PROJECT R8827 [FTR PART 6]
APPENDIX 5: Draft paper on vector transmission studies

Transmission of *Cassava brown streak virus* by *Bemisia tabaci* (Gennadius)

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Key words: Cassava brown streak disease, *Manihot esculenta*, *Bemisia tabaci*, *B. afer*, RT-PCR, vector, whitefly.

Abstract
Cassava brown streak disease (CBSD) occurs mainly in the coastal areas of eastern Africa. Although the disease was first reported in 1936, the pathogen was characterised only recently as *Cassava brown streak virus* (CBSV), belonging to the genus *Ipomovirus*, family *Potyviridae*. In this study, we have confirmed for the first time the vector transmission of CBSV by the whitefly, *Bemisia tabaci* (Gennadius) in laboratory conditions. Transmission to test plants was confirmed by the detection of CBSV by RT-PCR. Spread of CBSD in field coincided with increased whitefly numbers, further supporting the evidence that *B. tabaci* was the vector of CBSV. In the laboratory experiments, transmission occurred at low levels (maximum 22%). The optimum conditions and factors required for efficient CBSV transmission are far from understood. The aim of this study is to report the first successful transmission of CBSV by *B. tabaci*, so that future studies on transmission and epidemiology can be directed towards this insect.

Introduction

Cassava brown streak disease (CBSD) was first described in the former Tanganyika territory, now Tanzania (Storey, 1936). The disease is endemic in East African coastal cassava-growing areas from southern Kenya, through Tanzania to the Zambezi river in Mozambique, and occurs also, in some inland areas of Malawi, up to an altitude of about 3500 feet (1050 m) above sea level (Nichols, 1950; Sauti & Chipungu, 1993; Hillocks *et al*., 2002; see review by Hillocks & Jennings, 2003).

Symptoms of CBSD on cassava are leaf chlorosis in a characteristic pattern of feathering along the veins and in severe infection, stem necrosis. In extreme cases, stem necrosis results in shoot die back (Jennings, 1960). Unlike cassava mosaic disease (CMD) with which it is often associated, symptoms of CBSD may be found also on the roots, as a yellow/brown, corky necrosis in the starch-bearing tissue, making roots unfit for consumption. Losses in root weight of up to 70% were attributed to CBSD in susceptible cultivars (Hillocks *et al*., 2001). This can have a serious impact on food security, as the extent of the loss caused does not become apparent until the crop is harvested.

Although CBSD was first reported nearly 70 years ago, there is inadequate information about the disease, and it was shown only recently to be caused by *Cassava brown streak virus* (CBSV) belonging to the genus *Ipomovirus* of the family *Potyviridae* (Monger *et al*., 2001b). The causal agent was graft- (Storey, 1936) and mechanically-transmissible from cassava to a number of herbaceous hosts (Lister, 1959). It was suggested previously that CBSV was insect transmitted and that the most likely vector was the whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) (Storey, 1939; Bock, 1994). Another whitefly species *Bemisia afer* (Priesner & Hosny), although less abundant than *B. tabaci*, reaches its highest population densities in some areas where CBSD incidence is highest (Robertson, 1987; Munthali, 1992). For this reason, Bock (1994) believed that *B. afer* might be the most likely vector. Previous transmission tests with both species of whitefly and with some species of aphid were unsuccessful (Lennon *et al*., 1986; Brunt *et al*., 1990; Bock, 1994).

While the vector of CBSV has continued to be elusive, disease spread in the field is sometimes rapid and disease incidences of over 50% are common in some fields in the coastal areas of Tanzania and Mozambique (Jennings, 1960; Thresh and Mbwana, 1998; Hillocks *et al*., 1999). There is renewed interest in CBSD, as, due to the root necrosis symptom, it is a more important cause of crop loss than was previously believed. Successful screening for disease resistance requires an understanding of inoculum potential which in turn, requires that we know how the virus is transmitted. In this paper we describe for the first time the identification of a whitefly (*B. tabaci*) vector of CBSV.

Materials and methods

Generation of CBSV-free cassava plants, and RT-PCRCuttings from disease-free cassava plants cv. Albert (CBSD susceptible) were collected in fields at Kibaha, Tanzania. Plants were grown from cuttings in the quarantine glasshouse at Natural Resources Institute (NRI), UK, and observed for six months for the expression of CBSD symptoms. Plants were indexed for CBSV and its absence was confirmed by RT-PCR (Monger *et al*., 2001a) using the One Step RT-PCR Kit (Qiagen, UK). Nodes from CBSV-free plants were used for the multiplication by *in vitro* propagation (meristem tissue culture) (Frison, 1994). Absence of CBSV in tissue cultured plants

was further confirmed by RT-PCR and growing them again for six months. Plants obtained in this way were used for CBSV transmission experiments.

Vector transmission in glasshouse and insectary

Four CBSV transmission experiments were carried out under controlled conditions, using *B. tabaci* (ex. Namulonge, Uganda) and *B. afer* (ex. Entebbe, Uganda and Zanzibar, Tanzania) colonies (Maruthi *et al*., 2001) in the NRI insectary. Whiteflies were collected from colonies and allowed to feed for 48 hr within a clip-cage placed at the tip of a cassava leaf showing typical symptoms of CBSD. Viruliferous *B. tabaci* and *B. afer* adults were transferred onto disease-free cassava plants of cv. Albert, separately and together for 48 h of virus inoculation. Target plants were inoculated 1-3 times with 15 day intervals, using 15-60 whiteflies, depending on the type of experiment (see Table 1).

Further, vector transmission studies were initiated in the glasshouse at Kibaha Research Station, Tanzania (see Table 1). About 100 adults each of *B. afer* and *B. tabaci* collected in the field were introduced into different cages (dimension 2' x 2' x 3') containing one young CBSV-infected cassava plant (virus donor) and three virus-free plants cv. Albert (virus recipient). For each whitefly species three cages were maintained. Two control cages included whiteflies and virus-free plants only.

Similar transmission experiments were carried out in cages (dimension 1.5' x 1.5' x 2.5') at the NRI insectary using *B. tabaci* and *B. afer* colonies. About 1000 adults of *B. tabaci* and 500 *B. afer* were released into separate cages containing one CBSV-infected and three virus-free cassava plants cv. Albert. The set up was maintained for five months at 25±2 ^{o}C and 50% RH. The experiment was repeated using *N. benthamiana* as virus recipient plants.

Transmission by whitefly collected from the field

Adults of *B. tabaci*, *B. afer*, thrips and beetles (*Cybocephulus* spp.) were collected separately from cassava plants infected with CBSV at four different locations in Tanzania (Kibaha, Zigowale, Zanzibar and Chenika). About 5-20 insects were transferred into the glass tubes containing disease-free tissue cultured plants cv. Albert and the insects were allowed to feed for 5 days. The plantlets were then transferred into a soil-medium and maintained in the quarantine glasshouse at NRI and observed for symptom expression.

Seed transmission

Cassava seeds were collected from diseased and healthy plants of cv. Albert in fields at Kibaha, Tanzania in 2002. Seeds were planted in pots containing soil and compost (John Innes No. 2, UK) in equal proportions in the glasshouse at NRI. Eighty-nine and 32 plants grown from seeds taken from diseased and apparently healthy plants, respectively, were maintained for the expression of symptoms (see Table 1). After six months, plants were pruned at the base to allow the new growth to express symptoms. Thirty plants were selected randomly and tested for the infection of CBSV by RT-PCR.

Field trials on disease spread in relation to whitefly populations

Cuttings of a CBSD-susceptible cv. Mreteta were collected from plants without disease symptoms. Nine-hundred plants were grown from cuttings in a plot of thirty rows, each with 30 plants, at the spacing of 1m x 1m in December 1998 at Kibaha, coastal Tanzania. Two months earlier, two spreader rows were planted in the direction

of prevailing wind at one end of the plot using cuttings taken from plants showing symptoms of CBSD. The plot was isolated by more than 200 m from other cassava fields. During the first month after sprouting occurred, any plants that showed symptoms of CBSD or CMD were removed on the basis that the infection was derived from the cutting. Any new infections appeared were recorded from the second month after sprouting. The trial was repeated at Naliendele, southern Tanzania in January 1999. Whitefly populations were recorded monthly in the plots by counting the number of adults on the top two fully expanded leaves of the uppermost shoot on thirty plants on a diagonal line across the plot.

Results

Vector transmission in glasshouse and insectary

In the first controlled transmission experiment at NRI, three out of 15 (20%) cassava plants each inoculated by a mixed population of *B. afer* and *B. tabaci* produced typical CBSD symptoms (Table 1). Infection of CBSV in cassava plants was confirmed by the detection of virus using RT-PCR (Fig 1).

The number of days required from inoculation to symptom appearance varied; the first plant showed symptoms 20 days after first inoculation while the remaining two plants showed symptoms 40 days after inoculation. Symptoms on vector inoculated plants were similar to those seen on cutting-infected plants. Initial symptoms appeared in the form of yellowing of tertiary veins, which later extended into secondary and primary veins. This was followed by leaf chlorosis in a characteristic pattern of feathering and necrosis on stem.

It was not conclusive, however, whether one or both whitefly species were required for transmission. In order to investigate this, the experiment was repeated, but with *B. tabaci* and *B. afer* separately and together. The protocol was similar to that of the first experiment, except that the number of inoculations was reduced to two. None of the plants inoculated by *B. tabaci* and *B. afer* separately and together, however, became infected in the second experiment.

Further transmission experiments carried out (the third and fourth) were the exact repetition of the second experiment (Table 1) with the addition of a treatment in which *N. benthamiana* replaced cassava as the test host. Two (20%) cassava plants that became infected with CBSV in the third experiment were both inoculated by *B. tabaci*. The infection by CBSV was confirmed by the detection of virus by RT-PCR. None of the *N. benthamiana* plants was infected due to the apparent inability of cassava whiteflies to feed on *N. benthamiana* and their death within 12 h after feeding. Attempts for the establishment of cassava *B. tabaci* and *B. afer* on *N. benthamiana* were unsuccessful.

In the cage experiments conducted in Tanzania, two out of nine test plants (22%) developed symptoms of CBSD (Table 1). The infections occurred in separate cages containing *B. tabaci* which reproduced well on the donor plant. None of the plants in the *B. afer* cage developed symptoms. However, the comparison with *B. afer* was not valid as this species did not reproduce well and populations in the cages declined.

None of the cassava and *N. benthamiana* plants produced symptoms at NRI in experiments where whitefly were released into the cages [conducted before the clip-cage experiments]. Similar to the glasshouse studies of Tanzania *B. afer* numbers declined in cages at NRI. Both the whitefly species did not develop to substantial numbers in cages with *N. benthamiana* as virus recipient plants.

Transmission by whitefly collected from the field
None of the plantlets exposed to whitefly collected in the field from cassava showing symptoms of CBSD, developed symptoms when grown-on in the glasshouse.

Seed transmission
None of the plants grown from seed collected in Tanzania from plants showing leaf symptoms of CBSD, showed symptoms of CBSD after six months in the NRI glasshouse. The plants grown alongside that were obtained from diseased cuttings showed typical symptoms. Absence of CBSV was confirmed in 30 randomly selected plants tested by RT-PCR. The new growth obtained after pruning of plants did not show symptoms.

Field trials on disease spread in relation to whitefly populations
Whitefly population changes and CBSD development at Kibaha and Naliendele in 1999 are shown in Fig. 2. At Kibaha, the largest increase in the proportion of infected plants occurred in April, coinciding with the peak in total whitefly populations. Whitefly numbers declined almost to zero after July when there was no further spread of CBSD. When the scoring for CBSD and whitefly population counts began in March at Naliendele, the whitefly populations were already high and declined in April and the beginning of May, rising again in May and June (Fig. 2). The number of whitefly increased again in June with a concomitant increase in new infections of CBSD. By July around 22% of plants were showing CBSD symptoms at Kibaha and 17% at Naliendele. Although large numbers of symptomless plants remained in the plot, no new infections appeared after July when, during the dry season, whitefly numbers fall to between 0 and one or two per 30 plants.

Discussion
CBSV was transmitted on three different occasions out of seven attempts made using whiteflies in laboratory experiments. The rate of transmission (maximum 22%) achieved in laboratory studies was lower than that of the transmission rates of cucumber vein yellowing virus (CVYV) (55%) (Mansour and Al-Musa, 1993), a new member of the genus *Ipomovirus* (Lecoq *et al*., 2000). The rate was also low relative to high incidence and rates of spread of CBSD observed in the field (Hillocks and Jennings, 2003).

The low transmission rate and inconsistency of the results may be due to technical difficulties in our transmission protocols and because all the conditions that determine efficient vector transmission have not been understood. Firstly, the titre of virus may be very low in cassava plants grown in glasshouse conditions that are different from the field situation. Secondly and possibly more importantly, the feeding behaviour of *B. tabaci* on cassava plants might have a great effect on CBSV transmission. More than 90% of *B. tabaci* feed on top five leaves of cassava plants in field (Maruthi *et al*., 2004) but the most obvious CBSD symptoms and presumably higher virus titre appear on the lower leaves. Successful transmission achieved in experiments at NRI where clip-cages were used may have been because the method allows the whitefly to be placed at the tips of the most viruliferous leaves. Environmental conditions may also be significant. For instance, CVYV has been shown to produce 100% infection in tobacco plants exposed to 37 ^{0}C while plants kept at 16 ^{0}C were not infected (D. Janssen, personal communication). Future studies therefore should be aimed at addressing these problems by allowing *B. tabaci* to feed on the most symptomatic leaves in fields of Tanzania using clip cages, thus providing

ready access to virus for whiteflies and removing any laboratory effects. Now that the *B. tabaci* has been identified as vector the resources and experiments should be channelled for understanding CBSV-*B. tabaci* relationships in order to understand the epidemiology of this important disease. Although when the two whitefly species were separated, successful virus transmission was achieved only with *B. tabaci*, this does not eliminate the possibility that under suitable conditions, *B. afer* might also transmit CBSD.

In southern Tanzania where the main period for planting cassava is November – January, whitefly populations reach their maximum during the period February – May [sometimes into June], when there is plenty of fresh green cassava growth. Following the appearance of CBSD symptoms derived from the planting of infected cuttings, the main period of new [secondary] infections coincides with peak whitefly populations. During the dry season between May/June and September, whitefly numbers on cassava decrease with the decline in young green shoots and new infection of CBSD are rarely seen.

Both CMD and CBSD can spread rapidly in the field from cutting-borne infection foci, and it has been observed that the seasons in which the spread of CBSD is greatest are also those in which whitefly populations are high (R. J. Hillocks, unpublished). In the cassava growing seasons of 2002 and 2003, when whitefly numbers were low in Tanzania, little spread of CBSD occurred. This contrasted with the 2004 season with high whitefly numbers in February/March and rapid spread of CBSD. The apparent association between peaks in new infections of CBSD and peaks in whitefly populations was confirmed in our field trials. Although the results from those trials presented herein, do not on their own prove that whitefly is the vector of CBSV, they become more convincing now that transmission by *B. tabaci* has been confirmed.

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Figure legends

Figure 1. Gel electrophoresis photograph of RT-PCR products obtained using CBSV10 and CBSV 11 primers for the detection of CBSV in *B. tabaci* inoculated cassava plants. Lanes 1 to 3 represent cassava plants inoculated with CBSV by *B. tabaci*, lane 4 cassava plant cutting infected, and 5 to 8 cassava plants that did not show symptoms. Lane 9 represents a tobacco plant mechanically inoculated with CBSV and lane 10 is negative control (water). M = Molecular weight marker (1 kb Gibco-BRL).

Figure 2. New incidences of CBSD and mean monthly whitefly populations at (A) Kibaha and (B) Naliendele in 1999.

Table 1. Summary of different types of experiments carried out for the identification of vector of CBSV

Sl. No.	Experiment type	Location	Whitefly species or treatment	No. of replications	No. of inoculations	Total No. of insects inoculated	Total No. of cassava plants infected/ inoculated
1	1st controlled transmission	Insectary, NRI, UK	*B. tabaci* + *B. afer*	3	3	120 (60 each)	3/15
2	2nd controlled transmission	Insectary, NRI, UK	*B. tabaci*	3	2	40	0/12
			B. afer	3	2	40	0/12
			B. tabaci + *B. afer*	3	2	60 (30 each)	0/15
3	3rd controlled transmission	Insectary, NRI, UK	*B. tabaci*	3	2	40	2/10
			B. afer	3	2	40	0/10
			B. tabaci + *B. afer*	3	2	60 (30 each)	0/10
			Control - uninoculated	1	-	-	0/3
4	4th controlled transmission	Insectary, NRI, UK	*B. tabaci*	3	2	40	0/45
			B. afer	3	2	40	0/45
			Control - uninoculated	1	-	-	0/20
5	Cage transmission	Glasshouse, Kibaha, Tanzania	*B. tabaci*	3	1	100	2/9
			B. afer	3	1	100	0/9
			1 control each for *B. tabaci* & *B. afer*	1	1	100	0/6
6	Cage transmission	Insectary, NRI, UK	*B. tabaci*	3	1	1000	0/9
			B. afer	3	1	500	0/9
			1 control each for *B. tabaci* & *B. afer*	1	1	500	0/6

7	Field transmission (tissue culture)	Field, Kibaha, Tanzania + Insectary, NRI, UK	*B. tabaci*	1	1	15-20	0/20
			B. afer	1	1	15-20	0/20
			B. tabaci + *B. afer*	1	1	30 (15 each)	0/15
			Beetles	1	1	15	0/12
			Thrips	1	1	5	0/7
			Control - uninoculated	1	-	-	0/12
8	Seed transmission	Glasshouse, NRI, UK	Seeds from diseased plants	5	-	-	0/89
			Seeds from healthy plants	3	-	-	0/32
10	Field trials	Kibaha, Tanzania	-	1	-	-	207/900
		Naliendele, Tanzania	-	1	-	-	151/900

Fig. 1.

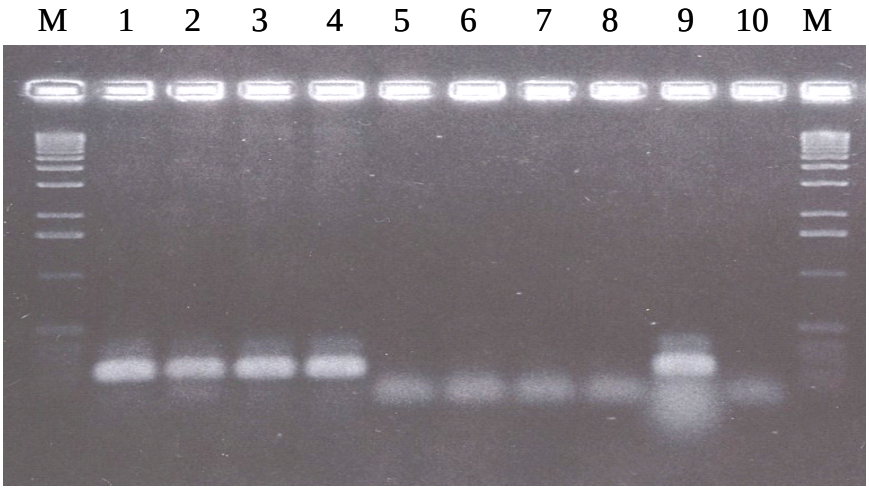
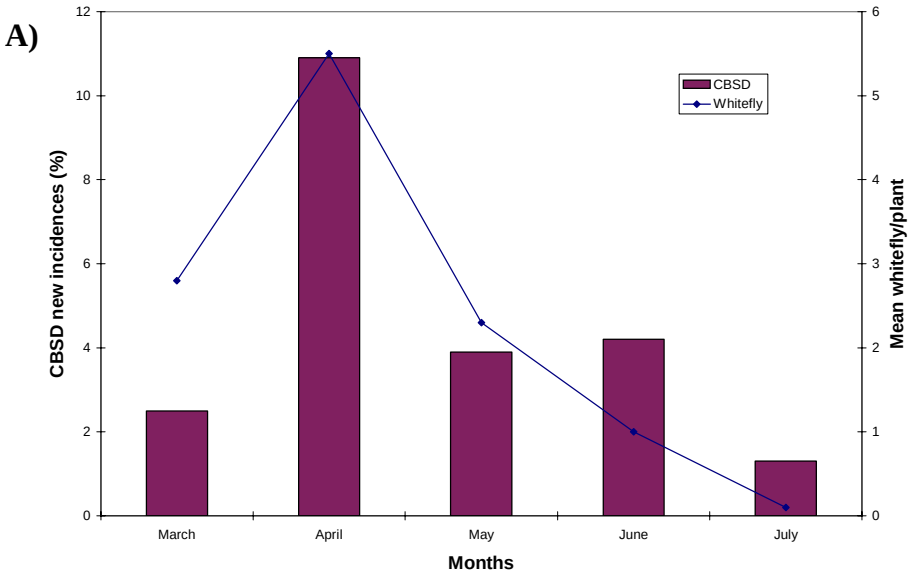
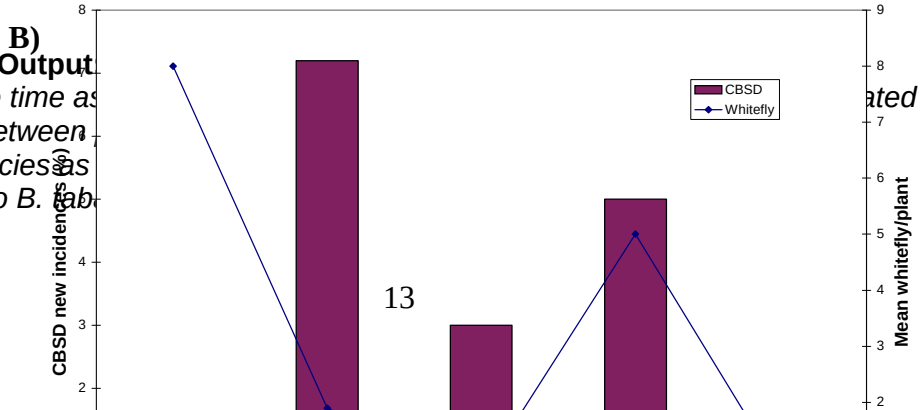


Fig. 2.





Additional Output

At the same time as ... ated variability between ... whitefly species as compared to B. tab...

Achievements

Bionomics, morphometric measurements and partial mitochondrial cytochrome oxidase I gene (mtCOI) sequences were examined for a population of *Bemisia afer* (Priesner & Hosny) (Hemiptera; Aleyrodidae) collected from cassava in Uganda. The development of the eggs, first to fourth instar nymphs and adults required respectively, 12, 31 and 10 days, with a total life duration of ~53 days on the cassava variety Ebwanateraka. Each female laid one egg per day for 13 days and ~40% of the eggs failed to develop into adults. The male: female sex ratio was ~1:4. The length and width of the four nymphal instars were positively correlated (correlation coefficient = 0.97). Females were bigger than males and they differed significantly based on body length ($P < 0.001$) and width ($P < 0.001$). Overlap in their body sizes, however, make the identification on size uncertain. The *B. afer* population shared 68% mtCOI sequence (817 nucleotides) identity with a cassava *B. tabaci* population (Namulonge) from Uganda.

Table 4. Comparison of mean *B. afer* and *B. tabaci* numbers on top and bottom most five cassava leaves recorded at four different locations in Tanzania

Location	No. of plants counted	Crop age (months)	No. on top five leaves		No. on bottom five leaves	
			B. tabaci	***B. afer***	***B. tabaci***	***B. afer***
Kibaha	26	4	41.6±8.96	0.03±0.03	21.0±4.00	0.5±0.15
Zigowale	39	6	6.6±1.11	0.0±0.00	0.2±0.09	0.05±0.03
Chenika	32	12	13.3±1.56	0.4±0.13	0.1±0.07	1.2±0.28
Zanzibar	18	12	69.2±8.42	0.1±0.07	8.8±1.70	9.9±1.69

Numbers of *B. afer* and *B. tabaci* adults varied between fields in Tanzania (Table). Populations of both *B. afer* and *B. tabaci* were highest in Zanzibar and lowest at Zogowale. The proportion of *B. afer* to *B. tabaci* was low on the top five leaves of cassava plants whereas it varied on the bottom five leaves. Fewer *B. afer* than *B. tabaci* were found on the bottom leaves at Kibaha and Zogowale compared to Chanika and Zanzibar. The overall ratio of *B. afer* to *B. tabaci* remained low, which was 1:13. About 96% of the *B. afer* adults were found on the bottom leaves, while the majority of *B. tabaci* were at the top. There was no direct correlation between age of the crop and whitefly numbers. It is notable that the majority (96%) of *B. afer* adults were feeding, and presumably ovipositing, on the yellowing senescent cassava leaves. This observation has important implications on the population development because *B. afer* requires a relatively longer developmental period (egg to adult hatch 43 days) than *B. tabaci* (15-20 days). The older cassava leaves normally drop off the plant quickly during the prolonged dry conditions in the field, removing many *B. afer* eggs and nymphs. *B. afer* population growth is therefore limited under the dry conditions. This distribution pattern of *B. afer* living of on older leaves, may be due to competition for feeding sites with the more aggressive *B. tabaci*, but this requires further investigation.

Partial mtCOI sequences of *B. afer* are available in the EMBL, DDJB and GenBank sequence databases under the accession number AF418673. Based on mtCOI sequences, *B. afer* was similar at 68% to a population of *B. tabaci* from Namulonge, Uganda (GenBank number AF418669). BLAST search for mtCOI sequences identified one other *B. afer* sequence of length 784 nucleotides in the database

(AY057218). The *B. afer* mtCOI sequences of this study shared 96% nucleotide identity with those sequences in the database.