Session 4

Diagnostics for Lower Fungi, Bacteria and Nematodes

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MOLECULAR DIAGNOSTICS OF PLANT AND INSECT PARASITIC NEMATODES

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ABSTRACT

The Internally Transcribed Spacer region (ITS) of the ribosomal DNA gene repeats is evaluated as a "genetic yardstick" for nematode identification. Individual nematodes can be added directly to Polymerase Chain Reaction (PCR) mixtures, and the amplified DNA is assessed by product size and sequence on high resolution agarose gels. The size of the ITS region is generally consistent for species within a genus, but varies among genera. Digestion of the amplified product by restriction endonucleases often produces species specific patterns for both agricultural pest species and nematodes from unmanaged ecosystems. The gel patterns, species photographs, descriptions, and distributions are being added to a database available through the World Wide Web.

INTRODUCTION

Current estimates of the number of nematode species range from 50,000 to over 1,000,000 (May, 1988) Although the majority of nematodes are not pest species, the diversity of the phylum creates a challenging task for nematode identification. Agricultural pest species are typically microscopic, soil-dwelling animals, possessing few diagnostic characters, which are usually discriminated by subtle morphological differences. These sometimes intractable taxonomic traits are often cited as justification for a molecular approach to nematode taxonomy (Powers, 1992). To this biological justification could be added the concern regarding the general loss of taxonomic expertise in nematology (Ferris, 1994) as well as the increased emphasis on identification of all components of the soil ecosystem (Bongers, 1990; Groombridge, 1992). Molecular diagnostics could provide greater precision for the identification of a wider range of nematodes, at the same time increasing accessibility of the diagnostic skills and tools.

HOW CAN MOLECULAR TECHNIQUES HELP?

PCR has turned what was once considered a serious obstacle in nematode identification (small size) to an advantage. Fortuitously, most soil-dwelling nematodes are approximately a millimeter in size, composed of 1,000 -2,000 cells. When added to a PCR reaction mixture, individual nematodes provide a suitable amount of DNA template without overwhelming the reaction with inhibiting cellular debris (Powers & Harris, 1993). With the appropriate oligonucleotide primers, a

discrete amplification product can be produced and evaluated within hours of removing the nematode from the soil. A second feature of nematodes, their extreme genetic diversity, can also be exploited for identification. Hundreds of millions of years of evolution has resulted in groups of nematodes that may appear similar at the morphological level, yet divergence at the DNA level is extensive. Genetic variation in the form of nucleotide substitutions or insertions and deletions offer convenient genetic markers for identification. Theoretically, these markers could provide an identification protocol that is rapid, sensitive, simple, and applicable to all nematodes. As such, they would constitute a "genetic yardstick" for species identification.

THE GENETIC YARDSTICK CONCEPT

The concept of a genetic yardstick has several interpretations (Avise, 1994; Lymbery, 1992).

1. As a standard unit to objectively measure all organisms. In this case there is no special link to a specific taxonomic status, the yardstick is just a common method to evaluate taxa of interest. Allozymes are a good example of a method used to evaluate organisms ranging from plants to animals. Subsequently it was demonstrated that often (but by no means always) the interspecific genetic distances exceeded a certain value (Avise & Aquadro, 1982). When these values are used as a point of reference to assess species boundaries, the genetic concept acquires a second, more specific, meaning.

2. As a common means to evaluate species; providing a criteria by which to make judgments about species status. Low estimates of genetic distance, based on allozymes analysis, have frequently been viewed as support for conspecific status. Its widely recognized that distance estimates can only serve as a rough guide to species status (Avise, 1994) and that the potentially diverse modes of speciation could lead to equally diverse genetic distances (Harrison, 1991). Yet, estimates of genetic distance are increasingly used in the fields of conservation biology and ecology to determine the "uniqueness" of populations (Volger *et al.*,1993; Bowen *et al.*,1991). Therefore, while it is naive to expect a single molecule to serve as a reference for all species, in a group like nematodes where 90 % of the species may be unknown or poorly characterized, even an imperfect genetic yardstick for species will aid diagnostic efforts.

The ITS Region

One potential genetic yardstick is the Internally Transcribed Spacer (ITS) region of the ribosomal DNA genes. The 18S, 5.8S, 28S ribosomal genes are linked together as a series of repeating genes separated by noncoding "spacer" DNA. The spacer DNA between the 18S and 5.8S, and 5.8S and 28S ribosomal genes are referred to as ITS1 and ITS2 respectively (Figure 1). From a diagnostic perspective, these spacer regions have the desirable characteristic of relatively rapid evolution, such that spacer regions from different species are generally quite genetically divergent. Yet the flanking ribosomal genes are highly conserved facilitating the design of PCR amplification

primers of taxonomically wide application. The primer set used in our laboratory to amplify ITS1 has produced a product in tests with nematodes from over 30 genera in both Adenophorean and Secernentean classes. Amplified products often vary in size among nematode genera due to insertions and deletions in the spacer region. When amplified ITS1 products are compared on agarose gels, this size variation provides a convenient initial screening for nematode identity (Figure 2). Nematodes species in the same genus, typically have similar sized amplification products, whereas size variation between genera is common.

A second level of resolution among amplified products of the ITS1 region is provided by endonuclease digestion and electrophoretic separation of the digestion products. Since ITS1 amplification products of congeneric species comigrate on agarose gels, discrimination at this taxonomic level requires a comparison of digestion patterns. Figure 3 shows a digestion pattern of amplified ITS1 DNA from 15 individual nematodes in the genus *Helicotylenchus* (spiral nematodes) from three populations. Nematodes were extracted from a soil sample of three plant hosts (bluegrass, maize, and big bluestem), added to a PCR reaction with the ITS1 primer set, evaluated initially on an agarose gel, and then the amplified product was digested with the restriction endonuclease *Hinf*I and evaluated on a second agarose gel. The genetic differences are readily apparent between these species that are otherwise virtually indistinguishable morphologically. An increasing number of plant and insect parasitic nematodes are being assayed by this method and a database of ITS1 profiles is being developed for online electronic access:
(http://ianrwww.unl.edu/ianr/plntpath/nematode/wormhome/ wormdiag.htm).

Large genetic differences are not necessary in order to discriminate nematodes by this PCR-RFLP method. *Heterorhabditis indicus* can be distinguished from other tropical Heterorhabditid species by ITS1 amplification and digestion with *Taq*I. Nucleotide sequencing of this region has shown that *H. indicus* differs from the other tropical species by a single nucleotide substitution which has created the restriction site (Adams, Burnell and Powers, unpublished). In contrast, the root knot nematode species *Meloidogyne chitwoodi* and *M. hapla* differ in over 15 % of their nucleotide sites in ITS1. (Powers &Adams, submitted). At least three restriction site differences are fixed for the discrimination of the two potato cyst nematodes, *Globodera pallida* and *G.rostochiensis*. (Fleming & Powers, 1995). Other cyst species in the genus *Heterodera* exhibit a wide range of genetic distances (Ferris *et al*, 1993;1994). For example, the extensive differences between the oat cyst nematode *Heterodera avenae* and the sugar beet cyst nematode *H. schachtii,* make alignment of nucleotide sequences challenging (Ferris *et al*., 1994). However, within the *H. schachtii* species group, the clover cyst nematode *H. trifolli* and the soybean cyst nematode *H. glycines* differ by only a few fixed nucleotide substitutions (Ferris *et al*., 1993). One of these substitutions affects a *Fok*I restriction site which permits discrimination of the soybean and sugar beet cyst nematodes (Powers, personal observation). It does not appear that the ITS region will aid identification of the three major mitotically parthenogenetic root-knot nematode species. *Meloidogyne arenaria, M. incognita,* and *M. javanica* have identical nucleotide sequences in this region (Powers & Adams, submitted).

DIAGNOSTIC TARGETS

The lack of species specific nucleotide differences in the ITS region in three of the major *Meloidogyne* species demonstrates that no single genetic locus will be sufficient for all diagnostic determinations. In many cases, the diagnostic target may exist at the subspecific taxonomic level or necessitate the recognition of distinct clonal lineages of parthenogenetic species. Examples of discriminations that will require more exhaustive molecular comparisons for diagnosis include pathotypes of potato cyst nematode, host races of soybean cyst, root-knot nematode races, cold adapted strains of insect parasitic nematodes and viruliferous versus non-virus transmitting isolates of *Xiphinema.* Kaplan and coworkers (personal communication) have recently systematically screened over 300 RAPD PCR primers and found six PCR products that differentiated the citrus parasite *Radopholus citrophilus* from *R. similis.* Sequencing of several of these products resulted in the creation of new sets of primers that amplify size specific products for each species. Similar approaches, using an initial RAPD screening to uncover diagnostic DNA polymorphism, are being tested on numerous plant and insect parasitic nematodes. It is conceivable that within several years primers will be developed for identification of many of the currently important agricultural pest species. However, it is important to remember that today's pest species represent a small fraction of the total parasitic nematode diversity. The application of a genetic yardstick approach coupled with rapid, convenient access to nematode databases may decrease the amount of time necessary to address future nematode problems.

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Figure 1. The Internally Transcribed Spacer Region of the ribosomal DNA. This repeating series of genes consists of three ribosomal coding genes flanked by a nontranscribed spacer region (NTS) and separated by internal transcribed spacers (ITS).

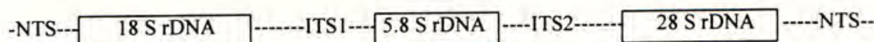


Figure 2. ITS1 size variation among plant parasitic nematodes. Each lane in this 1.0% agarose gel contains the PCR amplification product of a single nematode. The size of these amplification products ranges from 0.45 kb to approximately 1.1 kb. C1=*Criconemella* sp.; H1=*Heterodera schachtii*; H2=*Heterodera trifolii*; G1=*Globodera tabacum*; H3=*Heterodera mani*; M1=*Meloidogyne mayaguensis*; M2=*Meloidogyne konaensis*; P1=*Pratylenchus agilis*; P2=*Pratylenchus* sp.; T1=*Tylenchus* sp.; E1=*Helicotylenchus* sp.; Q1=*Quinisulcius acutus*; H1=*Hoplolaimus galeatus*; B1=*Belonolaimus longicaudatus*; X1=*Xiphinema* sp.; L1=*Longidorus breviannulatus*

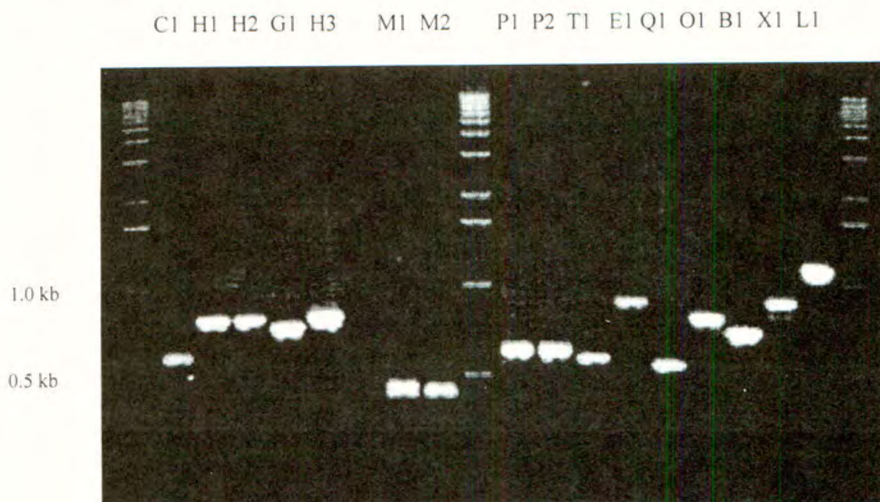
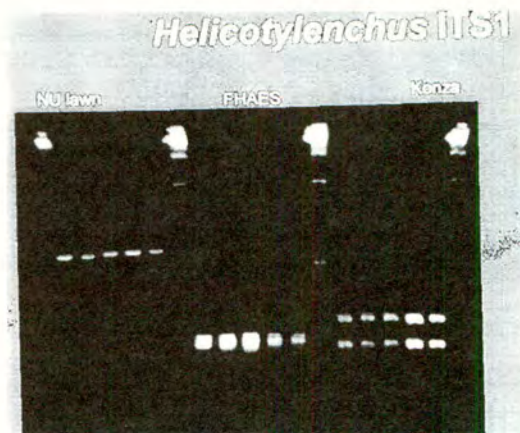


Figure 3. ITS1 digestion patterns from populations of spiral nematodes. NU lawn is an isolate from a bluegrass lawn on the University of Nebraska campus. FHAES is from maize on the Fort Hayes Agricultural Experiment Station in Kansas. Konza is from Indiangrass on the Konza Tallgrass Prairie Reserve in Kansas.



PRE-HARVEST DETECTION OF BACTERIAL AND FUNGAL ROTS OF STORED ONION BULBS.

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ABSTRACT

Rotting of stored onions can result in financial loss to growers. The major causes of these rots are *Pseudomonas gladioli* pv. *alliicola* and *Botrytis allii*. Both pathogens are disseminated in growing crops but the diseases are only expressed in stored bulbs. The ability to determine the incidence of bulb infection before the crop is harvested would allow the grower to assess the storage potential of a crop and manage sales of bulbs in order to minimise losses. A test was developed for the simultaneous detection of both pathogens from bulbs sampled three weeks pre-harvest. The test employs an indirect ELISA to identify soluble antigens extracted from bulb neck tissue by a freeze-thaw procedure. There was good correlation between pre-harvest test results and the incidence of rotting in stored bulbs.

INTRODUCTION

The viability of the onion industry requires that bulbs are stored for periods of up to seven months from the date of harvest. Following harvest, bulbs are stacked in drying bins and surface dried using forced air heated up to 30°C. The temperature is then reduced to 25-30°C and recirculated air at 65-75% R.H. is blown through the stack until the necks of the bulbs are dry. At this stage the temperature is reduced progressively in steps to prevent condensation until the stack can be maintained using either ambient night air or refrigerated air cooled to -2°C depending on the length of storage required.

Rotting of stored bulbs can occur due to the presence of pathogenic micro-organisms following infection during the growing stage of the plants. In the United Kingdom the two main causal agents of storage rots are *Pseudomonas gladioli* pv. *alliicola* (*P.g.a.*), producing bacterial mushy rot, and *Botrytis allii* (*B.a.)* (syn. *B. aclada*), causing neck rot (Maude & Presly, 1977). The incidence of *P.g.a.* infection can rise during a season when high rainfall occurs during spring or early summer and the organism is spread between plants by rain splash. Plant debris and dust containing spores from previous harvests can be a source of *B.a.* infection (Maude, 1983).

Deterioration of bulbs can occur immediately after drying in the case of *P.g.a.*, which is favoured by high temperatures, or after several weeks in storage in the case of *B.a.* which grows at lower temperatures. Early signs of rot due to *P.g.a.* are softening of the bulbs and brown discoloration of internal tissue begining usually in the neck region. *B.a.* infection often produces accumulations of grey/black spores around the neck

region of diseased bulbs. Internally the bulbs generally remain firm but growth of the organism occurs between the scales leading to browning of the tissue.

Post-harvest management of onion crops would be more effective if knowledge of the presence of the two pathogens was available at the time of harvest. A rapid serological technique employing antiserum conjugated to *Staphylococcus aureus* is available for the detection of *P.g.a.* (Davies & Taylor, 1994, Davies *et al.*, 1996) but this method is not appropriate for *B.a.* Techniques for isolating and culturing the pathogens are also available but are time consuming and require expertise in identification. Also, for this purpose, tissue samples must be taken from an area of bulb containing viable pathogens. This can be difficult to locate during the early stages of infection or in the absence of obvious disease symptoms.

The ability to detect antigenic material of *B.a.* in onion tissue ahead of mycelial growth has been demonstrated *in vivo* using scales inoculated with *B.a.* and probed with polyclonal antibody raised to surface washings and cell wall and cytoplasmic extracts of this fungus (Linfield *et al.*, 1995). This paper describes the application of an enzyme-linked immunosorbent assay (ELISA) detecting *P.g.a.* and *B.a.* in two experimentally infected crops by testing a sample of bulbs lifted three weeks before the main crop was harvested.

MATERIALS AND METHODS

Preparation of polyclonal antibodies

Polyclonal antibody was prepared against surface washings of *B.a.* (IMI 292 066). The methods of producing the antibody, its sensitivity and specificity have been previously described (Linfield *et al.*, 1995). Antiserum against *P.g.a.* was prepared using an isolate (HRI 1898C) of proven pathogenicity. A female New Zealand White rabbit was immunized with 5 intramuscular injections at two week intervals each containing 0.5 ml of the bacterial suspension (10^{10} cell/ml) emulsified with 0.5 ml of Freund's incomplete adjuvant. Specificity of the antiserum was tested against 46 plant pathogenic bacteria, including the homologous isolate and one other pathogenic *P.g.a.* isolate, and representatives of species and pathovars of the genera *Agrobacterium, Bacillus, Clavibacter, Enterobacter, Erwinia, Flavobacterium, Lactobacillus, Pseudomonas, Rhizobium, Rhodococcus* and *Xanthomonas.*. Sensitivity was tested against serial dilutions of extract from experimentally infected bulb tissue.

Field experiment

Experimental crops were grown at Horticulture Research International sites at Kirton, Lincolnshire and Stockbridge House, Yorkshire. Undressed seed of cv. Hysam was sown to give a density of 45 plants/m^2. Plot sizes were 1.83 m x 10 m with five rows/plot and both sites were drilled during the third week of March 1994. Standard herbicide but no fungicide was applied to the growing crops. There were four treatments in a five replicate fully randomised design.

A. Untreated control
B. Plants sprayed with *P.g.a.* suspension
C. Plants sprayed with *B.a.* conidial suspension
D. Plants sprayed with both pathogens.

The *P.g.a.* inoculum was prepared from isolate HRI 1898C. Bacteria from 100 plates containing confluent growth at 27°C on King's medium B (King *et al.*, 1954) were harvested into 15 litres of autoclaved tap water giving a suspension of 1.5 X 10^9 organisms/ml. *B.a.* was grown on 100 plates of prune, lactose, yeast, streptomycin and erythromycin medium (PLYSE) (Maude, 1963) for 28 days at ambient temperature in daylight. Conidia were harvested into 15 litres of autoclaved tap water giving a suspension of 1.5 X 10^4 spores/ml. A further 15 litres of suspension was prepared containing both organisms at the concentrations given above. Tween 20 was added to each suspension to give a final concentration of 0.025 % wetting agent. The crops were treated eight weeks before harvest by spraying the inoculum suspension onto the lower foliage/neck region of the plants using a knapsack sprayer. The *P.g.a.* application (treatment B) was repeated one week later.

Fifty bulbs were sampled from each replicate plot three weeks before the proposed harvest date. These were lifted in a block across all five rows starting 1 m from the end of the plot. The remaining bulbs were harvested during the last week of August and dried and stored as described above. Stored bulbs (50/replicate) were sampled at one and four months post-harvest.

Preparation of bulbs, extraction of antigen and ELISA testing

The pre-harvest bulbs sampled from plots inoculated with *P.g.a.* and the bulbs inoculated with both pathogens were incubated at 30°C for two weeks to encourage multiplication of *P.g.a.* This incubation step was not used on the samples of stored bulbs. The number of bulbs in each sample showing visible signs of rot was recorded. A small portion of tissue was removed from five of the bulbs in each treatment to confirm the presence of the pathogens by culturing. Where possible, these samples were taken from bulbs showing obvious symptoms of disease. Antigen extraction was carried out on all bulbs (pre- and post-harvest) by removing the dry outer scales and cutting a 1 cm thick transverse slice from the neck region. This was placed into small sealable plastic bag containing 2 ml of 0.01M phosphate buffered saline (PBS) (pH 7.2). The samples were frozen at -20°C for a minimum of 48 hours and then thawed before the tissue inside the bag was crushed using a roller; the liquid phase was then used for ELISA.

Indirect ELISA was used for the serological testing. Liquid-phase bulb extract (100 µl) was incubated overnight at 2°C in single wells of a microtitre plate. The plates also contained positive controls prepared from a suspension of the organism in PBS, adjusted to 0.4 absorbance at 620 nm. Extracts from four healthy bulbs served as negative controls. After overnight incubation, plates were washed with PBS, blocked with 200 µl 5 % bovine serum albumin (Sigma A-7030) for one hour, and then washed with PBS. For the ELISA 100µl of either antiserum (diluted 1:400 for *B.a.* and to 1:800 for *P.g.a)* were added to each well. Plates were incubated on an orbital shaker at

37°C for one hour. Following a further wash, 100 µl of goat anti-rabbit IgG whole molecule antiserum, conjugated to alkaline phosphatase (Sigma A-8025) adjusted to 0.75 enzyme units/ml, was added and the plate incubated with shaking for 1 hour at 37°C. After a further wash, 100 µl of substrate containing 1 mg/ml p-nitrophenyl phosphate (Sigma N2765) in 1 M diethanolamine buffer, (pH 9.8) was added and absorbance values read at 405 nm after 1 hour incubation in the dark.

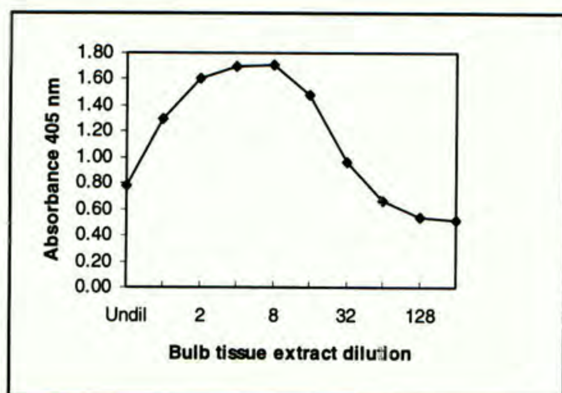
RESULTS

Specificity and sensitivity of *P.g.a.* antiserum

Both isolates of *P.g.a.* gave absorbance values of > 1.5 while all other bacteria tested gave values of < 0.5.

Testing the sensitivity of the antiserum using doubling dilutions of infected bulb tissue extract showed that stearic hinderance caused erratic results in undiluted extract. These results (Figure 1) suggest that a 1:16 dilution in PBS was the optimum bulb extract concentration and was used for all samples from the field experiments.

Figure 1. Absorbance curve produced using *P.g.a.* antiserum against doubling dilutions of infected bulb tissue.



Bulb tests

The percentages of bulbs from all replicates of each treatment found positive in ELISA and the percentages of selected bulbs from which the target organism was obtained by culturing are shown in Table 1. The percentages of bulbs showing visible signs of disease is shown in Table 2. One untreated replicate which showed high levels of *B.a.* by ELISA and culturing was excluded from the results shown. This anomaly is thought to result from spores spreading during spray application as this plot was sited downwind of two *B.a.* plots.

Table 1. Percentage incidence of *P.g.a.* and *B.a.* detected by ELISA and culturing in onion bulbs sampled three weeks pre-harvest, and one and four months post-harvest.

		Detection method					
			ELISA (1)			Culturing (2)	
Treatment		Preharvest	1 - Post	4 - Post	Preharvest	1 - Post	4 - Post
A	*P.g.a.*	0.4	0.4	0.4	2.0	4.0	4.0
	B.a.	0	0	0	0	0	0
B	*P.g.a.*	5.6	4.0	1.6	32.0	22.0	16.0
C	*B.a.*	18.6	16.0	17.4	92.0	100	100
D	*P.g.a.*	3.2	2.4	2.4	14.0	18.0	34.0
	B.a.	15.6	18.2	16.0	100	100	100

(1) out of 500 bulbs (five replicates of 50 at two sites)
(2) out of 50 bulbs (five replicates of five at 2 sites). Where possible these bulbs, which were from the same samples used for ELISA, were selected as they showed visible signs of disease.

Table 2. Percentage of bulbs with visible rot from the five replicates of each treatment at both sites (n = 500 bulbs).

Treatment	1 month post-harvest	4 month post-harvest
A. Untreated	0.4	0.8
B. *P.g.a.*	2.4	2.6
C. *B.a.*	16.0	20.6
D. *P.g.a.* + *B.a.*	15.2	19.4

DISCUSSION

The incidence of pathogens as detected by ELISA in the pre-harvest samples gave a good indication of the amount of disease developing in store. The percentage of bulbs from treatments C and D with visible rots at four months post-harvest was found to be higher then the level of *B.a.* detected by ELISA. At this stage many of the diseased bulbs had degenerated to pulpy tissue and secondary infection with opportunistic pathogens had occurred. The lower number of *P.g.a.* infected bulbs found at four months post-harvest compared to the number seen in the one month post-harvest sample (Treatment B) is presumed to be due to chance variation when the level of infection is near the limit of detection for the sample size examined. The higher percentages of bulbs found to be infected with either pathogen by culturing

(Table 1) was due to bias in sampling. Where possible these bulbs, which were selected from the 50 bulbs sampled from each replicate, were chosen as they showed visible signs of disease and were used to provide confirmation of the ELISA results. The results presented here are from one growing season only and verification is required.

This method permits the simultaneous analysis of onion bulbs for a bacterial and a fungal pathogen. It is based in part on the forecasting system for *P.g.a.* developed by Davies *et al.* (1994, 1996). If shown to be accurate and reliable this test system will allow growers to assess potential disease due to both pathogens and to make decisions on the disposal of the crop to minimise financial loss. Further work is required to develop a test for growers and crop consultants that does not need the sophisticated instrumentation used for microplate ELISA.

Additional information is needed to determine the optimum sample size of pre-harvest bulbs required to provide an accurate prediction of disease levels in store.

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APPLICATIONS OF PCR FOR THE DIAGNOSIS OF BACTERIAL RING ROT INFECTIONS IN POTATO

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ABSTRACT

Effective control of bacterial ring rot disease (*Clavibacter michiganensis* subsp. *sepedonicus*) depends greatly on the specific, sensitive and accurate detection of the pathogen in infected tubers. Visual assessment of the disease is difficult and bioassay tests using eggplant are time consuming and problematic. Serological methods are currently widely used but cross-reacting saprophytic bacteria may cause problems in certain cases. We used sequence information based on a genomic fragment isolated from *C. m. sepedonicus* to construct specific PCR primers to be used for the detection of *C. m. sepedonicus*. The PCR test was used as a parallel test method in combination with the immunofluorescence assay. Generally, both immunofluorescence and PCR methods gave the same positive and negative test results but in a few samples, where cross-reacting problems prevented reliable immunofluorescence detection, PCR test gave a clear negative results. Experiences of analysing routine samples suggest that when the PCR test is carefully performed, it is a reliable test system suitable for practical diagnosis of difficult samples.

INTRODUCTION

Clavibacter michiganensis subsp. sepedonicus is the causal agent of bacterial ring rot of potato. In Canada and Europe this pathogen is considered as a dangerous threat to the potato industry and restrictions have been set up on seed imports from countries were infections have occurred. This is largely due to the idea that the most effective way to control of this disease is to prevent it being introduced in the first place (Stead, 1993). However, history of the spread of potato ring rot pathogen suggests that prevention this disease by exclusion of imports is not an easy task. This bacterial pathogen is very difficult to detect reliably by visual inspection of symptoms because this disease can occur as latent infections. Further, current sampling strategies and diagnostic techniques do not allow to detect *C. m. sepedonicus* in latent infections with 100 % efficiency (Stead, 1993). Consequently, effective control of this pathogen greatly depends on the development of specific, sensitive and accurate detection systems. Improved diagnostic tools will be needed to the detection of latent infections from imported seeds, and in connection with disease control via eradication

to the sensitive detection of the presence of *C. m. sepedonicus* in potato storages. Occasional outbreaks of *C. m. sepedonicus* after the initiation of the eradication programme (Stead, 1993) suggests that currently used diagnostic methods are not sensitive enough in all situations.

In Canada and Europe, serological methods based on immunofluorescence (De Boer *et al.*, 1988, Baer & Gudmestadt, 1993) are widely used. However, detection of bacterial infections by immunofluorescence may have problems with cross-reacting saprophytic bacteria (De Boer & Wieczorek, 1984). Following the positive result found in serological tests, further analysis based on bacterial isolation and infection into eggplant is subsequently carried out. In practice, the eggplant test is time consuming and rather difficult to perform reliably. First attempts to replace previous diagnostic systems for *C. m. sepedonicus* detection were based on DNA hybridization using cloned DNA probes. Specific probes for *C. m. sepedonicus* detection was isolated by several groups (Johansen *et al.*, 1989 and Firrao, 1990). However, DNA hybridization methods turned out to be too tedious and too complicated for practical diagnosis of ring rot infections. Although DNA probes were specific, their sensitivity was not much better than those of used serological methods. The polymerase chain reaction (PCR) allows very specific, rapid and sensitive detection of DNA sequences and therefore holds a great potential for microbial diagnostics (Henson & French, 1993). The value of this technique for the detection of difficult infectious diseases has been demonstrated (Suzuki *et al.*, 1993) and the method is now accepted as a reliable diagnostic tool in the medical field, world wide. However, the PCR is only recently finding its way to plant pathogen diagnostics and few groups have tested its value for the detection of ring rot infections in potato. We have constructed specific PCR primers from genomic fragments of *C. m. sepedonicus* to be used as PCR-based diagnostics for the addition to current methods of detection of routine samples of *C. m. sepedonicus* during normal disease diagnostic inspection. Experiences of analysing practical composite samples of potatoes by immunofluorescence and PCR assay will be discussed in this paper.

MATERIALS AND METHODS

Specific primers have been designed from sequence information based on cloned DNA fragments isolated from *C. m. sepedonicus* genome (Kangasniemi *et al.*, 1996). Specificity of primers have been tested by amplification of DNA from several related bacterial pathogens. PCR assay using the Dynazyme DNA polymerase was done essentially as described by the manufacturer (Dynazyme, Finnzymes Co). PCR assays were performed with a DNA thermal cycler (MJ Research, Inc.) using 25 cycles as follows: 30 s (first cycle 2,0 min) denaturation at 94 °C, 30 s annealing temperature at 55 °C and 30 s primer extension at 72 °C. After the 25 cycle of amplification, the extension reaction was continued for another 10 min at 72 °C. All the reaction was loaded onto 1,4 % agarose gels containing 0,4 ug/ ml ethidium bromide for staining the gels. Two types of routine samples were used in this work. 200-400 potato tubers from 20 000-25 000 kg of potatoes were used to constitute a single sample in a latent infection test. Further, samples of single tubers with suspected ring-rot symptoms were also analyzed. For the latent infection test, 3-4 slices of potato was cut from the heel end of each potato. The slices were placed in a 1-litre container to which sterile distilled water was added, and shaken at 120 rev/min overnight.

10-100 ml of the liquid was taken, concentrated by two fold centrifigation and the resulting pellet was resuspended into 1 ml of buffer. Preparation of samples for serological tests followed the protocol in the Council Directive 93/85/EEC. The resulting sample was divided into two parts, one for IF and one for PCR, and used immediately or stored at -20 °C with glycerol. Indirect immunofluorescence was performed by both monoclonal and polyclonal antibodies. For PCR based detection, the sample was further extracted with one volume of phenol chloroform 1:1 and thereafter the sap was used directly or diluted 1:10 in Tris-EDTA buffer before amplification. Details of the sample preparation is described by Karjalainen *et al.*, (1995) and Kangasniemi *et al.*, (1996) and scheme for the detection system is shown in Figure 1.

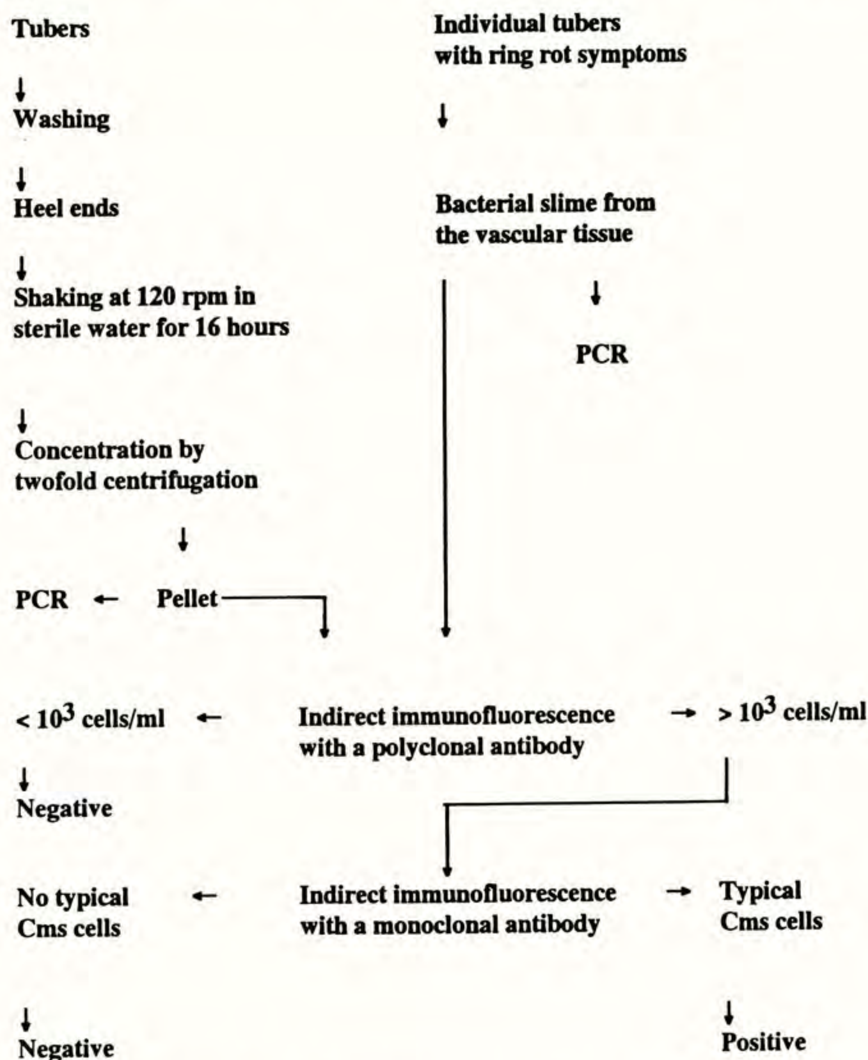

Fig.1. Schematic scheme for the detection system used for analysing potato tubers by indirect immunofluorescence and by PCR.

RESULTS AND DISCUSSION

In order to develop a PCR procedure suitable for analysing the latent infections in potato tubers from routine samples of large composite material, specific primers were successfully designed from sequence information of genomic fragments of *C. m. sepedonicus*. DNA of several related *Coryneform*-bacteria as well as *Erwinia* sp. bacteria were amplified by specific primers but only positive signal was produced by DNA from *C. m. sepedonicus*. We optimized a PCR procedure so that the annealing temperature was raised up to 55 °C which eliminated the nonspecific PCR products and the dilution of original tissue sap 1:10 improved further resolution. As a consequence of these improvements, a single, clear PCR product of 0,3 kb was detectable when tuber tissue was contaminated by *C. m. sepedonicus*. In addition to sample preparation for IF, only one simple phenol-chloroform purification step was needed for PCR analysis of samples.

For the testing of our PCR procedure for analysing practical samples for ring rot detection, two types of material were analyzed by both PCR and immunofluorescence methods. Composite samples consisting of 200-400 tubers from 20 000-25 000 kg of potatoes constituted a single sample in a latent infection test. Our results indicate that generally the same positive and negative results could be revealed in parallel tests with PCR and immunofluorescence. Most of the immunofluorescence positive samples tested in this research had quite a high amount of typically fluoresescing cells/microscope field. These samples were also positive in the PCR test. A few samples that were considered to be negative in the immunofluorescence test had also some fluoresescing cells/window and some of the samples had a high amount of cross-reacting atypical fluoresescing cells. These samples were difficult to analyze by serological methods, but the PCR test gave a clear negative result.

The eggplant bioassay has been traditionally used as a sensitive detection of *C. m. sepedonicus*. The level of sensitivity of 100 cells/ml have been reported (Olsson, 1976). However, several factors affect the reliability of bioassay test which reduce in many cases the sensitivity of detection by eggplant test (Janse & Vaerenbergh, 1987, Stead, 1993). The PCR procedure described here allows a detection limit less than 25 pq of *C. m. sepedonicus* DNA using only of 25 cycle of amplification. In a related PCR study, Hartung *et al.*, (1993) reported a detection limit of 25 pq of *Xanthomonas* sp. DNA which represented about 10 cells/reaction when Southern blots were made after electrophoresis and probed with biotinylated probe. Amplification of *Pseudomonas solanacearum* DNA by primers based on sequences from 16S rRNA enabled of 1-10 cells of bacteria to be detected by using 50 rounds of PCR cycle (Seal *et al.*, 1993). Recently, Li & De Boer (1995) showed that PCR primers based on sequence information of 16S rRNA allowed almost the same or better detection sensitivity of *C. m. sepedonicus* by PCR than by using IF-method. Detection of *C. m. sepedonicus* from stem samples by PCR test during the growing season may provide an effective method for preharvest assessment for the presence of the ring rot pathogen.

However, if the bacterial density is low, the bacteria will be difficult to detect by conventional methods (De Boer & Wieczorek, 1992). An ideal test system for such cases where very high sensitivity is required may be immuno-PCR (Sano *et al.*, 1992) or immunocapture-PCR (Wetzel *et al.* 1992). The advantage of immuno-PCR (or

immunocapture-PCR) is the considerable improvement of sensitivity over the direct PCR. Even a single antibody molecule can be detected by this method (Sano *et al.*, 1992). Improved sensitivity may be related to the observations that interference from plant substances may reduce the detection sensitivity of direct PCR by a factor of 10 (Wetzel *et al.*, 1992) while 250-fold increase in detection sensitivity was achieved by immunocapture-PCR of plum box virus detection apparently because interference of plant material in this test format cause less problems. Development for immuno-PCR or immunocapture-PCR for ring rot pathogen detection seems realistic alternative because antibodies are available to be used as capture probes. Additional advantage of immuno-PCR (immunocapture-PCR) is that it allows to the development of automated assay systems without the loss of sensitivity and therefore it holds a great potential for microbial diagnostics.

Application of PCR assay for the analysis of routine samples for the verification of difficult cases that cannot be resolved easily by serological methods seems possible. Specific fragments from different regions of *C. m. sepedonicus*, plasmid, 16 s rRNA and genomic fragments, provide a sequence information for designing primers for successful amplification. Available information of using PCR primers for ring rot pathogen detection consistently suggest their greater sensitivity over the serological tests. Consequently, PCR will be an attractive method for analysing stem samples or tubers where low concentration of bacteria cause problems with detection sensitivity by conventional methods. Serological methods and eggplant bioassays do not allow the detection of *C. m. sepedonicus* in latent infections with 100 % efficiency (Stead, 1993). PCR test with high sensitivity may be an important aid for resolving such difficult diagnostic cases in connection with controlling of disease spread by seed potato imports/exports and in eradication the disease. Successful application of PCR for eradication programme and testing imported/exported seeds requires that this very sensitive test is carefully performed. Our experience of using PCR as a diagnostic tool in routine laboratories suggest that when PCR is carefully performed in appropriate laboratory conditions and when several controls are included into the test procedure, the method is very reliable and gives reproducible results. There is always the danger of contamination that may yield false positives. However, when working with PCR, high working standards are required and this should be accepted in the laboratory before PCR assay will be used in diagnostic applications.

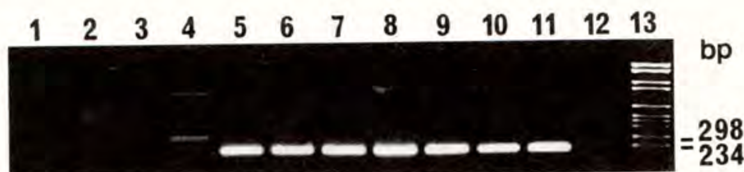

Fig.2. Example of detection systems of potato tuber sap containing ring rot pathogen based on PCR product analysis by gel electrophoresis. 1-4 negative and 5-10 positive samples; 11 positive and 12 negative controls.

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DETECTION AND IDENTIFICATION OF *PSEUDOMONAS SOLANACEARUM* AND OTHER PLANT-PATHOGENIC BACTERIA IN LESS-DEVELOPED COUNTRIES

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ABSTRACT

Different approaches have been taken to the development and adaptation of technology for the identification and characterisation of *Pseudomonas solanacearum,* causal agent of bacterial wilt, and other plant-pathogenic bacteria: serological (enzyme-linked immunosorbent assays using polyclonal and monoclonal antibodies), molecular (highly discriminatory probes and polymerase chain reaction) and biochemical ("conventional" techniques in kit format and metabolic profiling). As well as the technical problems that had to be addressed, the technology had to be suitable for use in tropical and sub-tropical countries with typically poor resources. Factors affecting the success of adaptation are discussed.

INTRODUCTION

Two streams of work within the research programme funded by the Natural Resources Research Department of the Overseas Development Administration (ODA) directed attention to developing diagnostics for *Pseudomonas solanacearum* which is one of the most damaging bacterial plant pathogens in the tropics and sub-tropics (Seal & Elphinstone, 1994). Firstly, serological and molecular diagnostics were developed for *P. solanacearum.* This followed the successful development of technology to detect the pathogen of Sumatra disease of cloves, *P. syzygii* (Eden-Green, 1994). Secondly, whereas some plant pathology laboratories overseas have the facilities and resources to use relatively advanced technology, many such laboratories lack experience in, and budgetary resources (or foreign exchange) for conventional tests; this has resulted in a general neglect of basic bacteriology (Black & Sweetmore, 1994). To overcome these problems the *BACTID* system was developed and adaptations were made to a commercial system for identification and characterisation by metabolic profiling. Special emphasis on the identification and characterisation of *P. solanacearum* brought the two streams together (Table 1).

Experience from the development and adaptation of the techniques overseas has shown that collectively they comprise a suite of technology that can be used for different purposes over a wide range of local situations. The factors affecting the choice of technique are discussed after the component techniques are described. A general review of the difficulties of adopting diagnostics in less-developed countries, and the requirements for such technology under different prevailing conditions, may be found in Black & Sweetmore (1995b).

Table 1. Diagnostic technology for plant-pathogenic bacteria, especially *P. solanacearum*.

Purpose	Appropriate technique
Identification of plant pathogens in pure culture at least to genus level	*BACTID* system
Identification and characterisation in pure culture to species level	Metabolic profiling: adapted *Biolog®* system
Detection and identification of *P. solanacearum* in plant tissue	ELISA with poly- or monoclonals PCR with 16S rDNA primers
Differentiation of *P. solanacearum* strains	RFLP DNA probe Serology with monoclonal antibodies
Detection of *P. solanacearum* in soil, or plant tissue containing inhibitors	ELISA with polyclonal antisera, or magnetic immunocapture-PCR

BACTID SYSTEM

The *BACTID* system was developed for identifying plant-pathogenic bacteria and eliminating saprobes using a streamlined approach to the choice of tests and media (*BACTID* scheme), compact format for the tests (*BACTID* kit) and supporting *BACTID* software. The various components of the system have been described in earlier publications (Black & Sweetmore, 1994, 1995a) and a complete package is due for publication shortly (Black *et al.*, 1996). Key features of the system are summarised below.

BACTID identification scheme and kit

Loosely based on the key by Bradbury (1970), the original use of the *BACTID* scheme was to follow the appropriate pathway for tests until an identification had been made. With each isolate this was likely to avoid unnecessary tests and hence save on media. In fact, when used with the *BACTID* kit, all the tests are usually done at the same time, or certain tests are pre-selected, but the quantities of media are very small (0.6-0.7 ml). The *BACTID* kit itself uses reusable microcentrifuge tubes with differently coloured caps to distinguish different media.

The degree of identification achieved varies with the type of bacteria; the principle being to provide adequate identification for practical diagnosis. Hence the core of the system is a suite of tests for identification roughly to the genus level. However, there are supplementary tests such as gelatin and starch hydrolysis and levan production, which aid confirmation or more detailed identification, e.g. *Xanthomonas campestris* pathovars and fluorescent pseudomonads. There is also a set of tests for RNA-group II *Pseudomonas* (*Burkholderia*) including *P. solanacearum*.

BACTID software

The *BACTID* software package is an expert system which will identify bacteria from results of the *BACTID* tests. Context-sensitive information (hypertext) is also provided on the principles and use of tests, full recipes for media and reagents and further identification of the bacterial groups.

METABOLIC PROFILING WITH *BIOLOG*

In the *Biolog®* system, a culture of the test bacterium is inoculated into the wells of a microtitre plate. The utilisation of 95 different organic substrates is determined using a redox colour reaction, giving a metabolic profile which, in general is unique to the taxon. This is compared with profiles of known bacterial taxa in a database using matching programs. The profiles can be also used to characterise isolates of one particular taxon for further classification work. Expensive equipment is not essential for basic use, which offsets the high costs of the plates (c. GB£3.50 per plate). This made the system attractive for evaluation in poorly-equipped laboratories.

By adapting the system in a number of ways, it has been made more accessible and usable in such situations (Black & Sweetmore, 1993, 1994), most significantly through the use of software from a third party (B*acterial Identifier* from Blackwell Science). Consequently, user-defined databases can be accessed at a fraction of the cost of the equivalent *Microlog®* software from *Biolog*. (Databases of representative tropical plant bacteria in either *Biolog* or *Bacterial Identifier* format are available from the senior author. *Bacterial Identifier* is no longer published, but enquiries may also be sent to the senior author.) The cost of the plates ultimately restricts its use, but if used in conjunction with the *BACTID* system, the wasting of expensive plates on unwanted saprophytes and other bacteria can be avoided.

SEROLOGICAL TECHNIQUES

Although serological assays have been used to detect and identify plant-pathogenic viruses for a number of years, the full potential of such techniques for diagnosing bacterial diseases has yet to be exploited. The main reason for this is that antibodies produced for many plant-pathogenic bacteria have generally lacked sensitivity and specificity. Both monoclonal and polyclonal antibodies have now been developed to *P. solanacearum*.

Polyclonal antibodies

Polyclonal antibodies to *P. solanacearum* were raised in female Dutch x Lop rabbits by immunising with glutaraldehyde-fixed whole cells. These polyclonals are extremely sensitive and have proved to be more than adequate at detecting and identifying *P. solanacearum* in a number of serological assays (Robinson-Smith, 1995).

Monoclonal antibodies

A number of monoclonal antibodies to *P. solanacearum* have been produced in mice using a variety of immunisation schedules (Robinson-Smith *et al.*, 1995). These monoclonals differ in specificity and although they lack the sensitivity needed for routine detection a panel may be used to differentiate isolates of *P. solanacearum* (Robinson-Smith, unpublished).

Methods of using the antisera

ELISA was chosen as the standard assay to be used overseas as, compared with other methods available (e.g. agglutination, precipitin and dip sticks), it offered the best

compromise between ease of use and sensitivity. An indirect ELISA, using a polyclonal and crude plant extracts was adopted and is described in Robinson-Smith (1995). This method is sensitive, being able to detect as few as 5×10^3 cells/ml, allowing detection of the disease before symptoms are apparent. Costs are also low (approximately GB£3.50 per 96 tests) making it ideal for use in less developed countries.

MOLECULAR TECHNIQUES

Polymerase Chain Reaction

Detection of bacteria by polymerase chain reaction (PCR) amplification using specific oligonucleotide primers has, in the past decade, been shown to be able to achieve sensitivities equal, and sometimes greater than selective plating for a range of plant-pathogenic bacteria. A primer set was designed to *P. solanacearum* 16S rRNA sequences, which resulted in reliable detection of 1×10^2 *in vitro*-cultured cells/ml by PCR amplification (Seal *et al.* 1993).

A common limitation of PCR technology is the inhibition of the thermostable DNA polymerase enzymes by compounds present in many plant extracts, leading to false negatives or low detection sensitivities. To overcome this problem without the use of expensive commercially-available purification resins, an alternative method combining serological and PCR technology has been developed for *P. solanacearum*. In this method *P. solanacearum* cells are removed from PCR-inhibitory compounds in samples by extraction with magnetic beads coated with polyclonal antisera for *P. solanacearum* (Seal, unpublished)

Highly discriminatory probes

To assess the genetic variability of the bacterial wilt pathogen within various regions and countries, a DNA probe has been identified that gives rise to over 40 different restriction fragment length polymorphism (RFLP) groups. In Mauritius, use of this probe has divided biovar 3 strains of the pathogen into 7 RFLP groups. Although the significance of these groups is not known, the method allows a panel of genetically distinct strains to be identified for use in screening programmes. This ensures that breeding lines are tested against isolates of *P. solanacearum* that are representative of the variation found within the area in which the crop is to be grown.

APPROPRIATE TECHNIQUES FOR EACH SITUATION

Due to the neglect of general bacteriology, it was observed that laboratory workers who could detect *P. solanacearum* in plant tissue by ELISA or PCR might not necessarily be able to recognise and work with *P. solanacearum* in culture. This is clearly important for recognition of false positives in serological and molecular work. The worker must be able to eliminate saprobic bacteria and other non-pathogens during isolation and correctly identify isolates of interest. This relies partly on good isolation technique (Lelliott & Stead, 1987) and partly on the availability of media and reagents for biochemical tests and/or selective media. *BACTID* provides a quick and easy solution to the problem of resources for preliminary identification, and has now been adapted in Zanzibar, Mauritius, Malaysia and

Zimbabwe with dissemination to other countries through workshops. The technique has proved to be very suitable for poorly-resourced laboratories, even those lacking constant electricity and water supply.

ELISA is a technology that has already been proven to be suitable for use in less developed countries, and some of the institutes to which it was transferred routinely use ELISA for virus detection. Transfer of the indirect ELISA test was therefore straightforward and is now used by many such laboratories for routine detection of *P. solanacearum*. As good antisera is produced to other plant-pathogenic bacteria, the ELISA technology transferred will be adapted to allow the detection of a wider range of bacteria.

The 16S rDNA PCR test for *P. solanacearum* is already being used successfully for screening and diagnostic purposes in a few European research and quarantine laboratories. A project to set up PCR equipment at laboratories in Malaysia, Mauritius and Zimbabwe was initiated in 1992. Local research staff in these laboratories have been trained in the PCR tests for *P. solanacearum* and it is hoped that these laboratories will, in the future, become regional diagnostic test centres, analysing samples posted from neighbouring countries. Technology transfer has to date made good progress and no insurmountable problems have been met (Seal, 1995).

These various technologies for detection and identification of *P. solanacearum* and other plant-pathogenic bacteria have been shown to be robust and proven to work under adverse conditions. The choice of method or methods will depend on the purpose of the diagnostic exercise and the laboratory conditions prevailing. *BACTID* provides methods for identification which can be used under the most basic conditions but requires pure cultures. For direct detection and identification of *P. solanacearum* in plant tissue and in soil, ELISA and PCR are available. The former requires less capital equipment but is less sensitive than PCR (for samples free of potent PCR-inhibitors). Also, although the polyclonal used in the ELISA test described is not as specific as the PCR primers, this is not a limiting factor for most diagnostic purposes. RFLP probes, metabolic profiling and monoclonal antibody analysis with sub-specific selectivity can be used to characterise *P. solanacearum*. However, despite RFLP probes currently offering the highest degree of discrimination, their use is least suitable to less-developed countries due to complex protocols and thermo-labile reagents being required.

Experience gained from adaptation of these technologies will be used in forthcoming work. In Tanzania and Malawi the aim will be to achieve wider adaptation of *BACTID*, ELISA and PCR technology and to extend the range of organisms covered by specific and confirmatory tests. Likely target organisms include *Xanthomonas* species in citrus, root crops and rice.

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PCR-BASED DETECTION OF *PHYTOPHTHORA* SPECIES IN HORTICULTURAL CROPS

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ABSTRACT

Accurate and rapid detection and diagnosis of *Phytophthora* diseases in horticultural crops is of key importance in the management of the disease. The minimising of disease spread via infected rootstock and a move away from prophylactic fungicide application are two major benefits of such a procedure. The Polymerase Chain Reaction (PCR) offers great potential as a highly specific tool to achieve this. This paper reports on the design and optimisation of such a PCR-based detection system based on ribosomal DNA sequences of several important *Phytophthora* species of horticultural crops. Specific and sensitive detection of the fungus in infected root material has now been achieved.

INTRODUCTION

The genus *Phytophthora* represents an important group of plant pathogenic fungi which are responsible for large scale losses of tropical and temperate crops. *Phytophthora* spp. typically have a very broad host range and despite the lack of aerial dispersal mechanisms, the ability to spread locally in drainage or irrigation water and persist as long-lived oospores in soil or planting material has resulted in a worldwide distribution of many species. They are a particular problem in vegetatively propagated horticultural crops, being spread on infected planting material.

The sustainability of both the propagation and cultivation stages of many horticultural crops is severely threatened by *Phytophthora* spp. In order to minimise disease problems in nurseries, plants are often treated prophylactically with high doses of fungicides which rather than solving the problem may actually exacerbate it. In order to minimise losses and "clean up" horticultural propagation systems it is important that a scheme for the accurate and specific detection and diagnosis of *Phytophthora* is established.

For example, *Phytophthora fragariae* is an important problem to raspberry and strawberry growers. Root infection results in red core of strawberry (caused by *P. fragariae* var *fragariae*) and root rot of raspberry (caused *by P. fragariae* var *rubi*). These diseases are major limiting factors in crop growth, requiring the application of fungicides which are expensive and potentially damaging to the environment. It is now recognised that worldwide spread of the strawberry and raspberry varieties of this pathogen has been brought about via the movement of infected rootstock (Duncan, 1993). In an effort to stem this it has been declared a quarantine organism in many countries, which means stocks must be guaranteed "disease free" before importation.

There are however many difficulties in detection and diagnosis of *Phytophthora* spp. The non-specific symptoms on the root, crown or stem base makes visual confirmation of the presence of *Phytophthora* difficult. Currently, detection and diagnosis of *Phytophthora* relies on visual inspection, bait testing (Duncan *et al*., 1993) or isolation of the fungus on selective media. The Duncan bait test has been successfully applied for many years, but is time consuming, taking 5-6 weeks. Visual inspection and isolation procedures require considerable experience for a correct identification of the *Phytophthora* species once in culture. Diagnostic tools based on polyclonal antibodies have also been developed which allow sensitive but generally not species specific detection of several pathogens. However, PCR primers to detect a single pathogen or many members of a group of related pathogens can be designed more quickly and at a lower cost than comparable serological techniques and have therefore considerable potential in a multi-purpose detection method.

USE OF RIBOSOMAL DNA IN PCR PRIMER DEVELOPMENT

The advantages of using nuclear ribosomal DNA (rDNA) sequences as target sites for PCR primers is now widely recognised. Within the rDNA subunit various levels of sequence conservation exist allowing phylogenetic separation at various levels of resolution from kingdoms through to individual species (Bruns *et al.,* 1991). There is an abundance of publications on rDNA sequence variation, sequences are rich in informative regions, mutation rates are known and many copies are present in each nucleus thus increasing the sensitivity of detection. Since one is looking at a very tightly defined region, species can be added to the analysis at any time resulting in an expanding sequence database.

Spacer regions of the ribosomal repeat unit which are not thought to play a functional role (Nues *et al*., 1994) , are less conserved than the ribosomal genes and have been reported to show interspecific variation in plants (Sun *et al*., 1994) and fungi (Lee & Taylor, 1992; Zambino & Szabo; 1993, Sherriff *et al.,* 1994). Therefore, for the purposes of molecular detection, it is possible to design primers with the appropriate level of specificity.

The following is a description of recent progress at SCRI on the molecular variation of ITS1 and ITS2 regions of *Phytophthora* spp. and their utility in the diagnosis and detection of *P. fragariae* and other species. Specific details of the procedures used and primers sequences will appear in subsequent papers.

PRIMER DESIGN

PCR primers designed for the amplification of fungal spacer regions (White *et al.,* 1990) were modified to allow efficient amplification of ITS 1 and ITS 2 regions from *Phytophthora* spp. The 300 bp ITS1 and 600 bp ITS2 PCR products were manually sequenced and aligned using multiple sequence alignment software on Seqnet (Daresbury Laboratory). The species sequenced so far are *P. fragariae* var. *fragariae*, *P. fragariae* var. *rubi*, *P. cambivora, P. cinnamomi, P. megasperma, P. nicotianae, P. cryptogea*, *P. citricola, P. drechsleri, P. infestans, P. idaei*, *P. pseudotsugae*, and *P. cactorum*. Both ITS1 and ITS2 regions were sufficiently conserved to allow an accurate alignment, some regions were identical in all species

tested and others showed variation at an intraspecific level.

Two distinct types of sequence were noted, those from non-papillate species sharing regions of homology not seen in papillate and semi-papillate species which formed a separate sub-group. This variability was used to designed primers specific for different *Phytophthora* species. As few as 3 base pairs differences at the 3' end of the primers allowed specific DNA amplification. To date, specific primer pairs have been designed for *P. fragariae, P. cambivora, P. cinnamomi* and *P. nicotianae.* They were tested against pure DNA from the 13 *Phytophthora* species detailed above. The primers designed for the species *P. fragariae* (including *P. fragariae* var *rubi* and *P. fragariae* var *fragariae*), *P. cinnamomi* and *P. cambivora* showed excellent specificity, resulting in amplification products from many isolates of that species but no amplification products from other species. The *P. nicotianae* primers also amplified a product from *P. infestans*, which as a papillate species, was shown to be closely related to *P. nicotianae* (unpublished results based on ITS sequence homology).

A comparison of 18S rDNA sequences of oomycetes on the EMBL database has allowed the design of a primer specific to members of the order Peronosporales, *i.e.* including *Pythium*, *Phytophthora* and the downy mildews. It can be used in conjunction with a reverse primer located in the beginning of the 28S gene, to amplify both ITS sequences. A nested PCR approach (Mac Manus & Jones, 1995), using the "peronosporales primers" in the first round and the species-specific ITS-based primers in the second round has been designed to improve the sensitivity of the PCR .

DETECTION OF THE PATHOGEN *IN PLANTA*

Using strawberry and raspberry plants infected with *P. fragariae* as a model system, a simple protocol was developed for the extraction of suitable DNA from roots for detection by PCR. The sensitivity of the technique was assessed as follows.

Heavily infected and healthy raspberry roots were mixed in the following ratios: 0:100; 20:80; 40:60; 60:40; 80:20 and 100:0 and DNA was extracted from the samples. Single-round PCR with primers specific for *P. fragariae* var. *rubi* detected the fungus in all samples containing infected roots regardless of the ratio of infected:healthy roots but some allowance must be made for the efficiency of extraction. It is far more difficult to grind in and extract DNA from healthy roots than from roots softened by infection with *P. fragariae* var. *rubi* . Therefore the efficiency of PCR detection of the pathogen could be overestimated.

In a separate test, by comparing the PCR signals obtained with several dilutions of the DNA from infected plants, *c.* 1/5 of the total amount of DNA extracted from the infected roots was reckoned to be that of the pathogen. This high proportion of fungal DNA may have been due in part to the degradation of plant DNA in necrotic or dead tissues.

To test the sensitivity of the test more critically, particularly when the tissues of the plant were not necrotic and the whole plant was not show any symptoms of infection, a time-scale experiment was done. DNA was extracted from strawberry root tissues at various intervals after inoculation with zoospores of *P. fragariae* var. *fragariae* with several DNA samples being

prepared for each sampling date. Using a single round PCR and the specific primers, sporadic detection was observed within three days of inoculation, and in all samples by seven days, at which stage symptoms were clearly visible on the root systems of the infected plants. To raise the level of sensitivity, the same DNA samples were tested using nested PCR with two sets of primers: the peronosporales specific primers in a first round PCR, and the *P. fragariae*-specific primers as the nested primers in a second round PCR. The pathogen was detected in all DNA samples within one day of inoculation when there were no symptoms visible on the roots. Only aniline blue staining for the fungus and observation of the root tissues under epifluorescent microscopy showed that the mycelium had started to invade root tissues.

Using the peronosporales specific primers in the first round PCR, it was possible also to test the same sample for the detection of several related pathogens. These primers amplify DNA of all *Phytophthora* species and the PCR product obtained from the first round PCR can be tested with several sets of specific primers designed for different species. *P. cactorum* is another pathogen of strawberry and *P. idaei* has been reported on raspberry (Kennedy & Duncan, 1995). Both pathogens have been detected on roots samples within which *P. fragariae* has also been detected (Lynn Surplus, personal communication).

DISCUSSION

There is now a system for the detection of individual species of *Phytophthora* in infected plant material that has great potential as a diagnostic tool. Indeed the specific detection of pathogen before the appearance of lesions on the plants represents a major step forward in diagnostics (detection + identification of *Phytophthora*), from existing procedures that take weeks to one which may be completed in a matter of days. More rapid disease diagnosis is a critical point in any integrated control package. It can prevent unnecessary spread of the disease and thereby prevent unnecessary applications of fungicide or allow more timely application of reduced dosages. A system of careful monitoring of propagation material and attention to improved phytosanitary conditions should result in an overall reduction in the incidence of *Phytophthora* in propagation stocks, the benefits of which will be passed on to growers.

To carry out larger scale detection programmes further work on the development of protocols for efficient amplification from zoospores will be necessary. Such work is now underway at SCRI and should result in a system of monitoring plant stocks via the trapping and detection of zoospores in drainage water from pots, water from recirculating irrigation systems or environmental monitoring of pathogen populations in streams or soil.

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SEROLOGICAL DETECTION OF *SPONGOSPORA SUBTERRANEA* F.SP. *SUBTERRANEA*

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ABSTRACT

A polyclonal antiserum has been produced against spore balls of *Spongospora subterranea* f.sp. *subterranea* prepared from potato tubers. It was able to detect as little as 0.02 spore balls in enzyme-linked immunosorbent assay (ELISA). It detected spore balls of different cultivar origin equally well. It also detected spores from different geographical origins. Western blot analysis revealed that the antiserum detected a number of different proteins the most distinct of which had a molecular weight of slightly less than 6.5 kDa. A technique was developed to suppress autofluorescence of spore balls allowing immunofluorescence studies to be carried out. Using this technique in conjunction with indirect FITC immunofluorescence, discrete bright fluorescent spots were visualised using the specific serum. With the non-specific serum only a very dull background fluorescence was evident.

INTRODUCTION

Powdery scab of potatoes is caused by infection of tubers by the obligate zoosporic parasite *Spongospora subterranea* f.sp. *subterranea*. The parasite also infects roots, stems and stolons producing wart-like galls. However, the scab-like lesions that are produced on tubers are the most damaging as they reduce the proportion of marketable crop, carry infection to subsequent crops when the potatoes are used as `seed' and release spore balls that remain viable in field soils for many years. *S. subterranea* is also important as the vector of potato mop-top furovirus (Jones & Harrison, 1969) which can cause severe damage in some potato cultivars. These diseases have become more important in parts of Europe over the last decade including the U.K. (Wale, 1987), Switzerland (Merz, 1993), Sweden (Rydén *et al.*, 1989), Finland (Kurppa, 1989), Holland (Turkenstein, pers. comm.) and also other parts of the world including Colombia (Sanudo & Jurado, 1990), USA (Mohan, pers comm), Australia (de Boer, 1991), India (Bhattacharyya *et al.*, 1985), Pakistan (Anon, 1991), New Zealand (Braithwaite *et al.*, 1994) and Turkey (Eraslan & Turhan, 1989). This increase in importance is thought to be due to the intensification of potato production, shorter rotations, increased irrigation of crops and spread of resting spores on seed stocks.

Control of powdery scab is difficult because of the persistence of the resting spores in soil and the difficulty in killing them. The only reliable form of control would be to plant clean seed into uncontaminated land. However, even though seed tubers with scabs may be identified by visual inspection, blemish-free tubers may be contaminated by contact with scabbed potatoes and symptomless infections have been reported (de Boer *et al.*, 1982; Diriwächter & Parbery, 1991). Sensitive and rapid serological detection of resting spores on tubers would provide a more reliable means of identifying contaminated tubers and could allow a certification scheme to be implemented.

Serology has been routinely used in the detection and quantification of viral and bacterial plant pathogens for many years, however until recently it has not been developed significantly for the detection of other plant pathogens. The serological detection of soil-borne filamentous fungi has progressed considerably only in the last 5-10 years (Dewey *et al.*, 1991). The use of antibodies to detect non-filamentous soil-borne fungi has not been as successful mainly due to the fact these fungi are obligate plant parasites and the isolation of pure fungal material as immunogens is consequently very difficult. Despite such difficulties Harrison *et al.* (1993) described the development of an enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of *S. subterranea* contaminating potato tubers. This paper describes the further development of serological techniques for the detection of *S. subterranea*.

MATERIALS AND METHODS

Preparation of spore ball material

Spore balls were used as immunogens and in most experiments were obtained from potato tubers cultivar Bintje grown in Switzerland. For additional tests, spore balls were collected from cv. Indira and Ukama grown in Switzerland, cv. Estima grown in Scotland and an experimental line (NDO 1496-1) grown in USA.

Production of antiserum

A New Zealand White rabbit was injected subcutaneously with 1 ml of spore ball preparation emulsified with 1 ml Freund's complete adjuvant. One and two weeks later further injections were made intramuscularly with 0.5 ml of spore ball preparation emulsified with 0.5 ml of Freund's incomplete adjuvant. A booster injection intramuscularly with 0.5 ml of spore ball preparation emulsified with 0.5 ml Freund's incomplete adjuvant was made seven months later. A final intramuscular booster injection was given after a further three months. Final blood serum samples were then collected and tested by ELISA. The γ - globulin containing fraction of the serum was prepared as described by Clark & Adams (1977).

ELISA

Plate trapped antigen (PTA) ELISA was used in all experiments. Prepared spore balls were ground with pestles and mortars in 0.05M sodium carbonate buffer (pH 9.6). Sample homogenates were pipetted into wells of microtitre plates (Nunc-Immuno Plate Maxi Sorp F96; Gibco Ltd., Uxbridge, UK) and incubated overnight (16h) at 6°C. Subsequently the plates were incubated for 2h at room temperature with raw serum. This was followed by goat anti-rabbit γ-globulin conjugated to alkaline phosphatase (Sigma Chemical Co.) for

3h at room temperature. Finally the plates were incubated with substrate (2-nitrophenyl phosphate (Sigma 104 phosphatase substrate tablets) in 10% diethanolamine adjusted to pH 9.8 with HCl) at room temperature and the optical absorbance at 405 nm (A_{405}) was measured.

Sensitivity of detection of resting spore balls

A sample of spore balls from potato cv. Bintje was weighed and quantified by counting in a haemocytometer slide. Dilutions containing 4000, 1000, 50, 12.5, 2.5, 0.5 and 0.1 spore balls/ml were then prepared. Scrapings were taken from the skin of a healthy Bintje potato using a scalpel and equivalent concentrations (g/ml) were prepared, ground and pipetted into wells of the ELISA plate as controls. ELISA was carried out as outlined previously.

Specificity of antiserum

Equal amounts (30 mg) of scrapings of uncontaminated tubers (cv. Bintje) and infected tubers (cv. Agria) taken as before, ground with a pestle and mortar or unground in 2 ml of 0.05M sodium carbonate buffer (pH 9.6) were tested by ELISA to determine the optimum method for preparing samples.

The ability of the antiserum to detect resting spores from different potato varieties was also tested. Samples of spore balls were collected from cultivars Bintje, Indira and Ukama and 30 mg of each was suspended in 2 ml of carbonate buffer and ground with pestles and mortars or left unground and tested by ELISA.

To determine whether the antiserum recognised resting spores of different geographical origin, spore ball preparations from Switzerland, Scotland and USA were quantified, ground with pestles and mortars in carbonate buffer and further diluted in carbonate buffer to give concentrations of 50, 5 and 0.5 spores/ml. These ground spore suspensions were then tested by ELISA as described earlier except that purified γ-globulin was used at 1μg/ml instead of raw serum.

Studies on the antigens detected by the antiserum

To characterise protein antigens, Western blot analysis of total spore ball proteins was carried out (Walsh *et al.*, in press). Samples were separated by electrophoresis on discontinuous denaturing polyacrylamide gels in a mini-gel apparatus. Gels were then Western blotted with the raw serum diluted or γ-globulin.

In order to visualise the position and distribution of antigens detected by the antiserum on and in spore balls, immunofluorescent studies were carried out. A technique was developed that eliminated autofluorescence. Spore balls treated with specific or non-specific raw sera followed by goat anti-rabbit IgG conjugated to FITC were viewed using a Carl Zeiss Standard RA microscope with a mercury light source and 2Fl filter combination specific for FITC.

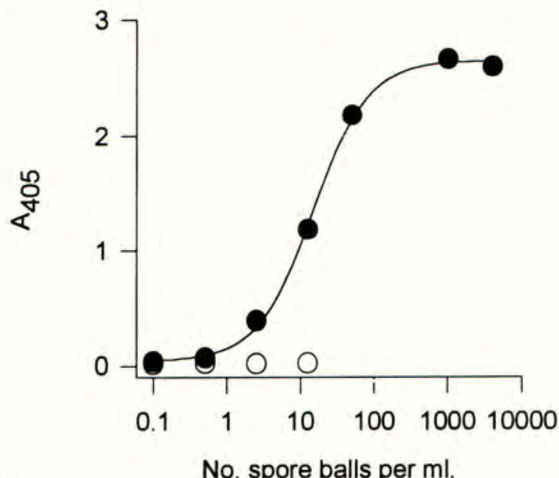
RESULTS

Sensitivity of detection of resting spore balls

The prepared and quantified spore balls reacted strongly in PTA-ELISA with the raw serum

from the final bleed and A_{405} values increased consistently with increased spore ball concentration (Fig. 1). The sample containing 0.02 spore balls (200µl of 0.1 spore balls/ml) gave an A_{405} of 0.04 whereas blank wells gave a value of 0.02. In contrast equivalent weights of scraping from uncontaminated potatoes gave low absorbence values (Fig. 1).

Figure 1. The relationship of the concentration of spore balls of *Spongospora subterranea* prepared from infected potatoes and equivalent concentrations of scrapings from a healthy potato with A_{405} in a PTA ELISA test (•) resting spores; (o) equivalent weights of scrapings from a healthy potato.



Specificity of antiserum

A comparison of the A_{405} obtained in PTA-ELISA for equal quantities of ground and unground scrapings showed that although grinding did not affect values for scrapings from uncontaminated potatoes (A_{405} = 0.13 with and without grinding) those of ground scrapings from infected potatoes were almost double those of unground (A_{405} = 0.18 not ground; 0.34 ground). In similar tests on equal weights of resting spore ball preparations from different potato cultivars there were remarkably no differences in A_{405} values for spores; grinding increased A_{405} values, but to a lesser extent than with scrapings (Table 1).

Table 1. Detection of spore balls of *Spongospora subterranea* obtained from different potato cultivars and the effect of grinding.

Cultivar origin	A_{405}*		
	Bintje	Indira	Ukama
Unground samples	0.48	0.48	0.48
Ground samples	0.55	0.56	0.56

Samples containing scrapings from healthy potatoes gave A_{405} of 0.13 and blank wells gave A_{405} of 0.00. * Mean value from 2 ELISA plate wells.

Tests on equal quantities of spore balls of different geographical origin demonstrated the ability of raw serum to detect these in PTA-ELISA although the A_{405} values obtained

showed differences between spores of different origins (A_{405} of Swiss spore balls @ 50/ml = 0.93; for Scottish spore balls @ 50/ml = 1.11; for USA spore balls @ 50/ml = 1.18).

Studies on the antigens detected by the antiserum
Western blotting revealed a number of bands with molecular weights of 25-80 kDa and a single band with a molecular weight of slightly less than 6.5 kDa that was particularly prominent.

In the immunofluorescent studies using specific serum, many bright fluorescent spots were seen within spore balls during microscopic examination. Focusing up and down suggested that the spots were internal rather than on the surface of the spore balls. In contrast spore balls treated with non-specific serum showed no such fluorescent spots, just a very dim general background fluorescence.

DISCUSSION

Further to the earlier description of serological detection of *S. subterranea* (Harrison *et al.*, 1993) this paper describes improvements in the sensitivity of serological detection of spore balls from tubers. There was a quantifiable relationship between the concentration of spore balls prepared from tubers and the A_{405} values obtained in ELISA. The assay detected 0.02 spore balls and was slightly more sensitive than the test described by Harrison *et al.*, (1993), perhaps due to differences in antisera titres or assay methods. The A_{405} values for equivalent weights of scrapings from uncontaminated potatoes were surprisingly low.

As grinding spore ball preparations and scrapings increased A_{405} values in comparison with unground samples, it is recommended that all samples should be ground prior to testing.

The ability to detect spore balls of different potato cultivar or geographical origin suggests that the antiserum has wide application. In addition to the detection of spore balls from Switzerland, Scotland, and USA the antiserum has also been shown to detect spore balls from Pakistan (Merz, pers. comm.) and Australia (Wilson, pers. comm.).

Results from Western blotting suggest that an alternative approach to producing a more specific antiserum might be to purify the prominent protein with a molecular weight of less than 6.5 kDa and use this as the immunogen. The presence of discrete brightly fluorescing spots in the immunofluorescence studies suggests that the most strongly recognised epitopes are localised either on the surface or more likely within spore balls. If each spot represented an individual spore (cyst), then each spore does not fluoresce to the same degree. Alternatively a component of the cytoplasm may fluoresce. The possibility that the antiserum detects something intimately associated with spore balls in addition to, or instead of spore balls themselves cannot be discounted.

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