Fifteenth Australian Weeds Conference

Additional European strains of \textit{Phragmidium violaceum} released for the biological control of blackberry

L. Morin$^{1,2}$, R. Aveyard$^2$, K.L. Batchelor$^3$, K.J. Evans$^{1,4}$, D. Hartley$^2$ and M. Jourdan$^5$

$^1$CRC for Australian Weed Management
$^2$CSIRO Entomology, GPO Box 1700, Canberra, Australian Capital Territory 2601, Australia
$^3$CSIRO Entomology, Floreat Park, Private Bag 5, PO Wembley, Western Australia 6913, Australia
$^4$TIAR, University of Tasmania, 13 St Johns Avenue, New Town, Tasmania 7008, Australia
$^5$CSIRO European Laboratory, Campus International de Baillarguet, 34980 Montferrier-sur-Lez, France

Summary  
Additional European strains of the leaf-rust fungus, \textit{Phragmidium violaceum} (Schultz) Winter have been approved for release in Australia to improve prospects of biologically controlling European blackberry (\textit{Rubus fruticosus} L. agg.) across the wide range of taxa and genotypes that exist. In tests prior to release, the additional strains were found to be pathogenic on all \textit{Rubus} genotypes tested except clones of \textit{Rubus laciniatus} Willd. Only one strain was capable at infecting clones of this species. Results from host-specificity tests concurred with previous findings that \textit{P. violaceum} does not pose a threat to commercial blackberry cultivars and Australian native \textit{Rubus} species. The strains have so far been released by CSIRO at a few sites in NSW and WA, as well as in Victoria by the Victorian Department of Primary Industries. A simple molecular diagnostic tool to distinguish additional European strains from existing populations of \textit{P. violaceum} in Australia is needed for monitoring their establishment after release. The challenges in developing such a tool are presented.

Keywords  
Biological control, rust fungus, \textit{Phragmidium violaceum}, European blackberry, \textit{Rubus fruticosus} agg., WoNS.

INTRODUCTION  
European blackberry is one of Australia’s Weeds of National Significance (Thorp and Lynch 2000). The leaf-rust fungus \textit{P. violaceum} is already present in Australia. It was introduced on at least two occasions: an unauthorised introduction of an uncharacterised population in the early 1980s and authorised releases of French strain F15 as a biological control agent in 1991 and 1992 (Bruzzese and Hasan 1986a). Comprehensive specificity testing was done prior to the authorised releases of this strain, using a pool of 15 isolates representing a broad genetic base of \textit{P. violaceum} (Bruzzese and Hasan 1986b).

Since its introduction the rust has provided useful control of European blackberry, although unsuitable climatic conditions and host resistance have limited effectiveness in some situations (Evans et al. 2005). Several taxa and genotypes of European blackberry have been found to occur in Australia (Evans et al. 2004b).

A ‘trap garden’ comprising different genotypes of \textit{R. fruticosus} agg. from Australia was established in 1999 in southern France to source additional virulent strains of \textit{P. violaceum} (Scott et al. 2002). Release of additional strains that infect the wide range of taxa and genotypes occurring in southern Australia may improve prospects of biologically controlling blackberry. Molecular analysis using SAMPL (selective amplification of microsatellite polymorphic loci) showed that rust strains collected from this garden were genetically different to accessions of the rust that currently exist in Australia and New Zealand (Gomez et al. 2006).

This paper summarises results of pathogenicity and host-specificity testing for eight purified strains collected from different blackberry genotypes in the French trap garden. It also reports on initial releases of these strains in NSW and Western Australia, but not on the additional releases that have since been made in Victoria (R. Adair, DPI Victoria pers. comm.). The paper also summarises attempts to develop a simple molecular diagnostic tool to differentiate additional strains from existing populations of \textit{P. violaceum}, as they cannot be distinguished morphologically. Such a tool is necessary for rapid screening of field-collected rust samples after release, to confirm establishment of additional strains. A comprehensive methodology with the full set of results will be published elsewhere.

MATERIALS AND METHODS  
Pathogenicity and host-specificity tests  
Eight purified rust strains collected from the French trap garden were bioassayed on a representative differential set of 12 Australian \textit{Rubus} genotypes (Evans et al. 2004a). The first series of tests were performed on detached leaflets. When inconclusive results were obtained, additional tests were performed on leaves still attached to plants. Pathogenicity of strains was also tested on...
a representative number of non-target plant species related to blackberry.

**Development of diagnostic molecular tool**

Published primers were used to amplify by polymerase chain reaction (PCR), various regions of the nuclear (ITS, IGS, beta-tubulin gene, elongation factor 1-alpha gene) and mitochondrial (Cox3, cytB, ATP6) genomes of a subset of the additional strains and Australian accessions of the rust. Amplified regions were then sequenced and compared.

A total of 54 RAPD (random amplification of polymorphic DNA) primers were screened to identify amplified genomic regions (‘bands’) that are specific either to the additional strains or the existing population of the rust in Australia. All RAPD primers were screened using a subset of DNA samples from each group. Two ‘bands’ were identified with two different RAPD primers as having highest potential to be used as diagnostic markers to distinguish the two groups. The ‘bands’ were excised, cloned and sequenced to generate SCAR (sequence-characterised amplified region) markers. These markers were then screened on the additional strains and Australian accessions of the rust.

**Releases**

The eight strains, as well as F15 strain that was introduced in the 1990s, were released either in autumn or spring 2004 at a few experimental field sites in the Tumut-Tumbarumba region in New South Wales (NSW) and the Manjimup region in Western Australia (WA) (Figure 1). Spores were suspended in water and sprayed onto the under-surface of leaves. Inoculated plants were misted with water and covered overnight with plastic sheets to maintain high humidity. On the day of inoculation, potted blackberry plants infected with each of the strains were also placed next to the inoculated areas at the release sites, to encourage additional infection. These plants were left in the field.

![Figure 1. Location of experimental sites where first releases of the eight additional strains of Phragmidium violaceum occurred in 2004.](image)

**RESULTS AND DISCUSSION**

**Pathogenicity and host-specificity tests**

Two different virulence phenotypes were observed among the additional strains. The strains were pathogenic on all **Rubus** genotypes tested except **Rubus laciniatus** (clones EB22 and KE1; Figure 2). Only one strain (G18-TG-00-4-1) was capable of infecting clones of this species. Results from host-specificity tests concurred with previous findings that **P. violaceum** does not pose a threat to commercial blackberry cultivars and Australian native **Rubus** species. None of the leaves of the blackberry cultivars Loch Ness and Chester that are popular among growers in Australia nor the native **Rubus** species that had not been tested previously (**R. nebulosus** A.R.Bean, **R. probus** L.H.Bailey, **R. queenslandicus** A.R.Bean), were susceptible to any of the strains tested.

**Development of diagnostic molecular tool**

We have faced major stumbling blocks in our attempts to develop a simple molecular tool to differentiate the additional strains from existing populations of **P. violaceum** in Australia. Availability of such a tool would facilitate and streamline monitoring of the additional strains following their release, against a background of established populations of other strain(s) of rust. ITS, IGS and Cox3 regions of representative samples successfully amplified but sequencing revealed no sites from which a PCR-based diagnostic tool could be designed. No amplification was obtained with the other regions primed.

During initial screening, only one of the SCAR markers (F08) identified showed promise for differentiating the additional strains from the existing
population of the rust in Australia (Figure 3). This marker was then pursued further by optimising PCR conditions and incorporating it in a duplex PCR test with a ITS primer (to provide a positive control for all samples). However, subsequent extensive screening of a wide range of samples revealed that F08 marker was sometimes detected in Australian rust accessions. The ISSR (Inter-Simple Sequence Repeats)-PCR method is now being investigated as a possible avenue to differentiate the additional strains from Australian accessions.

Releases Following approval from Biosecurity Australia, the additional strains were released for the first time in Australia in April 2004. Initial releases were limited to experimental sites in NSW and WA in order to trial various techniques and timing (spring or autumn).

Within a month of autumn releases in NSW, uredinial fructifying bodies (repetitive stage), likely to have arisen from successful infection by the strains released, were seen on plants within the inoculated areas. At that time, all other fructifying bodies on field plants were in the form of telia (overwintering stage), a sign that existing rust populations at the sites were developmentally ahead of the released strains. In WA, inoculated leaves of field plants had developed telia by seven weeks after inoculation. No rust infection was observed on young leaves of surrounding blackberry cane tips that were not artificially inoculated.

Signs of infection, in the form of uredinia, were also seen in inoculated areas within a month of spring releases. In NSW however, pycnia and aecia (fruiting bodies of the early stages in the rust life-cycle) were seen throughout the sites indicating that the existing rust population had already started to develop.

Samples of rust populations taken at experimental sites several months after releases are currently stored in anticipation that a suitable molecular diagnostic tool for rapid screening will be developed. This is the only way we can confirm with certainty that establishment has occurred at these sites. The SAMPL technique developed by Gomez et al. (2006) will be used for comprehensive monitoring of establishment and persistence of the additional strains following their future release at sites in Victoria, where extensive information on genetic structure of the existing population have been gathered in previous work (Gomez 2005).

CONCLUSION
Release of the additional strains originating from the French trap garden has potential to increase the genetic diversity and adaptation potential of the existing Australian population of *P. violaceum*. This could occur through recombination or an increase in effective population size and in turn improve the impact of this biological control agent on blackberry.

We are now planning to embark on a large-scale national release program, hence the imperative to develop a simple molecular diagnostic tool to confirm widespread establishment. In the meantime, extensive sampling at a few Victorian sites and screening using the technically demanding SAMPL procedure will provide data to demonstrate effectiveness of the release program.

Following establishment, the additional strains, or new recombinant genotypes, should spread naturally to other blackberry infestations within the regions. The strain(s) most adapted to particular areas should adversely impact blackberry populations over time through regular defoliation of plants.

ACKNOWLEDGMENTS
This project would not have been possible without the support of CSIRO Entomology, the CRC for Weed Management Systems (now known as CRC for Australian Weed Management), the Australian Government Department of Agriculture, Fisheries and Forestry, the Department of Agriculture of Western Australia, the Western Australian Department of Conservation and Land Management and the Riverina Highlands Weed Working Group. We are most grateful to our stakeholders in the Tumut-Tumbarumba and Manjimup regions for assistance in facilitating the project. We also wish to thank all staff of our respective organisations that provided assistance.

REFERENCES
Fifteenth Australian Weeds Conference


