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Development of a multiprong real-time PCR assay for the detection of the grapevine pathogen *Xylophilus ampelinus*

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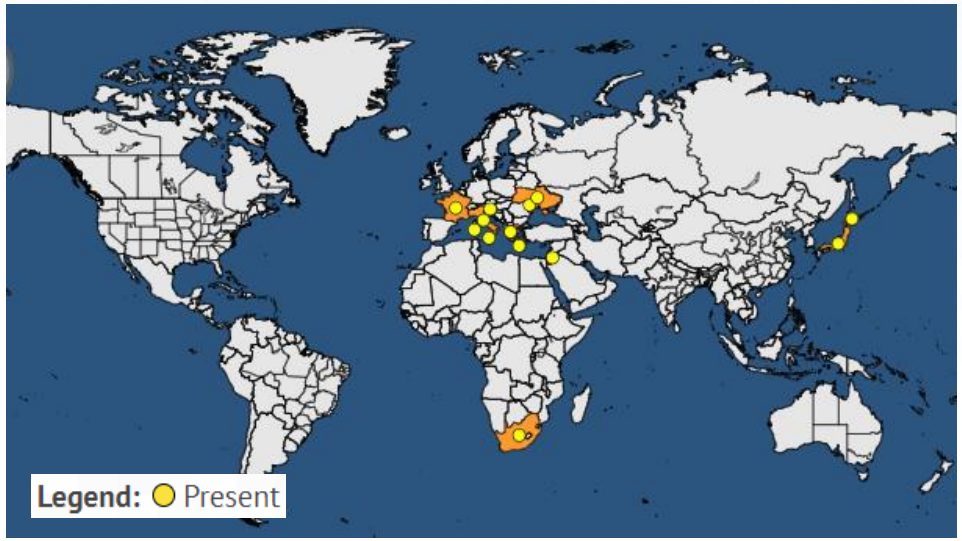
Xylophilus ampelinus

- A causative agent of bacterial blight in grapevines.
- Present in the southern parts of Europe (find also elsewhere e.g. South Africa and Japan).
- In Slovenia *X. ampelinus* was first confirmed in 2002.
- Since the year 2019 *X. ampelinus* has been listed as regulated non-quarantine pest in the EU.

Geographic distribution of *X. ampelinus*



https://gd.eppo.int/taxon/XANTAM/distribution, Last updated: 2025-12-01

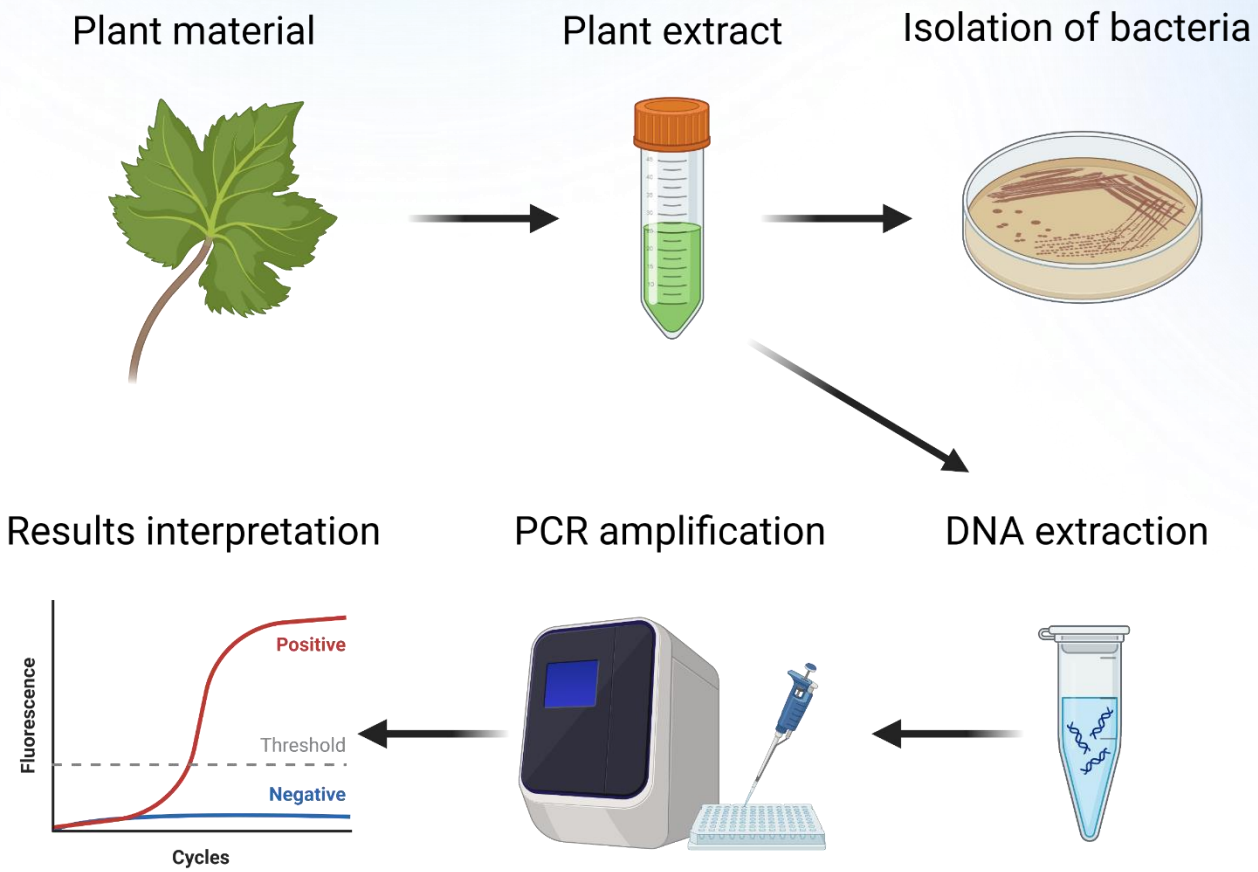
Symptoms of infection with *X. ampelinus*

Photo: Manca Pirc

Photo: Andjelka Prokić

Detection of *X. ampelinus*

- Due to difficulties in isolation of bacterial in pure culture molecular methods are particularly well-suited for the detection of *X. ampelinus* in routine diagnostics.
- Challenges:
  - Conflicting results between different tests
  - Testing of samples on which the tests were not previously validated (roots and grafted plants)
  - Testing of asymptomatic material
  - False positive or negative results could have serious repercussions



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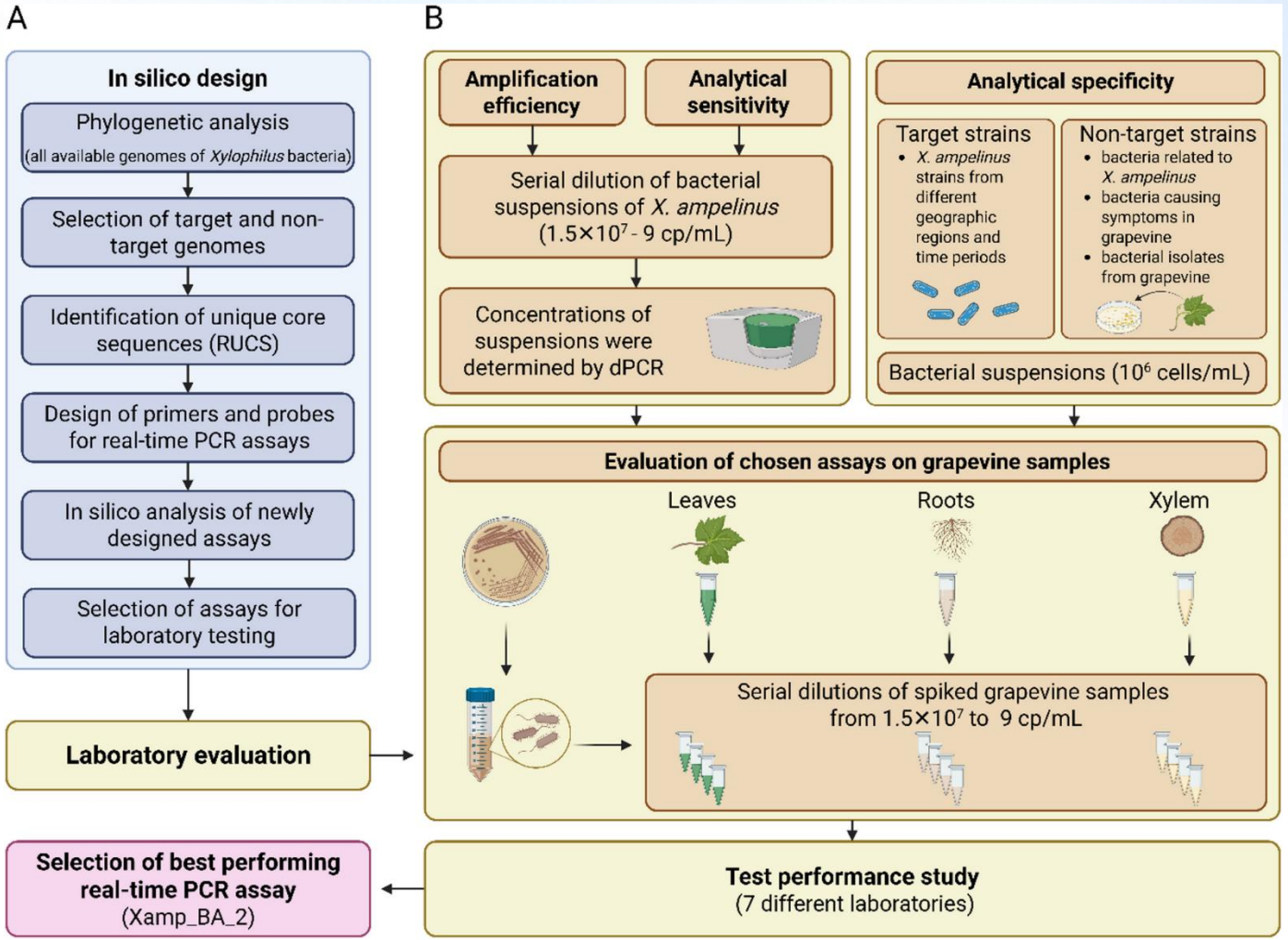
Study goals

- To design new quantitative real-time PCR tests for the detection of *X. ampelinus.*
- We decided to use publicly available genomic data to design real-time PCR tests.
- These tests should be able to reliably detect *X. ampelinus* with:
  - high specificity
  - high sensitivity
  - different grapevine samples
- To extend the use of existing qPCR (Dreo et al.) for the detection of *X. ampelinus* to other matrices

Study design

- In silico design and analysis
- Laboratory evaluation
- Selection of best performing tests



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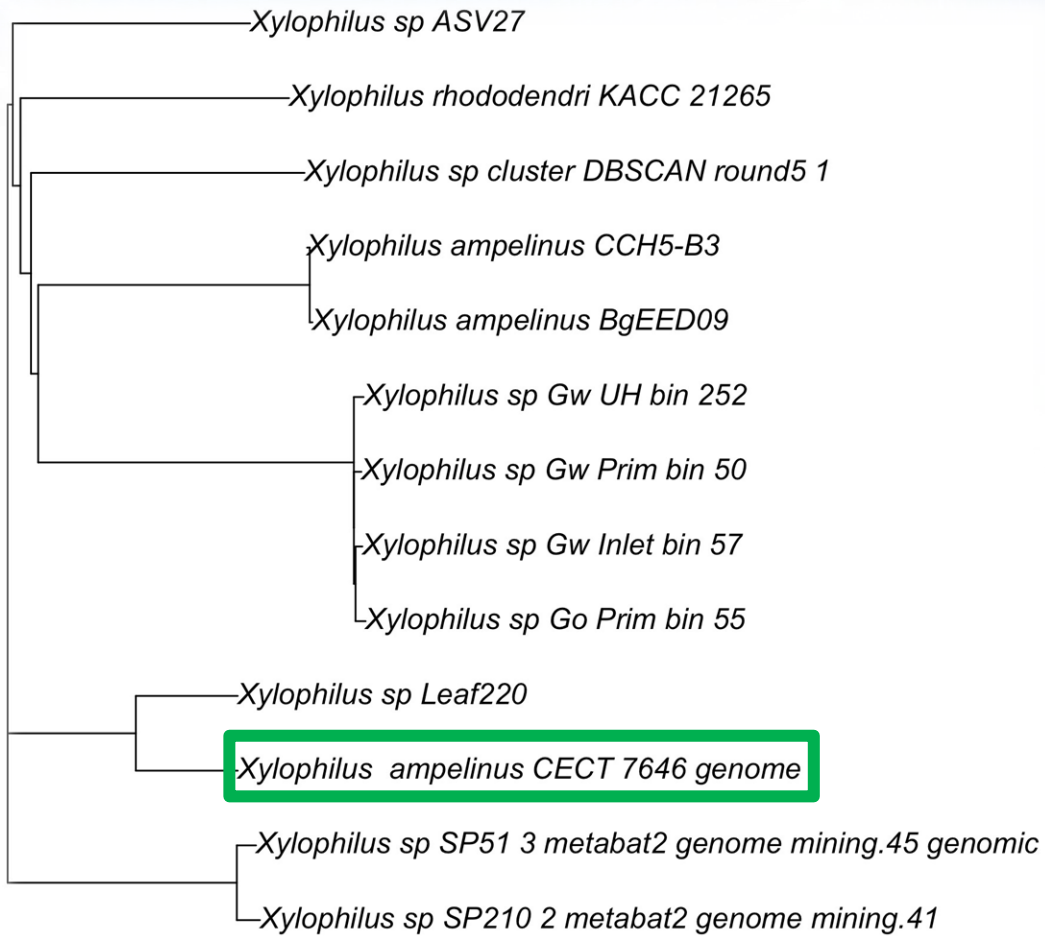
Determination of target and non-target genomes

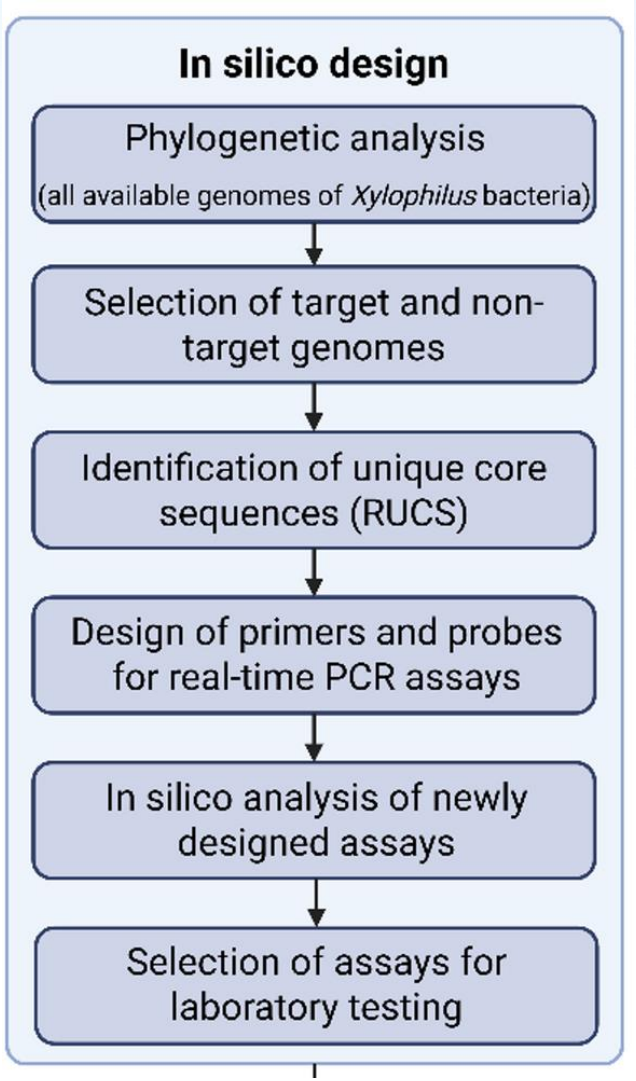
- All at the time available *Xylophilus* genomes
- Phylogenetic analysis is crucial as databases often include errors in the taxonomic classification
- Only one genome belongs to *X. ampelinus* – target
- Other genomes – non-target

Phylogenetic tree based on average nucleotide identity



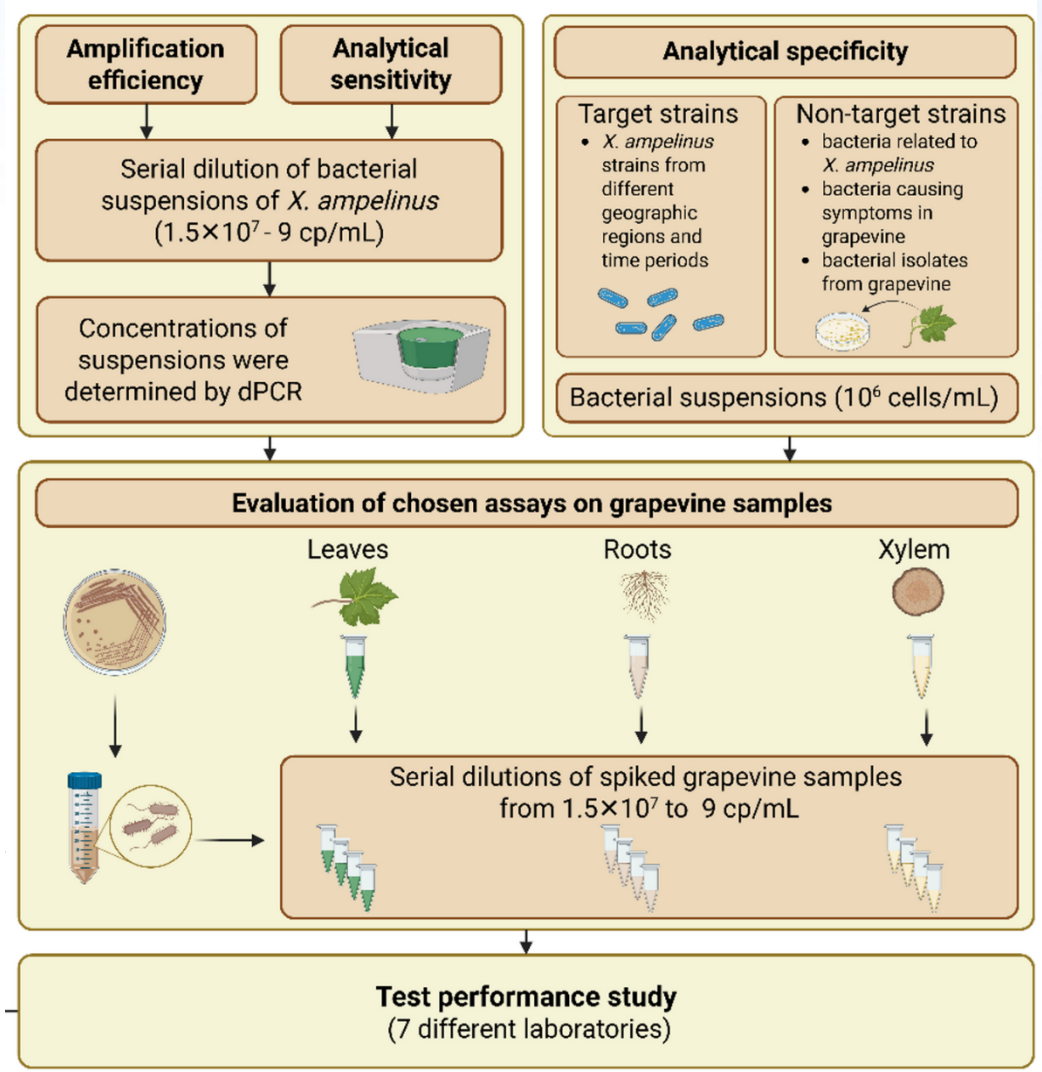
Target sequences and design of tests

- We successfully identified unique sequences present only in *X. ampelinus* and used them to design primers and probes.
- TaqMan chemistry based on hybridisation probes
- Together we designed 18 new real-time PCR tests.
- We excluded 4 tests during *in silico* analysis – prophages
- Amplified regions belonged to:
  - Known proteins
  - Hypothetical proteins
  - Non-coding
- Present in only one copy.



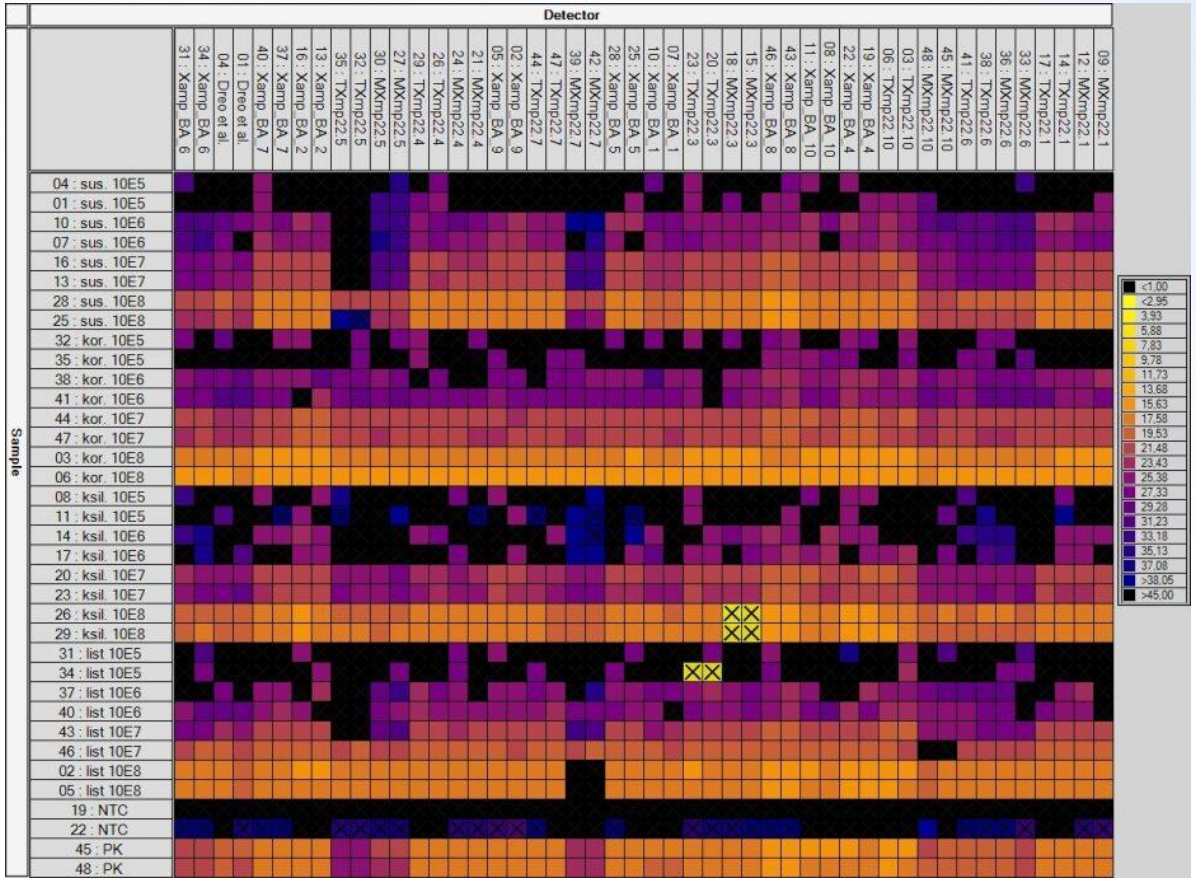
Laboratory validation of new tests

- In laboratory testing we evaluated:
  - Amplification efficiency
  - Sensitivity
  - Specificity
- How do new test perform in different matrices:
  - Leaves
  - Xylem
  - Roots
- Robustness of tests in different laboratories/equipment.



Amplification of target DNA

- For the high-throughput evaluation of amplification of newly designed
- real-time PCR tests we used **Fluidigm** system
- 48 tests × 48 samples = 2304 reactions on one chip
- We confirmed that our new tests successfully amplify DNA of *X. ampelinus*



Amplification efficiency & analytical sensitivity

- All newly designed test could amplify DNA of target organism but with different efficiency.
- Tests could detect *X. ampelinus* in concentration as low as 10^2 cp/mL.

Table 2 Performance characteristics of real-time PCR assays with DNA extracted from *X. ampelinus* suspensions in PBS

Assays	C_q(min–max)[a]	Linear range (cp/mL)[b]	Linear regression [c]			LOD95 [cp/mL]	C_q (1 cp/rxn)
			k	R^2	E		
Dreo	18.8–36.7	3.7×10^2-1.5×10^7	-3.66	0.996	0.88	1.28×10^2	36.72
TXmp22.2	18.9–37.7	3.7×10^2-1.5×10^7	-3.75	0.997	0.85	1.28×10^2	36.54
TXmp22.4	18.9–36.1	3.7×10^2-1.5×10^7	-3.56	0.992	0.91	1.77×10^3	36.27
TXmp22.5	20.2–38.1	3.7×10^2-1.5×10^7	-3.66	0.995	0.88	7.94×10^2	38.13
TXmp22.7	19.5–37.0	3.7×10^2-1.5×10^7	-3.65	0.997	0.88	1.28×10^2	37.37
TXmp22.10	20.9–34.2	6.2×10^3-1.5×10^7	-3.74	0.997	0.85	9.91×10^2	38.45
Xamp_BA_1	20.3–37.7	3.7×10^2-1.5×10^7	-3.61	0.996	0.89	1.28×10^2	38.09
Xamp_BA_2	20.9–39.1	3.7×10^2-1.5×10^7	-3.67	0.996	0.87	1.15×10^2	38.76
Xamp_BA_4	20.8–37.7	6.2×10^3-1.5×10^7	-4.76	0.998	0.62	9.91×10^2	38.22
Xamp_BA_5	19.2–32.2	6.2×10^3-1.5×10^7	-3.68	0.997	0.87	1.53×10^3	36.48
Xamp_BA_6	22.0–41.0	6.2×10^3-1.5×10^7	-3.64	0.992	0.88	9.91×10^2	40.24
Xamp_BA_7	19.0-36.6	3.7×10^2-1.5×10^7	-3.90	0.997	0.81	1.77×10^3	36.23
Xamp_BA_8	18.0-35.4	3.7×10^2-1.5×10^7	-3.53	0.996	0.92	1.28×10^2	35.66
Xamp_BA_9	18.9–31.6	6.2×10^3-1.5×10^7	-3.59	0.997	0.90	1.53×10^3	36.28
Xamp_BA_10	18.7–35.6	3.7×10^2-1.5×10^7	-3.70	0.997	0.86	1.28×10^2	35.88

[a] Minimal and maximal C_q values that still enable detection

[b] The range of concentrations for which C_q values were in linear relationship with logarithms of concentrations (determined by exploring slope values across sections of C_q values × log number of the cells)

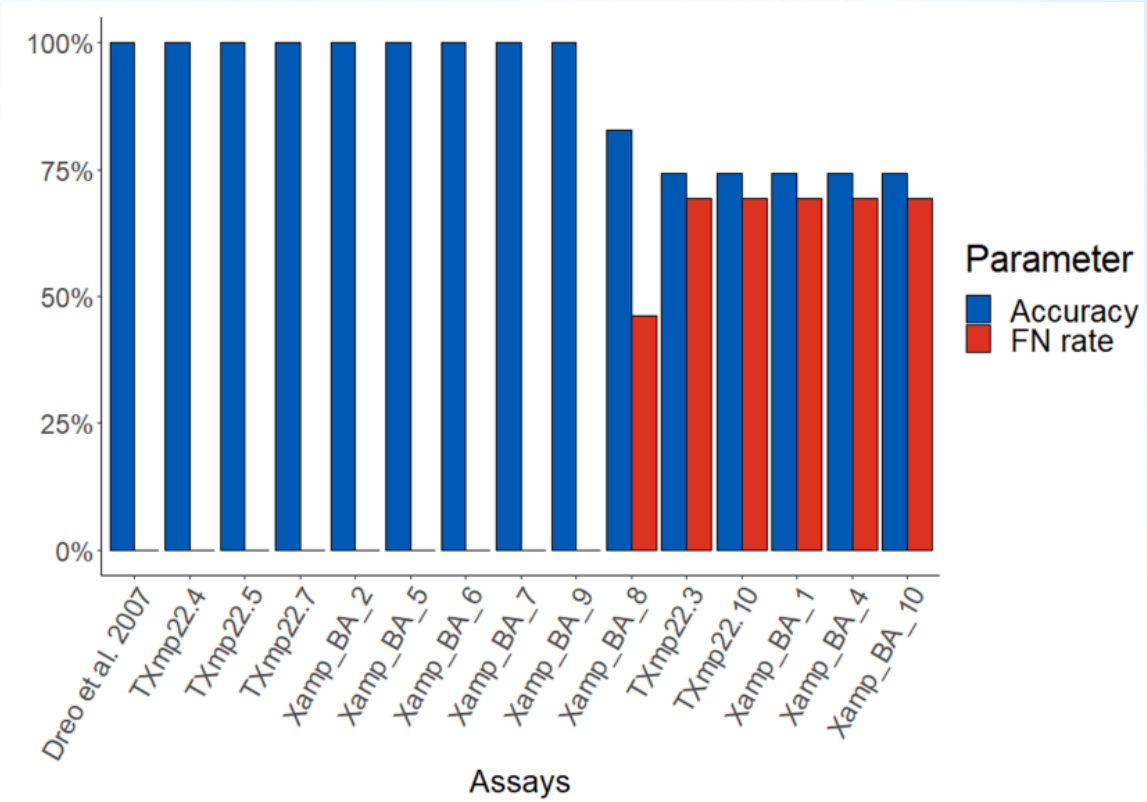
[c] Linear regression of all positive samples in a plot of C_q values against the logarithmic number of *X. ampelinus* cells: k = slope of the linear regression line; R^2 = average square regression coefficient; E = efficiency of amplification

Analytical specificity

- No false positive results.
- **Eight** of the new tests could detect all tested *X. ampelinus* strains.
- **Five** new tests selected for further evaluation.

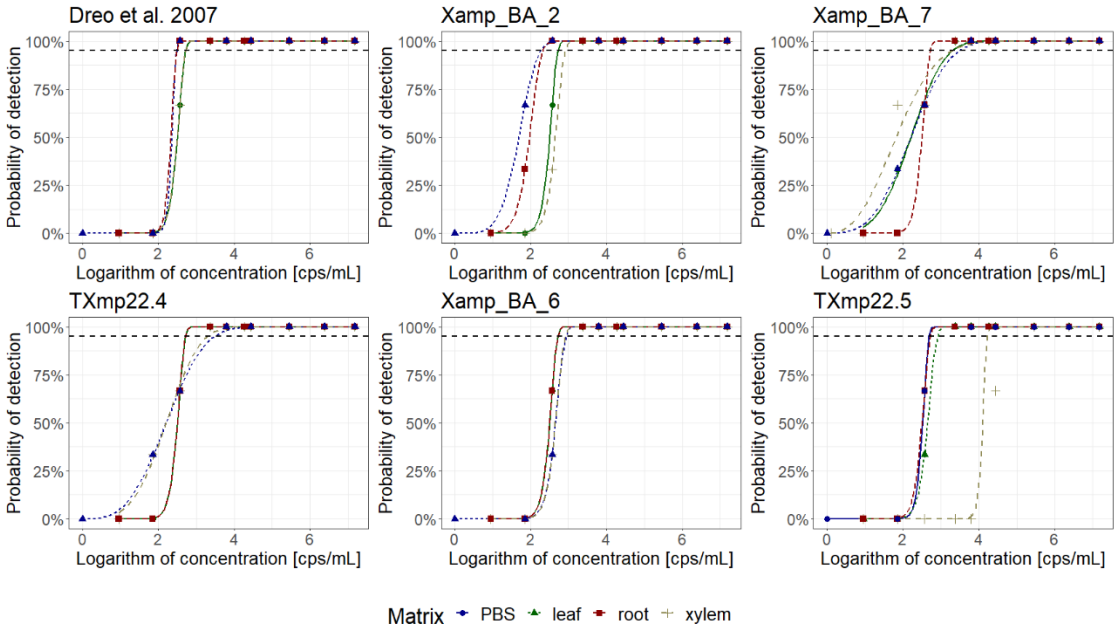
Selected tests

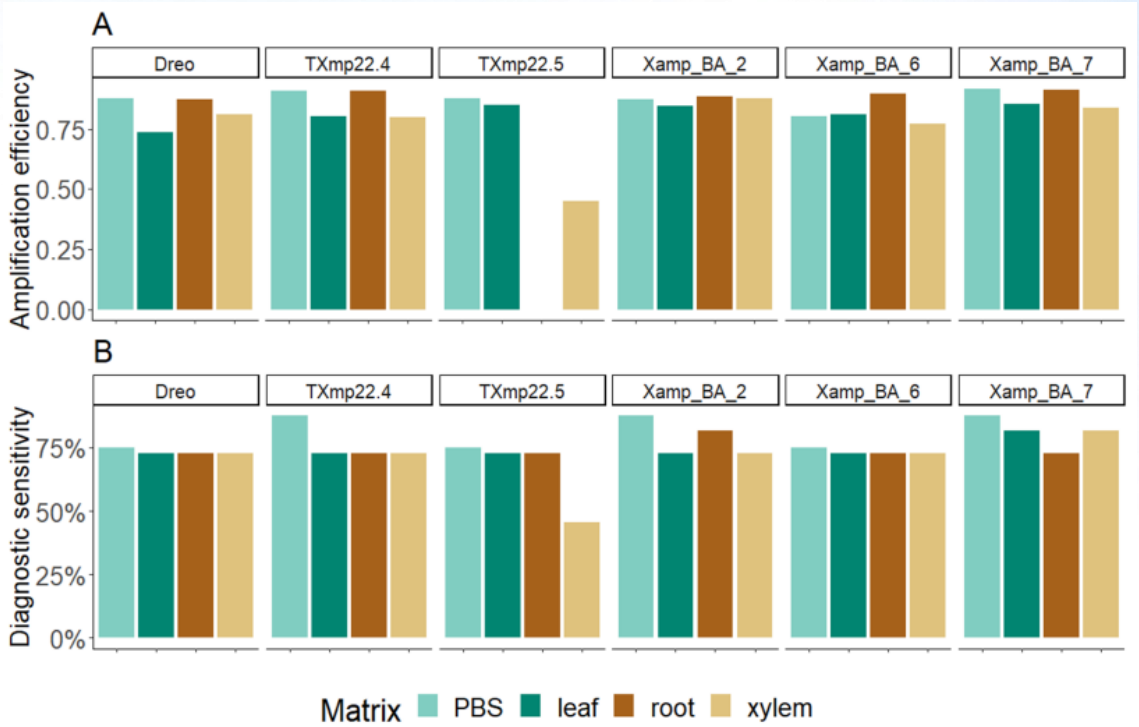
Test	Target sequence
TXmp22.4	Non-coding sequnce
TXmp22.5	ABC transporter permease
Xamp_BA_2	alanine racemase
Xamp_BA_6	Non-coding sequence
Xamp_BA_7	hypothetical protein



Performance of test in different matrices

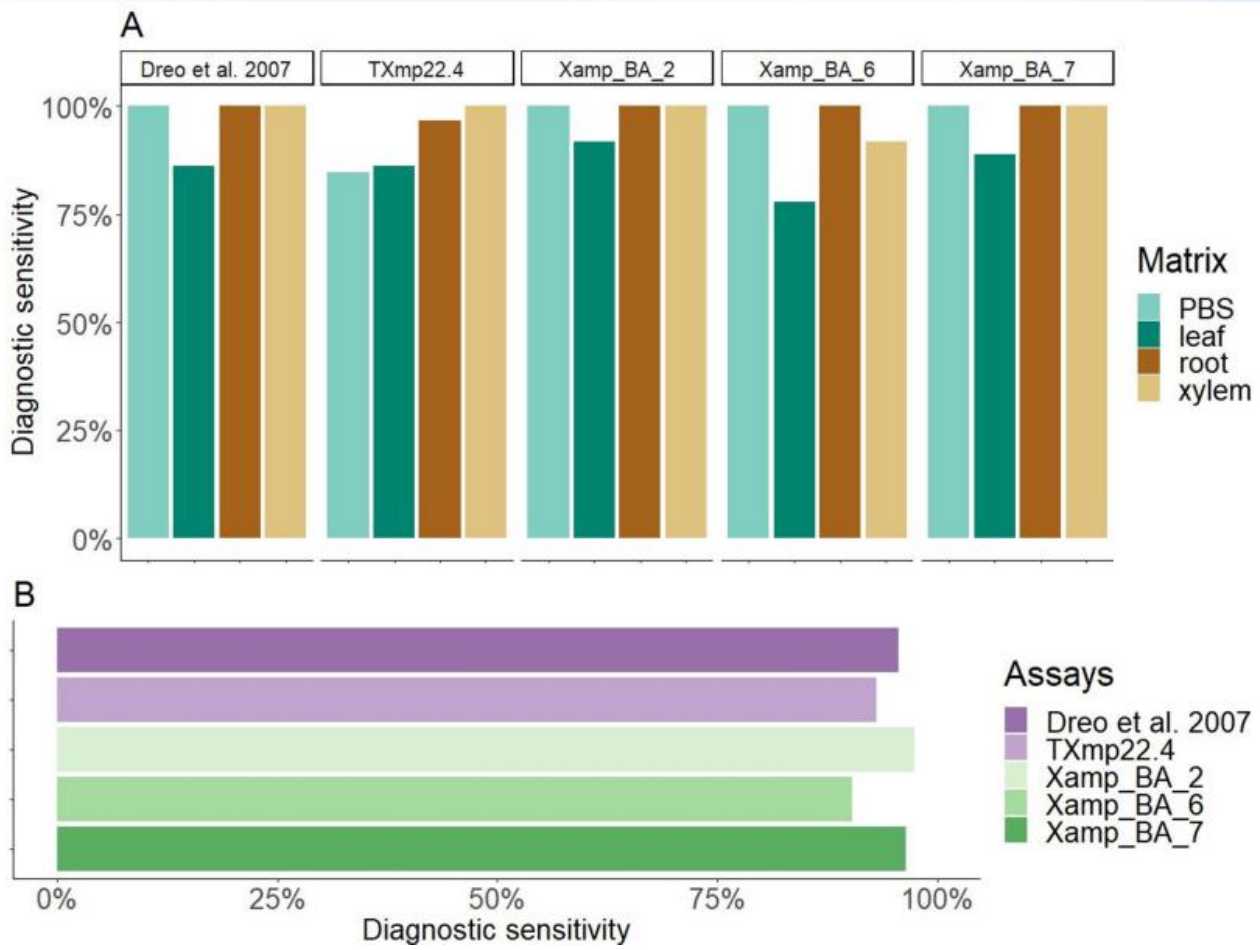
- **Four** new tests showed acceptable amplification efficiency and sensitivity (low as 10^2 cps/mL) across all matrices.
- Samples from leaves showed slightly lower efficiency.





Test performance study (TPS)

- To validate the performance of new tests in different laboratory settings we organised TPS with laboratories from multiple European countries
- Tests showed similar results across participating laboratories confirming results of previous testing.
- Leaves were again the most problematic sample type.
- One additional new test (Xamp_BA_6) was excluded leaving us with three new tests.



Conclusions

- We successfuly developed three new real-time PCR tests suitable for the detection of *X. ampelinus* in different grapevine samples
- We extended the use of existing real-time PCR Dreo et al. to matrices of roots and leaves
- We suggest the changes in EPPO protocol to include newly developed tests and extend the use of existing test
- Such tools for the reliable detection of plant pathogenic bacteria are crucial in preventing their spread in new areas through trade with plant material and preventing damage in agriculture.
- Laboratory testing remains a crucial part of the validation process for any new test before it can be used in routine diagnostic testing.

Aknowledgements

- Working group: Bacteriology and metrology
- Diagnostics of plant pathogenic bacteria
- Department of Biotechnology and Systems Biology
- National Institute of Biology

Laboratories that participated in TPS

NIB
crea Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria
anses
Agroscope
ersa Agenzia regionale per lo sviluppo rurale
REGIONE AUTONOMA FRIULI VENEZIA GIULIA
IPSP Istituto per la Protezione Sostenibile delle Piante Consiglio Nazionale delle Ricerche
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