EUPHRESCO Final Report

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Project Title (Acronym)
Development of pathotype-specific PCR tests for the identification of *Synchytrium endobioticum* pathotype 1 (SENDO D1)

Project Duration:

Start date:	01/01/2013
End date:	01/05/2014

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2. Executive Summary

Project Summary

Title:
Development of pathotype-specific PCR tests for the identification of Synchytrium endobioticum pathotype 1 (SENDO D1)

Summary:
Synchytrium endobioticum is a severe pathogen of potato causing wart disease. For this obligate fungus many pathotypes (races) exist, which are pathogenic or non-pathogenic to different cultivars of potato. To determine to which race an isolate belongs, two biological assays are used on a set of different potato cultivars: the Spieckermann and the Glynne-Lemmerzahl test. A differential set of cultivars has been recommended by EPPO (European Plant Protection Organization) for these tests. Drawbacks of these tests are that it can take up to several weeks to score the interaction and results are difficult to score and also often not conclusive. Therefore possibilities were investigated to look for molecular markers for race specificity.
Goal of the project was to develop an appropriate Taqman PCR protocol to reliably distinguish pathotype 1 from other pathotypes of *Synchytrium endobioticum*.

Objectives:
• To develop a Taqman PCR technique with acceptable specificity and reproducibility to distinguish *S. endobioticum* pathotype 1(D1) from the 'higher' pathotypes.
• To identify *S. endobioticum* pathotypes in reference collections of winter spores collected from green or composted ware material throughout Europe.

Results:
Eight isolates were analysed for looking for sequence differences using CRoPS.
Using the obtained sequence differences two molecular TaqMan PCR assays (P1 and P3) have been developed to distinguish:
P1: between pathotype 1 and non-pathotype 1 isolates of the pathogen and
P3: between pathotype 1 / 18 and pathotype 2 / 6
DNA of many isolates across Europe have been analysed. With the P1 assay all non-pathotype 1 isolates were correctly scored with the molecular TaqMan PCR method P1. Some pathotype 1 isolates were scored as non-pathotype 1.

Conclusions:
A molecular TaqMan PCR assay has been developed and validated. Several wart disease samples have been analysed. Non-pathotype 1 samples could be scored by the molecular assay as non-pathotype 1. Pathotype 1 samples were scored as pathotype 1 and some of them (isolates from the UK) were scored as non-pathotype 1. This means that there is sequence variation with the pathotype 1 population.

3. Report

Background

Potato wart disease (*Synchytrium endobioticum*) is an obligate pathogen that has been successfully controlled for a long period of time through regulation. The success of this regulatory control has been due to a policy of scheduling infested land, which can then not grow potatoes, and the availability and widespread use of cultivars resistant to the common European pathotype (Pathotype-1(D1)). More recently, EPPO descheduling protocols[1] have been developed that can be used to fully or partially deschedule land, primarily based on the length of time since the outbreak and testing soil for viable winter sporangia (direct examination and/or a bioassay using a highly susceptible cultivar). See also: EPPO Diagnostic Protocol for *Synchytrium endobioticum*.

Pathotype-1(D1) is the common pathotype found in Europe, but additional pathotypes previously restricted to parts of Eastern Europe have been spreading. The most important pathotypes in the EPPO region are 1(D1), 2(G1), 6(O1), 8(F1) and 18(T1). These can be differentiated using differential potato cultivars, as outlined in the EPPO Protocol. Since late 2008 there has been discussion about the validity of the Table used to identify pathotypes presented in the EPPO Diagnostic protocol (EPPO, 2008). More research/studies, including ring tests, are necessary here.

All the traditional diagnostic methods using bioassays are time consuming and labour intensive. Improved and more efficient, effective and harmonised approaches are highly desirable. Disadvantages of these bio-assays are:

- tester strains of *S. endobioticum* are not uniformly characterized throughout EU or are sparsely available
- loss of purity and vigour of differentials (mainly old cultivars are used)
- inconsistency between Spieckermann vs Lemmerzahl bioassays
- time-consuming and lengthy bio-assays for pathotype identification (2-3 months), which may first require an additional step if the wart material needs to be multiplied to ensure sufficient material is available for the tests (which may make the whole test over a year long).

<u>Pathotype identification:</u>

At the moment, different countries use a) different methods, and b) different potato cultivars to identify pathotypes in *S. endobioticum.* This obstructs clear communication, and might aggravate management control actions, once a new outbreak is identified in one of the countries. It would be desirable to introduce/implement one method for pathotyping with a validated set of differential cultivars. Additionally, the traditional method for pathotype identification using differential cultivars is very time consuming, labour intensive, expensive and takes a long time. Rapid and efficient molecular methods would be beneficial if developed and validated. In a project financed by the Dutch Ministry of Agriculture during 2007-2009, scientists have looked for genomic markers in *S. endobioticum* in order to distinguish pathotypes in PCR tests. From this project consistent SNP-markers (Single Nucleotide Polymorphism) were selected and successfully tested for

[1] OEPP/EPPO (1999) EPPO Standards PM 3/59 *Synchytrium endobioticum*: soil tests and descheduling of previously infested plots. *Bulletin OEPP/EPPO Bulletin* **29**, 225–231

pathotype specific identification on a standard tester set. A Taqman PCR test has being developed for routine identification purposes of pathotype 1(D1) of *S. endobioticum*. To ascertain specificity, reproducibility and selectivity of the method, a ring test on a well-known tester set and a diverse set of biologically well-characterized field isolates from different pathotype identity and geographical origin has been performed within another Euphresco project and lead by the dutch PPS.
This ringtest is not reported within this report.

Objectives and tasks of the project

• To develop a Taqman PCR technique with acceptable specificity and reproducibility to distinguish *S. endobioticum* pathotype 1(D1) from the 'higher' pathotypes.
• To identify *S. endobioticum* pathotypes in reference collections of winter spores collected from green or composted ware material throughout Europe.

Methods used and Results obtained

- **Materials and Methods**

Material has been collected by the Dutch Plant Protection Service and via the Euphresco network. Winterspores were collected from wart material and from compost. Two different DNA extraction procedures have been developed. The protocol can be viewed in detail on You Tube: http://www.youtube.com/watch?v=dZxqv8_6S9E
DNA extracts of 8 isolates were selected and sent to Keygene BV in Wageningen (The Netherlands) for CRoPS (Complexity reduction of Polymorphic Sequences) analysis. Whole Genome Amplification (WGA) was performed to amplify the genomic DNA extracted from winterspores. PCR and TaqMan PCRs were developed based upon obtained sequence differences between the different pathotypes.

- **Results**

Using the CRoPS approach In total 25 Mb of sequence information resulted in 243.440 good sequences for *S. endobioticum* with an average length of 133 bp. The results of the CRoPS analysis show that the number of sequences varied between 16.760 and 47.920 for the different SE isolates. The 243.440 sequences could be assembled to 14.660 contigs of which 85 (0.6%) contained one or more SNP with the criteria used. After removal of contigs derived from repetitive sequences and selection for a correlation with the race specificity, 9 informative loci remained which correlated with pathotype 1 (D1). PCR primers for these 9 loci were developed and tested on a large set of samples. All gave good amplification of these loci.
For two of 9 informative loci TaqMan PCR (P1 and P3) have been designed:
P1: to discriminate between pathotype -1 and non- pathotype 1 isolates.
P3: to discriminate between pathotype 1 / 18 and pathotype 2 / 6
DNA samples of many isolates have been tested with these two TaqMan PCRs with good results..

Testing the primers-probe set P1 on more isolates showed that the TaqMan PCR P1 discriminates clearly between pathotype -1(D1) and non pathotype -1(D1) isolates of *S. endobioticum*. There were some discrepancies in the scoring. Isolate SE8 was scored as non- pathotype 1. Biological scoring for that isolate was not performed in

the UK. Additional DNA samples were obtained from SASA (United Kingdom) collaborating in the Euphresco network on wart disease. TaqMan PCR for *Synchytrium endobioticum* and TaqMan PCR P1 was performed. Biological scoring of these isolates indicated that they all isolates were pathotype 1(D1). Sample WS1979 did not provide a TaqMan PCR scoring, possibly because there was not sufficient DNA for amplification or due to PCR inhibition.
The second TaqMan PCR test (set P3) was tested on a set of wart isolates. It showed that the TaqMan PCR P3 discriminates clearly between pathotype 1/18 and pathotype 2/6 isolates of *S. endobioticum*.
The developed TaqMan PCR P1 method has been evaluated in a ringtest within another part of Euphresco project (Bart vd Vossenberg et al.).

Discussion of results and their reliability

Based on the CRoPS results of the restricted set of 8 isolates, 9 potential SNPs were found (P1-P9), which could be potentially pathotype specific. Two candidate SNP sites (P1 and P3) were converted into TaqMan PCR assays. TaqMan PCR P1 discriminates between pathotype 1(D1) and non- pathotype 1(D1) isolates of *S. endobioticum* and TaqMan PCR P3 discriminates between pathotype 1/18 vs pathotype 2/6. Both markers could subsequently be tested on non-pure DNA of a larger collection of isolates using *S. endobioticum*-specific PCR primers. In combination with a whole genome amplification step, the specific SNP markers could be screened using only 25 winterspores and even in the presence of contaminating DNA. Also DNA isolated directly from wart material could be analysed with the TaqMan PCR P1 test and pathotype identity (pathotype 1(D1) or non- pathotype 1(D1)) could be determined. A slightly modified version of the TaqMan PCR P1 (mainly involving changes in the labelling dyes for the probes has been successfully validated and implemented at HLB laboratory in Wijster, The Netherlands. The test showed a 100% correlation with the bioassays from Dutch and German isolates. In analysing more DNA samples of potato wart material from different regions in Europe it appeared that the TaqMan P1 PCR test found that all isolates that were characterized as non- pathotype 1(D1) were correctly identified but that some pathotype 1(D1) isolates showed the non- pathotype 1(D1) TaqMan profile. This indicates that within the pathotype 1(D1) population more genetic variation exists. As our markers are based on association only (identity by descent) and not based on functional polymorphism, for instance in an avirulence gene, this is likely to occur. We therefore suggest the use of multiple markers for the molecular scoring and a thorough evaluation of the regional diversity before applying the molecular test. For Dutch and German isolates all isolates were characterized correctly for isolates that show the non- pathotype 1(D1) profile we recommend to use cultivars that differentiate between pathotype 1(D1) to confirm the result of the molecular test.
More research is needed to unravel the genetic structure of this obligate fungus. Next Generation Sequence technology may play an interesting role to find more SNPs and define the genetic relatedness between all isolates of *S. endobioticum*. To find pathotype specific markers, the biological scoring of the pathotypes is still a problem, since a differential set of potato cultivars has not been agreed on yet.
Only the difference between pathotype 1(D1) and non- pathotype 1(D1) isolates can easily be distinguished in using cv. Deodara, Tomensa or Eersteling (Anon, EPPO 2004) as a differential.

Main conclusions

The TaqMan PCR P1 assay allows direct screening of samples obtained during quarantine screening, without the need for a labor intensive, time-consuming and costly propagation of the pathogen at an appropriate biosafety facility, and are useful tools for the screening of larger collections of *S. endobioticum* samples in future research.

Papers, other publications and dissemination activities

Publications:

Peter J.M. Bonants, Marga P.E. van Gent-Pelzer, Gerard C.M. van Leeuwen and Theo A.J. van der Lee. A real-time TaqMan PCR assay to discriminate between race 1 (D1) and non-race-1 (D1) isolates of *Synchytrium endobioticum*. Submitted.

Presentations:

Peter Bonants, Marga van Gent and Theo van der Lee. Detection and identification of *Synchytrium endobioticum*, causal agent of wart disease in potato. EPPO workshop on wart disease. Moscow Russia, 14 February 2012.

Peter Bonants, Marga van Gent and Theo van der Lee. Detection and identification of *Synchytrium endobioticum,* causal agent of wart disease in potato
Euphresco meeting wart disease St Petersburg, 3 October 2013

Marga van Gent-Pelzer Theo van der Lee, and Peter Bonants. *Synchytrium endobioticum*; SNP's mining on data generated till now. Euphresco meeting wart disease Edinburgh, 16 April 2014

Theo van der Lee, Marga van Gent-Pelzer, Henri van der Geest and Peter Bonants. Assembly of the *Synchytrium endobioticum* genome. Euphresco meeting wart disease Edinburgh, 16 April 2014

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EPPO diagnostic Protocol for *Synchytrium endobioticum:* *http://www.eppo.org/QUARANTINE/fungi/Synchytrium_endobioticum/pm7-28(1)%20SYNCEN%20web.pdf*

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Van Leeuwen GCM, Wander JGN, Lamers J, Meffert JP, Van den Boogert PHJF, and Baayen RP, 2005. Direct examination of soil for sporangia of *Synchytrium endobioticum* using chloroform, calcium chloride and zinc sulphate as extraction reagents. *Bulletin OEPP/EPPO Bulletin* **35**, 25–31.

Appendices

Publication of this report is allowed after publication of the submitted paper.