DNA MICROARRAYS ON AGAROSE-COATED GLASS SLIDES FOR PLANT PATHOGEN IDENTIFICATION

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ABSTRACT

Agarose-coated glass slides were used as a platform for microarray analysis applied to plant pathogen identification. The agarose substrate combines the desirable features of fluorescence detection and high DNA immobilization capacity, in contrast to nylon membranes, which are unsuitable for fluorescent detection, and standard glass microarray slides, which have a low capacity for immobilization. Oligonucleotide probes were immobilized on the agarose substrate, then hybridized to fluorescently labeled sample DNA. Agarose concentration and hybridizing DNA length affected hybridization efficiency. Probes arrayed on the agarose distinguished *Didymella bryoniae* and *Botrytis cinerea* from each other with no cross reaction. No interference from other common greenhouse plant pathogens was found. Results compared favorably with those obtained on nylon membranes, and surpassed those achieved on the commercially available glass substrate. Agarose-coated slides are easily produced, and with the use of a manual arrayer, are an inexpensive alternative to commercial microarrays for small scale applications.

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CHAPTER 1: INTRODUCTION

1.1 DNA arrays for identification of microorganisms

1.1.1 DNA microarrays

To date, DNA microarrays are mainly used for gene expression analysis (Schena, 2003). However, another emerging application of microarray technology is typing of organisms to the level of species or strain, allowing rapid identification of the components of a microbial system or identification of disease organisms (Chizhikov *et al.*, 2001; Wilson *et al.*, 2002; Al-Khaldi *et al.*, 2002; Call *et al.*, 2003). The array format permits large scale screening for the presence of any number of different microorganisms or gene sequences and is well suited to the detection and identification of pathogenic organisms.

DNA arrays consist of a platform or substrate (for example, a microscope slide), upon which is arrayed DNA of interest. Identification of organisms by DNA array depends upon hybridization of the DNA, immobilized on the substrate, to sample DNA. The immobilized DNA, called probes, is selected to be specific to organisms of interest. Since many probes can be immobilized upon the substrate, sequential or simultaneous identification of many organisms is theoretically possible, on one slide or on duplicate slides. The immobilized DNA can take the form of oligonucleotides, cDNA, or PCR products. Either the probe, or (more commonly) the sample DNA, can be labeled for later detection. Successful hybridization is indicated by detection of the label on the substrate at the locations of particular probes.

Microarrays are defined by feature (spot) size in contrast to macroarrays. Microarrays can contain thousands of features, each less than 1mm in size (Schena, 2003); macroarrays are less dense, with features greater than 1 mm in size. In addition, microarrays are constructed on a

rigid substrate such as glass and use fluorescence detection, macroarrays are usually constructed on nylon or nitrocellulose membranes and use radioactive or chemiluminescence detection. Both types of array can be used to identify organisms.

1.1.2 Detection and diagnosis of plant disease

Plant disease is a major problem for the British Columbia greenhouse vegetable industry, causing serious economic losses each year. The major crops, peppers, lettuce, cucumber and tomatoes, are susceptible to a variety of fungal, bacterial and viral organisms. Plant diseases are controlled by chemical agents or by manipulation of environmental parameters such as humidity and temperature. Chemical agents such as fungicides may cause harvesting restrictions or pollution concerns and can be harmful to desirable insects such as bumble bees used for pollination and parasitic insects used for biological control of insect pests (Greenhouse Vegetable Production Guide, 1996). As an example of environmental disease control, increased ventilation to reduce high humidity, which is conducive to many plant diseases, can interfere with carbon dioxide enrichment, which may require restricted ventilation. In addition, nutritional controls that stress plants by pushing production to maximize fruits and flowers, increase susceptibility to some diseases (Jarvis, 1989). Therefore, efficient disease control will limit the need to apply measures that may conflict with other crop concerns.

Efficient disease control for greenhouse crops requires rapid identification of disease microorganisms. Conventional means of detection and diagnosis of plant disease rely on visual inspection of disease symptoms, and cultural and microscopic characteristics of the organisms associated with these symptoms. Symptoms may be obvious and cause little confusion; however, ambiguous symptoms may be present requiring a more thorough examination. In addition, it may not be clear which organism is responsible for a disease, since diseased tissue can also support harmless organisms. Identification of fungal organisms requires expert knowledge of morphology and cultural characteristics, and the presence of their reproductive structures is

usually needed. Fungi produce spores asexually (anamorphic stage) and/or sexually (teleomorphic stage) from different structures. Frequently fungi will produce only one reproductive state readily in culture, or none at all. For instance, *Didymella bryoniae (*anamorph *Phoma cucurbitacearum*), causal agent of gummy stem blight of cucumbers, readily produces pycnidia (anamorphic reproductive structures) and conidia (spores) in culture, but more rarely, perithecia and ascospores (teleomorphic structures). These pycnidia and conidia are not easily distinguished from those of other *Phoma* species (Keinath *et al.*, 1995) (Fig. 1a). *Botrytis cinerea* (Fig. 1b), an ubiquitous organism, can be a minor or major problem depending upon the crop and environmental conditions, and can coexist in the same lesions as other pathogens. In addition, identification of *Botrytis* to the species level is complicated by variation within the genus (Chilvers *et al.*, 2004). Culturing organisms in order to examine them uncontaminated by other organisms, or to generate the reproductive structures needed for precise identification, requires some time for growth to occur (several days to weeks). Cultures can become overgrown by co-existing harmless organisms if the growth rates of these organisms are much greater than those of the organisms of interest. Obligate parasites such as the powdery mildews will not grow in culture and must be collected and examined in tissue from field samples.

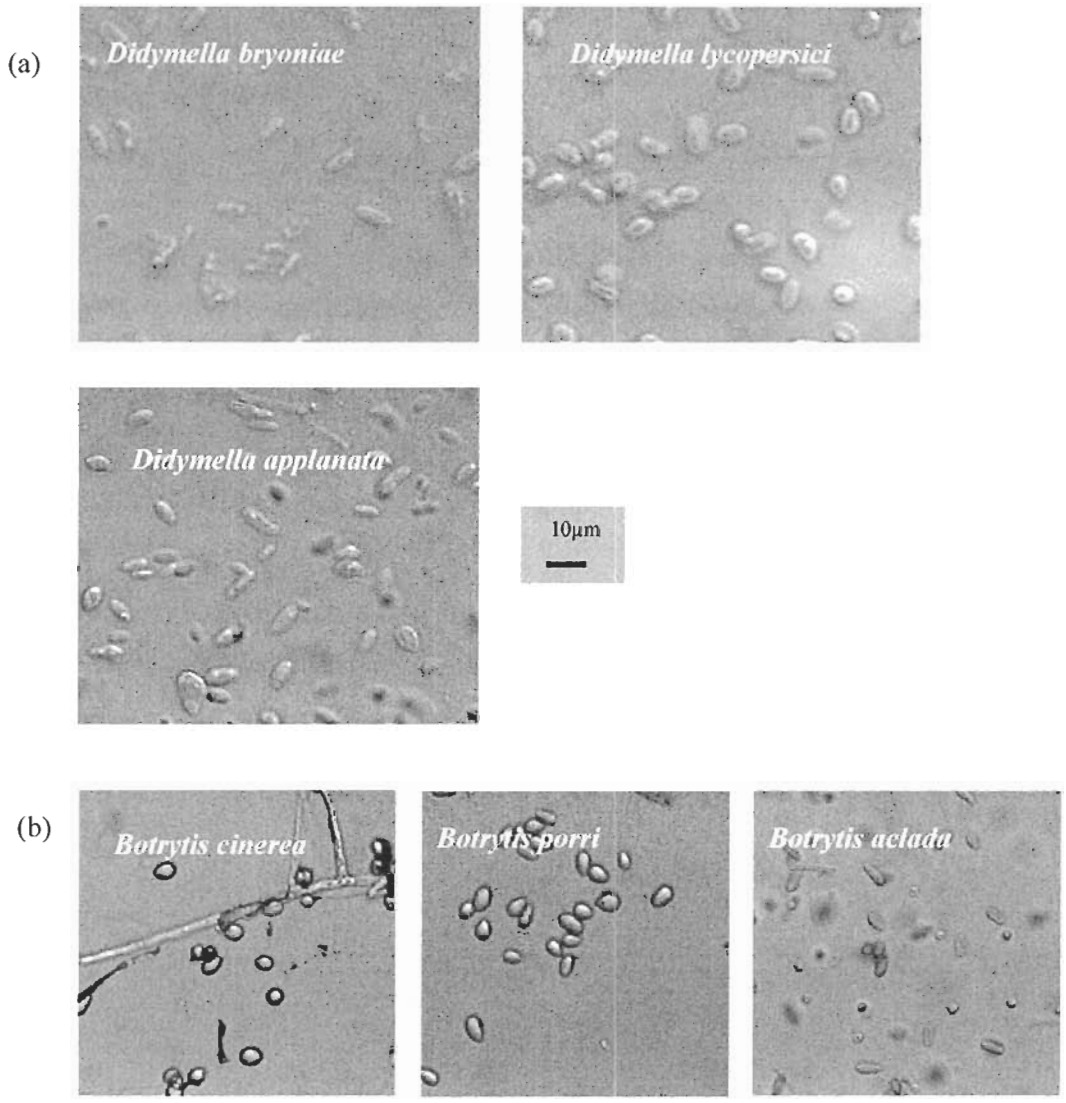
These disadvantages of traditional identification techniques can be overcome with the use of molecular methods of identification. Implementation of molecular techniques requires no particular knowledge of a microorganism's microscopic or other characteristics and typically takes a few hours or at most a couple of days. The presence of reproductive structures is not required. When examining mixtures of microorganisms, molecular identification methods are capable of showing the presence or absence of particular microorganisms, or of identifying all organisms present. Organisms can often be identified by direct testing of diseased plant tissue or environmental samples (such as water samples).

Figure 1 Micrographs of conidia of *Didymella* and *Botrytis* species

Conidia of different species of the same genus are often difficult to distinguish from one another, increasing the problem of identification by morphological characteristics. Infection is often caused by one species of a genus only, therefore identification to species level is important for decisions regarding disease control measures. Molecular tests (requiring no examination of morphological structures) have been developed that distinguish *D. bryoniae* from other *Didymella* species (Koch & Utkhede, 2002), and *B. cinerea* from other *Botrytis* species (Mathur & Utkhede, 2002).

(a) Conidia of three species of *Didymella*. *D. bryoniae* infects greenhouse cucumber (and other cucurbits), *D. lycopersici* infects greenhouse tomato, *D. applanata* infects raspberry and its spores could be found in nearby greenhouses.

(b) Conidia of three species of *Botrytis*. *B. cinerea* infects many crops, including greenhouse crops; *B. porri* and *B. aclada* infect *Allium* species such as onion, only.



Molecular means of identification of microorganisms can be divided into two groups: those based on antibodies and those based on nucleic acids. Serological methods such as enzyme-linked immunosorbent assay (ELISA) and immunofluorescence (IF) use antibodies to detect particular antigens, and have been in common use to identify some species of bacteria and viruses since the 1970s (Schaad *et al.*, 2003). More recently, ELISA kits have been developed that identify a few species of fungi.

Nucleic acid based identification techniques include the polymerase chain reaction (PCR) (Ioos and Frey, 2000; Ghignone *et al.*, 2003; Schaad *et al.*, 2003), dot blots (Koch & Utkhede, 2002; Mathur & Utkhede, 2002), macroarrays (Lievens *et al.*, 2003; Fessehaie *et al.*, 2003; Hong *et al.*, 2004), and microarrays (Cho and Tiedje, 2001; Al-Khaldi *et al.*, 2002; Borucki *et al.*, 2003).

PCR methods are highly sensitive and rapid. They are capable of identifying fungi from single spores (Lee & Taylor, 1990; Williams *et al.*, 2001). With appropriate choice of primers, PCR can be used to identify organisms to any taxonomic level. It is capable of identifying organisms in crude environmental samples, such as diseased plant tissue or air samples, without prior culturing (Henson & French, 1993; MacNeil *et al.*, 1995). PCR also allows identification of more than one organism at the same time, if combinations of primer pairs are provided for a multiplex reaction. Multiplexing however is limited to simultaneous amplification of the DNA of only a few organisms because of complications arising from primer-primer interactions, problems with accurate size discrimination by electrophoresis, and skewed amplification results (Lin *et al.*, 1996; Warsen *et al.*, 2004; Vora *et al.*, 2004).

PCR can be used to provide starting material for other molecular tests. These include direct sequencing of the PCR product, and hybridization reactions such as dot blots (in which PCR products are denatured, immobilized upon nylon membranes, and hybridized to labeled oligonucleotide probes) and array analysis (in which DNA probes are immobilized upon a

substrate, usually glass slides or nylon membranes, and hybridized to denatured and labeled PCR product). Dot blots permit the testing of many samples at once for a particular microorganism. Array analysis (or reverse dot blot) on the other hand is capable of determining the identity of many organisms at the same time. It is well suited to identifying single organisms or the organisms within sample mixtures, such as would be obtained with water, soil and air samples. It is ideally suited to screening environmental samples for the presence of pathogens.

For this study, PCR, followed by hybridization on an array, was used to identify fungal microorganisms of interest found in a greenhouse environment.

1.1.3 Aim of this study

The aim of this study was to determine the suitability of agarose (coated onto microscope slides) as a microarray substrate for identification of common greenhouse fungal pathogens. For this purpose, two common greenhouse pathogens were chosen for study, with the goal of detecting these organisms and differentiating them from each other without cross reaction. In addition, a comparison was desired with a commonly used commercial substrate – slides using the same amine-aldehyde oligonucleotide attachment chemistry (CSS slides (CEL, Inc.)) as agarose-coated slides were chosen for this purpose.

Agarose was chosen because of reports of its usefulness as a substrate with a porous nature (Afanassiev *et al.*, 2000; Wang *et al.*, 2002), capable of retaining greater amounts of immobilized DNA than the usual 2-D glass substrates used for microarray work. Although there are very few studies demonstrating successful hybridization of PCR products to immobilized oligonucleotides on an agarose surface (Wang *et al.*, 2002), several studies have reported hybridization of oligonucleotides to their complements (oligonucleotides of the same length) (Afanassiev *et al.*, 2000, Dufva *et al.*, 2004). Hybridization of immobilized PCR products to complementary oligonucleotides (Proudnikov *et al.*, 1998) as well as hybridization of fragmented

c-DNA, RNA or PCR products to immobilized oligonucleotides (Guschin *et al.*, 1997, Bavykin *et al.*, 2001) has been reported on another porous substrate, polyacrylamide. Agarose was also chosen as a possible substrate because of its reported usefulness with devices that utilize electric current to direct oligonucleotides to particular sites on a silicon platform and to greatly enhance the rate at which hybridization between immobilized DNA and sample DNA in solution takes place (Sosnowski *et al.*, 1997; Edman *et al.*, 1997). Agarose is also compatible with fluorescence imaging, a requirement for the very small feature size and high densities that are desirable for microarray applications.

1.2 Overview of DNA arrays

1.2.1 Primers and probes

Extracted DNA is usually present at concentrations too low to be detected directly in microarray applications, therefore amplification of sample DNA by PCR is performed first. In addition, the PCR step also permits labeling of a sample to enable detection.

A common region for amplification is the ribosomal gene locus, present in all organisms. The ribosomal genes contain areas of varying sequence conservation, and primers can be designed that amplify at different taxonomic levels. For example, primers are available that amplify only the fungal DNA present in a sample (White *et al.*, 1990; Gardes and Bruns, 1993). This preferential, but non-specific, amplification will exclude in this case, bacterial, insect, plant or any other undesired DNA. A particular advantage of the ribosomal genes is their presence in the genome in many copies, often 100s, which increases the sensitivity of PCR-based detection of organisms present in low numbers.

Hybridization reactions (in which sample DNA is hybridized to oligonucleotide probes) depend upon selection of appropriate probes via analysis of sequence information, which is typically available in the GenBank and other databases. If information for a particular organism

or gene of interest is unavailable, then DNA sequencing of the gene of interest is required. For the ribosomal genes, the less conserved regions are used to select probes that are specific to a particular species; the more conserved regions can be used to select probes that will detect at a higher taxonomic level. Sequences chosen for similarity using the BLAST (Basic Local Alignment Research Tool, Altschul *et al.*, 1990) program are aligned with multiple alignment software such as ClustalW (Chenna *et al.*, 2003) in order to choose probe sequences unique to the organism of interest.

1.2.2 Printing of arrays

Array printing is the process by which molecules of interest are placed on the array substrate at discrete locations or spots. Spot variables include density, size, regularity, reactivity and purity (Schena, 2003). Density (distances between spots) depends upon spot size and the capability of the spotting apparatus; the resolving power of the detection device should also be considered. Regularity (of spacing) is required in order to easily identify probe locations and for ease of automated or semi-automated data analysis. The reactivity of the molecules present within a spot ideally is 100%, but commonly is reduced to ~ 50% due to drying of sample on the array surface and chemical, thermal or enzymatic damage (Schena, 2003). Spot purity is reduced by contaminants such as unremoved PCR reactants (primers and nucleotides) and sample carryover, or during manufacturing processes such as photolithography, with incomplete synthesis of products. Spot purity is increased by use of substrates that selectively bind tagged molecules – for instance aldehyde surfaces, which bind amine-modified oligonucleotides. Purity is important for the specificity of reactions (such as hybridization) taking place on the array surface.

Variables to be considered when choosing a printing process are affordability, ease of implementation and throughput. Printing tools typically available to the researcher range from hand-held devices for manual application to automated printing robots; these cost a few thousand

to several tens of thousands of dollars. Devices intended for commercial use can cost hundreds of thousands of dollars. Manual devices offer greater ease of implementation than robotic equipment, however throughput is low.

1.2.3 DNA labeling

Fluorescent labeling is the method of choice for microarray applications. Sample fluorescent labeling can take two forms. In the direct form, fluorescent labels are incorporated into the sample DNA and these labels are then directly detected. In the indirect form, labels are incorporated into the sample DNA and, after hybridization, these labels are further complexed with fluorescent tag(s). The label can be a component of the PCR process that has been tagged for later detection - either labeled nucleotides (Borucki *et al.*, 2003; Cho and Tiedje, 2001) or labeled primer (Yershov *et al.*, 1996; Call *et al.*, 2001a; Tang *et al.*, 2004; Shepard *et al.*, 2005). Alternatively, the sample can be labeled post-PCR in a tailing reaction with terminal transferase and labeled nucleotide (Wilson *et al.*, 2002). The simplest and least expensive technique is the use of end-labeled primer that is directly detected. These advantages are offset by reduced sensitivity – each labeled strand of nucleic acid contains one fluorescent molecule only.

1.2.4 Fluorescence

Fluorophores are molecules capable of absorbing light and re-emitting light of a longer wavelength. Absorbed photons convert a fluorescent molecule into an excited state; fluorescence occurs when the molecule relaxes back to the ground state (by release of a photon). The difference in wavelength between absorbed light and emitted light is called the Stokes' shift, and allows emitted light to be distinguished from incident light. The greater the Stokes' shift, the easier this process becomes; therefore dyes with high Stokes' shifts are valued for microarray applications.

The efficiency of the absorption and emission process is reduced when an excited molecule returns to its ground state via processes other than fluorescence, such as quenching (in which nearby molecules absorb energy), and fluorescence energy transfer (in which energy is transferred to another fluorophore). The efficiency of fluorescent emission is described by the quantum yield (Q): the ratio of the number of photons emitted to the number of photons absorbed. The fluorescent intensity of a dye is proportional to the product of the dye's molar extinction coefficient (ε, defined as the absorbance of a 1M solution of a pure solute at a specific wavelength), its quantum yield, and its concentration (M).

Dyes with quite different values for ε and Q may be similar in brightness: for instance fluorescein with ε=70,000 $cm^{-1}M^{-1}$ and Q=0.9 has a brightness similar to Cy3, which has values of 200,000 $cm^{-1}M^{-1}$ and 0.3, respectively (Haugland, 2002).

Fluorescent molecules are susceptible to photobleaching, whereby repeated excitation damages the molecules. It is a cumulative effect whose rate is a function of excitation intensity (Song *et al.*, 1995). Susceptibility to this phenomenon varies amongst different fluorescent molecules.

There are many fluorescent dyes available, encompassing a wide range of absorption and emission wavelengths. Structurally, they have in common conjugated carbon bonds (double bonds on every other carbon atom), which are responsible for their fluorescence properties. Single nucleotides or oligonucleotides can be tagged with these dyes and incorporated into sample DNA during or after PCR.

1.2.5 Signal detection

Two types of equipment exist for detection of fluorescence signal from microarrays – scanners and imagers. Scanners, as the name suggests, scan back and forth over an array, gathering one pixel of information at a time. Imagers, on the other hand, take a photograph of the array or a large part of the array, and collect all the data in one step.

In both devices, light is passed through an excitation filter that selects the correct excitation wavelength for the fluorophore in use; this filtered light is then directed onto the sample. Emitted light from the sample passes through an emission filter, which removes excitation and other undesired wavelengths, to a lens that focuses the emitted light onto the detector. For scanners, this detector is a photomultiplier tube; for imagers, this detector is a camera (usually a charge-coupled device) containing a matrix of photosensors (Schena, 2003). Signal data is then gathered by computer.

Pixel size in scanners can usually be selected from a variety of settings – *e.g.*,, 10 μm to 1000 μm. Lower pixel size increases the resolution of the resulting image but causes scanning time to increase. In general, the pixel size is chosen to be equal to 1/10 or less of the diameter of each feature, for instance, spots 500 μm in diameter should be scanned at a resolution of 50 μm or less, yielding about 80 pixels of information per spot. Pixel size in imagers is not selected but is a feature of the detection device. Most imagers use pixel sizes of 7 – 12 μm, with larger pixels capable of storing more charge. Each unit of charge is converted into one count; therefore the larger the pixel , the greater the dynamic range. However, smaller pixels give higher image resolution. Thus, images with the highest resolution will have a lower dynamic range (range of signal intensities that can be detected).

The advantage of imagers over scanners is much decreased detection time, since all data is collected in one imaging step, typically 10 – 60 seconds. Scanners have the advantage of superior dynamic range and sensitivity.

1.2.6 Data analysis

Scanners and imagers acquire data from the entire microarray surface. To be useful, data related to spots must be distinguished from data related to background. Background signal can be due to reflection from, or intrinsic fluorescence of the substrate, to non-specific binding, and any

spurious signal. In addition, saturated signals (signals greater than the capacity of the imaging device to record) should be avoided, since values above the saturation level are indistinguishable. Saturation can usually be eliminated by choosing a lower voltage setting for the photomultiplier tube in the case of scanners, or reduced image capture time for imagers.

Quantitation of array data is performed by computer; however, some aspects of the signal segmentation process can be performed manually if desired. Manual selection of spots is useful for small arrays, and requires the user to manually enclose each spot (with ovals, rectangles, or irregular polygons). This process can be undertaken manually because the signal quantitation software provides an image of the array that can be adjusted such that faint spots are readily seen. The simplest way to quantify this data is to sum the values for all pixels in the selected area. Compensation can be made if desired for contribution of background fluorescence to spot fluorescence. The quantitation software will also adjust the image if desired to show false color or black/white reversal of the array image.

1.2.7 Substrates

For microarray analysis, probe molecules are immobilized upon a solid, rigid and flat substrate. Other desirable qualities for a microarray substrate are uniformity of the surface treatment (required for probe immobilization) and low background fluorescence. Microscope slides coated with polylysine were used for the first microarray experiments (Schena, 2003), and the slide format remains the most common form of substrate (with improved smoothness over ordinary microscope slides). It is possible to use glass slides without further modification as a substrate since nucleic acids can adsorb directly onto the glass surface (Call *et al.*, 2001b). However, typically the slides are coated in some way to increase attachment of probe molecules. Organo silane compounds are commonly used to attach two types of reactive groups to the glass surface – amine and aldehyde. In each case, one end of the silane molecule bonds to the glass surface, leaving the reactive amine and aldehyde groups of the compound free to bind nucleic

acids. Reactive amines create a positively charged surface that will electrostatically bind negatively charged nucleic acids. This surface is well suited to longer strands of nucleic acids, such as c-DNA, PCR products and long (>70-mer) oligonucleotides, which become bound at several points along their lengths. Shorter oligonucleotides experience non-specific attachment along the entire length of the molecules, causing steric hindrance that interferes with hybridization reactions (Schena, 2003). Aldehyde surfaces are better suited to short strands of nucleic acids but require a modification of the oligonucleotide by attachment (to the 5' phosphate group) of an amine group linked to a carbon spacer. The oligonucleotides therefore are attached by one end to the substrate with the rest of the molecule free to react as required. The attached amine groups react with the aldehyde surface by forming covalent Schiff linkages.

Organo silane compounds create a reactive surface that is essentially 2-dimensional, *i.e.*, one layer of probe molecules can become attached to the surface. Another class of coating materials adds some depth to the surface layer, creating a 3-dimensional volume in which attachment and subsequent reactions, such as hybridization, can take place. A 3-dimensional surface allows probe molecules to become layered on a particular area, and the consequent layering of signal molecules leads to an increase in signal intensity. Polyacrylamide has been used as a matrix for immobilization of oligonucleotides for subsequent hybridization (Yershov *et al.*, 1996; Guschin *et al.*, 1997; Rehman *et al.*, 1999; Bavykin *et al.*, 2001, Barsky *et al.*, 2002). The polyacrylamide, which contains active groups such as aldehydes for attachment of probes, is formed into discrete pads on the surface of a microscope slide, and provides a solution-like environment in which reactions can take place. A commercially available glass slide, 3-D Link™, which is coated with a long-chain, amine-reactive polymer forming a 3-dimensional substrate, showed much higher attachment and reaction efficiencies than several 2-dimensional substrates in a trial analyzing minisequencing and immobilization chemistries (Lindroos *et al.*, 2001) on oligonucleotide microarrays. Agarose microbeads, containing avidin attachment sites,

have been used to create arrays on a silicon device of biotinylated oligonucleotide capture probes that hybridized complementary oligonucleotides and easily distinguished single mismatches (Ali *et al.*, 2003).

Agarose has also been used to produce a 3-dimensional layer on glass slides that is stable during hybridization reactions and suitable for immobilization of oligonucleotide probes (Afanassiev *et al.*, 2000). Attachment sites within the layer are formed by oxidation of the agarose with $NaIO_{4,}$, creating aldehyde groups. It was reported that compared to a 2-D substrate in common use (CEL Inc. silylated slides (CSS)), hybridization of complementary oligonucleotides on agarose-coated slides gave much higher signals (Afanassiev *et al.*, 2000). In another study, comparing hybridization (of complementary oligonucleotides) on several amine-reactive substrates (including agarose-coated slides), the signal:noise ratio was highest on the agarose-coated slides (Dufva *et al.*, 2004). That study also determined that the agarose substrate could discriminate single nucleotide polymorphisms in 60-base targets using 21- and 25-base probes.

Agarose-coated glass slides have also been successfully used for hybridization of immobilized molecular beacons to unlabeled sample DNA (Wang *et al.*, 2002). Compared with glutaraldehyde-coated slides, which provide a planar surface for attachment, discrimination between single centrally located base mismatches in 16 base probes, and perfectly matched probe, was much higher for probes immobilized on agarose-coated slides. This discrimination approached values obtained in solution thus indicating that the agarose layer provides a solution-like environment.

CHAPTER 2: RESULTS AND DISCUSSION

2.1 PCR using primer sequences from the ribosomal genes

The ribosomal genes contain conserved areas that can be used to amplify groups of organisms, such as septate fungi (Phylum Dikaryomycota; fungi with cells divided from each other by a wall). Amplifying the DNA, in a single reaction, of organisms of interest in a mixture, is useful for identification by a diagnostic microarray. Such an array can be designed to simultaneously or sequentially identify many organisms. Several sequences within the ribosomal genes are useful as primers for amplification of the DNA of fungal organisms (White *et al.*, 1990; Gardes and Bruns, 1993), some of these primers amplify plant DNA as well. The relative positions of these primer sequences within the ribosomal DNA is shown in Fig. 2; their sequences are shown in Table 1a. Several combinations of primers were tested on plant and fungal tissues to determine the pairs most suited to specific amplification of fungal DNA in the presence of plant DNA (Table 1b, Fig. 3). Primers NS5, ITS4, ITS2 and UNLO28S22 are complementary to plant DNA (Figs. 3a, 3b and 3c, lanes 3) as well as fungal DNA, (Figs. 3a, 3b and 3c, lanes 4 and 5). All combinations of these primers yielded visible bands for uninfected plant tissue; for the combination NS5 and ITS2, two bands were clearly visible in one of the infected tissue samples – one of these bands is amplified plant DNA, the other is amplified fungal DNA (Fig. 3c, lane 2). However fungal DNA only was amplified by the following combinations of primers: ITS2 in combination with ITS5 (Fig. 3d, right side) or ITS1F (Fig. 3e, left side), UNLO28S22 in combination with ITS1F (Fig. 3d, left side) or ITS5 (Fig. 3e, right side), and ITS4 in combination with ITS1F or ITS5 (Fig. 3f). These results indicate that ITS1F and ITS5 are fungus specific. Any combination of these primers that did not amplify plant DNA was suitable for this study; in addition, a PCR product that spans both internally transcribed

spacers makes it easier to choose unique probe sequences. Therefore, the combination ITS1F and UNLO28S22 was chosen for initial tests of the agarose substrate.

Figure 2 Relative positions of several primers within the ribosomal genes (not to scale)

Primers are found within the well conserved areas of the ribosomal genes (18S, 5.8S and 28S rRNA). The scale indicates the approximate locations (base number) of the forward and reverse primers relative to the beginning of the 18S rRNA gene. Forward primers are shown with right-facing arrows.

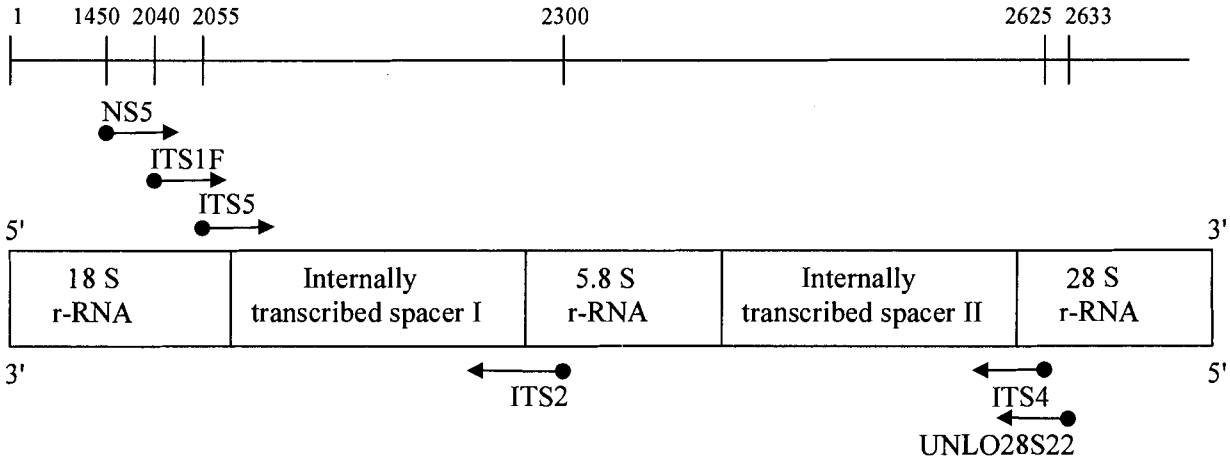


Table 1 Primers and primer combinations tested

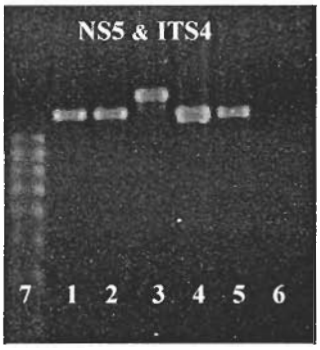
(a) Primers tested

Primer	Sequence	Reference
NS5	AACTTAAAGGAATTGACGGAAG	White *et al.*, 1990
ITS1F	CTTGGTCATTTAGAGGAAGTAA	Gardes and Bruns, 1993
ITS5	GGAAGTAAAAGTCGTAACAAGG	White *et al*, 1990
ITS2	GCTGCGTTCTTCATCGATGC	White *et al.*, 1990
ITS4	TCCTCCGCTTATTGATATGC	White *et al.*, 1990
UNLO28S22	GTTTCTTTTCCTCCGCTTATTGATATG	Bakkeren *et al*, 2000

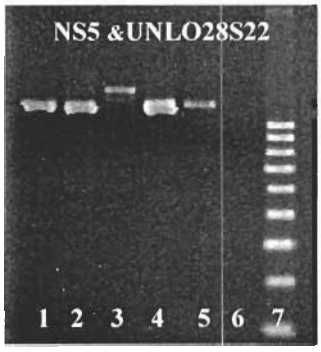
(b) Primer combinations tested

Primer combination	illustration	approximate size of amplicon	amplifies
NS5 and ITS4	Fig. 3a	1170 bp	Plant and fungal DNA
NS5 and UNLO28S22	3b	1200 bp	Plant and fungal DNA
NS5 and ITS2	3c	850 bp	Plant and fungal DNA
ITS1F and UNLO28S22	3d(left)	600 bp	Fungal DNA
ITS1F and ITS2	3e(left)	260 bp	Fungal DNA
ITS1F and ITS4	3f(left)	590 bp	Fungal DNA
ITS5 and ITS2	3d(right)	240 bp	Fungal DNA
ITS5 and UNLO28S22	3e(right)	580 bp	Fungal DNA
ITS5 and ITS4	3f(right)	570 bp	Fungal DNA

Figure 3 Amplification results – DNA extracted from pure culture and from diseased and healthy plant tissue

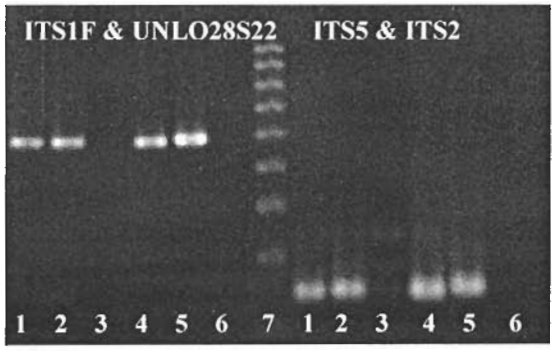


(a)



(b)

(c)



(d)

a-f:

Lane 1 plant tissue infected with *D. bryoniae*

Lane 2 plant tissue infected with *B. cinerea*

Lane 3 uninfected plant tissue

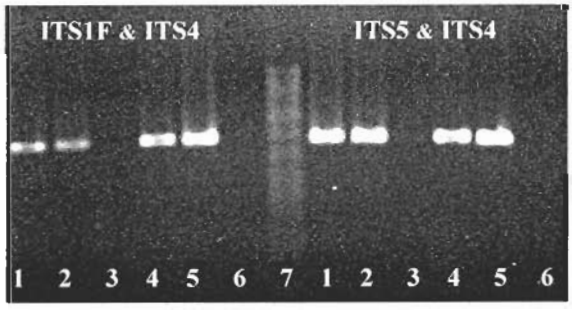
Lane 4 pure culture – *D. bryoniae*

Lane 5 pure culture – *B. cinerea*

Lane 6 water blank (no DNA)

Lane 7 KB size ladder

(e)



(f)

2.2 Probe sequences

Due to varying conservation of portions of the ribosomal genes and internally transcribed spacers, this region is useful for finding probe sequences for identification of fungal (and other) microorganisms. The ribosomal gene region is useful for other purposes as well (such as taxonomic studies), and has been sequenced for thousands of organisms. This sequence information is available on the GenBank (National Centre for Biotechnology Information, Bethesda, MD) database. Sequences obtained from GenBank for *D. bryoniae* and *B. cinerea* are shown in Figure 4. Probe sequences are capitalized, in bold and underlined.

2.3 Activation of the agarose surface

Agarose is a polysaccharide consisting of repeating units of the disaccharide agarobiose, which is composed of D-galactose and 3,6-anhydro-L-galactose (systematic name:(1→4)-3,6-anhydro-α-L-galactopyranosyl-(1→3)-β-D-galactopyranan). Upon oxidation with $NaIO_4$, this disaccharide can form aldehyde groups (Fig. 5a) wherever terminal D-galactose groups are present. Terminal galactose occurs at one end of each agarose polymer, and at the ends of branches of the polymer. These aldehyde groups can be used to link other molecules, such as DNA or protein, to the agarose via covalent linkages. Amine-modified oligonucleotides can become attached to agarose via formation of covalent Schiff base linkages between the amine modifications and the aldehyde groups (Fig. 5b). A Schiff base is defined as the compound formed in a condensation reaction between an amine and an aldehyde or ketone. A further reaction with $NaBH_4$ reduces the Schiff linkage, forming an irreversible bond (Materials & Methods 3.6).

The effects on oligonucleotide retention of using unactivated slides and not reducing the Schiff linkage were tested using slides coated with 0.05% agarose and arrayed with 10 μM

Figure 4 Locations of probe sequences within the ribosomal genes

(a) Ribosomal rRNA genes for *Didymella bryoniae* containing the probe sequences D6 and ITS3 (capitalized and underlined)
(b) Ribosomal rRNA genes for *Botrytis cinerea* containing the probe sequences B1 and ITS3 (capitalized and underlined)

The locations of the sequences for primers ITS1F and UNLO28S22 are also shown (bold italics). Note that the locations of probes B1 and D6 are almost the same relative to the probe ITS3. Probe ITS3 is the complement of primer ITS2. The alignment of these sequences to other species of *Didymella* and *Botrytis* are shown in figure 21. Sequences for these genes were obtained from the GenBank database.

(a) ***Didymella bryoniae*** 18S ribosomal RNA gene, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and 26S ribosomal RNA gene, partial sequence
ACCESSION AF297228

rRNA	<1..54	/product="18S ribosomal RNA"
misc_RNA	55..195	/note="internal transcribed spacer 1"
rRNA	196..353	/product="5.8S ribosomal RNA"
misc_RNA	354..510	/note="internal transcribed spacer 2"
rRNA	511..>553	/product="26S ribosomal RNA"

PROBE D6

primer ITS1F cttg gtcatttaga

```
 1  ggaagtaaaa gtcgtaacaa ggtttccgta ggtgaacctg cggaaggatc attacctaga
61  gttgcgggct ttgcctgcca tctcttaccc atgtcttttg agtaccttcg tttcctcggc
121 gggttcgccC GCCGATTGGA CAAAACTTAA Acccttttgta attgaaatca gcgtctgaaa
181 aaaacataat agttacaact ttcaacaacg gatctcttgg ttctgGCATC GATGAAGAAC
241 GCAGcgaaat gcgataagta gtgtgaattg cagaattcag tgaatcatcg aatctttgaa
301 cgcacattgc gccccttggt attccatggg gcatgcctgt tcgagcgtca tttgtacctt
361 caagctttgc ttggtgttgg gtgtttgtct cgcctctgcg cgcagactcg cctcaaaacg
421 attggcagcc ggcgtattga tttcggagcg cagtacatct cgcgctttgc actcacaacg
481 acgacgtcca aaaagtacat tttttacact cttgacctcg gatcaggtag ggatacccgc
541 tgaacttaag catatcaataagcggaggaaaagaaac
```

Primer UNLO28S22 complement

PROBE ITS3

(b) ***Botrytis cinerea*** gene for 5.8S ribosomal RNA, internal transcribed spacer 1 and internal transcribed spacer 2.
ACCESSION Z73765

rRNA	<1..3	/product="18S ribosomal RNA"
rRNA	4..149	/note="internal transcribed spacer 1"
rRNA	150..306	/product="5.8S ribosomal RNA"
rRNA	307..450	/note="internal transcribed spacer 2"
rRNA	451..>481	/product="28S ribosomal RNA"

PROBE B1

```
1...ttacagagtt catgcccgaa agggtagacc tcccaccctt tgtattatt actttgttgc
61  tttggcgagc tgccttcggg ccttgtatgc tCGCCAGAGA ATACCAAAAC TCttttatt
121 aatgtcgtct gagtactata taatagttaa aactttcaac aacggatctc ttggttctgG
181 CATCGATGAA GAACGCAGcg aaatgcgata agtaatgtga attgcagaat tcagtgaatc
241 atcgaatcta tgaacgcaca ttgcgccccc tggtattccg gggggcatgc ctgttcgagc
301 gtcatttcaa ccctcaagct tagcttggta ttgagtctat gtcagtaatg gcaggctcta
361 aaatcagtgg cggcgccgct gggtcctgaa cgtagtaata tctctcgtta caggttctcg
421 gtgtgcttct gccaaaaccc aaatttttct atggttgacc tcggatcagg tagggatacc
481 c...........catatcaataagcggaggaaaagaaac
```

PROBE ITS3

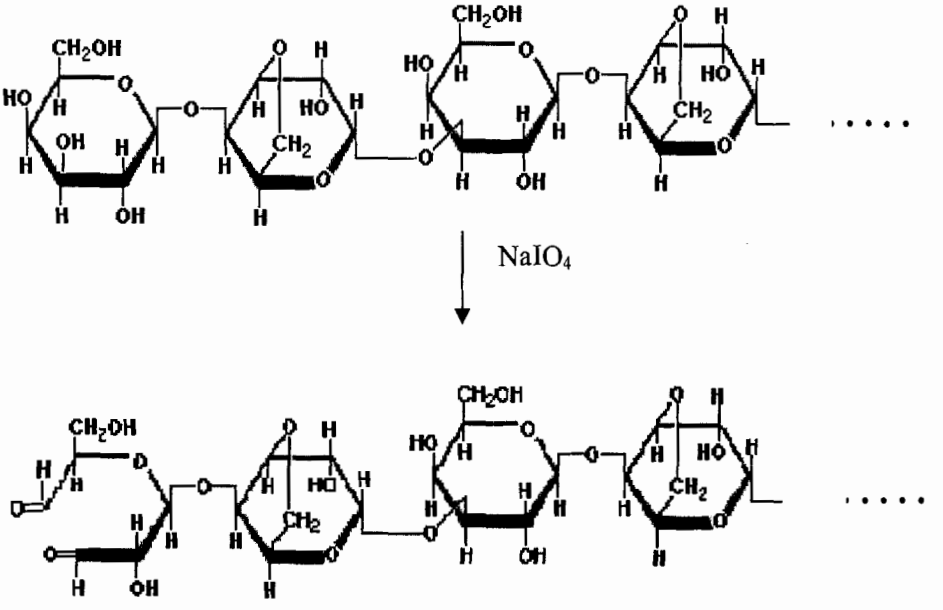
Primer UNLO28S22 complement

Figure 5 Activation of the agarose layer and attachment of amine groups

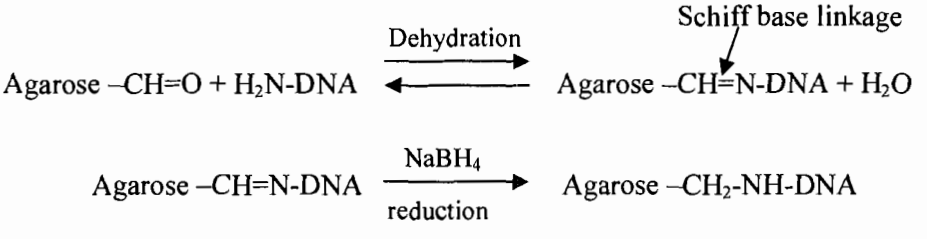
a) formation of aldehyde groups at terminal D-galactose groups of the agarose polymer, by oxidation with $NaIO_4$

b) formation and reduction of the Schiff linkage

a)



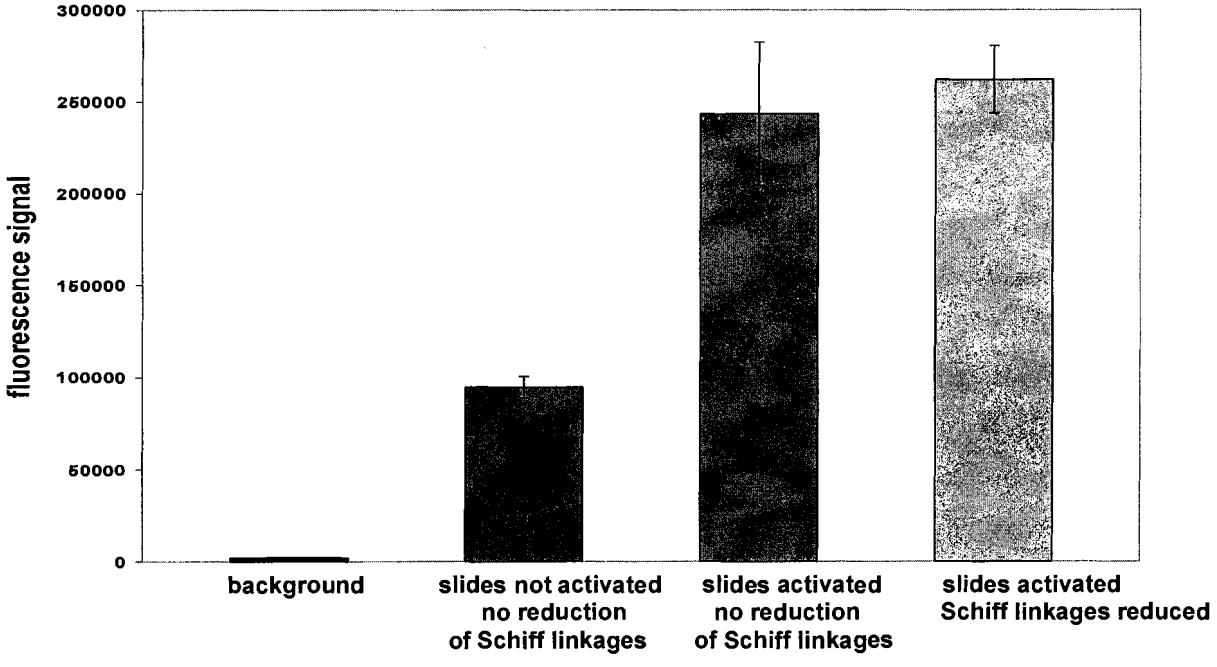
(b)



amine-modified, fluorescein labeled probe D6 (A-D6-Fl) (Fig. 6). All slides were washed after oligonucleotide spotting, as if they had gone through the Schiff linkage reduction procedure. Unactivated slides were able to retain oligonucleotide, yielding signal greatly above background levels. However, activation of the agarose increased signal from immobilized oligonucleotide ~ 2.5 X. Reduction of the Schiff base did not increase the amount of retained oligonucleotide. This is probably because, although unreduced Schiff base linkages are reversible, the equilibrium for this reaction lies far to the right. The reduction reaction also reduces any free aldehyde groups within the agarose, to unreactive alcohols (Schena, 2003) and thus acts as a blocking agent.

Figure 6 Retention of oligonucleotide probes on non-activated vs activated agarose-coated slides

Two slides per treatment, spotted at 48 locations with 10 μM A-D6-Fl, were measured. After application of the oligonucleotide, slides were placed in a dessicator overnight (to optimize formation of Schiff linkages). The two slides that were used to test the effect of Schiff linkage reduction were soaked in $NaBH_4$ solution. All slides were then put through a washing procedure of 2x1minute 0.2% SDS, 1x1minute RO water, 1x5minutes RO water. Mean values and errors of the mean are shown. Mean background for all slides is shown (error bar of 14% does not show at this scale).



2.4 Retention of amine-modified oligonucleotides

2.4.1 On differing aldehyde functionalized surfaces

Three levels of agarose concentration for agarose-coated slides as well as one commercially available aldehyde glass slide substrate, CSS, were tested for retention of amine-modified, fluorescein labeled oligonucleotide (A-D6-Fl) molecules after the Schiff reduction procedure and washes to remove unattached molecules. An array of A-D6-Fl spots was applied using a manual arrayer, and immobilized on each slide (Materials & Methods 3.6). In addition, 48 locations between spots were evaluated for a measure of background. An image of each substrate with its immobilized oligonucleotides is shown in Fig. 7a. Signal, and therefore retention, of oligonucleotide was highest on slides coated with 0.5% agarose (Fig. 7b). Background signal was also highest on 0.5% agarose-coated slides; this increase was negligible compared to spot signal (data not shown). Fluorescence intensity of the immobilized oligonucleotides on CSS slides was midway between that found on 0.005% and 0.05% agarose-coated slides (Fig. 7b). There was right skewing and wider distributions of signal values on the 0.05% and 0.005% agarose slides, as illustrated by frequency distributions (Fig. 7c). These distributions were obtained by determining differences from the means for each signal value, arranging these values in intervals, and counting the numbers of values within each interval. The results shown in this figure demonstrate that agarose concentration is an important factor when preparing the agarose microarray substrate, influencing retention of probe molecules.

2.4.2 Spot size

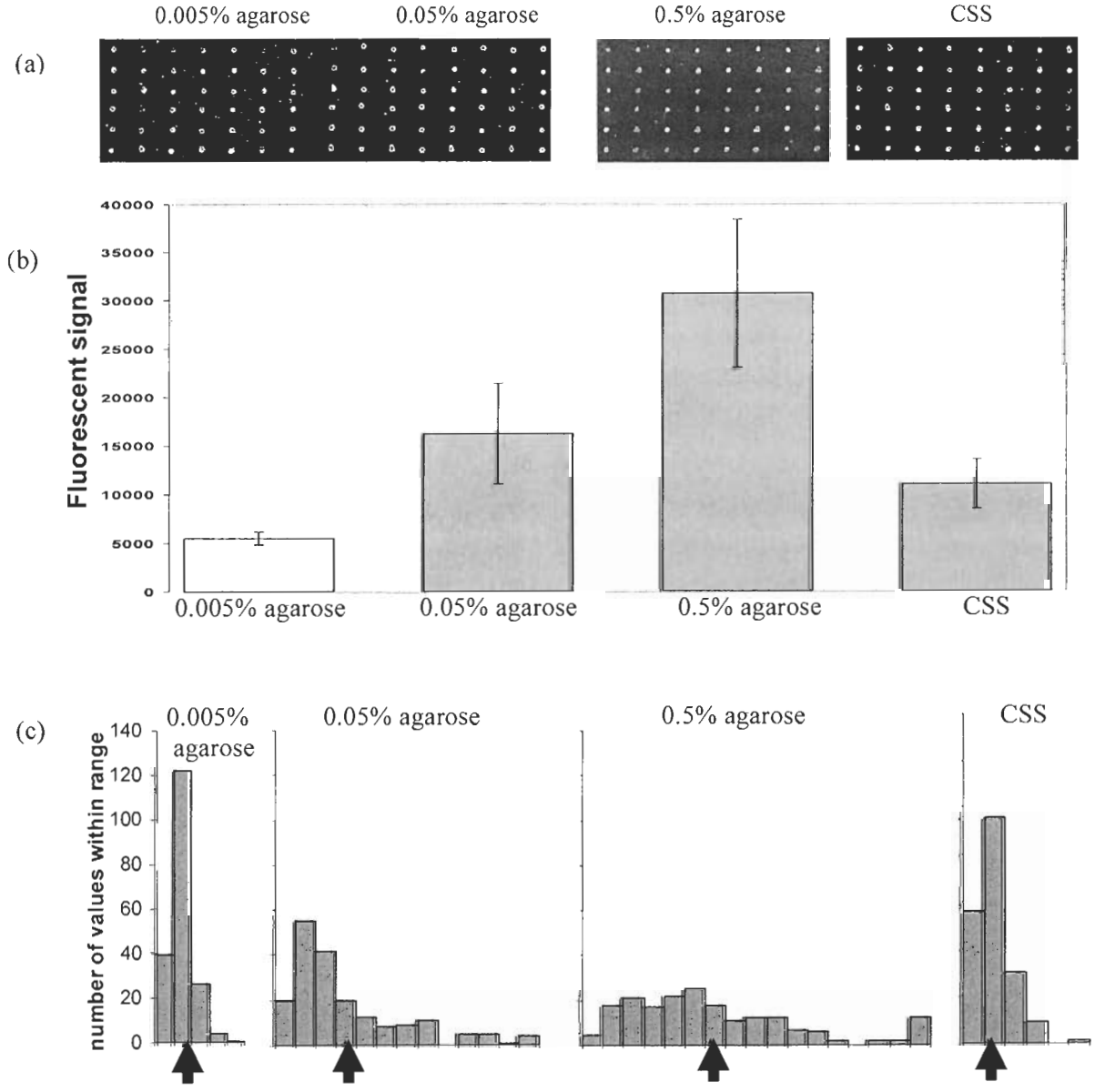
Spot (or feature) spread is a function of the spotting solution viscosity and substrate surface wettability (Smith & Reichert, 2003; Schena, 2003) and is defined as the difference between the sizes of the printing pin and the printed spot. Spot size was compared (Table 3) on two substrates – CSS slides and 0.05% agarose-coated slides by measuring the spot sizes formed

Figure 7 Retention of DNA oligonucleotide on various substrates

Four slides for each substrate were measured. Each slide was spotted with an 8 x 6 array of 10 μM amine-modified, fluorescein labeled oligonucleotide D6; oligonucleotide was immobilized upon the slides. Washes were performed to remove unattached oligonucleotide.

(a) representative slides (b) mean values and standard errors of the means for spot signal intensities, illustrating the slide to slide variation. Background signal was very low (1% - 3% of spot signal).

(c) differences from the mean for all 192 signal values found on the four slides for each substrate, illustrating the spot to spot variation, the same intervals were used for each substrate. The arrows indicate the interval where the difference from the mean approximates zero; points to the left of the arrow within each distribution represent values that are less than the mean, points to the right represent values greater than the mean



on these surfaces after application of 10 μM A-D6-Fl using the manual arrayer (with no further steps). Pin head diameter on this apparatus is ~ 300 μm. Spot size (area) on 0.05% agarose was ~10% greater than on the CSS slides, however signal was more than 200% greater. It appears that much more substance becomes deposited on the agarose slides compared to CSS slides. This may partly account for the increased retention on the agarose slides.

Table 2 Variation in deposition of oligonucleotide according to substrate

Spot parameters*	slide type			
	CSS		0.05% agarose	
	value	r.s.e.	value	r.s.e.
spot size (pixels)	108	2	131	9
spot size diameter (μm)	1180	1	1290	4.5
signal (x 10^5)	7.7	2	19.1	29

*mean values and relative standard error for three slides per treatment

2.4.3 Percent retention of applied oligonucleotide

The amount of applied oligonucleotide that is actually retained on 0.05% agarose-coated and CSS slides was estimated by comparing signal for a range of applied concentrations (0.01 to 100 μM) of A-D6-Fl on unwashed slides (Fig. 8), and slides washed to remove unattached oligonucleotides (Table 3). Signal values after washes were ~ 1% of the values before washes (data not shown).

A linear curve relating log(signal(before washes)) to log(applied amount) was generated and used to estimate apparent amount remaining after washing. This formula is shown in Fig. 8. Retention of applied oligonucleotides on both substrates was very low. For instance, on CSS slides, retention of applied 100 μM was ~ 0.06%, and on 0.05% agarose, 0.4%. On CSS slides, similar absolute amounts of 100 μM and 10 μM applied oligonucleotide were retained, while

application of 1 μM resulted in a smaller absolute amount retained. These figures indicate that the limits of retention are reached on CSS slides by the application of oligonucleotide at a concentration between 10 and 100 μM, and that application of higher concentrations does not lead to greater retention. On agarose-coated slides, there was a trend toward greater absolute retention from application of 100 μM than from application of 10 μM, illustrating the greater capacity of agarose-coated slides for oligonucleotide retention.

Figure 8 Curve relating amount of fluorescein to fluorescent signal generated

CSS and 0.05% agarose coated slides were arrayed with a dilution series of A-D6-Fl. 3 CSS and 8 agarose coated slides were arrayed using 100, 10, 1, 0.1 and 0.01 μM A-D6-Fl (12 spots each). An additional 2 slides for each treatment were arrayed using 50, 25,10, 5, 2.5, 1 and 0.5 μM A-D6-Fl (24 spots each). Slides were scanned with no further treatment (no washes). Data for CSS and for agarose coated slides was combined. The tool used for arraying applies 20 – 70 nl for each spot. Assuming 70 nl is deposited on agarose coated slides, then about 30 nl is deposited upon CSS slides (Table 2, compare ratios of signal). These volumes were used to estimate amounts (femtomoles) of A-D6-Fl that were applied in each spot. Logs of these amounts were graphed against logs of the signals obtained for these amounts, and an equation describing the relationship was generated. This equation was applied to the data shown in Table 3.

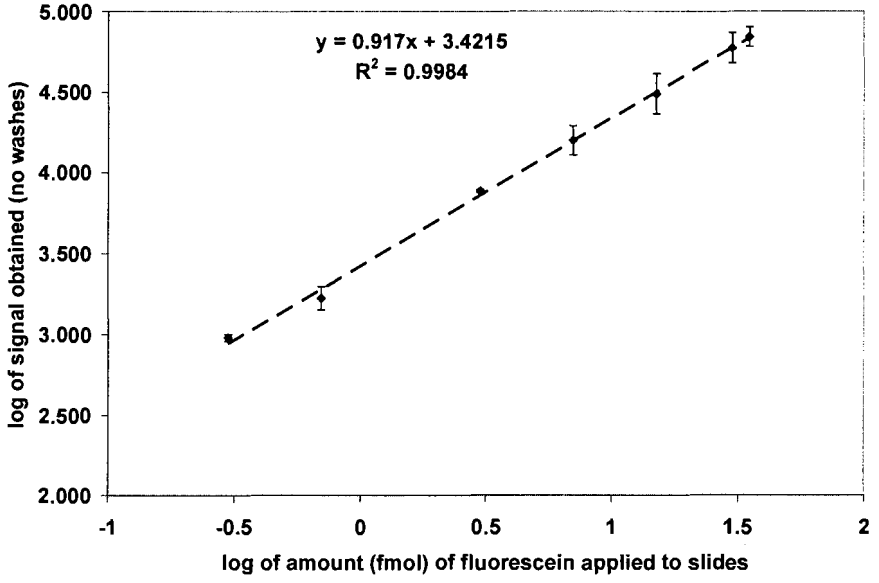


Table 3 Percent retention of oligonucleotide probe on washed slides

	CSS slides*	0.05% agarose coated slides*
(a) applied conc. (μM)	100	100
applied amount (fmol)	3000	7000
signal post wash	4687 ± 13%	55862 ± 67%
apparent amount (fmol) remaining post wash	1.61 – 2.14	8.33 – 48.8
% retention	0.062 ± 0.009	0.40 ± 0.29
(b) applied conc. (μM)	10	10
applied amount (fmol)	300	700
signal post wash	6052 ± 10%	15273 ± 63%
apparent amount (fmol) remaining post wash	2.20 – 2.74	2.29 – 11.7
% retention	0.82 ± 0.09	0.97 ± 0.66
(c) applied conc. (μM)	1	1
applied amount (fmol)	30	70
signal post wash	1916 ± 16%	1326 ± 51%
apparent amount (fmol) remaining post wash	0.58 – 0.83	0.22 – 0.74
% retention	2.4 ± 0.4	0.7 ± 0.4

*A set of slides prepared at the same time as the slides described in Figure 8 underwent post immobilization procedures (Schiff linkage reduction and rinses to remove unattached oligonucleotide). Slides were then scanned. On these slides, the spots prepared using 0.1μM and 0.01μM A-D6-Fl could not be seen; however, spots prepared using 100, 10 and 1 μM A-D6-Fl were visible. The intensities of these spots were measured. Using the equation generated by the figure 8 graph, the intensities of these spots (signal post wash) was converted to the amount of fluorescein at these spot locations (apparent amount (fmol) remaining postwash).

Applied amount = (applied concentration) x (applied volume); applied volume was estimated at 30 nl for CSS slides and 70 nl for agarose-coated slides.

$$\% \text{ retention} = \frac{\text{apparent amount remaining postwash}}{\text{applied amount}} \times 100\%$$

2.4.4 Uniformity of oligonucleotide application

Since there was considerable spot to spot signal variation on the agarose-coated slides and to a lesser degree on the CSS slides, the contributions to variation caused by the spotting procedure and agarose thickness were investigated. Agarose-coated slides (0.05%) and CSS slides were spotted with an array of 48 spots of 10 μM A-D6-Fl, dried, and scanned with no further treatment. Variation on each agarose-coated slide averaged 35% relative standard deviation, and on each CSS slides 19%. Background signal was more variable on the agarose-coated slides (19% vs 11%). After linkage reduction and removal of unattached oligonucleotides, the same slides were rescanned. Variation on agarose slides increased to 47%, and on CSS slides remained similar. The variation in background signal did not change.

The increased variability in background and probe signal on agarose-coated slides compared to CSS slides may be due to variations in the amount of agarose at different locations on the slide. The native fluorescence of agarose was found to increase with concentration (Table 4). Although signal variation on agarose-coated slides prepared manually is higher than on CSS slides, this increase is offset by the greater retention capacity of the agarose substrate.

Table 4 Native fluorescence of different concentrations of agarose coating

Concentration (%)	Mean signal[1]	r.s.e.[2]
1.0	395	11
0.5	331	8
0.1	219	3
0.05	230	6
0.01	191	5

[1]Signal was measured at 48 locations arranged in a 6x8 array, on three slides for each concentration of agarose coating. Slides were activated, dried, and scanned with no further treatment. [2]r.s.e. = relative standard error (relative error of the means for each slide)

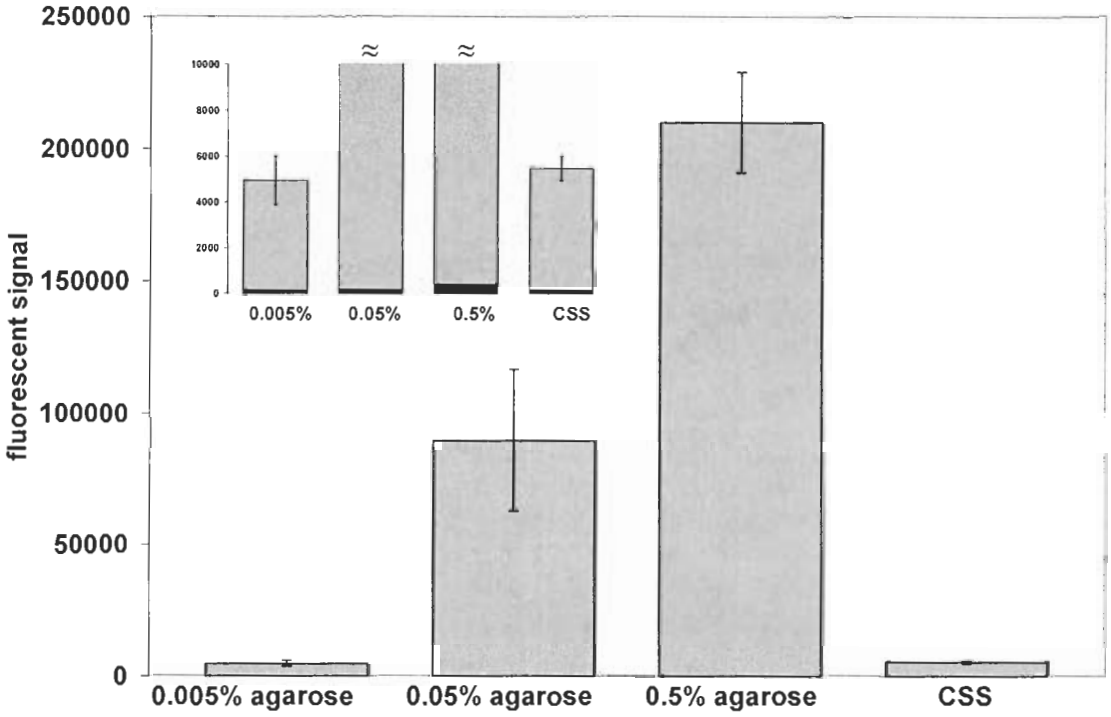
2.5 Hybridization of complementary oligonucleotides

2.5.1 On differing aldehyde functionalized surfaces

Four surfaces were compared for signal intensity obtained after hybridization between complementary oligonucleotides (Fig. 9). These surfaces were 0.5%, 0.05%, 0.005% agarose-coated slides and CSS slides. Signal intensity increased with agarose concentration. Signal on CSS slides was similar to signal on the lowest concentration of agarose (0.005%). Background signal was very low on all substrates (Fig. 9 inset). The much greater spot signal obtained on 0.05% and 0.5% agarose-coated slides resulted in a 17-fold increase in signal:noise ratio for these substrates over CSS slides and slides coated with 0.005% agarose.

As shown in Table 3, fluorescence signal due to retained 100 μM A-D6-Fl on 0.05% agarose-coated slides was ~ 6X in excess of that on CSS slides. However, from Fig. 9, signal due to hybridization of 100 μM A-D6 and 10 μM (D6)'-Fl on 0.05% agarose was almost 20-fold in excess of that on CSS slides. This is an indication that inhibition of hybridization is occurring on the CSS slides. CSS slides provide a planar surface for probe immobilization, agarose-coated slides provide a 3-dimensional layer, therefore similar signals due to immobilized oligonucleotide for these substrates (Fig.7b, Table 3b) indicate greater probe crowding on the CSS surface. Reduced steric interference could allow more pairing of complementary oligonucleotides in the agarose layer, increasing the efficiency of hybridization. This efficiency is similar on the 0.5% agarose-coated slides, where signals due to immobilized oligonucleotide (Fig. 7b) and to hybridization (Fig.9) are both approximately twice that on 0.05% agarose-coated slides.

Figure 9 Hybridization of complementary oligonucleotides on various substrates.



Three slides for each substrate were measured. Each slide contained an array of 48 spots (6X8) of immobilized oligonucleotide A-D6 (the concentration applied was 100 μM) and was hybridized to 10 μM (D6)'-Fl. Mean values and standard error of the means for the three slides are shown. Inset is graph expanded to illustrate proportion of signal due to background (black band) for each substrate (0.05 and 0.5% agarose substrates are off-scale). Hybridization temperature was 42°C and duration:was 16 hrs.

2.5.2 Effect of probe concentration on hybridization signal

To evaluate the effect of probe concentration on hybridization, the probe molecules A-D6 were immobilized on slides coated with 0.05% agarose and on CSS slides, at concentrations ranging from 10 – 250 μM. Complementary fluorescein labeled target (10 μM (D6)'-Fl) was then applied to the slides for hybridization (Fig. 10). Hybridization signal on CSS slides increased as probe concentration increased, however increasing probe concentrations above 25 μM (log=1.4) led to only slight increases in hybridization signal. In contrast, on agarose-coated slides, increasing probe concentration resulted in enhanced hybridization signal, in a linear trend. As shown in Table 3, on CSS slides, the absolute amount of oligonucleotide that became attached was similar for both 100 μM and 10 μM applied concentrations, therefore hybridization signals for these two concentrations of immobilized oligonucleotide would also be similar. Increased retention on CSS slides with increased concentration of applied oligonucleotide ceases at some point between 10 μM and 100 μM and accounts for saturation of the hybridization signal. On slides coated with 0.05% agarose, however, the retention limits are reached at concentrations greater than 100 μM of applied oligonucleotide, leading to increasing hybridization signal with increasing applied probe concentrations in the limits examined.

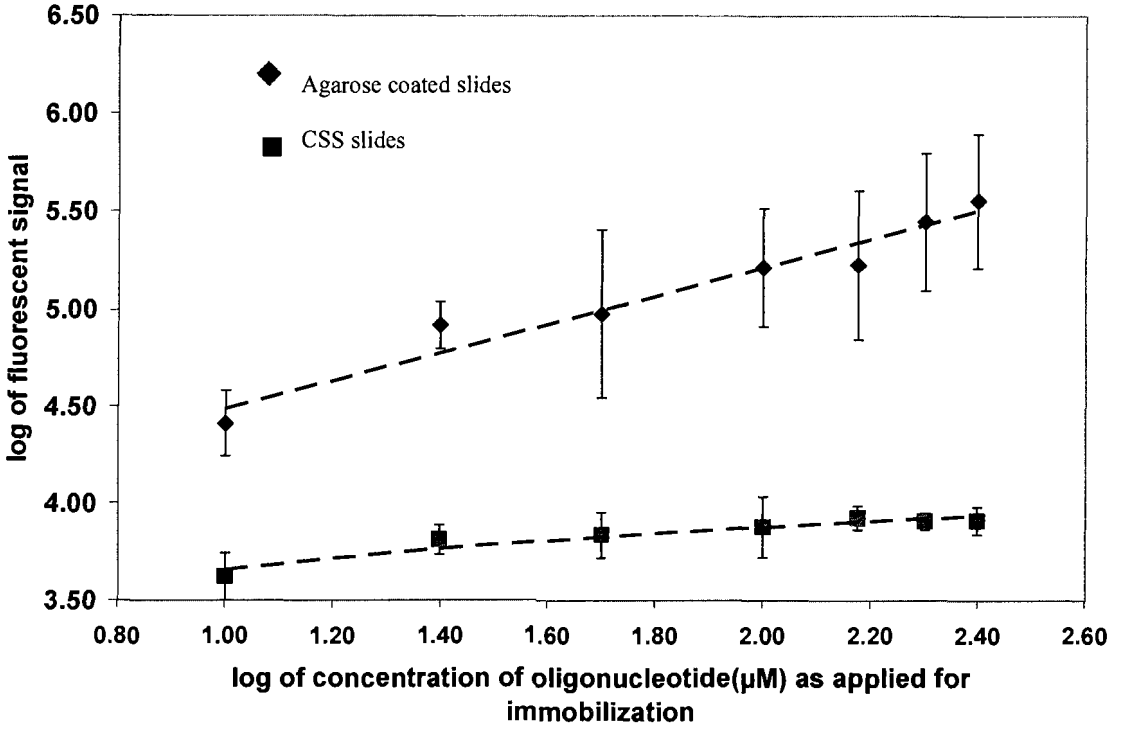
2.6 Hybridization of PCR products

2.6.1 Effect of length of hybridizing DNA and thickness of agarose layer

Initial hybridization tests were carried out on slides coated with 0.5% agarose and containing probes A-D6, A-ITS3 and A-B1 (Materials & Methods 3.4), which were hybridized to (D6)'-Fl (22 bases), or to PCR product (590 bases) derived from pure culture of *D. bryoniae* using primers ITS1F and UNLO28S22-Fl. (D6)'-Fl gave the desired results, binding clearly with A-D6, and showing only very slight non-specific binding to A-B1 and A-ITS3 (data not shown). However, the PCR product yielded non-specific results, reacting with all probes (data not shown).

Figure 10 Immobilized probe concentration vs hybridization signal

A dilution series of A-D6 was applied to CSS and 0.05% agarose-coated slides. 3 slides for each treatment were arrayed using 250, 100, 50, 25 and 10 μM A-D6 (24 spots each). An additional 3 slides for each treatment were arrayed using 250, 200, 150, 100 50, 25 and 10 μM A-D6-Fl (24 spots each). After immobilization of the oligonucleotide, the Schiff linkages were reduced and slides were washed to remove unattached oligonucleotide. Slides were then hybridized overnight at 42°C to 10 μM (D6)'-Fl. After post-hybridization washes, the slides were scanned and signals measured. Logs of applied concentrations were graphed against logs of the signals obtained for these concentrations after hybridization. No further increase in signal is seen on CSS slides at applied concentrations greater than 100 μM (log=2); whereas on the agarose-coated slides, overall signal is much greater, and continues to increase at applied concentrations greater than 100 μM.



Tests on slides coated with with 0.05% and 0.005% agarose were also unsuccessful – hybridization with PCR product yielded little or no signal (data not shown).

Other researchers have reported successful hybridization of 25-mer immobilized oligonucleotide to its 25-mer complement (Afanassiev *et al.*, 2000) and of 21 – 60-mer immobilized oligonucleotides to 60-mer target oligonucleotides (Dufva *et al.*, 2004) on slides coated with 1% agarose. However, it was mentioned that difficulties were encountered for hybridization of longer strands of DNA to the immobilized probe although the details were not reported (Afanassiev *et al.*, 2000). Wang *et al.* (2002) reported hybridization of 213 bp unlabeled PCR product to matched and mismatched molecular beacons on 1% agarose, with a much worse discrimination ratio compared to hybridization of the same beacons to 16-mer complementary oligonucleotide. These reported results and the preliminary results of the present study suggested that diffusion into and out of the agarose layer could be problematic for long DNA molecules. This possibility has also been suggested by other workers using another porous substrate, acrylamide (Yershov *et al.*, 1996; Guschin *et al.*, 1997; Barsky *et al.*, 2002), and who have found difficulties with diffusion into this substrate of DNA longer than ~ 200 - 250 nt long.

Therefore, for this work, short PCR product was generated using the primers ITS1F and fluorescently labeled ITS2 (ITS2-Fl), which yield products of ~ 260 bp (see Table 1). Hybridization of a short PCR product (259 bp), and a long one (590 bp), to probes immobilized on slides coated with 0.05% agarose, was compared. Positive hybridization results were obtained for the short product, whereas the results for the longer PCR product were indistinguishable from background (Fig.11).

Testing of short PCR product (264 bp) derived from pure culture of *B. cinerea* on slides coated with 0.5%, 0.05% and 0.005% agarose was also performed (Fig.12). Specific (no cross

Figure 11 Effect of length of PCR product on hybridization efficiency

PCR products of 259 bp (short PCR product) or 590 bp (long PCR product) derived from *D. bryoniae* were hybridized to the probe A-D6 on 0.05% agarose-coated slides. 6 blocks of 12 spots were measured of hybridization to A-D6; error bars show the standard error of the means obtained for each block of spots. Inset illustrates the contrast of specific hybridization (short PCR product to a block of 12 A-D6 spots), shown next to another block (of 12 spots of A-B1) where hybridization did not occur.

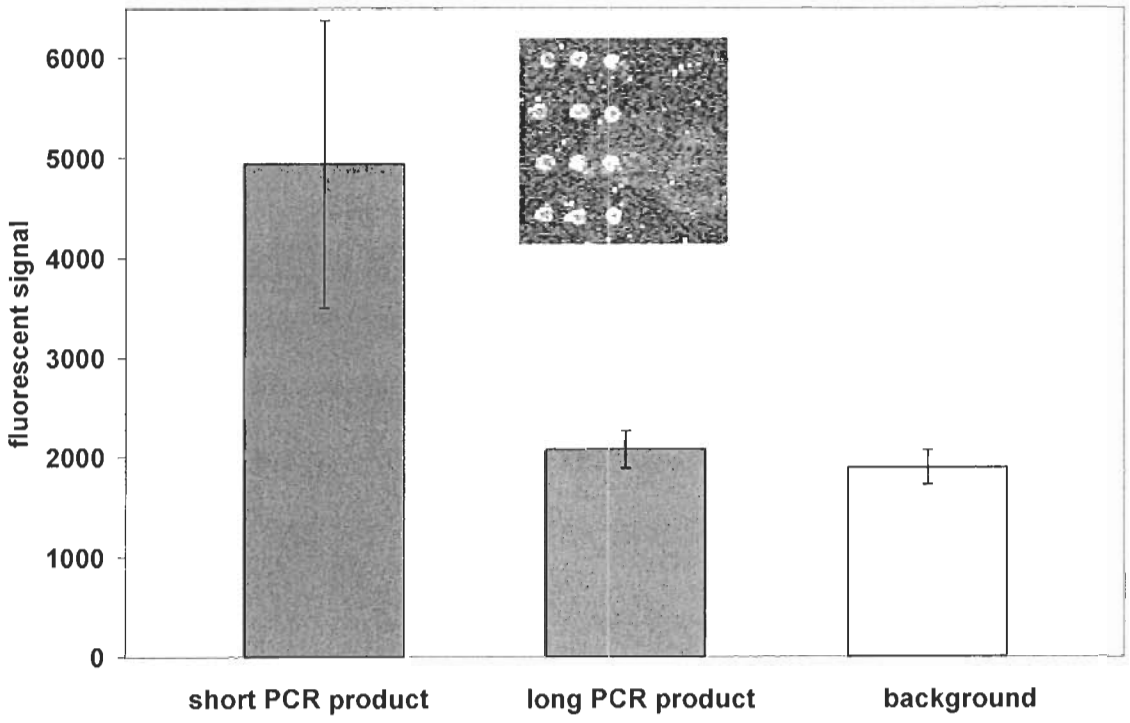
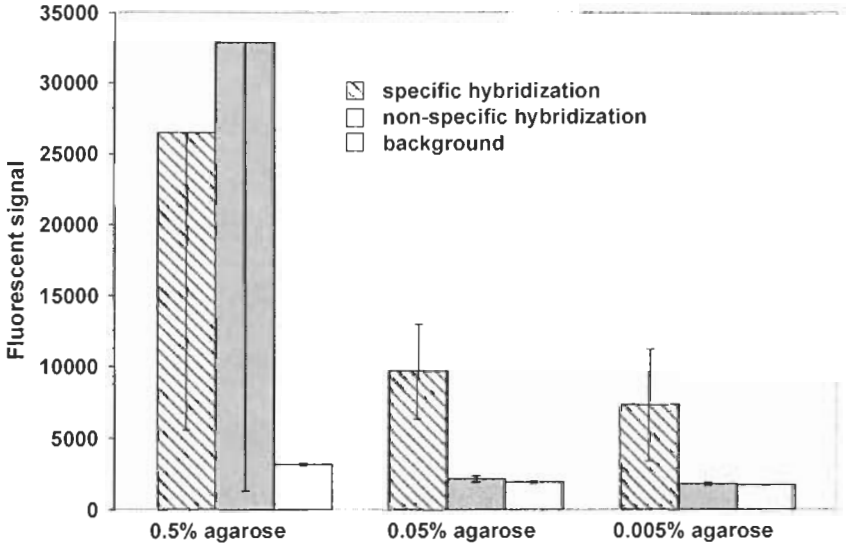


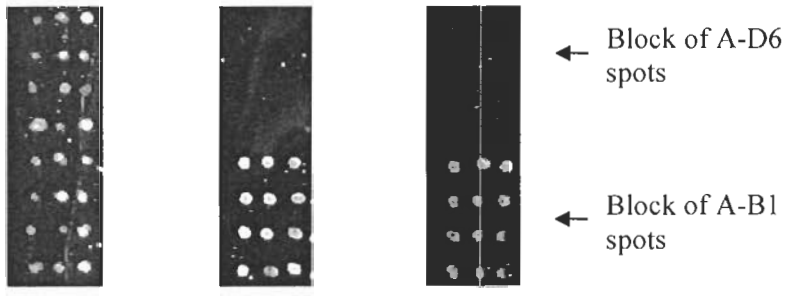
Figure 12 Effect of concentration of agarose coating on hybridization efficiency

(a) PCR product of 264 bp derived from *B. cinerea* was hybridized to A-D6 and A-B1 immobilized on agarose-coated slides. (a) Specific hybridization = hybridization to B1, non-specific hybridization = hybridization to D6, background = signal where no immobilized probe was present. Error bars are standard error of the means obtained for each block of spots (0.5% - 2 blocks; 0.05%, 0.005% - 4 blocks) (b) representative images of a portion of each slide used to generate the graphical data. All slides contained the same arrangement of oligonucleotides.

(a)



(b)



0.5% agarose 0.05% agarose 0.005% agarose

reactions) results were found on 0.05% agarose-coated slides as well as on 0.005% agarose-coated slides. Slides coated with 0.0005% agarose also showed specific binding, however the signal was quite weak (data not shown). However, as before when using long PCR product, non-specific (and highly variable) results were obained on 0.5% agarose, apparently because of indiscriminate retention of PCR product by the probes within the agarose layer. These results are an indication that agarose concentration also influences the success of hybridization (as well as influencing probe retention).

On the CSS slides, no hybridization could be seen between short PCR product and species specific probes (data not shown). However, hybridization between short PCR product (of *D. bryoniae* and *B. cinerea*) and immobilized ITS3 occurred weakly on this substrate (Fig. 13). Signal obtained after hybridization of PCR product and probe ITS3 on CSS slides was less than $1/10^{th}$ that obtained on slides coated with 0.05% agarose. On CSS slides, this hybridization signal could barely be distinguished from background. However, hybidization between complementary oligonucleotides could be easily seen on the CSS slides, although at much lower levels than on 0.05% and 0.5% agarose-coated slides (Fig. 9).

Hybridizations previously carried out on nylon membranes (Materials & Methods 3.6 and 3.7) confirm the results obtained with these probes on agarose-coated slides. Membranes containing immobilized probes A-D6, A-D7 (also specific to *D. bryoniae*), A-B1, A-ITS3, and a detection and immobilization control, D6-DIG, were hybridized to PCR products derived from *D. bryoniae* and *B. cinerea* (Fig. 14). Positive results were obtained for the species-specific probes and ITS3 with little or no cross-reaction. In contrast to results on agarose-coated slides using fluorescent detection, once a certain threshold has been reached on nylon membranes using chemiluminescent detection, greater signal yields a larger image rather than a more intense image. The same problem occurs with radioactive labeling (Schena, 2003; Machl *et al.*, 2002)

Figure 13 Hybridization on CSS slides compared to agarose-coated slides

Hybridization of *D. bryoniae* and *B. cinerea* PCR products to probe ITS3 was measured for four sets of spots on one slide each for each substrate. Error bars show standard error of the means (and do not always show at this scale). Background on each slide is also shown. Although the signal: background ratio on CSS slides is quite low, signal could be visually distinguished from the background.

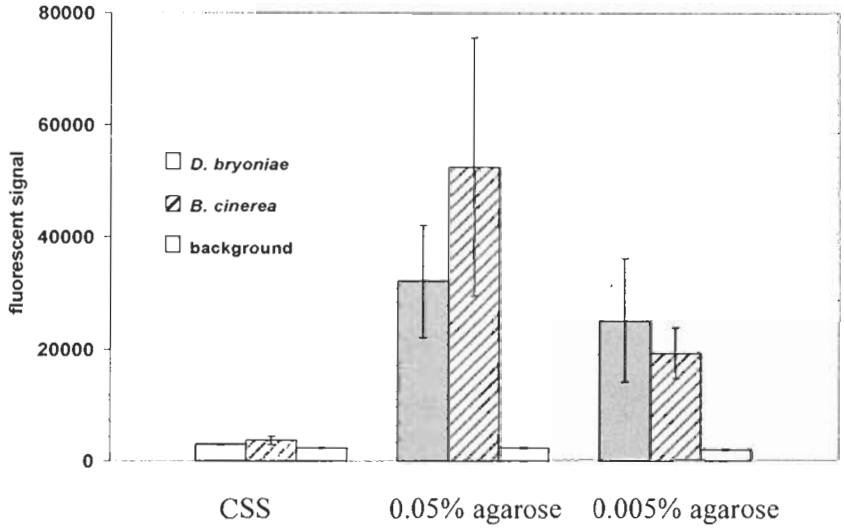
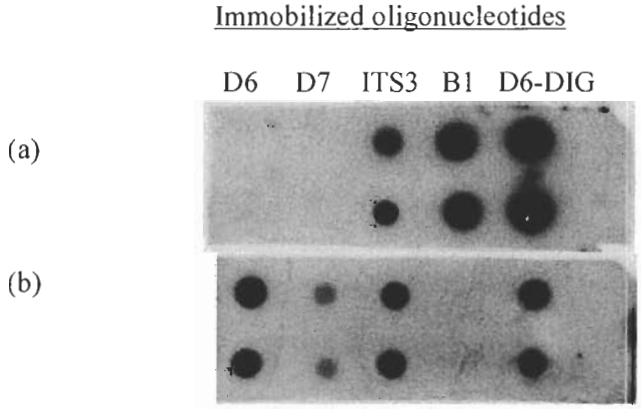


Figure 14 Hybridization on nylon membranes

PCR product from pure culture, generated with primers ITS1F and UNLO28S22, and digoxigenin-labeled nucleotides, was hybridized to membranes containing immobilized oligonucleotides. D7 is specific to *D. bryoniae*. D6-DIG is a digoxigenin-labeled immobilization and detection control. (a) *B. cinerea* PCR product (b) *D. bryoniae* PCR product



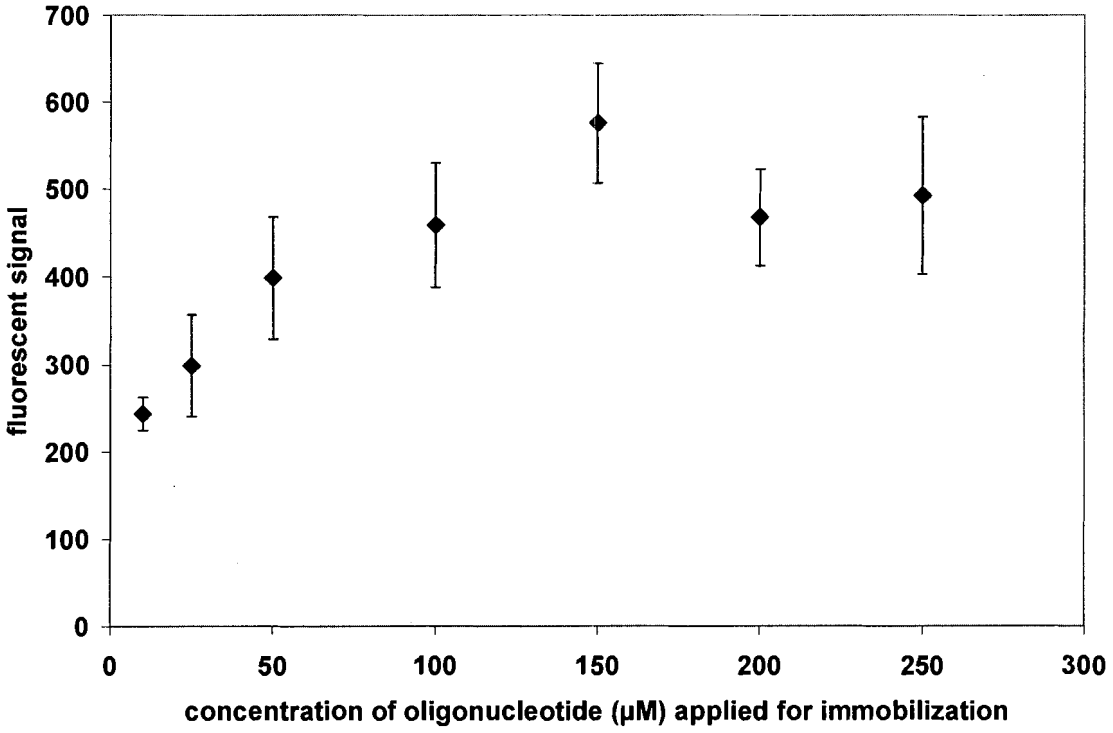
and limits the spot density that can be achieved on nylon membranes with these detection methods.

2.6.2 Effect of probe concentration on hybridization of PCR product

In order to study the effect of probe concentration on hybridization efficiency (Fig. 15), short PCR product of *D. bryoniae* was hybridized to the same range of immobilized oligonucleotide, A-D6, on 0.05% agarose-coated slides, as described for complementary oligonucleotides (Fig. 10). Fig. 15 shows a similar effect as Fig. 10 – there was a trend toward increasing hybridization signal with increasing concentration of oligonucleotide (applied for immobilization) up to 100 - 150 μM. In contrast however to Fig. 10, no further increase in signal could be seen after this point.

There are at least two types of interference that could lead to this reduced signal. Self-quenching of signal can occur (Lakowicz *et al.*, 2002) and is a well-known problem with the dye fluorescein; the excited energy instead of being released as light, is captured by nearby fluorescein molecules in a resonant energy transfer process. This phenomenon is increased by increasing density of fluorescein molecules, which will occur if more hybridization is occurring with greater concentrations of immobilized probe. However, self-quenching did not appear to occur for hybridization of complementary oligonucleotide, therefore this explanation is unlikely in this case. More likely, inhibition of hybridization is occurring, due to the increased DNA target size causing more steric interference. At increased densities of probe molecules, the target molecules encounter greater difficulties accessing the immobilized molecules (Zammatteo *et al.*, 2000; Peterson *et al.*, 2001; Schena, 2003). In addition, interactions between immobilized molecules (ds and ss) can destabilize the duplexes that do form, leading to increasing reduction in T_m as the hybridization reaction proceeds and the concentration of duplexes on the substrate surface increases (Piunno *et al.*, 1999). Increased density of the immobilized probe molecules

Figure 15 Hybridization of PCR product to a range of probe concentrations on 0.05% agarose. Two slides containing a dilution series of A-D6 were prepared. The concentrations applied were 250, 200, 150, 100, 50, 25 and 10 μM in two sets of 12 spots on each slide. Slides were hybridized overnight at 42°C to PCR product derived from *D. bryoniae* using primers ITS1F and ITS2-Fl. Error bars show the error of the means (of the hybridization signals) obtained for the four sets of spots for each applied concentration.



was reported to decrease the T_m (Piunno *et al.*, 1999) and increase specificity (Erickson *et al.*, 2003) (by increasing the stringency of the hybridization reaction).

Therefore, the discrepancy in hybridization (to varying concentrations of immobilized probe) results of complementary oligonucleotide, and of PCR product, is partly accounted for by the much greater size of the target DNA when PCR product is used (259 bp vs 22bp).

For subsequent experiments, the use of 100 μM oligonucleotide probe concentration was continued.

2.6.3 Hybridization times and temperatures

Several hybridization temperatures and durations were tested to determine optimal values for these parameters. Four temperatures (25°C, 37°C, 42°C and 48°C), and four durations (1/2 hr., 1 hr., 4 hrs. and 16 hrs.) were tested for hybridization of short PCR products of *D. bryoniae* and *B. cinerea* to immobilized probes on the array.

Temperature affected *D. bryoniae*:D6 hybridization with an optimum temperature ranging from 37°C to 42°C (Fig. 16a). *B.cinerea*:B1 hybridization was not sensitive to temperature in the range tested. Calculated T_m values by Sigma-Genosys, supplier of the oligonucleotides, using the 'nearest neighbor' (Breslauer *et al.*, 1986) method, were 62.4°C for B1 and 66.6°C for D6. These calculations apply to oligonucleotides in solution. There is experimental evidence that upon immobilization, the T_m will become reduced (Piunno *et al.*, 1999; Peterson *et al.*, 2000). T_m is defined as the temperature at which 50% of the oligonucleotide probe and its perfect complement are in duplex. For immobilized probes, T_d is calculated using the Wallace rule (Wallace *et al.*, 1979). T_d is defined as T_m, except that the probe is immobilized.

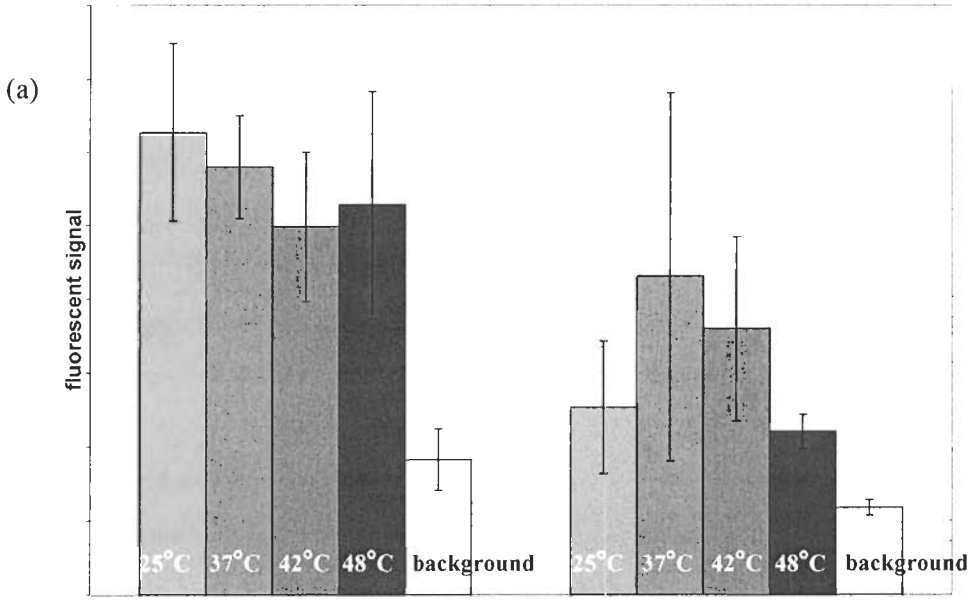
$$T_d = 2°C(A+T) + 4°C(G+C)$$

Figure 16 Effect of time and temperature on hybridization signal

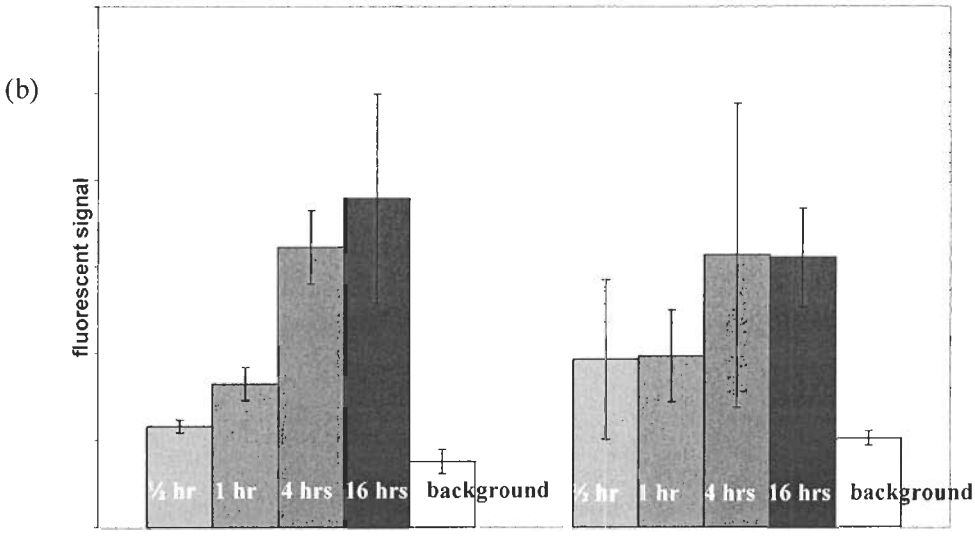
Two slides for each temperature and each duration were prepared for each species, containing immobilized probes A-B1 and A-D6 (applied at 100 μM, in 2 blocks of 12 spots per slide). Slides were hybridized to PCR product of *Botrytis cinerea* or *Didymella bryoniae* and subjected to the various times and temperatures. Means and errors of the means for the four sets of spots for each treatment are shown.

(a) hybridization temperature (duration 16 hours)

(b) hybridization duration (temperature 42°C)



Hybridization of *Botrytis cinerea* to B1 Hybridization of *Didymella bryoniae* to D6



Hybridization of *Botrytis cinerea* to B1 Hybridization of *Didymella bryoniae* to D6

Applying the Wallace rule to the oligonucleotides B1 and D6 yields the same value for both: 62°C. It should be noted that both formulas ('nearest neighbor' method and the Wallace rule) yield estimates only of T_m and T_d. A temperature of 48°C appeared to be too stringent for optimal *D. bryoniae*:D6 hybridization. Therefore, the true T_d value for D6 is likely lower than for B1.

Hybridization signal increased with duration of hybridization from ½ hour to 4 hours (Fig. 16b). There was little or no increase in signal at 16 hours duration. Therefore, at least four hours hybridization time is required to obtain the greatest amount of signal, with little or no gain in signal if longer hybridization times are chosen.

2.6.4 Hybridization kinetics

Within a substrate volume, hybridization efficiency depends upon (1) the availability of the immobilized probe molecules to the target (sample DNA) molecules and (2) the interaction between the probe and target molecules. The former depends upon the rate of diffusion of target molecules in solution to the substrate boundary, the rate of diffusion into the substrate, and the time required for even distribution of target molecules within the substrate (Sorokin *et al.*, 2003). Shorter sample DNA is more available than longer sample DNA for hybridization to the immobilized probe because of the greater ease of diffusion of smaller molecules, both into a substrate volume (such as a gel, Rubina *et al.*, 2004) and along the surface of a substrate with its immobilized probes (Chan *et al.*, 1995). The second factor, the interaction between probe and target molecules, is affected by the density of immobilized probe (Chan *et al.*, 1995; Sorokin *et al.*, 2003), as well as probe and target molecule lengths (Chan *et al.*, 1995)., and hybridization conditions such as temperature and target molecule concentration (Erickson *et al.*, 2003).

Hybridization of the sample molecule (S) and probe molecule (P) can be represented as follows:

$$S + P \Leftrightarrow S{\cdot}P \qquad (1)$$

where S = single-stranded sample DNA (target) complementary to the probe, P = immobilized probe, and S·P = hybridized target-probe duplex. The S·P duplex may dissociate to form S and P, and so the above reaction is reversible. In addition, when the sample molecule S is a single-stranded molecule from denatured PCR product, reannealing of S with its complementary strand occurs as follows:

$$S + S' \Leftrightarrow S{\cdot}S' \qquad (2)$$

The rate of sample-probe hybridization is dependent upon sample and probe concentrations (second order kinetics) and can be expressed as:

$$\text{rate} = k[S][P] - k'[S{\cdot}P] \qquad (3)$$

where k is the rate constant of association, and k' is the rate constant of dissociation in the reversible reaction of equation 1.

As the hybridization reaction proceeds, [S] and [P] decrease and [S·P] increases. The rate of hybridization can be expressed in differential form:

$$\text{rate} = d[S{\cdot}P]/dt \qquad (4)$$

Therefore, the rate can be measured by following the change in concentration of [S·P] with time.

In the case of hybridization with a perfect sequence match between S and P, k' (of equation 3) is negligible, and the rate of sample-probe hybridization is simplified as follows:

$$d[S{\cdot}P]/dt = k[S][P] \qquad (5)$$

Since the rate of target-probe hybridization is dependent upon [S] and [P], increasing [S] by increasing the concentration of sample PCR product (from which S is derived), should

increase the hybridization rate. Note that [S] available to the probe is reduced by the reannealing reaction given in equation 2. The hybridization rate can also be enhanced by increasing the rate constant, k. This is affected by experimental conditions such as hybridization temperature, salt concentration of hybridization buffer, as well as the nature of the DNA molecules such as the GC content of the probe and length of the target-probe duplex.

The formula describing the rate constant for reannealing strands of DNA in solution is:

$$k = k_n L^{0.5}/N \qquad (6)$$

(Wetmur & Davidson, 1968) where k_n = nucleation rate, L = DNA length and N = complexity of the DNA. Complexity of the genomic DNA is constant for a given set of organisms such as fungi. Reannealing occurs with the formation of several nucleation sites (where a few bases anneal) along the length of the strands, followed by a rapid 'zippering' that completes the annealing process. The rate of formation of nucleation sites, k_n, is dependent upon the hybridization conditions, such as temperature and salt concentration.

During hybridization of denatured PCR product to immobilized probe on an array, reannealing of the single-stranded DNA to its complementary strand, as shown in equation 2, competes with sample-probe hybridization. This competition becomes more severe as the length of the sample DNA increases. Longer DNA strands reanneal more quickly than shorter strands, with the annealing rate proportional to the square root of the strand length (equation 6). Moreover, other factors are also at play when comparing hybridization in solution and hybridization to an immobilized probe. Experimental evidence has shown that the T_m is reduced for immobilized dsDNA compared to dsDNA free in solution (Piunno *et al.*, 1999; Peterson *et al.*, 2000). This would further reduce probe-sample hybridization, favoring the undesirable reannealing reaction in the hybridization solution.

A comparison can be made for hybridization between immobilized probe D6 and denatured PCR product of either 590 bases (long product) or 259 bases (short product). Since

hybridization experimental conditions are the same for both processes, k_n values for reannealing and hybridization are the same. However, the differing lengths of DNA will affect how quickly the competing reaction of reannealing takes place, and how quickly this reaction occurs compared to the hybridization of probe to sample. Using equation 6, the factors describing the effect of DNA length are: $22^{1/2} \approx 5$ (probe-sample hybridization), $259^{1/2} \approx 16$ (reannealing of short PCR product strands), and $590^{1/2} \approx 24$ (reannealing of long PCR product strands). The long and short strands should reanneal about 5 times and 3 times respectively more quickly than the probe-sample duplex. The long strands should reanneal 50% more quickly than the short strands.

Considering the foregoing, the following steps can be taken to improve hybridization efficiency. (1) Reduce the competition due to reannealing strands by using sample DNA that is as short as possible. Two ways to achieve this is to use primers that will yield a shorter product, or to fragment the DNA after PCR. (2) Treat the sample DNA so that only the strand complementary to the probe is applied to the array for hybridization.

Sequence mismatches of sample and probe will also affect the sample-probe hybridization rate. Since the T_m is reduced for mismatched target-probe duplexes (Piunno *et al.*, 1999; Peterson *et al.*, 2000), $k'[S \cdot P]$ of equation 3 will increase for these duplexes, reducing the net rate of hybridization. This will favor the rate of reannealing over the rate of hybridization, thereby increasing the specificity of the reaction.

In conclusion, shorter DNA diffuses faster into the agarose layer, and is more available for hybridization. In addition, when both strands of sample DNA are applied for hybridization, shorter DNA may increase hybridization sensitivity (by reducing the reannealing rate of complementary strands relative to the target-probe hybridization rate for perfect matches). However, longer sample DNA may increase the specificity of the array (by increasing the rate of reannealing of target strands over that of hybridization of target to probe when a target-probe mismatch occurs). These factors need to be considered when developing a microarray protocol.

2.6.5 Hybridization with tissue sample DNA

Further testing was conducted with slides coated with 0.05% agarose, an immobilized probe concentration of 100 μM, hybridization time of 16 hours, hybridization temperature of 42°C or 48 °C, and various PCR products of ~ 260 bp derived by using primers ITS1F and ITS2-Fl. Each slide contained oligonucleotides arranged in blocks of 12 spots, replicated two or four times. The arrays identified *B. cinerea* (Fig. 17b) and *D. bryoniae* (Fig. 17c) from diseased cucumber stem tissue, and distinguished these organisms from each other with no cross reaction.

The DNA from a sample of uninfected cucumber stem tissue yielded a PCR product (confirmed by gel electrophoresis, data not shown). Upon hybridization to the array, hybridization occurred with the positive control probe, ITS3, but not to either species specific probe, D6 or B1 (Fig. 17d). The results from pure cultures and plant tissue infected with these organisms is summarized graphically in Fig. 18. This graph shows that the ratio of signal:background was ~ 2 – 4. Hybridizations that may have occurred at non-specific locations could not be distinguished from background.

Hybridization reactions with PCR products of the common greenhouse pathogens, *Sphaerotheca fuliginea* (causing powdery mildew of cucumber), *Erysiphe orontii* (causing powdery mildew of tomato), *Verticillium albo-atrum* and *V. dahliae* (causing wilt diseases of greenhouse crops), *Cladosporium cucumerinum* (causing cucumber scab), *Fusarium subglutinans* (a sweet pepper pathogen), and *Penicillium* sp. (from infected tomato) were positive for the control probe ITS3 and negative for B1 and D6 (Fig. 19).

Hybridization with PCR products of two other species of *Didymella*, *D. lycopersici*, which infects tomatoes, and *D. applanata*, which causes spur blight of raspberries, were positive for the control probe, and negative for B1 and D6 (Fig. 20a). Alignment of these sequences

Figure 17 Hybridization of DNA from diseased and non-diseased plant tissue to a low density microarray

(a) arrangement of probes in 12 spot blocks: B1 – *specific to B. cinerea*, D6 – specific to *D. bryoniae*, ITS3 – hybridization control, D6-F1 – immobilization, detection control (b) cucumber stem tissue showing symptoms of grey mold caused by *B. cinerea* (left); expected pattern (right) (c) cucumber stem tissue showing symptoms of gummy stem blight caused by *D.bryoniae* (left); expected pattern (right) (d) cucumber stem tissue showing no disease symptoms (left); expected pattern for fungal species other than *D. bryoniae* and *B. cinerea* (right)

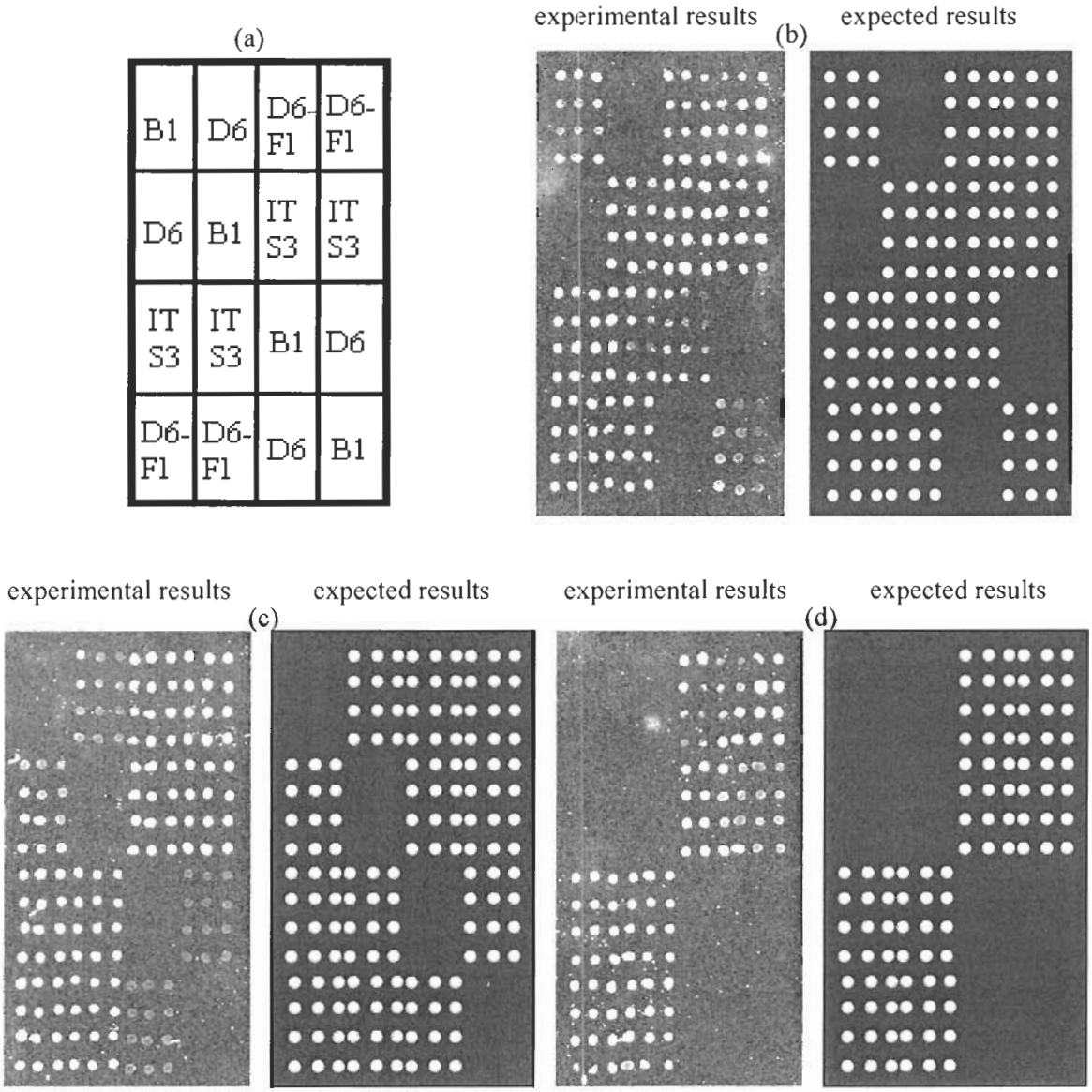


Figure 18 Hybridization signals of sample DNA extracted from pure culture and from infected plant tissue

One slide for each treatment was prepared, containing 4 sets of 12 spots of A-B1 and A-D6 immobilized probes (at an applied concentration of 100 μM). Slides were hybridized to PCR products derived for pure culture or infected plant tissue (using primers ITS1F and ITS2-Fl). Hybridization temperature for *B. cinerea* culture or infected tissue was 48°C; *D. bryoniae* was hybridized at 42°C. Areas where non-specific hybridization occurred(*i.e.*, hybridization of *B. cinerea* to probe A-D6, or *D. bryoniae* to A-B1) could not be distinguished from background. Means and errors of the means for the four sets of spots for each treatment are shown.

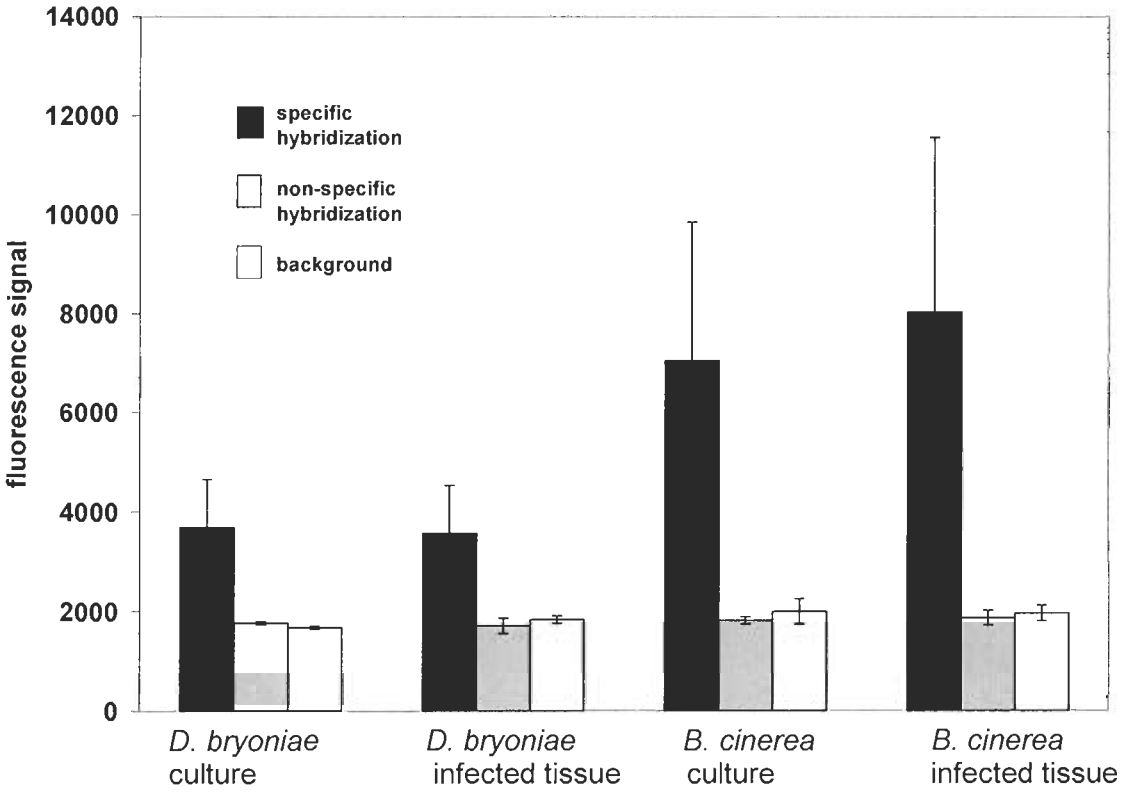
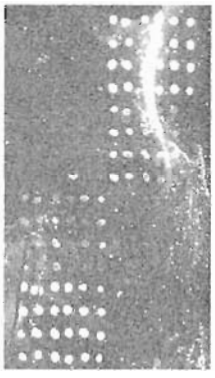
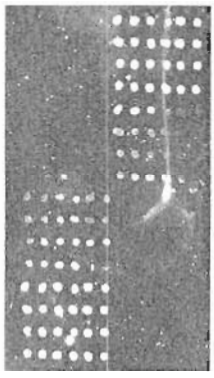


Figure 19 Hybridization of common greenhouse pathogens

Slides were prepared (one slide for each species) using the illustrated patterns for probe locations. Each probe block contained 12 spots of the particular probe. Slides were hybridized overnight at 42°C to PCR products derived from pure culture of the shown species (using primers ITS1F and ITS2-Fl). None of these species hybridized to the probes A-B1 or A-D6, and all hybridized to the control probe A-ITS3.

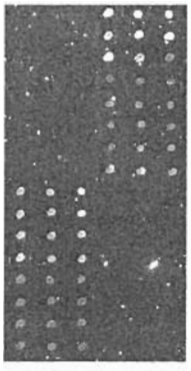


Sphaerotheca fuliginea

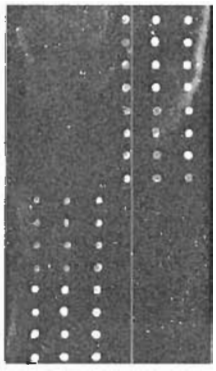


Cladosporium cucumerinum

B1	D6	D6-Fl	D6-Fl
D6	B1	ITS3	ITS3
ITS3	ITS3	B1	D6
D6-Fl	D6-Fl	D6	B1

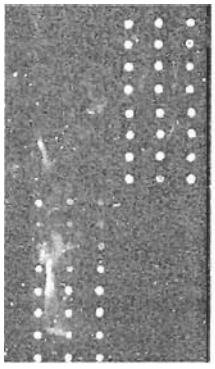


Erysiphe orontii

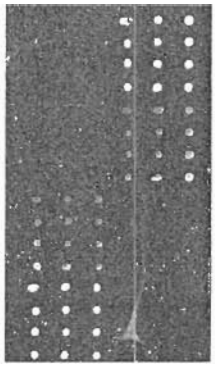


Penicillium sp.

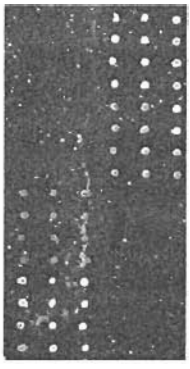
D6	D6-Fl
B1	ITS3
ITS3	D6
D6-Fl	B1



Verticillium albo-atrum



V. dahliae



Fusarium subglutinans

Figure 20 Hybridization results for species of *Didymella* and *Botrytis* and isolates of *D. bryoniae* and *B. cinerea*

Slides were prepared (one slide per species or isolate) using the illustrated pattern for probe locations. Each probe block contained 12 spots. Slides were hybridized overnight at 42°C (*Didymella* species) or 48°C (*Botrytis* species) to PCR products (primers ITS1F and ITS2-FI) derived from pure culture of the shown species or isolates. Only isolates of *D. bryoniae* hybridized to the probe A-D6, and only isolates of *B. cinerea* hybridized to the probe A-B1. Closely related species did not hybridize. (a) species of *Didymella* (b) isolates of *D. bryoniae* (c) species of *Botrytis* (d) isolates of *B. cinerea*

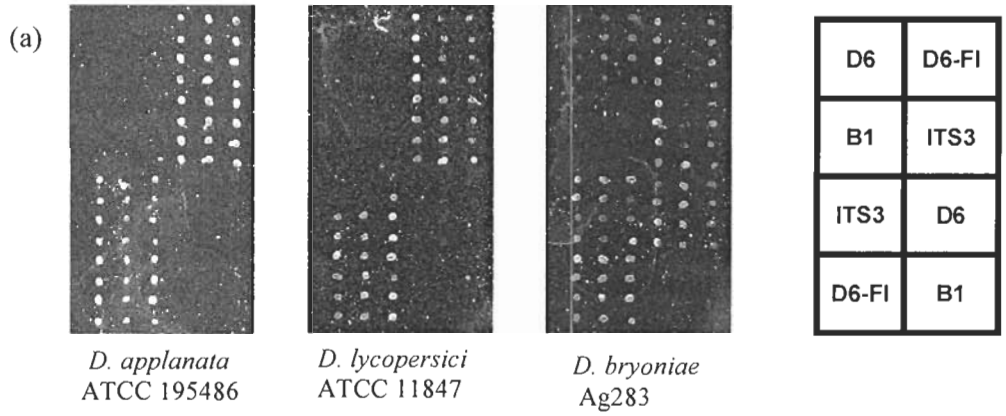


Figure 21 Alignments of *Didymella* species and *Botrytis* species

Sequences were obtained from GenBank for *Didymella bryoniae* and *Botrytis cinerea*, and species closely related to each of these. Each was aligned with the related species using Accelrys (San Diego, CA) DS Gene software. The bars indicates the locations of the probes

(a) *D. bryoniae*, probe D6 (b) *B. cinerea*, probe B1

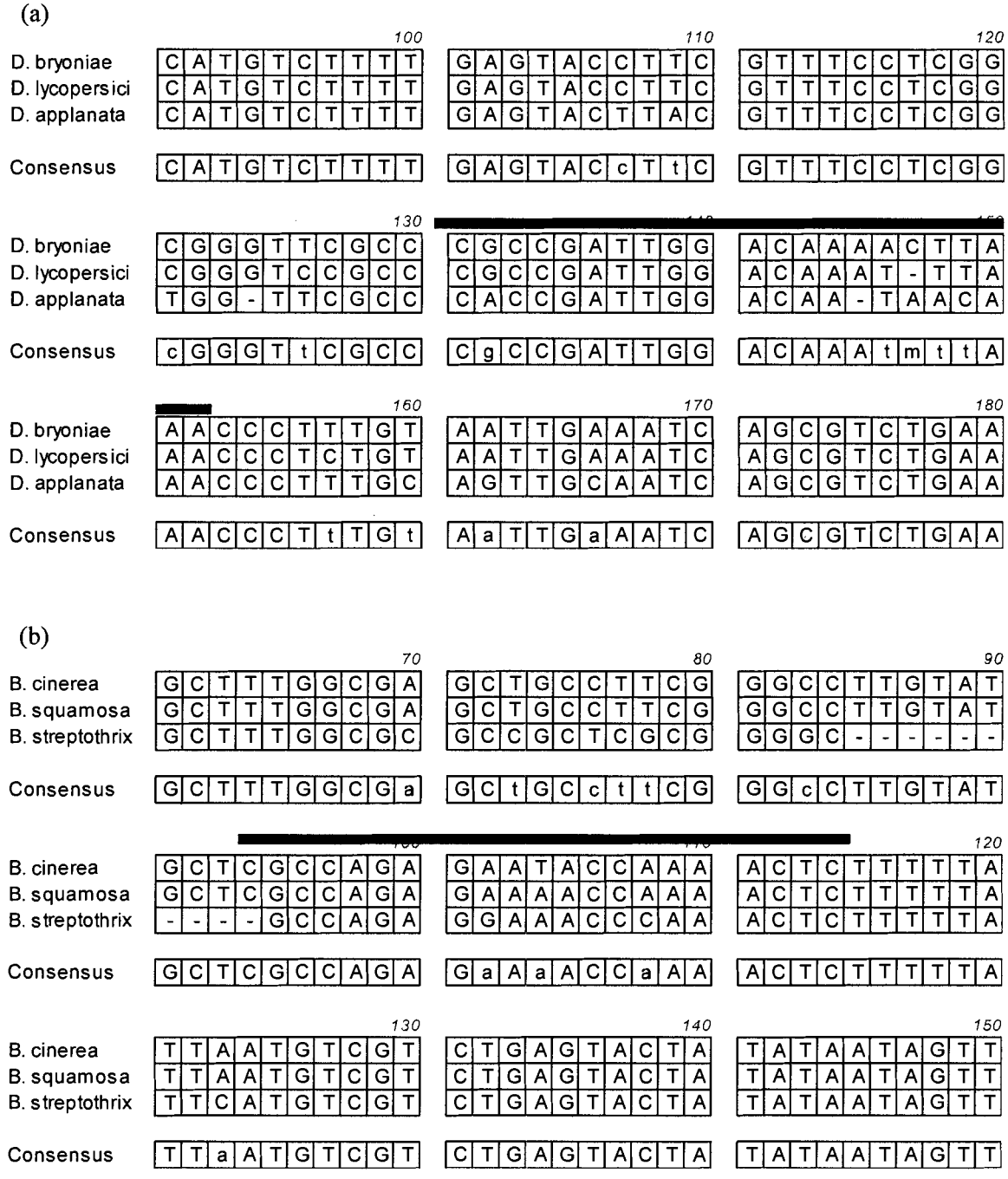
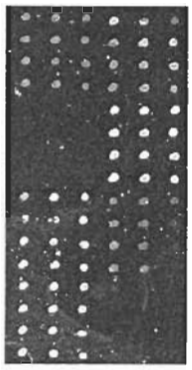
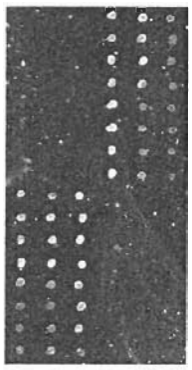


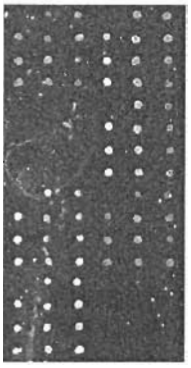
Figure 22 Commercial greenhouse samples



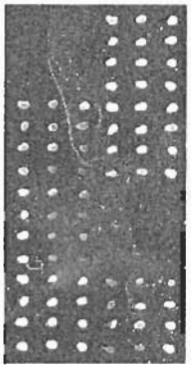
Ivar sample #1,
cucumber stem tissue



Ivar sample #2,
cucumber stem tissue



Ivar sample #3,
cucumber fruit tissue



Canagro sample,
tomato fruit tissue

Arrangement of immobilized probe in 12-spot blocks

D6	D6-FI
B1	ITS3
ITS3	D6
D6-FI	B1

(sequences obtained from GenBank) reveal that the sequence for *D. lycopersici* differs by two bp from the probe D6; *D. applanata* differs by 6 bp at this location (Fig. 21a).

Hybridization results for two other species of *Botrytis*, *B. squamosa* and *B. streptothrix*, were negative for B1 (and D6), and positive for the hybridization control, ITS3 (Fig. 20c). Comparison of sequences (obtained from GenBank) for these organisms show a one base mismatch between *B. cinerea* and *B. squamosa* for the B1 probe area, and 3 mismatches for *B. streptothrix* (Fig. 21b).

Other isolates of *D. bryoniae* and *B. cinerea* were also tested. This step is necessary for probe and array development because isolates obtained from widely dispersed locations may differ slightly from each other in DNA sequences. These other isolates gave positive results with their respective probes (Fig. 20b,d), with no non-specific results.

Commercial greenhouse samples suspected of gummy stem blight infection (*D. bryoniae*) or gray mold (*B. cinerea*) were tested by microarray (Fig. 22). Two of these samples were confirmed culturally and microscopically to be *D. bryoniae* (Ivar samples #1 and #3). Ivar sample #2 was found to be a species of *Fusarium*.

The results obtained for all samples are summarized in Table 5.

Table 5 Summary of hybridization results of tissue samples

Sample – pure culture	hybridization to D6	hybridization to B1
Didymella bryoniae, 4 isolates	**yes**	no
Botrytis cinerea, 3 isolates	no	**yes**
D. applanata	no	no
D. lycopersici	no	no
B. squamosa	no	no
B. streptothrix	no	no
Penicillium sp.	no	no
Cladosporium cucumerinum	no	no
Verticillium albo-atrum	no	no
V. dahliae	no	no
Erysiphe orontii	no	no
Sphaerotheca fuliginea	no	no
Fusarium subglutinans	no	no
Sample – diseased plant tissue diagnosed culturally with:		
Gummy stem blight	**yes**	no
Gray mold	no	**yes**
Fusarium sp.	no	no

2.7 Summary

The usefulness of agarose coated slides for the identification of microorganisms in a microarray format has been demonstrated by this study. The dynamic range of hybridized probe-complementary oligonucleotide that can be achieved is broader on agarose-coated slides than on CSS slides. Manipulation of immobilized probe concentrations may allow regulation of signal intensities from probe-sample duplexes with differing hybridization optimal conditions.

The agarose coated substrate was successfully used to identify and differentiate, without cross reaction, two plant pathogens (*Didymella bryoniae* and *Botrytis cinerea*) in pure culture and from diseased plant tissue. Agarose thickness and sample DNA length were determined to be

important parameters affecting hybridization efficiency. In addition, the superior sensitivity of this substrate for detecting hybridization, compared to a commercially available commonly used microarray substrate that utilized the same attachment chemistry, was also demonstrated. Probes immobilized on this substrate were able to discriminate a one-base mismatch from a perfect match.

The identification of microorganisms by microarray provides an accurate and rapid method of determining the organisms present in diseased plant tissue, and offers the possibility of identifying organisms present in mixtures. Culturing of tissue to isolate organisms of interest prior to DNA extraction is not necessarily required for diagnosis by microarray, considerably shortening the time required to make an identification. This is in contrast to conventional microscopic and cultural methods of identification, which usually require isolation and culturing of organisms in order to examine reproductive structures. In addition, similarities in microscopic structures among species can lead to errors in identification. Identification by microarray reduces or eliminates this ambiguity because of the unique DNA sequences present on the microarray.

Agarose coated slides are easily produced, and with the use of a manual arrayer, are an inexpensive alternative to commercial arrays for small scale applications.

2.8 Future work

The porous nature of the agarose layer, while increasing the capacity of the slide surface for immobilized probes and for the absolute amount of hybridization that can take place, also limits the size of sample DNA that can be applied to the slide for hybridization. This study showed that, on slides coated with 0.05% agarose, DNA of 590 bp was not able to hybridize with probes immobilized within the agarose layer. However DNA of 259 bp could successfully hybridize on this substrate. The phenomenon of improved hybridization with smaller target molecules is not limited to agarose or 3-D substrates, but is common to microarray substrates

(Wilson *et al.*, 2002; Schena, 2003). Smaller molecules diffuse more readily than large ones, and the formation of secondary structures is reduced.

These shorter strands of DNA were obtained by using primers that amplified a shorter region of the ribosomal genes of septate fungi. These short regions however reduce the possibility of finding the unique sequences required for identification of microorganisms to the species level. Other regions of the genome can also be chosen, however, finding primers that will amplify these regions simultaneously in several species can be problematic. In microarray identification of microorganisms, general primers are desirable because they enable amplification of some or all of the species present within mixtures, such as air or water samples, or diseased tissue supporting more than one organism.

Fragmentation of DNA can be used to yield sample DNA of a more favorable size. It is necessary to ensure that the fragments are not too small, leading to undesirably higher rates of non-specific hybridization.

Long strands of DNA (such as genomic DNA) can be fragmented mechanically by several means including (1) passage through a narrow orifice such as a syringe needle (2) by use of a nebulizer, or (3) by sonication. However, these methods yield a range of sizes of DNA, some or all of which will still be too large for diffusion into an agarose layer, leading to reduced sensitivity.

DNA can be digested with DNase I, a nuclease that cleaves phosphodiester bonds on one strand at a time, with a preferred cleavage site size of 4 – 6 bp (Sutton *et al.*, 1997). Extensive digestion yields fragments sizes varying from mononucleotides to dodecanucleotides (Vanecko and Laskowski, 1961), however, varying incubation time, temperature and enzyme concentration can produce larger fragments.

Restriction enzymes can also be used to cut DNA – this will give more reproducible fragment sizes. An enzyme could be chosen that cuts at a frequency that yields, on average, fragments of the desired size. One commonly used enzyme, Alu I, cuts at 5'AG↓CT, and therefore, on average, cuts a strand every 256 bp. This is a suitable size for the agarose substrate described in this study. Several other enzymes are available with 4-base recognition sites, and could be combined to yield smaller fragments. The enzyme CviJI cuts at RG↓CY, and theoretically would yield fragments on average 64 bp in length.

Future work for this application of PCR followed by hybridization on agarose-coated slides, requires optimization of sample DNA size and labeling protocol. Improvement of variation in substrate thickness is needed. Slides can be mechanically coated by spin-coating although this technique results in a very thin layer of agarose. Reduction of time required for hybridization is also desirable. Dynamic hybridization techniques can greatly reduce the time required for hybridization. Electronic hybridization reduces the hybridization time by electrically attracting target molecules to probe locations and requires construction of slides with electrodes (Schena, 2003). A flow-through arrangement whereby sample DNA flows over the immobilized probes will also speed up the rate of hybridization by greatly increasing the diffusion rate of target DNA (McQuain *et al.*, 2004).

PCR described in this paper requires primers that are to some degree specific, in this case, specific to fungi. However, random amplification approaches have been used with some success (Vora *et al.*, 2004). The advantage of using random primers is amplification of DNA of all organisms within a mixture; the disadvantage is generation of amplifed fragments that do not contain the sequence of interest (complementary to the immobilized probe sequence). This disadvantage would reduce the sensitivity of the procedure.

Oligonucleotides specific to other organisms can easily be added to the array. These organisms can include other fungi of the phylum Dikaryomycota (septate fungi, of which

Didymella and *Botrytis* are members), as well as other fungal phyla such as the Oomycota (of which *Pythium* species are common greenhouse plant pathogens). These other phyla, however, require other sets of primers (for the ribosomal DNA). Inclusion of bacterial oligonucleotides on the array also necessitates the use of different sets of primers. Sets of these primers can be combined in multiplex PCR, possibly leading to simultaneous amplification of the DNA of diverse organisms.

When developing the array, sensitivity and specificity of the array for each organism can be assessed. Sensitivity is the likelihood that positive results are found, it can also be thought of as the lowest amount of an organism or its DNA that will give a positive result. Specificity is the likelihood that positive results are true. Both parameters can be expressed mathematically, and calculated for a particular protocol.

$$\text{Sensitivity} = \frac{\text{Probability (true positive result)}}{\text{Probability(true positive result)} + \text{probability(false negative result)}}$$

$$\text{Specificity} = \frac{\text{Probability (true positive result)}}{\text{Probability(true positive result)} + \text{Probability(false positive result)}}$$

To assess sensitivity, DNA of organisms of interest can be prepared in one or more ways, amplified, and applied to the microarray for hybridization. Each protocol can be used to generate sensitivity values for each organism tested, or simply used to identify the best protocols for each organism.

Some methods for assessing sensitivity are described: (1) Extract genomic DNA from a pure culture of a particular organism, prepare a dilution series of the genomic DNA, and amplify a set volume from each dilution. (2) Prepare a spore dilution series for one particular organism, extract genomic DNA from a set volume for each spore concentration, and amplify. (3) Extract genomic DNA from pure cultures of several organisms, combine the genomic DNAs in several

ways, and amplify a set volume from each combination. (4) Prepare spore suspensions for several organisms, combine these suspensions in several ways, extract genomic DNA from each combination, and amplify.

Method #1 should give an estimate of the amount of genomic DNA that is required to obtain visible PCR product (and positive hybridization results). Method #2 should provide direct information about numbers of cells (spores) required to yield enough DNA for a visible amplification product. Methods #3 and #4 should provide information about the effects of organism mixtures on PCR product concentrations achieved for each organism, and the problems associated with dilution of one organism by another. Methods #1 and #3 provide information in an indirect fashion, by assuming equal efficiency of DNA extraction and amplification for each organism and using calculations (from the concentration of genomic DNA) to estimate the numbers of cells required to yield positive hybridization results. Methods #2 and #4 provide direct information about the capacity of the particular protocols used to yield positive hybridization results, and the numbers of cells actually required.

The first two methods, especially method #2, are the most practical, since the most common use of the array is likely to be tests of infected plant tissue, in which one pathogen is overwhelmingly present. When testing the sensitivity of the array for many organisms, method #1 may be more useful because much less time is required to complete each assay.

To assess the specificity of an array for a particular organism, account must be made of the stringency of the hybridization reaction. For instance, a reaction that yields false positives may be corrected by raising the temperature of the hybridization reaction. This however, may cause a loss of true positive results (*i.e.*, reduced sensitivity) for tests (of other organisms) requiring less stringency. Therefore, tests assessing specificity should be carried out at a range of hybridization conditions.

Tests for several organisms can be run using a particular assessment method, with the results obtained used to calculate sensitivity and specificity values. In this way, a particular

protocol can be compared for several organisms. These values are very useful if the diagnostic microarray is intended for use with mixtures of organisms. In practice, however, hybridization tests can be optimized for each organism, such that the probabilities of false negative or false positive results are very low.

CHAPTER 3: MATERIALS AND METHODS

3.1 Fungal isolates and plant samples

Fungal species used in this study consisted of *Didymella bryoniae*, *Botrytis cinerea*, 2 other species of *Didymella* – *D. lycopersici* and *D. applanata*, 2 other species of *Botrytis* – *B. streptothrix* and *B. squamosa*, and several other fungal plant pathogens. These are listed in Table 6. *Didymella* spp. were grown in cucumber-dextrose broth (boiled extract of five large cucumber leaves in ~ 1L of water, with the addition of 0.6% dextrose) on a shaker at room temperature for seven – ten days. Other species (except powdery mildews) were grown similarly in potato-dextrose broth. The resulting fungal mats were collected by suction-filtration in a Buchner funnel and washed to remove nutrient medium. Spores of powdery mildew of cucumber (*Sphaerotheca fuliginea*) were collected by shaking heavily infected cucumber leaves over paper. Powdery mildew of tomato (*Erysiphe orontii*) spores were collected from tomato leaves by scraping. Infected and uninfected plant samples were obtained from greenhouses at the Pacific Agriculture Research Centre at Agassiz, B.C., and from commercial greenhouses located in B.C.

3.2 DNA extraction

Approximately 200 mg tissue samples were taken for DNA extraction, which was performed using the Fast DNA® kit and the FastPrep® homogenizer from QBiogene (Carlsbad, CA), and their protocol for fungi. This procedure homogenizes sample by extremely vigorous shaking in the presence of a lysing matrix. Following centrifugation, the DNA is cleaned by the GENECLEAN™ (Qbiogene) procedure, using reagents contained in the kit.

Table 6 Fungal isolates examined in this study

Species	Isolate	Origin	Common host
Didymella bryoniae	Ag*283	British Columbia	cucumber
D. bryoniae	ATCC**56275	Netherlands	cucumber
D. bryoniae	ATCC 60646	Crete	cucumber
Phoma cucurbitacearum	DAOM^ 216033	Quebec	cucumber
D. applanata	DAOM 195486	British Columbia	raspberry
D. lycopersici	ATCC 11847	Germany	tomato
Botrytis cinerea	Ag 100	British Columbia	many crops
B. cinerea	DAOM 226636	Ontario	many crops
B. cinerea	DAOM 196802	Quebec	many crops
B. streptothrix	DAOM 37579	Ontario	---
B. squamosa	DAOM 211643	Ontario	onion
Verticillium albo-atrum	DAOM 191497	Alberta	tomato
V. dahliae	DAOM 22573	British Columbia	tomato
Cladosporium cucumerinum	DAOM 146982	Ontario	melon
Penicillium sp.	Ag 335	British Columbia	tomato
Sphaerotheca fuliginea	obligate***	British Columbia	cucumber
Erysiphe orontii	obligate	British Columbia	tomato
Fusarium subglutinans	Ag 344	British Columbia	pepper

*Ag = Pacific Agriculture Research Centre, Agassiz, B.C. culture collection
^DAOM = Canadian Collection of Fungal Cultures
**ATCC = American Type Culture Collection
***obligate = requires a living host

3.3 DNA amplification and labeling

PCR reagent concentrations in 20 µl volumes were 1X reaction buffer, 100 µM each of dATP, dGTP, dCTP and dTTP, 500 nM of each primer, 1.25U Gold Taq polymerase (GeneSys Ltd.), and 1 µl DNA template, overlaid with 1 drop of mineral oil. Thermal cycling parameters were 29 cycles of 45 seconds at 94°C, 45 seconds at 58°C and 45 seconds at 72°C followed by a single 10 minute cycle at 72°C. PCR results were visualized by electrophoresis of 1 ul aliquots on 1% mini-gels, followed by staining in an ethidium bromide bath (0.5 ug ethidium bromide/ml water) and photography under UV light. For low levels of initial template, two rounds of amplification can be performed, see Appendix 1.

DNA was labeled during PCR by inclusion of a primer labeled at the 5' end with fluorescein. This primer was either fluorescein modified ITS2 or fluorescein modified UNLO28S22. Primers were obtained from Sigma-Aldrich (Oakville, ON). Some other labeling methods are described in Appendix 2.

After PCR, excess nucleotides and primers were removed by treatment of the PCR product with the Qiaquick PCR purification kit (Qiagen, Inc. , Mississauga, ON).

For tests on nylon membranes, DNA was labeled during PCR by incorporation of digoxigenin labeled nucleotide, DIG-d-ATP (Roche Diagnostics, Laval, QC), the primers were ITS1F and UNLO28S22. Purification of the PCR samples was not needed.

3.4 Oligonucleotide probes

The probe sequence used to detect *Didymella bryoniae* was D6 (CGCCGATTGGACAAAACTTAAA) (Koch and Utkhede, 2002), and to detect *Botrytis cinerea* was B1 (CGCCAGAGAATACCAAAACTC) (Mathur and Utkhede, 2002). A positive hybridization control sequence, ITS3 (GCATCGATGAAGAACGCAG) (White *et al.*, 1990) was used for some tests. It is complementary to the primer ITS2 used for PCR amplification. Fluorescein labeled oligonucleotide D6 (D6-Fl) at a concentration of 10 µM was also applied as a

control to verify that immobilization had taken place. All probes were modified by attachment of a 5' amine group (A-). The modified probes were obtained from Sigma-Aldrich (Oakville, ON).

3.5 Substrate preparation

Plain precleaned glass slides (VWR) were coated with 1 ml of 0.5, 0.05, 0.005, or 0.0005% agarose (EMD Omnipur, VWR, Mississauga, ON) prepared in reverse osmosis (RO) water. The molten agarose was applied by pipettor at a temperature of ~ 70°C (to the slides resting on a level surface), and the slides were dried at 50°C for at least one hour. The agarose-coated slides were then activated by soaking in a bath of 20mM $NaIO_4$ for ½ hour, rinsed 2x1minute in RO water, and dried at 50°C. A commercially available aldehyde-functionalized slide (CSS, CEL Associates) designed to covalently bind single or double stranded DNA via a Schiff linkage was also tested. Some testing was also performed on nylon membranes (Roche Diagnostics, Laval, QC). Another type of agarose (glyoxal agarose) was briefly tested, results are described in Appendix 3.

3.6 Microarray construction

Amine-modified oligonucleotides (Sigma-Aldrich) were applied to each slide at a concentration of 100 μM (except A-D6-Fl, 10 μM) in spotting buffer (0.15 M NaCl, 0.1 M $NaHCO_3$, pH 8.5) using a hand-held arrayer (Schleicher & Schuell (Keene, NH) microCASTer™, 8 pin format). The specifications for this arrayer state that it will apply a maximum of 768 spots of ~ 650 μm in diameter at a centre to centre horizontal spacing of ~ 1.2 mm and vertical spacing of ~ 0.8 mm. For some tests, a single oligonucleotide was employed, applied in an array of 48 spots over the entire slide. For testing with fungal samples, different oligonucleotides were applied as blocks of 12 spots and each block was replicated on each slide 2 or 4 times. After application of oligonucleotides, slides were incubated overnight at room temperature in a dessicator. Slides were then soaked in sodium borohydride solution (0.05 g

$NaBH_4$, in 30 ml PBS, 10 ml ethanol) for 5 minutes, rinsed 2 x 1 minute in 0.2% SDS, 2 x 1 minute in RO water, and dried at 50°C.

Dufva *et al.* (2004) found with their protocol (in which slides were soaked for several hours in water after gellation of the agarose and before drying) that slides coated with 0.5% and 0.25% agarose were more difficult to fabricate than slides coated with 1% agarose because of fragility, easy detachment of the agarose and cracking during drying. For the current study, slides were routinely coated with 1 ml aliquots of 0.05% and lower concentrations of agarose without difficulty. However, problems were encountered with detachment of the agarose during reduction with $NaBH_4$, in initial tests using 1% and 0.5% agarose-coated slides. The H_2 bubbles generated during this procedure appeared to lift sections of the agarose away from the glass surface, which would lead to easy detachment of the entire agarose layer. With the much lower concentrations of agarose (0.05% and lower) subsequently used, detachment was never a problem with this blocking procedure.

In preliminary testing, this attachment protocol for oligonucleotides to a solid substrate was compared to avidin-biotin attachment (see Appendix 3).

For tests on nylon membranes, 1µl aliquots of 100 µM amine-modified oligonucleotide in 3XSSC were applied by pipettor and fixed to the membranes by baking at 120°C for ½ hour.

3.7 Hybridization

For sample analysis, microarrays were constructed on slides coated with 0.05% agarose and on CSS slides using the species specific probes A-D6 and A-B1, the hybridization control probe A-ITS3, and the immobilization/detection control, A-D6-Fl. Fluorescein labeled sample DNA from *D. bryoniae* and *B. cinerea* fungal cultures, from plant tissue infected with these organisms and showing disease symptoms, from uninfected plant tissue, and from fungal cultures of several other pathogens, was hybridized to these arrays. As a control, hybridizations with fluorescein labeled oligonucleotide complementary to D6 ((D6)'-Fl) were also conducted.

Purified PCR samples, or control oligonucleotide ((D6)'-Fl, 10 μM), were diluted 10X in hybridization buffer (5XSSC, 0.2% SDS) to yield 50 μl solution, denatured at 95°C for 10 minutes then quickly cooled on ice. The entire sample was applied to the area of a slide containing the immobilized oligonucleotides, covered with a glass coverslip (60 mm x 25 mm), and incubated overnight in a humid chamber at 42°C or 48°C. After hybridization, slides were rinsed at room temperature for 3 minutes in 2X SSC, 0.1% SDS, 1 minute in 0.1X SSC, 0.1% SDS, ½ minute in RO water, then dried at 50°C.

On nylon membranes, hybridization was conducted using DIG-labeled DNA from *D. bryoniae* and *B. cinerea* fungal cultures. Membranes were pre-hybridized for 1 hour at 42°C in hybridization buffer (5XSSC, 0.1% sarcosine, 0.02% SDS, 1% skim milk powder blocking agent). Ten μl DIG-labeled PCR product in 5 ml hybridization buffer was denatured in boiling water for 10 minutes, then hybridized to the membranes overnight at 42°C. Membranes were then rinsed at room temperature for 15 minutes in 2XSSC, 0.1% SDS and at 42°C for one hour in 2XSSC, 0.1% SDS

3.8 Data acquisition

Fluorescent signal was measured with a Typhoon 9410 scanner (Amersham Biosciences) at an excitation wavelength of 488 nm, emission wavelength of 526 nm, resolution of 100 μm, various PMT voltages, and analyzed using ImageQuant software. If necessary, values obtained at a particular PMT voltage can be converted to equivalent values for another voltage (Appendix 4). Most values were quantified by summing the intensities of each pixel within a standardized area, equal for each spot and smaller than total area of the spot (14 pixels for each spot). Data for percent attachment of applied oligonucleotide was obtained using 'spotfinder', which uses software to find spots according to set parameters, and sums the total intensity. Background fluorescence was not removed.

For nylon membranes, anti-DIG-alkaline phosphatase and an alkaline phosphatase substrate, CDP* (disodium 4-chloro-3-(methoxyspiro{1,2-dioxetane-3,2'-(5-chloro)tricyclo[3.3.1.1]decan}-4-yl)phenyl phosphate); Roche Diagnostics, Laval, QC), were used according to manufacturer's instructions to produce a chemiluminescent signal that was recorded by exposing the membranes to X-ray film.

APPENDIX 1:
DOUBLE AMPLIFICATION

Introduction

Initial amounts of DNA template can be very low – this may be the case with air and water samples, and in plant tissues with suspected but symptomless infection. One round of amplification by PCR yields very small amounts of product. To increase yields, a further round of amplification can be performed using PCR product of the first round as template.

Materials and methods

Combinations of PCR primers (Table 1a) in unnested, semi-nested and nested formats were tested for yield on DNA extracted from air samples and from pure culture of *D. bryoniae*. One µl aliquots of 1st round product were used as template for the 2nd round. Amplification conditions and visualization of results were as described in Section 3.3.

Results and discussion

Some possible combinations were not tested: for instance, the primer ITS4 was dropped from further consideration because it complements plant DNA and its sequence coincides with that of the primer UNLO28S22. Some results of double amplifications for two sets of amplifications derived from two 1st round primer combinations are shown in Figure 23. These images show that double unnested amplifications on impure samples such as those derived from air samples, yield very low levels of PCR product. Results are tabulated in Table 7 for all combinations tested.

During PCR, a plateau effect occurs after a number of cycles, where further cycles do not yield more product. Some reasons for this effect are reagent depletion, preferential annealing

of amplifed DNA to its complementary strand rather than to primers as the concentration of amplifed DNA increases and that of primers decreases, and accumulation of pyrophosphates, which poison the reaction. When a single species of DNA was the template for the 1st round (*i.e.*, set of cycles), nested, semi-nested, and unnested 2nd PCR rounds amplified efficiently (Fig.23a, lanes 3; Fig. 23b, lanes 6). This is in contrast to DNA extracted from the air samples (therefore a mixture of DNA species), where an unnested 2nd round of amplification yielded very little product (Fig. 23a(i), lanes 1,2; Fig 23b(i), lanes 1-5). For both types of DNA (single species and mixed species), semi-nested and nested amplifications proceeded efficiently in the 2nd round, yielding appreciably greater amounts of product than a single round alone (Fig. 23a(ii, iii); Fig. 23b(ii – iv)). Since reagent depletion and accumulation of pyrophosphates are not factors because an aliquot of the 1st round template is transferred to fresh PCR reagent mixture, competition of primers for annealing sites may be a problem for mixed template. Inhibition of PCR due to the presence of inhibitory compounds in the DNA extracted from air samples is also not a factor, since semi-nested and nested amplifications proceeded smoothly.

Conclusion

Low levels of initial DNA template require an additional set of amplifications in order to yield sufficient PCR product for visualization by gel electrophoresis, and of adequate concentration for subsequent hybridization reactions. This can easily be achieved using semi-nested or nested amplifications. Unnested amplifications of the air samples did not yield adequate product. Any of the sets of primers tested, in a nested or semi-nested format, yields enough PCR product for subsequent testing.

Table 7 Amplification results for two rounds of amplification

Initial template – air sampler DNA

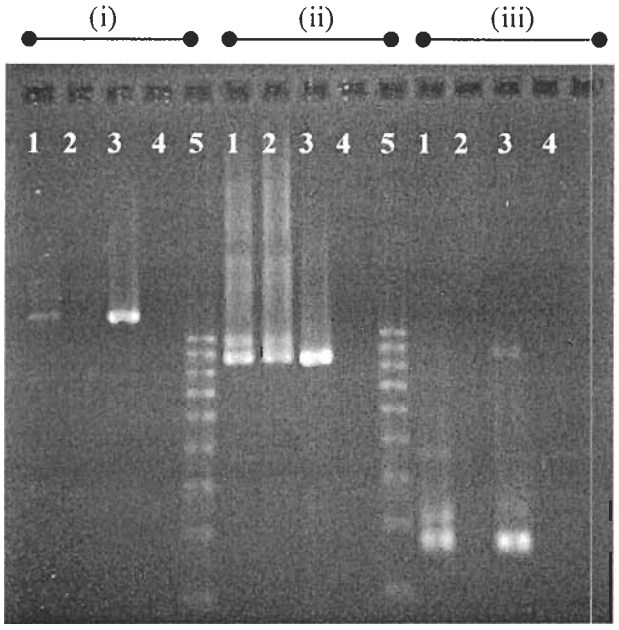
	primers, round 1	primers, round 2	format	amplification results	Figure
1	NS5 & UNLO28S22	NS5 & UNLO28S22	unnested	poor*	23a (i)
2	NS5 & UNLO28S22	NS5 & ITS2	semi-nested	good**	
3	NS5 & UNLO28S22	ITS1F & UNLO28S22	semi-nested	good	23a (ii)
4	NS5 & UNLO28S22	ITS1F & ITS2	nested	good	23a (iii)
5	NS5 & ITS2	NS5 & ITS2	unnested	poor	
6	ITS1F & UNLO28S22	ITS1F & UNLO28S22	unnested	poor	23b (i)
7	ITS1F & UNLO28S22	ITS1F & ITS2	semi-nested	good	23b (iii)
8	ITS1F & UNLO28S22	ITS5 & UNLO28S22	semi-nested	good	23b (iv)
9	ITS1F & UNLO28S22	ITS5 & ITS2	nested	good	23b (ii)
10	ITS1F & ITS2	ITS1F & ITS2	unnested	poor	
11	ITS1F & ITS2	ITS5 & ITS2	semi-nested	good	
12	ITS5 & UNLO28S22	ITS5 & UNLO28S22	unnested	poor	
13	ITS5 & UNLO28S22	ITS5 & ITS2	semi-nested	good	
14	ITS5 & ITS2	ITS5 & ITS2	unnested	poor	

*'poor' indicates that the product could not be visualized with gel electrophoresis, or that much less product was obtained compared to that obtained for DNA derived from a pure culture

** 'good' indicates that the product obtained could be visualized as well as the product obtained using DNA derived from a pure culture as template

Figure 23 Some results of double amplification of low levels of DNA template

(a)



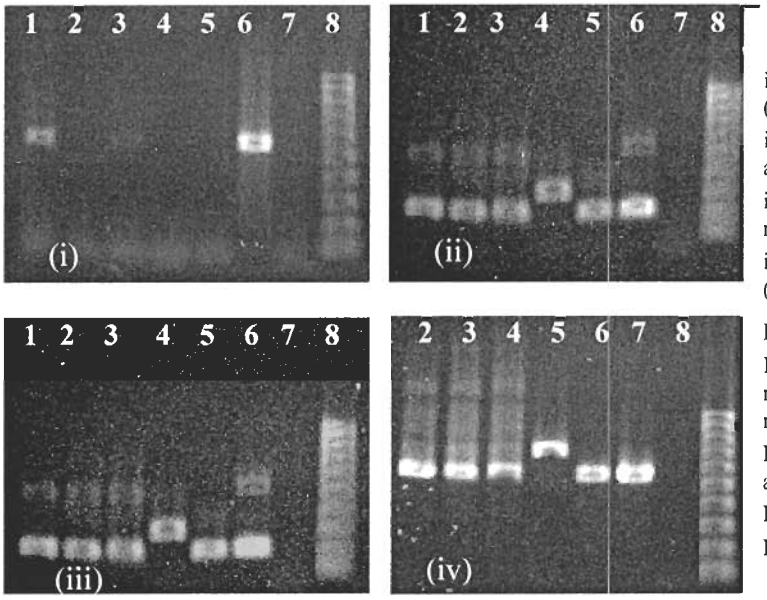
1st round primers: NS5 & UNLO28S22
(i) 2nd round - NS5 & UNLO28S22 (unnested amplification)

(ii) 2nd round - ITS1F & UNLO28S22 (semi-nested amplification)

(iii) 2nd round - ITS1F & ITS2 (nested amplification)

DNA template (PCR product of 1st round)
Lanes 1- DNA extr. from air sampler (2 hr run time)
Lanes 2- DNA extr. from air sampler (1/2 hr run time)
Lanes 3- Genomic DNA (*D. bryoniae*) diluted 10000X
Lanes 4- water blank (doubly amplified)
Lanes 5- KB ladder

(b)



1st round primers: ITS1F & UNLO28S22

i) 2nd round -ITS1F & UNLO28S22 (unnested amplification)

ii) 2nd round - ITS5 & ITS2 (nested amplification)

iii) 2nd round - ITS1F & ITS2 (semi-nested amplification)

iv) 2nd round - ITS5 & UNLO28S22 (semi-nested amplification)

DNA template (PCR product of 1st round)
Lanes 1 – 5 template from air sampler run 2hr., 1hr., ½ hr., ¼ hr., 5 min. respectively
Lane 6 Genomic DNA (doubly amplified)
Lane 7 Water blank (doubly amplified)
Lane 8 KB ladder

APPENDIX 2:
DNA LABELING

Introduction

Initial testing of the agarose substrate used 0.5% agarose-coated slides and DNA labeled during PCR by incorporation of a labeled primer. The primers yielded a product of ~ 600 bp (long PCR product). This study has shown that 0.5% agarose-coated slides and long PCR product does not yield useful hybridization results. However, during initial testing, an alternative explanation for the poor results was considered – failure to incorporate adequate label into the sample DNA. Therefore, a variety of labeling methods were compared.

Sample DNA can be directly labeled in several different ways. Three common methods are: during PCR by the use of labeled nucleotides, during PCR via a labeled primer, or after PCR by incorporation of a tail of labeled nucleotides in the presence of terminal transferase. These protocols alone and in combination were briefly assessed; yields were not quantified but were roughly compared by visualization on the gels.

Materials and methods

Incorporation of labeled primer took place as described in Section 3.3. For incorporation of labeled nucleotides, some of the d-TTP was replaced with fluorescein labeled d-UTP, to yield final concentrations of 5 μM Fl-dUTP and 95 μM dTTP. After labeling (during PCR), unused nucleotides and primers were removed. The tailing reaction took place post-PCR; unused nucleotides and primer of the PCR reaction were first removed. Sample was tailed with Fluorescein-12-dUTP (cat.#1373242, Roche Diagostics) using terminal transferase (cat.#3333566, Roche Diagnostics) and the manufacturer's standard tailing protocol. All

methods of labeling yielded product that could be visualized by gel electrophoresis (Fig. 24). Multiple labeling procedures increased yields (not shown).

Conclusion

Since it was apparent that the use of labeled primer was a successful means of labeling the DNA, this procedure was followed for subsequent testing. The other two methods have the following disadvantages : incorporation of labeled nucleotide is expensive relative to incorporation of labeled primer, and post-PCR tailing requires an extra step in procedure.

Figure 24 Gel electrophoresis image of labeled PCR products (fluorescence scan)

Sample DNA (long product) of *D. bryoniae* except lane 6 (*B. cinerea*)

Labeling method:

lane 1 labeled nucleotides

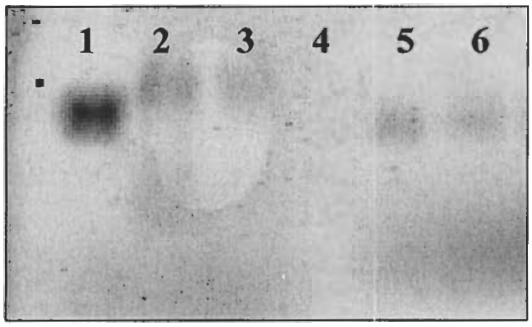
lane 2 labeled nucleotides and tailing

lane 3 tailing

lane 4 unlabeled DNA

lane 5 labeled primer

lane 6 labeled primer



APPENDIX 3:
OLIGONUCLEOTIDE ATTACHMENT CHEMISTRY AND TYPE OF AGAROSE

Introduction

Two protocols for attachment of oligonucleotides to agarose-coated glass slides were evaluated: direct attachment of amine-modified oligonucleotide to the agarose surface, and an indirect attachment where biotinylated oligonucleotide was attached to avidin, which was attached to the agarose surface. In addition, a comparison was made between two types of agarose: glyoxal agarose, which comes preactivated and contains reactive aldehyde groups, and plain agarose, activated after application onto the slide. These were compared for retention of probe oligonucleotide, and for suitability as a substrate upon which hybridization of complementary oligonucleotides can take place.

Materials and methods

For amine-aldehyde attachment, 18 - 1µl aliquots of 10 µM A-D6-Fl (C6 linker; 0.1M $NaHCO_3$/0.15M NaCl buffer, pH 8.5) oligonucleotide were spotted (in a 6 x 3 array arrangement) on slides coated with 0.5% glyoxal agarose (Biowhittacker Molecular Applications), or plain agarose (activated), allowed to dry at room temperature, incubated overnight at high humidity, and dried at 37°C for ½ hour. Slides were then soaked for 5 minutes in sodium borohydride solution, followed by rinsing twice for one minute in 0.05% Tween 20 in PBS, twice for one minute in RO water, and dried.

For biotin-avidin attachment, slides coated with 0.5% glyoxal agarose or plain agarose (activated) were first spotted with 18 - 1µl aliquots of avidin (0.1% in PBS) in the same 6 x 3 array arrangement, then treated as for aldehyde-amine attachment. Ten µM 5'-biotin-modified,

3'-fluorescein-labeled poly-thymidine oligonucleotide (B-T12-Fl; C7 linker; Sigma-Aldrich) in PBS was then applied as 2μl droplets (in order to ensure complete coverage of the smaller avidin droplets) onto the locations corresponding to avidin placement, incubated at high humidity for 3 hours, rinsed twice for one minute in PBS, once for one minute in RO water and dried at 37°C.

To compare hybridization on the two types of agarose, 32 spots of 100 μM amine-modified oligonucleotide (A-D6) were applied, using the hand-held microarrayer, to slides coated with 0.5% plain or glyoxal agarose. Preparation of this type of slide is described in Sections 3.7 and 3.8. Hybridization as described in Section 3.9, using 10 μM (D6)'-Fl was then performed.

Results and discussion

Signal values for the avidin-biotin system were ~ 50% higher on plain agarose than on glyoxal agarose; with the amine-aldehyde system signal values were ~ 30% greater on glyoxal agarose than on plain agarose (Fig. 25a). Retention of 10 μM A-D6-Fl was repeated on slides coated with 0.5% glyoxal agarose or plain agarose, using the handheld microarrayer to apply the oligonucleotide. No differences in amount of oligonucleotide retained were found for the two types of agarose (data not shown).

Fluorescent signal from hybridized oligonucleotides was ~ 40% higher on glyoxal agarose than on plain agarose (Fig. 25b). Relative to background, spot signals on both substrates were very high, therefore a 40% increase did not significantly increase discrimination of the spot from its background.

Conclusion

Because there was no clear-cut advantage to either probe immobilization protocol, the more easily implemented one (amine-aldehyde) was chosen for further work. Since glyoxal agarose is much more expensive than plain agarose (~ 50X), did not result in much signal

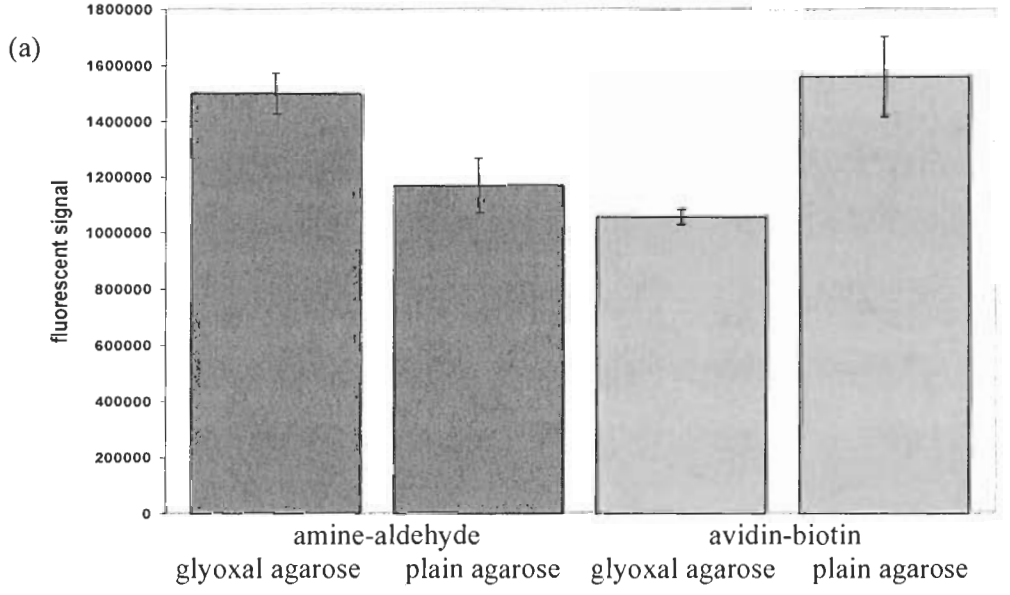
improvement, and saved just 1 hour of procedure time, further testing with agarose-coated slides took place using plain agarose.

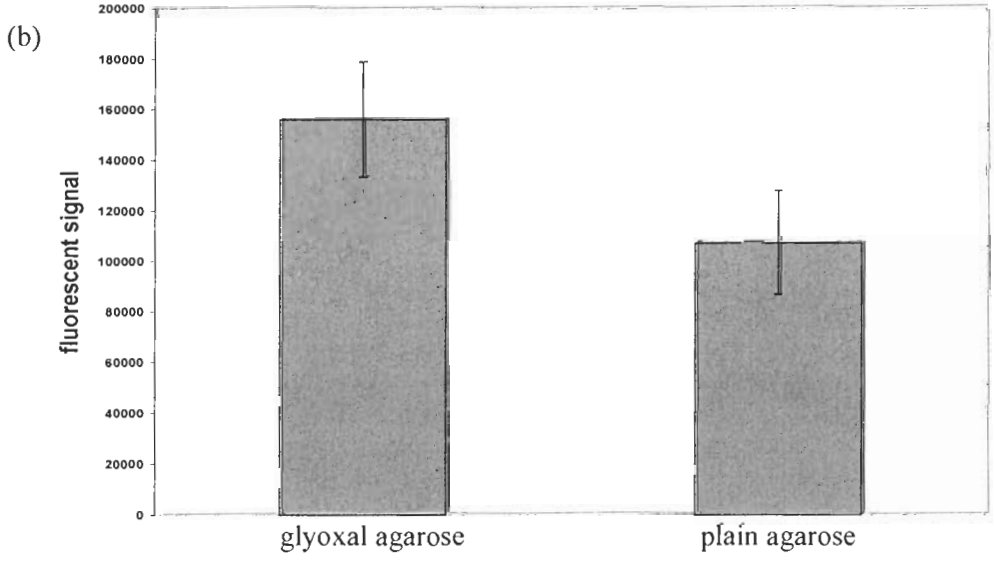
Figure 25 Attachment and hybridization on two types of agarose.

Four slides for each treatment were measured. Mean values and errors of the means (slide to slide variation) for each treatment are shown.

(a) amine-aldehyde attachment vs avidin-biotin attachment

(b) hybridization of complementary oligonucleotides

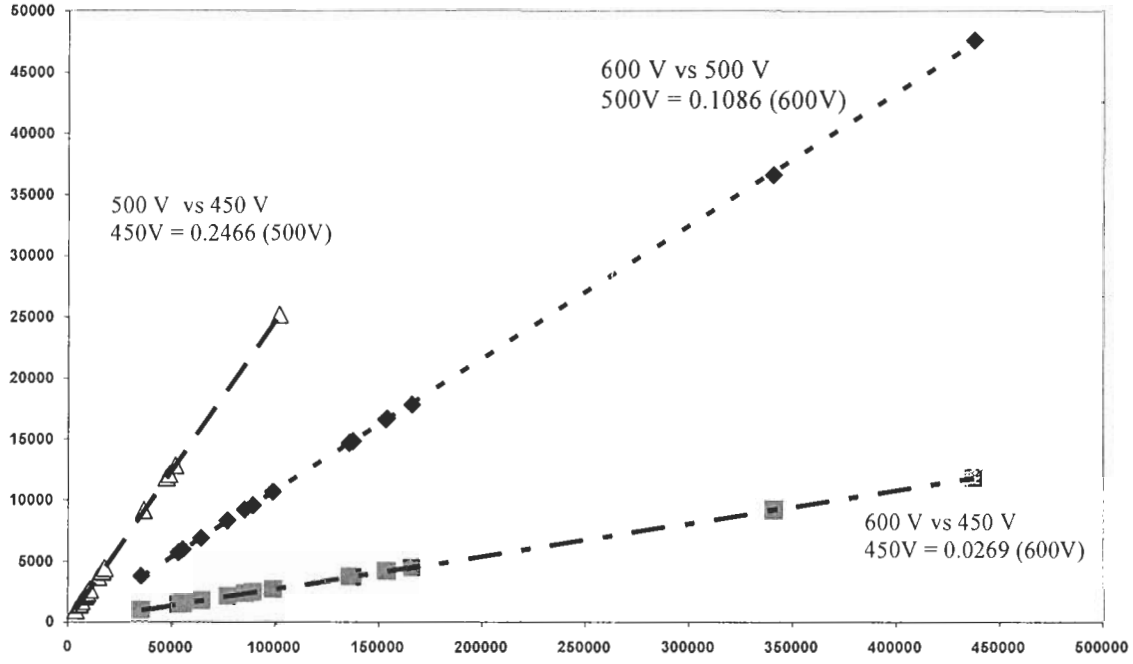




APPENDIX 4:
SIGNAL RELATIONSHIPS AT DIFFERENT PMT VOLTAGES

Fluorescence curves are useful when comparing fluorescence readings at different PMT voltages. The graph illustrates some examples of signal:signal relationships.

Figure 26 Signal at different PMT voltages



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