Information pest: *Xanthomonas axonopodis* pv. *allii*

Xanthomonas axonopodis pv. *allii* (Xaa) is the causal agent of bacterial blight of onion (*Allium cepa*) and other *Allium* species. First reported in 1978, the disease has been emerging since the 1990s. It is included in the A1 list of pathogens of the European and Mediterranean Plant Protection Organization (EPPO).

The bacterium can contaminate seeds, which are a major source of inoculum. Seed trade is responsible for long-distance dissemination. Yield losses ranging from 20 to 50% have been recorded under conditions conducive to efficient development of disease.

On onion, lesions consist of lenticular water-soaked leaf spots which turn into dry chlorotic lesions that eventually coalesce. When disease is severe, leaf dieback can occur, resulting in a reduction of bulb size.

Introduction

The PCR Xaa set has been developed by Qualiplante according to Robène-Soustrade et al. (2010). Qualiplante supplies this PCR set under CIRAD license.

License cirad

(French patent N° 2951 459 - Spain and Holland patents N° 2 491 136 - USA patent N° 13/503.116).

This method is also referred in the Appendix 2 of the PM7/128 Xanthomonas axonopodis pv. allii, European and Mediterranean Plant Protection Organization Bulletin (2016) 46 (3), 429-443.

The PCR primers used in this Duplex Nested End-Point PCR method are designed to target two sequences showing sequence similarity with bacterial genes encoding the PilW/PilX proteins (PIL marker) and the *avrRxv* virulence gene (AVR marker).

Validation data of the method are available from a test performance study realized in 2014 by ANSES and CIRAD. The performance characteristics obtained are:

- Analytical sensitivity: 100% of samples were detected as positive at a concentration of $1x10^3$ cfu mL^{-1}. At $1x10^2$ cfu mL^{-1}, 81% samples tested positive.
- Analytical specificity: 100% inclusivity - 89% exclusivity (when considering the taxa). No amplification was obtained for any unrelated phytopathogenic bacteria or for any saprophytic bacteria commonly isolated from onion leaves and seeds. Cross-reactions with a few strains classified in *X. axonopodis* genetic subgroup 9.1 or 9.2: *X. axonopodis* pv. *begoniae*, *X. axonopodis* pv. *vesicatoria*, *X. axonopodis* pv. *citrumelo*, *X. axonopodis* pv. *cassavae*, *X. axonopodis* pv. *desmodii*, *X. axonopodis* pv. *desmodiiganetici*, *X. axonopodis* pv. *phyllanthi*, *X. axonopodis* pv. *tamarindi* and *X. axonopodis* pv. *lespedezae*, and also two strains of *X. vasicola* pv. *musacearum*. Depending on the pathovar, one or both markers were observed. The probability of finding these pathovars on onion plants or seeds is negligible because the capacity to induce symptoms on onion plants is specific to *X. axonopodis* pv. *allii* strains.
- Repeatability: from $1x10^4$ to $1x10^3$ cfu mL^{-1}: 100%. At $1x10^2$ cfu mL^{-1} accordance: 70%.
- Reproducibility: 100%.

The PCR set of Qualiplante was tested in the same study and the same results were obtained in terms of specificity, sensitivity and reproducibility.

This product should be used only for research purposes.

Intended use

The PCR set is validated for the detection of Xaa in Duplex Nested End-Point PCR.

Suitable tissues are pure cultures, seeds and infected tissues.

Identification of *X. axonopodis* pv. *allii* strains pathogenic to *Allium* species can be achieved only with the first PCR. For the detection of *X. axonopodis* pv. *allii* from asymptomatic plant material, the nested-PCR protocol is required because the second PCR round greatly increases sensitivity.

Set format and content

Two sets are available for 24 and 96 tests.

Article N°	Product name
7Xaa--P2	PCR Xaa set 24
7Xaa--P9	PCR Xaa set 96

Content	set 24	set 96
Direct Master Mix	24 tests 7Xaa--P2-DM-	2x48 tests 7Xaa--P9-DM-
Nested Master Mix	24 tests 7Xaa--P2-NM-	2x48 tests 7Xaa--P9-NM-
Positive Control	3 tests 7Xaa--P2-PC-	8 tests 7Xaa--P9-PC-
Negative Control	3 tests 7Xaa--P2-NC-	8 tests 7Xaa--P9-NC-

Storage conditions

This set can be shipped at room temperature but upon receipt it should be stored immediately at the recommended storage temperature: **from -30 ° C to -10 ° C**.
Avoid prolonged exposure to light and repeated freeze and thaw cycles.

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Shelf life

If the set is correctly stored, at constant-temperature freezer, its performance is guaranteed until the expiration date indicated on the tubes label.

Materials and equipment (not provided)

- DNA extraction tools and reagents
- Nuclease-free filter tips and micropipettes
- Optical grade nuclease-free tubes/plate
- Disposable latex or vinyl gloves
- DNA ladder and loading-dye buffer
- PCR thermal cycler
- Agarose gel reagents and apparatus

Nucleic acids extraction

Extract DNA from samples according to your usual protocol. Upon request, Qualiplante can recommend you an extraction method.

FIRST PCR

Reaction set-up

a) Slowly thaw Direct Master Mix by placing it on ice or at 4°C.
b) Shake briefly Direct Master Mix and spin down the liquid.
c) Add 23 µl of Direct Master Mix (without DNA template) to each PCR tubes or wells of an optical-grade PCR plate.
d) Add 2 µl of DNA template to the Direct Master Mix. Do not forget to prepare a PCR tube or well of a PCR plate for the Positive Control and the Negative Control.

Components	Volume/PCR tube or well
DNA template or Positive control or Negative control	2 µl
Direct Master Mix	23 µl
Total Volume / PCR tube or well	25 µl

In order to confirm the absence of any reagent's contamination, we strongly recommend including a no-template control (e.g. DEPC water) in the assay.

Run and thermal cycling

a) Seal carefully the PCR tubes or PCR plate. Centrifuge briefly to collect components at the bottom of the PCR tubes or wells of the plate. Protect from light before thermocycling.
b) Load the PCR tubes or plate into the PCR thermal cycler and follow the thermal cycling below:

Steps	Temp (°C)	Time	Cycle(s)
Initial denaturation	95°C	5 min	1
Denaturation	95°C	1 min	40
Annealing	63°C	1 min	
Elongation	72°C	2 min	
Final elongation	72°C	5 min	1
Storage	4°C	∞	-

Dilution of 1st PCR products

Using DNase and RNase free PCR tubes, **dilute to 1:100** in sterile water each PCR product for samples, Positive Control and Negative Control obtained during the previous step.

Shake and spin down each tube.

SECOND PCR

Reaction set-up

a) Slowly thaw Nested Master Mix by placing it on ice or at 4°C.
b) Shake briefly Nested Master Mix and spin down the liquid.
c) Add 24 µl of Nested Master Mix (without DNA template) to each PCR tubes or wells of an optical-grade PCR plate.
d) Add 1 µl of **diluted 1st PCR products** to the Nested Master Mix. Do not forget to prepare a PCR tube or well of a PCR plate for the 1st PCR products obtained with Positive Control and Negative Control.

Components	Volume/PCR tube or well
PCR products from the 1st PCR diluted to 1:100 (*)	1 µl
Nested Master Mix	24 µl
Total Volume / PCR tube or well	25 µl

(*) See the section "Dilution of 1st PCR products"

Run and thermal cycling

a) Seal carefully the PCR tubes or PCR plate. Centrifuge briefly to collect components at the bottom of the PCR tubes or wells of the plate. Protect from light before thermocycling.
b) Load the PCR tubes or plate into the PCR thermal cycler and follow the thermal cycling below:

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Steps	Temp (°C)	Time	Cycle(s)
Initial denaturation	95°C	5 min	1
Denaturation	95°C	30 sec	30
Annealing	57°C	30 sec	
Elongation	72°C	40 sec	
Final elongation	72°C	5 min	1
Storage	4°C	∞	-

Agarose gel electrophoresis

Prepare an agarose gel **at 3% w/v in 1X-TAE buffer.**

Gel loading:
- load the DNA ladder (for example 1'000 bp DNA step ladder).
- load 10 µl of PCR products from the previous step adding the loading dye buffer (*not provided in the set*).

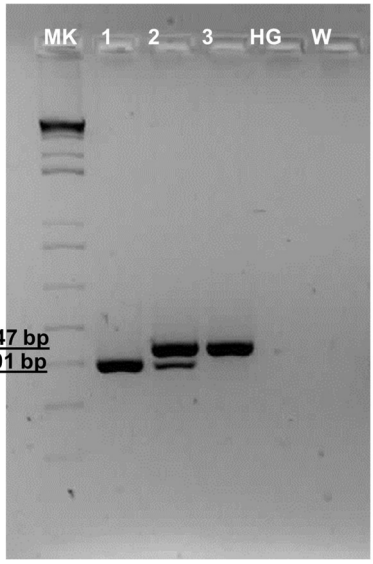
Run: run the gel electrophoresis for 70 minutes at 90V.

Results analysis

ANALYSIS VALIDATION

The analysis is validated when:
- ✓ a DNA fragment of 447 bp (corresponding to the PIL marker of Xaa) and/or a DNA fragment of 401 bp (corresponding to AVR marker of Xaa) is/are visible in the positive control lane.
- ✓ none DNA fragment are visible in the negative control lane.

The picture below represents a 1X-TAE 3% agarose gel showing the DNA amplification of Xaa.



MK: DNA ladder - **1:** Xaa infected sample, AVR marker - **2:** Xaa infected sample, AVR and PIL marker, or Positive Control of the set - **3:** Xaa infected sample, PIL marker - **HG:** healthy sample or Negative Control of the set - **W**: No template control.

RESULTS INTERPRETATION

- ✓ A sample is **positive** when:
  - a DNA specific fragment of 447 bp is present in the PCR reaction; in this case, the sample is infected by Xaa (PIL marker).
  - a DNA specific fragment of 401 bp is present in the PCR reaction; in this case, the sample is infected by Xaa (AVR marker).
  - a DNA specific fragment of 447 bp and 401 bp are present in the PCR reaction; in this case, the sample is infected by Xaa (PIL and AVR marker).
- ✓ A sample is **negative** when no fragment is present in the PCR reaction.

The table below summarizes the results interpretation:

Fragment size		Interpretation
447 bp	**401 bp**	
		NEGATIVE
✓		**POS.** PIL marker
	✓	**POS.** AVR marker
✓	✓	**POS.** PIL and AVR marker

POS: infected sample – NEGATIVE: healthy sample

Special handling instructions

This set was designed to be used by laboratory staff trained to follow the usual molecular biology precautions. Always perform the tests in a nuclease-free work environment. Always wear gloves when handling samples containing DNA and the components of the set. Do not touch any set components with an ungloved hand. Use appropriate laboratory disposable parts. Use nuclease-free tubes and filter tips to avoid degradation and cross-contamination. Do not use components from sets with different batch numbers in the same test procedure. Do not interchange reagents with other sets. To avoid cross-contamination, use separate rooms for (a) nucleic acids extraction, (b) preparation of the Master Mix and (c) amplification. To avoid cross-contamination and obtain reliable results, it is essential to strictly follow the protocol in this manual. Avoid unnecessary freeze-thaw cycles of the set components. Do not use reagents after their expiration date.

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Troubleshooting

Post-PCR data analysis shows no amplification, or amplification plots look grossly abnormal:

Possible causes	Corrective actions
Evaporation of the sample due to inadequate sealing of the plate	Repeat the test using the appropriate tools to seal correctly the plate
Consumables are not appropriate for the method	Repeat the test using consumables recommended by the thermal cycler supplier
The quality of nucleic acid extracted is low	Repeat the extraction step. Ensure that the method of extraction has been performed correctly. In any doubt, please, contact us
Abnormal amplification	Centrifuge the plate briefly to spin down the contents and eliminate any air bubbles

No amplification reaction is observed in the positive control well, while other samples are positive:

Possible causes	Corrective actions
The positive control provided with the set was not added into the reaction well	Repeat the test. If the problem persists, please, contact us

An amplification plot is observed in the negative control well:

Possible causes	Corrective actions
Contamination of the negative control or the Master Mix with target-positive nucleic acid	Repeat the test by applying appropriate quality procedures to prevent contamination. Seal the plate correctly

Warranty and Responsibilities

Qualiplante SAS guarantees the buyer exclusively concerning the quality of reagents and of the components used to produce the Sets. Any product not fulfilling the specifications included in the product sheet will be replaced. This warranty limits Qualiplante SAS responsibility to the replacement of the product. No other warranties, of any kind, express or implied-are provided by Qualiplante SAS.
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