

# *Xanthomonas fragariae* National Diagnostic Protocol

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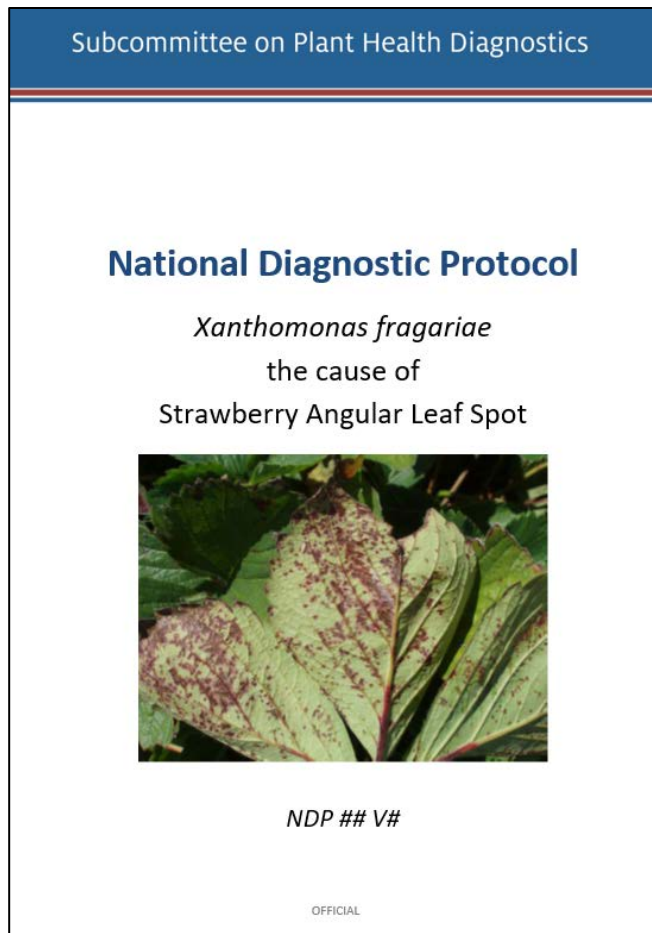
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# What does an NDP look like? And what is in it?



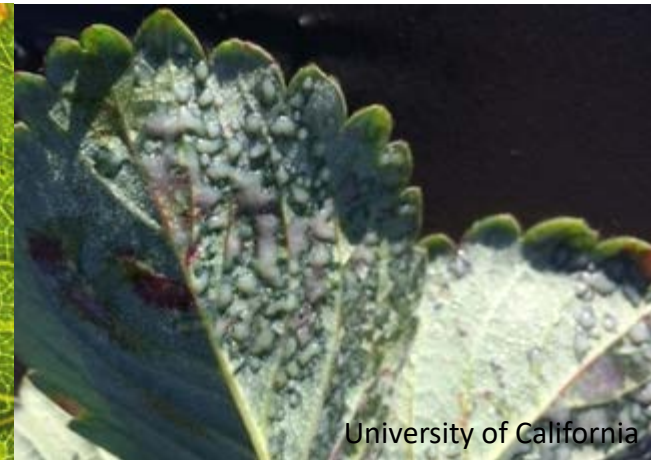
- ▾ National Diagnostic Protocol
  - ▾ 1 INTRODUCTION
    - 1.1 Host range
  - ▾ 2 Taxonomic Information
    - 2.1 Taxonomic description
  - ▾ 3 Detection
    - 3.1 Symptomatic Tissue
    - 3.2 Asymptomatic tissue
    - 3.3 Symptoms
  - ▾ 4 Identification
    - 4.1 Morphological identification.
    - 4.2 Biochemical methods
    - 4.3 Pathogenicity testing
    - 4.4 Molecular tests
    - 4.5 Reference material
  - 5 ContactS for further information
  - 6 Acknowledgements
  - 7 References
  - ▾ 8 Appendices
    - 8.1 Media and buffers
  - ▾ 9 DIAGNOSTIC PROCEDURES TO SUPPORT SURVEILLANCE
    - 9.1 Introduction
    - 9.2 Sampling
    - 9.3 In Field Tests
    - 9.4 Laboratory Tests
    - 9.5 Acknowledgements
    - 9.6 References

# 1. *Xanthomonas fragariae*

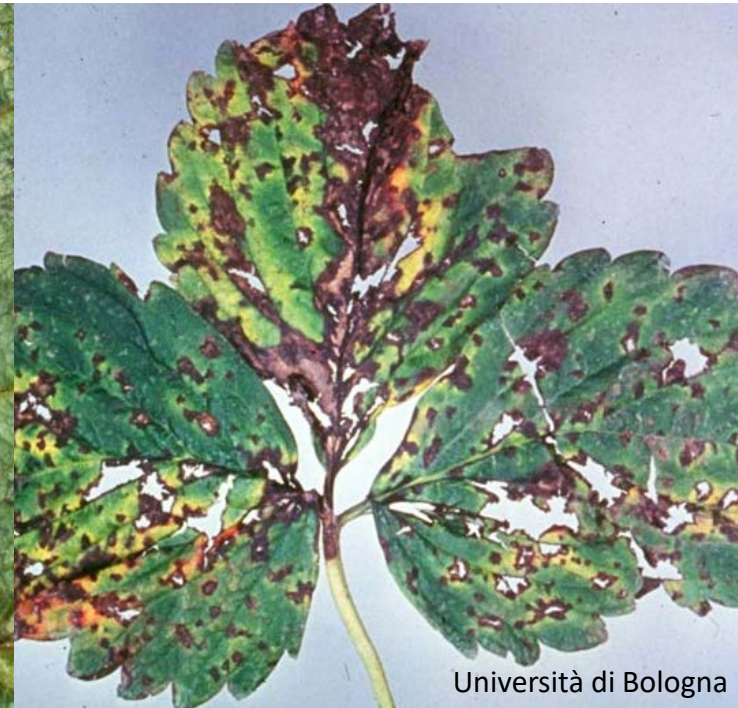
- Causes Angular Leaf Spot of strawberry
- Bacterial pathogen
- Host specific
- *X. fragariae* is a notifiable and quarantinable pathogen
- Losses from
  - Decreased productivity/yields
  - Unmarketable fruit with calyx infections



## 3.3 Symptoms – Early infection



## 3.3 Symptoms – Late infection



## 3.3 Symptoms - Fruit



- Severe infections
- Blossom blight
- Fruits do not typically get infected
- Calyx turns brown/black = unmarketable

## 1.2 Disease cycle

- Preferred conditions
  - Prevalent in autumn and spring.
  - >90% humidity
- Transmission
  - Asymptomatic planting stock
  - Aerosols, rain and irrigation systems
  - Contaminated equipment and machinery
  - Prolonged survival in soil and plant debris



# NDP technical aspects

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### 3. Sample collection

- Keep samples dry, wrapped in paper towel, contained in a plastic bag/ziplock.
- Keep sample cool (fridge, not freezer)
- Process ASAP



## 3.1 Sample selection - Symptomatic

- Leaves with initial water-soaked spots are preferred as samples for the diagnosis – helps with isolation
- Leaves with dry spots and with or without exudates can be used.
- Crown tissue or whole plants



## 3.1 Sample selection - Asymptomatic

- Several entire plants for sampling



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## 3.2 Sampling preparation - Symptomatic

- Focus on
  - Early stage symptoms (water-soaked spots in the lower part of the leaves) if available.
  - Lesion margins
  - Discoloured leaf veins and stems
  - Crown



## 3.2 Sampling preparation - Asymptomatic

- Several entire plants – small amounts of leaf, petioles and crown tissues
- Or bacterial concentration method
  - 30 g sample in 150 mL of PBS
  - Agitation, centrifugation, pellet resuspension, dilutions and extractions



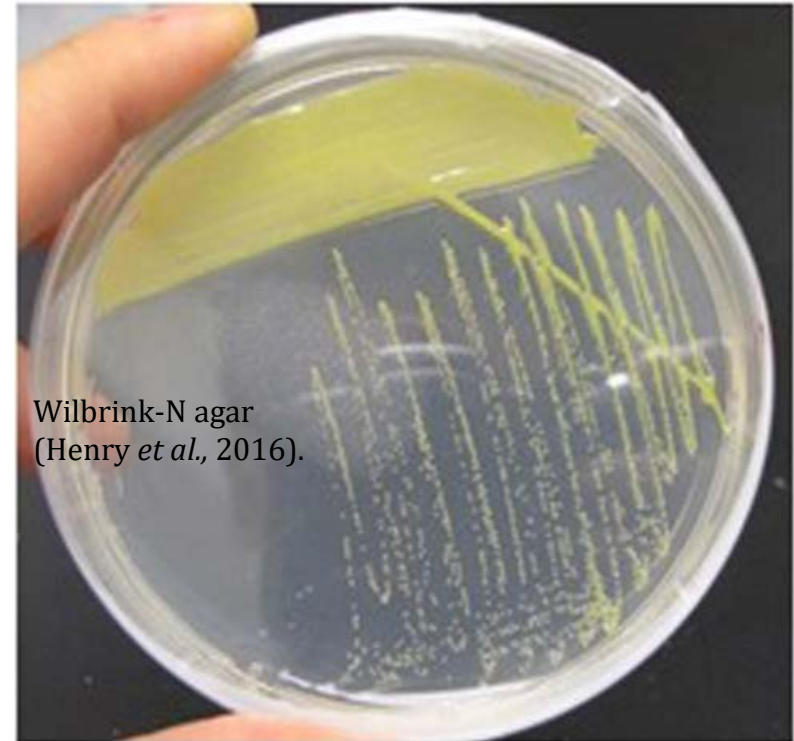
## 4.1 Isolations

- Best chance at pathogen isolation – leaves with young lesions where possible or margins of older lesions.
- Surface sterilise material with 70% ethanol, use sterile blades
- Ooze test in sterile water, microscope examination
- Wilbrink-N medium, YPGA or SPA agar
  - 10-fold serial dilutions from 1:10 → 1:10,000



## 4.1 Isolations

- Incubation: 25-28°C, examine after 3 days
- *X. fragariae* is slow growing – microscopic at 3 days.
- The large colonies at 3 days are not *X. fragariae*
- Final observation at 7 days
- NDP contains the biochemical profile and path testing options (4.2, 4.3).



## 4.4 Extraction methods

- IPPC best recommendation:
- **DNeasy Plant Mini Kit (Qiagen), as modified for phytoplasma DNA** extraction (Lopez *et al.*, 2005), best results the ring test (SMT-4-CT98-2252)
- Other kits tested in this project validation
  - DNeasy Plant Kit (Qiagen)
  - Magnetic DNA purification for Food (Promega)
  - MagJET Plant Genomic DNA (Thermofisher)
- Suspect colonies
  - Crude method
  - Blood and Tissue (Qiagen)
  - Genomic DNA Purification Kit (Promega)
- Key points
  - Extraction methods in your lab that successfully extract strawberry plant material and bacterial DNA
  - Extraction control: healthy strawberry plant material

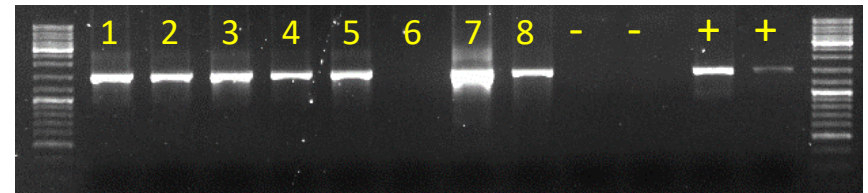
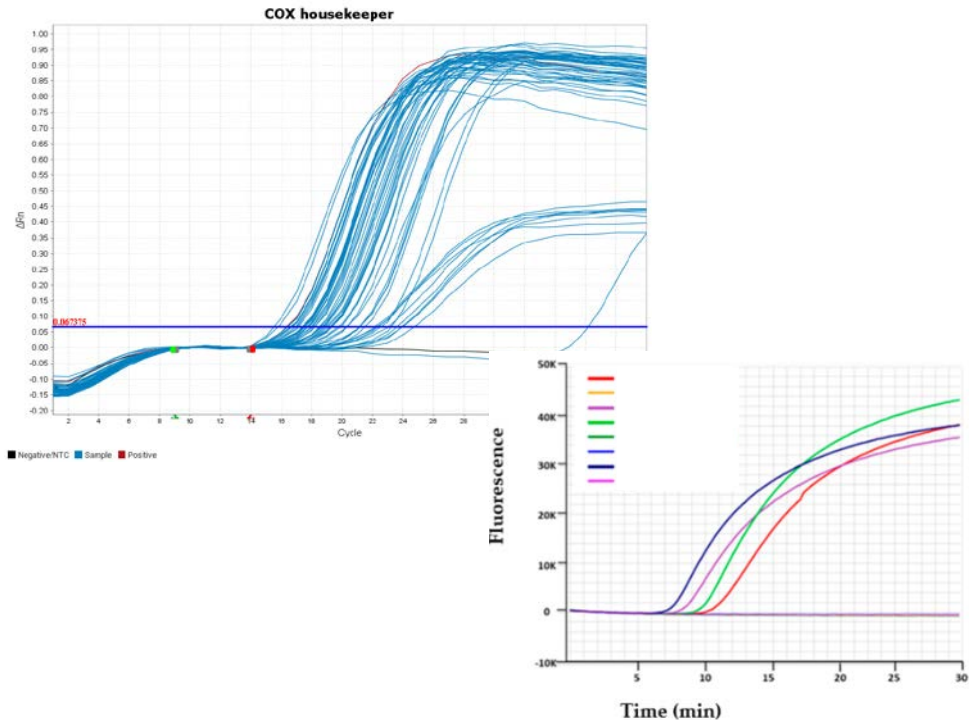
## 4.5 Reference material

- Available from:
  - DAR (NSW)
  - ICMP (NZ)
  - DSM (Germany)
- Synthetic constructs
  - Multiple targets



## 4.4 Molecular methods

- Has your extraction worked?
- Molecular housekeeper controls in NDP
  - Weller *et al.*, (2000) COX qPCR
  - Tomlinson *et al.*, (2013) COX LAMP
  - Weisburg *et al.*, (1991) 16S PCR



## 4.4 Molecular methods - Pathogen

- Extensive molecular screening
- High levels of scrutiny
- Back up your tests – some assays don't work
- Triplicates
- Testing panels
  - *X. fragariae*
    - *in-planta* sensitivity
  - *Xanthomonas* sp.
  - Strawberry host DNA – diagnostic and backyard samples
  - VIC, QLD, NSW

## 4.4 Molecular methods - Pathogen

- Recommended primary screening
- Weller *et al.*, (2007)
  - qPCR
  - Target: *X. fragariae* gyrB
- Wang & Turechek (2016)
  - Loop-mediated isothermal amplification (LAMP) – Can also be used in-field!
  - Target: *X. fragariae* random amplified polymorphic DNA (RAPD) fragment
- Kit used, primer list and concentrations, reaction set up and cycle conditions in NDP
- Result interpretation in NDP



## 4.4 Confirmatory molecular

- Back up tests to confirm a positive detection
  - Pooler *et al.*, (1996) PCR
  - Hartung and Pooler (1997) PCR
  - Moltmann and Zimmermann (2005)
- Run dilutions of extracts

## 4.4 Sequencing

- Sanger sequencing of anything amplified (NOT LAMP)
  - Gel extractions for multiplex
- Need an isolate to confirm presence of *X. fragariae*
- MLSA – *gyrB*, *ropD*, *dnaK*
- HTS – short or long read platforms

A	C	C	G	C	G	A	G	A	T	T	T	C	C	G	C	T	G	T	A	C	G	C	C	T	G	G	T	G	G	C
A	C	C	G	C	G	A	G	A	T	T	T	C	C	G	C	T	G	T	A	C	G	C	C	T	G	G	T	G	G	C
A	C	C	G	C	G	A	G	A	T	T	T	C	C	G	C	G	A	T	C	C	G	C	C	T	G	G	T	T	G	C
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A	T	C	G	C	G	A	A	G	T	C	T	C	C	G	C	G	C	A	T	C	G	C	C	T	G	A	T	C	G	C

## 9. Diagnostics to support surveillance

- In field tests and extractions
  - Plant Material DNA Extraction Kit (Optigene)
  - Cellulose disk method
  - LAMP
- Surveillance
  - Increase capacity
    - Column extractions (low)
    - Extraction robots (Qiacube or Kingfisher) – high up to 1000's of extractions
  - Reference back to lab tests in previous NDP sections



## Wrap up

- Enormous amount of work done by Jodie
- EMAI team for validation
  
- Jodie Crowder
- David Lovelock
- Rachel Mann
- Linda Zheng
- Fiona Constable



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