# A diagnostic tool for improved detection of *Xanthomonas fragariae* using a rapid and highly specific LAMP assay designed with comparative genomics

Michael Gétaz[(1)], Andreas Bühlmann[(2)], Pierre H.H. Schneeberger[(3)], Cinzia van Malderghem[(4)], Brion Duffy[(1)], Martine Maes[(4)], Joël F. Pothier[(1)], Bart Cottyn[(4)]

(1) Zürich University of Applied Sciences (ZHAW), Environmental Genomics and Systems Biology Research Group, Institute of Natural Resource Sciences, Wädenswil, Switzerland
(2) Agroscope Changins-Wädenswil, Department of Epidemiology and Molecular Diagnostics, Wädenswil, Switzerland
(3) Swiss tropical and Public Health Institute, Institute of Epidemiology and Public Health, Ecosystem Health Sciences, Basel, Switzerland
(4) Plant Sciences Unit - Crop Protection, Institute for Agricultural and Fisheries Research (ILVO), Merelbeke, Belgium

Zurich University of Applied Sciences
zhaw
Life Sciences and Facility Management
Institute of Natural Resource Sciences

## Introduction

The Gram-negative bacterium ***Xanthomonas fragariae*** is under **quarantine status in Europe** in order to limit its propagation as it causes a disease named **angular leaf spot** on strawberry (**Fig. 1**), reducing plant yield and economic loss for fruit production. The damages can be particularly severe under protected cultivations with high density plots aided by high humidity and sprinkler irrigation systems.

First observed in the USA in 1960, the bacterial disease has spread worldwide in strawberry growing regions **without effective treatment** against it. Bacterial spread is helped by **symptomless infected plant** material and therefore is not always detectable by visual inspection. An early and specific detection as well as a reliable identification is crucial for efficient plant disease management.

Methods based on specific DNA sequences already exist (nested PCR and qPCR) but need to be performed in laboratories, due to equipment requirements. We developed a rapid and highly specific **loop-mediated isothermal amplification (LAMP)**, which technological advances allow to perform analyses on a portable device (**Fig. 2**) and offers more flexibility regarding the place of analyses. This technology could also reduce time between sampling and sample analysis and therefore improve quick control measures with simply interpretable results with **minimal training requirements**.


**Fig. 1:** Angular leaf spots located on naturally infected plants of strawberry. The spots represented here are showing variability in terms of severity of infection, from light green water soaked leaf spots (A) or small necrosis (B) to a fading leaf.

**Fig. 2:** LAMP portable device (Genie II, Optigene Ltd.) working on battery was taken out of the laboratory for a field trial experiment where results could directly be visualized on the screen of the machine within 20 min..

## Results

The genome information of 27 available *Xanthomonas* strains including their plasmids were used to perform a **comparative genomic analysis**. Using the unique *X. fragariae* genome LMG 25863 available, it was possible to highlight a **specific target** that were tested for specificity in order to **design the LAMP primers**.

**Table 1:** Non-exhaustive list of *Xanthomonas* species and pathovar tested for specificity investigation.

| Species | Number of tested strains | Host plant | LAMP assay amplification |
|---|---|---|---|
| *Xanthomonas fragariae* | 37 | Strawberry | + |
| *Xanthomonas albilineans* | 1 | Sugarcane | - |
| *Xanthomonas alfalfae* subsp. *alfalfa* | 1 | Lucerne | - |
| *Xanthomonas arboricola* sp. | 4 | Strawberry | - |
| *Xanthomonas arboricola* pv. *corylina* | 28 | Hazelnut | - |
| *Xanthomonas arboricola* pv. *fragariae* | 16 | Strawberry | - |
| *Xanthomonas arboricola* pv. *juglandis* | 1 | Walnut | - |
| *Xanthomonas arboricola* pv. *pruni* | 1 | Prunus | - |
| *Xanthomonas axonopodis* | 1 | Citrus | - |
| *Xanthomonas axonopodis* pv. *aurantifolii* | 1 | Citrus | - |
| *Xanthomonas axonopodis* pv. *citrumelo* | 1 | Citrus | - |
| *Xanthomonas axonopodis* pv. *phaseoli var. fuscans* | 1 | Bean | - |
| *Xanthomonas bromi* | 1 | Brome grass | - |
| *Xanthomonas campestris* pv. *campestris* | 1 | Brassicaceae | - |
| *Xanthomonas campestris* pv. *vesicatoria* | 1 | Pepper | - |
| *Xanthomonas cassavae* | 1 | Cassava | - |
| *Xanthomonas citri* pv. *citri* | 1 | Citrus | - |
| *Xanthomonas citri* pv. *malvacearum* | 1 | Cotton | - |
| *Xanthomonas codiaei* | 1 | Geranium | - |
| *Xanthomonas cucurbitae* | 2 | Pumpkin | - |
| *Xanthomonas cynarae* | 1 | Artichoke | - |
| *Xanthomonas gardneri* | 1 | Pepper, tomato | - |
| *Xanthomonas hortorum* pv. *hederae* | 2 | Ivy | - |
| *Xanthomonas hyacinthi* | 1 | Hyacinth | - |
| *Xanthomonas melonis* | 1 | Melon | - |
| *Xanthomonas oryzae* pv. *oryzae* | 1 | Rice | - |
| *Xanthomonas perforans* | 1 | Tomato | - |
| *Xanthomonas pisi* | 1 | Pea | - |
| *Xanthomonas sacchari* | 1 | Sugarcane | - |
| *Xanthomonas theicola* | 1 | Tea plants | - |
| *Xanthomonas translucens* pv. *translucens* | 1 | Barley | - |
| *Xanthomonas vasicola* pv. *holcicola* | 1 | Great millet | - |
| *Xanthomonas vesicatoria* | 1 | Tomato | - |

The **Specifity** of the primer set and the target was tested on a comprehensive collection of *X. fragariae* isolates (*n = 37*) originated from distinct geographically origin, as well as other *Xanthomonas* species and pathovars (*n = 79*) and different bacterial species (*n = 11*) including species possibly found on strawberry plants (**Table 1**).

**Sensitivity** analyses of the *X. fragariae* LAMP assay for **pure DNA** reached **500 fg** out of a serial dilutions going from 5 pg to 5 fg (**Fig.3**). **Crude material** was also tested and sensitivity reached of **$10^5$ CFU ml-1** out of a serial dilutions between $10^8$ and $10^3$ CFU $ml^{-1}$. Sensitivities of both preparation methods were not affected by added plant material as it was thought that **inhibitors of the LAMP reaction** could play a role in the reaction.

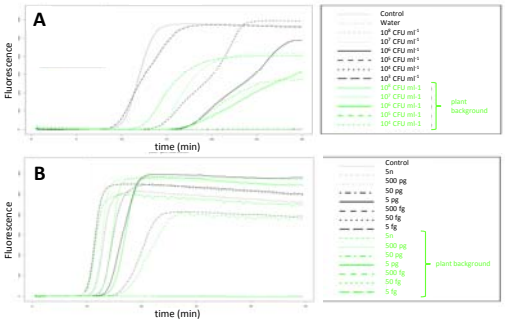


**Fig. 3:** Sensitivity of the LAMP assay for both crude material (A) and pure DNA (B) preparations. Both preparation methods had an identical serial dilutions with addition of plant material as background in order to test potential plant inhibition. For crude material (A), results were obtained using boiled cells dilutions of the *X. fragariae* strain LMG 25863 ranging from $10^8$ CFU $ml^{-1}$ to $10^3$ CFU $ml^{-1}$. The assay sensitivity reached a detection limit of $10^5$ CFU $ml^{-1}$ for crude material. For pure DNA (B), the tested serial dilution ranging from 5 pg to 5 fg highlighted a detection limit of 500 fg. Plant background did not interfere with assay sensitivity and all positive reaction were detected within 7 to 17 min.

## Conclusion

The designed LAMP assay shows a **great specificity for *X. fragariae*** and all positive reaction were **detected within 7 to 17 minutes** regarding bacterial concentration.

Sensitivity was performed with pure bacterial DNA but also crude material to get closer to natural conditions. The **detection limit was determined to be 500 fg** corresponding to about 100 genome copies of *X. fragariae*. Addition of **plant material did not interfere** with the reaction. Validation with naturally infected plants confirmed that **the assay detects *X. fragariae* from leaves with various severities of symptoms**.

This assay allows to screen samples independently from a laboratory, giving more flexibility and therefore **reducing time between sampling and results**. This could **help to intercept contaminated plant material** before further spread.

**Validation** test using **naturally infected strawberry plants** was performed with the LAMP assay in order to control if visible symptoms similar to leaf spots with different severities could be detected. An evaluation of the quantification could be obtained with the serial dilution of crude material (**Fig. 4**).


**Fig. 4:** These pictures (A – E) of naturally infected plants leaves are showing various severities of symptoms. The LAMP assay was performed taking 50 mg of leaf where spot appear. Since amplification time was correlated with quantity of bacteria included in the reaction. Approximations were the following: (A and B) 50 – 500 pg in reaction, (C and D) 5 ng in reaction and (E) more than 5 ng in reaction.

Michael Gétaz – michael.getaz@zhaw.ch
Tel. +41 58 934 50 97
ZHAW Zürich University of Applied Sciences
Institute of Natural Resource Sciences
Environmental Genomics and System Biology Research Group
Einsiedlerstrasse 31, 8820 Wädenswil, Switzerland
http://www.zhaw.ch/iunr/EGSB

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