Volume: 4, Issue: 1, (June, 2022), pp -20-28
agri.rjt.ac.lk/sljae

SRI LANKAN JOURNAL OF AGRICULTURE AND ECOSYSTEMS *e*ISSN: 2673-1401

SLJAE

ORIGINAL ARTICLE

Investigation of the Presence of *Xylella fastidiosa*: A Quarantine Pathogen in Potential Host Plants in Sri Lanka

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DOI: http://doi.org/10.4038/sljae.v4i1.76

Abstract

Xylella fastidiosa, a gram-negative, xylem-inhabiting devastating plant pathogenic bacterium is reported in around 600 host plant species. *Xylella fastidiosa* is a regulated-quarantine pathogen in the European Union where the confirmation of pathogen-free certification is required in international trade. At present, Taiwan is the only country in Asia with a long-term presence of this bacterium. Hence there may be a risk of introduction into Sri Lanka through international trade. Therefore, an investigation was conducted from December 2020 to March 2021 to find out the presence of *X. fastidiosa* in potential hosts in Sri Lanka. A total number of 54 composite samples were obtained from 23 potential host plant species declared in the regulation (EU) 2020/1201 of 14 August 2020 including ornamental plant species from nine districts where most of the export nurseries are scattered. These were tested for the presence of *X. fastidiosa* by PCR using specific primers targeting the conserved genomic region of the RNA polymerase sigma factor of the rpoD gene of 733 bp. The positive DNA was sourced from the French collection of Plant Associated Bacteria, CIRM-CFBP for comparison. All local samples were found to be free from *X. fastidiosa*. These preliminary investigation results are considered as an encouraging indication, considering that *X. fastidiosa* was not found in Sri Lanka, at least in the surveyed areas. However, frequent studies are needed to be conducted to monitor the pathogen invasion.

Keywords: *Polymerase chain reaction, Xylella fastidiosa, Quarantine pest, International trade, Sri Lanka*

Date of Submission: 22.01.2021 Date of Acceptance:19.06.2022

1. Introduction

One of the most significant emerging plant diseases threats worldwide is caused by the bacterium *Xylella fastidiosa* (Wells et al. 1987) which is a fastidious Gram-negative, xylem-limited, vector-transmitted, rod-shaped bacterium with rippled cell walls (CABI 2021). *Xylella fastidiosa* has a broad host range of 595 host plant species including ornamental plants which are included in the list of the latest update of the plant database of the European Food Safety Authority (Rapicavoli et al. 2018; European Food Safety Authority 2020). Until recently, it was mainly distributed throughout the Americas but now outbreaks in Asia and Europe are reported (ISPM 27).

Numerous species of xylem sap-sucking insects (leafhoppers /Cicadellidae) are known to be vectors of this bacterium (Ortin et al. 2016). The European Food Safety Authority suggests that all xylem sap feeders should be regarded as potential vectors of *X. fastidiosa* (Elbeaino et al. 2014). There are four subspecies of *X. fastidiosa*. They are *Xylella fastidiosa fastidiosa*, *Xylella fastidiosa pauca*, *Xylella fastidiosa* multiplex and *Xylella fastidiosa sandyi* (Schaad et al. 2004; Schuenzel et al. 2005), which have evolved in distinct geographical regions. This each subspecies shows a high degree of host specificity (Almeida 2015).

Considering all of the above facts it has direct impacts on crop production and indirect effects on upstream or downstream economic sectors in Europe (https://ec.europa.eu). Accordingly, *X. fastidious* has been declared a quarantine pest by the European Commission and highlighted serious concerns on the possibility of entering this pathogen to the other part of the world from countries where the pathogen is present.

Export of host plants from Sri Lanka (Annex 1 of the regulation of EU 2020/1201 of 14 August 2020) would be no longer possible until the submission of the latest scientific and technical information after conducting a survey by proving the plants are free from the specified pest. If we are unable to do so, it will severely affect the export industry, especially the exportation of ornamental plants to European countries.

In addition, *X. fastidiosa* spp. is no longer limited to a few countries and its long-term presence in Taiwan raises questions about its potential distribution in Asia including Sri Lanka. The main dispersal pathway is the movement of infected and potentially-infected asymptomatic plant materials from areas where the pathogen occurs (International symposium on *X. fastidiosa:* summary and key learnings 2017). Records of CABI revealed that Sri Lanka is free from *X. fastidiosa*. However, international transit of plants and plant-based products due to the open economy and international conventions signed and ratified would pave the path for the movement of serious quarantine pests even *X. fastidiosa*.

It has a great negative impact on not only the export industry but also the vegetation in Sri Lanka. This is because Sri Lanka is a hotspot for biodiversity. Furthermore, civilians who directly or indirectly depend on the agriculture sector could be faced a very difficult situation in the

future if *X. fastidiosa* is established following introduction from another country through commerce. This would negatively affect natural vegetation and lead to the extinction of the fauna of Sri Lanka. The main danger is that *X. fastidiosa* could become established in natural vegetation which would then act as a reservoir for infection of other susceptible crops and forest trees (CABI 2021).

Considering the high biosecurity risk imposed by the bacterium and its possible impact on the export industry of Sri Lanka, this study was designed to investigate the presence of the bacterium in potential hosts in different geographical regions of Sri Lanka.

2. Materials and Methods

Sample Collection

A preliminary survey was conducted from December 2020 to March 2021 on the presence of *X. fastidiosa* in potential hosts as per the guidelines of ISPM 27 and EPPO PM 7/24(4). A total of 54 fresh leaf samples of 23 species of potential host plants of *X. fastidiosa* were collected from Gampaha, Kandy, Kurunegala, Puttalam, Anuradhapura Polonnaruwa, Monaragala, Matale and Badulla districts in Sri Lanka (Table 1) where most of the export nurseries are scattered. Samples were collected from production sites of plant and plant products as well. Production sites were physically inspected by authorized plant quarantine officers and samples of both symptomatic and asymptomatic plants were randomly collected from all sites. Composite samples were formed by mixing multiple random samples of the same species accordingly.

Collected samples which were clearly labeled for traceability of information, (plant species, sample number, sampling date and photographs) were transported to the laboratory under the cool condition with silica gel to avoid degradation of pathogen DNA (Wilkie et al. 2013). Plant samples were immediately kept at -80 °C after preparation.

Table 1: Information on the collected samples

District	Location	Scientific name and reference number
Gampaha	Katunayake Katana Gampaha Dunagaha Badalgama	*Citrus sinensis* (NPQS 259), *Ipomea batatas* (NPQS 260), *Ayerrhora carambola* (NPQS 262), *Alternanthera sessilis* (NPQS 263), *Allamanda cathartica* (NPQS 265), *Coffea arabica* (NPQS 268), *Persea americana* (NPQS 382), *Phoenix reclinata* (NPQS 383), *Ixora armeniaca* (NPQS 384) *Muntingia calabura* (NPQS 270), *Citrus limon* (NPQS 272), *Citrus crenatifolia* (NPQS 273)
Kandy	Gampola Kundasale	*Morinda citrifolia* (NPQS 281), *Coffea arabica* (NPQS 282), *Piper nigrum* (NPQS 284), *Nerium oleander* (NPQS 289), *Citrus aurantifolia* (NPQS 378)
Badulla	Boralanda	*Lavendula angustifolia* (NPQS 285, NPQS 286, NPQS 287, NPQS 288), *Jasminum grandiflora* (NPQS 385), *Nandina domestica* (NPQS 386)
Kurunegala	Mawathagama Giriulla Pannala	*Vitis vinifera* (NPQS 316), *Alternanthara dentate* (NPQS 317), *Muntingia calabura* (NPQS 318), *Muntingia calabura* (NPQS 319), *Rosa pascali* (NPQS 320), *Citrus crenatifolia* (NPQS 348), *Citrus sinensis* (NPQS 350), *Alternanthera sessilis* (NPQS 352), *Passiflora edulis,* (NPQS 381)
Puttalam	Chilaw Marawila	*Rosa elina*. (NPQS 321), *Persea americana* (NPQS 322), *Ipomoea batatas* (NPQS 323), *Citrus medica* (NPQS 324), *Citrus reticulate* (NPQS 325), *Cannaceae zingiberales* (NPQS 327), *Senna auriculata* (NPQS 328)
Anuradhapua	Anuradhapura	*Sorghum halepense* (NPQS 314), *Citrus aurantifolia.* (NPQS 341), *Citrus sinensis* (NPQS 342), *Citrus reticulate* (NPQS 343), *Citrus paradisi* (NPQS 344), *Citrus reticulata* (NPQS 379, NPQS 380)
Polonnaruwa	Polonnaruwa	*Citrus paradisi*. (NPQS 370), *Sorghum halepense* (NPQS 371), *Persea americana* (NPQS 372)
Matale	Galewela, Ukuwela Nalanda	*Murraya koenigii* (NPQS 373), *Nerium oleander* (NPQS 376), *Murraya koenigii* (NPQS 374), *Citrus sinensis* (NPQS 375)
Moneragala	Bibile	*Citrus crenatifolia* (NPQS 377)

Detection of X. fastidiosa through PCR

Conventional PCR, the test based on Minsavage et al. (1994) was used to detect *X. fastidiosa* from all collected samples.

Polymerase chain reaction

A total of 54 samples from host plants (Table 1) were randomly selected and tested by PCR for the detection of *X. fastidiosa*. Total DNA was extracted from midribs, petioles, leaf basal part and twigs (0.5–1g) using the CTAB method (EPPO PM 7/24(4)). For PCR, the RST31/RST33 set of primers targeting the rpoD gene (forward 5' –GCGTTAATTTTCGAA reverse GTGATTCGATTGC-3') was used. Reactions were conducted in a final volume of 25 µL, using 0.15 µL of 5U μL^{-1} GoTaq polymerase (Promega), 0.3 µM each of forward and reverse primer, and 2 µL of total DNA template (50 ngµL-1). Thermo cycling conditions were as follows: 95 °C for 1 min followed by 40 cycles of (95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s) and a final step of 72 °C for 5 min (EPPO PM 7/24(4), ISPM 27). Since *X. fastidiosa* has not been previously reported in Sri Lanka, positive samples of test DNA of X. *fastidiosa* were procured from the French collection of Plant Associated Bacteria, CIRM-CFBP. Altogether, 54 samples were subjected to PCR with two replicates. In addition, both positive and negative controls were used. The PCR products were analyzed by electrophoresis using Major science, MBE-150, UK, Blue gel electrophoresis system in 1% 1X Tris-Acetate-EDTA (TAE)-agarose gels and DNA bands visualized by staining the gel in ethidium bromide using Vilber Lourmat, France gel documentation system supported by Vision-Capt software (peqlab.co.uk).

3. Results and Discussion

The positive controls reacted positively, whereas no bands were observed with the negative controls. Moreover, no amplified DNA was obtained from any of the tested samples confirming the absence of the bacterium in those samples.

Gel electrophoresis pictures clearly showed that the DNA of all test samples has not yielded any amplification of the desired fragment size of 733 bp which is specific to *X. fastidiosa*. All positive tests were amplified and given the desired fragment size. Gel pictures of the representative samples are shown in Fig. 1, 2 and 3. No apparent PCR amplification of DNAs from a collection of several plant-pathogenic or saprophytic bacteria as well as from non-infected plant extracts occurred with this primer (Minsavage et al. 1994). Furthermore, a comparative analysis of different molecular and serological methods conducted by Waliullah et al. 2019) for the detection of X. *fastidiosa* in blueberry, confirmed the validity of the RST31/33 primer used in the detection. As per his experiment, the primer set HL5 and HL6 which needed a concentration as low as 1 pg/ µl of DNA (~350 copies) were seen to be superior to RST 31/33 primers in detecting *X. fastidiosa*.

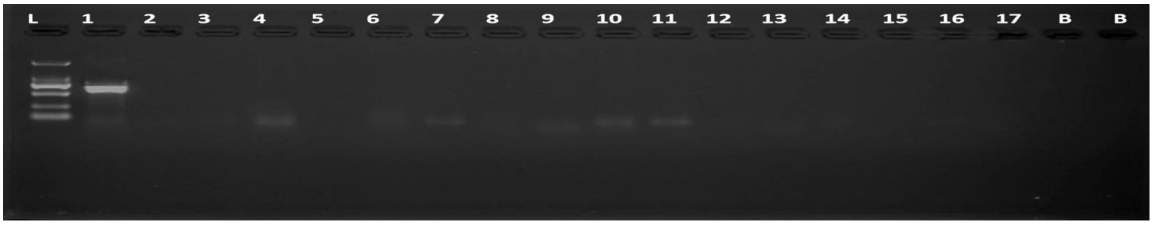

Figure 1: Gel electrophoresis image
L- 2000D DNA marker, 1-Positive control (*X. fastidiosa,*733bp), 2 & 12 –NPQS 316, 3&16 – NPQS 317,4 & 10 – NPQS 324, 5 &15- NPQS 319, 6 & 14 – NPQS 322, 7 & 11 –NPQS 348, 8 & 17 – NPQS 259 & B –Negative control.

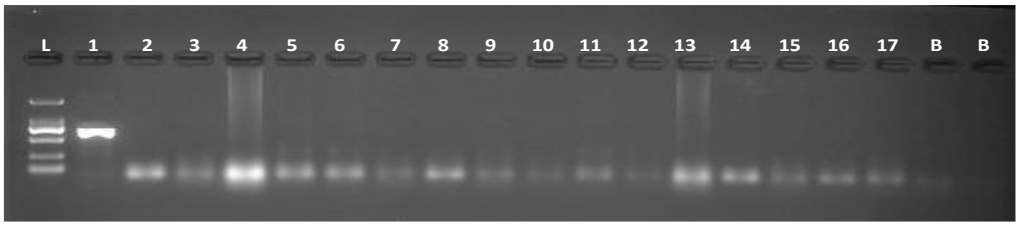

Figure 2: Gel electrophoresis image
L-2000D DNA marker, 1- Positive control (*X. fastidiosa,*733bp), 2 & 14 – NPQS 341, 3 & 15 – NPQS 323, 4 & 13 – NPQS 325, 5 & 11 – NPQS 318 , 6 & 17 – NPQS 268, 7 & 10 –NPQS 320, 8 & 16 - NPQS 321, 9 &12 – NPQS 328 & B – Negative control.

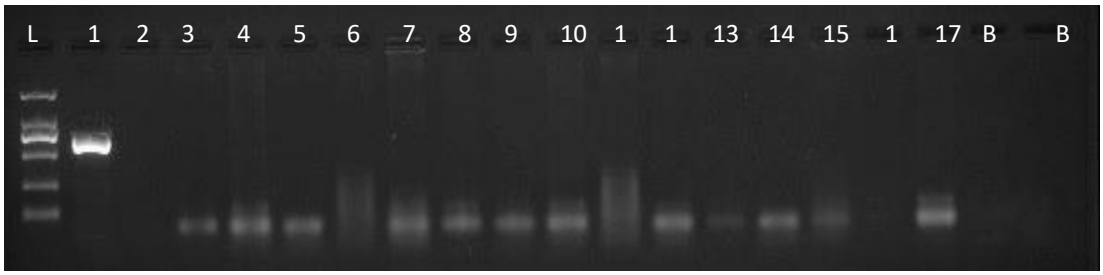

Figure 3: Gel electrophoresis image
L-2000D DNA Marker, 1- Positive control (*X. fastidiosa,*733bp), 2 & 16 –NPQS 282, 3 & 13 –NPQS 327, 4 & 10 – NPQS 350, 5 & 14 –NPQS 270, 6 & 11 –NPQS 284, 7 & 15 –NPQS 265, 8 & 17 – NPQS 342, 9 & 12 – NPQS 262 & B - Negative control

However, at this low concentration, the Conventional PCR (C-PCR) product was hardly visible suggesting that the more applicable detection limit for C-PCR is about 1.25 pg/µL of DNA (~440 copies) that can be achieved by the primer set RST 31/33.

The used primer (RST31/33) which was verified and validated by European and Mediterranean Plant Protection Organization (EPPO) diagnostic protocols and International Plant Protection Convention (IPPC 2006) is widely accepted and recommended for the detection of the bacterium in quarantine programs targeting the amplification of a 733-bp fragment of the *rpo*D gene, which codes for an RNA polymerase sigma-70 factor (EPPO, 2019; Minsavage et al. 1994). Further, it is widely applied in many laboratories for the detection of *X. fastidiosa* in different host plants and vectors (ISPM 27).

Further, EcoRI and RsaI restriction enzymes can be utilized to identify the genetic polymorphism among the strains of *X. fastidiosa* (Minsavage et al. 1994) The molecular-based diagnosis was undertaken for the detection of the pathogen since the concentration of the bacterium in many hosts may be very low, the method like PCR is at least 100-fold more sensitive than the Enzyme-Linked Immunosorbent Assay (ELISA) and LAMP method. Therefore, the sensitivity of the PCR method for detecting *X. fastidiosa* in potential hosts can successfully be used not only in research activities and but also for this type of disease diagnostic programs (Minsavage et al. 1994).

Furthermore, it is important to select the appropriate diagnostic tool to detect the pathogen most reliably. From a diagnostic utility standpoint, the benefits of any technique to detect causal agent of diseases depends on the simplicity, specificity, sensitivity, robustness, cost-effectiveness, and suitability of the tool under any circumstances (Waliullah et al. 2019). The molecular-based diagnostic tool that was adopted is the best method for the detection and identification of this particular pathogen in the most reliable and efficient way as per available literature.

Although, this investigation was started as an immediate step to safeguard the international trade, further studies are to be planned with different types of primers specific to the pathogen and Real-Time- PCR techniques to meet the EU regulations that may change from time to time.

The main entry pathway of *X. fastidiosa* in a country is trade. The movement of plants and insect vectors are important sources for the spread of the specified pathogen. Sri Lankan plant quarantine sector must be vigilant to prevent the entering of this quarantine pathogen into the country in any way as the management of this devastating disease is challenging and it often requires early detection of the pathogen to reduce major losses which may occur subsequently in different aspects.

4. Conclusions

Thus, this study verifies that potential hosts tested are free from *X. fastidiosa*. However,

frequent studies in different regions are needed for the confirmation of the country's freedom.

5. Acknowledgment

I would like to express my special thanks of gratitude to the officers of the Plant Pathology Division of National Plant Quarantine Service and Mr. Hiranya Athukorala and Ms. Ayesha Sandaruwani, students of Uva Wellassa University of Sri Lanka who assisted in the collection and preparation of samples for the analysis.

Conflicts of Interest: The authors declare that there are no conflicts of interest regarding the publication of this paper.

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