Final Report

RALSTO-ID
Determination of specificity and test performance study of current and novel molecular methods for *Ralstonia solanacearum* (brown rot of potatoes) and identification of methods allowing for detection of non-European strains of brown rot

Project Duration:

Start date:	01/05/2013
End date:	30/04/2015

1. Research Consortium Partners

Coordinator – Partner 1			
Organisation	National Reference Center, National Plant Protection Organisaton, the Netherlands		
Name of Contact (incl. Title)	Ir. Napoleon Tjou-Tam-Sin	**Gender**:	M
Job Title	Researcher		
Postal Address	P.O. Box 9102, 6700 HC; Wageningen; the Netherlands		
E-mail	n.tjou-tam-sin@nvwa.nl		
Phone	0031(0)651246794		

Partner 2			
Organisation	Institute of Agricultural and Fisheries Research		
Name of Contact (incl. Title)	Dr. Johan van Vaerenbergh	**Gender**:	M
Job Title	Researcher		
Postal Address	Burg. Van Gansberghelaan 96; 9820 Merelbeke; Belgium		
E-mail	johan.vanvaerenbergh@ilvo.vlaanderen.be		

Partner 3			
Organisation	Agence Nationale de Sécurité Sanitaire; Laboratoire de la santé des végétaux; Unité Ravageurs et Agent Pathogènes Tropicaux		
Name of Contact (incl. Title)	Dr. Gilles Cellier	**Gender**:	M
Job Title	Researcher		
Postal Address	7 chemin de l'Irat - Ligne Paradis; 97410 SAINT PIERRE; Ile de la Réunion		
E-mail	gilles.cellier@anses.fr		

Partner 4			
Organisation	National Institute of Biology		
Name of Contact (incl. Title)	Dr. Tanja Dreo	**Gender**:	F
Job Title	Researcher		
Postal Address	Vecna pot 111; 1000 Ljubljana; Slovenia		
E-mail	tanja.dreo@nib.si		

Partner 5			
Organisation	Instituto Valenciano de Investigaciones Agrarias, Apartado Oficial		
Name of Contact (incl. Title)	Dr. Maria Lopez	**Gender**:	F
Job Title	Researcher		
Postal Address	46113 Moncada Valencia; Spain		
E-mail	mlopez@ivia.es		

Partner 6			
Organisation	Plant Protection Central Research Institute		
Name of Contact (incl. Title)	Dr. Aynur Karahan	**Gender**:	F
Job Title	Researcher		
Postal Address	35040 Bornova; Izmir; Turkey		
E-mail	akarahan@zmmae.gov.tr		

Partner 7			
Organisation	Food and Environment Research Agency		
Name of Contact (incl. Title)	Dr. John Elphinstone	**Gender**:	M
Job Title	Researcher		
Postal Address	Sand Hutton, YO41 1LZ York, United Kingdom		
E-mail	john.elphinstone@fera.gsi.gov.uk		

2. Executive Summary

Within the EU Council Directive 2006/63/EC standard tests for detection and diagnosis of *Ralstonia solanacearum* (Rsol) are described. Based on the requirements of 2006/63/EC, samples found positive in the detection tests must be further tested for identity verification and for verification of their virulence by a pathogenicity test. This pathogenicity test must be performed on test plants within confined conditions (quarantine glasshouses), making the procedure time consuming and expensive.
If the pathogenicity of Rsol isolates could be confirmed by a molecular laboratory test targeting Rsol pathogenicity factors, the costs would be significantly reduced and the speed of testing should be enormously increased.

The main aim within this EUPHRESCO project was initially to develop and validate a molecular test, targeting pathogenicity factors, for the detection and identification of Rsol. After development and validation, the test would be further evaluated in a test performance study (TPS).
However, as no funding was granted for the main part of the project (namely the test development and validation), evaluation by a test performance study, was cancelled.

As an alternative, three smaller projects were initiated by project members, which address issues related to the Rsol test protocols. To avoid research duplication, different aspects of diagnostic method development and implementation were tackled by different project partners. The aspects studied were:

(1) Occurrence of intraspecific diversity of Rsol populations on local scale in the agro-ecological environment. Fundamental knowledge on diversity of Rsol was gained by characterizing Rsol populations in plant and water ecosystems in various geographic locations in Spain. Populations were characterized using several methods, namely: biochemical and metabolic profiling, serological methods and molecular methods. The genetic diversity showed to reflect the geographical origin within the country.

(2) Development of a new test method that covers a wide range of Rsol variants. The Rsol species taxon, known to possess a considerable variation, was proven to be a species complex, and is hence referred to as the "*Ralstonia solanacearum* species complex" (Rssc). In order to develop a method that covers 17 major groups of interest in the Rssc, in a single multiplex reaction, microarray technology (ArrayTube) was used. Validation data of the developed protocol showed strong intra-repeatability, inter-repeatability, and reproducibility as well as good specificity. A hierarchical analysis of the probe groups is suitable for an accurate characterization. Compared with single marker detection tests, the developed method efficiently combines several tests by testing large numbers of phylogenetic markers in a single reaction assay. This custom microarray (RsscAT) represents a significant improvement in the epidemiological monitoring of Rssc strains worldwide, and it has the potential to provide insights for phylogenetic incongruence of Rssc strains based on the host of isolation and may be used to detect emergent strains.

(3) To evaluate test options for the EU test protocols (as laid down in the EU Council Directive 2006/63/EC) for Rsol and *Clavibacter michiganensis* subsp. *sepedonicus* (Cms), the performance of real-time PCRs was compared with the performance of IF (Immuno-fluorescense). There was also a multiplex real-time PCR for detection of both Rsol and Cms included in this study. Performance data of the real-time PCR tests from this study together with the results from the previously performed EUPHRESCO inter-laboratory comparisons (Van Vaerenbergh *et al.*, 2017) form a profound basis to include real-time PCR as option in the EU Council Directives 2006/63/EC and 2006/56/EC for latent detection of Rsol and Cms. This test methodology has shown to be fast, sensitive and specific. The need for an update

of the EU council directives for detection of Rsol and Cms was pointed out to the EU standing committee. The addition of real-time PCR tests in both control directives will have great benefits for diagnostic laboratories, NPPOs and trade in terms of reliable and fast throughput in inspections and surveys.

The results from these projects are published in three papers: one on the diversity of Rsol in relation to geographic location (Caruso *et al*., *2017*), one on development of a tube-Wise Diagnostic Microarray for the Multiplex Characterization of Rsol *(*Cellier *et al*., 2017*)* and one on performance criteria of diagnostic methods for Rsol (Vreeburg *et al*., 2016). In addition, the results from earlier EUPHRESCO interlaboratory test comparison projects were recently published by Van Vaerenbergh *et al*. (2017), which confirm the results of this actual project topic published in the paper by Vreeburg *et al*., (2016).

3. Report

This report provides EUPHRESCO with an overview of the outputs of the RALSTO-ID project. More details are available in the three published papers on the different sub-projects (Caruso *et al.,* 2017; Cellier *et al.,* 2017; Vreeburg *et al.,* 2016).

Project aims

The main goal of this project was to improve efficiency and reliability of Rsol test protocols. Specific objectives were:

(1) to characterize Rsol populations in ecosystems and to gain fundamental knowledge on the diversity of the pathogen in relation to geographic location.

(2) to develop a diagnostic protocol for a wide variety of strains from the Rssc allowing the detection of the 17 major groups of interest in the Rssc, in a single multiplex reaction.

(3) to compare the performance of the real-time PCRs (for Rsol and Cms) with IF using a large number of routine potato tuber samples, including samples with proven infection with either Rsol or Cms.

Description of the main activities

Outbreaks of Rsol occasionally occur within EU/EPPO members states. These outbreaks can generally be related to two main infection routes, namely: (1) infections by a wide variety of strains from the *Ralstonia solanacearum* species complex (Rssc) imported into the EU/EPPO region via propagative plant material (e.g. cuttings, plants for planting), and originating from diverse areas with a warm climate; and (2) introductions of transient EU/EPPO populations of the Rsol strain adapted to temperate climatic zones (Rsol, phylotype II, Race 3, biovar 2) into production systems. Outbreaks of Rsol, phylotype II, Race 3, biovar 2 within countries of the EU/EPPO region have most often occurred within potato or tomato crops. Infections by a wide variety of strains from the Rssc have mostly occurred in ornamental crops, which are often grown in protected cultivation systems. Testing of plant material to prevent introductions via both infection routes require appropriate methods. Within this EUPRESCO project three studies were performed, namely:

(1) characterisation of Rsol populations within plant and water ecosystems in Spain. The following is highlighted: on different taxonomical levels, knowledge of methodology to assess diversity of Rsol populations is relevant. Therefore it is important to gain insight in levels of pathogen diversity within ecosystems. A study on the characterization of intraspecific diversity of Rsol populations in Spain was performed to gain more knowledge on the diversity of Rsol isolates. Isolates from different regions in Spain were phenotypically and genotypically analysed to determine their relatedness, using different methodology, such as indirect-ELISA, biochemical analysis, amplified fragment length polymorphism (AFLP) and sequence analysis.

(2) test development for the wide variety of strains from the Rssc. The following is highlighted: the complexity of the Rssc represents a challenge for the accurate characterization of epidemiological strains by official services and research laboratories. The majority of protocols focus only on a narrow range of strains. The main drawback associated with the current methods for detection and characterization of Rssc strains is their reliance on combining different protocols to properly characterize the strains at the ecotype level, which requires time and money. A standard protocol which characterizes multiple major groups of interest in the Rssc, in a single multiplex reaction, would bring much benefit in terms of efficiency.
Within this project we aimed to develop a protocol for detection of 17 important groups of the Rssc. Development of a multiplex detection test was based on the microarray technology by TubeWise.

(3) evaluation of test options for the EU Rsol and Cms test protocols. The following is highlighted: the EU Council Directives 2006/63/EC and 2006/56/EC, which amend the Annexes of Council Directives 98/57/EC and 93/85/EC on the control of Rsol and Cms

respectively, describe standard tests for detection of Rsol and Cms (Anonymous, 1993, 1998, 2006a and 2006b). These tests include an immunofluorescence (IF) test or conventional PCR as first screening tests. IF has proven itself as robust, cheap and reasonably specific. Although IF holds many advantages, this technique largely depends on high quality antisera and well trained personnel for examination of the slides with stained bacteria. Conventional PCR requires clean DNA and the visualisation of the amplification products on gel. Real-time (TaqMan®) PCR is considered to be a good alternative first screening method by many diagnosticians, being specific and sensitive, with less processing steps than conventional PCR but there was a lack of performance data to confirm that. Before using real-time PCR in official routine screening for potato brown rot and ringrot within EU member states, information was needed on the performance of these real-time PCR tests.
Within this study, the performance of different real-time PCR tests was compared to immunofluorescence tests currently used. The IF-tests for detecting Cms, or Rsol were performed according to the EU Council Directives 2006/56/EC and 2006/63/EC (Anonymous, 2006a and 2006b), respectively. Real-time PCRs used in this study to detect Cms and Rsol are those described by Schaad *et al*. (1999) and Weller *et al*. (2000), and real-time PCR adapted from Weller *et al*. (2000) respectively. Additionally, a multiplex real-time PCR for detection of both Rsol and Cms, described by Massart *et al*. (2014), was used.

For details on material and methods used we refer to the respective papers resulting from these studies, by Caruso *et al*. (2017), Cellier *et al*. (2017), and Vreeburg *et al*. (2016).

Results

Three independent studies were performed in this EUPHRESCO project to improve the efficiency and reliability of diagnostic protocols on Rsol. Here we list the main results of those three projects. For details on the results from the studies within this EUPHRESCO topic we refer to the respective papers on these studies, by Caruso *et al*. (2017), Cellier *et al*. (2017), and Vreeburg *et al*. (2016). The main findings were:

(1) The analysis of diversity of Rsol isolates in Spain showed low genetic variability among the Rsol strains. Phylogenetic analysis, AFLP analysis and rep-PCR showed low variability between strains. However, there is variation between different geographic regions within Spain, suggesting multiple introductions of Rsol isolates from distinct locations in Spain.
(2) The microarray was developed to evaluate diverse bacterial strains and represents an affordable, accurate, fast, and user-friendly diagnostic tool with a high potential for standardization and routine diagnostic testing use. Design of the microarray components resulted in a highly sensitive method that can be used to identify samples belonging to the 17 mayor epidemiological groups of Rsol. Only a few of the phylotype II strains belonging to the banana infecting moko group gave false positive results. This suggests that in general the microarray that was developed by Cellier *et al.,* (2017) is specific and sensitive enough to be used in diagnostics and/or characterization studies on the RSsc isolates.
(3) Comparison of different Real-time PCR methods with immunofluorescence showed that Real-time PCR is as sensitive as immunofluorescence. Real time PCR also has the added benefit of a lower dependence on highly trained personnel and specific, high quality antisera batches. However, it has to be mentioned that the sensitivity of a real-time PCR protocol also depends on the DNA extraction method used. In this study it was observed that using one specific DNA extraction protocol could increase the real-time PCR sensitivity by up to 100x as compared to another DNA extraction protocol.

Conclusions

(1) Genetic diversity of Rsol populations within plant and water ecosystems in Spain was showed to reflect the geographical origin within the country (Caruso, *et al*., 2017). It can be concluded that multiple introductions of the Rssc in Spain have taken place.
Some of the Spanish strains probably originate from import of contaminated potatoes from other European countries, whereas other strains might have a stronger link with potato samples from third countries. However, the analysed Spanish Rssc strains do not present a very high degree of genetic diversity either, certainly compared to the Andean countries where the potato and Rssc originates from.

(2) The test development for the detection of strains from the Rssc complex resulted in an efficient method using microarray technology to detect a wide variety of Rssc strains. Due to its strong intra-repeatability, inter-repeatability, and reproducibility as well as good specificity, this microarray is specific and sensitive enough to be used in diagnostics and/or characterization studies. Compared with single marker detection tests, the method described in the paper by Cellier *et al*. (2017) addresses efficiently the issue of combining several tests by testing a large number of phylogenetic markers in a single reaction assay. This custom microarray (RsscAT) represents a significant improvement in the epidemiological monitoring of Rssc strains worldwide, and it has the potential to provide insights for phylogenetic incongruence of Rssc strains based on the host of isolation and may be used to indicate potentially emergent strains.

(3) The study on test options for the EU testing protocols shows that real-time PCR is a good addition for routine screening of potato tubers for (latent) infections of Rsol and Cms. Also results published by Van Vaerenbergh *et al*. (2017) reporting Euphresco inter-laboratory comparisons (2009–2012) on detection of Rsol and Cms, support the conclusion that inclusion of real-time PCR as detection method for Rsol and Cms in the EU regulations is strongly advisable. This test methodology has shown to be a fast, sensitive and specific technique. There are more real-time PCRs for Rsol and Cms available besides the real-time PCRs used in this study. Real-time PCRs for Rsol have been designed to be either generic for multiple phylotypes, like the real-time PCR of Weller *et al*. (2000) used in this study, or specific for only a subset of the different phylotypes or biovars. A generic PCR will be advantageous in the light of the international trade of potatoes and other potentially Rsol bearing plants and plant products. To exploit the benefits of real-time PCR testing for Rsol and Cms, the need was pointed out to the EU standing committee to update the EU council directives for detection of Rsol and Cms. When the concerning control directives are supplemented with real-time PCR tests it will bring great benefits for diagnostic laboratories, NPPOs and trade in terms of reliable and fast throughput in inspections and surveys.

Recommendations

Concerning follow-up research, the following is suggested: within the diagnostic test procedure, samples found positive in the detection tests must be confirmed by identity verification tests and a pathogenicity test. This pathogenicity test causes high costs because of the needed facilities. Additionally, it requires a long throughput time. To reduce necessity of costly services and to speed-up the test process even more after inclusion of real-time PCR tests it would mean a great progress if the pathogenicity of Rsol could be confirmed by a laboratory test which targets genetic pathogenicity factors of the pathogen as was originally planned for this topic. A new initiative on this topic is already initiated within the EUPHRESCO network.

Furthermore, upon proven fit for purpose based on performance data, the real-time PCR methods should be brought to the attention of the EU Standing Committee, as suitable options to be included in the EU Council Directives for the detection of Rsol and Cms. However, inclusion of these tests in the respective EU Council Directives is not a goal to be achieved within the span of this project.

Publications

Caruso, P., Biosca, E.G., Bertolini, E., Marco-Noales, E., Gorris, M.T., Licciardello, C. and López, M.M. (2017) Genetic diversity reflects geographical origin of *Ralstonia solanacearum* strains isolated from plant and water sources in Spain. International Microbiology 20(4):155-164.

Cellier, G., Arribat, S., Chiroleu, F., Prior, P., and Robène, I. (2017). Tube-wise diagnostic microarray for the multiplex characterization of the complex plant pathogen Ralstonia solanacearum. Frontiers in Plant Science 8, 821.

Vreeburg, R.A.M., Bergsma-Vlami, M., Bollema, R.M., de Haan E.G., Kooman-Gersmann, M., Smits-Mastebroek, L., Tameling, W.I.L., Tjou-Tam-Sin N.N.A., van de Vossenberg, B.T.L.H. and Janse, J.D. (2016). Performance of real-time PCR and immunofluorescence for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* and *Ralstonia solanacearum* in potato tubers in routine testing. EPPO Bulletin 46, 112-121.

Acknowledgements

The test performance study was supported by the Ministry of Economic Affairs, Netherlands Food and Consumer Product Safety Authority [grant number OS2012208]. Further we acknowledge Mr Bram de Hoop, Delegation Leader of the Netherlands at SCOPAFF – Plant Health, who pointed-out to the EU standing committee, the need to update the EU council directives for detection of Rsol and Cms based on the results of the study performed in this project.

References

- Anonymous (1993) Council directive 93/85/EC. Official Journal of the European Communities L-259, 1-25.
- Anonymous (1998) Council directive 98/63/EC. Official Journal of the European Communities L-235, 1-39.
- Anonymous (2006a) Council directive 2006/56/EC. Official Journal of the European Communities L-182, 1-43.
- Anonymous (2006b) Council directive 2006/63/EC. Official Journal of the European Communities L-206, 1-106.
- Caruso, P., Biosca, E.G., Bertolini, E., Marco-Noales, E., Gorris, M.T., Licciardello, C. and López, M.M. (2017) Genetic diversity reflects geographical origin of *Ralstonia solanacearum* strains isolated from plant and water sources in Spain. International Microbiology 20(4):155-164.
- Cellier, G., Arribat, S., Chiroleu, F., Prior, P., and Robène, I. (2017). Tube-wise diagnostic microarray for the multiplex characterization of the complex plant pathogen Ralstonia solanacearum. Frontiers in Plant Science 8, 821.
- Massart S, Nagy C & Jijakli MH (2014) Development of the simultaneous detection of *Ralstonia solanacearum* race 3 and *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers by a multiplex real-time PCR assay. European Journal of Plant Pathology 138, 29-37.
- Schaad NW, Berthier-Schaad Y, Sechler A & Knorr D (1999) Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers by BIO-PCR and an automated real-time fluorescence detection system. Plant Disease 83, 1092-1100.

- Van Vaerenbergh J, Müller P, Elphinstone JG, Vreeburg RAM & Janse JD (2017) Euphresco inter-laboratory comparison (2009–2012) on detection of *Clavibacter michiganensis* subsp. *sepedonicus* and *Ralstonia solanacearum* in potato tubers: proposal to include TaqMan real-time PCR as a primary (core) screening test in EU/EPPO standard methods. EPPO bulletin 47, 24-32.
- Vreeburg, R.A.M., Bergsma-Vlami, M., Bollema, R.M., de Haan E.G., Kooman-Gersmann, M., Smits-Mastebroek, L., Tameling, W.I.L., Tjou-Tam-Sin N.N.A., van de Vossenberg, B.T.L.H. and Janse, J.D. (2016). Performance of real-time PCR and immunofluorescence for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* and *Ralstonia solanacearum* in potato tubers in routine testing. EPPO Bulletin 46, 112-121.
- Weller SA, Elphinstone JG, Smith NC, Boonham N & Stead DE (2000) Detection of *Ralstonia solanacearum* strains with a quantitative, mulitplex, real-time, fluorogenic PCR (TaqMan) assay. Applied and Environmental Microbiology 66, 2853-2858.

Appendices
Frequently used abbreviations:

Cms = *Clavibacter michiganensis* subsp. *sepedonicus*

Rsol = *Ralstonia solanacearum*

Rssc = *Ralstonia solanacearum* species complex.