Development of an advanced PCR technique to detect grapevine trunk diseases

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Summary

The grapevine trunk is a host to a number of fungi and yeasts. A sample of wood from any grapevine will more than likely harbour several different fungal species (see Figure 1). Some fungi cause diseases such as Eutypa, esca, Petri disease, blackfoot and deadarm, but most fungi appear to be harmless or may even be beneficial to the vine. These fungi are commonly called endophytes. Healthy vines can have a greater number of fungal species inside them than those vines that are of poor health. Oddly enough, it is often the pathogenic fungi that have the potential to cause these diseases and such fungi can be found in healthy, productive vines (Halleen et al., 2003). Some pathogenic fungi may be suppressed by endophytes, or lack a stress event to trigger disease. Some diseases such as esca require a combination of at least two pathogenic species before disease symptoms appear (Mugnai et al., 1999).

To date, PCR technology can detect the presence or absence of disease-causing fungi and only one at species at a time can be tested. This provides only a partial view of what may really be happening inside a vine. A relatively new PCR method, called t-RFLP, used to measure fungal populations in soil, has been adapted in the viticulture industry to measure the entire fungal population inside a vine, including pathogens and endophytes. Not only does this method have enormous potential as a research tool, but it has been developed to screen vines for the most common fungal pathogens.

Figure 1. Cross sections through a grapevine cordons. The various discolorations are evidence of a range of fungal inhabitants in the wood.

Current diagnostic technology for fungal diseases

Currently, there are two major methods available for the identification of fungal pathogens. The most common method is morphological identification. This involves taking samples of grapevine tissue and placing them on nutrient-rich agar plates. Over a period of several days (or weeks) the fungi present in the grapevine will grow and thus be examined under a microscope for a number of identifying features. Such features can include the spore shape, type and size, colour, the growth rate of the fungi and the organisation of the fruiting structures which produce the spores.

Due to the fact that several fungi species may be present in a single vine wood sample this particular identification process is not only timely but also requires expert knowledge (as many fungi look very similar to the untrained eye). Because different fungi grow at different rates, the faster growing fungi will outgrow the slower species. If the pathogenic fungus is slow growing, as is *Phaeomoniella chlamydospora*, it can be easily missed. The other drawback to this type of identification is that not all fungi will grow out of the wood sample onto the agar, so are never detected.

The second method is PCR based. In this method DNA is extracted from grapevine tissue and specific primers are used in an attempt to amplify DNA. The analysis is simple. If a DNA band appears, then the fungus is present. Current tests usually detect only one fungal pathogen at a time, and consequently if you wish to test for several different fungi the test will have to be repeated for each individual species (Groenwald *et al.,* 2000, Tegli *et al.,* 2000). This disadvantage can be overcome by using a multiplex PCR, currently under development at Corbans Viticulture, which can detect multiple targets in a single test e.g. *Eutypa lata, Phomopsis viticola* and *Phaeomoniella chlamydospora* (Shiller *et al.,* 2007).

The t-RFLP method

A variant on the PCR method called “t-RFLP” (terminal label Restriction Fragment Length Polymorphism) is currently being developed at Corbans Viticulture. This technique is commonly used in microbial ecology to investigate bacterial and fungal communities in soil and water. We have adapted it to measure whole fungal populations in grapevine samples. This method uses universal PCR primers to target all the fungi in a sample, not just the pathogens. Because multiple species are often present, this technique takes the mixed PCR product and separates out the different species thus enabling precise identification.

In our adaptation of this technique, xylem wood is cut from the grapevine and DNA is extracted. PCR is then used to select part of the DNA from any fungi present in the extraction. As new copies of the fungal DNA are made, a fluorescent coloured dye is added to both ends (different colours for each end). The DNA is then cut with a restriction enzyme so two fragments exist, each with a coloured tag. The position of the cut varies with different fungal species, thus the sizes (after cutting) are also different.

This cutting process is then repeated with another different enzyme resulting in each fungus producing four different sized DNA fragments (Figure 2).

The fragments are then fed through a machine which sorts the fragments by size, using the coloured tag to identify each DNA fragment. The analysis of this data is done with a computer program written by Fitzjohn and Dickie (2007) from Landcare Research. This program (TRAMPR) matches the unique size patterns of the DNA fragments produced in a mixed sample, to an existing database of known fungi.

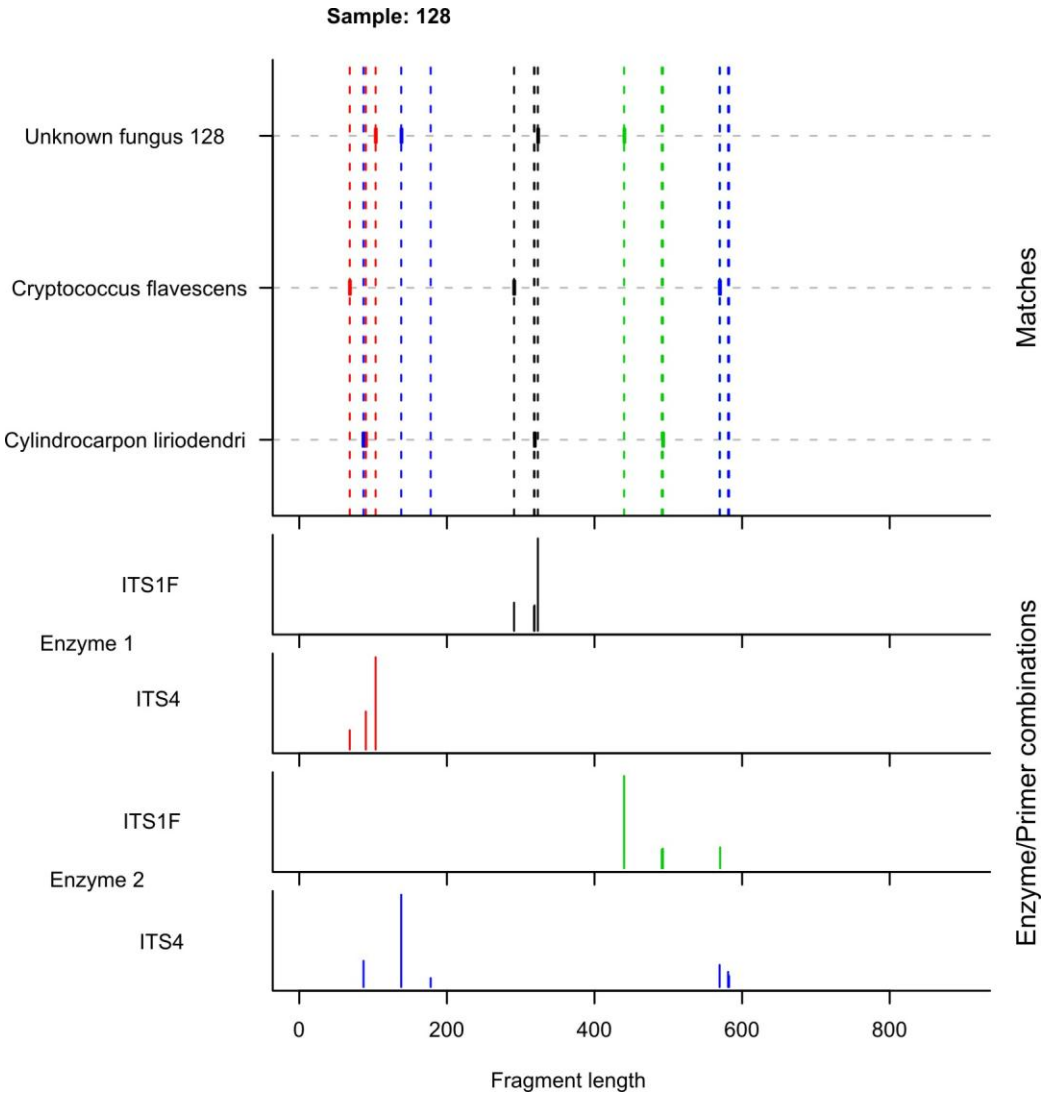

Figure 2. Results of t-RFLP performed on grapevine wood. The coloured peaks in the lower part of the figure correspond to a DNA fragment length (after cutting). Each fungus produces one peak only of each colour, and the different combination of peaks is specific to a fungal species. In the upper portion of the figure TRAMPR has scanned the database to match peaks and identified the fungi present. In this sample are

Cylindrocarpon liriodendri (a pathogen), Cryptococcus flavescens (a harmless yeast), and another species which has yet to be identified in our database.

t-RFLP as a diagnostic tool

The backbone to this system is the database which consists of pure cultures of fungi that have previously been found in grapevines and put through the t-RFLP process. The database developed by Corbans Viticulture contains all the major pathogens as well as commonly occurring harmless species. With the restriction enzymes selected, each known pathogen found in grapevines has a unique set of fragments, whilst the endophytes can be identified to at least the genus level.

The sensitivity of this t-RFLP method enables the detection of all fungal species present, providing greater scope when compared to existing diagnostic tools that have been developed to date. It may prove useful in detecting emerging pathogens, incursions from imported vines or disease caused by multiple infections of different fungi.

During the development of this particular method, t-RFLP has been used alongside the traditional culturing technique, and has produced similar results (in regards to known pathogens), whilst also providing additional information on endophytes and fungal diversity inside vines after various treatments. The t-RFLP method takes only four days, whereas the culturing technique can take several weeks depending on the growth rate of the pathogen.

t-RFLP as a research tool

Ongoing research in this area at Corbans Viticulture includes comparing fungal populations in grapevines from different viticulture regions in New Zealand. We have also examined the succession of fungi following the hot water treatment of grapevines, which has allowed us to monitor how long this treatment is effective for. Furthermore t-RFLP has been used to track changes in fungal populations in vines after exposure to a pathogen. With the current importance of sustainable viticulture, t-RFLP is being used to track biocontrol agents and beneficial organisms in both plants and soil to monitor their effectiveness at colonising vines with beneficials and preventing pathogen infections.

In the future, t-RFLP could also be used to monitor the effect of environmental stresses on fungal populations in the vines. Similar methods may be used for a range of other horticultural crops.

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Biographies

Dr. Bevan Weir has a BTech degree in Biotechnology from the University of Auckland, and graduated with a PhD in 2007. His doctoral thesis examined the nitrogen-fixing soil bacteria known as “rhizobia” in native New Zealand legumes. Bevan is now using his knowledge and experience in PCR technology to develop new methods to study fungal populations in grapevines. Bevan Weir can be contacted at weirb@landcareresearch.co.nz

Dr. Anna Graham completed her PhD at the University of Auckland on factors affecting the quality of grafted grapevines. She is currently the Research and Innovation manager for Corbans Viticulture in Auckland and has worked on development of new PCR tools for research on changes in fungal populations in vines after chemical, biological and hot water treatments and more cost-effective diagnosis of fungal diseases in grapevines. Anna Graham can be contacted at anna@corbansviticulture.co.nz