NATIONAL Wine & Grape INDUSTRY CENTRE

Australian Government

Australian Grape and Wine Authority

Development of rapid and sensitive molecular diagnostic tools to detect trunk disease pathogens from environmental and plant materials.

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FINAL REPORT to

AUSTRALIAN GRAPE AND WINE AUTHORITY

Project Number: **GWT 1421**

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Research Organisation: **National Wine and Grape Industry Centre, Charles Sturt University**

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1. ACKNOWLEDGEMENTS

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I thank Dr. Jose Urbez Torres for sharing his expertise in molecular diagnostics and the SuRDC Plant Pathology Team for the technical support.

2. ABSTRACT

Dr. Regina Billones-Baaijens travelled to Canada in March – June, 2016 to undergo training on new molecular techniques and conduct collaborative research with Dr. Jose R. Úrbez Torres at the Summerland Research and Development Centre (SuRDC), Agriculture and Agri-Food Canada, Summerland, British Columbia. This travel was financially supported by a National Wine and Grape Industry Centre (NWGIC) Research Fellowship Grant; a Wine Australia Travel Bursary with in-kind contribution from SuRDC. The output of this travel will contribute to one of the objectives of a current Wine Australia-funded project, SAR1205 'Practical management of grapevine trunk diseases (GTD) and the newly approved project, SAR1601 "Grapevine trunk disease management for vineyard longevity in diverse climates of Australia". These projects aim to ascertain spore dispersal of GTD pathogens and to determine the climatic conditions that favour the release of these pathogens. The new project also aims to quantify levels of GTD pathogens in nursery propagation material and determine different stress conditions that trigger disease expression in vineyards. The 3-month collaborative research period at SuRDC included hands-on training on (a) DNA macroarray; (b) Droplet Digital PCR (ddPCR); and (c) specificity test and qPCR optimisation of Eutypa dieback (ED) and Botryosphaeria dieback (BD) multi-species primers developed for the GTD project (SAR1205). The DNA macroarray and ddPCR are rapid and specific tools for the identification, detection and quantification of plant pathogens. The training at SuRDC has resulted in enhanced skills in the development of rapid and accurate molecular tools for the detection and quantification of GTD pathogens in the environment. In addition, experience was gained to optimise and troubleshoot molecular assays that will fast track the analyses of spore trap samples collected for the spore trapping studies in Australia. The skills gained in Canada will allow researchers at the NWGIC to adopt these DNA-based diagnostic tools for future GTD research subject to the availability of equipment. The transfer of technologies between Canadian and Australian researchers builds on collaboration established through previous exchanges between the countries by Drs Urbez Torres and Mark Sosnowski (SARDI) and will contribute to the development of control strategies for grapevine trunk diseases that is considered a serious threat to the sustainability of both wine industries.

3. EXECUTIVE SUMMARY

Eutypa dieback (ED) and botryosphaeria dieback (BD) are serious diseases of grapevines worldwide causing cankers, dieback and eventually the death of vines. These grapevine trunk diseases rank in the top five priority diseases of the Australian wine grape industry (GWR08/04). The diseases persist in affected vines from season to season, causing long term decline. They are becoming more prevalent as vineyards age and are a threat to the Australian wine industry, which contributes $40 billion to the economy.

Dr Billones-Baaijens is one of the primary investigators for the on-going research in the project: 'Practical management of grapevine trunk diseases (SAR1205) with Dr. Sandra Savocchia as her project supervisor and Dr. Mark Sosnowski (SARDI) as the national project leader. This collaborative project between the National Wine and Grape Industry Centre (NWGIC) at Charles Sturt University and the South Australian Research and Development Institute (SARDI) is funded by Wine Australia. One of the objectives of this research is to trap and detect the spores of trunk disease pathogens from different wine growing regions in Australia using rapid and highly sensitive DNA-based molecular tools. Multi-species primers have been developed to detect and quantify ED and BD pathogens from the environment. These multi-species primers were shown to be specific and sensitive in the detection and quantification of ED and BD inoculum from environmental spore-trap samples. These primers will also be used in the newly approved Wine Australia project "Grapevine trunk disease management for vineyard longevity in diverse climates of Australia". The new project also aims to quantify levels of GTD pathogens in nursery propagation material and determine different stress conditions that trigger disease expression in vineyards.

The purpose of the travel to Canada was to undergo training on new molecular techniques and conduct collaborative research with Dr. Jose R. Úrbez Torres at the Agriculture and Agri-Food Canada Research Centre located in Summerland, British Columbia. Dr. Úrbez Torres is a highly respected scientist in the taxonomy and epidemiology of grapevine trunk disease pathogens and has established collaborative links with the Australian project team through previous visits to Australia and by Dr Sosnowski to Summerland. This travel was funded through the NWGIC Research Fellowship Program with co-contribution from a Wine Australia Travel Bursary. SuRDC's in-kind contribution (~$18,000) covered the cost for the molecular reagents and other consumables, professional in-kind contribution for Dr. Urbez-Torres and his technical staff that provided assistance for the laboratory work.

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The main outputs of this travel included:

(a) **Hands-on training on DNA macroarray.** This DNA-based diagnostic technique is a rapid and specific tool to detect and identify plant pathogens in a single test. This technique was successfully developed by Dr. Urbez Torres and his team to detect young vine decline (YVD) pathogens from nursery plant materials in British Columbia. Dr Billones-Baaijens work with Dr. Urbez-Torres and his technical staff included the testing the DNA macroarray that they recently developed for detecting ED pathogens. The training included the activation and blotting of the probes on the membranes; buffer preparation; hydrisation of samples; membrane imaging and data analysis. Dr Billones-Baaijens also underwent training on the development and design of DNA macroarray probes using different software.

(b) **Hands-on training on digital Droplet PCR (ddPCR)**. Droplet Digital PCR (ddPCR) is the latest technology for quantitative DNA analysis. It is widely reported to be highly sensitive compared to qPCR and allows accurate quantification of nucleic acids without the need for reference standard curves. The partitioning of samples into droplets also separates any potential contaminants from target templates, limiting their amplification within the droplet which would affect quantification. The ddPCR system further allows multiplexing or detection of multiple species of pathogens using a DNA binding dye called SYBR green. Multiplex ddPCR of ED and BD multi-species primers (SAR1205) has been successfully optimised by Dr. Urbez Torres and his team at SuRDC for the analyses of their spore trap samples. Therefore, the multiplex ddPCR could be used to fast track analyses of environmental samples in SAR1601 if appropriate equipment can be obtained, ultimately reducing cost for consumables and labour by 50%. Dr Billones-Baaijens' ddPCR training included sample preparation, droplet generation, equipment operation and data analysis.

(c) **Testing the specificity of the multi-species primers (SAR1205) using international ED and BD isolates.** The two multi-species primers were able to detect 70 ED and BD isolates from Canada, USA and other international isolates from the SuRDC collection but did not amplify DNA from any of the non-target species. These results confirmed the previous tests conducted at NWGIC demonstrating they are highly specific to the ED and BD target species.

(d) **Optimisation of multi-species primers for qPCR.** The multi-species primers were optimised for qPCR using gradient annealing temperatures and primer concentrations. The ED multi-species primers were found to be highly specific to the target species and were highly suitable for qPCR using a fluorescent DNA binding dye called SYBR green. However, the BD multi-species primers were found to produce primer dimers which are PCR by-products that formed when two primers bind with each other usually when the DNA templates in the PCR are very low. Since SYBR green dye can attach to any double stranded DNA including primer dimers, this can result in false quantification of non-specific PCR products. To resolve this problem, a multi-species probe specific for BD pathogens was further developed by Dr Billones-Baaijens. This multi-species probe (TaqMan) uses a different fluorescence chemistry from SYBR green and do not bind to primer dimers. Tests showed the BD probe was highly specific and can accurately quantify BD pathogens and this probe is now currently being used for qPCR analysis for BD spores from spore tape samples.

(e) **Extension.** Dr Billones-Baaijens presented a one-hour seminar entitled “Grapevine Trunk Disease problems down under” to the local Okanagan Valley wine growers and SuRDC technical staff. The seminar received positive feedback from the growers and technical staff and allowed the transfer of information on management of grapevine trunk diseases between the Australian and Canadian wine industries.

In summary, the DNA macroarray was found to be a rapid and highly sensitive technique to detect several GTD pathogen species in a single sample. This technology is highly suitable for detecting pathogens from nursery plant materials and other environmental samples. The DNA macroarray for ED pathogens developed by Dr. Urbez Torres will allow the identification of all ED species present in spore tape samples. However, the DNA macroarray for BD pathogens has not been developed to date. The ddPCR was also found to be more sensitive and easy to optimise as this technology is more sensitive than qPCR and less prone to contamination. However, both DNA macroarray and ddPCR require special equipment which are currently not available at NWGIC. The knowledge that Dr Billones-Baaijens has gained in using these molecular techniques will allow the adoption of these technology for future GTD research in Australia.

The travel to Canada has allowed Dr Billones-Baaijens to enhance her skills in the development of rapid and accurate molecular tools for the detection of GTD pathogens in the environment. It has further enhanced her skills in optimisation and troubleshooting of molecular assays that will fast track the analyses of spore trap samples collected from different wine growing regions enabling the identification of critical periods for spore release. This will allow the development of industry recommendations particularly in pruning practices and fungicide applications to prevent infections by these pathogens. This travel also contributed in the transfer of technologies and exchange of GTD isolates between Canada and Australia and to the development of control strategies for grapevine trunk diseases that threaten the sustainability of the wine industry for both countries.

4. BACKGROUND

Eutypa dieback (ED) and botryosphaeria dieback (BD) are major trunk diseases, causing significant yield reduction and threatening the sustainability of Australian vineyards. The spores of ED and BD pathogens that primarily infect grapevine pruning wounds are generally dispersed by rain splash and travel some distances depending on wind speed. Data on the patterns of spore release by ED and BD pathogens in different climatic regions of Australia are lacking.

Previous studies on spore dispersal from other countries relied primarily on conventional techniques like microscopy and plating that are extremely time consuming, less accurate and less sensitive in detecting these pathogens from environmental samples. Therefore, there is a need for the development of rapid and highly sensitive DNA-based molecular tools that will fast track the analyses of these spore trap samples and lead to achieving the milestone of the research.

Spore-trapping in different climatic wine regions of Australia using Burkard spore traps is currently on-going. This 3-year study investigates the spore dispersal patterns of ED and BD pathogens in South Australia (Barossa Valley and Coonawarra) and New South Wales (Hunter Valley and Griffith). These spore trapping studies will be further continued in the recently approved Wine Australia project "Grapevine trunk disease management for vineyard longevity in diverse climates of Australia" (SAR 1601).

Multi-species primers have been developed to specifically detect and quantify the spores of ED and BD pathogens and these have been used to analyse the spore tapes from the different regions. The two multi-species primers are rapid and inexpensive tools in detecting and quantifying multiple ED and BD pathogens in a single qPCR run. However, due to its multiple targets, these primers cannot discriminate the different species within a sample and determine which species are the most prevalent and wide-spread in Australian vineyards.

Dr. Urbez Torres and his research team in British Columbia have recently developed a DNA-based molecular diagnostic tool (DNA macroarray) for rapid and precise detection of multiple species in a single sample. This technology has already been tested and shown to detect several species of pathogens causing young vine decline from a single nursery planting material including Petri/Esca disease and Black foot. His team also recently developed DNA-probes to detect several Diatrypaceae species associated with Eutypa dieback. These probes will allow the identification of different ED pathogens in a single sample. The probes to detect BD pathogens are still to be developed. Combining the macroarray with the qPCR in the spore trapping studies in Australia would allow the quantification as well as identification of the most prevalent and abundant ED and BD pathogens present in different wine growing regions in Australia. Furthermore, worldwide there is an emerging concern of nursery planting materials being potential sources of infection of these trunk pathogens. However, information on the prevalence and distribution of these diseases in Australian nurseries is lacking. The hands-on training gained on the DNA macro-array can be potentially used in future research to investigate the presence of these pathogens in Australian nurseries and to understand their potential spread within the nursery system. This training will also facilitate the collaboration with Dr. Jose Urbez Torres and his research team in the development of DNA-probes targeting BD pathogens.

Preliminary tests using Australian BD isolates showed they are highly specific to their target pathogens. Testing these primers with the Canadian and other international isolates will further confirm its specificity to their target species. Furthermore, the optimisation of these multi-primers for quantitative PCR (qPCR) and suitability for droplet digital PCR (ddPCR) will further demonstrate their suitability for quantitative DNA analysis that is essential for the analyses of spore trap samples.

This travel contributed to the first objective of project SAR1205 ‘Practical management of grapevine trunk diseases’, which is to ascertain spore dispersal throughout the pruning season and to determine the climatic conditions that favour the release of these pathogen. These spore trapping studies will be continued in the following Wine Australia-funded project: Grapevine trunk disease management for vineyard longevity in diverse climates in Australia (2017-2020). The new project also aims to quantify levels of GTD pathogens in nursery propagation material and to determine different stress conditions that have the potential to trigger disease expression in vineyards.

5. RESEARCH OUTPUTS:

(a) Hands-on training on DNA macro-array.

The DNA macroarray tool is a rapid and sensitive DNA-based detection technique that can simultaneously identify multiple microorganisms in a single test (Figure 1). This technology is based on the design of species-specific probes that are fixed on a nylon membrane and are hybridised with a PCR product from a single sample. The DNA macroarray for the detection and identification of fungal pathogens causing young vine decline was successfully developed in British Columbia (Urbez Torres et al. 2015). The macrorarray for the ED fungi is currently being developed by Dr. Urbez Torres and his team while the macroarray for BD is yet to be developed.

The DNA macro-array for ED pathogens can be potentially incorporated in the analyses of spore trap samples as this will allow the identification of individual Diatrypaceae species trapped in vineyards. However, it requires some funding to purchase specialised equipment particularly the hybridisation oven and the chemiluminiescent imaging system which is currently not funded in the new project. The team will investigate further funding sources to potentially secure the equipment needed to allow the adoption of this technology for future GTD research in Australia. Dr. Billones-Baaijens can also potentially work collaboratively with Dr. Urbez Torres in the development of DNA macroarray for BD pathogens at NWGIC.

(b) Hands-on training on Droplet Digital PCR

Droplet Digital polymerase chain reaction (ddPCR) is a new technology for precise absolute quantification of nucleic acid target sequences by combining the water-oil emulsion droplet technology with microfluids (Figure 2). This new system is now becoming a popular alternative to quantitative PCR technology.

The current qPCR system at the NWGIC allows quantification of the pathogens of interest from environmental samples. However ddPCR is more sensitive and less costly than qPCR and the procedure is more time efficient and less prone to contamination issues. In addition, the current qPCR procedure requires multiple reference standard curves to obtain accurate quantification for each individual species of a pathogen. The standard curves are difficult to develop as most of the pathogen species do not produce enough material *in vitro*.

Dr. Billones-Baaijens' training on the ddPCR system at SuRDC allowed accurate quantification of nucleic acids without the need for reference standard curves. The ddPCR technology has also been widely reported to be highly sensitive compared to qPCR and pre-amplification of templates is not necessary. This will minimise issues with contamination from environmental samples. The partitioning of samples into droplets also separates any potential contamination from target templates and limits its amplification within the droplet, thus, it does not affect quantification.

Multiplex ddPCR of the ED and BD multi-species primers developed in SAR1205 has been successfully optimised by Dr. Urbez Torres and his team at SuRDC for the analyses of their spore trap samples. The use of multiplex ddPCR would fast track the analyses of environmental samples in Australia and reducing the cost by 50% compared with qPCR. If the equipment can be acquired, Dr. Billones-Baaijens' new knowledge on ddPCR will allow adoption of this system for SAR1601 which aims to quantify pathogen levels in nursery plant materials and determine the thresholds that result in disease expression.

(c) Testing primer specificity on international ED and BD isolates.

The ED and BD multi-species primers developed by Dr Billones-Baaijens were able to amplify the DNA from 70 Diatrypaceae and Botryosphaeriaceae isolates from Canada, US and other international isolates. These primers did not amplify any of the non-target species demonstrating that they are highly specific to ED and BD pathogens.

(d) Optimisation of qPCR

The multi-species primers were optimised for qPCR using gradient annealing temperatures and primer concentrations. The ED multi-species primers were found to be highly specific to the target species and were suitable for qPCR using a DNA fluorescent dye called SYBR green. However, the BD multi-species primers had issues with primer dimer formation. Primer dimers are PCR by products that form when the two primers bind with each other particularly when DNA concentrations in the PCR are very low. Since the SYBR green dyes bind to any double stranded DNA including primer dimers, this can result in false quantification of non-specific PCR products. To resolve this problem, a multi-species probe specific for BD pathogens was further developed. This probe uses a different fluorescent chemistry (TaqMan) and do not bind to double stranded DNA like SYBR green, thus, resolves primer dimer problems. Tests showed the BD probe was highly specific and accurate in quantifying BD pathogens and has been adopted for qPCR analysis for BD from spore tape samples in SAR1205.

(e) **Extension.** Dr Billones-Baaijens' seminar entitled "Grapevine trunk Disease problems down under" was well received by the Okanagan Valley winegrowers and the SuRDC technical staff. Dr Billones-Baaijens' also visited some vineyards with Dr. Jose Urbez Torres to observe the spore-trapping technology used by SuRDC that can be potentially adopted for future GTD research (Figure 3).

6. SUMMARY

This 3-month collaborative project with Dr. Úrbez Torres has enhanced Dr Billones-Baaijens' skills in molecular techniques for plant disease diagnosis, fungal taxonomy and genetics. In addition she gained further skills in optimisation and troubleshooting of molecular assays that will assist in fast tracking the analyses of spore trap samples collected from Australian wine growing regions and to identify the critical periods for spore release. The data generated from these spore-trapping studies will be disseminated via oral presentations to growers and industry personnel. All data generated will be used to develop industry recommendations.

This collaborative research also generated scientific data on DNA-based detection of different grapevine trunk disease pathogens from environmental samples and plant materials. It is envisaged that this data will be publishable in prestigious international journals on which Dr Billones-Baaijens will be one of the primary authors. These data will be presented at local and international trunk disease workshops and other scientific fora that will gain the Australian trunk disease team recognition and will assist in forming collaborations with the local and international scientific community involved in grapevine trunk disease research. The transfer of technologies between Canadian and Australian researchers has built a good relationship between research organisations in the two countries and will contribute to the development of control strategies for grapevine trunk diseases that is a serious threat to the sustainability of both wine industries.

7. FIGURES

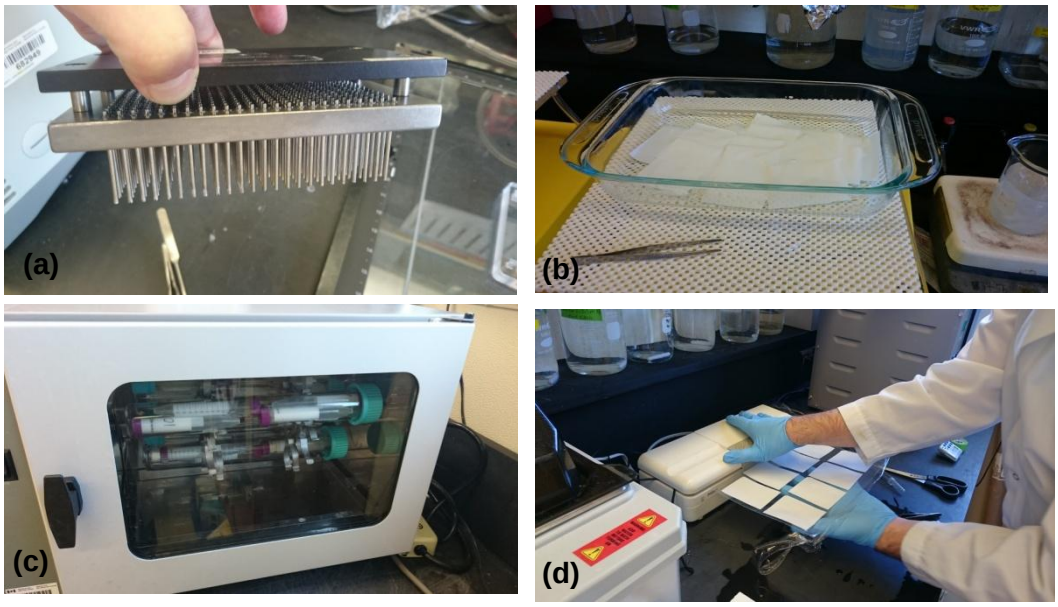


Figure 1. DNA macroarray (a) The replicator pin used for blotting the probes on the membrane; (b) probe-blotted membranes are soaked in blocking solution to prevent non-specific binding of non-target species; (c) membranes are submerged in the DNA samples and placed inside the hybridisation oven to allow samples to hybridise with the probes; (d) the hybridised membranes being processed for imaging using the Chemiluminescent imaging system.

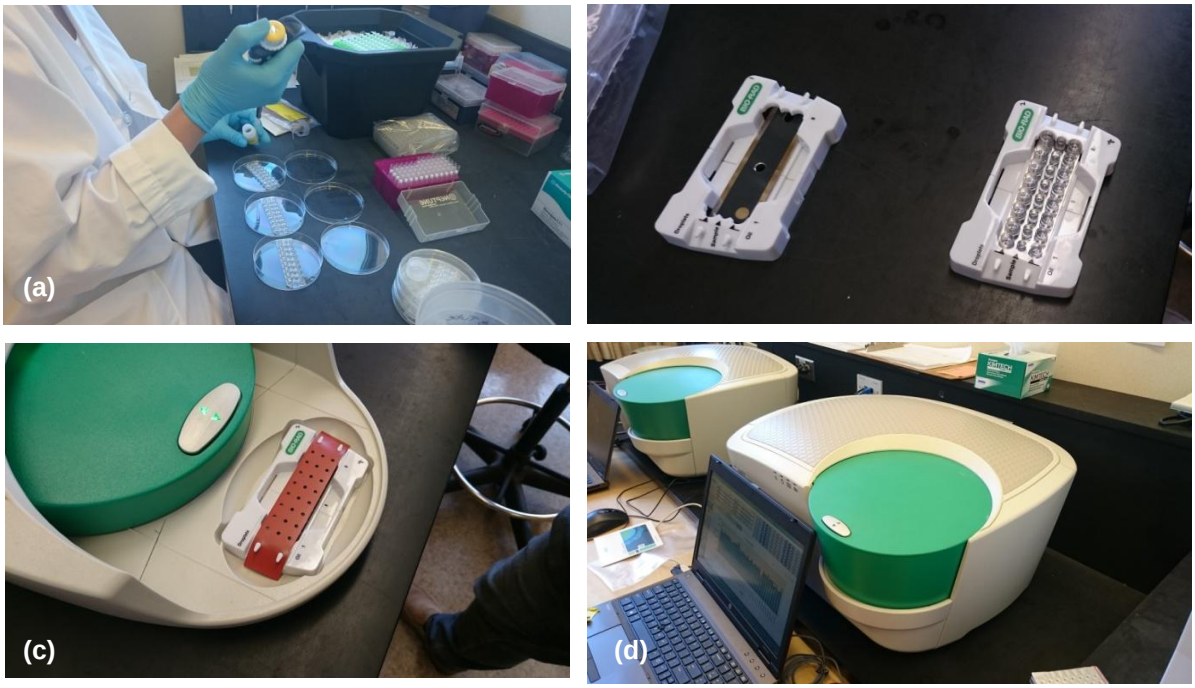


Figure 2. (a) Preparation of ddPCR reactions; (b) cassettes used for generating droplets; (c) cassette mounted inside the droplet generator; (d) ddPCR reader used for quantification of PCR products.

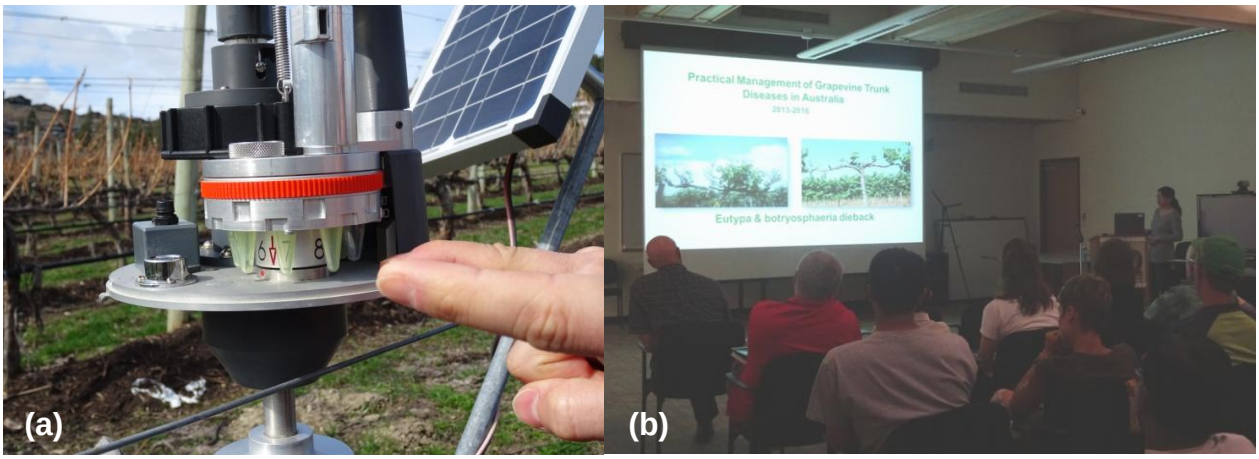


Figure 3. (a) The SuRDC Burkard spore-trap that make use of microtubes instead of Melinex tapes to collect spores in Okanagan Valley vineyards, BC; (b) Dr. Regina Billones-Baaijens presenting a seminar entitled “Grapevine Trunk Disease problems down under” to the Okanagan Valley wine growers and scientific staff at the Summerland Research and Development Centre, Summerland, BC, Canada.