Final Report

GWRDC Travel Report - "Early diagnosis of Eutypa dieback in grapevine"
Project UA 01/06

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Purpose of travel
To undertake research in the laboratory of Dr Russell Molyneux at the United States Department of Agriculture (USDA) Western Regional Research Center in Albany, California as part of CRCV project 2.2.

Objectives
1. To gain experience in the analysis of secondary metabolites produced by *Eutypa lata* by working with scientists in the American Vineyard Foundation (AVF) Eutypa research program at the USDA.
2. To gain an understanding of what secondary metabolites are produced by Australian isolates of *E. lata*, and which of these metabolites are responsible for causing foliar symptoms.
3. To compare secondary metabolite production between American and Australian isolates of known pathogenicity.
4. To develop spectroscopic techniques allowing for the early detection of Eutypa dieback in grapevines.
5. To further the collaboration which is being established between researchers in the Cooperative Research Centre for Viticulture (CRCV) and the AVF on the management of Eutypa dieback.

Background
One of the primary aims of project 2.2.2 within the CRCV is to develop molecular and spectroscopic techniques to enable the early detection of *E. lata* in infected grapevines. Characteristic foliar symptoms of Eutypa dieback have been reported to be due to translocatable toxins which are produced by the fungus. In particular, eutypine, an acetylenic phenol secondary metabolite eutypine has been implicated (Tey Rulh *et al*. 1991). Hence an original aim of the CRCV project was to develop techniques for the detection of eutypine in infected grapevines.

However, Dr Russell Molyneux and co-workers in the AVF project have demonstrated that eutypine is not solely responsible for phytotoxicity in grapevines, and that the foliar symptoms of Eutypa dieback may be the result of a suite of related compounds produced by the fungus (Molyneux *et al*. *in press*). In their preliminary analysis of secondary metabolite production from culture filtrates of 12 *E. lata* isolates, Russell Molyneux and Noreen Mahoney found only five which produced eutypine. More detailed analysis of three isolates from California and Italy, grown on two culture media, revealed significant differences in amount and composition of acetylenic phenol metabolites both among isolates, and between media. Eutypine was detected in only one of the three isolates.

Russell and Noreen had access to three isolates of *E. lata* only, and a major aim of the collaboration was to compare secondary metabolite production between Australian and American isolates of the fungus (objective 2), and to determine whether the trends observed by these researchers were supported following the analysis of a wider range of isolates. The majority of

the research carried out by whilst Richard was in California used the original three isolates and a further eight isolates originating in Australia, France and New Zealand. In addition, Dr Mette Creaser (SARDI, CRCV) forwarded five Australian isolates which she had assessed for pathogenicity in the glasshouse.

Research

The composition of the culture medium is known to influence secondary metabolite production in fungi. Here, production of acetylenic phenol metabolites was analysed using 11 *E. lata* isolates grown on four different culture media (objectives 2 and 3). The media tested were potato dextrose broth (PDB) and malt yeast broth (MYB), both used previously by Russell and Noreen, Pezet's medium (used by French researchers in the analysis of secondary metabolite production by *E. lata*) and Vogel's medium (which Richard had used previously to grow the fungus for DNA extraction). All isolates were grown under both stationary and shaking conditions. In addition, two of the broths (PDB and Vogel's) were solidified by the addition of agar in order to compare the effect of solid and liquid media. Secondary metabolites were extracted from both 20 and 30 day-old cultures, and subjected to HPLC analysis using procedures developed by Molyneux *et al.*

A wide range of secondary metabolites was detected, and production was strongly influenced by both the isolate of fungus and the type of medium used. Two isolates consistently produced high levels of metabolite in the majority of media examined and six isolates produced significant quantities of metabolite on some of the media tested. The remaining three isolates consistently produced very little or no acetylenic metabolites, however, under certain conditions these isolates were also capable of metabolite production.

Medium type strongly influenced metabolite production, with a single isolate producing different metabolites on different media. Only four of the eleven isolates produced eutypine, but none of these isolates did so in more than one medium. The secondary metabolite most consistently produced was eutypinol, a compound which French researchers have postulated is a detoxification product of eutypine. Nine of the isolates produced eutypinol in a range of media. A wide variety of other compounds, some of which had been found in other studies (Molyneux et *al*. *in press*), and some of which had not previously been detected, were identified. The structures of these novel compounds have not yet been elucidated, however, this will be achieved in the future by employing NMR spectroscopy. None of these compounds were produced by all isolates. Metabolite production was strongly influenced by the use of stationary or shaking conditions, and solid or liquid medium, indicating that environmental conditions are important in determining the type and quantity of secondary metabolites produced.

Because of the differences observed in metabolite production on different culture media, a second set of trials was initiated using either grapevine wood or grapevine wood extracts as a substrate for fungal growth, in order to mimic natural conditions, and to determine the consistency among isolates. Cultures were grown on a liquid grapevine extract, a solidified grapevine extract, and directly on moist, ground grapevine wood. Treatments were set up with and without the addition of sucrose, in an attempt to boost metabolite production.

Metabolite yields were very low on wood extracts containing no additional sucrose, extremely high in those treatments to which sucrose was added. As with artificial culture media, eutypinol was the metabolite most consistently produced, although several as yet unidentified compounds were also produced in significant quantities. Once again, no common compounds were produced by all 11 isolates. Metabolite production was generally consistent among isolates, although some still did not produce significant quantities.

To try to determine which compounds were responsible for the expression of foliar symptoms (objective 2), culture filtrates from all 11 isolates, grown on all four artificial media, were

concentrated and spotted onto discs excised from young grapevine leaves. No correlation was apparent between secondary metabolite production and filtrate toxicity, suggesting that eutypine and the related compounds we detected by HPLC are not solely responsible for foliar symptoms of Eutypa dieback.

Conclusions

During the 4 months spent at the USDA we were able to gain a better understanding of secondary metabolite production by *E. lata*. Richard has gained valuable experience in the analysis of secondary metabolites produced by *E. lata* (objective 1), and will be able to continue aspects of this research whilst in Adelaide. Findings support the hypothesis that eutypine is not the sole compound responsible for foliar symptoms of Eutypa dieback. Secondary metabolite production varied significantly between different fungal isolates, and was also very dependent upon the type of medium on which the fungus was cultured. Metabolite production was similar between Californian and Australian isolates (objectives 2 and 3). More consistent results were obtained on grapevine wood extracts than on standard laboratory media, although some isolates still did not produce secondary metabolites on grapevine media.

Future work

Both AVF and CRCV personnel are very keen to continue this collaboration. Russell and Noreen will examine more American isolates; particularly isolates from the Gallo Winery collection. Richard will continue to examine metabolite production from a wider variety of Australian isolates in Adelaide and will send extracts to Russell and Noreen for analysis, including liquid chromatography/mass spectrometry (LCMS), and possible identification of metabolites/putative toxins. Analysis of a wider range of isolates will enable us to determine whether eutypinol, which was present in nine isolates, or another as yet unidentified compound, will be suitable for use as a chemical marker for the early diagnosis of Eutypa dieback. Richard will conduct pathogenicity tests on the Australian isolates examined and will send foliage and wood samples of artificially inoculated grapevine cuttings to Russell to determine whether compounds detected in artificial culture are produced when *E. lata* is grown *in planta* (objective 3). Secondary metabolite production by isolates of known pathogenicity, provided by Dr Mette Creaser (SARDI), will also be analysed (objective 3). Concurrently, Russell and Noreen will examine field material collected from the Napa Valley in spring (objectives 2 and 4). Growth of the fungus and production of metabolites in solidified media containing wood from tolerant and susceptible cultivars will be compared to examine differences in production of potential toxins. Richard will continue DNA analysis of the isolates used in the biochemical studies, to examine correlation between banding patterns and metabolites produced.

Acknowledgements

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References

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