Final Report

Project title
Interlaboratory test performance studies for identification of *Ralstonia solanacearum* and molecular confirmation of its virulence

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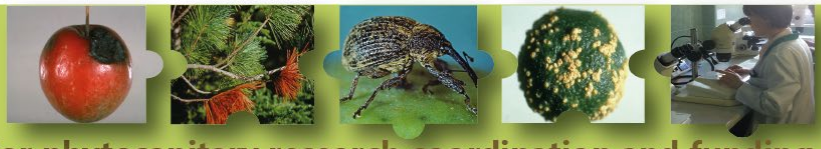
Contents



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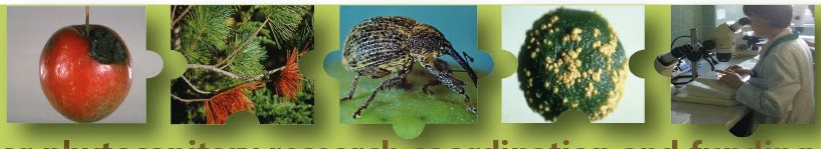

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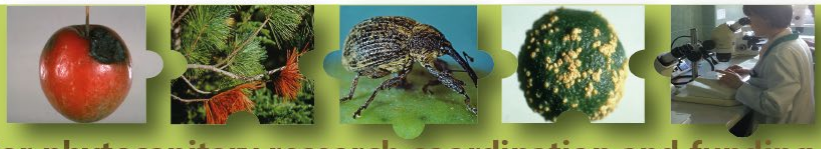

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2. Short project report

2.1. Executive summary

The EU directive 2006/63/EC describes a detailed protocol for the official testing of *Ralstonia solanacearum,* that is internationally recognized and has been implemented in many diagnostic laboratories across Europe and beyond. In this protocol, the confirmation of the identity of the bacterium is performed by a laborious, time-consuming and expensive pathogenicity test. Additionally, the described protocols involved in the official testing of *Ralstonia solanacearum* (for detection and/or identification) require a lot of time, a high level of quarantine measures, and a high degree of expertise. The aim of this project was to develop, and evaluate molecular diagnostic methods for the detection and identification of *Ralstonia solanacearum* and verification of its virulence that would be faster, more specific and robust. Based on the current taxonomic insights, the high degree of heterogenity present inside the *Ralstonia solanacearum* species complex (RSSC) has resulted in the clear distinction of three new species inside this complex; the requirement defined in this Euphresco project was that the new molecular tests could be optimally implemented for the detection and identification of *Ralstonia solanacearum* and *Ralstonia pseudosolanacearum*, but not of *Ralstonia syzygii*.
The two main objectives of the project were:
1) To identify *Ralstonia solanacearum* virulence genes, and subsequently develop a PCR test on those virulence genes. The development of such a test could substitute the pathogenicity test required by EU directive 2006/63/EC for complete diagnosis of *Ralstonia solanacearum*.
2) To verify other molecular methods to detect or identify *Ralstonia solanacearum* strains. These methods include the real-time Loop-mediated isothermal amplication test (LAMP) and the recombinase-polymerase amplification assay (RPA).

2.2. Project aims and objectives

The bacterium *Ralstonia solanacearum* causes severe economic damage in a wide range of agriculturally important crops, amongst which are tomato, potato, anthurium, curcuma, and rose (Tjou-Tam-Sin *et al*., 2017). *Ralstonia solanacearum* is a heterogeneous species, which has been classified according to many different principles. The most recent phylogenetic research by Safni *et al*., 2014 and confirmed by Prior *et al*., 2016, has concluded, that the species complex consists of three distinct species: *Ralstonia solanacearum, Ralstonia pseudosolanacearum*, and *Ralstonia syzygii*. In this report, we will use the name *Ralstonia solanacearum* for the species complex, unless specifically mentioned otherwise. Additionally, the research on this project did not include *Ralstonia syzygii* as one of the targets.

Because of the serious threat to agriculture, *Ralstonia solanacearum* is regulated in many countries, including North America and the European Union. In the EU, the methods used to reliably detect and identify *Ralstonia solanacearum* are laid down in the EU directive 2006/63/EC. This document comprehensively describes which procedures must be followed, but these procedures often require a high level of expertise, a high level of quarantine measures, and are time consuming. Specifically, the need for isolation and a pathogenicity test in this procedure are a bottle neck for rapid identification. Hence, the project aimed to develop an alternative method to replace the pathogenicity test. In addition, other molecular methods were verified to increase the number of tools that can reliably detect or identify *Ralstonia solanacearum* in a rapid way.

As an alternative to the pathogenicity test, the first objective of the project was to develop and validate a PCR test based on *Ralstonia solanacearum* virulence genes. To this end, RNA sequence analysis of different *Ralstonia* genes was performed to determine expression of these genes during pathogenesis. In addition, genes previously described in literature to be

expressed during *Ralstonia* pathogenesis in potato were evaluated for their potential as targets for a pathogenicity test. In total, 7 candidate genes were identified, which are expressed during pathogenesis. However, of these seven genes, no candidate was present in all *Ralstonia solanacearum* and *Ralstonia pseudosolanacearum* genomes, but absent in *Ralstonia syzygii*. Additionally, the expression of these candidate genes was poor, making them unsuitable as targets for a pathogenicity test. Hence, the project partners were unsuccessful in developing a PCR on *Ralstonia solanacearum* virulence genes as alternative for the pathogenicity test.

The second objective of this project was to evaluate and validate a real-time Loop mediated isothermal amplification test (real-time LAMP) as an additional quick and easy-to-use method for the detection of *Ralstonia solanacearum*. At the project proposal stage, RPA was also considered as an alternative, but only real-time LAMP was investigated during the project. In total, four different DNA isolation protocols were tested for their performance in combination with the real-time LAMP test. The performance of real-time LAMP test in combination with different DNA isolation protocols was compared to the performance of three other tests currently used in the diagnosis of *Ralstonia solanacearum*: Immunofluorescence antibody staining (IFAS), conventional PCR and real-time PCR. The results showed that performance of real-time LAMP was comparable or even better than that of currently used IFAS, conventional PCR, and real-time PCR tests. Hence, real-time LAMP is a good alternative method compared to other molecular and serological techniques currently used for the diagnosis of *Ralstonia solanacearum*. As for DNA isolation protocols, most protocols seem to perform well for potato extract, but there were differences based on the matrix.

2.3. Description of the main activities

Within the scope of this project, two main activities were undertaken to develop and validate tools and protocols to decrease the time needed for the diagnosis of *Ralstonia solanacearum*. More specifically, the consortium tried to develop a PCR test on pathogenicity factor genes as a substitute for the time-consuming pathogenicity test. Additionally, a real-time LAMP test for the detection of *Ralstonia solanacearum* was evaluated. The abovementioned activities were made possible by the collection of a panel of test strains from participating countries.

To develop a PCR-based protocol, which could substitute the pathogenicity test, candidate genes are needed, that are expressed when the pathogen is infecting the plant. Hence, virulence factors are the ideal candidates as targets for such a test. Examples of *Ralstonia solanacearum* virulence factors are genes encoding inorganic nitrogen reduction, and genes involved in production of extracellular polysaccharides (Dalsing, 2015; Denny, 2006). Two criteria are relevant in the selection of these candidate genes: the targets need to be present across a broad range of *Ralstonia solanacearum sensu lato* strains, and the genes must be actively expressed during plant-pathogen interactions.

To identify suitable candidate genes for the detection of virulent strains of *Ralstonia solanacearum in vivo*, two *Ralstonia solanacearum* strains (rose isolate PD7123 = IPO4001 and potato isolate PD2763=IPO1828) were grown on Selective Medium South Africa (SMSA), and SMSA with added tomato juice agar or tomato leaf extract. The added tomato extracts would induce the production of virulence factors as previous studies by Meng *et al.,* 2015 demonstrated. Hence, comparison of the gene expression between the two media would identify genes induced in the presence of a host plant. From growth curves of these media, the best time for RNA extraction was determined, and subsequently RNA was extracted. RNA samples were sequenced by RNAseq analysis, and subsequently analyzed for differences in expression between the two media.

A second approach was to screen the genomes generated in the previous RNA expression experiment for the presence of candidate genes associated with virulence, as postulated by Ailloud *et al*. (2015). These authors identified 227 candidate genes. Among the prototype genomes for each phylotype, these candidates were screened for presence in phylotypes I, IIA, IIB and III and absence in phylotype IV. When suitable candidates were identified, primers and probes would have to be generated.

The second objective of the project was to develop and validate a real-time LAMP test for direct detection of *Ralstonia solanacearum*. To this aim, we compared the test sensitivity to standard serological Immunofluorescence Antibody Staining (IFAS) and different molecular methods (conventional PCR and RT-PCR). In addition, we also evaluated the efficiency of different DNA extraction protocols from different matrices (potato tuber, soil and irrigation water) artificially inoculated with the pathogen.

To test the efficiency of DNA extraction, four different protocols were evaluated: the boiling method at 96°C, the BioSprint 15 DNA Plant Kit, the QIAGEN DNeasy® Plant Mini Spin Columns Kit, and CTAB/Chloforom-Isoamyl Alcohol DNA extraction protocol. The pathogen was isolated from naturally infected potato tubers, grown on SMSA (Elphinstone *et al*., 1996) and used to inoculate the three matrices of interest. Potato extracts were artificially inoculated by serial dilutions of *R. solanacearum* from 10^7 to 10 cfu/ml. Samples were processed to isolate DNA, remove inhibitors and improve the sensitivity of detection techniques.

Detection of the bacterium was subsequently done using four different tests: IFAS test, conventional PCR, real-time PCR and real-time LAMP. IFAS test was carried out using polyclonal antibodies for *R. solanacearum* (Janse, 1988; Bellstedt and Van der Merwe, 1989) and the secondary antiserum conjugated with fluorescin isothyocyanate (Janse, 1988; Balabel, 2014). Conventional PCR was performed according to the protocol by Pastrik and Maiss, 2000, and analysed with gel electrophoresis. Real-time PCR reactions were performed according to the protocol by Weller *et al*., 2000. Real-time LAMP reactions were performed with primers according to the protocol by Kubota *et al*., 2008. The optimized reaction mixtures contained 1 µM of the inner primers FIP and BIP, 0.1 µM of the outer primers F3 and B3, 0.5 µM of the loop primers LF and LB and 3 µl template DNA. A volume of 30 µl of mineral oil was added into MicroAmp tubes, that contains LAMP mix to avoid contamination. LAMP amplifications were carried out at 65°C for a time of 40 min. Fluorescence readings were recorded using the channel optimized for fluorescein (FAM) every 1 min.

2.4. Main results

2.4.1. Development of a PCR method on *Ralstonia solanacearum* virulence genes

To determine which *Ralstonia solanacearum* genes have induced expression levels during pathogenesis, gene expression was compared between colonies grown on SMSA medium and colonies grown on SMSA medium enriched with tomato leaf extracts. Details of this novel procedure developed within this project are available in Sedighian *et al*., 2020. Subsequently, RNA samples were processed by the in-house sequencing facility at BioScience, Wageningen Plant Research. Sequence data received were analyzed for quantity and quality. From all growth conditions, large numbers of reads were obtained, ranging from 20 to 30 million and more than 90% passed the Q30 quality criterion. However, upon mapping of the reads to the genome of either of the isolates, a strange pattern was observed. Many of the mapped reads started or ended as fixed positions, suggesting a non-random distribution of reads in the data set. Subsequent analyses indicated that a large portion of the reads mapped to rRNA, indicating that the protocol for removal of rRNA before library construction was not successful.

As seen in Figure 1, the vast majority of reads mapped to one of the four copies of the rDNA. These reads are shown in yellow as they could have been mapped to any of the other three rRNA loci in the genome. As Figure 1 shows, only very few reads mapped to genes adjacent to this rRNA locus. This observation is representative for the entire genome.

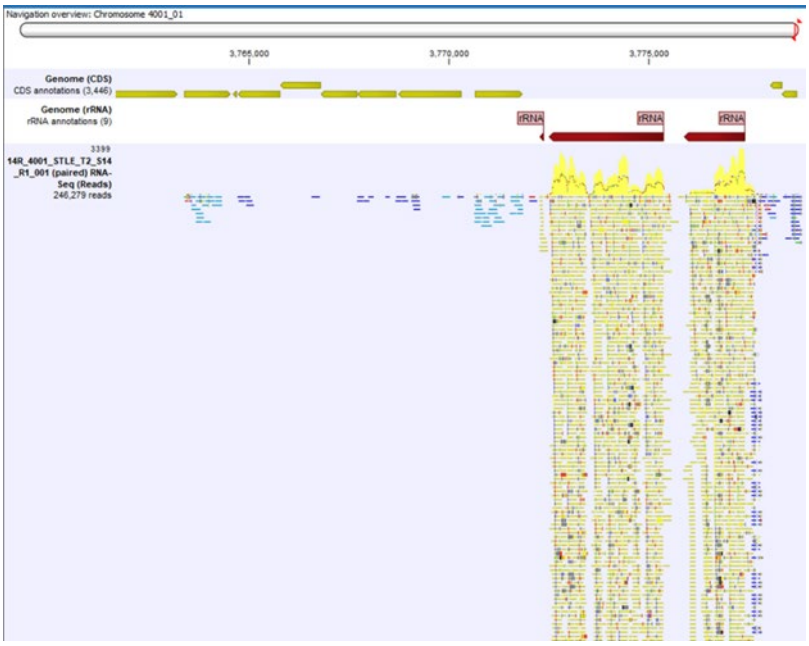

Fig. 1. *Biased mapping of reads to the 16S and 23S rDNA genes. Whereas many reads mapped to these two genes, only very few reads mapped to genes adjacent to these loci.*

The low coverage of reads across the genome did not allow to investigate whether there are genes that show differences in expression for the growth conditions used in the experiment.
An alternative approach was to screen the genomes generated in part A1 for the presence of candidate genes associated with virulence, as postulated by Ailloud *et al*. (2015). These authors identified 227 candidate genes. Among the prototype genomes for each phylotype, these candidates were screened for presence in phylotypes I, IIA, IIB and III and absence in phylotype IV.

Table 1. *Presence/absence of seven candidate genes that obey the rule that they are present in all phylotypes except phylotype IV. Annotation of candidates is shown in dark green and absence or presence for the different phylotypes is shown in red of light green, respectively.*

Family	Description	Phylotype I GMI1000	IIA CFBP2957	III CMR15	IIB Po82	IV PSI07
RipG4	F-box LRR protein GALA4					
RipO1						
RipQ						
RipS1	SKWP1					
RipAF1	Putative ADP-ribosyltransferase					
RipAP	Ankyrin Repeats					
RipAV						

Screening the 138 *Ralstonia* genomes (genomes generated in A1 along with several genomes obtained from the public domain) for these seven candidate effector genes led to the results depicted in the following (table 1):

- *ripAF1* is present in only a very few isolates (n=11) belonging to IIA a=or IIB;

- *ripO1* was found in some strains of I, most phylotype IIA and 75 out of 79 phylotype IIB strains;
- *ripG4* is present in one isolate of phylotype I, 7 out of 9 isolates of phylotype IIA and all isolates of phylotype IIB (n=79);
- *ripQ* is also present in all phylotype IIB strains and in some strains of phylotype I (n=1), phylotype IIA (6 of 9) and in all three strains of phylotype III;
- *ripAV* was detected in all phylotype I and phylotype III strains and the vast majority of IIA (6 of 9) and phylotype IIB strains (74 out of 79);
- *ripS1* was found in nearly all phylotype I, IIA, IIB and III strains, but also in half of the phylotype IV strains (3 of 6);
- *ripAP* was absent in all phylotype IV isolates but could not be detected in one isolate of phylotype I (15-021) and one isolate of phylotype IIA (strain K60).

Expression levels of all these candidates are presented in Table 2, showing some variation between the potato and the rose isolate. However, the research by Sedighian *et al.*, 2020, showed that RNA expression in the isolates used in this study varies over time. In general, the levels of expression were very low.

***Table 2**. Expression levels of candidate genes*

	IPO1828	IPO4001	1828 expression	4001 expression
RIP_S1	NGGAALNO_01311	FFGALPJL_00612	12.8	6.41
RIP_G4	(NGGAALNO_02614)???	FFGALPJL_02357	1.23	0.46
RIP_O1	NGGAALNO_01082	FFGALPJL_03601	3.60	5.15
RIP_AV	---	FFGALPJL_04020		1.16
RIP_AF1	---	FFGALPJL_04105		1.11
RIP_AP	NGGAALNO_00845	FFGALPJL_04501	0.66	2.14
RIP_Q	NGGAALNO_00889	FFGALPJL_04560	0.79	3.00

2.4.2. Evaluation of a real-time LAMP test

2.4.2.1. Detection of *R. solanacearum* in inoculated potato tubers by IFAS, PCR and LAMP

To evaluate the performance of a real-time LAMP test for the detection and identification of *Ralstonia solanacearum*, we compared its performance with IFAS, PCR and real-time PCR tests currently used in *Ralstonia solanacearum* identification. Additionally, 4 DNA extraction protocols were compared for their performance in combination with the molecular methods used in this study. The IFAS results showed no variation between different substances either for cell morphology or degree of fluorescence. Cells showed short rod-shaped morphology and were stained evenly as bright green fluorescent. The detection limit of the IFAS method was determined at 2.7×10^5 cfu/mL in crude samples. The detection limit of the molecular methods (conv. PCR, real-time PCR, and real-time LAMP) ranged between 2.7×10^3 and 2.7×10^6 cfu/mL.

Table 3 shows the results of the statistical analysis applied to the CT values obtained from real-time PCR analyses carried out on DNA samples using different extraction methods. The DNA was extracted from potato tubers artificially inoculated with the bacterium at concentrations between 10^5 and 10^7 cfu/mL.

***Table 3.** Results of least significant difference (LSD) test at P<0.05 applied to real-time PCR tests.*

Means± Standard deviation / t grouping				
Cell concentration (CFU/ml)	Crude (Boiling at 96°C)	BioSprint 15 DNA Plant Kit	QIAGEN DNeasy® Plant Mini Spin Columns Kit	CTAB
2.7×10^{7}	0	22.81±0.09 B	28.47±0.45 A	21.49±0.36 C
2.7×10^{6}	26.05±0.35 C	25.60±0.04 D	32.68±0.03 A	30.40±0.25 B
2.7×10^{5}	32.44±0.00 B	30.84±2.86 B	33.49±0.23 A, B	38.20±0.02 A

2.4.2.2. Detection of *R. solanacearum* in inoculated irrigation water

In samples of inoculated irrigation water, the IFAS test showed a detection limit of 5×10^{4} cfu/mL in crude samples. The PCR detection limit was 5×10^{5} cfu/mL using the boiling method at 96°C, 5×10^{4} cfu/mL using BioSprint 15 DNA Plant kit, and 5×10^{3} cfu/mL using QIAGEN DNeasy® Plant Mini Spin Columns kit and CTAB. Detection limit of real time PCR was 5×10^{6} cfu/mL using the boiling method at 96°C, and 5×10^{5} cfu/mL using BioSprint 15 DNA Plant kit. Sensitivity of real-time PCR for the detection of *R. solanacearum* in inoculated irrigation water was improved using QIAGEN DNeasy® Plant Mini Spin Columns kit and CTAB protocol with a higher detection limit of 5×10^{4} cfu/mL. Table 4 shows results of the statistical analysis applied to the CT values obtained from real-time PCR analyses carried out on DNA samples using the four different extraction methods. DNA extraction was from irrigation water artificially inoculated with the bacterium at concentrations between 10^{4} and 10^{6} CFU/ml.

The best sensitivity of real-time LAMP was achieved using BioSprint 15 DNA Plant kit and CTAB method with the detection limit equal to 5×10^{3} cfu/mL. The limit was equal to 5×10^{5} cfu/mL using boiling and 5×10^{4} cfu/mL using QIAGEN DNeasy® Plant Mini Spin Columns kit.

Table 4. *Results of least significant difference (LSD) test at P<0.05 applied to real-time PCR analysis*

Means± Standard deviation / t grouping				
Cell concentration (CFU/ml)	Crude (Boiling at 96°C)	BioSprint 15 DNA Plant Kit	QIAGEN DNeasy® Plant Mini Spin Columns Kit	CTAB
5×10^{6}	30.45±0.27 A	29.76±0.29 A	25.89±0.19 B	27.40±1.71 B
5×10^{5}	0	30.03±0.02 A	28.70±0.04 C	29.51±0.21B
5×10^{4}	0	0	35.24±1.09 A	33.97±0.18 A

2.4.2.3. Detection of *R. solanacearum* in inoculated clay soil

The IFAS test provided higher sensitivity for the detection of *R. solanacearum* in inoculated clay soil samples, with detection limit to 5×10^{6} cfu/g in crude samples. The CTAB method was the only DNA extraction method able to recover DNA from clay soil to be used in conventional and real-time amplification reactions which have shown the same degree of sensitivity with a level equal to 1,2 $x10^{6}$ cfu/g. Conversely, DNA extraction for the real-time LAMP test was carried out with all methods that showed a detection limit equal to 1.25×10^{4} cfu/g using BioSprint 15 DNA Plant kit, 1.25×10^{5} cfu/g using QIAGEN DNeasy® Plant Mini Spin Columns kit, and 1.25×10^{7} cfu/g using CTAB. No results were obtained using the boiling method.

2.4.3. Test Performance studies

Part of this project was the evaluation of the two tests, PCR on virulence genes and the real-time LAMP, amongst different laboratories to determine their performance. However, the TPS of the PCR protocol on *Ralstonia solanacearum* virulence genes was not carried out, as no suitable candidate genes were identified. Similarly, the TPS on the evaluation of the real-time

LAMP test was not carried out, as there was not enough time left after evaluation of the protocol to organize this TPS. Hence, no results were obtained from any of the test performance studies.

2.5. Conclusions and recommendations to policy makers

2.5.1. Development of a PCR on *Ralstonia* virulence genes

To identify genes related to virulence as targets for a molecular test, *Ralstonia solanacearum* strains were screened for the presence of virulence genes. In a first approach, differences in gene expression between strains grown on artificial medium and strains grown on virulence inducing medium were compared. However, RNAseq analysis revealed a non-random distribution of reads, rendering the data set unsuitable for further analysis. Moreover, both isolates reacted differently to the treatments. Potentially interesting candidates emerging from differential expression analyses were a cluster of genes present in the rose strain and absent in the potato strain. Unfortunately, this cluster showed was derived from a bacteriophage insertion into the genome of PD7123 as was shown by BLASTx.

In a second approach, reference genomes of *Ralstonia solanacearum* were screened for 229 genes previously identified by Ailloud *et al*., 2015, which were shown to be involved in pathogenesis. Among this set, there were seven candidate effectors, which were further evaluated as candidates for development of a PCR on virulence genes. However, none of the candidate effectors was shown to be present in *Ralstonia solanacearum* and *Ralstonia pseudosolanacearum*, but absent in *Ralstonia syzygii* strains. The *ripAP* gene seemed to be the best candidate, with absence in only one strain out of 41 *R. pseudosolanacearum* and one out of nine *R. solanacearum* IIA strains. In addition, effector candidate genes were poorly expressed, making them unsuitable as targets for a PCR on virulence genes. Hence, the results of the RNA screening of *Ralstonia solanacearum* genomes yielded no positive results. As no test was developed, no recommendation can be given out of this research line.

2.5.2. Validation of a real-time LAMP test

The present study has shown the remarkable potential of real-time LAMP in the detection of *R. solanacearum* from different matrices (potato tuber, irrigation water and clay soil). For potato tuber matrices, the most sensitive diagnostic test was the real-time LAMP using either the boiling method and DNA extraction by BioSprint 15 DNA Plant kit or CTAB protocol. The detection limit achieved by all these methods was 2.7×10^3 cfu/mL. The detection limit for conventional PCR, real-time PCR and IFAS was on average shown to be 100-fold lower compared to real-time LAMP.

For the matrix water, the most sensitive diagnostic tests with a detection limit equal to 5×10^3 cfu/mL, were the real-time LAMP with DNA extraction by BioSprint 15 DNA Plant kit and CTAB; and the PCR with DNA extraction by QIAGEN DNeasy® Plant Mini Spin Columns kit and CTAB. Both tests showed the lowest sensitivity using the Boiling method (5×10^5 cfu/mL). Conversely, the real-time PCR was the least sensitive with a detection limit equal to 5×10^4 cfu/mL (DNA extraction by QIAGEN DNeasy® Plant Mini Spin Columns kit and CTAB) and equal to 5×10^6 cfu/mL (using the boiling method).

Clay soils was the most difficult matrix for pathogen detection. The boiling method was not efficient for DNA extraction and amplification by the 3 molecular tests. As for the potato tuber matrix, real-time LAMP was the most sensitive test for detection of *R. solanacearum* in combination with DNA extraction by BioSprint 15 DNA Plant kit (1.25×10^4 cfu/mL). PCR and real-time PCR showed a lower sensitivity ($1,2\ x10^6$ cfu/mL) with the CTAB protocol as the only efficient DNA extraction protocol.

Overall, the real-time LAMP was shown to be the most sensitive diagnostic method in this study for the detection of *R. solanacearum* from potato tuber and soil matrices showing the detection limit between 2.7×10^3 and 1.25×10^4 cfu/mL, respectively. Moreover, it offers numerous advantages: it is rapid, cost-efficient and user-friendly. In addition, it is less influenced by inhibitors derived from culture medium, plant tissues and soil compared to PCR (Ebbinghaus *et al*., 2012), permitting less stringent DNA extraction procedures than those required for PCR and real-time PCR (Kiddle *et al*., 2012). This means, that the LAMP test could be a valuable tool for the testing of *Ralstonia solanacearum* and should be recommended in international standards. However, a specific technical protocol is recommended for each tested matrix. Hence, more research is needed to further investigate the use of LAMP, which should focus on improving the DNA extraction methods.

As for the DNA extraction methods, a difference was observed among the tested matrices: in potato tuber matrix, all DNA extraction methods were highly sensitive with the exception of the QIAGEN DNeasy® Plant Mini Spin Columns kit; in the irrigation water matrix, the best results were obtained with DNA extraction by BioSprint 15 DNA Plant kit or CTAB protocol in clay soil matrix, the most sensitive DNA extraction method was by BioSprint 15 DNA Plant kit. For the DNA extraction method by QIAGEN DNeasy® Plant Mini Spin Columns kit is not performing well in association with the real-time LAMP using all the tested matrices. The BioSprint 15 DNA Plant kit seems to give the best overall results for these matrices. However, further validation should be performed to conclusively decide, which methods are to be recommended for analysis.

The IFAS test was able to detect *R. solanacearum* in different matrices as crude samples and showed satisfactory efficiency and sensitivity. Additionally, it is high-throughput and less sophisticated. However, there are some drawbacks, such as the high time consumption, the use of more human resources, and the decreased specificity due to cross reactions resulting in false positive results. Hence, the IFAS test is still considered one of the most reliable tests for the detection of *Ralstonia solanacearum*, but other methods, such as real-time PCR and real-time LAMP should be considered as alternatives for addition to international standards.

2.6. Benefits from trans-national cooperation

The benefits from this international project are most notably the sharing of knowledge and resources between the different project partners.

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3. Publications

3.1. Article(s) for publication in the EPPO Bulletin

None.

3.2. Article for publication in the EPPO Reporting Service

None.

3.3. Article(s) for publication in other journals

- Sedighian N, Mendes O, Poleij L, Bonants P, & van der Wolf J (2020). Detection of Ralstonia pseudosolanacearum in drain water based on concentration, enrichment and the use of a duplex TaqMan PCR test. *EPPO Bulletin*.
- Additional publications by Van der Wolf *et al*. (PRI, NL) and Valentini *et al*. (CIHEAM-IAMB, IT) linked to this project are in preparation.

4. Open Euphresco data

None.