An artificial inoculation protocol for *Uromycladium acaciae*, cause of a serious disease of *Acacia mearnsii* in Southern Africa

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Abstract

Uromycladium acaciae is the cause of a severe wattle rust epidemic in plantations of *Acacia mearnsii* (black wattle) in Southern Africa. Research on the biology of this damaging rust is assisting in the development of control strategies. One strategy under investigation is the identification and deployment of resistant lines of *A. mearnsii*. Selection of resistant families currently relies on large-scale, time-consuming and expensive field trials. In this study, we present a detailed artificial inoculation protocol for *U. acaciae*, which can be used to screen for resistance. The results of an experiment that used the protocol to screen the relative resistance of twelve families of *A. mearnsii* to *U. acaciae* are also presented. The developed artificial inoculation

protocol can also be used to investigate several other aspects of this host-pathogen system.

Key words

Fungi, relative resistance, relative susceptibility, resistance screening, phenotype trait selection, breeding

Introduction

Native to south-eastern Australia, *Acacia mearnsii* (black wattle) has been grown in Southern Africa since the 1850s for tannin (extracted from its bark) and for timber (Moreno Chan et al. 2015). In South Africa, plantations of *A. mearnsii* cover c. 110,000 ha and annual bark extract and wood chip exports have an estimated value of more than US$150 million (Moreno Chan et al. 2015). The black wattle industry directly employs an estimated 36,000 people (Dunlop 2002).

The black wattle industry in Southern Africa is threatened by the recent emergence of *Uromycladium acaciae*, the causal agent of wattle rust (McTaggart et al. 2015). Symptoms of the disease include rachis and rachilla (leaf midrib) malformation, matting of leaves and severe stunting of growth (McTaggart et al. 2015; Fraser et al. 2017). Severe symptoms of the rust were first observed in the KwaZulu-Natal Midlands in 2013 (McTaggart et al. 2015), but now occur in plantations throughout KwaZulu-Natal, Mpumalanga and Swaziland. Growth loss estimates of 20 - 40% in one year have been reported (Little and Payn 2016).

Little and Payn (2016) showed that application of azoxystrobin-based fungicides was effective in reducing the impact of the rust. However, the continued use of large amounts of fungicide is neither economically sustainable nor environmentally desireable. Instead chemical control should constitute only one part of a broader

integrated pest management strategy for the rust. This strategy should also incorporate effective forest hygiene (e.g. the use of disease free material in the establishment of plantations), appropriate silvicultural practises (e.g. timely pruning and weeding) and the development and deployment of resistant or tolerant lines of *A. mearnsii*.

Field trials are currently being used to screen the relative resistance and susceptibility of different families of *A. mearnsii* to *U. acaciae* for breeding programmes. These trials depend on natural infections and are, therefore, situated adjacent to infected plantations, which act as a source of inoculum. Although these field trials produce valuable data, they are expensive and time consuming to establish, maintain and assess. An alternative method to screen for resistance, which solves many of these problems, is to artificially inoculate young *A. mearnsii* plants with teliospores of *U. acaciae* and to maintain them under controlled conditions that are conducive to infection. The resistance levels of families can then be assessed when symptoms develop. However, this is dependent on the development of an optimised artificial inoculation protocol.

Fraser et al. (2017) identified the optimal environmental conditions for the infection of *A. mearnsii* by *U. acaciae*. Fraser et al. (2017) showed that only young *A. mearnsii* tissue was susceptible to infection and that a 48 hour dew period at 15–20°C was optimal for infection. In this Research Note, we first present a detailed artificial inoculation protocol for *U. acaciae* based on our experience of working with the pathogen and the work reported in Fraser et al. (2017). We then present the details of a proof of concept experiment that used this protocol to screen the relative resistance of twelve families of *A. mearnsii* to infection by *U. acaciae*.

Artificial inoculation protocol

Preparation of inoculum

To establish a single pustule isolate of *U. acaciae*, teliospores should be scraped from a single telium with a sterile scalpel and suspended in 1–5 ml distilled water with Tween 20 (0.05%; DWT). The suspension should then be applied onto young tissue of healthy plants of *A. mearnsii* with a sterile paintbrush and the plants incubated as described below. When telia appear, teliospores should be harvested and re-applied to healthy plants to bulk-up inoculum. Large telia on stems and branches continue to produce teliospores for several months, therefore teliospores can be harvested with a sterile scalpel every two to three weeks for an extended period. Teliospores can be stored at 4°C after drying in silica gel (spores stored in this way have been shown to germinate after 5 months, and to cause infections after 2–3 months) or can be re-inoculated onto healthy plants. Several rounds of re-inoculations will be required to produce the amount of teliospores needed for large scale experiments. As well as harvesting teliospores with a sterile scalpel, teliospores can also be harvested directly into suspension by placing host material with abundant telia in DWT and stirring with a magnetic stirrer. Host material with telia can be stored in the same manner as teliospores.

For inoculations, the concentration of teliospore suspensions are estimated with a haemocytometer and adjusted to approximately 10^5 spores ml^{-1}. As a guide, 5 ml of suspension is prepared per plant, although the amount needed varies depending on the size of the plants to be inoculated. An estimate of the amount of spore suspension required for each inoculation can be attained when mock-inoculating the negative-control plants with DWT (see below).

Both *U. acaciae*-infected and healthy plants of *A. mearnsii* can be kept in the same phytotrons (glasshouses) without cross-contamination. This may be a result of the proposed rain-splash dispersal mechanism of *U. acaciae* (Fraser et al. 2017). Cross contamination will likely occur, however, if infected and healthy plants are kept in nurseries or larger greenhouses that have overhead watering systems and are open to wind and air turbulence. In the phytotron, attempts are also made to avoid wetting telia during watering operations, to both stop the spread of teliospores and limit the development of any mycoparasites.

Preparation of plant material

Plants are held in a 20°C phytotron (16–24°C) under natural light before and after inoculations. Plants are routinely pruned back to 10–20 cm, to make them more manageable and allow a better fit in dew chambers after inoculation (see below). Only young plant tissues (not fully expanded leaves) are susceptible to infection by *U. acaciae* (Fraser et al. 2017), so any pruning is done at least two weeks before inoculations to allow the development of fresh tissue. Plants obtained from commercial nurseries may already be infected, as was our experience. Therefore, where possible, plants should be raised from seed or cuttings in-house under hygienic conditions.

Inoculation

If a large number of plants are to be inoculated then an airbrush (such as those produced by Iwata, Portland, Oregon) is used to apply the teliospore suspension. If only a few plants are to be inoculated then a paintbrush is used. Teliospore suspensions are applied to young expanding tissue, rather than fully expanded leaves. Plants are sprayed until just before the point of spray run off. Plants can be inoculated individually, or if in seedling trays, a whole tray can be inoculated at once. If inoculating a whole tray, inoculum is distributed evenly across all plants. This is accomplished by

turning the tray and spraying the plants from all angles (coating both abaxial and adaxial surfaces). After inoculation, plants are placed in a dew chamber (described below). To simulate South African field conditions, where rain usually falls in the late afternoon, all inoculations are performed in the late afternoon, however the impact of inoculation timing has not been tested. Before inoculation, negative-control plants are mock-inoculated with DWT and placed in a dew chamber. To check the viability of the inoculum, 100 µl aliquots of the teliospore suspension are incubated on glass slides on 1.5% water agar plates under the same conditions as the inoculated plants. Germination is assessed after 24 hours. The airbrush is cleaned before and after each inoculation by running DWT, followed by 100% ethanol, through the system.

If screening the relative resistance of families or clones of *A. mearnsii*, the different families or clones should be randomised across all trays before inoculation. Similarly, all families should be screened at the same time. However, if inoculations need to be staggered, then each family should be represented in each inoculation. Over longer time periods (e.g. when screening newly selected families), seedlings from representative families, used in earlier inoculations, should be included. Likewise, in experiments examining other factors, each treatment should be randomised across trays and inoculations. These are especially important notes, given the amount of variation in mean disease severity between repeat experiments reported by Fraser at al. (2017).

Incubation

After inoculation, and while still wet, plants are placed in a dew chamber at 15–20°C for 48 hours. Dew chambers used at the University of Pretoria consist of a large sealed transparent autoclave bag, which has 1–2 cm of water at the bottom and has been moistened internally with a spray of distilled water. After 48 hours the plants are

removed from dew chambers and placed in a phytotron or greenhouse with a temperature of 16–23°C under natural light. The critical period for infection to occur is the first 48 hours after inoculation. Very few symptoms have been observed to develop on plants that are held at 25°C during this period (Fraser et al. 2017). However, symptoms and telia developed on plants held at 25°C after infection at 20°C. The optimal temperature, relative humidity and leaf-wetness frequency/duration for disease development and sporulation have not been identified.

Disease scoring

The first symptoms of rust on inoculated plants can appear two weeks after inoculation. Spermogonia and telia develop two to five weeks after inoculation. Fraser et al. (2017) assessed incidence (proportion of plants with telia) and severity (number of telia per plant) five to six weeks after inoculation. However, there was large variation in the number (and size) of telia observed and a severity scoring system from 0-5 (Table 1; Fig. 1) was developed using material from the resistance screening experiment described below.

Resistance screening experiment

Materials and Methods

To provide proof of concept, the artificial inoculation protocol described above was used to screen the relative resistance of twelve families of *A. mearnsii*. These families, along with several others, had been putatively identified as resistant or susceptible in field trials established by the Institute for Commercial Forest Research (ICFR) at several sites in the province of KwaZulu-Natal (Moreno Chan J, ICFR, unpublished data). Rust severity was assessed in the field trials at ages between four to seven

Table 1 Rust severity scoring system for *Uromycladium acaciae* on *Acacia mearnsii* (see Fig. 1 for further details)

Rust Score	Description
0	No spermogonia or telia
1	Sporadic small telia without leaf malformation (covering <10% of the leaf)
2	Sporadic large telia and possible leaf malformation (covering <10% of the leaf)
3	Telia common and possible leaf malformation (covering 10-40% of the leaf)
4	Telia abundant and possible leaf malformation (covering 41-80% of the leaf)
5	Telia highly abundant and possible leaf malformation (covering 81-100% of the leaf)

Figure 1 Rust severity scoring system for *Uromycladium acaciae* on *Acacia mearnsii*. Rust was scored on the three leaves immediately below latest flush (see Table 1 for scoring system). a, d, e, h, i, l abaxial surfaces of leaves demonstrating each severity score (in ascending order). b, c, f, g, j, k close up of the abaxial surface of leaves serving as examples of each severity score (in ascending order).

months using a scale ranging from 0 to 4, where 0 = no rust and 4 = very severe rust (Table S1). More details of these field trials will be published in a later manuscript.

Seedlings from the selected families were reared at the ICFR Nursery in Pietermaritzburg, from September 2016 to the beginning of the experiment in April 2017. The seedlings were back-pruned six weeks before the start of the experiment.

Twenty seedlings of each family were randomised across five seedling trays (one seedling of each family per block, four blocks per tray), inoculated and incubated following the protocol outlined above. Four seedlings of each family acted as negative (non-inoculated) controls. Seedlings were inoculated with a suspension of teliospores (8.5×10^4 spores ml^{-1}; 4 ml per seedling) from a local single pustule isolate (PREM 61766; Fraser et al. 2017). Seedlings were incubated in dew chambers at 18°C for two days before being transferred to a growth room with a mean temperature of 21°C (18-22°C), mean relative humidity of 67% (38-90%) and day length of 12 hours (14 000 lux). Seedlings were then watered daily. Disease incidence and severity were assessed after five weeks on up to three branches per seedling. On each branch, rust severity was scored on up to three fully expanded leaves immediately below the flush (Fig. 1) following the scoring system shown in Table 1. Individual leaf scores were averaged to give a mean rust score for each seedling.

All statistical analyses were undertaken in R (R Core Team 2014). The effect of inoculation on rust severity (compared to negative control plants) was assessed with the Mann-Whitney U Test. The effect of family on the severity of inoculated seedlings was assessed with ANOVA, followed by a Tukey HSD test. Linear modelling was used to compare mean family rust severity scores from the artificial inoculation experiment and the field trials.

Results and Discussion

The severity of rust on inoculated *A. mearnsii* varied significantly between families (Fig. 2; ANOVA, $F = 20.48$, d.f. = 11, $P < 0.001$). Severity was lowest on the SP4-71 family (mean 0.3 ± 0.1) and greatest on the SP2-48 family (mean 2.92 ± 0.2) (Fig. 2). These results demonstrate that the artificial inoculation protocol can be used to distinguish between resistant and susceptible material of *A. mearnsii*. The results also show that more resistant genotypes of *A. mearnsii* are present in the wattle breeding programme.

There was a polynomial relationship between field and artificial inoculation mean family severities (Fig. 3). A strong positive relationship in severity was seen for families with lower susceptibility, but the relationship plateaued for the more susceptible families. The different scoring systems used in artificial inoculations and field trials (Table 1 and S1) may explain this relationship. The scoring system used in artificial inoculations may not be sensitive enough to distinguish highly susceptible families from each other, though this is of little concern, as it clearly identified the families with low susceptibility, which are of interest to the wattle-breeding programme.

Although telia of *U. acaciae* were observed on negative control seedlings (mean severity score of 0.9 ± 0.1), the severity of rust was significantly greater on inoculated seedlings (mean severity score of 2.0 ± 0.1) (Mann-Whitney U Test, $W = 1990.5$, $P < 0.001$). A severity score of 2 was not exceeded on any of the control plants. These results conclusively show that most of the rust observed resulted from the inoculations and not from contamination in the nursery.

The protocol described above is similar to that described by Morris (1991) for *Uromycladium tepperianum*. Morris (1991) introduced *U. tepperianum* as a biocontrol

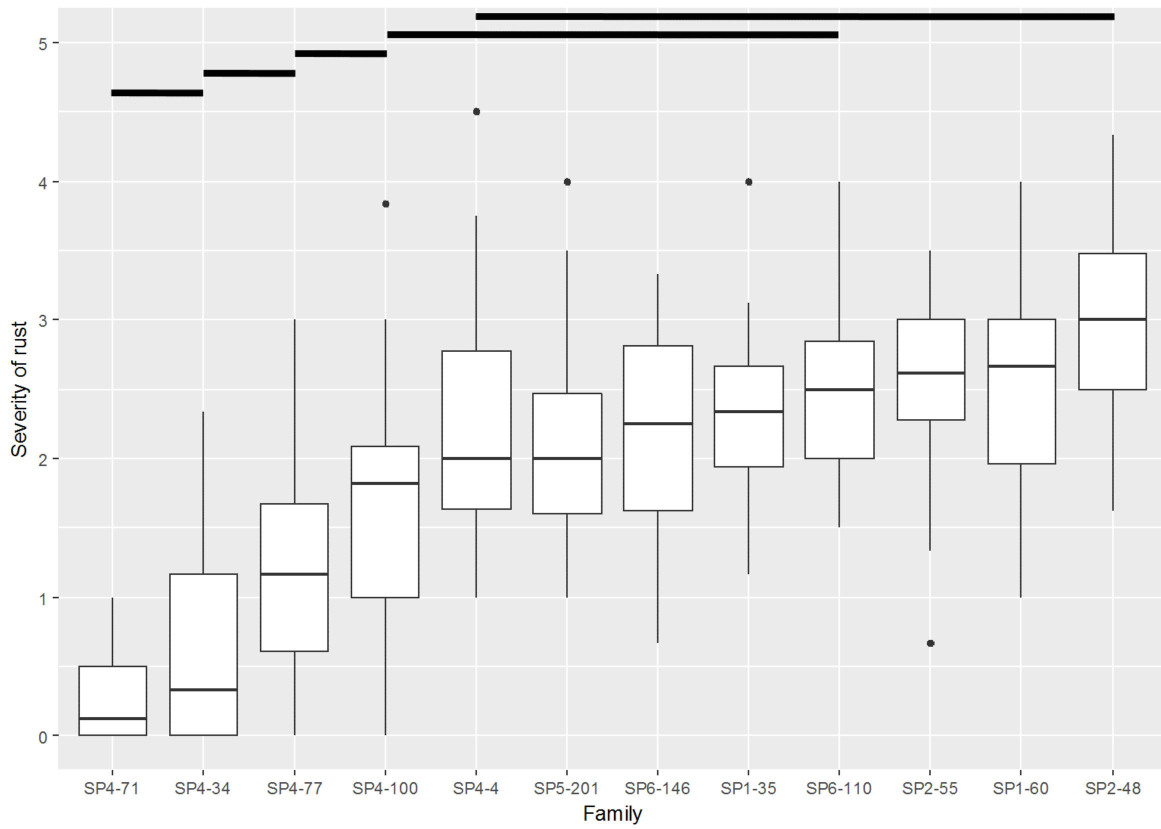

Figure 2 Variation in susceptibility to *Uromycladium acaciae* for twelve families of *Acacia mearnsii*. Families below the same dark black horizontal bar do not differ significantly (Tukey HSD, $P > 0.05$).

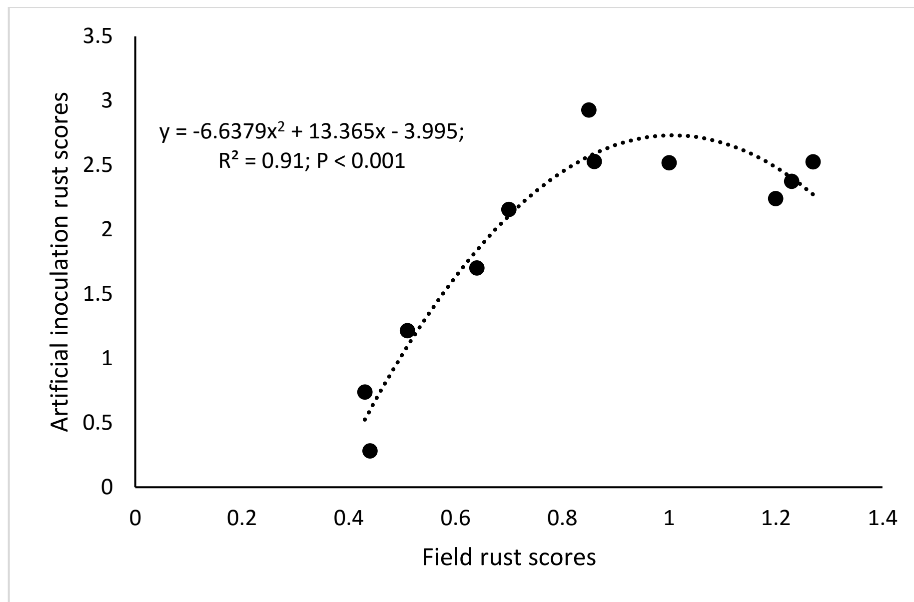

Figure 3 Severity of *Uromycladium acaciae* on families of *Acacia mearnsii* in field trials and controlled artificial inoculations. Each dot represents the mean rust severity for a family. See Table 1 for details of the scoring system used in artificial inoculations and Table S1 for that used in field trials.

agent of *Acacia saligna*, an invasive weed in South Africa. Teliospores were harvested from seedlings in the greenhouse, which had been inoculated previously with an isolate from Western Australia. Teliospores were suspended in 0.05% Tween 80 at a concentration of 10^5 spores ml^{-1}. Young tip growth of *A. saligna* was then inoculated and covered with foil-lined plastic bags for 24 hr.

Results of the resistance screening experiment demonstrated the value of this artificial inoculation protocol to wattle breeders. However, the importance of several factors remain unknown and there is room for further optimisation of the protocol. Although the optimum environmental conditions for infection have been identified (Fraser et al. 2017), the impact of environmental variables on disease development and sporulation is unknown. Optimum temperatures for development and sporulation have been demonstrated for several rust species, including *Phakopsora pachyrhizi* (Kochman 1979). Melching et al. (1989), who also studied *P. pachyrhizi*, showed that more frequent dew periods after infection lead to the development of more lesions. The minimum temperature for infection by *U. acaciae* needs to be identified, although it is unlikely to be far below 15°C, given that few basidiospores are produced at 10°C (Fraser et al. 2017). The effect of light on infection by *U. acaciae* is also unknown. Infection by *Austropuccinia psidii* (syn. *Puccinia psidii*) has been shown to only occur under low light levels or darkness (References in Glen et al. 2007). All inoculations with *U. acaciae* to date have been carried out in the late afternoon/early evening and it is unclear if this is important. Experiments should also seek to identify the optimum inoculum load. Yeh et al. (1982) showed that increased inoculum concentrations of *P. pachyrhizi* led to a greater production of telia.

As well as providing a very useful screening tool for wattle breeders to assess relative resistance, the artificial inoculation protocol described here will also enable the

investigation of several other aspects of this host-pathogen system. These would for example include, the infection and growth processes of *U. acaciae*, the impact of infection by *U. acaciae* on plant growth and physiology, the amount of variation in the virulence or aggressiveness between different populations or isolates of *U. acaciae*, the efficacy of different fungicides or biocontrol agents in controlling the rust, and the identification of resistance mechanisms and markers.

The identification of resistance mechanisms in *A. mearnsii* using this artificial protocol could speed up resistance screening further. Markers, such as quantitative trait loci, associated with resistance mechanisms could be used to rapidly screen for resistance, and replace the artificial inoculation protocol outlined here. Marker assisted selection using molecular markers, such as SNPs (single nucleotide polymorphisms) and SSRs (simple sequence repeats), may prove more efficient than phenotype trait selection (Aktar-Uz-Zaman et al. 2017). In other rust systems, molecular markers have been identified that are associated with mechanisms of both qualitative resistance (Aktar-Uz-Zaman et al. 2017) and quantitative resistance (Ganthaler et al. 2017).

Conclusion

Uromycladium acaciae is the cause of a serious rust disease on *Acacia mearnsii* in Southern Africa. The development of an artificial inoculation protocol for resistance screening, presented here, will greatly assist the wattle-breeding programme. The value of this protocol was demonstrated in a resistance screening experiment. Some unknowns, namely the impact of environmental factors on disease development and sporulation, have yet to be determined. Results from experiments on these factors can be used to further optimise the protocol. Until this is done, the current protocol can be used to produce valuable data for the wattle-breeding programme. The protocol can also be used to investigate several other aspects of this host-pathogen system.

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Supplementary Material

Table S1. Rust severity scoring system used for Uromycladium acaciae on Acacia mearnsii in field trials (J Moreno Chan, ICFR, unpublished).

Score	Rust severity	Percentage of the branch covered with telia
0	Rust not present	No rust
1	Low	1 – 25%
2	Moderate	26 – 50%
3	Severe	51 – 75%
4	Very severe	>75%