Characterization and detection of *Phytophthora fragariae* in plant, water and soil by molecular methods*

P. J. M. Bonants, M. P. E. van Gent-Pelzer and M. Hagenaar-de Weerdt

Plant Research International, PO Box 16, 6700 AA Wageningen (The Netherlands); e-mail: p.j.m.bonants@ipo.wag-ur.nl

Phytophthora fragariae var. *fragariae* is pathogenic to strawberry only and is an EPPO A2 quarantine pest. Seventy-five isolates of *P. fragariae* from all over the world have been characterized by amplified fragment length polymorphism (AFLP) analysis. However, a sensitive, specific and fast method of detection is now needed, and polymerase chain reaction (PCR)-based methods have now been set up. Based upon sequences of the ITS (internal transcribed spacer) regions of rDNA of several *Phytophthora* species, PCR primers specific for *P. fragariae* have been developed together with SCRI (Dundee, GB). These primers were tested on DNA extracted from water, soil and plant material. A nested PCR procedure has been included in the currently used bait test to improve sensitivity and reliability. Also several methods have been studied to detect the amplicon of the PCR reaction: gel electrophoresis, DIAPOPS (detection of immobilized amplified products in a one-phase system), PCR-ELISA, TaqMan and molecular beacon. With the last two methods, using the ABI 7700 detection system, quantification of target DNA is possible.

Introduction

Identification and detection of plant pathogens is an important issue, especially when quarantine pests are involved. A zero tolerance is required for these organisms. Sensitivity and specificity are the main issues in this field. Fast and reliable measures are needed, because export of plant material cannot tolerate long waiting periods before the results are known.

Phytophthora fragariae var. *fragariae*, which is pathogenic only to strawberry, is an EU and EPPO quarantine pest (A2 status). The DNA fingerprinting method AFLP (amplified fragment length polymorphism) (Vos *et al.*, 1995) has been used for correct identification of the fungus. Generation of the AFLP DNA fingerprinting patterns of different isolates of the fungus gave the possibility of constructing a dendrogram in which genetic relatedness can be derived.

However, the method nowadays used for detection (Duncan test) is a bait test with several drawbacks. The interval between the start of the test and the results (5–6 weeks) is too long and experience of morphological characterization is needed. A much faster method has been developed based upon polymerase chain reaction (PCR). Sequences of the internal transcribed spacer (ITS) regions of rDNA have been shown to differentiate *Phytophthora* species efficiently (Lee & Taylor, 1992; Cooke & Duncan, 1997). Based upon these ITS sequences, primers specific for *P. fragariae* have been developed. Previous research has shown that the fungus can specifically and sensitively be detected in roots with nested PCR (Bonants *et al.*, 1996, 1997), as well as in water samples. This nested PCR procedure was used to screen many root and water samples from bait tests.

Different methods to detect the PCR amplicons have been developed to avoid traditional gel electrophoresis. In this study, the following methods have been compared: gel electrophoresis, DIAPOPS (detection of immobilized amplified products in a one-phase system), PCR-ELISA, Taqman and Molecular Beacon. The last two methods were developed to detect PCR amplicons by fluorescence in real time. The principle of these methods are summarized in Figs 1–4.

Materials and methods

Fungus

Several isolates of *P. fragariae* were kindly supplied by E. van de Weg (Plant Research International, Wageningen, NL) and D. Cooke (SCRI, Invergowrie, Dundee, GB). Zoospore suspensions were prepared as previously described (Bonants *et al.*, 1997).

DNA isolation

DNA isolations from mycelium, water samples, zoospore suspensions and from strawberry root samples were performed as described before (Bonants *et al.*, 1997).

* Paper presented at the EPPO Conference on diagnostic techniques for plant pests, Wageningen (NL), 2000-02-01/04.

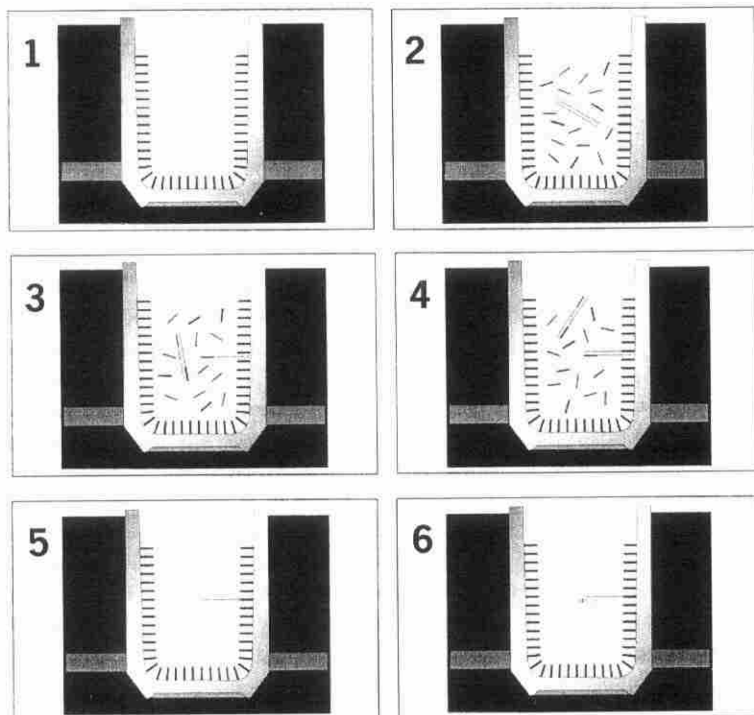

Fig. 1 Principle of DIAPOPS: (1) covalent binding of one PCR primer to the wall of a microtitre plate well; (2–4) second round of PCR in a microtitre plate (amplification, elongation of bound primer, generation of bound and unbound amplicons); (5) hybridization of biotinylated detection probe (CPB2) to bound amplicon; and (6) binding of streptavidin alkaline phosphatase conjugate to biotinylated probe and finally measurement of coloration with alkaline phosphatase substrate (4-MUP).

AFLP

AFLP analysis was performed as previously described (Bonants *et al.*, 1999). AFLP patterns were analysed with ImageMaster software (Amersham Pharmacia Biotech). Only reproducible bands were incorporated in the analysis. A similarity matrix was constructed using the method of Nei & Li (1979). UPGMA cluster analysis of binary data was performed with Treecon software (van de Peer & de Wachter, 1994).

PCR methods

Nested PCR, gelelectrophoresis

Nested PCR experiments were performed as previously described (Bonants *et al.*, 1997). Primers DC6/I4 (Lee & Taylor, 1992) and DC1/B5 (Bonants *et al.*, 1997) are described in Fig. 5. PCR buffer: 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$ (Boehringer); dNTP 60 μM; 0.6 μM of each primer; 1 U *Taq* polymerase (Boehringer). Conditions for the first PCR: 2 min 94°C, 35 cycles of 30 s at 94°C, 30 s at

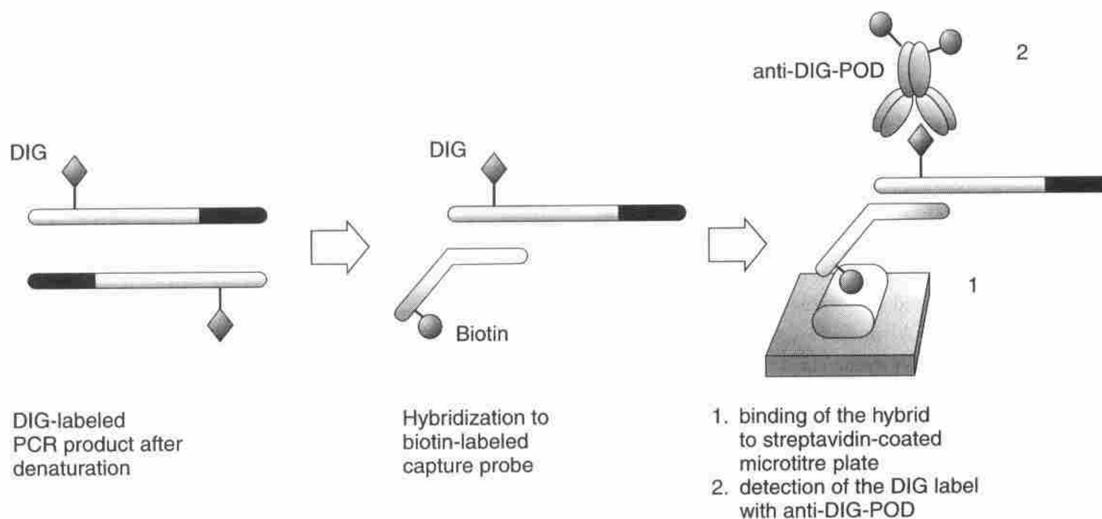

Fig. 2 Principle of PCR-ELISA: incorporation of DIGdUTP in the second round of PCR; denaturation of the dsPCR product; capture of DIG-labelled PCR products with biotinylated capture probe (CPB2); binding of captured products onto streptavidin-coated microtitre plates; treatment with anti-DIG-conjugate labelled with peroxidase (POD); enzymatic coloration with ABTS (peroxidase substrate).

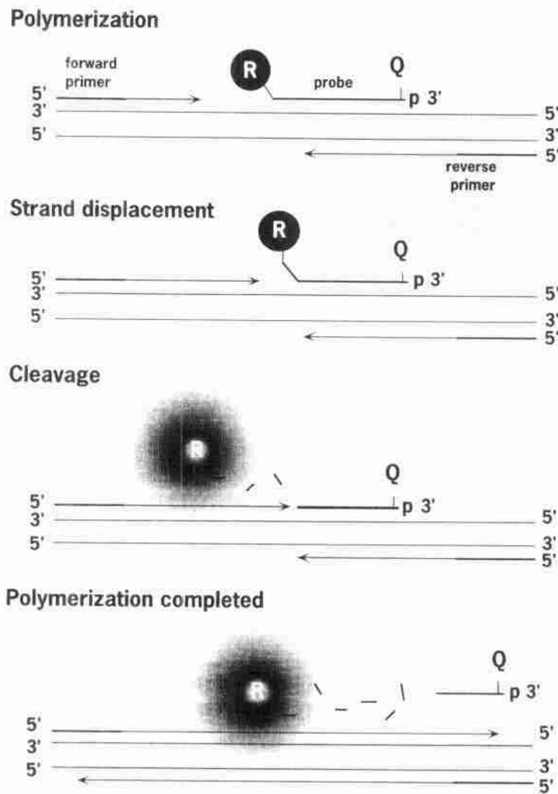

Fig. 3 Principle of TaqMan: real-time monitoring of fluorescence during PCR in which a TaqMan probe, which also binds to the target amplicon, is degraded during PCR. The probe contains a fluorescent group and a quencher molecule on both ends of an oligonucleotide. The fluorescence is quenched due to the close proximity of both groups. The TaqMan probe is added to the PCR reaction mix and binds to the PCR amplicon and is degraded by 5′-exonuclease activity of the *Taq* polymerase. Then the fluorescent group comes into solution and fluorescence is no longer quenched which results in increase of fluorescence. Real-time measurement of the fluorescence is possible during PCR in an ABI Prism 7700 Sequence Detection system (Perkin-Elmer). In this way quantification is possible.

57°C, 60 s at 72°C and a final 10 min at 72°C. For the second PCR reaction, 5 μL 1 : 20 diluted amplicon was used as a template. Conditions for the second PCR: 2 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 65°C, 60 s at 72°C and a final 10 min at 72°C. Next, 5 μL was run on 1.0% agarose gel. Bands were visualized by ethidium-bromide staining.

DIAPOPS

NucleoLink microtitreplates (Nunc A/S) were coated with primer P10T-B5 as recommended by NUNC A/S. Then, 45 μL of PCR mix and 5 μL of template were added to each well. The final concentration was: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% gelatine, 0.1% Tween 20, 5% DMSO, 200 μM of each dNTP, 0.5 μM primer DC1 and 0.06 μM primer B5, 1 U *Taq* polymerase (Boehringer). Primers are described in Fig. 5. Conditions for PCR: 3 min at 94°C, 40 cycles of 10 s at 94°C, 15 s at 65°C, 10 s at 72°C and a final 5 min at 72°C. PCR reactions were run in a PE 9600. PCR amplicon was detected by hybridization with biotinylated probes (50 nM Biot-B5 and 50 nM CPB2). Alkaline phosphatase-conjugated streptavidin (1 : 3000) was added and finally 100 μL 1 mM 4-MUP substrate in 1 M diethanolamine pH 9.8, 1 mM $MgCl_2$. Fluorescence was measured after 30 min incubation at 50°C in a fluorescence plate reader (excitation 355 nm, emission 460 nm).

PCR-ELISA

DIG-dUTP (Boehringer) was added to PCR reactions. PCR-ELISA experiments were performed as previously described (Bonants *et al.*, 1997). Primers and probe are described in Fig. 5.

TaqMan

Next, 45 μL of PCR mix and 5 μL of template were added to each tube. The final concentration in this 50-μL mix was: TaqMan-buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 4 mM $MgCl_2$), 60 μM dNTP, 0.6 μM of each primer (DC1/MP5), 0.2 μM of TaqMan-probe (Perkin-Elmer); 2.5 U AmpliTaq Gold (Perkin-Elmer); Conditions for PCR: 10 min 95°C, 35 cycles of 15 s at 95°C, 1.5 min at 60°C. TaqMan probe and primers are described in Fig. 5. PCR experiments were performed and fluorescence was measured in an ABI 7700 (Perkin-Elmer).

Molecular beacon

Next, 20 μL of PCR mix and 5 μL of template were added to each tube. The final concentration was: PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 60 μM dNTP, 0.6 μM of each primer (DC1/MP3), 0.2 μM Molecular Beacon; 0.75 μM ROX; 1 U *Taq* polymerase (Boehringer). Conditions for PCR: 2 min at 95°C, 35 cycles of 30 s at 95°C, 1 min at 60°C, 1 min at 72°C. Molecular beacon and primers are described in Fig. 5. PCR experiments were performed and fluorescence was measured in an ABI 7700 (Perkin-Elmer).

Results

The relatively new molecular method AFLP was used for identification of *P. fragariae* (Fig. 6). The AFLP patterns for several isolates using a fluorescent sequencing system are shown in Fig. 7. The patterns showed great homology between isolates. Many products were the same. This should be indeed the case because all isolates were *P. fragariae*. However, some fragments appeared for some isolates and not others. After preparation of a similarity matrix, a dendrogram was constructed in which genetic relatedness could be seen (Fig. 8).

For detection purposes, PCR primers were developed together with SCRI (D. Cooke and J. Duncan) which can be used to amplify the fungus in infected material in a specific and very sensitive way. Figure 5 shows the primers and probes which were developed from ITS sequences of

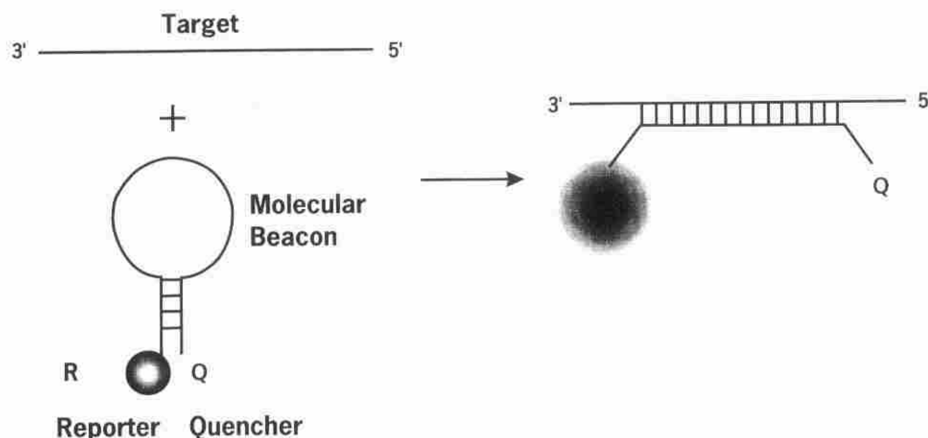

Fig. 4 Principle of Molecular Beacon: a fluorescence-labelled circular probe which binds during PCR to the target amplicon causes an increase of fluorescence. The molecular beacon contains a loop structure and a stem structure. The loop contains the complementary sequence of the target (in this case *Phytophthora fragariae*). The stem consists of a 6–7-base dsDNA sequence. At the 5′ end and at the 3′ end, fluorogenic groups are attached. In its closed form, the molecular beacon does not fluorescence because of quenching. When the molecular beacon binds to its target it opens and the fluorescence increases. When included in the PCR reaction, the MB binds during the annealing step to the target sequence and the MB starts to fluoresce. This probe can also be used for quantification of a template DNA concentration using the ABI 7700 (Perkin-Elmer).

rDNA. Single-round PCR can be performed but the more sensitive nested procedure can also be used. In this way, two sets of primers are used in two separate and successive PCR reactions.

The currently used detection test is a bait test in which infected material is incubated in soil or tap water with a bait plant on top of it. After incubation for a long period, the bait plant is tested for the presence of oospores. We investigated the presence of zoospores (which infect the strawberry roots) in the baiting system with water as a substrate by a nested PCR procedure. A dilution series of zoospores from 20 000 to 2 zoospores was made and DNA isolated from this series was tested with nested PCR. As many as 20 zoospores could be detected (Bonants *et al.*, 1997) using gel electrophoresis to detect the PCR amplicon.

Fluorescence measurements with a TaqMan PCR are shown in Fig. 9. From these amplification curves, one can calculate the C_t-value, which is the number of PCR cycles necessary to increase fluorescence above background. Figure 10 shows C_t-values represented vs. template DNA concentration. The C_t-value increases with decreasing amounts of template DNA. More PCR cycles are needed to

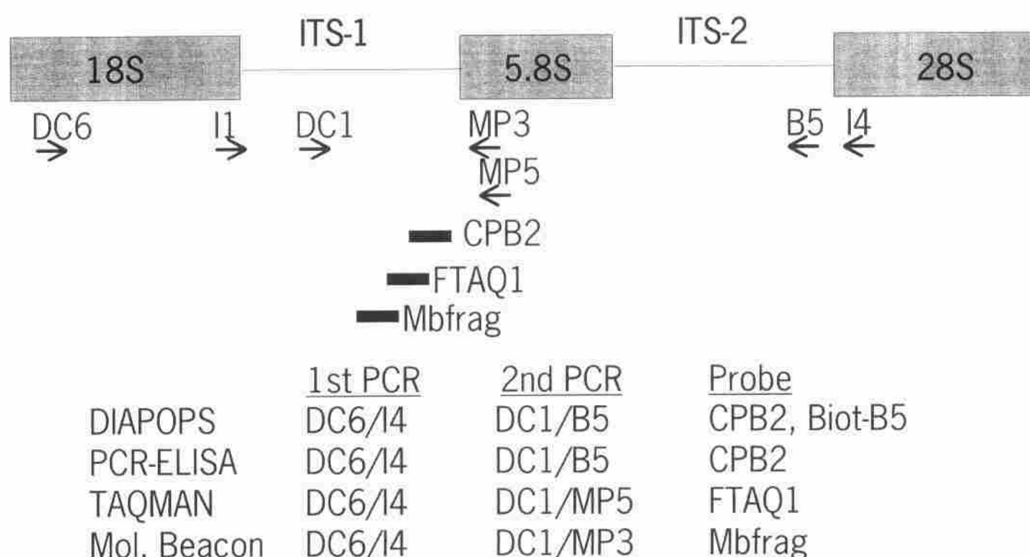

Fig. 5 Scheme of an rDNA unit with primers and probes used in this study.

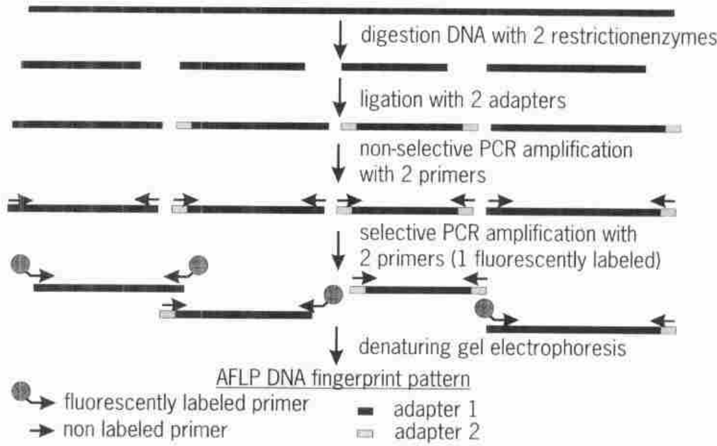

Fig. 6 Schematic representation of the AFLP procedure.

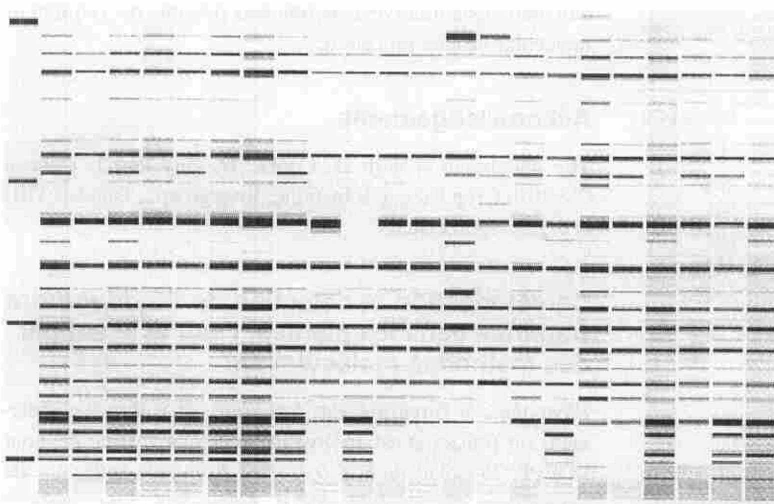

Fig. 7 AFLP DNA fingerprinting patterns of several isolates of *Phytophthora fragariae*.

increase fluorescence above background values. In this way quantification of target DNA is possible. It was also observed that nested PCR was far more sensitive than a single PCR.

The alternative detection methods were tested on the same dilution series of *P. fragariae* DNA with single and with nested PCR. Using gel electrophoresis, we detected 100 ag of DNA with nested PCR. The same amount of DNA was detected with PCR-ELISA and TaqMan. DIAPOPS was less sensitive, only detecting 10 fg. With single PCR, the detection limit was 1 pg DNA. Again, nested PCR was far more sensitive than a single PCR. Comparison between TaqMan and molecular beacon for nested PCR of *P. fragariae* showed equal sensitivity. PCR experiments were performed with the molecular beacon included in single and nested PCR reactions with DNA samples from roots, zoospore suspensions and water. C_t-values of these PCR reactions are shown in Table 1.

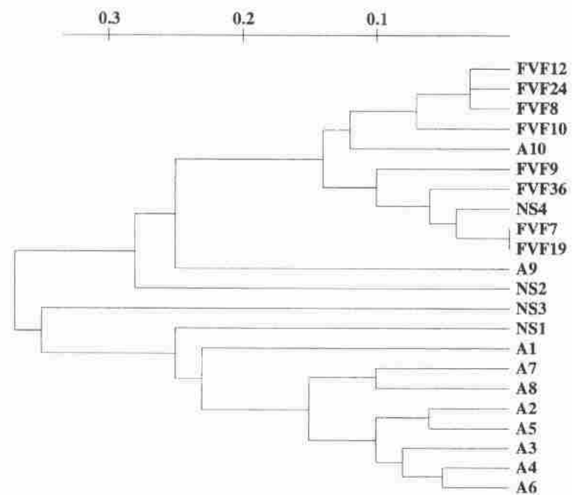

Fig. 8 Dendrogram of AFLP analysis of several *Phytophthora fragariae* isolates.

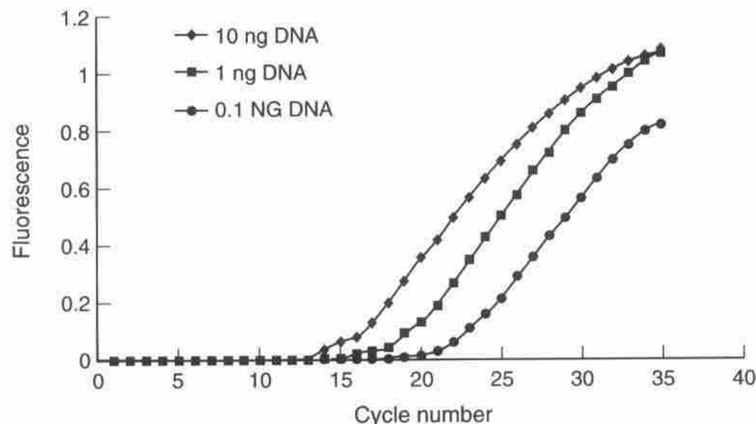

Fig. 9 Amplification plots with TaqMan probe (single-round PCR) for three different *Phytophthora fragariae* DNA concentrations.

Table 1 C_t-values of molecular beacon experiments with single and nested PCR with different DNA templates (DNA, zoospores, water samples, root samples)

Sample	PCR	Nested PCR
1 ng DNA *Phytophthora fragariae*	23.2	8.2
250 zoospores *Phytophthora fragariae*	ND	20.8
Water sample 1	18.0	ND
Water sample 2	16.1	ND
Root sample 1	27	11.8
Root sample 2	31.2	12.7

Conclusions

AFLP was confirmed as a suitable method for correct identification of *P. fragariae* isolates. It was also found that the fungus could be detected in a sensitive and specific manner by PCR, using several different methods for detection of the PCR amplicon. Quantitative detection was possible by TaqMan or molecular beacon procedures.

Acknowledgements

The collaboration with D. Cooke, D. Guy and J. Duncan (Scottish Crop Research Institute, Invergowrie, Dundee, GB) is highly appreciated.

Caractérisation et détection de *Phytophthora fragariae* dans les plantes, l'eau et le sol par des méthodes moléculaires

Phytophthora fragariae var. *fragariae* est pathogène seulement sur fraisier et est un organisme de quarantaine A2 pour l'OEPP. 75 isolats de *P. f. fragariae* du monde entier ont été caractérisés par analyse AFLP. Cependant, une méthode sensible, spécifique et rapide est désormais nécessaire et des

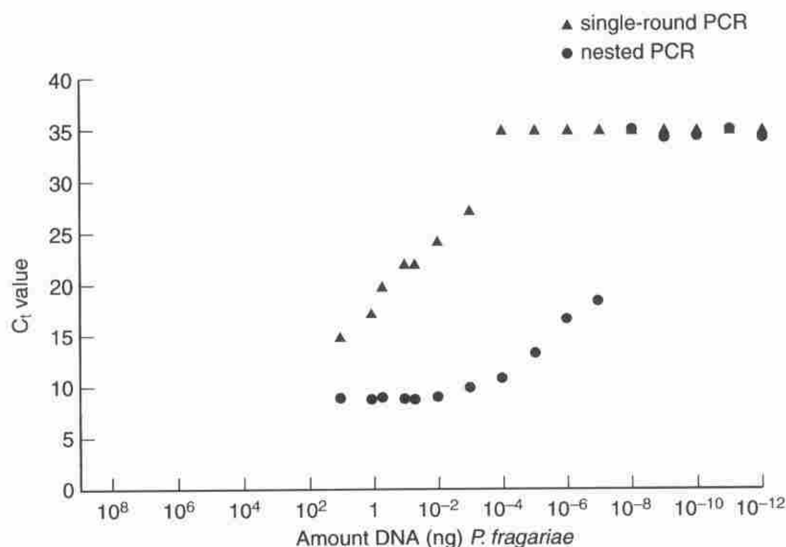

Fig. 10 Graphical representation of C_t-values of TaqMan experiments for a dilution series of *Phytophthora fragariae* DNA for single-round and nested PCR.

méthodes de PCR ont été mises au point. D'après les séquences des régions ITS (internal transcribed spacer) de l'ADNr de plusieurs espèces de *Phytophthora*, des amorces de PCR spécifiques pour *P. fragariae* ont été préparées en collaboration avec le SCRI (Dundee, Ecosse). Ces amorces ont été testées sur de l'ADN extrait de l'eau, du sol et de matériel végétal. Une procédure de PCR ''emboîtée'' a été incorporée au test par appât utilisée actuellement pour améliorer sa sensibilité et sa fiabilité. Plusieurs méthodes ont été étudiées pour détecter l'amplicon de la réaction de PCR: électrophorèse sur gel, DIAPOPS, PCR-ELISA, TAQMAN et balise moléculaire. Ces deux dernières méthodes permettent la quantification de l'ADN cible à l'aide du système de détection ABI 7700.

Характеризация и выявление *Phytophthora fragariae* в растениях, в воде и в почве молекулярными методами

Phytophthora fragariae var. *fragariae* патогеничен только к землянике и числится в списке A2 карантинных вредителей ЕОЗР. 75 изолятов *P. f. fragariae* со всего мира были охарактеризованы методом AFLP. Однако сейчас необходимы чувствительные, специфичные и оперативные методы выявления, поэтому была отработана методика на базе PCR. На основе последовательностей областей Внутренних Транскрибированных Спейсеров (ITS) рДНК нескольких видов *Phytophthora* совместно с SCRI (Данди, Шотландия) были разработаны праймеры PCR, специфичные к *P. fragariae*. Эти праймеры были проверены на ДНК, извлеченной из воды, почвы и растительного материала. «Встроенная» процедура PCR в настоящее время включена в используемое тестирование приманки, что позволяет улучшить ее чувствительность и надежность. Исследуется также несколько методов, позволяющих обнаружить ампликон реакции PCR: гель-электрофорез, DIAPOPS, PCR-ELISA, TAQMAN и Молекулярный Маяк. Два последних метода при использовании системы выявления ABI 7700 позволяют также проводить количественный анализ целевой ДНК.

References

Bonants PJM, Van Gent-Pelzer MPE, Hagenaar-De Weerdt M, Cooke DEL, Lacourt I & Duncan JM (1996) The use of nested PCR for the detection of *Phytophthora fragariae* in strawberry. In *Developments in Plant Pathology, Proceedings of the 4th International Symposium of the European Foundation for Plant Pathology* (eds Dehne HW *et al.*), pp. 43–45. Kluwer, Dordrecht (NL).

Bonants P, Hagenaar-De Weerdt M, Van Gent-Pelzer MPE, Lacourt I, Cooke DEL & Duncan JM (1997) Detection and identification of *Phytophthora fragariae* by the polymerase chain reaction. *European Journal of Plant Pathology* **103**, 345–355.

Bonants P, Hagenaar-De Weerdt M, Kema G, Van Den Boogert P, Waalwijk C & Baayen R (1999) AFLP DNA Fingerprinting of plant-pathogenic fungi at IPO-DLO. *Petria* **9**, 97–104.

Cooke DEL & Duncan JM (1997) Phylogenetic analysis of *Phytophthora* species based on ITS1 and ITS2 sequences of the ribosomal RNA gene repeat. *Mycological Research* **101**, 667–677.

Lee SB & Taylor JW (1992) Phylogeny of five fungus-like protoctistan *Phytophthora* species, inferred from the internal transcribed spacers of ribosomal DNA. *Molecular Biological Evolution* **9**, 636–653.

Nei M & Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences USA* **76**, 5269–5273.

van de Peer Y & de Wachter R (1994) Treecon for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Computional Application Biosciences* **10**, 569–570.

Vos P, Hogers R, Bleeker M, Reijans M, Van De Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M & Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**, 4407–4414.