Validation of a real-time PCR method for the detection of *Phytophthora ramorum*[1]

A. Chandelier[1], K. Ivors[2], M. Garbelotto[3], J. Zini[1], F. Laurent[1] and M. Cavelier[1]

[1]*Walloon Agricultural Research Centre, Department of Biological Control and Plant Genetic Resources, rue de Liroux, 4, B-5030 Gembloux (Belgium); e-mail: chandelier@cra.wallonie.be*

[2]*Department of Plant Pathology, North Carolina State University, 455 Research Drive, Fletcher, North Carolina 28732 (USA)*

[3]*Ecosystem Sciences Division, ESPM, University of California, 151 Hilgard Hall #3110, Berkeley, California 94720 (USA)*

To validate a real-time PCR method for the detection of *Phytophthora ramorum*, an intra-laboratory procedure was developed. The specificity of the TaqMan probe/primer sets was determined by carrying out real-time PCR on total DNA extracted from pure culture of several *Phytophthora* species. The limit of detection and the potential effects of plant substrates were evaluated by conducting the test on total DNA from healthy plant materials (*Rhododendron* spp., *Viburnum* spp. and *Pieris* spp.) spiked with known amounts of *P. ramorum* genomic DNA. The PCR efficiency was estimated through the linear regression of the dilution curve. Precision of the TaqMan assay was assessed on material from a single artificially infected plant (*Rhododendron* spp.). Two kinds of tissues were tested: a severely infected twig and an apparently healthy leaf. Intra-assay repeatability was evaluated on 10 replicates of the same DNA sample analysed in a single assay. Inter-assay reproducibility was evaluated on the same DNA sample amplified over five separate assays while the intersample reproducibility was evaluated on separate DNA extractions of four samples from both plant tissues amplified in a single assay.

Introduction

In the United States, *Phytophthora ramorum* Werres, De Cock & Man in′ t Veld is the causal agent of a disease known as sudden oak death (Rizzo *et al*., 2002). Lethal infections have been reported on various tree species, including *Lithocarpus densiflora*, *Quercus agrifolia*, *Q. kellogii* and *Q. parvula var. shrevei*. The main symptoms are trunk cankers with occasional exudation of brown or black colour. Foliar infections have also been reported on shrubs, in particular *Vaccinium* spp., *Umbellaria* spp. and *Rhododendron* spp., as these species support sporulation by the pathogen (Davidson *et al*., 2005; Rizzo & Garbelotto, 2003; Tooley *et al*., 2004). In Europe, *P. ramorum* was first identified in 1993 on *Rhododendron* and *Viburnum* spp. in the Netherlands and Germany (Werres *et al*., 2001). Since then, it has been identified on a wide range of woody and ornamental plants (for a review, http://www.eppo.org/QUARANTINE/Alert_List/fungi/oak_death.htm). In Belgium, it was isolated for the first time in 2002 from an imported *Viburnum bodnantense* nursery plant (De Merlier *et al*., 2003).

The geographical origin of *P. ramorum* remains unclear. Amplified Fragment Length Polymorphism (AFLP) analyses (Ivors *et al*., 2004) showed that the North-American and European isolates constituted distinct phylogenetic lines, with the large majority of the American isolates (75%) representing a homogeneous group (clonal population) while European isolates being slightly more variable. European isolates also differ from the American isolates by phenotypic features (Brasier, 2003), however, sequences of the ITS (Internal Transcribed Spacer) region of the rDNA (ribosomal DNA) are identical.

Since 2002, EU emergency measures (Commission Decision 2002/757/EC amended in 2004) have been applied to prevent the introduction and dispersion of *P. ramorum* in Europe. In such a context, the availability of an accurate and reliable detection method is essential to limit the risk of false positive or false negative results. Morphological identification is generally the conventional method for the detection of pathogenic fungi. However, this is a time-consuming process which can not always be adapted to the detection of *P. ramorum*. The frequency of successful detection by culturing depends on the environmental conditions and can be influenced by the host plant substrate (woody vs. herbaceous tissue) (Garbelotto & Rizzo, 2005; Hayden *et al*., 2004). In addition, *P. ramorum* is slow-growing in culture and is easily overgrown by other fungi and/or bacteria, even on *Phytophthora* selective media. Therefore, culturing is not always a suitable method for detecting low levels of *P. ramorum* in asymptomatic tissues. Due to such difficulties, improved diagnostic tests are needed for quarantine purposes. Polymerase chain reaction (PCR) has long been used to detect *Phytophthora* species (Ristaino *et al*., 1998), and most recently *P. ramorum* (Kroon *et al*., 2004; Martin *et al*., 2004). PCR offers several advantages over traditional microbiological methods: micro-organisms do not need to be cultivated, and the technique is

[1]Paper presented at the EPPO Conference on *Phytophthora ramorum* and other forest pests (Falmouth, Cornwall, GB, 2005-10-05/07).

Table 1 List of the *Phytophthora* species used in this study and Ct values obtained with the real-time PCR carried out on genomic DNA samples extracted from mycelium. The Ct values were determined at a threshold of 0.5

Strain #	Species	Original host	Year	Country	Collection	Ct
2200	*Phytophthora alni*	*Alnus glutinosa*	2000	Belgium	CRA-W[1]	40.0
2203	*Phytophthora cinnamomi*	*Quercus rubra*	1998	France	INRA[2]	40.0
2207	*Phytophthora cambivora*	*Castanea* sp.	1994	France	INRA[2]	40.0
2216	*Phytophthora fragariae*	*Rubus* sp.	nd	France	LNPV[3]	40.0
2281	*Phytophthora cryptogea*	*Gerba* sp.	1971	Netherlands	MUCL[4]	40.0
2294	*Phytophthora citricola*	Soil	2001	Belgium	CRA-W[1]	40.0
2338	*Phytophthora ramorum* (A2)	*Viburnum* sp.	2002	Belgium	CRA-W[1]	18.0
2384	*Phytophthora europaea*	Soil	nd	France	INRA[5]	40.0
2387	*Phytophthora ramorum* (A1)	*Rhododendron*	2002	Belgium	CRA-W[1]	16.2
2542	*Phytophthora cactorum*	*Fragaria* sp.	2003	Belgium	CRA-W[1]	35.9
3194	*Phytophthora quercina*	*Quercus* sp.	nd	Germany	CBS[6]	40.0
3201	*Phytophthora citricola*	*Kalmia* sp.	2004	Belgium	CRA-W[1]	38.3
3203	*Phytophthora cactorum*	Soil	2004	Belgium	CRA-W[1]	36.3
3205	*Phytophthora gonapodyides*	Soil	2004	Belgium	CRA-W[1]	40.0
3213	*Phytophthora* sp.	*Aesculus* sp.	2004	Belgium	CRA-W[1]	40.0

[1]Collection from the laboratory (CRA-W, Belgium).
[2]Dr Robin, INRA-Bordeaux (France).
[3]Dr Ioos, LNPV-Nancy (France).
[4]MUCL collection, Louvain-La-Neuve (Belgium).
[5]Dr Delatour, INRA-Nancy (France).
[6]CBS collection, Utrecht (the Netherlands).
nd: not determined.

highly sensitive, rapid and specific. Real-time PCR combines the advantages of the conventional PCR with a real-time detection of the amplification products, and can be adapted to high throughput analyses. Several real-time PCR assays have been developed for the detection of *P. ramorum* (Hayden *et al*., 2004; Ivors *et al*., 2002). The objective of this study was to develop an intra-laboratory validation procedure to assess the reliability of the TaqMan assay developed by Ivors *et al*. (2002) in a context of laboratories being certified according to the standard EN ISO/IEC 17025.

Materials and methods

DNA extraction

Approximately 3 g of plant tissues were ground in liquid nitrogen and an aliquot (approximately 100 mg) transferred into a 1.5-mL microcentrifuge tube containing 250 µL extraction buffer [Tris HCl pH 8.0 100 mM; EDTA 20 mM; NaCl 1.4 M; CTAB 2%; PVP K-30 2%] and 40 µL Proteinase K (1 mg/mL). The tubes were placed for 2 h at 55°C with occasional swirling and then centrifuged for 2 min at 6000 *g*. The supernatant was transferred into a new tube and DNA extraction was carried out with the High Pure Template Preparation Kit (Roche Diagnostic GmbH, Germany) according to the manufacturer's instructions. Mycelium was recovered from pure culture of different *Phytophthora* species (Table 1) cultivated on V8-agar (12 h light/12 h dark, 22°C). Genomic DNA was extracted by the same procedure as that described for plant tissues, and DNA concentrations were estimated spectrophotometrically.

PCR amplification

The TaqMan probe (labelled at the 5′ end with 6-FAM and at the 3′ end with a black-hole quencher) and primer sets, both derived from the ITS region, were those designed by Ivors *et al*. (2002). For the real-time PCR assay, an aliquot of 5 µL of DNA was included in each 25 µL PCR reaction (1 × reaction buffer [qPCR MasterMix, Eurogentec, Belgium]; 0.2 µM of each PCR primer [Invitrogen, Life Technologies Ltd], and 0.2 µM TaqMan probe [Eurogentec, Belgium]). Amplifications were done in an Applied Biosystem ABI Prism 7000 Sequence Detection System using the following conditions (10 min at 95°C and 40 cycles of 95°C for 15 s, 60.5°C for 1 min). The rhodamine derivation ROX present in the PCR master mix was used as a passive reference to normalize the signal. The threshold cycle (Ct) corresponded to the cycle at which the fluorescent signal exceeded the background. Ct values were estimated at a threshold value of 0.5 in all cases (baseline start and end cycles, 2–15). Two negative water controls were included with each PCR run. The first water control was treated as the samples to analyse (DNA extraction step). The second control corresponded to the PCR water control. A DNA sample extracted from a *P. ramorum* infected plant (*Rhododendron* spp.) was also used as a positive control to monitor the performance of the PCR. The nested conventional PCR developed by Hayden *et al*. (2004) was used to confirm the results obtained with the real-time PCR assay validated in this study. The amplicon (291 bp) was separated by electrophoresis in 2% agarose gel stained with ethidium bromide.

Table 2 Comparison of Ct values obtained with the real-time PCR carried out on total DNA from different species of healthy plants (non-spiked = NS) and from total DNA spiked (S) with 50 and 5 fg of *P. ramorum* genomic DNA. The Ct values are determined at a threshold of 0.5

	Rhododendron (leaf)			*Viburnum* (green twig)			*Viburnum* (woody twig)			*Pieris* (green twig/leaf)		
Replicates	NS	S 50	S 5	NS	S 50	S 5	NS	S 50	S 5	NS	S 50	S 5
1	37.2	33.1	36.3	38.4	34.0	37.6	37.5	31.6	34.0	39.5	33.7	34.7
2	38.7	33.6	35.5	37.7	33.5	35.5	37.0	32.0	33.0	37.0	32.5	34.2
3	40.0	33.4	35.7	37.1	33.9	35.5	37.8	31.4	33.1	38.2	33.0	35.1
4	39.5	34.4	36.2	37.2	33.9	35.2	40.0	31.8	35.2	38.0	33.5	36.3
5	37.6	33.6	35.4	40.0	33.4	36.2	37.8	32.2	36.9	39.2	32.9	35.4
6	39.0	34.2	36.0	38.6	33.5	38.6	37.3	31.9	34.2	38.2	33.5	35.9
Mean	38.7	33.7	35.9	38.2	33.7	36.4	37.9	31.8	34.4	38.3	33.2	35.3
SD[1]	1.1	0.5	0.4	1.1	0.3	1.4	1.1	0.3	1.4	0.9	0.5	0.8
NCt[2]	35.4	–	–	34.9	–	–	34.7	–	–	35.6	–	–
Blank PCR[3]	40.0	–	–	40.0	–	–	40	–	–	40.0	–	–
Blank EXT[4]	40.0	–	–	38.6	–	–	40	–	–	40.0	–	–
C+[5]	17.6	–	–	17.6	–	–	17.9	–	–	17.1	–	–

[1]SD = standard deviation.
[2]NCt = the Ct value above which a sample is considered as negative. It is calculated according to the equation [Mean – 3 ∗ SD].
[3]Blank PCR = PCR carried out with ultrapure water in place of DNA.
[4]Blank EXT = PCR carried out with a water sample treated identically to the unknown samples (it follows all the steps of the DNA extraction).
[5]C + = PCR carried out with total DNA from healthy plant spiked with 5 ng *P. ramorum* genomic DNA (mean of 6 replicates).

Validation experiments

Assessment of PCR, limit of detection and PCR efficiency
Real-time PCR assays were conducted on six replicates of total DNA extracted from leaves and twigs of three plant species (*Rhododendron* spp., *Viburnum* spp. and *Pieris* spp.). These DNA samples were spiked with known amounts of genomic DNA extracted from a pure culture of *Phytophthora ramorum* (5 ng, 500 pg, 50 pg, 5 pg, 500 fg, 50 fg and 5 fg). The three nursery plants were selected among genera which have been reported as the most susceptible to *P. ramorum* under EU conditions.

Test specificity
Real-time PCR was performed on total DNA extracted from pure cultures of the different *Phytophthora* cultures listed in Table 1. These species were chosen due to their potential presence on ornamental or woody plants found in Europe. *P. lateralis*, which is genetically close to *P. ramorum*, was not tested in the experiment as it has a very limited host range.

Test precision
A *Rhododendron* plant was inoculated in a greenhouse by inserting a 5 mm agar plug from the margin of an actively growing V8-culture of *P. ramorum* (isolate 2387, Table 1) into a bark wound created out on the stem. Necrotic lesions were observed on the twig after four to five days. Two kinds of tissues were collected: a part of the twig with necrosis just above the inoculation point and an adjacent asymptomatic leaf. The validation experiment was based on a protocol described by Winton *et al*. (2001). Both plant tissues were separated into four subsamples for the DNA extraction step. The intra-assay repeatability was evaluated on 10 replicates of one of the DNA subsamples analysed in a single assay. The inter-assay reproducibility was assessed on the same DNA subsample five separate times while the inter-sample reproducibility was determined on separate extractions of the four subsamples amplified a single time. Statistical analysis was carried out using SAS software (SAS Institute, Cary, NC Version 8.2).

Results and discussion

Assessment of PCR, limit of detection and PCR efficiency

The Ct values for the non spiked and spiked plant extract samples were compared (Table 2). The mean Ct value observed for the different kinds of non spiked plant substrates was close to 38. However, some of the replicates gave lower values (as an example, Ct = 37.2 for one of the replicates of *Rhododendron* total DNA). Results were confirmed by nested PCR carried out on the same samples (data not shown).

In order to establish the Ct value above which a sample is considered as negative (NCt), an equation was chosen empirically to take into account the variability of the Ct values observed for a healthy plant:

$$NCt = M - 3 * SD$$

in which M is the mean Ct value calculated from the 6 replicates of healthy plant DNA, and SD is the corresponding standard deviation. For all the plant substrates analysed, the NCt was close to 35. Therefore, samples with Ct values below 35 should be considered as positive. However, for the samples with Ct values around the NCt (Ct = 34–36), results were considered

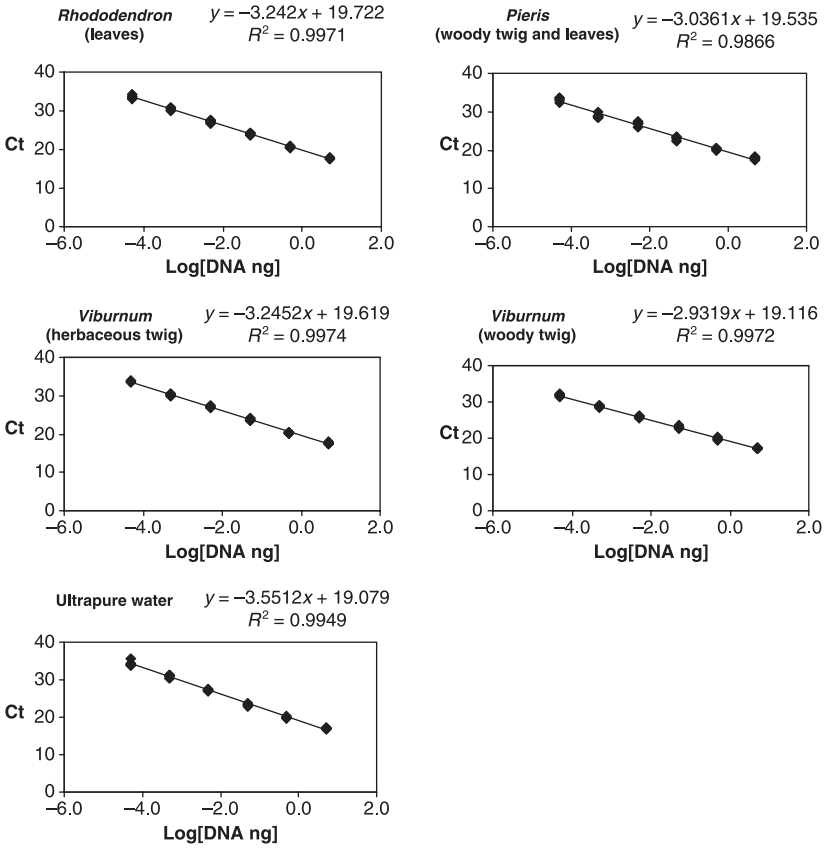

Fig. 1 Standard curves showing amplification of successive 10-fold dilutions of *P. ramorum* genomic DNA (from 5 ng to 50 fg) in total DNA extracted from different plant substrates and in water. Ct values were determined at a threshold of 0.5. Six replicates were tested for each dilution.

questionable. In such conditions, a new PCR is performed. If the result is still uncertain, the sample is considered negative.

According to the AFNOR standard XP V 03-020-2 (2003), the limit of detection (LOD) corresponds to the smallest target DNA concentration for which six replicates give a positive result. Considering the NCt values obtained for the different plant substrates, the method here described detects at least 50 fg of pathogen DNA (Table 2).

The PCR efficiency was estimated through the linear regression of the dilution curve. Similar calibration curves were obtained when *P. ramorum* genomic DNA was diluted in sterile water or in plant extract DNA (Fig. 1). The determination coefficients were $R = 0.997$ (*Rhododendron*, *Viburnum* herbaceous and woody tissues) and $R = 0.987$ (*Pieris*). When the Ct values corresponding to a concentration in target DNA of 5 fg were discarded (value out of the linear phase), the slopes of the standard curves in *Rhododendron* and in *Viburnum (*herbaceous tissues) total DNA were –3.24 in both cases. This value is close to that of a PCR with an efficiency of approximately 100% (–3.30). In contrast, a slope of greater than –3.30 was observed when the dilutions were carried out in DNA extracted from woody tissues (*Viburnum*, –2.93; *Pieris*, –3.04). The slope of the standard curve in water was less than –3.30 (–3.55), indicative of a reaction efficiency < 1. Generally, PCR reactions do not reach 100% efficiency due to experimental limitations.

Test specificity

As shown in Table 1, only the *P. ramorum* isolates (A1 and A2 mating types) were unambiguously detected. As all the *Phytophthora* DNA samples evaluated in this study were checked for DNA viability by carrying out PCR with the generic primers ITS1 and ITS4 (White *et al*., 1990), which amplify a part of the rDNA (data not shown), failure of the DNA extraction could be excluded as the reason for the negative results. The Ct values obtained with *P. cactorum* total DNA were close to the NCt for the two isolates evaluated, suggesting a slight cross-reaction. However, the DNA concentrations used in the experiment were very high (approximately 5–10 ng) and do not correspond to those found in naturally infected samples.

Test precision

Mean Ct values among the three experiments designed to assess the precision of the real-time PCR assay are shown in Table 3. The Kruskal–Wallis test applied to the data revealed significant differences between the different plant tissues ($P < 0.001$). In contrast, the results were not significantly different between experiments, regardless of the plant tissue (global probability = 0.8505). The Bartlett test indicated homogeneity of variances between the experiments for the twig ($P = 0.6104$) but not for the leaf ($P = 0.0121$).

Table 3 Mean Ct values obtained with the real-time PCR carried out on total DNA extracted from two kinds of tissues from an artificially infected *Rhododendron* spp. SD = standard deviation. N = number of replicates

		Twig with necrosis		Leaf without necrosis	
	N	Mean	SD	Mean	SD
Intra-assay	10	20.4	0.2	31.3	0.4
Inter-assay	5	20.3	0.2	31.5	0.5
Inter-samples	4	20.7	0.3	30.5	1.4

The greatest standard deviation observed for the inter-samples assay on an apparently healthy leaf (SD = 1.4) suggests this is due to the DNA extraction rather than the real-time PCR assay, which is critical for samples with low levels of target DNA.

Conclusion

For implementation of a test procedure for routine use, it is essential to set up a validation methodology. The results of this study indicate that the real-time PCR method developed by Ivors *et al*. (2002) could be used as a rapid screening method for detection of *P. ramorum* in plant tissues. The DNA extraction method used in our laboratory is well adapted to herbaceous tissues. The results with woody tissues, which are generally known to contain compounds inhibiting the PCR reaction (Ippolito *et al*., 2002), should be improved, notably by further purifying the DNA or diluting DNA extracts. Nevertheless, with all the plant tissues evaluated, the limit of detection reached 50 fg, which probably corresponds to very low concentration in *P. ramorum* as the test was found to detect the presence of the pathogen in asymptomatic tissues. From a qualitative point of view, this suggests that the test can be used without any further modification, even for DNA samples extracted from woody material, at least for the plant substrates evaluated in this study. Calibration experiments with known amounts of target DNA demonstrated that starting quantities varying over 5–6 orders of magnitude could be detected with DNA extracted from herbaceous tissues. This suggests that the TaqMan assay could also be used for quantitative analyses in epidemiological studies.

During the year 2004, 63 samples were analysed at the Agricultural Research Centre in Belgium using a microbiological method adapted to the isolation of *Phytophthora* species from woody material (De Merlier *et al*., 2005) and the real-time PCR method described here. For 79% of the samples analysed, both methods gave identical results. In addition, all the samples which were positive with culturing were also positive using the molecular method (i.e. no sample positive with the microbiological method and negative with the PCR). In contrast, a few samples were found positive with the TaqMan assay and negative with the isolation method. All ambiguous samples with Ct values around the NCt were declared positive with the nested PCR assay developed by Hayden *et al*. (2004). These results highlight the difficulty of isolation of *P. ramorum*, notably due to the great variability of host plants and environmental effects. These ambiguous results could also be explained by the status of the pathogen (living or dead). However, even in the last case, a positive result suggests that the plant analysed has been in contact with the pathogen. In such conditions and considering the legal status of *P. ramorum* as a quarantine organism and its implications, the result should be considered as positive as a precautionary measure. Therefore, provided that the different controls give unambiguous results, and that the potential effect of plant substrate on the PCR reliability has been evaluated, samples with positive PCR results should be considered as positive without any further microbiological confirmation. Otherwise, certain PCR positive samples would be declared as negative, although they harbour *P. ramorum*. For critical samples (Ct value between 34 and 36), new reactions should be performed. An additional molecular method which targets another region of the genome could also be a consideration.

Acknowledgements

We are grateful to Viviane Planchon from the Walloon Agricultural Research Centre, Section Biometry, Data management and Agrometeorology for the statistical analysis of data.

Validation d'une méthode PCR en temps réel pour la détection de *Phytophthora ramorum*

Afin de valider une méthode PCR en temps réel pour la détection de *Phytophthora ramorum*, une procédure intra-laboratoire a été développée. La spécificité des ensembles sonde TaqMan /amorce a été déterminée en réalisant une PCR en temps réel sur de l'ADN total extrait d'une culture pure de plusieurs espèces de *Phytophthora*. La limite de détection et les effets potentiels des matrices végétales ont été évalués en conduisant le test sur de l'ADN total du matériel végétal sain (*Rhododendron* spp., *Viburnum* spp. et *Pieris* spp.) enrichi avec des quantités connues d'ADN génomique de *P. ramorum*. L'efficacité de la PCR a été estimée par la régression linéaire de la courbe de dilution. La précision de l'analyse TaqMan a été évaluée sur du matériel provenant d'une seule plante artificiellement infectée (*Rhododendron* spp.). Deux types de tissus ont été testés : un rameau sévèrement infecté et une feuille apparemment saine. La répétabilité intra-analyse a été évaluée sur dix répétitions du même échantillon d'ADN analysé dans un seul test. La reproductibilité inter-analyses a été évaluée sur le même échantillon d'ADN amplifié dans cinq tests séparés alors que la reproductibilité inter-échantillons a été évaluée sur des extractions d'ADN distinctes de quatre échantillons provenant des deux types de tissus végétaux amplifiées lors d'un seul test.

Апробация ПЦР в реальном времени – метод для обнаружения *Phytophthora ramorum*

Для апробации ПЦР в реальном времени в качестве метода для обнаружения *Phytophthora ramorum* была

разработана внутрилабораторная процедура. Специфика наборов зонд/праймер TaqMan была определена с помощью ПЦР в реальном времени на полной ДНК, экстрагированной из чистой культуры нескольких видов *Phytophthora*. Оценка предела обнаружения и потенциальных эффектов субстратов растений проводилась путем тестирования на полной ДНК, полученный из здорового растительного материала (*Rhododendron* spp., *Viburnum* spp. и *Pieris* spp.), заточенной известными количествами геномной ДНК *P. ramorum*. Эффективность ПЦР оценивалась по линейной регрессии кривой растворения. Точность анализа TaqMan оценивалась на материале от одного искусственно инфицированного растения (*Rhododendron* spp.). Тестировались два типа тканей: сильно инфицированный побег и внешне здоровый лист. Воспроизводимость анализов оценивалась на десяти репликатах того же самого образца ДНК, проанализированного в едином тесте. Воспроизводимость анализов оценивалась на том же самом образце ДНК, амплифицированном в ходе более пяти отдельных анализов, в то время как воспроизводимость между образцами оценивалась на раздельных экстрагированиях ДНК четырех образцов из обеих амплифицированных тканей растения в едином анализе.

References

AFNOR Standard XP-V-03-020-2 (2003) Détection et quantification des organismes végétaux génétiquement modifiés et produits dérivés. Partie 2: Méthodes basées sur la réaction de polymérisation en chaîne, pp. 1–20. AFNOR (FR).

Brasier CM (2003) Sudden Oak Death: *Phytophthora ramorum* exhibits transatlantic differences. *Mycological Research* **107**, 258–259.

Davidson JM, Wickland AC, Patterson HA, Folk KR & Rizzo DM (2005) Transmission of *Phytophthora ramorum* in mixed-evergreen forest in California. *Phytopathology* **95**, 587–596.

De Merlier D, Chandelier A & Cavelier M (2003) First report of *Phytophthora ramorum* in Belgium. *Plant Disease* **87**, 203.

De Merlier D, Chandelier A, Debruxelles N, Noldus M, Laurent F, Dufays E, Claessens H & Cavelier M (2005) Characterisation of Alder *Phytophthora* isolates from Wallonia and development of SCAR primers for their specific detection. *Journal of Phytopathology* **153**, 99–107.

Garbelotto M & Rizzo D (2005) A California-based chronological review (1995–2004) of research on *Phytophthora ramorum*, the causal agent of Sudden Oak Death. *Phytopathologia Mediterranea* **44**, no. 2.

Hayden KJ, Rizzo D, Tse J & Garbelotto M (2004) Detection and quantification of *Phytophthora ramorum* from California forests using real-time polymerase chain reaction assay. *Phytopathology* **94**, 1075–1083.

Ippolito A, Schena L & Nigro F (2002) Detection of *Phytophthora nicotianae* and *P. citrophthora* in citrus roots and soil by nested PCR. *European Journal of Plant Pathology* **108**, 855–868.

Ivors KL, Hayden KJ, Bonants PJM, Rizzo DM & Garbelotto M (2004) AFLP and phylogenetic analyses of North American and European populations of *Phytophthora ramorum*. *Mycological Research* **108**, 378–392.

Ivors K, Tse J & Garbelotto M (2002) TaqMan PCR for detection of *Phytophthora* DNA in environmental plant samples. *Proceedings of 'Sudden Oak Death, a Science Symposium'*, p. 56. Monterey, California (USA).

Kroon LPNM, Vestappen ECP, Kox LFF, Flier WG & Bonants PM (2004) A rapid diagnostic test to distinguish between American and European populations of *Phytophthora ramorum*. *Phytopathology* **94**, 613–620.

Martin FN, Tooley PW & Blomquist C (2004) Molecular detection of *Phytophthora ramorum*, the causal agent of Sudden Oak Death in California, and two additional species commonly recovered from diseased plant material. *Phytopathology* **94**, 621–631.

Ristaino JB, Madritch M, Trout CL & Parra G (1998) PCR amplification of ribosomal DNA for species identification in the plant pathogen genus *Phytophthora*. *Applied and Environmental Microbiology* **64**, 948–954.

Rizzo D & Garbelotto M (2003) Sudden Oak Death: endangering California and Oregon forest ecosystems. *Frontiers in Ecology and the Environment* **1**, 197–204.

Rizzo D, Garbelotto M, Davidson JM, Slaghter GW & Koike ST (2002) *Phytophthora ramorum* as the cause of extensive mortality of *Quercus* spp. & *Lithocarpus densiflora* in California. *Plant Disease* **86**, 205–214.

Tooley PW, Kyde KL & Englander L (2004) Susceptibility of selected Ericaceous ornamental host species to *Phytophthora ramorum*. *Plant Disease* **88**, 993–999.

Werres S, Marwitz R, Man in′ t, Veld WA, De Cock AWAM, Bonants P, de Weerdt M, Themann K, Ilieva E & Baayen RP (2001) *Phytophthora ramorum* sp. nov., a new pathogen on *Rhododendron* and *Viburnum*. *Mycological Research* **105**, 1155–1165.

White TJ, Bruns T, Lee S & Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols, a guide to methods and applications* (ed. Innis MA, Gelfand DH, Sminsky JJ & White JJ), pp. 315–322. Academic Press, San Diego (US).

Winton LM, Stone JK, Watrud LS & Hansen EM (2001) Simultaneous one-tube quantification of host and pathogen DNA with real-time polymerase chain reaction. *Phytopathology* **92**, 112–116.