Variation in *Septocyta ruborum* and its potential for biological control of European blackberry

Robin J. Adair[1], Janita Baguant[2] and Ann C. Lawrie[2]
[1] Department of Primary Industries, PO Box 48, Frankston VIC
[1] Current address: Australis Biological, PO Box 151, Bittern VIC 3918
[2] RMIT University, PO Box 71, Bundoora VIC 3083
(robin.adair@australisbiological.com.au)

Summary The fungus *Septocyta ruborum* causes a destructive necrosis of floricanes in blackberry (*Rubus fruticosus*) in Europe and is a potential candidate for biological control in Australia. Over 100 accessions of *S. ruborum* from Europe were analysed for variation in pathogenicity and virulence. Virulence varied among accessions within *R. fruticosus* 'species'. Accessions screened for genetic diversity using (internal transcribed spacer (ITS) sequencing and RAPD (random amplification of polymorphic DNA) yielded identical ITS sequences, but three of 120 RAPD primers showed high levels of polymorphism. Accessions clustered into five main groups, but these were not related exclusively to virulence or geography. Some accessions from northern Europe and one accession from Hungary clustered apart from the main group. The large amount of polymorphism in both virulence and genetics suggests that *S. ruborum* reproduces sexually or that it has subdivided into many races. The large genetic variation offers considerable potential for selection of host-specific forms, an advantage in biological control.

Keywords Pathogen, coelomycete, purple blotch disease, Australia.

INTRODUCTION

The variable impact of *Phragmidium violaceum* (Schultz) Winter (blackberry rust fungus) on European blackberry (*Rubus* species) in Australia suggests additional organisms are required to provide effective biological control of this highly invasive weed. The cane pathogen *Septocyta ruborum* (Lib.) Petr. is specific to *Rubus* where it causes mortality of infected hosts and is under evaluation as a new biological control agent. The pathogen is widespread in Europe where it infects *Rubus* species predominantly in the sections Rubus and Corylifolii.

The coelomycete *S. ruborum* causes severe stem dieback, necrosis and eventually death in floricanes of *Rubus fruticosus* L. sp. agg. The disease is systemic with early infection occurring on primocanes and foliage. Stem symptoms commence as small spots or patches, but advance to large dark purple lesions that occur over most of the stem. The disease infects vascular bundles and migrates through host tissue *via* xylem vessels. Apart from a dubious record from *Potentilla* (Feige *et al*. 2001), *S. ruborum* only infects *Rubus*, where species are mainly within the *R. fruticosus* spp. agg., including *R. anglocandicans* A. Newton (the main type in Australia) or cultivars with *R. fruticosus* in their pedigrees are susceptible. *R. fruticosus* is highly invasive and problematic in agricultural and natural ecosystems in Australia and is subject to targeted biological control. *S. ruborum* infects commercial blackberry cultivars in North America and was recently detected in New Zealand, where it appears confined to clones of Youngberry and Boysenberry. Pathotypes of *S. ruborum* that infect wild *R. fruticosus*, but have minimal impact on key commercial cultivars of blackberry are sought to augment biological control using the rust fungus *P. violaceum*.

We analyse the genetic variation of isolates of *S. ruborum* across its natural distribution in Europe, and New Zealand to determine the extent of polymorphism, and their virulence on *R. fruticosus*, in order to predict the pathogen's potential as a selective biological control agent.

MATERIALS AND METHODS

Inoculum preparation Stem sections (3–4 cm long × 0.2–0.25 cm wide) of *R. anglocandicans* A. Newton, a widespread and abundant species in SE Australia, were surface sterilised with 70% ethanol and rinsed in sterile water. Accessions were plated onto potato dextrose agar and maintained at 20°C with 12 h light/day until sporulation. Spore suspensions were prepared by flooding the culture with 2 mL of sterile water then scraping the top layer of the colony. The suspension was filtered and adjusted to 1×10^7 spores/mL.

Virulence Stem sections of *R. anglocandicans* (3–4 cm long × 0.2–0.25 cm wide) from the mid-section of healthy canes were surface sterilised with 70% ethanol and rinsed in sterile water. Stems were dipped in spore suspensions for 10 s, incubated in Petri dishes lined with moist sterile filter paper at 20°C and scored for

disease severity up to 63 days post-inoculation (dpi). Accessions inducing complete necrosis or coverage with pycnidia were considered virulent. Time to infection was also used as an indicator of virulence. Data were analysed using a non-parametric (Kruskal-Wallis) test.

Stratification Stems were prepared as for virulence testing. Stems were exposed to 4°C for 3, 6, 9, 12 and 15 days then maintained at 20°C. Control stems were maintained at 20°C until infection symptoms were expressed.

Molecular analysis *Septocyta ruborum* cultures were transferred to potato dextrose broth on a shaker (200 rpm) at room temperature. Mycelia from uncontaminated accessions were transferred to 1.5 mL Eppendorf tubes and centrifuged at 800 rpm for 20 min. The supernatant was discarded and the pellet washed in sterile distilled water. Mycelia were ground with sterile glass beads and DNA was extracted with a Qiagen DNeasy Plant MiniKit following the manufacturer's instructions. DNA was precipitated with 3 M sodium acetate (pH 5.2) and cold ethanol (-20°C). DNA pellets were washed with 70% ethanol and dried in a speed vacuum for 4 minutes. DNA was transported from France to Australia (under permit) and resuspended in sterile PCR grade water. The primer pair ITS1F and ITS4 were used to target the ITS spacer region of genomic DNA according to Gardes and Bruns (1993). PCR reactions were performed with one cycle of denaturation at 94°C for 10 min, 35 cycles of denaturation of: 94°C for 30 s, annealing at 55ºC for 30 s and extension at 70°C for 1 min, with a final extension of 72°C for 10 min. The PCR products from three accessions were selected (according to different RFLP patterns with the PCR product), purified using a QIAquick PCR purification kit and sequenced using a ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit; the sequencing product was precipitated using the manufacturer's protocol and analysed at Micromon (Monash University). The primers OPB-1, OPB-5 and OPB-6 (Operon) were selected for RAPD analysis. PCR reactions for all accessions were performed on a Thermohybaid PCR Express thermal cycler: 1 cycle at 94°C for 12 min, 45 cycles of denaturation at 94°C for 1 min, annealing at 36°C and extension at 72°C for 2 min, and a final extension at 72°C for 10 min. The total 25 µL PCR product was loaded onto a 1.4% agarose gel and run at 100 V for 45–60 min.

Statistical analysis Principal components analysis was used to produce a score plot of RAPD products of *S. ruborum* accessions based on the first two components. A dendrogram with complete linkage and squared Euclidean distance was also produced, using Minitab Version 14.

RESULTS

Virulence There was a significant difference in virulence amongst accessions of *S. ruborum* (Kruskal-Wallis, H = 509.7, P <0.001). Three accessions were highly virulent at 45 dpi, while eight accessions did not cause any disease or only minor infection symptoms. There were significant differences in virulence among the geographical origin of accessions (Austria, England, France, Hungary, Scotland, Norway) (H = 62.17, P <0.001). The most virulent accessions of *S. ruborum* were from England, but others were also found from Italy and France.

Effect of stratification Control stems inoculated with *S. ruborum* and maintained at 20°C (no stratification) developed pycnidia in necrotic regions at 20 days post inoculation (dpi) and cirrhi at 25 dpi. Stratified stems developed pycnidia slightly later than controls at 20–25 dpi.

Genetic diversity All ITS sequences were identical and highly similar to those of *Septoria* species on GenBank using a Blastn search. By contrast, high levels of polymorphism were detected in European populations of *S. ruborum* using RAPD markers (Figures 1 and 2). Most isolates belonged to a clade of closely related accessions from England, France and Italy. A second clade contained seven isolates from Britain, Italy and Hungary. Other English and Hungarian isolates were main outliers in separate clades (Figures 1 and 2). Clades could not be related to virulence of *S. ruborum* as shown in the stem assay, to the geographical origin or to the *Rubus* host species.

DISCUSSION

Septocyta ruborum in the field has variable virulence on *R. anglocandicans*, the dominant invasive *Rubus* species in southern Australia. Stem piece assays *in vitro* were successful low-cost assays showing the wide range of virulence in isolates of *S. ruborum* from across Europe and in selecting a few highly virulent isolates for whole-plant inoculations. The lack of correlation with the geographical origin of accessions or with the natural hosts of the pathogen in Europe suggests high genetic variation in the pathogen and an equal likelihood of finding virulent isolates on any of the *Rubus* species.

In Europe, pycnidia and conidia of *S. ruborum* are produced on stems after winter, where stratification is a precursor to sporogenesis (Koellreuter 1952). We

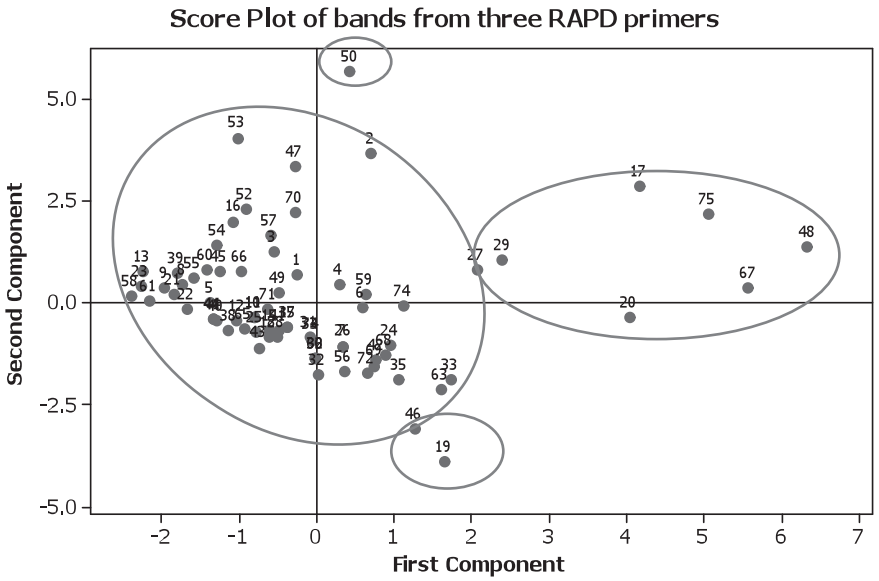

Figure 1. Principal components (correlation analysis) plot of 20 RAPD bands from isolates of *Septocyta ruborum*. Isolates 1-4 Austria, 5-29 England, 30-46 France, 47-56 Hungary, 57-73 Italy, 74 Norway, 75 Scotland. Clades are shown.

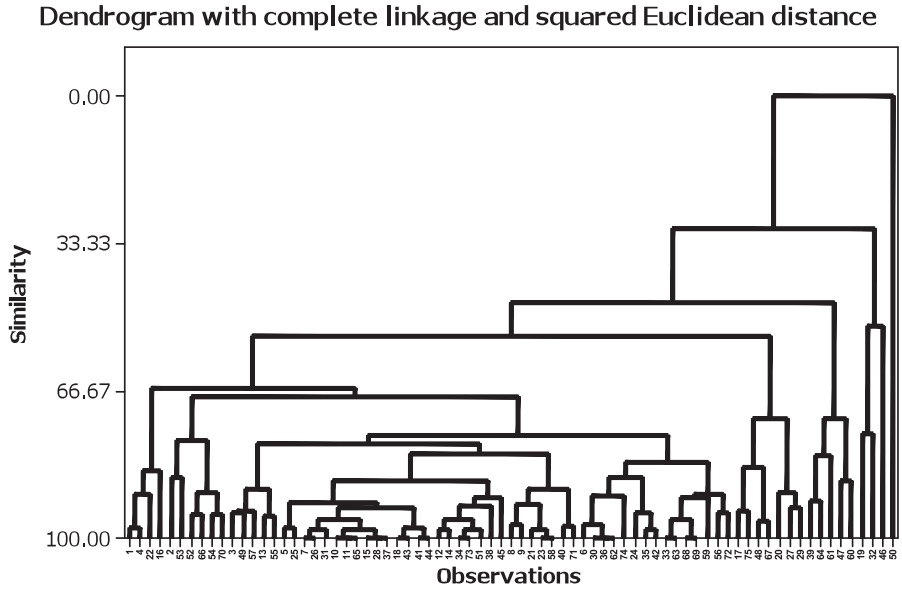

Figure 2. Dendrogram based on multivariate analysis with complete linkage and squared Euclidean distance of 20 RAPD bands from isolates of *Septocyta ruborum*. Isolates are as in Figure 1.

found no evidence for a stratification requirement on excised stems, but acknowledge that responses are likely to vary considerably between whole plant and *in vitro* material.

Subsequent repeated attempts to induce infection by *S. ruborum* on whole potted plants of *R. anglocandicans* or *R. ulmifolius* Schott with a range of pre- and post-inoculation treatments were unsuccessful

(Baguant 2006, Baguant unpublished 2008). Whole plant inoculation techniques are critical for establishing the host range, selection of suitable virulent pathotypes and describing the biology and ecology of *S. ruborum.* This is the priority for the future development of *S. ruborum* as a potential biological control agent for invasive blackberry in Australia.

RAPD markers also indicate considerable intraspecific diversity amongst European populations of *S. ruborum* and also indicate that the centre of the biodiversity is France, Italy and England. It will be interesting to compare the genetics of the isolate(s) found recently in New Zealand with those from Europe as it may indicate its origin, a matter of importance in quarantine and biosecurity. The high level of genetic variation indicates that sexual reproduction may occur in *S. ruborum*, but the teleomorph has not yet been found. Several explanations are possible: the pathogen has lost this form of reproduction in its recent evolution; the teleomorph occurs on hosts other than *Rubus*; *S. ruborum* is an anamorph of another related sexually reproducing species; or the sexual stage is elusive and difficult to find. Resolution of this aspect of the biology of *S. ruborum* is a priority for the development of this pathogen for biological control purposes.

The polymorphism in genetics and variation in virulence of *S. ruborum* suggest there is potential for the selection of pathotypes of the disease that have host selectivity. This will be important for the selection of forms that are virulent against invasive *Rubus,* but have minimal impact on important commercial *Rubus* cultivars.

ACKNOWLEDGMENTS

We thank Dr Mireille Jourdan and other staff of CSIRO at Montpellier, France, where the virulence assays were conducted.

REFERENCES

Baguant, J. (2006). 'A new approach to biological control of blackberry'. Hons. Thesis, Royal Melbourne Institute of Technology University.

Koellreuter, J. (1950). Morphologie und biologie von *Rhabdospora ramealis* (Desm. et Rob.) Sacc. *Phytopathologische Zeitschrift* 17, 129-160.

Gardes, M. and Bruns, T.D. (1993). ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology* 2, 113-118.

Feige, G.B., Ale-Agha, N. And Nagel, B. (2001). Microfungi on Rosaceae in eastern Westphalia (Sauerland, Northrhine-Westphalia, Germany). In, Proceedings of the 53rd International Symposium on Crop Protection. Belgium, 8 May 2001, pp. 117-132.