J. Appl. Genet. 45(1), 2004, pp. 3-15

Review article

Fungal molecular diagnostics: a mini review

Simon D. ATKINS, Ian M. CLARK

Rothamsted Research, Harpenden, Hertfordshire, UK

Abstract. Conventional methods to identify fungi have often relied on identification of disease symptoms, isolation and culturing of environmental organisms, and laboratory identification by morphology and biochemical tests. Although these methods are still fundamental there is an increasing move towards molecular diagnostics of fungi in all fields. In this review, some of the molecular approaches to fungal diagnostics based on polymerase chain reaction (PCR) and DNA/RNA probe technology are discussed. This includes several technological advances in PCR-based methods for the detection, identification and quantification of fungi including real-time PCR which has been successfully used to provide rapid, quantitative data on fungal species from environmental samples. PCR and probe based methods have provided new tools for the enumeration of fungal species, but it is still necessary to combine the new technology with more conventional methods to gain a fuller understanding of interactions occurring in the environment. Since its introduction in the mid 1980's PCR has provided many molecular diagnostic tools, some of which are discussed within this review, and with the advances in micro-array technology and real-time PCR methods the future is bright for the development of accurate, quantitative diagnostic tools that can provide information not only on individual fungal species but also on whole communities.

Key words: fungi, micro-array, molecular identification, PCR, real-time PCR.

Introduction

The ability to accurately identify an organism is fundamental to all aspects of fungal diagnostics and epidemiology whether this is in the field of plant pathology, medical science, environmental studies or biological control. In phytopathology

Received: April 4, 2003. Accepted: May 26, 2003.
Correspondence: S.D. ATKINS, Rothamsted Research, Harpenden, Hertfordshire, UK AL5 2JQ, email: Simon.atkins@bbsrc.ac.uk

early identification of the causative agent of disease is paramount in order to recognise the pathogen, and implement regulations involving control and quarantine. Conventional methods often rely on identification of disease symptoms, isolation and culturing of environmental organisms, and laboratory identification by morphology and biochemical tests. These methods, although the cornerstone of fungal diagnostics, can lead to problems in identification, resulting in incorrect interpretation, diagnosis and ultimately treatment. The methods rely on experienced, skilled laboratory staff, the ability of the organism to be cultured, are time consuming, non-quantitative, prone to contamination and error and in the case of plant and medical pathology often delay treatment.

New, rapid screening methods are being developed and increasingly used in all aspects of fungal diagnostics. These methods include immunological methods, DNA/RNA probe technology and polymerase chain reactions (PCR) technology. How these methods have been implemented in plant pathology has been intensively reviewed, most recently by McCartney et al. (2003). This review concentrates on the use of PCR technology for fungal diagnostics. The past decade has seen many advances in fungal molecular diagnostics, the most prolific being in PCR technology. In contrast to more conventional methods, samples can be tested directly, and isolates do not require culturing. They are rapid, highly specific and can be used to detect minute quantities of fungal DNA from environmental samples before symptoms occur, therefore, allowing implementation of early control methods. With improved DNA extraction methods from environmental samples available, and with adequate controls, PCRs can be run routinely and do not require a high level of expertise for interpretation of the results. PCR technology can also provide very accurate quantitative data supplying the necessary additional information required for control and quarantine decisions, and for assessing how effective fungal agents are in the case of biological control. The ability to design PCR primers to target specific regions of DNA has lead to a greater understanding of fungal ecology, fungal-plant interactions, fungal-pest interactions and fungal-fungal interactions. As more information becomes available on fungal genomics and gene function, the greater the scope of PCR technologies becomes.

As yet, these technologies are still laboratory based, but the aim is to develop PCR/probe based detection kits that can be taken into the field to assess: fungal diseases; the ability of a soil to be suppressive against particular pest through naturally occurring fungal antagonists; or to monitor and assess released fungal strains, providing a new and exciting field of fungal molecular diagnostics.

PCR technology

The development of PCR technology relies on three fundamental steps: 1) the selection of a specific target region of DNA/RNA to identify the fungus; 2) extraction of total community DNA/RNA from the environmental sample; 3) a method to identify the presence of the target DNA/RNA region in the sample.

Selection of target DNA

There are two avenues to this application, selection of the target DNA using specific sequence information from databases, allowing primers to be designed across conserved and variable regions, or cloning and sequencing of arbitrary parts of the fungal genome. The process of primer design is reviewed by MARCHESI (2001). The main area for the development of fungal diagnostics is ribosomal genes (WHITE et al. 1990), present in all organisms and at high copy numbers aiding detection and the sensitivity of the PCR reaction.

The fungal nuclear ribosomal DNA (rDNA) consist of three genes, the large subunit gene (25S), the small subunit gene (18S) and the 5.8S gene, separated by internal transcribed spacer (ITS) regions, in a unit repeated many times. The ITS region is an area of particular importance to fungal diagnostics. It has areas of high conservation and areas of high variability and is an ideal starter for the development of specific PCR primers for identification of fungal species. Universal primers (WHITE et al. 1990) are available for fungi that isolate the regions of the ITS, once cloned these sequences can be compared to the wealth of other sequences in the sequence database and diagnostic primers developed for a particular fungus. The degree of variability within the ITS region, however, may not be sufficient to discriminate sufficiently between fungal species and biovars in some cases, although ATKINS et al. (2003a) discriminated between two varieties of the same nematophagous fungus *Pochonia chlamydosporia* using primers based on the ITS region.

Although the ITS region is the main target, other genes are becoming more widely studied, in particular the β-tubulin gene (FRAAIJE et al. 1999, HIRSCH et al. 2000), and mating type genes (DYER et al. 2001, FOSTER et al. 2002). Development of taxon-specific primers based on these genes is routine and there are many examples in the literature where they have provided discriminative probes and primers (MCCARTNEY et al. 2003). In some cases where the data provided is inadequate, screening arbitrary regions of the genome is often the next step (SCHESSER et al. 1991, MUTASA et al. 1995). Design of probes or primers based on sequence information from this data is often less discriminating as the regions are arbitrarily chosen and there is often little in the database to compare the sequence to, therefore, primers and probes designed can often recognise other organisms.

Once primers have been selected they need to be screened to demonstrate that they are selective for a particular taxon and that false positives do not occur. Figure 1 demonstrates the use of specific primers to detect three fungal species *Pochonia chlamydosporia, Plectosphaerella cucumerina* and *Paecilomyces lilacinus* from soil.

It is more difficult to design PCR based primers that are isolate specific, although with the rapid increase in the size of DNA databases and advances in PCR technology it is becoming easier. The identification of isolates is particularly im-

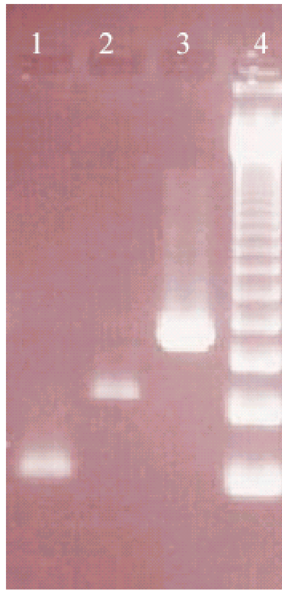

Figure 1. Detection of *Paecilomyces lilacinus* (Lane 1) *Pochonia chlamydosporia* (Lane 2) *Plectosphaerella cucumerina* (Lane 3): Lane 4 = 123 bp marker (Roche, UK). All primers designed to function at 60°C

portant when monitoring released strains and if no isolate specific primer set is available then a combination of techniques is required. Firstly isolates need to be cultured to allow DNA profiling (only effective when DNA is extracted from pure culture). Once DNA has been extracted a DNA profile can be generated. A number of primers exist for this such as RAPDs (random amplified polymorphic DNA: WELSH and MCCLELLAND 1990), REPs (repetitive extragenic palindromic: HIGGINS et al. 1982) and ERICS (enterobacterial intergenic consensus: DE BRUIJN 1992, ARORA et al. 1996). Interpretation and review of these methods is outlined by BRIDGE and ARORA (1998). These profiles can be compared to other isolates in the genus and specific isolates monitored. Figure 2 demonstrates this. Figure 2A shows the use of selective primers to confirm that isolates taken were *P. chlamydosporia* as described by HIRSCH et al. 2000. In figure 2B the gel shows an ERIC PRC profile of *P. chlamydosporia* isolates re-isolated from a field plot where the fungus had been added to control potato cyst nematodes (PCN). All but one of the isolates (Figure 2B; Lane 5) had the profile of the released organism (Lane 2). Lane 5 demonstrates the profile of an indigenous isolate. DNA profiling allows isolates to be discriminated, without this technology fungal isolates could not be identified and knowledge about how populations interact, or how a released isolate competes and survives in the environment could not be ascertained.

DNA extraction protocols

A large number of extraction methods exist for fungi, and the method chosen depends upon the need for rapid extraction, DNA purity and the type of environmental sample the DNA is to be extracted from. For simplicity DNA can be directly extracted from fungal colonies removed from an agar plate and boiled with an extraction buffer (KLIMYUK et al. 1993). This enables DNA to be extracted from a large number of colonies in a short period, and the diluted DNA extract is sufficiently pure for PCR. Better quality DNA is however required for total community extracts when processing environmental samples or when large volumes of DNA are needed, or for long term storage. DNA is extracted from fungal myce-

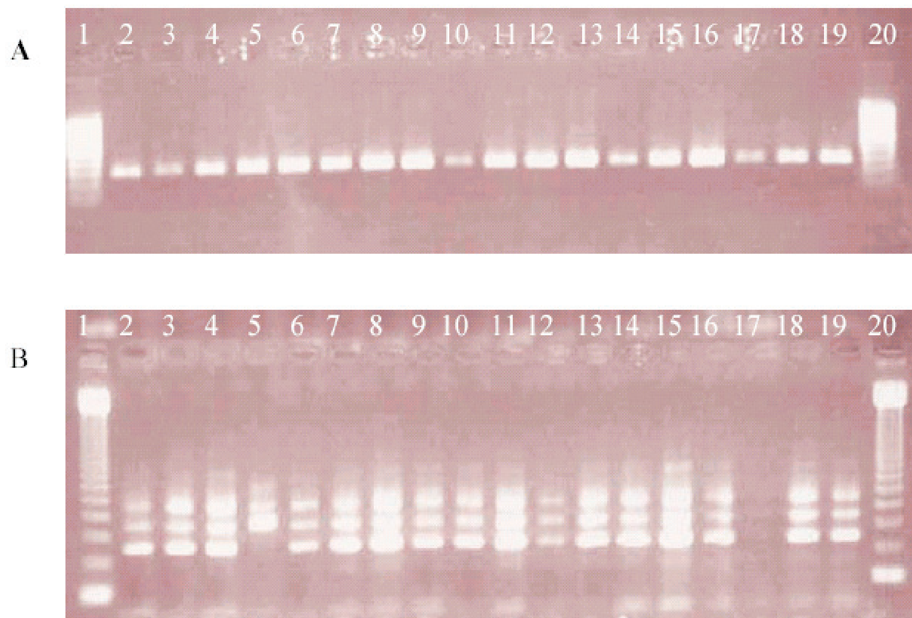

Figure 2. Detection of *Pochonia chlamydosporia* isolate RES280 from colonies isolated from soil. A) Use of selective primers to confirm isolates to be *P. chlamydosporia* (HIRSCH et al. 2000): Lanes 1 and 20 = 100 bp marker; Lane 2 = positive control Isolate RES280; Lanes 3-19 = *P. chlamydosporia* isolates. B) ERIC PCR of isolates to confirm isolate status: Lanes 1 and 20 = 100 bp marker; Lane 2 = positive control Isolate RES280; Lanes 3-19 = *P. chlamydosporia* isolates

lium by a number of physical methods, grinding in liquid nitrogen, bead beating and use of cell wall degrading enzymes, often with detergents such as sodium dodecyl sulphate (SDS). DNA is then purified by phenol and phenol/chloroform washes to remove cell components and DNA degrading enzymes. DNA is precipitated by isopropanol precipitation and salts removed by washing DNA in ethanol and air-drying. The addition of RNase is often used to remove RNA from the sample. The method used in our laboratories is that of LEE and TAYLOR (1990). Co-extraction methods to extract both DNA and RNA from soil exist and allow identification and activity (GRIFFITHS et al. 2000) of fungal species. Extraction of fungal DNA from environmental samples such as plant tissue, or soil is now often kit based for ease and reliability, and a large range of kits exist from a number of companies (Dynal's DNA direct, Mo Bio Laboratories, Inc. Soil DNA isolation kit, Qiagen DNeasy range are examples). The choice of kit is often subjective to the laboratory, for soil we use the soil DNA extraction kit (Mo-Bio Laboratories, UK), and extraction from plant material is done using a DNeasy™ Plant mini kit (Qiagen, UK).

DNA probing technology

This technology uses probes with sequence homology to target DNA and has provided the backbone to most of the current knowledge and still has many uses. DNA probes (either short oligos, or large fragments of DNA several hundred base pairs long) can be used to detect specific sequences in DNA or RNA samples, and can be used for detection and activity studies. The probe is labelled either with a radioactive isotope or a chemiluminescence reporter. Once bound to the DNA sample, which is immobilised on a nitrocellulose or nylon membrane, the probe is detected by exposure to X-ray film (SOUTHERN 1975).

Although PCR technology has reduced the detection time and greatly increased the levels of detection compared to hybridisation techniques for the majority of fungal diagnostics purposes, it still has a very important role to play. It can answer several questions such as copy number, presence or absence of restriction sites, insertion of foreign DNA elements and other roles reviewed in SAMBROOK et al. (1989). Subtractive hybridisation is exploited to demonstrate the activity of genes switched on as a response to a particular stimulus, house-keeping genes that are present in the control sample are removed leaving only those genes that are active as a result of the stimulus, therefore, fungal-host interactions can be more closely studied and gene activity assessed (TOMKINS et al. 1999).

Microarray technology is a method currently under much review and was first introduced by SCHENA et al. in 1995. Initially the advent of high density microarrays made it possible to measure the expression levels simultaneously of all or most genes in a given genome using cDNA as the probe target. However, with the advent of oligonucleotide arrays they can, in theory, be custom made to identify the presence and relative quantity of specific fungal species/isolates using ITS or ribosomal RNA information, as well as functional genes i.e. for pathogenicity and resistance. Here, DNA from an environmental sample can be screened against several hundred to several thousand oligoprobes fixed to a support. The environmental DNA is labelled and the resulting patterns can be compared to a reference set of known organisms. Therefore, this technology would enable all fungi in a particular sample to be detected and quantified. Scaling up of this technology enables hundreds or thousands of different organisms to be screened for simultaneously in the same sample (SERVICE 1998, LIPSHUTZ et al. 1999, WANG 2000). However, the complexity of environmental samples, soil having an estimated 30 million different genes in each gram compared to an estimated 30,000 genes in the human genome, may greatly increase the level of false positives and background, thereby necessitating pre-selective methodologies to isolate specific groups of DNA/RNA. The use of fluorescent probes has allowed direct *in situ* analysis of organisms, even those that are unculturable, increasing the knowledge of interactions occurring. This technique is refereed to as fluorescent *in situ* hybridisation (FISH) and has been used in the study of fungal interactions (LI et al. 1997, SPEAR et al. 1999, SCHRODER et al. 2000).

PCR

PCR is a fundamental part of fungal molecular diagnostics. Since its introduction in the mid 1980's it has become the cornerstone of DNA technology and has spawned an increasing number of associated technologies. PCR is the enzymatic exponential amplification of a specific target region using short primers, leading to detectable amounts of amplified DNA from one or a few original sequences. Conventional PCR is not quantitative but is qualitative and has been used to detect, monitor and identify fungi from a whole range of environmental samples and is the core of fungal molecular diagnostics. Associated technologies include methods that increase sensitivity and are more discriminative such as nested PCR in which a second round of PCR using a separate primer set internal to the first round increases amplification of a specific region in the first PCR amplified target gene. This is not only more specific, but is also much more sensitive allowing the detection of the target DNA several fold lower than conventional PCR. Reverse transcription PCR (rt PCR) exploits the use of the enzyme reverse transcriptase to convert RNA to cDNA before PCR amplification. This method enables gene activity to be investigated and is an important step forward in understanding gene function and activity. Detection of several different fungal isolates in the same PCR reaction can be achieved if the highly specific primers are designed to anneal at the same temperature and the PCR products are designed to be of different sizes to allow discrimination (Figure 1). Fluorescent *in situ* PCR uses fluorescently labelled primers or probes to detect and localise fungi within fixed environmental samples after semi-permeabilizing (STERFLINGER et al. 1998). Following PCR the fluorescence from the labelled primers/probe is detected using a confocal microscopy. This technique not only allows the direct detection of organisms from an environmental sample but also shows its spatial distribution and interactions with host and other organisms. BAGO et al. (1998), utilised *in situ* PCR to detect and localise arbuscular mycorrhizal infections. The scope of PCR technology is endless, it can be used to investigate a single species (Figure 1), or to investigate whole communities (HEUER et al. 1997, RANJARD et al. 2001, DAHLLOF 2002).

Quantification of PCR

One of the main limitations of PCR has been quantification. PCR is ideal for detection of small amounts of target signal, but without quantification, decisions on whether to treat fungal disease, or to assess the effect of the fungus are delayed until more conventional methods of quantification such as culturing on agar plates can be performed. Techniques have now been developed that allow quantification of PCR target signals. As outlined above, fluorescent *in situ* PCR provides a direct method of quantification of a fungus from a fixed sample. Other methods include competitive PCR. This method involves an additional target sequence being added to the PCR mixture. The PCR product is of different size to the fungal DNA

to be PCR'ed, but is recognised by the same primers and competes for them hence the referral to competitive PCR. The added target is serial diluted across a range of PCR reactions and the level is previously calculated. Quantification is done visually on a gel, when the ratio of both target signals is the same then the level of unknown fungal DNA matches the quantity of the added DNA and, therefore, the level of fungus in the sample can be quantified. This method has been used to successfully quantify the nematophagous fungus *Pochonia chlamydosporia* from soil (MAUCHLINE et al. 2002).

Real-time PCR

A less time consuming process involves the use of real-time PCR. During real-time PCR, the accumulation of PCR products is measured automatically during each cycle in a closed tube format using an integrated cycler/fluorimeter. Direct measurement of the accumulated PCR product allows the phases of the reaction to be monitored. The initial amount of target DNA in the reaction can be related to a cycle threshold (*ct*) defined as the cycle number at which there is a statistically significant increase in fluorescence. Target DNA can then be quantified by construction of a calibration curve that relates *ct* to known amounts of template DNA. Figure 3 shows an example of a calibration curve for real-time PCR based on a serial dilution of DNA from the nematophagous fungus *Plectosphaeralla cucumerina*. DNA was extracted from two field sites in the UK where PCN populations were naturally declining, Spalding and Ely. The level of *P. cucumerina* DNA was quantified and the population of the fungus was ascertained by comparison of the *ct* value to the standard curve. The real-time data correlated well with other more conventional methods to assess the population of the fungus (ATKINS et al. 2003b). PCR products can be monitored using either fluorescent DNA intercalating dyes such as SYBR Green I, or sequence specific probe based assays using TaqMan probes (WITTWER et al. 1997) and molecular beacons (TYAGI et al. 1998*)*. The use of intercalating dyes is a cheaper option, but is less specific as the dye binds to all double-stranded DNA present, and primer dimers can result in a false reading. Sequence specific probes add a greater degree of specificity to the reaction (LIVAK et al. 1995). The probe consists of a fluorescent reporter dye and a quencher, once the probe has bound and the *Taq* enzyme elongates the target gene the reporter dye is released and fluorescence detected. The specificity of the probe allows single nucleotide polymorphisms (SNPs) to be targeted (TYAGI et al. 1998*)*, therefore, allowing isolate specific probes to be designed. The use of probes with different reporter dyes allows isolates to be monitored together, a massive step forward in trying to understand how isolates interact, and trying to understand the complexity of populations. Currently, Lux™ Fluorgenic primers are replacing TaqMan probes in real-time PCR technology to provide a cheaper, reliable method with the specificity of TaqMan probes without some of the restraints (NAZARENKO et al. 2002). Real-time PCR has been used

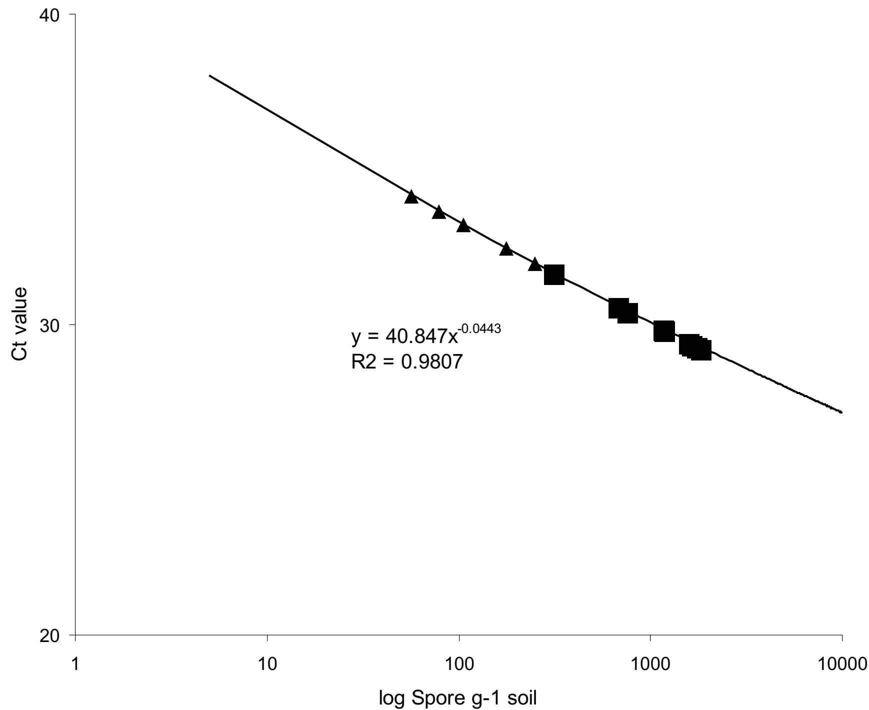

Figure 3. Standard curve for quantification of the nematophagous fungus *Plectosphaerella cucumerina* based on ct values from a serial dilution of DNA. Plotted are ct values from soil samples taken from two field sites in the UK, Ely (▲) and Spalding (■) where nematode populations were being controled

successfully to quantify a number of plant pathogens (BOHM et al. 1999, BATES et al. 2001, CULLEN et al. 2001, WINTON et al. 2002) and the technique has much promise for the future.

Discussion

Fungal diagnostics has increased dramatically with the introduction of molecular tools, in particular that of PCR. There are many applications for this technology and the aim now is to produce products that can detect fungal isolates at source without the need for samples to be taken back to the laboratory for processing. Advances in automation of many of these processes means that rapid screening of samples can now be undertaken, and commercialisation of detection of fungal pathogens subject to government regulations on fungal disease levels and quarantine are now feasible. PCR technology has many applications in plant pathology,

extensively reviewed by McCartney et al. (2003). These areas include diagnosis and quantification of fungal diseases, study of fungicide resistance, study of pathogen mating-types, identification of virulent pathotypes and detection of airborne pathogen inoculum.

PCR technology has opened up many avenues of investigation, not only in plant pathology, but in all aspects of mycology. Yet it must be stated that this technology is only a tool and must be combined with other techniques to try and gain a wider picture of what may be occurring. For example, to detect and quantify a fungus from soil does not necessarily mean that it is having an effect on, say, the pest nematode population although reports have indicated that this is so (Atkins et al. 2002). Of more scientific relevance is, therefore, the identification of fungi found within nematode eggs added as a bait to the soil. Here, PCR plays an important role in identifying the presence of a particular fungus from individual eggs and, therefore, its role in suppression of the pest population can be assessed. This is an example of where the combination of a number of techniques is required to derive a conclusion and results from direct PCR on total community DNA may be misleading (Atkins et al. 2003a).

Since the introduction of PCR in the mid 1980's the technology has greatly increased, becoming more widespread and affordable. Each year the technology is improved and new aspects are considered and tested. The future holds many challenges, but will prove to be an exciting time. Molecular diagnostics is making a huge impact on research in mycology, and, in time, its importance will increase in the monitoring, detection, quantification and identification of fungi, leading to better management and control of plant, animal and human disease, and investigation of beneficial fungi.

Acknowledgements. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the UK.

REFERENCES

Arora D.K., Hirsch P.R., Kerry B.R. (1996). PCR-based molecular discrimination of *Verticillium chlamydosporium* isolates. Mycol. Res. 7: 801-809.

Atkins S.D., Sosnowska D., Evans V.J., Clark I.M., Hirsch P.R., Kerry B.R. (2002). Investigation of three nematophagous fungi in two potato cyst nematode suppressive soils. IOBC Bulletin (in press).

Atkins S.D., Hidalgo-Diaz L., Clark I.M., Morton C.O., Montes De Oca N., Gray P.A., Kerry B.R. (2003a). Approaches for monitoring the release of *Pochonia chlamydosporia* var. *catenulata,* a biological control agent of root-knot nematodes. Mycol. Res. 107: 206-212.

Atkins S.D., Clark I.M., Sosnowska D., Hirsch P.R., Kerry B.R. (2003b). Detection and quantification of *Plectosphaerella cucumerina*, a potential biological control agent of potato cyst nematodes, by using conventional PCR, real-time PCR, selective media, and baiting. Appl. Environ. Microbiol. 69: 4788-4793.

BAGO B., PICHE Y., SIMON L. (1998). Fluorescently-primed *in situ* PCR in arbuscular mycorrhizas. Mycol. Res. 102: 1540-1544.

BATES J.A., TAYLOR E.J.A., KENYON D.M., THOMAS J.E. (2001). The application of real-time PCR to the identification, detection and quantification of *Pyrenophora* species in barley seed. Mol. Plant Pathol. 2: 49-57.

BOHM J., HAHN A., SCHUBERT R., BAHNWEG G., ADLER N., NECHWATAL J., OEHLANN R., OSSWALD W. (1999). Real-time quantitative PCR: DNA determination in isolated spores of the mycorrhizal fungus *Glomus mosseae* and monitoring of *Phytophthora infestans* and *Phytopthora citricola* in their respective host plants. J. Phytopathol. 147: 409-416.

BRIDGE P.D., ARORA D.K. (1998). Interpretation of PCR methods for species definition. In: Applications in PCR Mycology (P.D. Bridge, D.A. Arora, eds.). CABI Publishing UK: 63-84.

CULLEN D.W., LEES A.K., TOTH I.K., DUNCAN J. M. (2001). Conventional PCR and real-time PCR detection of *Helminthosporium solani* in soil and on potato tubers. Eur. J. Plant Pathol. 107: 387-398.

DAHLLOF I. (2002). Molecular community analysis of microbial diversity. Curr. Opinion Biotechnol. 13: 213-217.

DE BRUIJN F.J. (1992). Use of repetitive (Repetitive Extragenic Palindromic and Enterobacterial Repetitive Intergenic Consensus) sequences and the Polymerase Chain Reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. 58: 2169- 2187.

DYER P.S., FUMEAUX P.A., DOUHAN G., MURRAY T.D. (2001). A multiplex PCR test for determination of mating type applied to the plant pathogens *Tapesia yallundae* and *Tapesia acuformis.* Fungal Genetic Biology 33: 173-180.

FOSTER S.J., ASHBY A.M., FITT B.D.L. (2002). Improved PCR-based assays for pre-symptomatic diagnosis of light leaf spot and determination of mating type of *Pyrenopeziza brassicae* on winter oilseed rape. Eur. J. Plant Pathol. 108: 374-383.

FRAAIJE B., LOVLL D.J., ROHEL E.A., HOLLOMON D.W. (1999). Rapid detection and diagnosis of *Septoria tritci* epidemics in wheat using a polymerase chain reaction/Pico green assay. J. Appl. Microbiol. 86: 701-708.

GRIFFITHS R.L., WHITELEY A.S., O'DONNELL A.G., BAILEY M.J. (2000). Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA and rRNA-based microbial community composition. Appl. Environ. Microbiol. 66: 5488-5491.

HEUER H., KRSEK M., BAKER P., SMALLA K., WELLINGTON E.M.H. (1997). Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl. Environ. Microbiol. 63: 3233-3241.

HIGGINS C.F., AMES G.F., BARNES W.M., CLEMENT J.M., HOFUNG M. (1982). A novel inter-cistonic regulatory element of prokaryotic operons. Nature 298: 760-762.

HIRSCH P.R., MAUCHLINE T.H., MENDUM T.A., KERRY B.R. (2000). Detection of the nematophagous fungus *Verticillium chlamydosporium* in nematode-infested plant roots using PCR. Mycol. Res. 104: 435-439.

KLIMYUK V.I., CARROLL B.J., THOMAS C.M., JONES J.D.G. (1993). Alkali treatment for rapid preparation of plant material for reliable PCR analysis. Plant Journal 3: 493-494.

LEE S.B., TAYLOR J.W. (1990). Isolation of DNA from fungal mycelia and single spores In: PCR Protocols: A guide to Methods and Applications. (M.A. Innis, D.H. Gelfand, J.S. Sninsky, T.J. White, eds.). Academic Press London, UK: 282-287.

LI S., SPEAR R.N., ANDREWS J.H. (1997). Quantitative fluorescent *in situ* hybridisation of *Aureobasidium pullulans* on microscopic slides and leaf surfaces. Appl. Environ. Microbiol. 63: 3261-3267.

LIPSHUTZ R.A., FODER S.P.A., GINGERAS T.R., LOCKHART D.J. (1999). High density oligonucleotide arrays. Nature Genetics Supplement 21: 20-24.

LIVAK K.J., FLOOD S.J.A., MARMERO J., GIUSTI W., DEETZ K. (1995). Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR products and nucleic acid hybridisation. PCR Methods and Applications 4: 357-362.

MARCHESI J.R. (2001). Primer design for PCR amplification of environmental DNA targets. In: Environmental Molecular Microbiology: Protocols and Applications (P.A. Rochelle, ed.). Horizon Scientific Press, Wymondham, UK: 43-54.

MAUCHLINE T.H., KERRY B.R., HIRSCH P.R. (2002). Quantification in soil and the rhizosphere of the nematophagous fungus *Verticillium chlamydosporium* by competitive PCR and comparison with selective plating. Appl. Environ. Microbiol. 68: 1846-1853.

MCCARTNEY H.A., FOSTER, S.J., FRAAIJE B.A., WARD E. (2003). Molecular diagnostics for fungal plant pathogens. Pesticide Management Science 59: 129-142.

MUTASA E.S., CHWARSZCZYNSKA D., ADAMS M.J., WARD E., ASHER M.J.C. (1995). Development of PCR for the detection of *Polymyxa beae* in sugar beet roots and its application in field studies. Physiol. Molecular Plant Pathol. 47: 303-313.

NAZARENKO I., LOWE B., DARFLER M., IKONOMI P., SCHUSTER D., RASHTCHIAN A. (2002). Multiplex quantitative PCR using self-quenched primers with a single fluorophore. Nucleic Acids Res. 30: e37.

RANJARD L., POLY F., LATA J.C., MOUGEL C., THIOULOUSE J., NAZARET S. (2001). Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: Biological and Methodological variability. Appl. Environ. Microbiol. 67: 4479-4487.

SAMBROOK J., FRITSCH E.F., MANIATIS T. (1989). Molecular cloning. A laboratory manual. Cold Spring Harbour Laboratories, Cold Spring Harbour, USA.

SCHENA M., SHALON D., DAVIS R.W., BROWN P.O. (1995). Quantitative monitoring of gene-expression patterns with a complementary-DNA microarray. Science 270: 467-470.

SCHESSER K., LUDER A., HENSON J.M. (1991). Use of polymerase chain reaction to detect the take-all fungus, *Gaeumannomyces graminis,* in infected wheat plants. Appl. Environ. Microbiol. 57: 553-556.

SCHRODER S., HAIN M., STERFLINGER K. (2000). Colorimetric *in situ* hybridisation (CISH) with digoxigen-labeled oligonucleotide probes in autofluorescent hyphomycetes. Intern. J. Microbiol. 3: 183-186.

SERVICE R.F. (1998). Microchip arrays put DNA on the spot. Science (Washington) 282: 396-399.

SOUTHERN E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503-517.

SPEAR R.N., LI S., NORDHIEM E.V., ANDREWS J.H. (1999). Quantitative imaging and statistical analysis of fluorescence *in situ* hybridisation (FISH) of *Aureobasidium pullulans.* J. Microb. Methods 35: 101-110.

STERFLINGER K., KRUMBIEN W.E., SCHWIERTZ A. (1998). A protocol for PCR *in situ* hybridisation of hyphomycetes. Intern. J. Microbiol. 1: 217-220.

TOMKINS J.P., MAHALINGAM R., SMITH H., GOICOECHEA J.L., KNAP H.T., WING R.A. (1999). A bacterial artificial chromosome library for soybean P1437654 and identification of clones associated with cyst nematode resistance. Plant Mol. Biol. 41: 25-32.

TYAGI S., BRATU D.P., KRAMMER F.R. (1998). Multicolor molecular beacons for allele discrimination. Nature Biotechnology 16: 44-53.

WANG J. (2000). From DNA biosensors to gene chips. Nucleic Acids Res. 28: 3011-3016.

WELSH J., MCCLELLAND M. (1990). Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18: 7213-7218.

WHITE T.J., BRUN 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. (M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White, eds.). Academic Press, San Diego, USA: 315-320.

WINTON L.M., STONE J.K., WATRUD L.S., HANSEN E.M. (2002). Simultaneous one-tube quantification of host and pathogen DNA with real-time polymerase chain reaction. Phytopathology 92: 112-116.

WITTWER C.T., HERMANN M.G., MOSS A.A., RASMUSSEN R.P. (1997). Continuous fluorescence monitoring of rapid cycle DNA amplification. BioTechniques 22: 130-138.