of the integrated deoxyribonucleic acid sequences, the technique that combines both serological and genomic detection, called immuno-capture Polymerase chain reaction (IC-PCR) is used as the “gold standard” for BSV indexing. However, like any other molecular methods, IC-PCR has limitations such as the inability of the antiserum to capture all BSV isolates [13]; the use of several primer sets in detection of the virus, that are unlikely to detect the entire diversity of the BSV sequences; and the false positives due to presence of contaminants, that arise from carryover of deoxyribonucleic acid remaining in capture tubes. Due to the aforementioned reasons, an alternative sequence-independent detection method called Rolling Circle Amplification (RCA), was developed [5]. The method has a dual purpose of specific detection of only the circular *Badnavirus* genome discrimination of episomal and integrated viral deoxyribonucleic acids. The

RCA is also very sensitive to very low titer of deoxyribonucleic acids [19,20].

It has been previously reported that BSV can be transmitted by several mealy-bug species [21-24]. Their results revealed that *Planococcus citri* [25,26] and *P. ficus* [25] have the highest transmission efficiency of 100% and 80% respectively. However, *Dymicoccus brevipes* [23], a pineapple mealy-bug species [27] has the lowest BSV transmission efficiency of 20%. The contradicting results amongst mealy-bug species in transmission of BSV can be associated with differences in receptors of the vector that interact with the capsid protein of the virus during acquisition access feeding [28]. According to [28] latent and retention time of the plant viruses in their vector, affects the efficiency and mode of transmission. Several species of mealy-bugs have been reported worldwide [21-24]. Nonetheless, only five mealy-bug species have been used worldwide in the greenhouse experiments as vector for BSV [21-24,29]. The results of the related studies [21,23] revealed that *D. brevipes* [27], *P. citri* [25,26]*, P. ficus* [25], and *Saccharicoccus sacchari* [27] are potential vectors of BSV. However, *Paracoccus burnerae* (Common name oleander mealy-bug, Homiptera; Planococcidae) has been recently reported as an additional vector of BSV [29]. Mealy-bug vectors including *P. burnerae* can successfully acquire BSV within time range of 5 min to 12 hours as reported previously [21,22,29,30]. However, no information is available concerning to the latent and retention period of the BSV in the *P. burnerae* vectors. In our study, we performed experimental trials on the latent and retention time of BSV in the newly reported vector (*P. burnerae*) [29]; to establish transmission mode of the virus at ambient temperatures (9-30℃). The objectives of this study were achieved by determining both the latent and retention periods of the BSV in its vector using both IC-PCR and RCA techniques.

2. MATERIALS AND METHODS

2.1 Screening of Virus-source Plants and Receptor Plants

BSV infected banana plantlets were obtained from infected banana germplasm materials at Kenya Agricultural Research Institute (KARI), Njoro greenhouse and BSV-free banana plantlets were obtained from healthy germplasm at KARI, Njoro tissue culture laboratory. The BSV status of all plantlets was confirmed by IC-PCR using the standard protocol [12,29,31-33] and RCA as described previously [5,29,34,33]. The two methods were used to overcome the high variability of the BSV at both serological and genomic level [4,9,12-15], and to detect low viral concentration in inoculated plantlet tissues by RCA technique [19,20]. Each selected banana plantlet was established in a 2 kg polythene bag and used at 4-leaf stage.

2.2 Collection of Mealy-bugs

Mealy-bugs were collected under the pseudostem sheaths and on the roots of the infected banana plants as described [21,29]. Vector collections were done in infected banana fields at KARI, Kisii Research Centre. The mealy-bug specimens were identified to the species level based primarily on adult female morphological features. After collection, the insects were reared on pumpkin fruits placed in black cages which provided dark conditions, and an ambient temperature of 9-30℃ necessary for their optimal development. The advantages of using pumpkin fruits include the fact that fruit is not a BSV host and mealy-bugs are easy to remove from hard skinned pumpkins for inoculation experiments, thus facilitating the determination of the latent and retention time. To prevent contamination by crawling insects, the cage was placed on a pan containing soapy water. Since mealy-bugs have a life cycle of 4-6 weeks, the mealy-bug colonies were reared for four months to achieve the necessary number of specimens for the tests.

2.3 Transmission Trials to Determine the Latent Period of BSV in *P. burnerae* Vector in Greenhouse

More than one hundred second instars of *P. burnerae* cultured in the rearing cages were subjected to the virus source plants in different clip cages for ten days in the greenhouse of 9-30℃ to guarantee them acquire the virus. The female mealy-bug instars were then transferred on to the pumpkin fruits using camel hair brush. The pumpkin fruit was placed on the plastic bowl in black cage where the second instars were sampled from, during inoculation access period. Sampling was done at regular intervals, every day for a period of 3 days (i.e day 1, 2 and 3). Two sets with randomly picked 30-40 second instars of *P. burnerae* were sampled. One set was used for assaying the existence of BSV in mealy-bug instars (in case of inoculation failure) using the RCA technique. The second set was

fed on virus-free plants in clip cages in the greenhouse for inoculation access time of 14 days. The cages were always placed on the plates containing soapy water to prevent ants from colonizing the mealy-bug cultures. This experiment was carried out in triplicates. After 14 days of inoculation period, the leaf samples of virus inoculated plants were sampled; deoxyribonucleic acid was isolated as described elsewhere [5,29,34,35] and assayed for the presence of BSV using RCA technique as described [5,29,34,33,36]. Restriction fragment length polymorphism and gel electrophoresis were performed as described [5,29,34,33,36]. The relationships between isolate types were determined using the restriction fragment data. The data was presented as tables and figures representing the success and failure of BSV transmission by *P. burnerae* during experimental trials. Data generated from this study was compared with the standard characteristics of different mode of transmission of viruses by their vectors as described [28].

2.4 Transmission Trials to Estimate the Retention Period of BSV in the *P. burnerae* Vector in Greenhouse

More than two hundred non-viruliferous *P. burnerae* first instars in the rearing cages were fed on the virus source plants (same cultivar) in a black cage to guarantee them acquire the virus within the greenhouse of 9-30℃. After 10 days, the mealy-bug instars from the cage were transferred to another cage, placed on the pumpkin fruit (on a bowl) for seven days and then they were sampled to determine the retention period. Camel hair brush was used to remove the mealy-bug instars from the infected plants on to the pumpkin fruit. About 30-40 mealy-bug instars from the virus source plant cage were sampled randomly and tested for the presence of BSV using RCA technique. Thereafter, more than 30 instars of *P. burnerae* previously preserved for seven days were sampled every day for a week and were fed on the clean plantlets, for inoculation access time. The leaves of inoculated banana plants by the viruliferous instars were sampled; deoxyribonucleic acids were extracted as described [5,29,35] and diagnosed for the presence of BSV using the RCA technique as described [5,29]. Forty *P. burnerae* instars on the inoculated plants were also sampled as well; deoxyribonucleic acid was extracted as described [29,34]. Restriction fragment length polymorphism and gel electrophoresis were performed as described [5,29,34,33,36]. The relationships between isolate types were determined using the restriction fragment data. The data was presented as tables and figures representing the success and failure of BSV transmission by *P. burnerae* during experimental trials. Data generated from this study was compared with the standard characteristics of different mode of transmission of the viruses by their vectors as described [28].

3. RESULTS AND DISCUSSION

3.1 Screening of Virus-source Plants and Receptor Plants

The screening of the virus source plants and receptor plants corroborated those obtained in previous reports [29]. Chirume cultivar tested positive for BSV with both IC-PCR and RCA, while Cavendish cultivar tested negative for the virus with the same detection methods as previously published [29]. Both Chirume and Cavendish cultivars are triple A-genome containing cultivars, that lack the endogenous activatable BSV sequences [5,8,37].

3.2 Transmission Trials to Determine the Latent Period of BSV in *P. burnerae* Vector

Results from the study revealed that *P. burnerae* instars had no latent period (Table 1 and Fig. 1), since the vector was able to transmit the virus immediately after acquisition. This implies that BSV does not undergo any biochemical modification on its capsid protein in the vector's gut during transmission process.

Virus-vector specificity involves an interaction between virus capsid proteins and membranes of the vector salivary membranes [38]; and this interaction determines the latent period of the virus in its vector. During this period, the viruses replicate and increased their numbers in the vector. However, no data exists on the latent period of BSV isolates in the known vectors of mealy-bug species. The latent period allows the virus to acquire the ability to infect the host. In other vector transmitted viruses, for example the *Potyvirus*, a helper component proteinase (HC-Pro) encoded by viral deoxyribonucleic acid has been shown to be a key protein during transmission process [39].

The data collected in this study suggested that BSV is transmitted by the *P. burnerae* instars in a

non-circulative mode of transmission, which can be classified into two modes: - non-persistent and semi-persistent modes of transmission. The two modes of transmission do not require a latent period for transmissibility of the virus. The viruses transmitted through these two modes of transmission are found along the gut of the vector and do not cross the vectors gut membranes into the heamolyph and/or cells [28].

3.3 Transmission Trials at 9-30℃ Conditions to Estimate the Retention Time of BSV in *P. burnerae*

The retention period of BSV was successfully estimated using the RCA technique. The results revealed that *P. burnerae* instars were able to retain the virus for four days after acquisition access time (Table 2 and Fig. 2). Thereafter, the vector could not cause any infection during inoculation access period to a healthy banana plant. A 7.4 kb RCA products was identified in *P. burnerae* that retained the BSV within the four days. However, the BSV was not detected after four days in the viruliferous *P. burnerae* instars. Results from other studies on the mode of transmission of BSV by mealy-bugs species have revealed that the vectors can retain the virus for five days [22], which is within the range of the semi-persistent mode of transmission. However, the previous results [22] were contrast with those of this study, which could be due to the differences in the viral loads in the viruliferous instars depending on the factors prevailing during acquisition access period, and the vector species used in the experiments. The

Table 1. Transmission trials at 9-30℃ conditions t o determine the latent period of *Banana streak virus* (BSV) in *P. burnerae* vector

Source of acquisition	mealy bugs	Acquisition time	Latent time	Inoculation time	Assaying for BSV by RCA		
					Replicates		
					1	2	3
Chirume	30-40	10 days	1	14 days	+ve	+ve	+ve
Chirume	30-40	10 days	2	14 days	+ve	+ve	+ve
Chirume	30-40	10 days	3	14 days	+ve	+ve	+ve
Controls							
Mysore	ND	ND	ND	ND	+ve	+ve	+ve
Water/healthy plant	ND	ND	ND	ND	-ve	-ve	-ve

ND-Positive and Negative controls not fed on by the viruliferous mealy-bugs. The inoculated banana tested positive with RCA for Banana streak virus after three days of acquisition feeding

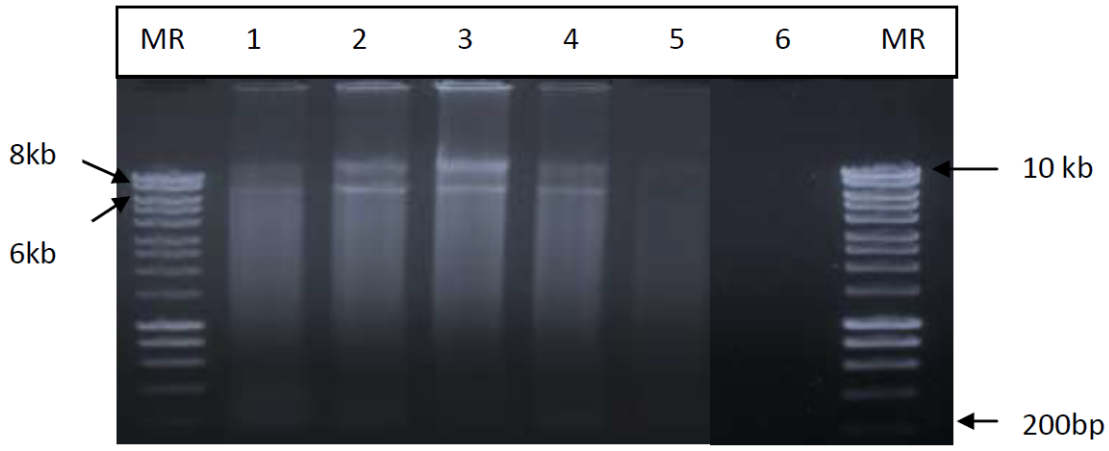

Fig. 1. TempliPhi products for DNA from plants exposed to infected Mealy bug (Latent period)
Lane MR- represent Molecular marker (Hyperladder™Bioline) Lanes 1-3 Plant DNA from samples inoculated by viruliferous instars after 1-3 days of acquisition access period; Lane 4-Positive control; Lane 5- Negative control (Healthy plant DNA) and Lane 6- Negative control (water)

mode of transmission of BSV by its' vectors, mealy-bug species, was proposed to be semi-persistent by [13,21]. The data from this study based on retention time of BSV in mealy-bug do agree with their suggestion. In the semi-persistent mode of transmission, the virus is retained in the vector for a minimum of five minutes and a maximum time of less than a week [28,40]. From the results of this study, it can be hypothesized that the BSV do not replicate in the gut of the vector because the viral load in vectoring insect could not go beyond four days in the *P. burnerae* vector. If the virus replicated in the vector, it could be transmitted for a longer period than five days due to the increase in the number of the viral particles in the vector and thus this could result in persistent mode of transmission. However, further experimental approaches including *in situ* localization of the virus in the vector need to be performed to test the hypothesis.

The pineapple and sugarcane mealy-bug species are known to retain the virus for up to five and six days, respectively, after transfer from the virus sources [21]. Retention period of three to four days of BSV by mealy-bug vectors has been reported previously [30,41], which corroborates with the results obtained of this study. The results obtained in this study and those from elsewhere seem to rule out non-persistent and propagative modes of transmission of BSV by the studied mealy-bug species.

Table 2. Transmission trial results of the retention time of the BSV in *P. burnerae* at 9-30ºC conditions

Source of BSV acquisition	mealy bugs	Acquisition time	Latent time	Inoculation period	Detection of BSV by RCA		
					Replicates		
					1	2	3
Chirume	30	10 days	1	4 days	+ve	+ve	+ve
Chirume	30	10 days	2	4 days	+ve	+ve	+ve
Chirume	32	10 days	3	4 days	+ve	+ve	+ve
Chirume	40	10 days	4	4 days	+ve	+ve	+ve
Chirume	35	10 days	5	4 days	-ve	-ve	-ve
Chirume	33	10 days	6	4 days	-ve	-ve	-ve
Chirume	40	10 days	7	4 days	-ve	-ve	-ve
Controls							
Mysore	N/A	N/A	N/A	N/A	+ve	+ve	+ve
Water/healthy plants	N/A	N/A	N/A	N/A	-ve	-ve	-ve

ND-Positive and negative controls not fed on by the viruliferous mealy-bugs

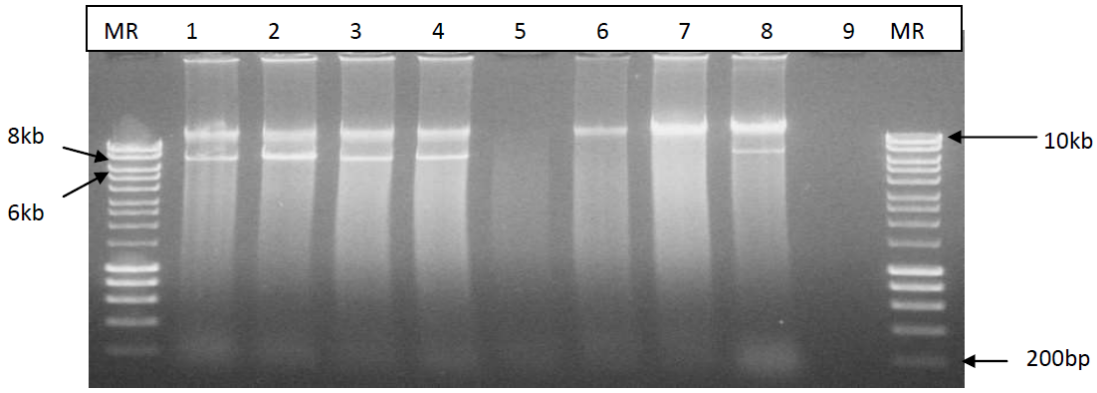

Fig. 2. TempliPhi products for DNA from banana plants exposed to viruliferous P. burnerae
Lane MR- Molecular marker (Hyperladder™1, Bioline); Lanes 1-7 represent DNA from plant samples inoculated by viruliferous P. burnerae and allowed to hold for 1, 2, 3, 4, 5, 6 and 7 days before inoculation, respectively; Lane 8 represents Positive control (Mysore); Lane 9 is a Negative control (water)

Studies with other virus vectors reveals that viruses destined for inoculation are retained at sites within the stylet and food canal or foregut depending on the mode of transmission. They indicate that virions retained at the distal tip of the stylet bundle are most likely to play a determining role in transmission [41-43]. However, one of the conundrums in transmission is that the binding of virions within the vector must be readily reversible. Considering that the food and salivary canals merge at the tip of the vector's stylet, salivation may function to enhance the release of bound virions and their delivery into plant cells [42,44]. This could be true in transmission of BSV by their vector. In other vector transmitted viruses, a primary determinant of both vector transmissibility and specificity is the viral capsid protein in a non-persistent and helper component protein in semi-persistent mode of transmission. These proteins contribute immensely to the retention time of vector transmitted virus. However, no information on the capsid protein of BSV that is involved in the transmission of the virus by mealy-bug species has been reported. It can be hypothesized that the ORF I and II gene products of BSV could be involved during virus-vector interaction. Within the insects feeding apparatus, the retention sites for semi-persistent viruses have been determined only in the leafhopper-transmitted viruses [45,46] and they are located in the foregut of the leafhopper. Similar data on semi-persistent mealy-bug transmitted viruses have not been reported; thus, information on the precise location of the mealy-bug receptor(s) recognized by the BSV Capsid protein domains is not known.

A distinguishing feature of semi-persistent transmission lies in the retention period of hours to days. One of the best characterized among the semi-persistent, vector transmitted viruses is the *Cauliflower mosaic virus*, the type member of the family *Caulimoviridae*, to which BSV belongs. The C*auliflower mosaic virus* has adopted a helper-dependent transmission strategy which determine the retention time of the virus in the vector, but with the added twist of requiring two viral-encoded, nonstructural proteins, P2 and P3 [46,47]. To date, no information on the helper dependent transmission strategy has been reported on the mealy-bug transmitted viruses such as *Badnavirus, Trichovirus* and *Closterovirus*. In addition, no information is available on BSV transmission strategy. However, a helper-dependent transmission strategy rather than Capsid mediated strategy could be involved during BSV transmission by the mealy-bugs, due to the fact that the vector was able to retain the virus for four days.

4. CONCLUSION

The *P. burnerae* can transmit BSV immediately after they acquire the virus with no latent period and can retain the virus for four days after acquisition access time. Thus, based on the standard characteristics of the different mode of transmission, the vector *P. burnerae* transmit the BSV semi-persistently, which can broadly be classified as non-circulative mode of transmission. However, further studies are needed to determine the proteins both for BSV and its vector that are involved in the transmission process. These findings can lead in future to the use of viral genes that encode for proteins that are defective, as a management strategy to prevent vector inoculation and successful transmission of BSV by its vectors. Further screening for plants encoding molecules (e.g. peptides) that are able to bind to cuticle protein receptors in the vector mouthparts may provide innovative virus management strategies by interfering with the process of virus retention.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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