Detection of Phytophthora species by MALDI-TOF Mass Spectrometry

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**Declaration**

This thesis is presented as part of the requirement for a doctorate of philosophy in biotechnology at Murdoch University. The work described in this thesis as its main content is my own work unless otherwise stated and has not been previously submitted for a degree at any tertiary education institution. The work was carried out under the supervision of Dr. P.A O’Brien.

[Signature]

Cornelia Charito Siricord
Abstract

*Phytophthora* diseases have caused worldwide economic, social and environmental impacts for decades. Once their presence is confirmed, they are difficult to eradicate. To reduce and manage the damage inflicted by the pathogen, fast and reliable disease management protocols are required. Tests that enable the rapid and reliable identification of the pathogen assist greatly in disease management.

*Phytophthora* species are traditionally not only detected by baiting but also by plating of symptomatic tissue on selective media. Species can be identified by the characteristics of the mycelium growing out of the bait. However, the method is low throughput, labour intensive, and prone to false negatives. An alternative approach would be to detect the pathogen by the presence of its DNA. This involves amplification of the pathogen DNA using Polymerase Chain Reaction (PCR) and detection of the amplification product. Detection is usually by agarose gel electrophoresis. However, this is also a labour intensive process involving pouring, loading, running, and staining of the gels. The aim of this thesis is to explore the use of Matrix Assisted Laser Desorption/ Ionisation Time-of-Flight (MALDI-TOF) mass spectrometry for detection of PCR products. This procedure enables the analysis of large numbers of samples within a very short time-frame as the average time for analysis of each sample is in the order of milliseconds.

The assay involves annealing an extension (genotyping) primer to the PCR product and its extension by a single nucleotide. The nature of the nucleotide added differentiates species as does the site to which the primer anneals. Multiple extension (genotyping) primers can be used together in a single reaction for detection of multiple species. In this project four genotyping primers (GPs) were designed from the ITS regions of
Phytophthora palmivora, Phytophthora cinnamomi, Phytophthora citricola, and Phytophthora cambivora.

The extension primers were tested for their specificity on the DNA of the target species. The four primers designed were specific for their intended targets except for GPPalm3 which in addition to being extended by ddT when tested with DNA from P. palmivora, was also extended by ddC when tested with DNA from other species of Phytophthora or Pythium.

These primers were also tested for their ability to detect multiple Phytophthora species in a single reaction (multiplexing). Mixtures of primers were added to mixed DNA templates and the primer extension reaction carried out. The primers were designed so that their masses were sufficiently different for them to be identified from a mixture. Six replicates were analysed for each reaction. In general only about 1-3 of the six replicates gave a positive reaction. This indicates that there may be some interference between primers, or that the presence of all four nucleotides interfered with the primer extension reaction. Increasing either the amount of enzyme, the amount of nucleotides or both did not improve the results.

The sensitivity of detection was tested by the addition of different amounts of mycelium to soil. The detection sensitivity depended on the primer pair used for PCR amplification. The ITS1/2 primer pair was more sensitive than the ITS1/4 pair. The limit of detection was 1 µg mycelium g soil\(^{-1}\). However using nested PCR, levels of sensitivity comparable to those obtained using the ITS1/2 primer pair could be achieved. Primers to other regions of the genome such as the beta cinnamomin elicitin gene gave very low levels of sensitivity compared to the ITS primers.
In comparison with DNA detection we found that the limit of detection using baiting was 4 µg mycelium g soil\(^{-1}\). Results below this limit were unreliable. The method suffered from the additional disadvantage that it took a long time in comparison to DNA detection.

DNA detection methods do not distinguish between living and dead organisms in the soil. However it can be hypothesised that DNA is unlikely to persist for any significant length of time in soil. To test this, we added plasmid DNA to soil and tested the persistence of this DNA using a variety of methods such as precipitation of labelled DNA, southern blotting and PCR amplification. It was found that in general, in soils from different ecosystems, the bulk of the DNA was undetectable after 24 hours. The rate of DNA breakdown differed with the soil type. In some soils, the added DNA was not detected even after 2 hours, whereas in others it could be observed after 10 hours. The detection depended on the method. Southern blotting showed that although DNA could be observed at 10 hours, by 24 hours it was completely degraded. In contrast a PCR product could be obtained from the soil extracts up to 24 hours. In a separate experiment, plasmid DNA was detectable over a 24 hour incubation period in 5 soil samples from 5 different sites. The results suggest that DNA is degraded rapidly in soil and is unlikely to persist longer than 24 hours.

The results in this thesis demonstrate that MALDI-TOF MS is a suitable alternative to agarose gel electrophoresis for analysis of PCR products. The technique is rapid, differentiates species from mixtures, is high-throughput and amenable to automation. Implementation will require further research to automate the primer extension assay to reduce the sensitivity to impurities in the DNA and to design parameters for sampling asymptomatic material.
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium Chloride</td>
</tr>
<tr>
<td>CALM</td>
<td>Dept of conservation and land management, Australia</td>
</tr>
<tr>
<td>CCRTP</td>
<td>Conservation and Research Centre for Tropical Plant Pathology</td>
</tr>
<tr>
<td>CSIRO</td>
<td>Centre of Scientific Investigation and Research Organisation</td>
</tr>
<tr>
<td>CSPD</td>
<td>Disodium 3-(4-methoxyspiro{1,2 dioxethane-3,2'-5'-chlolo}triclo[3.3.1.13,7]decan}-4-yl)phenylphosphate</td>
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<tr>
<td>CTAB</td>
<td>Cetyltrimethylammonium Bromide</td>
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<tr>
<td>ctDNA</td>
<td>Calf thymus DNA</td>
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<tr>
<td>Da</td>
<td>Dalton</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DIG probe</td>
<td>Digoxigenin labelled probe</td>
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<tr>
<td>dNTP</td>
<td>Deoxynucleoside Triphosphate</td>
</tr>
<tr>
<td>ddNTP</td>
<td>Di-deoxynucleoside Triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GP</td>
<td>Genotyping Primer</td>
</tr>
<tr>
<td>ITS</td>
<td>Internal Transcribed Spacers</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase pair</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar (10⁻³ M)</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-Assisted Laser Desorption/Ionization</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium Chloride</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
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<td>m/z</td>
<td>mass-to-charge ratio</td>
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<td>Polyclonalpolyribonucleotide-40</td>
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<tr>
<td>PVPP</td>
<td>Polyclonopolyribonucleotide</td>
</tr>
<tr>
<td>RAPD</td>
<td>Random Amplification Polymorphic DNA</td>
</tr>
<tr>
<td>rDNA</td>
<td>Ribosomal DNA</td>
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<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>SAP</td>
<td>Shrimp Alkaline Phosphatase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
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<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>SNUPE</td>
<td>Single Nucleotide Primer Extension</td>
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<tr>
<td>TAE</td>
<td>Tris, Acetate and EDTA buffer</td>
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<tr>
<td>TOF</td>
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<td>microliter</td>
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