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RESEARCH ARTICLE

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SURVEILLANCE STRATEGY FOR HUANGLONGBING OF CITRUS IN BAHIA THROUGH SENTINEL ROUTES

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ABSTRACT

Bahia is the second-largest orange producer in Brazil, with production predominantly based on traditional cultivation and management practices. However, this activity faces serious phytosanitary threats, requiring constant attention from agricultural defense authorities and integrated preventive actions. The objective of this study was to monitor the possible invasion of the bacterium *Candidatus Liberibacter* spp. (CLAs) through the collection of the vector insect *Diaphorina citri* along strategically distributed sentinel routes. Collections were carried out quarterly, and *D. citri* insects found on citrus plants and orange jasmine (*Murraya paniculata*) were collected using an entomological aspirator. The material obtained was sent to the Plant Virology Laboratory of Embrapa Cassava and Fruits for molecular analysis using Real-Time PCR (qPCR). Between 2019 and 2022, 7,648 adults were collected along Strategic Routes and 4,180 along Control Routes. All plant tissue samples and 99% of insect samples tested negative. Only two collections of psyllids in Western Bahia tested positive; however, the infection was not confirmed in the second sampling. Thus, Bahia maintains its status as an HLB-free area. Early detection of CLAs is essential for phytosanitary surveillance, allowing a rapid and efficient response to eradicate foci and prevent the spread of the devastating disease known as citrus huanglongbing.

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INTRODUCTION

Huanglongbing (HLB), also known as greening, is the most serious disease affecting citrus worldwide. First reported in China in 1929, HLB has since spread to more than 40 countries. In South and North America, the first records occurred between March 2004 and August 2005, in São Paulo (Brazil) and Florida (United States), respectively (Bové, 2006). In 1996, a rapid method was developed to detect *Candidatus Liberibacter* spp., the causal agent of HLB, using the polymerase chain reaction (PCR) technique, which relies on a single pair of primers. Later, this evolved into Nested-PCR, which employs two pairs of primers. This technique offers greater sensitivity and specificity, increasing the number of

bacterial DNA copies amplified (Jagoueix *et al.*, 1996; Hocquellet *et al.*, 1999; Hung *et al.*, 1999). In Bahia, the insect vector is easily found, particularly in urban areas, as it is associated with orange jasmine (*Murraya paniculata*), a host favorable for its development. Similar to citrus seedlings, the transport and commercialization of *Murraya* plants infected with *Ca. Liberibacter* pose a risk for HLB introduction in the state (Fancelli, 2020). Monitoring the psyllid vector in areas still free from HLB allows control decisions to be anticipated by up to 18 months—the estimated time for symptom manifestation in host plants. During this period, asymptomatic individuals can act as sources of bacterial inoculum if infected (Manjunath *et al.*, 2008). Another widely adopted method, particularly in Mexico, is the creation of Containment and Risk

Reduction Zones (ARCOS). This approach uses a web-based system integrating area organization, vector monitoring, assessment of insecticide use, and biological control (Mora-Aguilera *et al.*, 2014). A study by Flores-Sánchez *et al.* (2015) found that 15 months after the detection of *Candidatus Liberibacter asiaticus* in a Tahiti lime orchard in Tizimín, Yucatán, Mexico, HLB had a negative effect on fruit size, peel thickness, pH, juice volume, and weight. This highlights the importance of further research related to HLB. The Agricultural Defense Agency of Bahia (ADAB) operates through fixed and mobile barriers to inspect the movement of plants. The agency removes citrus and *Murraya* seedlings lacking the Phytosanitary Certificate of Origin (CFO), transported without the Plant Transit Permit (PTV), or produced in federal units where Greening or HLB occurs.

MATERIAL AND METHODS

The research was conducted from 2019 to 2022, with four annual collections of psyllids carried out along Strategic Routes (routes with high plant transit flow) and Control Routes (routes with high tourist flow) (Figure 01). Each collection point was georeferenced, allowing the creation of maps using the MapMarker and QGIS applications.

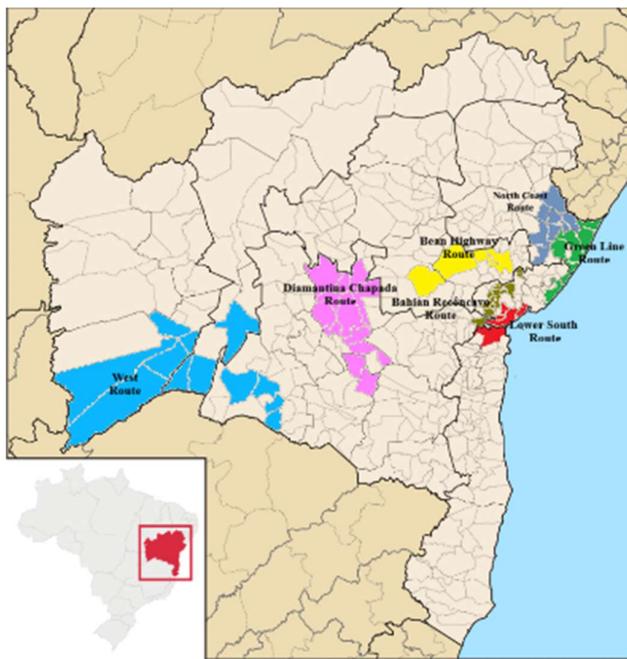


Figure 01 – Map of the Strategic Routes (West – blue, Chapada – pink, Recôncavo – yellow, and North Coast – moss green) and Control Routes (Linha Verde – olive green, Baixo Sul – red, and Estrada do Feijão – yellow). Application: MapMarker. Source: AMORIM, D. F. (2021).

The designation of the routes was based on the risk of HLB introduction, being classified as follows: Strategic Route – with a high probability of encountering insects carrying the citrus HLB bacterium, and Control Route – with a low probability of bacterial detection in insects. The following attributes were considered: intense movement of people and goods, proximity to states with the pest, continuous presence of susceptible hosts, tourist areas, and trade of propagation material in nurseries. With quarterly frequency, collections of the vector insect were carried out on orange jasmine (*Murraya paniculata*) and citrus plants found in nurseries, public areas, and private properties along the routes. Each sampling point

was georeferenced, and the host type recorded. Initially, all *Murraya* plants in the municipalities were mapped, and collections were concentrated in points with greater plant transit and tourist activity. At the beginning, sampling occurred in multiple points per municipality, collecting from all *Murraya* and/or citrus plants where adult psyllids were found. After mapping hosts, the protocol was standardized to one collection per municipality, limited to 20 adults per point, except in municipalities with nurseries and greenhouses, where all *Murraya* and citrus were sampled due to the higher risk of HLB introduction.

A total of 954 host plants were georeferenced: 88 citrus and 866 *Murraya*. Of these, 80 citrus and 292 *Murraya* were from Control Routes, and 8 citrus and 574 *Murraya* from Strategic Routes. Adults were collected with a manual aspirator, nymphs with a fine brush, and shoots with eggs were preserved in 95% ethanol. Samples were labeled with date, location, and code, and data such as coordinates, host, and insect stage were recorded in standardized spreadsheets. Samples were sent to the Plant Virology Laboratory of Embrapa Cassava and Fruits, where real-time PCR (qPCR) was performed to amplify the bacterial genomic fragment and assess DNA quality and concentration, following Abreu *et al.* (2020). Samples with 5–10 adults and/or 10 nymphs were transferred to 1.5 mL microtubes and washed three times with distilled water. qPCR tests used positive controls from Fundação Sylvio Moreira and negative controls (previous samples and water). Data were entered into Excel and analyzed quantitatively, counting psyllids by routes and municipalities to determine psyllids per municipality and psyllids per route. Graphs were generated using Excel, Paint, and QGIS, with adjustments for better visualization when converted to JPG images.

RESULTS AND DISCUSSION

As a result of monitoring the potential invasion of the HLB-causing bacterium along the routes, 11,828 specimens of *D. citri* were collected and analyzed by the EMBRAPA Laboratory (Table 01).

Routes	Georeferenced Points	Number of Insects Collected	Average per Point
Control			
Green Line Route	171	1.386	8,1
Lower South Route	97	1.799	18,55
Bean Highway Route	75	995	13,27
Subtotal	343	4.180	12,19
Strategic			
West	151	1.267	8,39
Diamantina Chapada	213	1.691	7,94
Bahian Recôncavo	39	3.808	97,64
North Coast	179	882	4,93
Subtotal	582	7.648	13,14

Table 01 – Quantification of mapped points and number of psyllids collected over four years in the State of Bahia. Strategic Routes: – West Bahia (Urandi, Pindaí, Guanambi, Palmas de Monte Alto, Carinhanha, Feira da Mata, Cocos, São Félix do Coribe, Santa Maria da Vitória, Bom Jesus da Lapa, and Coribe); – Chapada Diamantina (Andaraí, Mucugê, Ibicoara, Tanhaçu, Ituaçu, Abaíra, Boninal, Barra da Estíva, Piaçã, Seabra, Palmeiras, and Lençóis); – Bahian Recôncavo (Santo Antônio de Jesus, Laje, Conceição do Almeida, Sapeaçu, Cruz das Almas, Muritiba, Governador Mangabeira, Cachoeira, Jaguaripe, Feira de Santana, Conceição do Jacuípe, and Amélia Rodrigues); – North Coast (Alagoinhas, Entre Rios, Inhambuê, Olindina, Itapicuru, Rio Real, Jandaíra, Conde, and Esplanada). Control Routes: – Green Line (Lauro de Freitas, Camaçari, Mata de São João, Entre Rios, Esplanada, Conde, and Jandaíra); – Lower South (Valença, Jaguaripe, Aratuípe, Nazaré, Vera Cruz, and Itaparica); – Bean Highway (Itaberaba, Ipirá, Anguera, Serra Preta, and Feira de Santana).

Regarding the number of collections carried out over the past four years (Figure 02), in the Control Route during 2019, a lower number of insects was observed compared to subsequent years. This difference was not due to intrinsic factors but rather to the fact that, in 2019, only two collection trips were conducted, whereas in the following years, four quarterly collections were performed. Consequently, the number of psyllids collected was three times higher than in 2019.

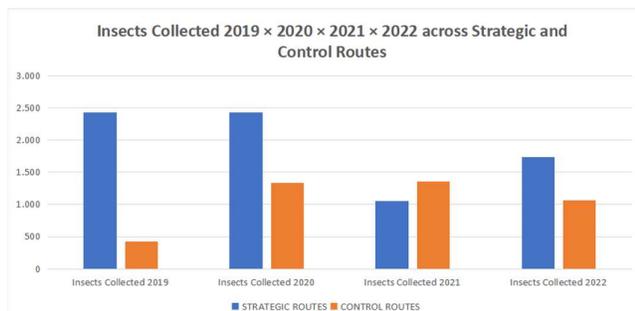


Figure 02. Number of insects collected over four years along the Strategic and Control Routes

On the other hand, in the Strategic Route during 2021, a lower number of insects were collected. This reduction can be explained by the atypical conditions caused by the COVID-19 pandemic, which prevented the completion of all four route expeditions (West, Chapada, Recôncavo, and North Coast), reducing psyllid collections by approximately 50%. Meanwhile, Figure 03 shows that the Recôncavo Route recorded a particularly high number of psyllids collected. This is mainly due to the proximity of *Murraya paniculata* host plants to large citrus-growing areas along the BR-101 highway. However, in 2021, collections decreased because of the pandemic peak, resulting in only three collection trips that year.

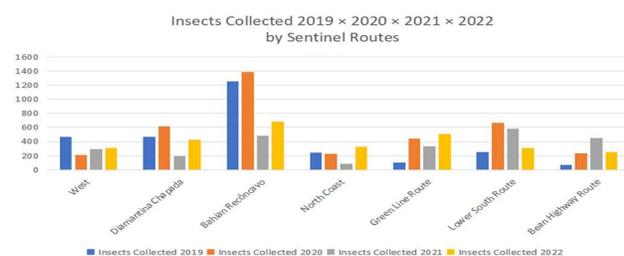


Figure 03. Psyllids collected over four years, shown separately for each route

In the Control Routes, many insects were collected, especially along the Green Line and Lower South routes, likely due to the high density of host plants in urban areas and nurseries. The thermal amplitude of these regions also favors *D. citri* populations by promoting frequent plant flushing. The Bahian Recôncavo exhibits a high frequency of citrus psyllid (ACP) cycles, as reported by Laranjeira *et al.* (2019), who noted that the development and occurrence of *D. citri* are influenced by altitude, temperature, humidity, and precipitation. Liu & Tsai (2000) also emphasized that the psyllid's life cycle is highly temperature dependent. In 2021, the Lower South region recorded the most insects, mainly in Valença-BA, which accounted for nearly half of all psyllids. This was linked to a 100-meter hedge of *Murraya paniculata*, managed year-round, dust-free, and irrigated, ensuring continuous flushing and ideal conditions for eggs, nymphs, and adults. According to Parra *et*

al. (2010), psyllid population growth is positively related to plant flushing. *D. citri* was consistently detected across all routes, including non-citrus-producing areas such as the Green Line and Bean Highway, confirming adequate route definition and underscoring the need for ADAB to intensify monitoring.

Among Strategic Routes, Recôncavo recorded the highest number of insects, followed by Chapada Diamantina. These citrus-producing regions show greater potential for bacterial spread if HLB occurs. Conversely, the North Coast route had the lowest captures, five times fewer than Recôncavo. In the Control Routes, Lower South and Green Line presented nearly double the insects of other routes. Though not citrus producing, the Bean Highway (995 insects) still exceeded the North Coast Strategic Route (882 insects), possibly because collections there were interrupted by the pandemic. Both Strategic and Control Routes had similar sample numbers, but Strategic Routes collected 1.8 times more insects, corroborating data from monitoring activities conducted in the Recôncavo region of Bahia, as reported by Sanches *et al.* (2018). This pattern is linked to the preferred host *Murraya paniculata*, which offers favorable feeding and reproduction conditions due to constant flushing (Abreu *et al.*, 2020). These findings agree with Halbert and Manjunath (2004), who identified *M. paniculata* as a key host for both the bacterium and the vector.

Although the Control Routes were initially expected to show low vector occurrence, consistent captures of *Diaphorina citri* were observed, likely due to the widespread presence of *Murraya paniculata* in residential and public areas. Consequently, the Lower South, Bean Highway, and Green Line routes present a high potential for Huanglongbing (HLB) establishment if infected vectors or propagative materials are introduced. After the samples were processed, two positive detections of *Candidatus Liberibacter asiaticus* were recorded in 2021 from *Murraya paniculata* (orange jasmine) plants collected in the municipalities of Bom Jesus da Lapa and São Félix do Coribe.

As shown in figure 4 and Table 2, samples RP1.9.1.1 (Ct = 22.34) and RP1.11.2 (Ct = 27.77) exhibited positive amplification for *Candidatus Liberibacter asiaticus*, confirming the pathogen's presence in insects collected along Positive Route 1 (Western Bahia), while the negative control showed no amplification and the positive control (Ct = 11.71) validated the assay. The variation in Ct values indicates differences in bacterial load among samples, with RP1.9.1.1 showing a higher concentration. Following initial confirmation, phytosanitary surveillance actions were implemented within a 4 km radius around the positive detection points, including local mobilization, counter-sampling, and vector control. The counter-samples tested negative, eliminating the need for host eradication but reinforcing the importance of maintaining continuous monitoring and preventive surveillance across sentinel routes.

Sample	Ct Value
Negative control	Not determined
Positive control	11,71
RP 1.9.1.1	22,34
RP 1.11.2	27,77

Table 02 – Quantification cycle (threshold cycle – Ct) values of the tested samples

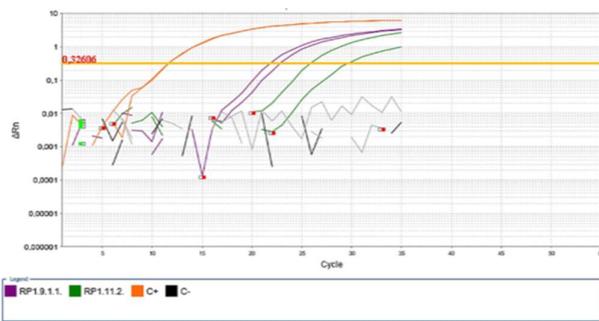


Figure 04 – Detection of *Ca. Liberibacter asiaticus* by quantitative real-time PCR (qPCR) in DNA samples extracted from *Diaphorina citri* collected along Positive Route 1 (Western Bahia). The tests of collected and control samples were performed in duplicate. The graph shows the amplification curves for each duplicate of the analyzed samples, according to the legend. (Information from EMBRAPA Cassava and Fruits).

To address the two suspected areas, it was not necessary to fully implement the protocols established in the HLB Contingency Plan, as the host plants were not eradicated, given that the counter-sample results were negative. In summary, the actions included: raising awareness among local authorities, establishing institutional partnerships, delimiting the central and surrounding areas, collecting new samples for molecular analysis, and adopting measures to control the insect vector. These actions were implemented within a 4 km radius from the location where the positive sample had been collected (Figure 05).

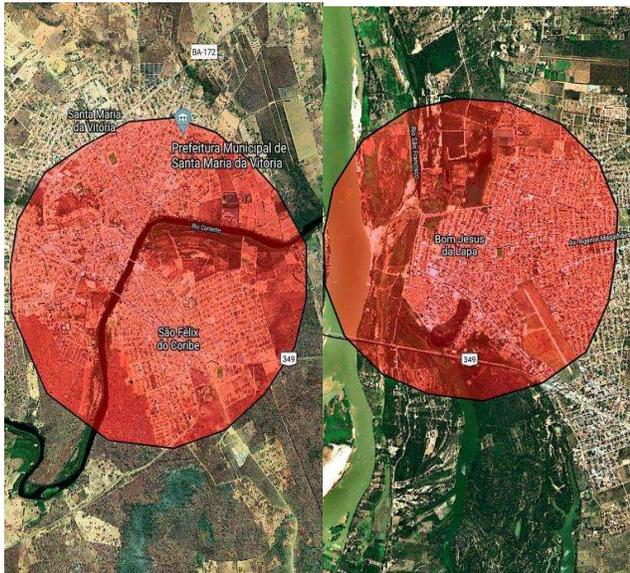


Figure 05. Delimitation of the focus areas in the urban zones of Bom Jesus da Lapa (right) and São Félix do Coribe (left).

In the municipalities of Bom Jesus da Lapa and São Félix do Coribe, actions were initiated with the following objectives:

- (i) Delimiting areas where phytosanitary measures would be applied. From the point where infectious insects were detected, a 4 km radius was established in the urban zones of both municipalities.
- (ii) Collecting plant material and insects to form new samples, which were then sent to the laboratory for qPCR testing.

The results were negative for all leaf samples from *Murraya* plants where infectious insects had previously been found. Thus, with the reanalysis of plant tissue and insect samples collected along the Western Route (2022), based on the 2021 samples, the negative results obtained almost a year later reaffirm Bahia's status as a territory free of citrus HLB. To define the risk of pest introduction and spread, the Green Line and Lower South Routes could be integrated into the Strategic Routes, as both contain significant numbers of hosts and vector insects.

They also include numerous gardens and nurseries with high propagative material traffic, increasing dissemination risk. These findings identified the routes most predisposed to HLB spread within Bahia, supporting strategies to strengthen phytosanitary surveillance. A similar study conducted by Abreu *et al.* (2020) detected the bacterium in two of 21 insect samples collected along the Chapada Diamantina Strategic Route. Despite this, five years later, no symptoms appeared in host plants, showing that the ADAB's surveillance and control strategy has been effective. This led to an increase in municipalities under surveillance along the Chapada route and inspections of all hosts in domestic orchards within a 4 km radius. These measures have helped maintain Bahia's HLB-free status. Another key aspect is that detections of infectious insects occurred in urban areas and in *Murraya* hosts, confirming the most likely entry pathway of the pest into Bahia. *Murraya* produces flushes throughout the year and is often found along routes with heavy plant traffic, favoring the introduction of infected propagative material. Therefore, inspection of plant transport on highways and in commercial establishments, particularly nurseries, should be intensified. The movement of propagative material is critical along the Green Line, Lower South, Recôncavo, Chapada, and Western Routes, which contain many orchards or lie near major highways with high cargo traffic from states where HLB occurs. It is not uncommon for fruit and ornamental seedlings to be intercepted and sold illegally. Thus, phytosanitary barriers remain essential tools for prevention and inspection, ensuring that Bahia continues free from Huanglongbing (HLB or Greening).

CONCLUSION

The monitoring of the bacterium *Candidatus Liberibacter* sp. along the sentinel routes detected suspicions only in Western Bahia, which were later not confirmed. The immediate action of ADAB prevented the formation of infection foci and strengthened the state's phytosanitary surveillance system. Given the prevalence of small-scale producers in the region, the introduction of HLB into the Bahian Recôncavo could lead to rapid bacterial spread due to property proximity and intense road traffic. Therefore, the continuous training of citrus growers is essential to ensure a quick and effective response. This study reinforced Bahia's agricultural defense system and consolidated partnerships with research institutions.

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