TECHNICAL INNOVATION FACTSHEET

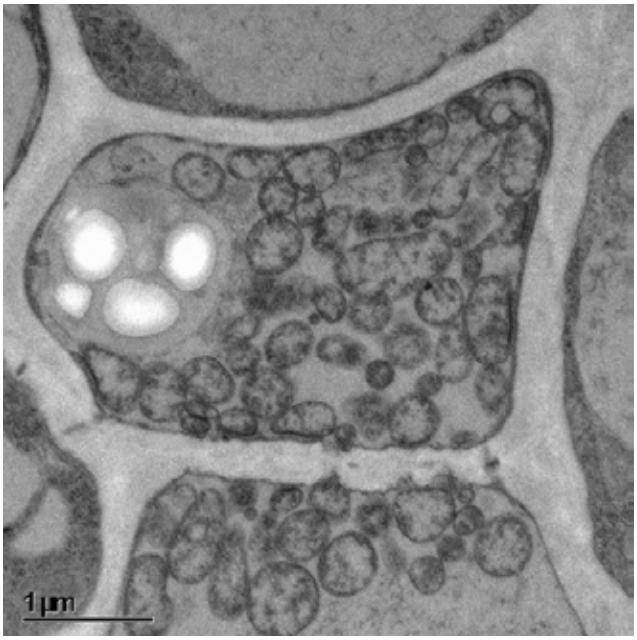
PREPARATION OF REFERENCE MATERIAL FOR MOLECULAR TESTING OF '*CANDIDATUS* PHYTOPLASMA SOLANI'

■ THE PROBLEM ADDRESSED

Lack of well-characterized and certified material for use as phytoplasma reference positive control

The important economic sector of world grapevine production of 74 million tons is threatened by several grapevine yellows (GY) diseases associated with the presence of phytoplasmas. There is presently a lack of availability of well-characterized and certified material suitable for use as reference positive controls in different diagnostic schemes, validation studies, performance studies and proficiency tests, which are all part of the management programs for fast and accurate detection of GY phytoplasmas.

'*Candidatus* Phytoplasma solani' associated with "bois noir", the most widespread disease of grapevine in the Euro-Mediterranean basin, was selected as a model. In the last 20 years, "bois noir" has become a major limiting factor in the European viticulture, which seriously affects quality and quantity of grapevine production with infection rates reaching 50–80% in some areas. In South Africa GY is associated with the presence of '*Ca.* P. asteris' while in Chile and in the other countries several diverse phytoplasmas are present. Molecular methods are needed to detect '*Ca.* P. solani' or '*Ca.* P. asteris' in low concentrations and in many samples and quantitative PCR is a commonly used method. It gives a numerical result which reflects the concentration of the target in the sample. However, these numerical values also depend on the specific reagents, their concentrations and the instrument used. Thus the results obtained in different laboratories are not directly comparable. A reference material can be used as a fixed point in all the studies and research making the results directly comparable among laboratories and, even more importantly, over the time. Certified reference materials are not available for plant pathogens. To overcome this difficulty, we have prepared two types of reference materials for phytoplasmas of grapevine.



• Transmission electron microscope picture of cross-section of a midrib of symptomatic *Catharanthus roseus* infected with '*Ca.* P. solani' (Photo: M. Tušek Žnidarič).

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■ THE PRACTICE/INNOVATION PROPOSED BY TROPICSAFE

Two new reference materials for grapevine phytoplasmas

Two types of reference materials have been prepared:

1) DNA sequences of the target region used for the quantitative PCR originally described by Hren et al. (2007), together with nucleotide data available in NCBI, were used for designing synthetic DNA, e.g. gBlocks, IDT. Synthetic double-stranded DNA of known size and amount is cheap and provides high amounts of the specific sequence target suitable for technical assessment of tests and as a positive control for testing,

2) DNA from grapevine naturally infected with '*Ca.* P. solani' was selected and protocols for its quantification by digital droplet PCR were designed for assigning reference values to the concentration of target sequences. This method enables absolute quantification without the need for standards and calibration.

■ HOW IS TROPICSAFE IMPLEMENTING IT?

Publically available sequence data and characterization of material with digital droplet PCR

Preparation of reference material is a challenging process requiring high accuracy, and it is particularly difficult, demanding and time consuming since the growth of phytoplasmas in artificial media is still not a routine method. The two types of reference materials prepared during the TROPICSAFE project are the prerequisites for further studies of grapevine infected with either '*Ca.* P. solani' or '*Ca.* P. asteris' that require reference positive test controls. Both approaches for preparation of reference material are easily transferrable to other phytoplasmas and DNA targets. Moreover, they have been applied to assigned values to reference materials used in international proficiency tests organized by the National Institute of Biology in Slovenia for a number of different pathogens.

The first reference material is based on the synthetic double-stranded DNA, for which the publically available sequence data were used. The known size and amount allows preparation of material with relatively well-defined target concentration without additional testing.

The second reference material, more similar to the diagnostic samples, is based on DNA samples from grapevine leaf veins infected with '*Ca.* P. solani'. Reference values of target sequence concentrations were assigned after the characterization of the material with digital droplet PCR, a gold standard method for absolute quantification.

> gBlock control on KT281865.1 *Candidatus* Phytoplasma solani isolate STOL3 1-acyl-sn-glycerol-3-phosphate acyltransferase gene, partial cds
ACGTAAAACAGCTTTAAGTTTAATAAAGGCAATTCCAAAAGTAAAAGCAGGTTTAGCGATG
GTTGTTTTTCCTGAAGGTGGTATTAAAGATCGAAATGATGAAGCAACGGTACCACTTTTAG
AGGGGTCTTTTAAAATTGCTTTTAAAACGCAAGCCGA

• Sequence of the proposed synthetic DNA control for the ´*Ca.* P. solani´ amplicon targeted by qPCR. Shaded areas correspond to primer and probe annealing sites, the sites recognized by the reagents used to detect the pathogen.

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■ HOW IS IT WORKING?

Potential of reference positive test controls and their transfer to other phytoplasmas and DNA targets

In a synthetic DNA reference material a potential drawback is that the synthesized DNA is target specific and therefore a test specific sequence; its known size and amount allow preparation of material with relatively well defined target concentration without additional testing. Based on the selected target sequence this reference material can be ordered commercially, which is an important advantage of this approach.

The plant material naturally infected with '*Ca.* P. solani' was characterized by digital droplet PCR (ddPCR) as a higher quality method in metrological and clinical fields for absolute quantification of target concentrations without the need for calibration. For the preparation of reference material, a current qPCR protocol was successfully transferred to the ddPCR format and used to determine absolute concentration of the target DNA copies in the samples. The protocol can be used to assign values to naturally infected samples or artificially prepared defined mixtures.

Either of the two types of reference material described here are fit for the purpose. A reference material with a known target concentration provides comparability over the tests and, more importantly, over the time as it can be repeatedly prepared with the same characteristics. The described approach is transferrable to other phytoplasmas and DNA targets.

	Synthetic dsADN (gBlock™)	Naturally infected samples
PROS	• High amounts of target sequence DNA • Well defined target concentration • Commercially available • Comparabile over tests	• Similar to the diagnostic samples • More commutable • Can be used in a variety of molecular tests • Provides information on the influence of DNA extraction
CONS	• Sequence specific • DNA extraction not needed	• Limited amounts and availability • DNA extraction needed

• Characteristics of the two types of reference materials prepared for the ´*Ca.* P. solani´ agent of the "bois noir" disease.

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KEY WORDS

Reference material, detection, plant pathogen, digital droplet PCR

FURTHER INFORMATION

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AUTHORSHIP

Tanja Dreo tanja.dreo@nib.si, **Špela Alič** spela.alic@nib.si, **Marina Dermastia** marina.dermastia@nib.si

National Institute of Biology, Department of Biotechnology and Systems Biology, Ljubljana, Slovenia

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