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Are stingless bees the primary vector in spread of banana *Xanthomonas* wilt in Central Uganda?

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ABSTRACT

Stingless bees were speculated to be the primary vectors in the spread of the bacterial agent responsible for banana *Xanthomonas* wilt (BXW) in Uganda. It was hypothesized that the vector entered the banana plant through the male flowers. We therefore determined the likely role of the bees in spread of *Xanthomonas campestris pv. musacearum* that causes BXW. We first documented species of stingless bees present in the banana farms, their nesting sites and foraging behavior. Then we documented the foraging behavior of *Hypotrigona gribodoi* - Magretti and *Plebeina hildebrandti* Friese reared in wooden observation hives where we offered banana sap, bacterial ooze and nectar. We then measured sugar concentration in Pisang Awak, the banana variety reported to be most susceptible to *Xanthomonas*. We tested how far stingless bees would fly to collect banana nectar through indirect recruitment experiments to experimental feeders with 11, 33, 48 and 54% sugar solutions. Our findings were: four species of stingless bees, *Hypotrigona gribodoi*, *Plebeina hildebrandti*, *Meliponula ferruginea* Lepeletier and *Meliponula* sp. foraged and nested within the banana farms. They collected nectar from both male and female banana flowers but spent more time on female flowers than on male flowers. They did not collect banana sap and bacterial ooze from scars of recently dehisced male flowers and at the nest entrance of the observation hives. The foraging distance of workers of *P. hildebrandti* was 1050 and 1215 m when sugar solutions 11 and 33% were offered, respectively. The foragers would therefore fly less than 1215 m from the nest to collect Pisang Awak nectar which had an average sugar concentration of 12.5%. Thus stingless bees were likely to get accidental contamination with *Xanthomonas campestris pv. musacearum* just like any other moving object or insects. If the bees got accidentally contaminated with *Xanthomonas*, saps and resins within the nest would decontaminate them.

Keywords: Stingless bees, vectors, *Xanthomonas*, sugar, nectar.

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INTRODUCTION

Stingless bees are known for their highly medicinal honey and as pollinators of several crops. However, in Uganda they were speculated to be the primary vectors of *Xanthomonas campestris* pv. *musacearum* (*Xcm*), the causal agent of banana xanthomonas wilt. *Xanthomonas* was speculated to enter the banana plant through moist scars of recently dehisced male flowers and floral bracts. Nevertheless, the speculation was not based on any study to support the hypothesis.

The speculation was based on the grounds that Banana Xanthomonas Wilt (BXW) in Uganda had similar symptoms to other banana bacterial diseases such as Moko whose vectors were insects (Eden-Green, 2004). In addition, *Xanthomonas* had been recovered from stingless bee's body (Tinzaara et al., 2006). However, no study had been done to show the role and mode of transmission of the bacteria by insects or to support the hypothesis that stingless bees were the primary vectors.

A study on the foraging behavior of the bees was therefore necessary to determine the likelihood and the means through which the bees could contract and spread the pathogen from a banana plant to another and back to the nest. The behavior of the bees within the nest was of special importance in determining the fate of the pathogen if brought back to the nest by the foragers. The possibility of the nest acting as a reservoir for the pathogen and a center for the disease spread required investigation. We therefore did a study in central Uganda to determine the possible role of stingless bees in the spread of banana *Xanthomonas* Wilt.

METHODOLOGY

Study area

The study was carried out for one year in farmlands planted with bananas in four districts, Luwero, Mpigi, Mukono and Wakiso in Central Uganda.

Determining stingless bee species and their nesting sites in the farmland

A stingless bee survey was done in selected farms in Luwero, Mpigi, Mukono and Wakiso districts in central Uganda. During the survey, all species of stingless bees visiting banana plants were documented and specimen captured for later identification at the National Museums of Kenya by Dr. Mary Gikungu. Their nesting sites were also recorded.

Foraging behavior and foraging duration in the banana farms

Stingless bees foraging behavior on bananas was recorded in Mukono and Wakiso districts. In Mukono, the foraging behavior was recorded in eight Pisang Awak banana plants (5 males, 3 females) for 84 h. In Wakiso, it was recorded on seven sweet banana plants (6 males, 1 female) and one Highland (Matooke) banana for 84 h. A forager was observed from the time it landed on the flowers until it left. Observations were done in one hour sessions from 8.00 to 18.00 h. The total time a bee spent on the flowers and the activities done before flying away were recorded. Observations were made and documented on which part of the banana plants were visited by bees. When a forager flew away from the banana plants, it was noted if it landed on banana plants within the vicinity. When a forager flew away, the observer waited for the next stingless bee forager to arrive on the flowers and observations began. The species of the forager under observation was recorded. Other insect visitors on the flowers were also recorded.

We observed if stingless bees would take up ooze of *Xanthomonas* infected banana plants and banana sap. Green fruits, bracts, male and female flowers were cut to allow sap to seep out, and then we observed if bees would take them up. We also observed whether bees would collect sap from naturally occurring scars on the flower stalks caused by fall of male flowers and bracts.

Determining the amount of nectar and sugar concentration produced by male and female Pisang Awak flowers

Using micro-pipets (1, 2, 5, 10 and 100 µl), nectar was extracted and volume measured, from 29 male and 8 female flowers, from eight Pisang Awak bananas in Mukono district. Using a hand held sugar refractometer, the nectar sugar in each flower was measured.

Foraging behavior of *Hypotrigona gribodoi* and *Plebeina hildebrandti* colonies reared in wooden observation hives

Three colonies of *Hypotrigona gribodoi* and four colonies of *Plebeina hildebrandti* were reared in wooden observation hives at Kawanda Agricultural Research Institute (KARI) in Uganda. The observation hives were wooden with transparent glass cover. The hives for *P. hildebrandti* measured 50 cm length, 20 cm width and 25 cm height, while for *H. gribodoi* measured 20 cm length, 10 cm width and 15 cm height. The hives had holes drilled on one side in which silicon tubes were inserted for exit and entrance of the bees. The bees were provided with fresh sap and old sap from healthy bananas, ooze from bananas infected with *Xanthomonas* and banana nectar at the nest entrance. To hold nectar, sap and ooze at the entrance, a silicon tube, 3.8 cm length and 2.5 cm width was cut open and placed at the edge of the stingless bees exit tube, such that it wrapped around it and left 1.3 cm extending beyond the exit tube (Figure 1).

Procedure

One at a time, two drops of nectar, sap, and ooze were placed within the 1.3 cm extension of the silicon tube. The nectar was from Pisang Awak male flowers with 11% sugar concentration. Immediately after placing either of the above substances, we counted for five minutes the numbers of bees that came to the entrance tube. That is immediately 0, 2.5 and 5 min. The bees that touched with antenna or took up the substances provided were recorded with the aid of a digital video camera. We did not differentiate whether it was the same bees coming to the entrance tube from inside the hive and returning or different bees that we counted at 0, 2.5 and 5 min. The silicon tube was changed every time a new substance was under observation. Observations were done for *H. gribodoi* and *P. hildebrandti*. The experiment was repeated three times for both ooze and sap.

To find out if bees would take old sap e.g. the sap which exudes from the scars caused by the fall (dehiscent) of male flowers and the flower bracts, three small containers were filled with banana sap and placed inside three *P. hildebrandti* hives for seven hours. The sap was squeezed from the base of male and female Pisang Awak flowers immediately after plucking them from the flower stalks. After seven hours we observed if the bees had taken up the sap. The three small containers were then removed and placed adjacent to the hives to find out if the foragers would take up the substances within them.

Plebeina hildebrandti foraging behavior and foraging distance

Procedure for recruitment of nest mates to experimental feeders (food sources)

These experiments were done to determine if the foragers would collect nectar from one banana plant or more during a single foraging trip. Three feeders were used to represent the banana plants. Small containers with cotton wool soaked with sugar solutions were used as the feeders. Cotton wool was provided to prevent bees from drowning in the sugar solution. The sugar solutions consisted of honey from *Apis mellifera*, sugar and water.

Using a feeder (feeder one), foragers were trained to come to the feeder at a distance of 30 m from the hive. On the assumption that bees scent marked feeder one during the training, a second feeder (feeder two) was placed adjacent to feeder one at 30 m from the hive. This was done to determine if bees would collect sugar

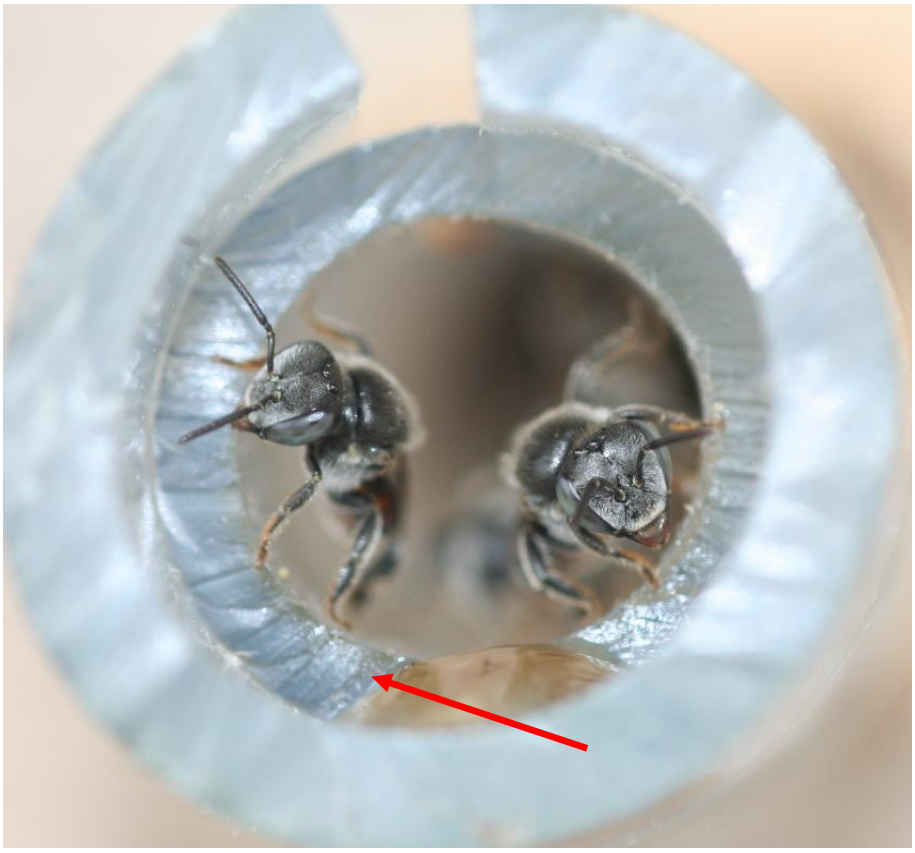

Figure 1. A 3.8 cm × 2.5 cm silicon tube cut open and placed at the edge of the hive exit tube, such that it wrapped around the exit tube and left 1.3 cm extending beyond the tube. Drops of nectar, sap and ooze (arrow) were placed on the 1.3 cm extension for uptake by stingless bees foragers.

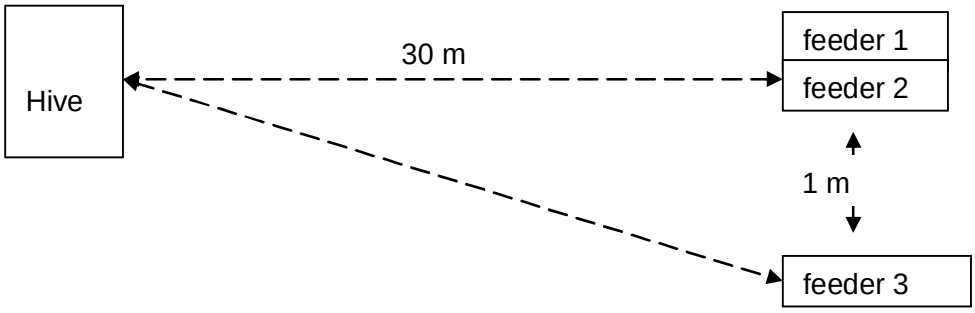

Figure 2. Feeder arrangement in an experiment to determine if *Plebeina hildebrandti* foragers recruited nest mates to a food source.

solution from feeder two which was not scented marked. A third feeder (feeder three) was placed one meter from feeder one and two, but equal distances (30 m) from the hive to determine if the bees would collect sugar solution from it (Figure 2). All the three feeders were identical small containers with cotton wool soaked with 40% sugar solution. Foragers arriving at the three feeders were counted and marked every 5 min for a period of 60 min. Bees arriving at feeder three were captured. Attention was paid on bees flying across the three feeders.

Procedure for training of the bees to experimental feeders

A few drops of the sugar solution were put at the silicon exit tube of a hive in which *P. hildebrandti* colony was reared. After the foragers took up the solution, the experimental feeder was placed in front of the exit tube. The feeder was in contact with the tube so that the bees which had previously taken up the sugar solution from the exit tube could find it. Foragers were allowed to take the sugar solution and return to the hive twice before the feeder together with the bees

on it could be moved for some centimeters from the entrance and when the bees were back on the feeder they were moved stepwise for 1 to 3 m up to 30 m from the hive.

Procedure for determining how far foragers would fly to collect sugar solutions of different concentrations

To determine *P. hildebrandti* foraging distance and if sugar concentration determined the distance flown, bees were trained to 11, 33, 48 and 54% sugar solutions. The feeder together with the bees on it was moved step by step from the hive up to the furthest distance when recruitment diminished and no more bees arrived on it. At every step new recruits were counted and marked on the thorax.

The choice of 11 and 33% sugar concentrations was based on the knowledge that Pisang Awak, the banana variety most susceptible to *Xanthomonas* had nectar sugar concentrations of 0 to 32%, with a mean of 12.5%. The choice of 48 and 54% was based on the fact that stingless bees profit mostly from nectars with over 40% sugar concentration.

RESULTS

Stingless bee species and their nesting sites in banana farms

Four stingless bee species, *Hypotrigona gribodoi*, *Plebeina hildebrandti*, *Meliponula ferruginea* (Lepeletier) and *Meliponula* sp. were recorded visiting banana flowers in all the four districts. *Meliponula* sp. was recorded only in Mukono district. *H. gribodoi nested* in cavities in trees, mud walls, blocked drainage pipes, electrical sockets and wooden poles. *P. hildebrandti* nested in occupied termite mounds. *M. ferruginea* nested in cavities in trees with 10 nests found in *Cupressus lucitanica*. *Meliponula* sp. nests were not found.

Foraging behavior and foraging duration in the banana farms

Four species of stingless bees collected nectar from banana flowers. They neither collected fresh sap from different parts of banana plant, nor sap from scars on the flower stalks caused by fall of male flowers and bracts. The foragers did not collect ooze from banana plants infected with *Xanthomonas*. More than one forager could easily access nectar in female flowers as the flowers opened widely (Figure 3a), while in male flowers, only one forager would collect nectar at a time, except for the small *Hypotrigona gribodoi*. Foragers had difficulties squeezing through the male flowers to reach the nectar as the flowers were compressed together and did not open widely (Figure 3b). As there were other animal visitors (honey bees, solitary bees, wasps, flies, beetles, drosophila, ants, weaver birds and sunbirds), competing for nectar or predating on other insects on the flowers, a stingless bee forager visited several flowers under one or more open bracts before leaving a banana plant. After collecting nectar, a bee would clean its wings and legs while seated on top of the flowers or bracts before flying away. In one occasion, a forager was observed flying from the banana plant under observation to a second banana in a separate stool hanging two meters away.

Although there was no significant difference on the duration that foragers belonging to the four species spent per visit to the flowers from morning to evening (One way ANOVA $F_{9, 247} = 1.13$ $P = 0.46$), there was a clear trend showing that the foragers spent more time on flowers between 0800 and 1400 h (Figure 4a). The foragers spent more time on female flowers than on male flowers (t-Test = 2.5, DF = 108, P = 0.01 (Figure 4b).

Pisang Awak nectar volume and sugar concentration

On average Pisang Awak had nectar with 12.5% sugar concentration with a range of 0 to 32%. The mean nectar volume per flower was 30.2 µl with a range of 0.5 to 102 µl. There was significantly larger volumes (Figure 5a) and higher nectar sugar concentration in male flowers than in female flowers during the day (t-Test = -3.2, DF = 41, P = 0.01 for nectar volume and t = -2.7, DF = 41, P = 0.01 for sugar concentration (Figure 5b).

Foraging behavior of *Hypotrigona gribodoi* and *Plebeina hildebrandti* colonies reared in wooden observation hives

Foragers of both *Hypotrigona gribodoi* and *Plebeina hildebrandti* collected Pisang Awak nectar with 11% sugar concentration offered at the nest entrance (Table 1). Foragers did not take up sap and ooze. When sap was introduced inside the hives in small containers, the bees did not take it. Instead they covered the sap with propolis collected from within the nests. When the containers were taken out, the bees collected back the propolis (Figure 6).

Recruitment and foraging distances for *Plebeina hildebrandti* foragers when offered 11, 33, 48 and 54% sugar solutions

Recruitment of nest mates to experimental feeders (food sources)

Significantly more bees arrived on feeder one, than two and three ($F_{2, 33} = 17.05$; $P < 0.01$) during the 60 min observation period. A total of 192 bees (93.2% of the total bees to the three feeders) arrived to feeder one, used in training the bees from the nest entrance up to 30 m from the hive. Twelve bees (5.83%) arrived to feeder two, which had not been used in training the bees, but was placed adjacent to feeder one. Two bees (0.97%) arrived at feeder three, which had not been used in training the

Figure 3. (a) A stingless bee forager collecting nectar from inside a female banana; (b) Two stingless bees try to reach nectar in a male flower. One forager (i) tries to get inside the flower while another (ii) tries to access nectar outside, at the flower base.

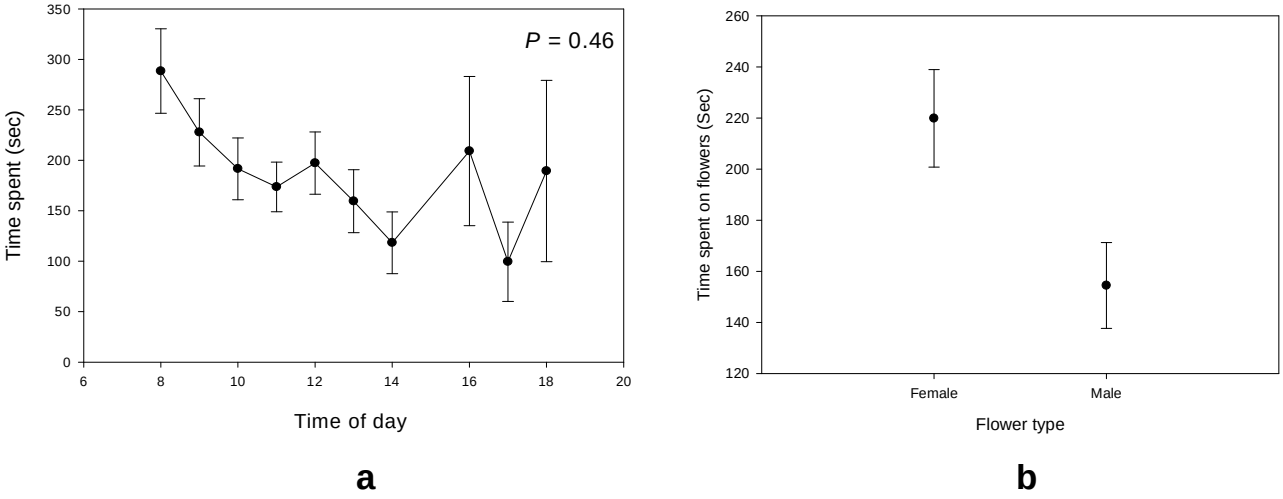

Figure 4. (a) Mean (± S.E) time spent per visit by four species of stingless bees' foragers on banana flowers from 8 A.M. to 6 P.M. One way ANOVA, P = 0.46**.** (b) Mean (± S.E) time spent by three species of stingless bees' foragers on male and female banana flowers. N = 110 0bservations, 48 on female and 61 on male flowers.

bees, but was placed one meter from feeders one and two (Figure 7). Of the 12 bees on feeder two, nine were directly from the nest while three had previously visited feeder one. The two bees that arrived at feeder three had previously visited feeder one.

Foragers recruited to the sugar solutions declined with increase in distance. For example to 48% sugar solution, at the hive entrance (0 m) 44 bees came to the feeder while at 1220 m from the hive only one bee came to the feeder. Recruitment of new comers stopped before foragers who had foraged earlier, on the sugar solution (experienced), stopped foraging. Foraging distance was dependent on the concentration of the sugar solution, with foragers flying shorter distances for low sugar concentration and longer distances for higher sugar concentration (Table 2).

DISCUSSION

Stingless bees foraging behavior and its implications to the spread of *Xanthomonas*

Four stingless bees species collected nectar from bananas in central Uganda. They collected nectar from both male and female Pisang Awak flowers. It was easier for the foragers to access nectar in female flowers as they opened widely in comparison to male flowers. This

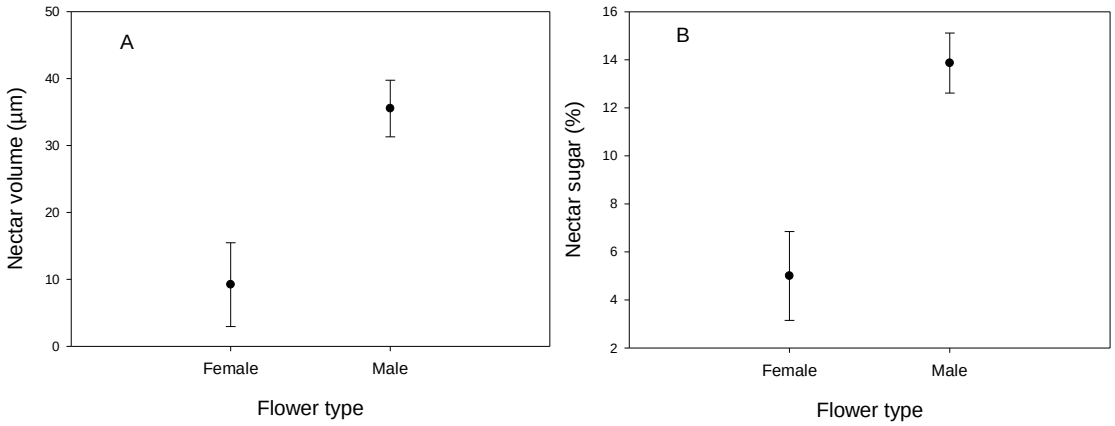

Figure 5. (a) Mean (± S.E) in (A) nectar volume in µl; and (b) nectar sugar (percentage) in male and female Pisang Awak flowers during the day. N = 43 flowers.

Table 1. Mean number of *Hypotrigona gribodoi* and *Plebeina hildebrandti* workers observed at the nest entrance tube in the presence of 11% Pisang Awak nectar, sap and ooze during three (5 min) observation periods, and the number of workers that either antenned or collected the nectar. N = Nectar, S = Sap, Oz = Ooze.

Species	Bees at the entrance tube			Bees antenning nectar, sap or ooze	Bees collecting nectar, sap or ooze
	0 min	2.5 min	5 min	In 5 min	In 5 min
H. gribodoi	1	5	8	4N, 1S, 3Oz	5N, 0S, 0 Oz
P. hildebrandti	1	10	10	1N, 2S, 0 Oz	6, 0S, 0 OZ

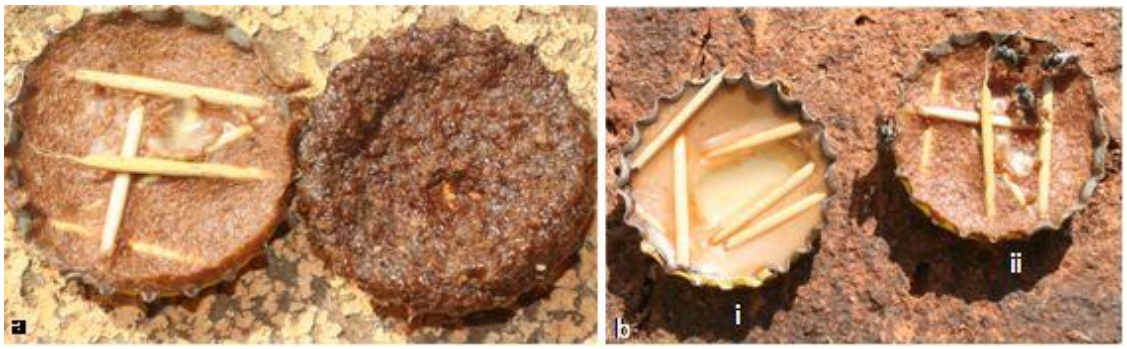

Figure 6. (a) Sap in small containers introduced inside *P. hildebrandti* hive showing propolis layer smeared on top. (bi) The bees did not touch sap put in a control container; (bii) Bees collecting propolis covering the container which had been put inside the hive.

allowed more than one forager to simultaneously collect nectar in a female flower. On the other hand, male flowers were narrow and so much compressed that it was difficult for the bees to get inside and only a single forager could get in at a time. As a result larger quantities of nectar were recorded in male flowers than in female flowers during the day. Due to nectar remaining in the male flowers for a longer time without being taken by insects, evaporation of water took place leading to higher sugar contents than in female flowers. Female flower morphology encouraged interactions of more than one forager, which could enhance the spread of *Xanthomonas* as compared to male flower morphology.

The foragers neither collected ooze from bananas infected with *Xanthomonas* nor sap from injured parts and moist scars caused when male flowers dehisced. Provision of sap, ooze and nectar to *H. gribodoi* and *P. hildebrandti* reared in wooden observation boxes confirmed that stingless bees did not collect sap and ooze from bananas. In fact, banana sap introduced inside the hives was embalmed with propolis. Embalming is normally done to predators and other intruders which die

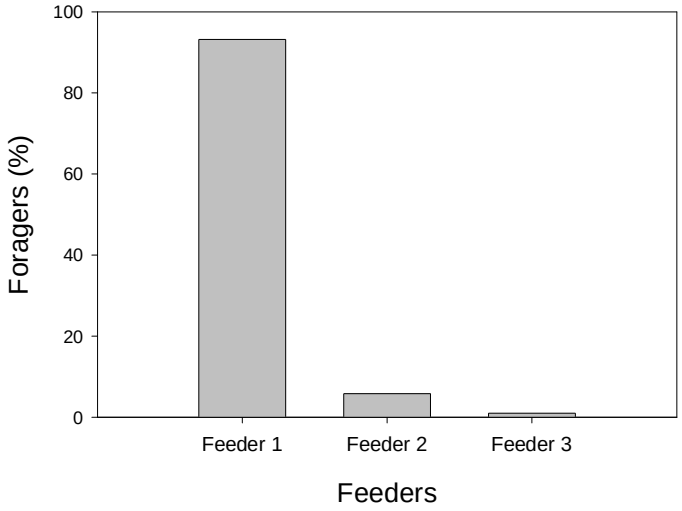

Figure 7. Percentage of *Plebeina hildebrandti* foragers arriving on three feeders with 40% sugar solution, placed 30 m from the hive, for a period of 60 min. Feeder 3 placed one meter from the other two.

Table 2. Distances from the hive at which recruitment of new nest mates and foraging by experienced *Plebeina hildebrandti* foragers stopped when provided with sugar solutions of varying concentrations.

Sugar concentration of the solutions (%)	Distance (m) at which recruitment of new comers stopped	Distance (m) at which experienced foragers stopped collecting the sugar solutions
11	1030	1050
33	1160	1200
48	1185	1220
54	1215	1230

inside stingless bee nests to prevent bacteria growth (Ghilsalberti, 1979). Therefore, if *Xanthomonas* is spread through scars caused by fall of male flowers, stingless bees were not primary vectors as they did not visit or collect any material from the scars. Other animal vectors or modes of transmission could be more important in the spread of the disease. For example, since Banana *Xanthomonas* Wilt is a vascular disease (Biruma et al., 2007), biting and sucking insects may be more important vectors than stingless bees.

Possible accidental contamination of stingless bees with *Xanthomonas*

There is a possibility of accidental contamination of stingless bees with *Xanthomonas* through body surface contact with bacterial ooze. In this case, the bacteria would be on their surface and not inside their body. Also if *Xanthomonas* was present in the nectar, stingless bees are likely to get contaminated, either through contact with the nectary or imbibing the nectar into the nectar stomach. In this case *Xanthomonas* would be swallowed into the nectar stomach. Some studies have reported presence of *Xanthomonas* in nectar (Ssekiwoko et al., 2006), while others found no *Xanthomonas* in nectar (Mwangi et al., 2007). If *Xanthomonas* was present in the nectar, the bacteria would either be carried to the bee nest or transported to another banana plant.

Fate of *Xanthomonas* inside the stingless bee nest

In the nest, nectar from foragers may undergo several pathways. One, it is fed to nest mates (trophalaxis), in which case the nectar is partly eaten and digested. In this case *Xanthomonas* is likely to be destroyed or it may come out with the waste products. Waste products in stingless bee nests are stored in one corner from where it's later carried out. Two, nectar is stored in nectar pots where it's dehydrated to honey with a high sugar concentration of about 70 to 80%. Nectar pots contain propolis (mixture of wax, plant resins and gums) as a constituent building material. Propolis has been shown to

have antimicrobial properties (Ghilsalberti 1979; Mereste and Mereste, 1988; Marcucci, 1995; Gilliam, 1997; De Campus et al., 1998). Therefore, *Xanthomonas* would be eliminated from inside stingless bee nests by propolis and from honey by the high sugar content through desiccation. Furthermore, the perennial colonies of stingless bees depend on food stores in humid tropical environments and species living in the soil employ mechanism for preservation and for protection of food stores from microorganisms (Gilliam et al., 1984, 1985). Honey bees and stingless bees in particular have an ancient relationship with *Bacillus* species which help to protect nest from microbial invasions. *Bacillus* species produce antimicrobial compounds that inhibit other microorganisms (Gilliam et al., 1984; Cano et al., 1994; Gilliam, 1997).

Fate of *Xanthomonas* in the banana fields

Personal observations in the field showed that foragers visited a single banana plant per foraging trip. From recruitment experiments foragers (93.2%) persistently visited feeder one, which had been used in recruiting the bees from the nest entrance, while only 5.83% arrived on feeder two placed adjacent to feeder one at 30 m from the hive. Persistency of foragers on feeder one for 60 min shows that, either the foragers were scent marking the feeder so that new comers only came to feeder one, or the foragers were guiding the new comers from the nest to the feeder. Therefore, even in banana plantations foragers from one nest would collect nectar from one banana plant and not each forager going to different plants. If *Xanthomonas* was present in a banana plant, it would be transported between the plant and the bee nest. Therefore the hypothesis that stingless bees are primary vectors as they live in large colonies, and are likely to spread *Xanthomonas* in mass need reexamination.

Plebeina hildebrandti forage distance

P. hildebrandti foraging range decreased with decrease in sugar concentration. For example for 11% sugar solution foraging stopped at 1050 m, for 33% at 1215 m, for 48% at 1220 m and for 54% at 1230 m. Roubik et al. (1995) pointed out that the nectar gathered by Meliponini averaged 44% sugar concentration with mean range of 20 to 61%. An indication that although *P. hildebrandti* collected nectar from banana flowers, bananas may not be a preferred choice for optimal foraging, hence the bees may not be willing to fly for long distances to collect banana nectar. Roubik et al. (1995) predicted that a sizeable proportion of flowers must have nectar of optimal sweetness for bees, regardless of flower nectar volume. Therefore Pisang Awak, the most susceptible banana variety to *Xanthomonas,* had copious nectar with a mean of 12.5 % sugar concentration, which is below optimal sweetness for the bees.

Extrapolating *Plebeina hildebrandti* foraging rage to the other stingless bees species

Three other species of stingless bees were found to collect nectar from banana flowers. *Hypotrigona gribodoi* (Magretti) is a small bee with a body size of 2 to 3 mm (Eardley, 2004), and likely to have a much shorter foraging distance than *P. hildebrandti,* which has a body size of 3.3 to 5.2 mm. *Meliponula ferruginea* (Lepeletier) with a body size of 5.1 to 5.9 mm (Eardley, 2004), and *Meliponula* sp*.* are slightly larger than *P. hilderbrandti*, hence likely to forage slightly further. Studies have shown that there is a correlation between the bee size and the maximum foraging distance (van Nieuwstadt and Iraheta, 1996; Araújo et al., 2004). Maximal flight distance for medium sized stingless bees is estimated at 1159 to 1710 m while that for larger stingless bees is estimated at 2 km (Araújo et al., 2004). This shows that if stingless bees were the vectors of *Xanthomonas*, they could only spread it locally and long distance transmission would be through other means which require investigations.

Conclusion

Stingless bees are likely to get accidental contamination with *X. campestris* pv*. musacearum* just like any other moving object or insect.

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