Developing Novel Genetic Techniques to Diagnose Bacteria Infection in Tropical Crops

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In the 1960's the long sought pathogens of plant 'yellows diseases' were found to be prokaryotic cellular microbes, the phytoplasmae.

Prior to this discovery, viruses were thought responsible for the damage caused to hundreds of plant species. Instead the culprits were found to be a type of bacteria, the phytoplasmae. Phytoplasmae are bacteria devoid of a cell wall, grouped in the class *Mollicutes* (Bertaccini *et al.*, 2005). Members of this class include another group of plant pathogens, the spiroplasmas. Both are characterised by their relatively small genome size (530-1350 kilobases).

Phytoplasmas are known to cause disease in hundreds of plant species worldwide. It is in the tropics where phytoplasmas cause the most devastating results (Cousin, 1995). Cape St Paul's Wilt, for example can strip coconut trees bare (see Figure 1).

New disease reports are published frequently and the range of hosts is expanding, encompassing economically important food, fibre, forage, fruit and ornamental plants.

Phytoplasma infections vary in severity, even destroying a crop of highly adapted, hardy trees. Currently there are no appropriate treatments for infected plants. Hence, the effects of phytoplasma diseases can be severe with dire economic consequences (Davies *et al.,* 2002).

Members of the family *Cicadellidae,* commonly known as 'leafhoppers' transmit many of the phytoplasmae.

Figure 1: *The devastating damage caused to coconut trees by Cape St Paul's Wilt.*

Phytoplasmas inhabit plant phloem; it is here that they propagate.

After feeding from the phloem of an infected plant these insects move on to feed from additional plants, carrying the infection from one plant to the next.

Although phytoplasma infections are systemic, pathological effects are seen in areas of the plant where the number of bacteria are highest (Kuske and Kirkpatrick, 1992). Not all plant species infected with phytoplasmae have disease symptoms, but commonly plants exhibit some or all of the following: virescence, phyllody, yellowing, witches'- broom, leaf roll and generalised decline (Bertaccini *et al.,* 2005) see p. 2 below. These are all indicative of significant alterations to plant hormone or growth regulator concentrations.

Currently, the phytoplasmas are classified in numbered groups (I to XI) according to several factors, including host plant, country of origin and phylogenetic

relationships. For example, the Aster Yellows are members of Group I and Tomato Big Bud (see figure 2) is in Group II. Typically they are named after the plant they were initially isolated from but phytoplasma do not specifically infect a single plant species. Furhtermore, there are contradictions regarding the phytoplasma group classification system following genetic analysis.

DNA consists of long sequences of paired bases called genes which code for a particular trait. Some of these gene sequences are consistent across bacteria but vary in their detailed sequence. These differences can be compared and used as a diagnostic test for a particular phytoplasma. Some studies suggest that after phylogenetic analysis of the 16S rRNA and IGS genes there are 15 different groups (Harrison *et al.,* 2002), others have delineated at least 20 (Wang *et al.,* 2003 and Seemüller, 1998).

Hence, it is necessary to devise a rapid, effective and efficient mechanism for detecting and identifying these bacteria. This investigation explored the potential conferred by genetic based techniques for phytoplasma diagnosis.

Much of the research into this field has concentrated on looking for differences in the phytoplasma 16S ribosomal gene, essentially the number of base pairs in a quantitative analysis. The 16S gene is present in two copies within the phytoplasma genome and can be amplified with ease using the polymerase chain reaction (PCR). However, the 16S gene is highly conserved in sequence between different phytoplasma isolates.
This investigation compared the diagnostic potential of the 16S gene with several other phytoplasma genes selected from an electronic database at the National Center for Biotechnology Information (NCBI) publicly available at: http://www.ncbi.nlm.nih.gov/).

Disease Symptoms

Virescence: A symptom in which green pigmentation occurs in plant tissues, not normally green, through chlorophyll formation.

Phyllody: Development of floral parts into leafy structures, as shown below.

Figure 2: an example of phyllody

Yellowing: The yellow colour of plant parts resulting from the excessive proportion of yellow pigments, in turn produced by the underdevelopment or partial destruction of the green pigments.

Witches' Broom: Wild, erratic, broom-like growth at the ends of shoots, stems or branches.

Leaf Roll: Curling of the leaves.

Figure 3: A Tomato Plant infected with Tomato Big Bud.

The other genes in addition to 16S were as follows:
i) the 16S/23S intergenic spacer region (IGS), a stretch of DNA found between the 23S and 16S rRNA genes;

ii) a peptide chain release factor gene (*pep;*

iii) an ATP-dependent RNA helicase (*helicase*); and finally,

iv) an elongation factor TU gene (*tuf*).

These gene sequences were generated by cutting complete DNA at certain points using specific enzymes. The restriction enzymes most suitable for providing differential fragments from phytoplasmas from each of the groups were selected using the NCBI database.

Experimental Methods
The first stage of the investigation involved the collection of DNA from infected plants and uninfected controls. The process of DNA extraction isolates DNA of both phytoplasma and plant origin. The plant DNA of interest is contained within chloroplasts, the energy converting organelles of plant cells. Chloroplast DNA is similar to that of bacteria, and hence serves as a useful comparison.

The DNA of known phytoplasmae were isolated from infected plant material using a commercially available DNA extraction kit.
Control samples were drawn from Napier Grass and Madagascan Periwinkle, two plants commonly infected by phytoplasmas.

In the second stage of the investigation, DNA extracted from the infected plants was amplified by using the polymerase chain reaction or PCR. PCR is a standardised technique in gene analysis to provide sufficient genetic material for detection. It works through the use of short lengths of DNA called primers that have a known sequence. Double stranded DNA is melted in a heating step exposing two single strands to which the primer can anneal.

For the final stage, two methods were compared for their ability to differentiate between gene sequences from different phytoplasmae. These were:

a) Restriction fragment length polymorphism (RFLP), and

c) Genetic fingerprinting (terminal restriction fragment length polymorphism or T-RFLP).

RFLP
DNA was cut into fragments at specific sites using enzymes. The pattern of cut DNA was viewed using gel electrophoresis (Lee *et al.,* 2002). Analysis of a known genomic sequence can show what size of fragments to expect depending upon the enzymes chosen for the cuts.

Figure 4 shows an example of the 16S rRNA gene from several phytoplasma isolates. There is little variation across the samples confirming that this gene is less useful for phytoplasma diagnosis.

Unfortunately, despite numerous attempts to select for suitable primer sites and PCR conditions, the non-ribosomal genes *pep, helicase* and *tuf* could not be generated.

Figure 5 shows the 16S rRNA gene (after PCR amplification) digested using *Mbo*I. Different patterns emerged for the different groups.

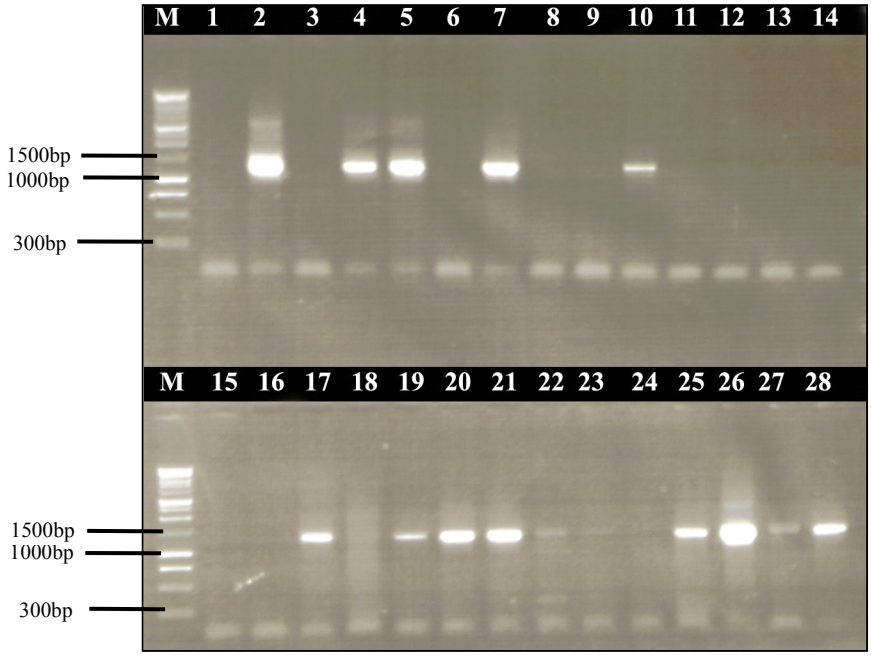

Figure 4: A gel image depicting the 16S rRNA phytoplasma gene from several phytoplasmae. The numbers represent a single DNA sample from different phyoplasmae. The brighter and thicker the band formed in a given well the more DNA there is present in the sample. M represents a 1kb DNA ladder; this is used in gel electrophoresis to determine the size of amplified DNA fragments. The ladder comprises fragments of known sizes (some of which are labelled in the diagram) measured in terms of DNA base pairs (bp). DNA bases are specific chemical structures which make up a length of DNA and are identical in all DNA regardless of its origin. The human genome has recently been sequenced providing a code comprising a specific order of the 3000 million DNA bases pairs.

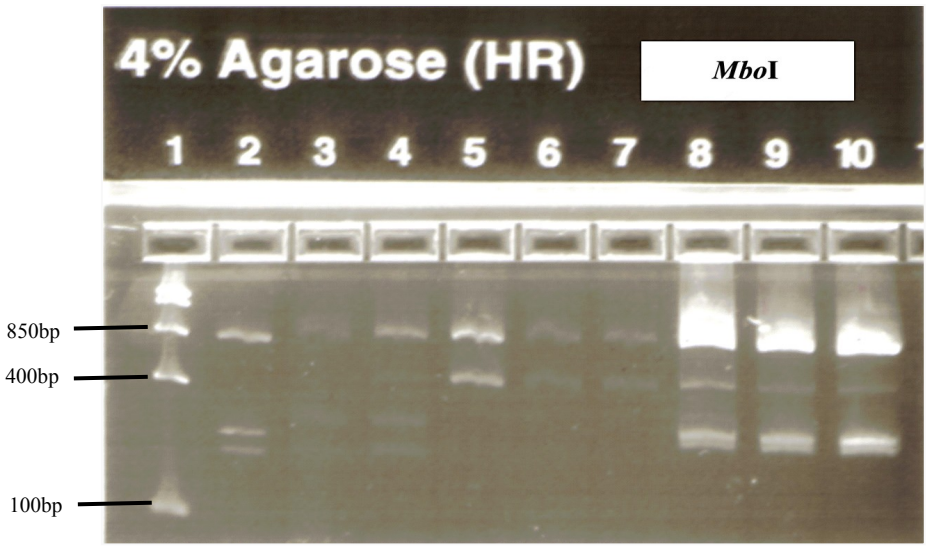

Figure 5: The gel image obtained following digestion of the 16S rRNA gene using *Mbo*I.

Lane	Organism	Group	Predicted 16S rRNA (bp)	Experimental 16S rRNA (bp)
2	Napier Grass Stunt	XI	28, 195, 239, 785	195, 239, 800
3	Brinjal Little Leaf	VI	195, 267, 786	195, 267, 786
4	Elms Yellows	V	195, 267, 786	195, 267, 467, 786
5	Aster Yellows Cactus	I	467, 785	467, 785
6	Aster Yellows Dwarf	I	467, 785	467, 785
N/A	Crotalaria Saltiana Phyllody	II	222, 241, 784	N/A
7	Aster Yellows Gladiolus	I	467, 785	467, 785
8	Tomato Big Bud	II	222, 241, 784	222, 241, 750, 785, 790, 850
9	Soybean Phyllody	II	241, 222, 784	222, 241, 748, 785, 790
10	Sweet Potato Little Leaf	II	222, 240, 785	222, 241, 770

Table 1. The table summarises the experimentally determined fragment sizes, along with those predicted from sequence information available and the group classification for 10 different phytoplasma isolates. Differences between groups regarding the fragment sizes obtained can be observed and used as, or as part of, a diagnostic protocol.

Figure 4 represents results obtained following the digestion of the IGS using *Mse*I.

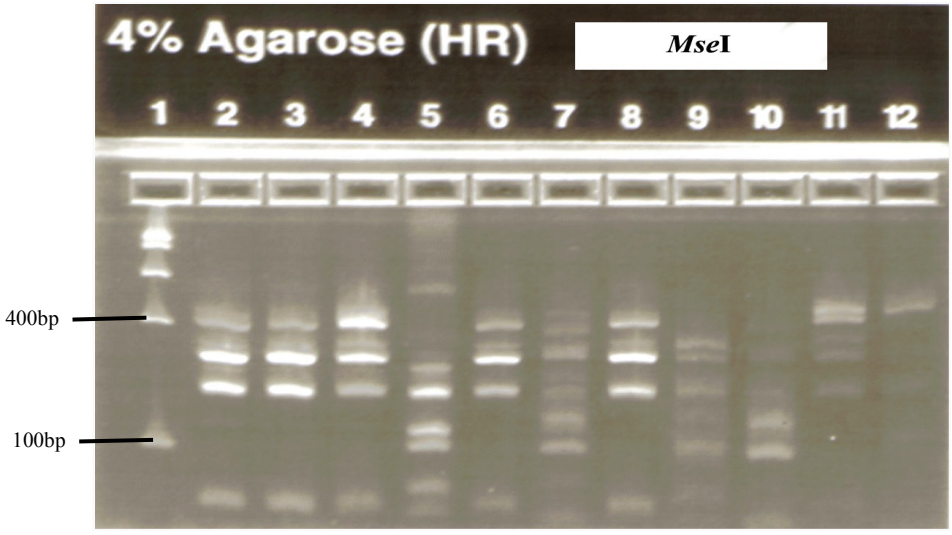

Figure 6: A second example of RFLP results obtained. These results correspond to digestion of the IGS using the enzyme *Mse*I. Comparing the different banding patterns achieved allows the assessment of the use of this technique using the given enzyme and IGS as a method for efficiently diagnosing the type of phytoplasma infection in a plant.

Lane	Organism	Group	Predicted IGS (bp)	Experimental IGS (bp)
2	Tomato Big Bud	II	56, 187, 277	56, 187, 277, 290, 400
3	Soybean Phyllody	II	56, 187, 277	56, 187, 277, 290, 400
4	Sweet Potato Little Leaf	II	56,187, 277	56, 187, 277, 290, 400
5	Napier Grass Stunt	XI	15, 49, 53, 65, 165, 175	49, 65, 100, 120, 175, 260, 780
6	Sweet Potato Little Leaf	II	56, 187, 277	56, 187, 277, 290, 400
7	Brinjal Little Leaf	VI	24, 34, 50, 73, 99, 103, 136	50, 73, 100, 120, 136, 277, 300, 350, 400, 490
8	Tomato Big Bud	II	56,187, 277	56, 187, 277, 290, 400
9	Aster Yellows Cactus	I	6, 9, 35, 50, 64, 89, 102, 186	64, 102, 186, 277, 290
10	Brinjal Little Leaf	VI	24, 34, 50, 73, 99, 103, 136	50, 103, 136, 187, 277
11	Crotalaria Saltiana Phyllody	II	56, 187, 277	56, 187, 277, 290, 370, 400, 470
12	Aster Yellows Hydrangea	I	6, 9, 35, 50, 64, 89, 102, 186	186, 470

Table 2. The table summarises the experimentally determined fragment sizes, along with those predicted from sequence information available and the group classification for 10 different phytoplasma isolates. Differences between groups regarding the fragment sizes obtained can be observed and used as, or as part of, a diagnostic protocol.

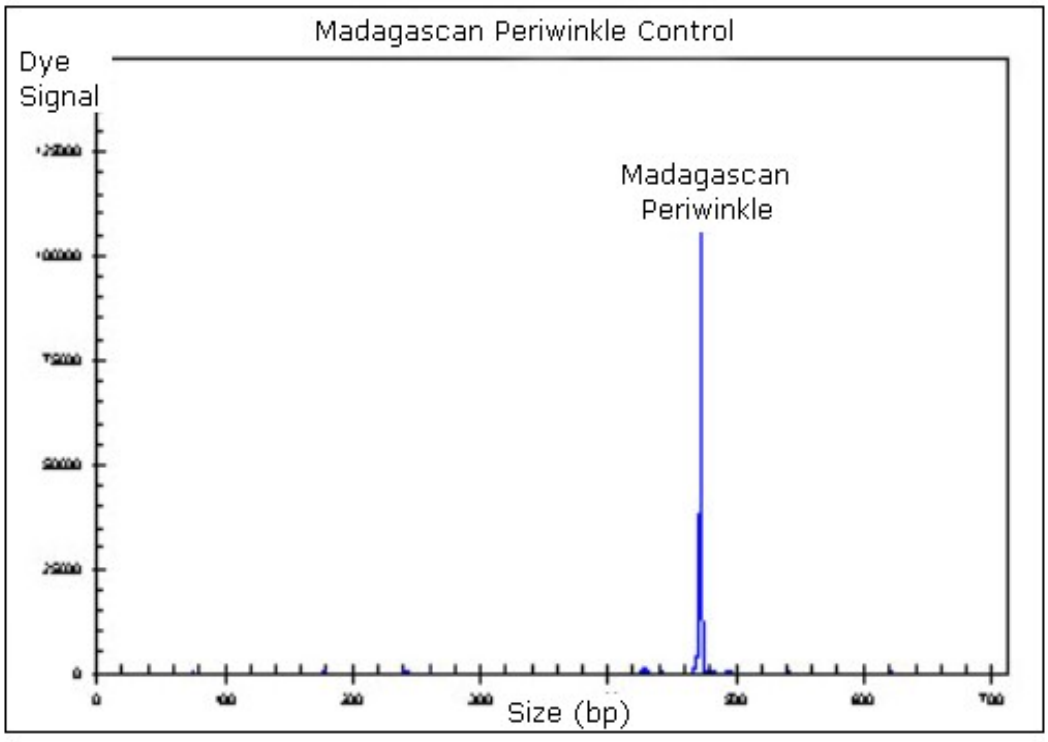

Figure 7: Graphical T-RFLP result for Madagascan Periwinkle control DNA, a single peak denotes plant DNA (fragment size of 470bp), an absence of other peaks represents a lack of phytoplasma DNA present.

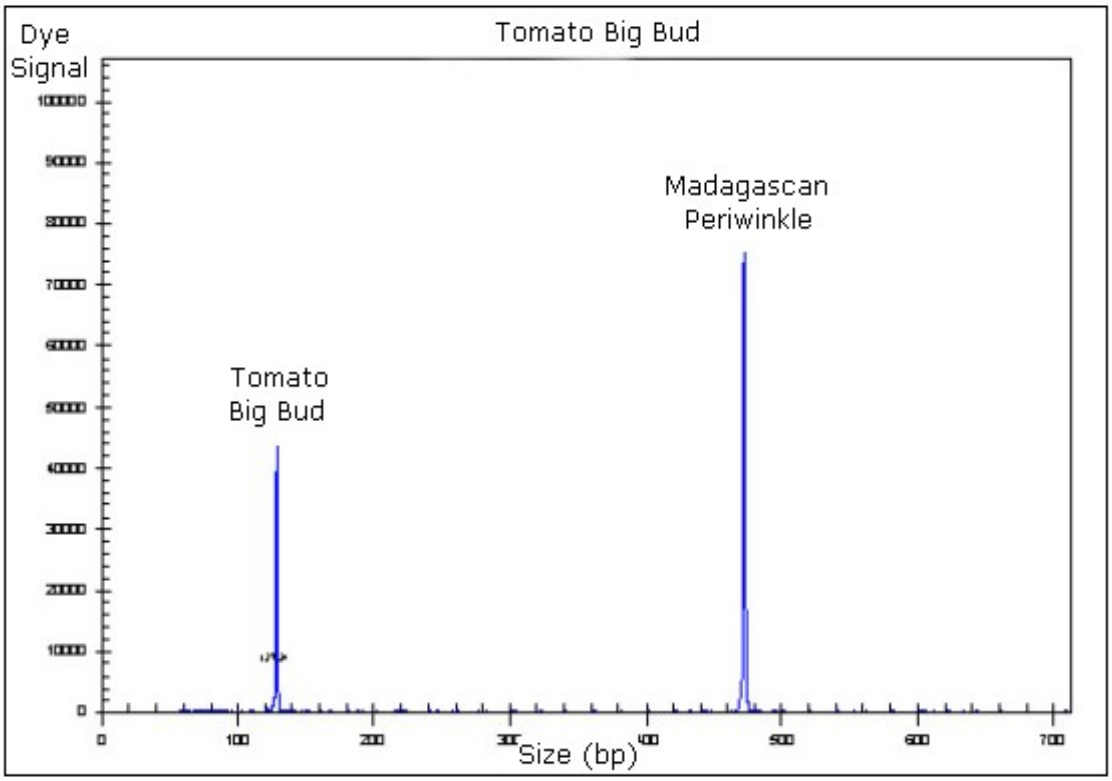

Figure 8: Graphical T-RFLP result for Tomato Big Bud, notice the larger peak denotes Madagascan Periwinkle (plant) DNA (fragment size of 470 nucleotides), while the peak depicting a fragment size of 129 nucleotides represents the Tomato Big Bud DNA present. This indicates the presence of the Tomato Big Bud bacteria in this sample from a Madagascan Periwinkle. The larger the phytoplasma specific peak, the higher the titre of phytoplasma present, indicating the usefulness of the T-RFLP protocol as a quantitative diagnostic technique.

The results suggests that PCR amplification has to be accompanied with RFLP using an enzyme that is specific to a particular gene sequence. Enzyme *Mbo*I appears the most appropriate enzyme for the 16S rRNA gene, able to resolve Groups I, II and XI. For IGS *Mse*I appears most suitable providing distinction between Groups I, II, VI and XI.

Results obtained with PCR amplification of the peptide chain release factor, ATP-dependent RNA helicase and elongation factor phytoplasma genes show that these sequences are inappropriate for the identification of infection due to problems isolating the desired DNA fragments. Other problems were encountered during amplification of the *tuf*, *pep* and *helicase* primers.

Genetic Fingerprinting (T-RFLP)

Terminal-restriction fragment length polymorphism (T-RFLP) is a direct DNA profiling method that usually targets genes that encode rRNA (Klamer *et al.,* 2002). This genetic fingerprinting method generates fluorescently labelled terminal restriction fragments (TRFs) of various lengths depending on the DNA sequence of the bacteria present and the enzyme used to cut the sequence. The results of T-RFLP are obtained by high-resolution gel electrophoresis on automated DNA sequencers. The laser scanning system of the DNA sequencer detects the labelled primer (Sakai *et al.,* 2004) and from this signal the sequencer can record corresponding fragment sizes and relative abundances. Resulting data is very easy to analyse, being presented as figures for

statistical analysis, and graphically for rapid visual interpretation.

DNA was digested with *Mse*I to generate 23S rRNA genes following PCR amplification. Figure 5 shows an example of PCR fragment size following this procedure for the Madagascan Periwinkle control, while Figure 6 provides an example of one of the results obtained using material isolated from an infected plant.

T-RFLP analysis can distinguish between plant and phytoplasma DNA, quantify levels of infection and identify isolates present even in mixed infections. However, T-RFLP analysis of the 23S rRNA gene alone fails to provide significant fragment differences between the phytoplasma groups. Although it supersedes RFLP in terms of providing a greater resolution and automated quantitative results, analysis of other fragment sizes may prove necessary for accurate identification.

Summary

Tropical crops can be severely affected by bacterial agents called phytoplasmae. The physical symptoms exhibited by plants infected with phytoplasmae cannot be used to identify a general phytoplasma infection.

This investigation has demonstrated the diagnostic potential of two genetic techniques RFLP and T-RFLP analysis following PCR amplification.

Although ribosomal genes, or the IGS region have been found to be the most appropriate regions of DNA for phytoplasma analysis, the possibility of locating a suitable non-ribosomal gene has yet to be discredited.

References

Bertaccini, A., Franova, J., Botti, S. and Tabanelli, D. (2005) Molecular characterization of phytoplasmas in lilies with fasciation in the Czech Republic. *FEMS Microbiology Letters,* **249**, 79-85.

Cousin, M. (1995) Phytoplasmas and phytoplasma diseases. *Agronomie,* **15**, 245-264.

Davies, H., G., Garcia-Rossi, D., Daoust, M. and Strong, D., R. (2002) The use of molecular assays to identify plant pathogenic organisms vectored by biological control agents. *BioControl,* **47**, 487-497.

Edel-Hermann, V., Dreumont, C., Pérez-Piqueres, A. and Steinberg, C. (2004) Terminal restriction fragment length polymorphism analysis of ribosomal RNA genes to assess changes in fungal community structure in soils. *FEMS Microbiology Ecology,* **47**, 397-404.

Harrison, N., A., Myrie, W., Jones, P., Carpio, M., L., Castillo, M., Doyle, M., M. and Oropeza, C. (2002) 16S rRNA interoperon sequence heterogeneity distinguishes strain populations of palm lethal yellowing phytoplasmas in the Caribbean region. *Association of Applied Biologists,* **141**, 183-193.

Klamer, M., Roberts, M., S., Levine, L., H., Drake, B., G. and Garland, J., L. (2002) Influence of Elevated CO_2 on the Fungal Community in a Coastal Scrub Oak Forest Soil Investigated with Terminal-Restriction Fragment Length Polymorphism Analysis. *Applied and Environmental Microbiology*, **68**, 4370-4376.

Kuske, C. and Kirkpatrick, B. (1992) Distribution and multiplication of Western Aster Yellows Mycoplasma-like organisms in *Catharanthus roseus* as determined by DNA Hybridization Analysis. *Phytopathology,* **82**, 457-462.

Lee, M., E., Grau, C., R., Lukaesko, L., A. and Lee, I., M. (2002) Identification of aster yellows phytoplasmas in soybean in Wisconsin based on RFLP analysis of PCR-

amplified products (16S rDNAs). *Canadian Journal of Plant Pathology,* **24**, 125-130.

NCBI Database (Revised 28th February 2006) National Center for Biotechnology Information, U.S. National Library of Medicine, National Institute of Health. Available at: http://www.ncbi.nih.gov/ [Accessed 21st March 2006].

Sakai, M., Matsuka, A., Komura, T. and Kanazawa, S. (2004) Application of a new PCR primer for terminal restriction fragment length polymorphism analysis of the bacterial communities in plant roots. *Journal of Microbiological Methods,* **59**, 81-89.

Seemüller, E., Marcone, C., Lauer, U., Ragozzino, A. and Goschl, M. (1998) Current status of molecular classification of the phytoplasmas. *Journal of Plant Pathology*, **80**, 23-36.

Wang, K., Hiruki, C. and Yah, F. (2003) Molecular evolution of phytoplasmas based on polymorphisms in the 16S rRNA genes and the 16/23S spacer regions. *Proceedings of the Japan Academy* **79**, 155-162.

Further Reading

Dickinson, M. (2003) *Molecular Plant Pathology*, 1st Edn. BIOS Scientific Publishers, London, 5. (*Excellent introduction to molecular diagnostic techniques and phytoplasma science).*

Phytoplasma -Vector.com. Available at: http://phytoplasma-vector.com/vectors/vectors.htm [Accessed 21st March 2006]. *(For a basic introduction to phytoplasmas and their vectors).*

You will find that the greatest interest in terms of research is into phytoplasma taxonomy and diagnosis. However, the following references highlight some other research fields currently being explored.

Bressan, Girolami and Boudon-Padieu, 2005. Reduced fitness of the leafhopper vector *Scaphoideus titanis* exposed to Flavescence doree phytoplasma. The Netherlands Entomological Society. 115, 283-290.

Carginale, et al., 2004. Identification of genes expressed in response to phytoplasma infection in leaves of *Prunus armeniaca* by messenger RNA differential display. Gene. 332, 29-34.

Favali, et al., 2004. *Catharanthus roseus* L. plants and explants infected with phytoplasmas: alkaloid production and structural observations. Protoplasma. 223, 45-51.

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Theresa is 21 years old and graduated top of her year in 2006 with a first class BSc. degree in Biology. She is especially interested in microbiology, particularly the field of pathology in both plants and animals. Theresa is due to begin a Masters in Biological Photography and Imaging also at the University of Nottingham, which she hopes will lead to a career combining her love of science, photography, communication and wildlife.