

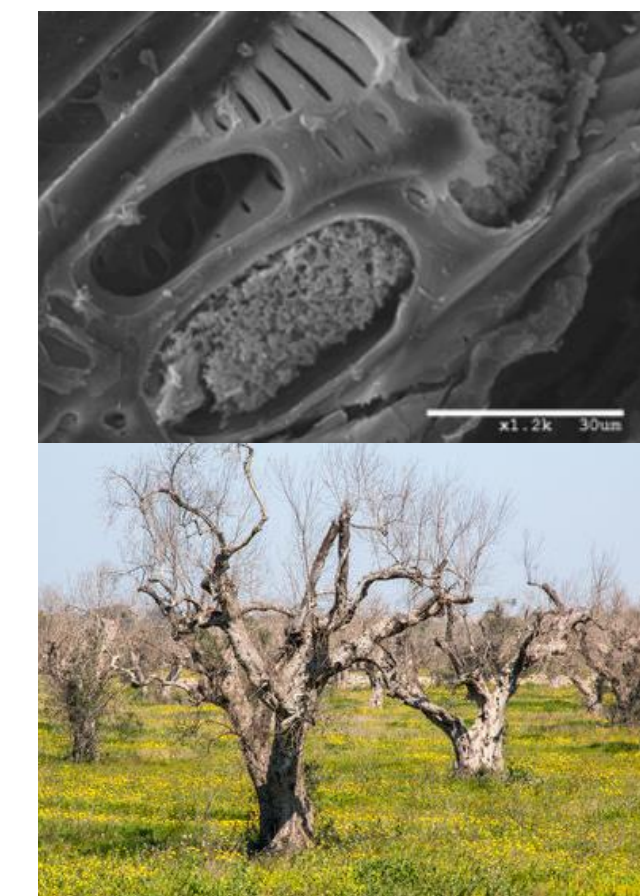
Advancing Reference Measurement Systems for *Xylella fastidiosa*: Development of Candidate Reference Measurement Method and Evaluation of DNA Extraction Methods



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Background *Xylella fastidiosa*, a causative agent of various plant diseases, poses a significant threat to crops such as olive, grapevine, plum, citrus, and peach. Spread by xylem-feeding insects, its containment remains a major agricultural challenge. Reliable detection is crucial for disease management, but *X. fastidiosa*'s slow growth limits traditional microbiological methods, making way for serological and molecular tests, including PCR, qPCR, and dPCR. Molecular methods, are known for their speed and sensitivity, but can be affected by plant matrix variability, including species, tissue type, and seasonal factors. Classified by European and Mediterranean Plant Protection Organization (EPPO) as an A1 pest with zero-tolerance regulations in many countries, laboratory diagnostics prioritize detecting *X. fastidiosa* at minimal concentrations. To ensure accuracy and comparability across laboratories, the establishment of a reference measurement procedure is thus essential.



Fritsch, A. et al., 2008, Plant Disease, 92:319-318, <https://www.efsa.europa.eu/en/press/news/180910>

EXPERIMENTAL PLAN

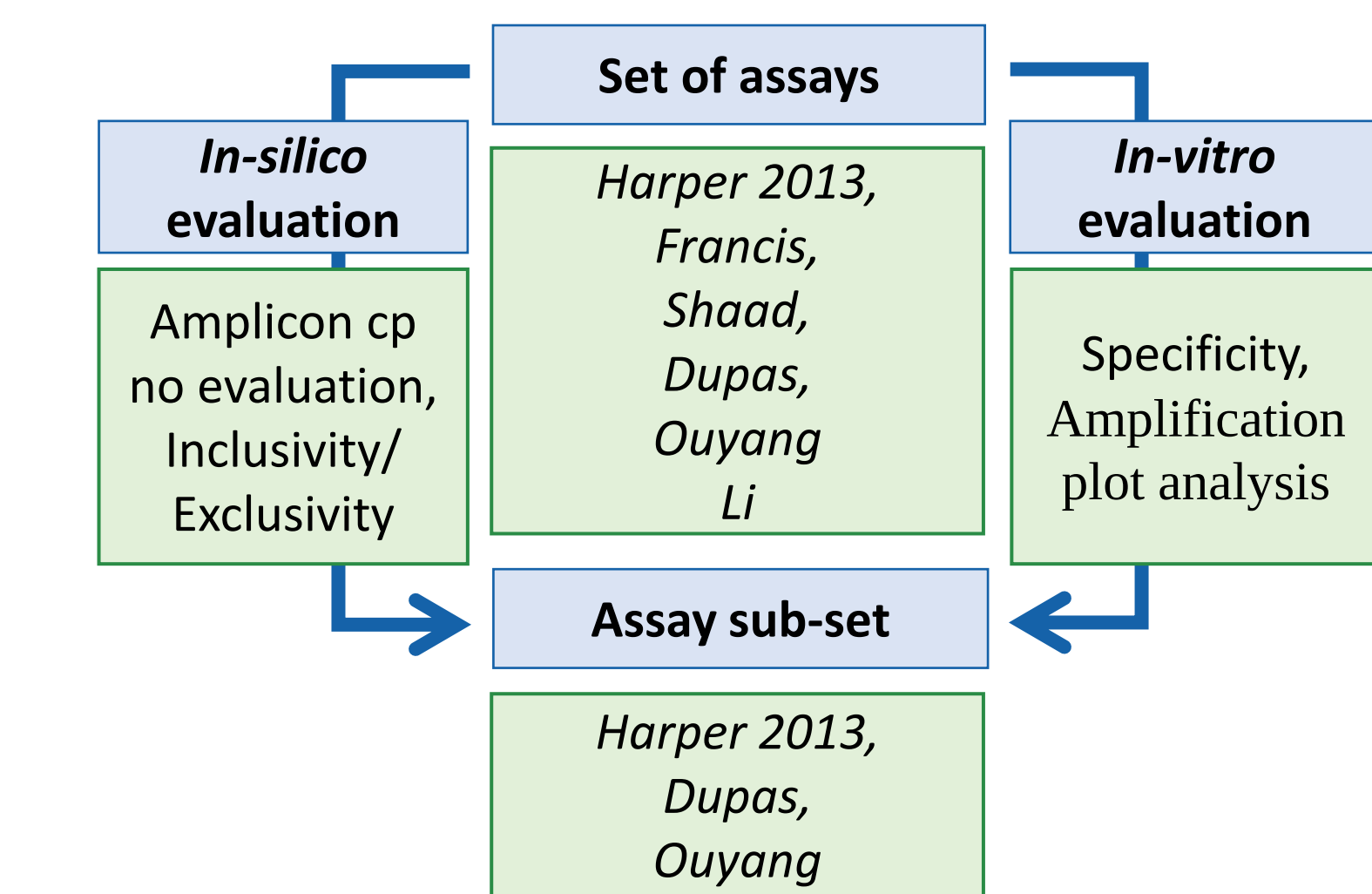


Fig 1: Schematics of candidate reference method selection.

A series of assays targeting *X. fastidiosa*, including those described in PM 7/24 (4) (EPPO, 2019), were evaluated both *in silico* and *in vitro* (Figure 1) to select the most suitable test for transfer to dPCR and assessment as a potential reference method. The test based on Harper et al. (2010, erratum 2013) was chosen and evaluated according to international guidelines, including PM 7/98 (5) (EPPO, 2021) and ISO/IEC 17025:2017 (Figure 2). To improve dPCR performance, the probe was modified with internal ZEN quencher, which effectively reduced background signals. Additionally, to assess DNA extraction methods for both yield and repeatability, a comprehensive experimental plan (Figure 3) was developed.

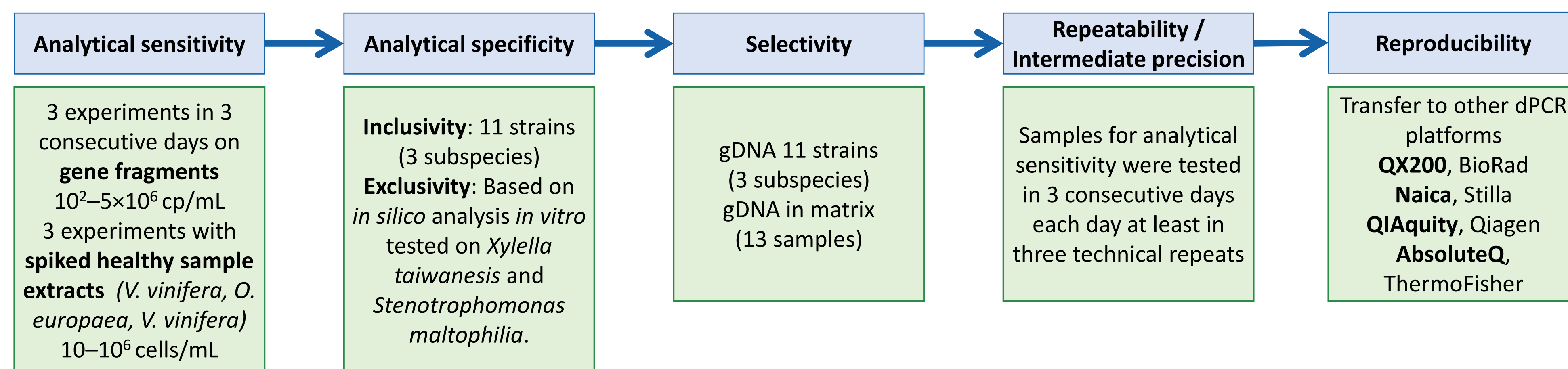


Fig 2: Schematics of candidate reference method evaluation.

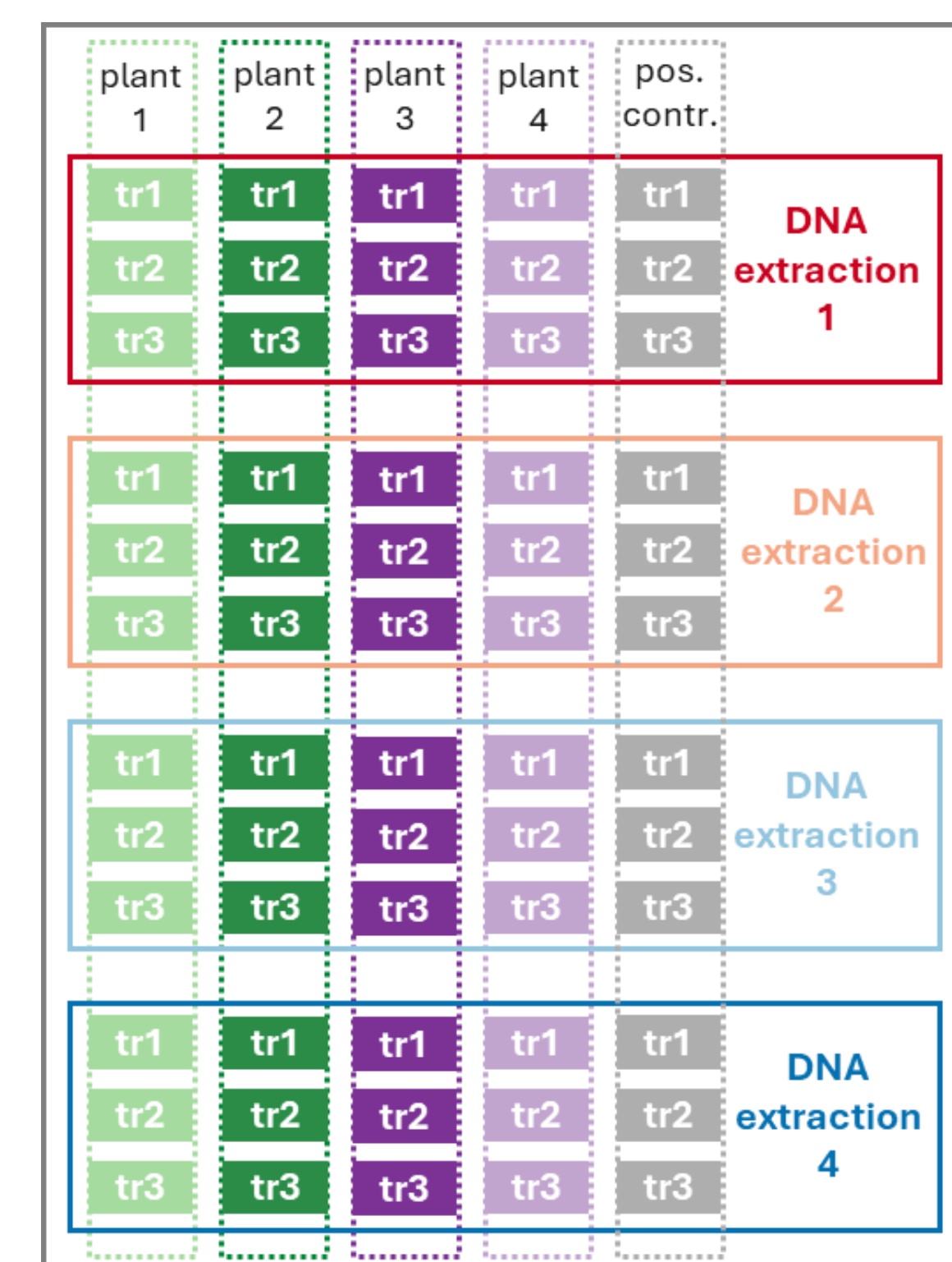


Fig 3: Schematic presentation of extractions.

Plant 1: Olive (*Olea europaea*) + *X. fastidiosa* ssp. *pauca*
Plant 2: Rosemary (*Rosmarinus officialis*) + *X. fastidiosa* ssp. *pauca*
Plant 3: Grape vine (*Vitis vinifera*) + *X. fastidiosa* ssp. *fastidiosa*
Plant 4: Myrtle-leaf milkwort (*Polygala myrtifolia*) + *X. fastidiosa* ssp. *multiplex*
Pos. contr.: positive control, *X. fastidiosa* ssp. *fastidiosa* suspension in PBS buffer
DNA extraction 1: QuickPick Plant DNA Kit
DNA extraction 2: CTAB (according to EPPO PM 7/24 (4))
DNA extraction 3: Modified DNeasy Mericon Food Standard Protocol
DNA extraction 4: Maxwell® RSC PureFood GMO and Authentication

CONCLUSIONS

- Plant diseases have significant societal and socioeconomic consequences, impacting agriculture and biodiversity.
- Well-evaluated reference methods are crucial for standardization of diagnostics and preventing the spread of plant pathogens, especially for high-risk quarantine pests.
- Digital PCR (dPCR) once again proven to be a highly accurate and reproducible technique, supporting its potential as a candidate reference measurement procedure (RMP) for pathogen detection. It enables reliable quantification and plays a key role in standardizing molecular diagnostics for plant pathogens.
- Extraction methods and plant matrices significantly impact *Xylella fastidiosa* quantification, with variations in yield and repeatability. *Olea europaea* yielded the lowest DNA concentration, highlighting the importance of appropriate extraction method selection for accurate detection.

RESULTS

Dilution	Experiment 1		Experiment 2		Experiment 3		Repeatability		Interm. precision	
	cp/rnx	CV %	cp/rnx	CV %	cp/rnx	CV %	cp/rnx	CV %	Mean	CV %
1	44064	2.58	43162	2.73	43208	1.70	43478	2.42	43478	1.17
2	4724	7.28	4500	1.25	4408	2.55	4547	5.44	4544	3.59
3	482	7.80	460	7.36	447	5.65	462	7.13	463	3.80
4	185	16.64	179	11.50	179	7.86	181	11.31	181	2.05
5	47	19.71	52	22.15	54	14.07	51	18.62	51	7.55
8	19	29.79	20	23.05	19	45.61	19	31.36	19	3.08
7	8	25.44	9	17.62	9	32.75	8	24.95	8	7.69
8	Neg.	NA	Neg.	NA	Neg.	NA	Neg.	NA	Neg.	NA

Table 1: Analytical sensitivity 1. Repeatability and intermediate precision of the method were first assessed using synthetic gene fragments. The limit of detection (LOD) was determined to be approximately 8 cp/rnx (or 10^3 cp/mL), while the limit of quantification (LOQ) was estimated < 51 cp/rnx (or 6.4×10^3 cp/mL). Intermediate precision was assessed $< 8\%$ across all dilutions.

	<i>Vitis vinifera</i>			<i>Rosmarinus officialis</i>		<i>Olea europaea</i>	
	Estimated	Mean cp/mL	CV %	Mean cp/mL	CV %	Mean cp/mL	CV %
10^6		2.25×10^6	3.41	2.34×10^6	35.41	1.03×10^5	0.78
10^5		1.91×10^5	4.49	2.14×10^5	4.88	1.35×10^4	6.60
5×10^4		9.71×10^4	3.62	1.27×10^5	9.25	5.28×10^3	12.51
10^4		2.53×10^4	5.46	2.33×10^4	4.16	2.22×10^3	14.57
5×10^3		1.55×10^4	8.10	2.16×10^4	3.60	Neg.	NA
10^3		1.42×10^3	17.36	1.96×10^3	28.98	Neg.	NA
5×10^2		1.16×10^3	35.84	2.28×10^3	30.43	Neg.	NA

Table 2: Analytical sensitivity 2. After LOD was assessed on gene fragments, additional assessment was performed on spiked healthy plant samples, here the limit of detection (LOD) was approximately 2×10^3 cp/mL (~ 18 cp/rnx), except for *Vitis vinifera*, where the LOD was higher, $\sim 10^3$ cp/mL (~ 8 cp/rnx). The results of this experiment point to challenges in nucleic acid extraction (see Figure 6).

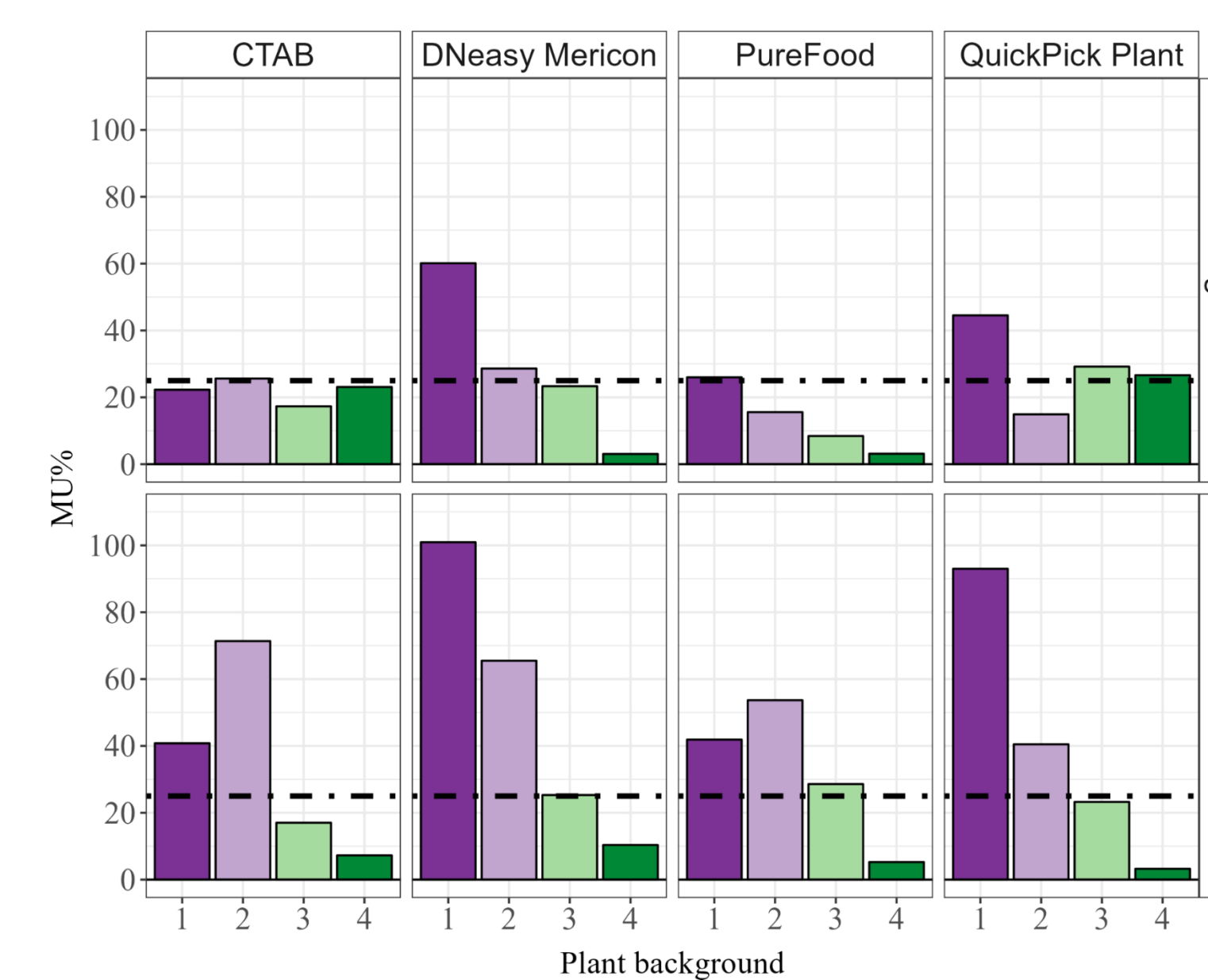


Fig 5: Measurement uncertainty (MU) of each extraction method. The 25 % target MU was met for *Vitis vinifera* and *Rosmarinus officialis*, for all extraction methods, and both concentrations, while *Polygala myrtifolia* exhibited high MU at low concentration and *Olea europaea* showed higher MU in most cases.

Fig 4: Reproducibility of the test on different dPCR platforms. Reproducibility is highlighted by the linearity of the test using four different platforms, where R^2 values were as follows: QX200 0.9989, Naica 0.9997, AbsoluteQ 0.9986 and QIAcuity 0.9978. LOD was at determined at ~ 1 cp/ μ L (QX200 ~ 12 cp/rnx, Naica ~ 4 cp/rnx, AboluteQ ~ 5 cp/rnx), except for QIAcuity, where it was determined at ~ 7 cp/ μ L (~ 33 cp/rnx).

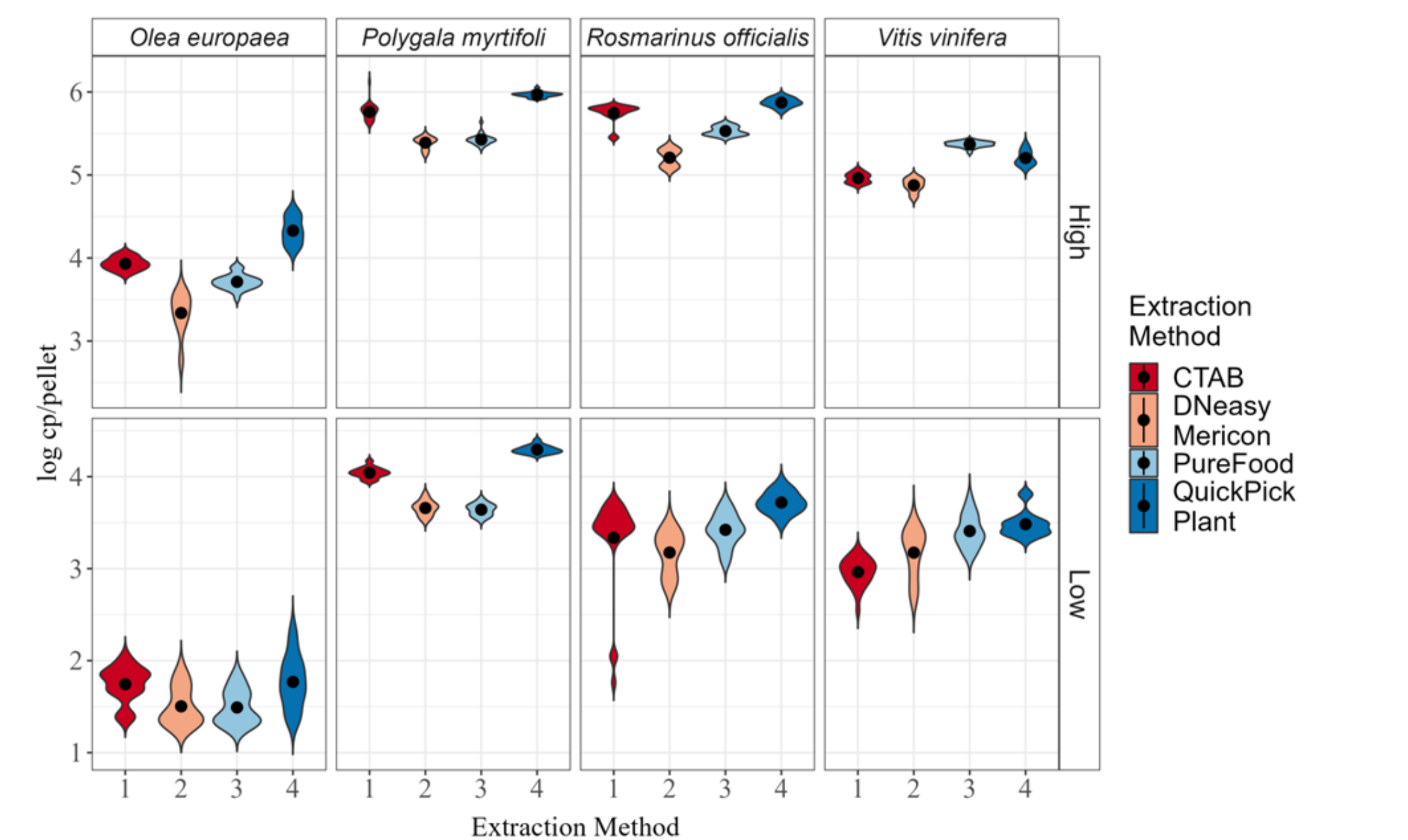


Fig 6: Yield and repeatability of four extraction methods across plant extracts spiked with *X. fastidiosa*. Each method was tested in triplicate over three days (nine extractions per method/plant), using two bacterial concentrations: high (5×10^5 cp/mL) and low (10^4 cp/mL). There is a significant difference in yield between extraction methods.