Eur J Plant Pathol (2010) 126:129–133
DOI 10.1007/s10658-009-9522-3

Improved real-time PCR assay for detection of the quarantine potato pathogen, *Synchytrium endobioticum*, in zonal centrifuge extracts from soil and in plants

Marga P. E. van Gent-Pelzer · Marjon Krijger · Peter J. M. Bonants

Received: 14 January 2009 / Accepted: 9 July 2009 / Published online: 28 July 2009

Abstract Real-time PCR was used for quantitative detection of the potato pathogen, *Synchytrium endobioticum*, in different substrates: zonal centrifuge extracts, warts and different plant parts of potato. Specific primers and a TaqMan probe, designed from the internal transcribed spacer region of the multi-copy rDNA gene were tested in extracts from artificially and naturally infested soil. Co-amplification of target DNA along with an internal competitor DNA fragment made the diagnostic assay more reliable by guarding against false negative results. A calibrations curve was created by spiking zonal centrifuge fractions of clean soil samples with a dilution series of winter spores. The Taqman assay was also performed on infected potato plant material (stolons) along with the detection of the cytochrome oxidase gene as a potato endogenous control. Sensitivity of the TaqMan assay was improved at least 100-fold and proved to be reliable for accurate diagnosis of the disease.

Keywords Detection · Identification · Potato wart disease · TaqMan PCR

M. P. E. van Gent-Pelzer · M. Krijger · P. J. M. Bonants (✉)
Plant Research International BV,
P.O. Box 69, 6700 AB Wageningen, The Netherlands
e-mail: peter.bonants@wur.nl

The chytridiomycete, *Synchytrium endobioticum*, is a plant pathogenic fungus that causes serious disease in potato in cool, moist climates world-wide. The fungus exhibits an obligate bio-interaction with plant tissue via zoospores for infection and winter spores for survival. Upon infection, the fungus induces its host to produce tumour-like outgrowth (warts) on tubers, stolons and sprouts. Warts consist of hypertrophic host cells each containing a single sporangium, which host the infectious zoospores (Hampson 1985; Hampson *et al*. 1994). In senescing wart tissue infectious zoospores are no longer produced and thick-walled resting sporangia or winter spores are formed and able to survive inter-host periods for up to 20 years (Hampson 1993). Regulations have been in force throughout the world to prevent inter-field spread of the fungus. According to the European and Mediterranean Plant Protection Organization (EPPO) infected fields should be scheduled for 20 years (EPPO) or longer if soil tests disclose the presence of *S. endobioticum.* In the European Union (EU), potato production is prohibited until the presence of the disease can no longer be demonstrated. Various methods have been developed for direct enumeration of winter spores in soil (Pratt 1976; Laidlaw 1985; Zelya and Melnik 1998) and adapted to the requirements of EPPO for routine use (Van Leeuwen *et al*. 2005). Basically, the methods are directed to separate winter spores from soil particles with a higher specific weight using sieving and subsequent centrifugation

techniques (Pratt 1976; van Leeuwen *et al*. 2005). The resulting extracts are subject to microscopic counting procedures (reviewed by Van Leeuwen *et al*. 2005). A zonal centrifuge (Hendrickx 1995) has become available for routine extraction of nematode eggs from soil samples. This technique has been adapted and further modified for routine winter spore extraction from 100 g soil samples as a possible alternative for the sieve-centrifugation method presently in use (Wander *et al*. 2007).

The ITS regions of the multi copy rDNA gene of *S. endobioticum* have been sequenced from which specific PCR primers and probes have been identified (Lévesque *et al*. 2001, 2002; Niepold and Stachewicz 2004; van den Boogert *et al*. 2005). The 18S region of rDNA has also been used to develop a detection method for *S. endobioticum* (Abdullahi *et al*. 2005). These developments in soil extraction and PCR technology were both a motivation to develop and validate a more sensitive and specific detection method for winter spores. In this study we used TaqMan PCR technology to develop a quantitative detection method for *S. endobioticum* winter spores in soil and plant extracts.

Genomic DNA of *S. endobioticum* was isolated from sporangia in fresh wart tissue using $CsCl_2$ (specific weight 1.4) centrifugation and the Ultra Clean Soil DNA extraction kit (UC kit; MoBio Laboratories Carlsbad, USA). DNA extracts directly from warts (75 mg fresh weight) were prepared using the Wizard® Magnetic DNA Purification System for Food (Promega, Southampton, UK). Positive TaqMan results showed the correct identification of *S. endobioticum* in the wart tissue (data not shown).

In another experiment a dilution series of winter spores was added to Zonal Centrifuge (ZC) fractions of uninfested soil (Vredepeel) obtained from PPO-AGV (Lelystad, The Netherlands). Winter spores (pathotype 2(G1)) were obtained from the collection of the Plant Protection Service, Wageningen, the Netherlands. Winter spores (0, 1, 5, 10 and 50) were individually transferred in small amounts of water (max. 10 µl) to clean ZC fractions (Wander *et al.* 2007) with a pipette under a stereomicroscope. Higher numbers of winter spores (100, 500 and 1000) were taken from a stock solution (5 winter spores μl^{-1}), the concentration of which was determined using high-density sporangial suspensions counted in disposable Glasstic slides with a 0.33 mm^2 grid (Hycor Biomedical Inc. Edinburgh, UK). Aliquots of 0.2 ml ZC soil extracts containing winter spores were transferred to a 2 ml beadbeat tube and DNA extraction was performed as described in van den Boogert *et al.* (2005).

Stolons and tubers of five different potato cultivars, grown for 12 weeks in pots with *S. endobioticum* (2500 ws kg^{-1}) infested soil, were visually scored for wart disease symptoms. Subsequently, stolons with and without wart symptoms were surface-sterilised before grinding under liquid nitrogen using a mortar and pestle. About 200–500 mg of the resulting powder was used for DNA extraction in quadruplicate using the Wizard® Magnetic 96 DNA Plant System kit (Promega) according to instructions of the manufacturer.

Table 1 Primers/probes used in this study

Synchytrium endobioticum (Sendo)	
Primer SendoITS2F:	TTTTTACGCTCACTTTTTTTAGAATGTT
Primer SendoITS2R:	TCTGCCTCACACACCACATACA
Sendo probe 2:	**FAM**-AATTCGAGTTTGTCAAAAGGTGTTTGTTGTGG-**Eclipse Darkquencher**
Internal Amplification Control (IAC) (Klerks *et al*. 2004)	
Primer FIAC:	TGGCCCTGTCCTTTTACCAG
Primer RIAC:	TTTTCGTTGGGATCTTTCGAA
IAC Probe pIAC:	**VIC** -AACCATTACCTGTCCACACAATCTGCCC-**Eclipse Darkquencher**
Internal Plant Control (IPC) (Weller *et al*. 2000)	
Primer COX F:	CGTCGCATTCCAGATTATCCA
Primer COX RW:	CAACTACGGATATATAAGRRCCRRAACTG
Probe COXSOL1511T:	**Yakima-Yellow**-AGGGCATTCCATCCAGCGTAAGCA-**Black Hole Quencher 1**

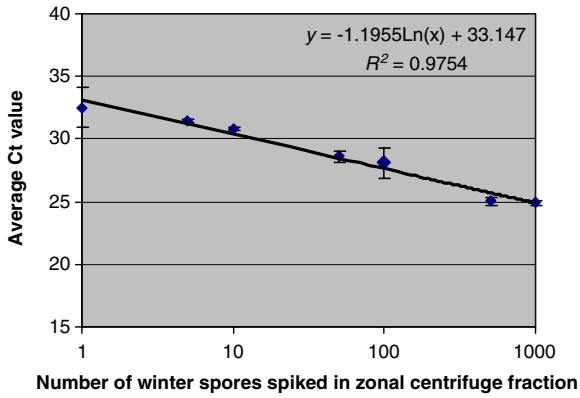

Fig. 1 TaqMan PCR calibration curve of a dilution series of *S. endobioticum* winter spores added to zonal centrifuge fractions of non-infested soil samples in triplicate

Forward and reverse primers and a FAM-labelled probe (Table 1) were designed to unique rDNA regions of the ITS2 sequence using Primer Express software (Applied Biosystems, Branchburg, NJ, USA). The real-time PCR used amplification components from a PCR kit, Premix Ex Taq (Takara Bio Europe SAS, Saint-Germain-en-Laye, France) and was carried out in optical plates/tubes and caps (Applied Biosystems, Foster City, U.S.A.). Each 30 µl PCR reaction contained 1x Premix Ex Taq and 0.6 µl ROX reference Dye (Takara), 250 nM of each SendoITS2F and SendoITS2R primer, 83.3 nM SendoTaqMan probe final concentration and 2% (1 or 2 µl) sample of the DNA template. Internal amplification control (IAC) primers (FIAC and RIAC) and probe (pIAC) were added to each PCR reaction to check amplification for inhibition and false negative results according to Klerks *et al*. (2004). The target DNA (12.5 fg) was a green fluorescent protein (gfp) construct in *Escherichia coli* containing plasmid DNA and genomic *E. coli* DNA. The PCR conditions were: a hot start for 10 min at 95°C, 40 cycles of 2-step amplification (15 s 95°C; 60 s 60°C). The real-time quantitative PCR was performed in an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, USA). The internal plant control (IPC) primers (COX F and COX RW) and probe (COXSOL1511T probe) were based on a previously described assay designed for the cytochrome oxidase 1 (COX) gene (Weller *et al*. 2000). The COX gene assay was carried

Table 2 TaqMan PCR detection on DNA extracts (in quadruplicate) from stolons of potato plants infected with *S. endobioticum*

Sample	Cultivar	Ct Sendo	Ct IPC	Ct IAC	Visual symptoms on tubers/stolons
V64-1	Russet Burbank	nd	20.54	33.62	No warts
V64-2		nd	21.01	nt	
V64-3		nd	20.86	nt	
V64-4		nd	20.44	nt	
V81-1	Karakter	nd	16.71	33.78	No warts
V81-2		38.78	nd	nt	
V81-3		nd	16.06	nt	
V81-4		nd	16.62	nt	
V76-1	Festien	27.39	16.34	33.97	No warts
V76-2		21.87	16.62	nt	
V76-3		23.12	16.73	nt	
V76-4		22.17	16.74	nt	
62-1	Fontane	21.94	17.74	32.87	Warts
62-2		21.18	17.50	nt	
62-3		22.39	18.24	nt	
62-4		20.22	16.73	nt	
V38-1	Maritiema	nd	16.72	35.05	Warts
V38-2		20.73	16.96	nt	
V38-3		27.42	17.14	nt	
V38-4		35.37	19.60	nt	
Neg C		nd	nd	33.37	
Pos C		22.33	nd	nt	

Pos Control=100 pg *S. endobioticum* DNA, Neg Control= MilliQ water

nd not detected; *nt* not tested

out in a separate tube to determine whether potato DNA was present in the extract. All primers and probe sequences and reporter/quencher dyes (Eurogentec) are shown in Table 1.

The specificity of the primers/probe combination was tested using four other Chytridiomycota (*Chrytridium confervea*, CBS675.73; *Rhizoclosmatium* sp.a JEL 347; *Rhizophydium* sp.a JEL 136; *Rhizophlyctis* sp. a BK 47-07) described by van den Boogert *et al*. (2005). No DNA amplification was observed with any of the species tested. In addition, BLAST analyses of the ITS 1 and 2 regions did not show any homology that closely matched the primers or probe sequences. Comparison with the previously published TaqMan PCR, using the same conditions for DNA extraction and the real-time PCR kit, showed that the current method detected 1 fg of genomic DNA of *S. endobioticum* compared to 100 fg (Van den Boogert *et al*. 2005). Amplicon size was 186 bp while the current real-time PCR amplifies a much smaller product of 84 bp. Amplification of smaller fragments is more efficient, resulting in lower detection limits.

TaqMan PCR was performed on the extracted DNA samples as described above. A calibration curve was developed from the TaqMan PCR analyses of the dilution series of *S. endobioticum* winter spores added to ZC soil fractions in triplicate (Fig. 1), showing that a single winter spore can be detected. All ninety DNA samples extracted from ZC fractions of infected field soil samples, confirmed by microscopic observation, were positive for *S. endobioticum* by TaqMan PCR (data not shown). The fungus could also be detected in stolons from plants with warts and also from symptomless plants (Table 2). Each cultivar is represented by four subsamples from one single plant. In these tests the IPC was not used in a multiplex setting because the high amount of plant DNA would interfere with the *S. endobioticum* detection. One sample (V81-2, Table 2) was a false negative for IPC since three other subsamples of V81 were clearly positive. *Synchytrium endobioticum* could not be detected in V81. Sub-sample V38-1 was negative for *S. endobioticum* while other sub-samples of the same plant were positive. Non-homogeneous distribution of the fungus may lead to differences in Ct values of sub-samples. The low amount of IAC target showed a relatively high Ct of 33.37 in water without DNA extracts from stolons. The presence of amplification-inhibiting agents will shift the Ct of IAC towards 40. A DNA sample that inhibits the IAC Taqman completely will be excluded for *S. endobioticum* detection.

The results obtained in this study illustrate the sensitive and specific identification of *S. endobioticum* from winter spores added to zonal centrifuge fractions of soil. The use of internal amplification control assays is vital for guarding against false negative results and improves the reliability of any routine diagnostic test. The TaqMan PCR is also suitable for detection of *S. endobioticum* in symptomatic and asymptomatic plant material. Potato cultivars with a high resistance level (cv. Russet Burbank, Karakter) to the pathotype used in this study showed no visual symptoms and *S. endobioticum* was not detected. The cv. Festien (medium resistant), also showed no visual symptoms of wart disease but *S. endobioticum* was detected by the real-time PCR assay. Mixed results were obtained with the susceptible cv. Maritiema, probably because of non-homogeneous distribution of the fungus in the tissue of the visibly-infected plant. In future the COX1 gene can be used to normalise the assay to measure the total amount of DNA in each plant extract. Similar amplification patterns (data not shown) were obtained on a different real-time amplification system (Smartcycler from Cepheid, Sunnyvale, CA, USA), and shows that on-site field diagnostics is also feasible and can therefore be used to support visual detection of *S. endobioticum* in potato plant tissue in the field as well as in the laboratory.

Acknowledgements This study was supported by the Dutch Ministry of Agriculture, Nature and Food Quality and by the EU through the FP6 project Portcheck (SSPE-CT-2004-502348). The authors thank Paul van den Boogert and Gerard van Leeuwen (Plant Protection Service, Wageningen) and Kees Bus and Jan Lamers (PPO-AGV, Lelystad) for valuable discussions and supplying winter spores of *Synchytrium endobioticum*, zonal centrifuge fractions and infected potato plants. The authors want also to thank the reviewer for his/her valuable comments and contributions to the manuscript.

References

Abdullahi, I., Koerbler, M., Stachewicz, H., & Winter, S. (2005). The 18S rDNA sequence of *Synchytrium endobioticum* and its utility in microarrays for the simultaneous detection of fungal and viral pathogens of potato. *Applied Microbiology and Biotechnology, 68*(3), 368–375.

Hampson, M. C. (1985). Sequence of events in the germination of the resting spore of *Synchytrium endobioticum*, European pathotype 2, the causal agent of potato wart disease. *Canadian Journal of Botany, 64*, 2144–2150.

Hampson, M. C. (1993). History, biology and control of potato wart disease in Canada. *Canadian Journal of Plant Pathology, 15*, 223–244.

Hampson, M. C., Yang, A. F., & Bal, A. K. (1994). Ultrastructure of *Synchytrium endobioticum* resting spores and enhancement of germination using snails. *Mycologia, 86*, 733–740.

Hendrickx, G. (1995). An automatic apparatus for extracting freeliving nematode stages from soil. *Nematologica, 41*, 308.

Klerks, M. M., Zijlstra, C., & van Bruggen, A. H. C. (2004). Comparison of real-time PCR methods for detection of *Salmonella enterica* and *Escherichia coli* O157:H7, and introduction of a general internal amplification control. *Journal of Microbiological Methods, 59*, 337–349.

Laidlaw, W. M. R. (1985). A method for the detection of the resting sporangia of potato wart disease (*Synchytrium endobioticum*) in the soil of old outbreak sites. *Potato Research, 28*, 223–232.

Levesque, C. A., de Jong, S. N., Ward, L. J., & DeBoer, S. H. (2001). Molecular phylogeny and detection of *Synchytrium endobioticum*, causal agent of potato wart. *Canadian Journal Plant Pathology, 23*, 200–201.

Levesque, C. A., Ward, L. J., de Jong, S. N., De Boer, S. N., van den Boogert, P. H. J. F., & Baayen, R. P. (2002). A new soil assay for *Synchytrium endobioticum* the causal agent of potato wart. *Phytopathology, 92*, S46.

Niepold, F., & Stachewicz, H. (2004). PCR-detection of *Synchytrium endobioticum* (Schilb.) Perc. Zeitschrift-fuer-Pflanzenkrankheiten-und-Pflanzenschutz. *Journal of Plant Diseases and Protection, 111*(4), 313–321.

Pratt, M. A. (1976). A wet-sieving and flotation technique for the detection of resting sporangia of *Synchytrium endobioticum* in soil. *Annals of Applied Biology, 82*, 21–29.

van den Boogert, P. H. J. F., van Gent-Pelzer, M. P. E., Bonants, P. J. M., de Boer, S. H., Wander, J. G. N., Levesque, C. A., et al. (2005). Development of PCR-based detection methods for the quarantine phytopathogen *Synchytrium endobioticum*, causal agent of potato wart disease. *European Journal of Plant Pathology, 113*(1), 47–57.

van Leeuwen, G. C. M., Wander, J. G. N., Lamers, J., Meffert, J. P., van den Boogert, P. H. J. F., & Baayen, R. P. (2005). Direct examination of soil for sporangia of *Synchytrium endobioticum* using chloroform, calcium chloride and zinc sulphate as extraction reagents. *EPPO Bulletin, 35*, 25–31.

Wander, J. G. N., van den Berg, W., van den Boogert, P. H. J. F., Lamers, J. G., van Leeuwen, G. C. M., Hendrickx, G., et al. (2007). Use of a novel technique, the Hendrickx centrifuge, for extracting winter sporangia of *Synchytrium endobioticum* from soil. *European Journal of Plant Pathology, 119*, 165–174.

Weller, S. A., Elphinstone, J. G., Smith, N. C., Boonham, N., & Stead, D. E. (2000). Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. *Applied Environmental Microbiology, 66*, 2853–2858.

Zelya, A. G., & Melnik, P. A. (1998). Detection methods for *Synchytrium endobioticum*. *Bulletin OEPP/EPPO Bulletin, 28*(4), 543–544.