Effect of temperature, leaf wetness and the developmental stage of host tissue on infection of *Acacia mearnsii* by *Uromycladium acaciae* (Pucciniales)

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Abstract

Uromycladium acaciae causes a serious rust disease in forest plantations of non-native *Acacia mearnsii* (black wattle) in South Africa. Little is known about the biology of *U. acaciae*, making disease control difficult. Germination studies and artificial inoculations were conducted to identify the optimal environmental conditions for infection by *U. acaciae*. Germination of teliospores, basidiospores, and urediniospores was assessed at seven temperatures, with or without light. The effect of temperature on infection was also assessed. As was the effect of dew period length on germination of teliospores, production of basidiospores and infection. Teliospores and urediniospores germinated between 5 and 30°C, with an optimum at 15–25°C. Basidiospores were produced and germinated at temperatures between 5 and 25°C, with an optimum at 15–20°C. The optimum temperature for infection by basidiospores was 15–20°C. All spore types germinated in 6–24 hours under optimal conditions. However, production of basidiospores was severely reduced if teliospore germination was interrupted by dry periods, even if teliospores were re-wetted. Symptoms and telia developed on only one plant exposed to a dew period of less than 12 hours, with a dew period of 48 hours found to be optimal. Artificial inoculation experiments showed that *U. acaciae* was only able to infect young, growing tissues. Results of this research were used to develop an artificial inoculation protocol for resistance screening and can be used in disease risk modelling and forecasting.

Key Words

Fungi, epidemiology, rust, climatic niche, Botrycephaleae, dispersal

Introduction

Acacia mearnsii (black wattle) is native to south-eastern Australia (Dunlop 2002). It is an important species in southern Africa, where it has been grown since the 1800s for timber and extraction~~s~~ of tannin from its bark (Chan et al. 2015). *Acacia mearnsii* is grown on c. 110 000 ha in South Africa, predominatly in mist belt areas of the KwaZulu-Natal and Mpumalanga Provinces (Chan et al. 2015). Bark extract and wood chip exports (for the production of high quality paper) from South Africa are estimated to be worth more than US$150 million per annum (Chan et al. 2015) and the industry is estimated to employ

36,000 people (Dunlop 2002). As well as being a very useful forestry species, *A. mearnsii* also grows in unmanaged jungle stands across the country and is seen by many as a highly destructive and invasive species (Beck and Dunlop 2002).

Wattle rust, caused by *Uromycladium acaciae*, is an emergent disease on *A. mearnsii* in southern Africa (McTaggart et al. 2015a). First observed in KwaZulu-Natal in 2013, symptoms of the disease include matting of leaves, pinnule and rachis malformation and severely stunted growth of seedlings and young trees (McTaggart et al. 2015a). Yield loss has been quantified in one study, which showed that the rust caused a reduction in growth of between 20–40% over one season (Little and Payn 2016). The disease has now spread across plantations in the KwaZulu-Natal and Mpumalanga Provinces and Swaziland and has also been observed in jungle stands in the Eastern Cape and Limpopo Provinces, as well as in the east of the Western Cape (Fraser et al. unpublished data).

Uromycladium acaciae was first described as *Uredo acaciae* on an Australian species of Acacia in New Zealand (Cooke 1890), but is native to Australia (McAlpine 1905, 1906). It appears to have been present in South Africa since at least the mid-1980s in its uredinial form, which was not associated with significant damage to plantations of *A. mearnsii* (Morris et al. 1988). It was originally thought to be *U. alpinum* based on its host range and the morphology of its urediniospores (Morris et al. 1988). Telia and spermogonia of *U. acaciae*, which were associated with severe damage to plantations of *A. mearnsii*, were not observed in South Africa until 2013 (McTaggart et al. 2015a). As spermogonia, uredinia and telia of the pathogen co-occurred on the same leaves, McTaggart et al. (2015a) concluded that they were all part of the lifecycle of *U. acaciae*. These were the first reports of spermogonia and uredinia *of U. acaciae,* which was previously thought to be microcyclic (Dick 2009; Berndt 2010). Interestingly, only the uredinial stage of U. acaciae is currently found in jungle stands in the west of the Western Cape (Fraser et al. unpublished data).

Uromycladium was originally established by McAlpine in 1905 and currently includes ten species native to Australia or Melanesia (McTaggart et al. 2015a). Nine species of *Uromycladium* infect Australian species of *Acacia*, while one species, *U. falcatarium*, infects the closely related *Paraserianthes falcataria* (syn. *Falcataria moluccana*) (Doungsa-ard et al. 2015). Three species, *U. falcatarium*, *U. notabile* and *U. tepperianum*, are associated with the development of large galls. The remaining seven species, including *U. acaciae*, cause leaf rust. All species of *Uromycladium* are autoecious (Cunningham 1923; Berndt 2010), but there is variation between species in the number of different life stages observed (McAlpine 1905, 1906; Dick 2009). Some species of *Uromycladium*, such as *U. tepperianum* and *U. falcatarium*, are microcyclic and produce only telia and spermogonia.

Other species, such as *U. robinsonii* and *U. simplex*, also produce uredinia (McAlpine 1905, 1906; Dick 2009; Doungsa-ard et al. 2015).

Very little is known about the biology or climatic niche of any species of *Uromycladium*. Most research has focused on species associated with the formation of highly conspicuous galls, particularly *U. tepperianum*. This species has a one year life cycle in both Australia and South Africa; infections occur in mid-winter, but symptoms, spermogonia and galls only develop the following autumn (Burges 1934; Morris 1997). Teliospores disperse in wind (Morris 1991, 1997) and germinate on the surfaces of susceptible plants to produce a penetration peg, which directly penetrates host epidermal cells, without the production of basidiospores (Morris 1987). Only young shoot tips and immature reproductive tissues are susceptible to infection (Morris 1997). Teliospores of *U. tepperianum* remain viable for weeks during winter, but are killed by a few hot, dry days in summer (Burges 1935). In comparison, the teliospores of *U. falcatarium* germinate on the surface of susceptible plants to produce basidiospores, which then germinate to produce penetration pegs that infect the plant directly through the epidermal cells (Rahayu 2010). Nothing is known about the infection behaviour of other species of *Uromycladium*.

An important first step in the development of control strategies for any emerging plant disease is to determine the climatic and environmental requirements of the causal agent (Yamaoka 2014). Identification of the environmental factors important at different life stages of *U. acaciae*, such as infection, will support the study of the epidemiology of this pathogen. This information will facilitate several possible methods of disease control. For example, the development of risk and forecasting models, informing responsible fungicide spraying regimes, and development of protocols for resistance screening of host genotypes and families through artificial inoculation. This paper presents the results of spore germination trials and artificial inoculation experiments, designed to elucidate the climatic and environmental niche of *U. acaciae*. Factors investigated included temperature, light, wetness period, spore density and the developmental stage of host tissue. This is the first report of any work of this type on a species of *Uromycladium*.

Materials and Methods

Source of plant and fungal material

Seedlings of *Acacia mearnsii* were obtained from Sappi's Richmond Nursery in late August 2015 and were potted on into 1.6 l pots with Earth2Earth potting medium (Mpumalanga). Seedlings were also grown from commercial seedlots provided by J. Chan (ICFR, Pietermaritzburg, KwaZulu-Natal). All seedlings were maintained in phytotrons at the University of Pretoria, Hatfield campus (natural light, 15–26°C) until needed. Seedlings were watered as necessary and routinely pruned back to c. 10–20 cm.

Samples of *A. mearnsii* with abundant telia of *Uromycladium acaciae* were collected from plantations at Hilton College (KwaZulu-Natal) in August 2015 and from plantations near Dundonald-A (Mpumalanga) between April and June 2016. Teliospores were scraped from

leaves with a sterile scalpel and dried in a desiccator (Silicon oxide gel) for 48 hours before being stored at 4–6°C until needed.

To establish a single-pustule culture of *U. acaciae*, teliospores from the Hilton College collection were suspended in 5 ml distilled water with Tween 60 (1.7 x 10^5 spores ml^{-1}) and applied to three healthy 4-month-old seedlings of *A. mearnsii* (c. 1.6 ml $plant^{-1}$) with a paintbrush at the beginning of December 2015. Inoculated seedlings, and three negative controls, were individually covered by plastic bags moistened internally with distilled water. The individual plants were placed together into a dew chamber, consisting of a large sealed autoclave bag that had been moistened with a spray of distilled water and had c. 2 cm of water at the bottom. The seedlings were incubated in the dew chamber for 48 hours at approximately 20°C (16–24°C) before being removed and placed in a phytotron (25°C; natural light). Spermogonia and telia developed within five weeks. Two months after the inoculation a single telial isolate of the rust was cultured for future experiments. Teliospores were scraped from one telium using a sterile scalpel, suspended in 4 ml of distilled water with Tween (0.5 × 10^5 spores ml^{-1}) and applied to three 6-month-old seedlings of *A. mearnsii* (c. 1.3 ml $plant^{-1}$) with a paintbrush. Seedlings were incubated as above and spermogonia and telia developed within 2–4 weeks. This isolate was maintained through re-inoculation of seedlings of *A. mearnsii* at the University of Pretoria Hatfield campus. A voucher specimen of the single telium isolate on *A. mearnsii* has been submitted to the dried herbarium collection (PREM) of the South African National Collection of Fungi (PREM 61766).

To obtain urediniospores for experiments, samples of *A. mearnsii* with abundant uredinia were collected from plantations near Lottering (Eastern Cape), Piet Retief (Mpumalanga) and Stellenbosch (Western Cape) between June and August 2016. Pinnules with uredinia (without telia) were collected, dried and stored at 4–6°C until needed.

Germination of teliospores and basidiospores

The effect of temperature and light

This experiment was repeated twice (Table 1). In the first experiment, teliospores were suspended in sterile distilled water (SDW), adjusted to 1 × 10^5 spores ml^{-1}, and aliquots of 100 µl were pipetted onto sterile slides on 1.5% water agar (WA) plates. The plates were incubated with or without light (c. 15 µmol m^{-2} s^{-1}) at one of seven temperatures (5, 10, 15, 20, 25, 30 and 35°C). Plates incubated in the dark were wrapped in aluminium foil. There were three replicate plates for each light and temperature treatment combination (42 plates in total). At each temperature, the position of light and dark replicates was randomised. Repeated measurements of teliospore germination were taken at 6, 12, and 24 hours by assessing the germination status of 100 spores under a compound microscope. Basidiospore germination was assessed at 24 hours. In all experiments, teliospores and basidiospores were considered to have germinated when basidia and germ tubes were longer than the width of the respective spores.

The design of the second experiment was the same as the first, with five exceptions. Teliospores had been stored an extra c. 3 months; there were four replicates per treatment combination; the presence of basidiospores was recorded at 6, 12 and 24 hours; the percentage of teliospores producing basidiospores was assessed at 24 hours; and, the germination of teliospores incubated at non-optimal temperatures (5, 30 and 35°C) for 24 hours was assessed after a further 24 hours at 20°C to ascertain whether they were still viable.

The effect of wetness period and drying

The effect of wetness period and drying on teliospore germination was investigated in two experiments that were each repeated twice (Table 1). In the first experiments, the period of wetness required for germination was investigated. Aliquots of teliospore suspension (100 µl; 1×10^5 spores ml^{-1}) in SDW were pipetted onto slides on 1.5% WA plates. Teliospore suspensions were then incubated in the dark at 20°C for different time periods (1, 2, 3, 4, 5 or 6 hours), before being dried in an air current and returned to the incubator in dry Petri dishes. A control treatment was kept wet for the entire 24 hours. Each treatment had four randomised replicates. Teliospore germination and basidiospore production was assessed 24 hours after the start of the experiment.

In the second set of experiments, the impact of different periods of drying was investigated. Teliospores in suspension (1×10^5 spores ml^{-1}) were exposed to different wetting and drying regimes at 20°C without light. These regimes consisted of a wet period, dry period and second wet period. Teliospores were imbibed for either one (no visible germination) or two hours (germination visibly underway) before being dried in an air current and incubated dry for one of six time periods (1, 3, 6, 12, 24, 48 hours). Teliospores were then re-wetted and incubated for 24 hours, before germination of teliospores and production of basidiospores were assessed. For this experiment, teliospore suspensions were applied to sterile slides on 1.5% WA plates. During the dry period, slides were incubated in Petri dishes without WA. During the second wet period, slides were again incubated on 1.5% WA plates. Each treatment had four randomised replicates.

The effect of spore density

A suspension of teliospores of *U. acaciae* was adjusted to five different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5×10^5 spores ml^{-1}). Aliquots of 100 µl from each suspension were then pipetted onto four replicate sterile slides on 1.5% WA plates, which were incubated in the dark at 20°C for 24 hours. Teliospore germination was assessed as above.

Infection by teliospores and basidiospores on *A. mearnsii*

The effect of temperature

An airbrush (LPH-80 spray gun connected to a IS 875HT Smart Jet Tubular Compressor, Iwata, Portland, Oregon) was used to inoculate 4-month-old seedlings of *A. mearnsii* with a suspension of teliospores adjusted to 0.8×10^5 spores ml^{-1}, in SDW with Tween 20. Inoculated seedlings were placed in a dew chamber (as described above). Plants were

incubated at one of three temperatures (15, 20 and 25°C) for 48 hours, before being removed from dew chambers, randomised and placed in a phytotron with natural light at 19–23°C. Ten inoculated plants were incubated at each temperature. The presence of symptoms was assessed 5 weeks after inoculation. The presence and number of telia on each plant was recorded. This experiment was repeated twice (see Table 1 for further details).

The effect of dew period

Using a paintbrush, 35 7-month-old *A. mearnsii* seedlings were inoculated with a suspension of teliospores adjusted to 0.5×10^5 spores ml^{-1}, in SDW with Tween 20. Concurrently, seven control plants were mock-inoculated with SDW. All plants were covered individually by moistened plastic bags and incubated in dew chambers (as described earlier). Five plants were incubated in dew chambers for 0, 3, 6, 12, 24, 48 or 96 hours within a phytotron with natural light at 16–23°C. After incubation, plants were randomised and maintained in the phytotron. The presence of symptoms was assessed 5 weeks after inoculation. The presence and number of telia on each plant was recorded. This experiment was repeated twice (see Table 1 for details of differences between the first and second experiment).

Effect of the developmental stage of host tissue

Using a paintbrush, four *A. mearnsii* seedlings were inoculated with a *U. acaciae* teliospore suspension, adjusted to 1×10^5 spores ml^{-1}, in SDW with Tween 20. The spore suspension was applied to all aerial parts of the plants. Concurrently, three control plants were mock-inoculated with SDW. All plants were covered individually by moistened plastic bags and incubated in dew chambers (as described earlier), for 48 hours in a phytotron with natural light at 16–23°C. Each plant was photographed at the time of inoculation to record the developmental stage of the leaves. The presence of symptoms was assessed 5 weeks after inoculation and photographic images were referenced to determine which developmental stages were susceptible to infection. This experiment was repeated three times (see Table 1 for details of differences between experiments).

Effect of temperature and light on urediniospore germination

Aliquots of 100 µl of an urediniospore suspension in sterile distilled water (SDW) with Tween 20, adjusted to 1×10^3 to 1×10^4 spores ml^{-1}, were spread onto a layer of 1.5% WA on sterile slides on 1.5% WA plates. Plates were incubated with or without light (c. 15 µmol m^{-2} s^{-1}) at one of seven temperatures (5, 10, 15, 20, 25, 30 and 35°C) for 24 hours. Plates incubated under the dark treatments were wrapped in aluminium foil. There were three replicate plates for each treatment combination (42 plates in total). Germination was assessed on 20–50 spores per replicate. Urediniospores were considered to have germinated when germ tubes were longer than the width of the spores. This experiment was repeated three times, once with spores from Mpumalanga, the Eastern Cape and the Western Cape, respectively (Table 1). In one experiment, the germination of

urediniospores incubated at non-optimal temperatures (5, 30 and 35°C) was assessed after a further 24 hours at 20°C, to determine whether they were still viable.

Statistical analyses

All statistical analyses were carried out in R software (R Core Team, 2014).

Spore germination and production proportions were arcsin or logistically transformed towards normality before analysis. Polynomial regression models (in the form, $y = a + bx + cx^2 + dx^3$) were created using the linear modelling (lm) function to assess the impact of temperature and light on spore germination and production. Linear modelling was also used to assess the effect of drying and spore density on spore germination and production. Experiment (repeat) was also included as an explanatory variable in analyses. Each analysis started with a maximal model (including all explanatory variables and their interactions) before identification of the minimal adequate model through model simplification and model comparison with ANOVA.

The generalised linear modelling (glm) function was used to analyse the effect of temperature on infection. A binomial error structure was fitted to analyse incidence data (presence or absence of telia). A quasipoisson error structure was fitted to analyse severity data (count of telia per plant). Temperature and experiment (repeat) were included as explanatory variables in analyses. The minimal adequate model was identified as above.

The generalised additive modelling (gam) function in the 'mgcv' package was used to analyse the effect of dew period on infection. A binomial error structure was fitted to analyse incidence data (presence or absence of telia). A quasipoisson error structure was fitted to analyse severity data (count of telia per plant). In both analyses, dew period was included as a non-parametric smoother. Experiment (repeat), dew period and their interaction were included as parametric variables in the maximal models, before identification of the minimal adequate model through model simplification and comparison.

Results

Germination of teliospores and basidiospores

The effect of temperature and light

Teliospores germinated within a temperature range of 5–30°C. However, germination halted rapidly at 30°C, leaving short basidia. No germination was observed at 35°C. The optimum temperature for teliospore germination was 15–25°C (Fig. 1A). There was a significant interaction between light, experiment and temperature (ANOVA, d.f. = 68, F = 6.45, P = 0.003), indicating that the effect of light was not consistent. In the first experiment, there was a significant interaction between light and temperature (ANOVA, d.f. = 29, F = 15.33, P < 0.001), with greater germination at 10°C under the light treatment than the dark treatment. However in the second experiment, there was no significant

interaction between light and temperature (ANOVA, $P > 0.05$), but germination was significantly greater under the light treatment (ANOVA, d.f. = 42, $F = 7.51$, $P = 0.009$).

Basidiospores, four per basidia (Fig. 2A), were produced at temperatures between 5 and 25°C under both the light and the dark treatments. Between 15 and 25°C, basidiospores were observed after 6 hours. At lower temperatures, basidiospores were not observed until 12 hours. The greatest production of basidiospores was observed at 15 and 20°C (Fig. 1B). There was a significant interaction between light and temperature (ANOVA, d.f. = 32, $F = 4.52$, $P = 0.042$), with greater production of basidiospores at 25°C under the dark treatment.

Because very few basidiospores were observed at 5°C, and none were observed at temperatures above 25°C, germination of basidiospores was only assessed at temperatures between 10 and 25°C. This is despite the observation of some germination at 5°C. The optimum temperature for germination of basidiospores was 15–20°C (Fig. 1C). Germination of basidiospores was significantly reduced under the light treatment (ANOVA, d.f. = 50, $F = 15.27$, $P < 0.001$) and in the second experiment (ANOVA, d.f. = 50, $F = 12.36$, $P < 0.001$).

Teliospores from the 5 and 30°C temperature treatments were able to germinate when incubated at 20°C (Fig. 3). This was not the case for teliospores originally incubated at 35°C, which were unable to germinate when returned to 20°C. Germination of teliospores, production of basidiospores and germination of basidiospores after the 5°C treatment were no different to the control. All three measures were, however, significantly reduced after the 30°C treatment (Tukey HSD, $P > 0.05$).

The effect of wetness period and drying

Germination of teliospores reached a maximum with a 2–5 hour period of wetness, however production of basidiospores required more than 6 hours to reach its maximum (Fig. 4). Similar results were observed in both repeats of this experiment.

Periods of drying significantly reduced teliospore germination and basidiospore production. Drying of teliospores had a greater impact on germination in the first repeat of this experiment than the second (ANOVA, d.f. = 106, $F = 17.33$, $P < 0.001$; Fig. 5A). In the first repeat, germination decreased rapidly with any dry period, whereas drying had less of an effect in the second experiment. Production of basidiospores was also significantly greater in the second repeat of this experiment (ANOVA, d.f. = 108, $F = 15.72$, $P < 0.001$; Fig. 5A). However, any period of drying rapidly reduced the production of basidiospores in both experiments (Fig. 5B). In both experiments, greater teliospore germination was observed with a two hour wet period than with a one hour wet period (ANOVA, d.f. = 106, $F = 6.32$, $P = 0.013$), however original wetness period had no impact on basidiospore production (ANOVA, $P > 0.05$).

The effect of spore density

The concentration of the teliospore suspensions had no significant effect on germination (linear regression, $P > 0.05$).

Teliospore and basidiospore infection experiments

Symptoms developed on inoculated *A. mearnsii* seedlings within five weeks in all infection experiments. Symptoms included the slight swelling of stems and rachises, leaf and rachis malformation (or twisting) and the development of spermogonia and telia (Fig. 6A). Telia developed on stems, rachises, rachilla (leaf midrib) and pinnules. Uredinia were not observed to have developed on any of the inoculated plants. Symptoms did not appear on any non-inoculated control seedlings.

The effect of temperature

Temperature had a highly significant impact on the infection of seedlings of *A. mearnsii* by basidiospores of *U. acaciae*. The incidence (Fig. 7A) and severity (Fig. 7B) of rust both decreased significantly with rising temperature ($P < 0.001$). Across both experiments, the greatest incidence and severity was seen after incubation at 15°C (80% plants with telia; mean 44.4 ± 15.0 telia per plant). In contrast, incidence and severity after incubation at 25°C was extremely low (10% plants with telia; mean 0.1 ± 0.1 telia per plant). Intermediate incidence and severity was observed after incubation at 20°C (70% plants with telia; mean 18.9 ± 8.9 telia per plant). Although the same pattern was seen across both experiments, experiment itself had a highly significant impact on rust severity (ANOVA, d.f. = 57, $F = 35.42$, $P < 0.001$). Significantly more telia were observed on plants in the second experiment (mean 39.7 ± 11.4 telia per plant; compared to only 2.6 ± 0.8 in the first experiment).

The effect of dew period

Dew period had a highly significant impact on the infection of *A. mearnsii* seedlings by basidiospores of *U. acaciae*. Incidence (Fig. 6B; $X^2 = 16.84$, $P = 0.001$) and severity (Fig. 6C; $F = 9.43$, $P < 0.001$) of rust both increased significantly with length of dew period up to 48 hours (Fig. 6A), before decreasing slightly. No telia were observed on plants incubated in a dew chamber for 0 or 3 hours, while telia were only observed on one plant incubated in a dew chamber for 6 hours. Incidence of rust was greatest after dew periods of 24–48 hours (100%, 90% plants with telia, respectively), while severity was greatest with a dew period of 48 hours (mean 69.0 ± 33.4 telia per plant). As was observed for the temperature experiments, the same pattern was seen across both dew period experiments, but experiment itself had a significant impact on rust severity (ANOVA, $F = 26.46$, $P < 0.001$). Significantly more telia were observed on plants in the second experiment (mean 32.9 ± 11.4 telia per plant; compared to only 4.9 ± 2.4 in the first experiment).

Effect of the developmental stage of host tissue

Uromycladium acaciae was only able to infect younger tissue of *A. mearnsii*. Fully expanded leaves were not susceptible to infection.

Effect of temperature and light on germination of urediniospores

Urediniospores germinated at 5–30°C (Fig. 8), there was no germination at 35°C. Generally the optimum temperature for germination of urediniospores was 15–25°C. However, there was a significant interaction between temperature and light (ANOVA, d.f. = 97, F 11.91, P < 0.001), with greater germination at 10°C under the light treatment than the dark treatment. There was also a significant interaction between temperature and experiment (ANOVA, d.f. = 98, F 10.89, P < 0.001), with slightly warmer optimal temperatures observed in experiment 3 and experiment 2.

Urediniospores incubated at 5°C germinated normally after being moved to 20°C (no significant difference from control; ANOVA, P > 0.05; Fig. 3). However, spores incubated at 30°C or above did not germinate after later incubation at 20°C.

Discussion

The optimal conditions for the infection of *Acacia mearnsii* by *Uromycladium acaciae* were successfully identified. Infection occurred with leaf wetness periods of at least 12 hours at 15–20°C. The rust was found to only infect developing leaves and stems; symptoms did not develop on inoculated fully expanded leaves. This research represents an important first step towards understanding the infection biology of *U. acaciae* and the development of effective control strategies.

Although these were the first experiments designed to identify optimal conditions for infection by a species of *Uromycladium*, there is some information available on the infection biology of *U. tepperianum* and *U. falcatarium*. The observation of infection of *A. mearnsii* by basidiospores demonstrated that *U. acaciae* is an autoecious rust, as has been proposed for all species of *Uromycladium* (Cunningham 1923; Berndt 2010). The production of basidiospores by germinating teliospores has been reported previously for *U. falcatarium* on *P. falcataria* in Malaysia (Rahayu et al. 2010). Under conditions of high humidity, teliospores of this species germinate to produce basidiospores after 10 hours. After a further 6 hours, basidiospores produce a penetration peg, which penetrates host cells directly through the epidermis (Rahayu et al. 2010). In contrast, teliospores of *U. tepperianum* do not produce basidiospores, but rather infect epidermal cells of *A. saligna* directly with penetration pegs after 12–24 hours at 20°C. As is the case with *U. acaciae* on *A. mearnsii*, only young expanding leaflets, phyllodes, stems and reproductive tissues of *A. saligna* are susceptible to infection by *U. tepperianum* (Morris 1991). Further work is needed to ascertain whether basidiospores of *U. acaciae* infect *A. mearnsii* directly through epidermal cells or through stomata and lenticels. If *U. acaciae* does infect through epidermal cells, this may explain why only young tissue is susceptible.

As detailed above, the time required for germination of teliospores and infection by *U. acaciae*, *U. falcatarium* and *U. tepperianum* is very similar. However, the period of disease development (time between inoculation and sporulation) varies markedly between the species. The 2–5 weeks reported here for *U. acaciae* is longer than the one week reported for *U. falcatarium* on *P. falcataria* in Malaysia (Rahayu et al. 2010), but much shorter than the 3–6 months reported for *U. tepperianum* in Western Australia (Morris 1987). The short development time for *U. acaciae* and *U. falcatarium*, may explain why these pathogens are so damaging. Both species will potentially always have abundant inoculum available when conditions are conducive for infection (more than 12 hours wetness at 15–20°C, in the case of *U. acaciae*). However, *U. tepperianum* is also hugely damaging to invasive *A. saligna* in the Western Cape province of South Africa, despite its long development period and seasonal production of teliospores (Wood and Morris 2007). *Uromycladium tepperianum* has a one-year life cycle in the field, spreading and infecting in mid-winter (the wet season in Mediterranean ecosystems), developing through summer and then sporulating the next winter (Morris 1991). The sporulation pattern of *U. acaciae* through the year is currently being investigated at field sites in the Mpumalanga province.

Apart from the lack of uredinia, the symptoms that arose in the artificial inoculation experiments described here were very similar to those observed in the field and described by McTaggart et al. (2015a). Common symptoms included the slight swelling of stems and rachises, leaf and rachis malformation and the development of spermogonia and telia (Fig. 7C). In the field, telia often become more conspicuous under wet conditions, when teliospores exude from telia in sticky brown masses, which are often described as “slime” by farmers and foresters. Leaves and pinnunles bearing telia often become matted under these conditions. Similarly, if telia on inoculated seedlings became wet during watering, they would become “slimy” and pinnule matting would occur. It is thought that this “slime” is caused by the swelling and bursting of hygroscopic cysts at the base of teliospores (Fig. 2A), which may release an adhesive substance into solution, helping teliospores adhere to the surface of the host. These cysts collapse after wetting, which probably explains why they were not described intact by McTaggart et al (2015a).

The presence of the “slimy” teliospore masses in the field and phytotron suggests that teliospores of *U. acaciae* may disperse through rain/water splash, rather than by wind as suggested by Dick (2009). This is in contrast to teliospores of *U. tepperianum* and *U. falcatarium*, which do not have a cyst and are thought to be wind dispersed (Morris 1997; Rahayu 2010). Water dispersal of teliospores has only been reported for one other rust fungi, *Chrysomyxa weirii* (spruce needle rust) (Crane et al. 2000). Future experiments that monitor sporulation will allow the investigation of the role of rainfall and other environmental variables in the dispersal of spores of *U. acaciae*. It would also be interesting to know whether the cyst-bearing teliospores of other rust species, such as *U. maritimum*, *U. robinsonii* and *U. simplex*, also disperse in water.

The main areas where *A. mearnsii* is grown commercially in South Africa are in the KwaZulu-Natal and Mpumalanga Provinces in the east of the country, where the climate is

characterised by wet summers and dry winters. Given the optimum conditions for infection reported here, 12 hours or more of leaf wetness at 15–20°C, it would appear that the period from October to March (Southern Hemisphere summer) with frequent rainfall (often in the evening) and average minimum (night) temperatures of approximately 15°C is ideal for infection by *U. acaciae*. However, as *A. mearnsii* is preferentially grown in mist belt areas, favourable conditions for infection may also occur during drier periods of the year. Further, as the optimal conditions for disease development and sporulation were not assessed here, more work is needed to fully identify the conditions favouring the development of epidemics of *U. acaciae*. What has been observed in the maintenance of the Hilton isolate of *U. acaciae* is that, although little infection will occur at 25°C, once infection has taken place (at 15–20°C) the rust can develop and sporulate in/on plants held at 25°C. This, combined with the fact that infection has a narrower optimum temperature than germination of teliospores, suggests that infection may be the most critical life stage in determining the climatic niche of this species. It is hoped that the data presented here will contribute to the development of disease risk maps and potentially in the development of disease forecasting that can be used to optimise the use of fungicides.

The results reported here have been used to design an artificial inoculation protocol for screening families and clones of *A. mearnsii* for resistance to *U. acaciae*. This protocol is currently being trialled with families of *A. mearnsii* putatively identified as susceptible or resistant to the rust in naturally infected field trials. It is important to optimise the artificial inoculation protocol further, as one clear observation from the infection experiments was that rust severity varied significantly between repeat experiments. There are several possible explanations for this variation (Table 1), including the use of different plant sources, age of plants, inoculum source, inoculation method, inoculum load and environmental conditions in the phytotron post inoculation. Future experiments should be designed to identify which of these variables are significant, particularly the impact of environmental conditions on disease development and sporulation. Importantly, although severity varied between experiments, the relationships between temperature and severity, and dew period and severity did not differ between repeat experiments, providing strong support that the optimal conditions for infection reported here are real. Importantlly, this artificial inoculation protocol can also be used to investigate several other areas of this host-pathogen system, such as the method of infection, the impact of the rust on plant growth, variation in the virulence/aggression of different isolates of *U. acaciae* and the efficacy of different fungicides or biocontrol agents. It can also be used to identify the minimum temperature for infection.

One of the main questions that arose from this work is why uredinia did not develop after infection by basidiospores. There are two possible explanations for this phenomenon. The first possibility is that there are two species of *Uromycladium* present on *A. mearnsii* in South Africa. The first species, a rust known only from uredinia, originally observed in 1984 in the Western Cape (Morris et al. 1988), which despite being observed occurring across South Africa has caused no obvious damage to trees. The second species, a more recently introduced and highly damaging rust that produces telia, and was

first observed in 2013 in KwaZulu-Natal (McTaggart et al. 2015a). The second possibility is that there is only one species of *Uromycladium* present on *A. mearnsii* in South Africa, which produces all spore stages (McTaggart et al. 2015a), but conditions in the phytotrons used in the described experiments are not conducive to the development of uredinia, only the development of spermogonia and telia. If the latter case is true, then there would need to have been a change in environmental (climatic) conditions or in the genetics of the rust (mutation or introduction of a new genotype), either of which triggered a switch in life cycle expression.

The presence of only uredinia on infected *A. mearnsii* in the west of the Western Cape, but the presence of uredinia, spermogonia and telia in other areas of South Africa, as outlined in the introduction, may offer support to the suggestion that there are two species of *Uromycladium* present on *A. mearnsii* in South Africa. Interestingly, there are some genetic differences between collections from the west of the Western Cape and the rest of South Africa (McTaggart et al. 2015a). There is a single nucleotide polymorphism (SNP) and two indels in the internal transcribed spacer (ITS) region, and one SNP in the large subunit (LSU) region of ribosomal DNA. However, McTaggart et al. (2015a) recovered these isolates as a phylogenetic species and attributed the nucleotide differences to intra-specific variation, which also occurs in other genera of rust fungi, such as *Endoraecium* (McTaggart et al. 2015b) and *Puccinia* (Demers et al. 2017). The comparison of the ITS and LSU regions, as well as other molecular markers, of more collections of uredinia from the west of the Western Cape, as well as uredinia and telia from other regions of South Africa will help to answer this important question.

The optimum conditions for infection of *A. mearnsii* by *U. acaciae* were identified as a leaf wetness period of 48 hours at 15–20°C. The rust was only able to infect developing tissues. Given that other life stages, such as germination, development and sporulation, appear to occur over a wider range of conditions, it is proposed that the infection process is the critical stage for determining the climatic niche of *U. acaciae*. Further work is needed to show this conclusively. The data presented here have been used to develop an artificial inoculation protocol for host resistance screening and will assist the development of disease risk modelling. As outlined in the discussion, further research is needed in several areas of this host-pathogen system, not least in elucidating the life-cycle of this important rust pathogen.

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Figure legends

Figure 1. Effect of temperature and light on the germination of teliospores of *Uromycladium acaciae* after 24 hours. **A**, teliospore germination; **B**, basidiospore production; **C**, basidiospore germination. Expt, experiment (repeat); D, dark; L, light.

Figure 2. Germination of spores of *Uromycladium acaciae*. **A**, production of a basidium by a teliospore, note the germ pores and hyaline hygroscopic cysts; **B**, production of basidiospores, four per basidia; **C**, germination of a basidiospore. Scale bars = 10 µm.

Figure 3. Viability of spores of *Uromycladium acaciae* after 24 hours in suspension at non-optimal temperatures (5, 30 and 35°C). Viability was assessed after incubation at 20°C for 24 hours. There was no teliospore germination after the 35°C treatment, and no urediniospore germination after the 30°C treatment. Error bars indicate one SE. Within the different response variables, treatments with the same letters do not differ significantly (ANOVA, Tukey HSD, $P > 0.05$).

Figure 4. The effect of length of period of wetness on the germination of teliospores and the production of basidiospores of *Uromycladium acaciae*.

Figure 5. The effect of drying periods on germination of spores of *Uromycladium acaciae*. Teliospore suspensions were wet for one or two hours (1 hr wet, 2 hr wet), before exposure to a period of drying and re-wetting for 24 hours. **A** teliospore germination; **B** basidiospore production. Expt, experiment (repeat).

Figure 6. The effect of dew period on the infection of seedlings of *Acacia mearnsii* by *Uromycladium acaciae*; **A**, brown telia and leaf malformation (twisting) symptoms five weeks after artificial inoculation and incubation in a dew chamber for 48 hours; **B**, incidence of rust (presence or absence of telia); **C**, severity of rust (experiment 1, circles, solid line; experiment 2, triangles, dashed line). Random noise has been added to the dew period data in **B** and **C** with the jitter function to improve data visualisation.

Figure 7. The effect of temperature on the infection of seedlings of *Acacia mearnsii* by *Uromycladium acaciae*; **A**, incidence of rust (presence or absence of telia); **B**, severity of rust (experiment 1, circles, solid line; experiment 2, triangles, dashed line). Random noise has been added to the temperature data with the jitter function to improve data visualisation.

Figure 8. The effect of light and temperature of the germination of urediniospores of *Uromycladium acaciae*.

Table 1. Details of *Uromycladium acaciae* germination and infection experiments

Experiment	Source of material[1]		Start date	Inoculation details[2]
	Acacia mearnsii seedlings	*Uromycaldium acaciae*		
Teliospore and basidiospore germination experiments				
Effect of temperature and light	-	Hilton, KZN; Aug 2015	15/09/15	10^5 spores ml^{-1}
	-	Hilton, KZN; Aug 2015	08/12/15	10^5 spores ml^{-1}
Effect of wetness period	-	Hilton, KZN; Aug 2015	09/02/16	10^5 spores ml^{-1}
	-	Dundonald-A, MP; May 2016	18/07/16	10^5 spores ml^{-1}
Effect of drying	-	Hilton, KZN; Aug 2015	18/12/15	10^5 spores ml^{-1}
	-	Dundonald-A, MP; May 2016	15/07/16	10^5 spores ml^{-1}
Effect of spore density	-	Hilton, KZN; Aug 2015	07/12/15	0.5-2.5 × 10^5 spores ml^{-1}
Teliospore and basidiospore infection experiments				
Effect of temperature	4-month-old (PSO-16 2007)	Hilton isolate; Nov 2016	28/11/16	0.8 × 10^5 spores ml^{-1}; 2 ml per plant; airbrush
	4-month-old (PSO-14 2014)	Hilton isolate; Nov 2016	04/12/16	0.5 × 10^5 spores ml^{-1}; 2 ml per plant; airbrush
Effect of dew period	7-month-old (Richmond)	Hilton, KZN; Aug 2015	11/03/16	0.5 × 10^5 spores ml^{-1}; 0.7 ml per plant; paintbrush
	4-month-old (PSO-16 2007; PSO-14 2014)	Hilton isolate; Nov 2016	05/12/16	0.6 × 10^5 spores ml^{-1}; 2 ml per plant; airbrush
Effect of development stage of host tissue	8-month-old (Richmond)	Dundonald-A, MP; Apr 2016	21/04/16	10^5 spores ml^{-1}; 3 ml per plant; paintbrush
	12-month-old (Richmond)	Dundonald-A, MP; May 2016	11/08/16	1.5 × 10^5 spores ml^{-1}; 25 ml per plant; airbrush

	12-month-old (Richmond)	Hilton isolate; Aug 2016	15/08/16	10^5 spores ml^{-1}; 1 ml per plant; paintbrush
Urediniospore germination experiments				
Effect of temperature and light	-	Lottering, EC; June 2016	22/07/16	10^3 spores ml^{-1}
	-	Piet Retief, MP; July 2016	05/08/16	10^4 spores ml^{-1}
	-	Stellenbosch, WC; Aug 2016[3]	23/08/16	5×10^3 spores ml^{-1}

[1] EC, Eastern Cape Province; KZN, KwaZulu-Natal Province; MP, Mpumalanga Province; WC, Western Cape Province. Date, date of collection.

[2] Spore suspension concentration, amount and application method (airbrush, LPH-80 spray gun connected to a IS 875HT Smart Jet Tubular Compressor, Iwata, Portland, Oregon).

[3] Also inoculated three 12-month-old *A. mearnsii* seedlings with this spore suspension with no symptom development. Another urediniospore inoculation on the 18 Oct 2016 was also unsuccessful.

Figure 1A

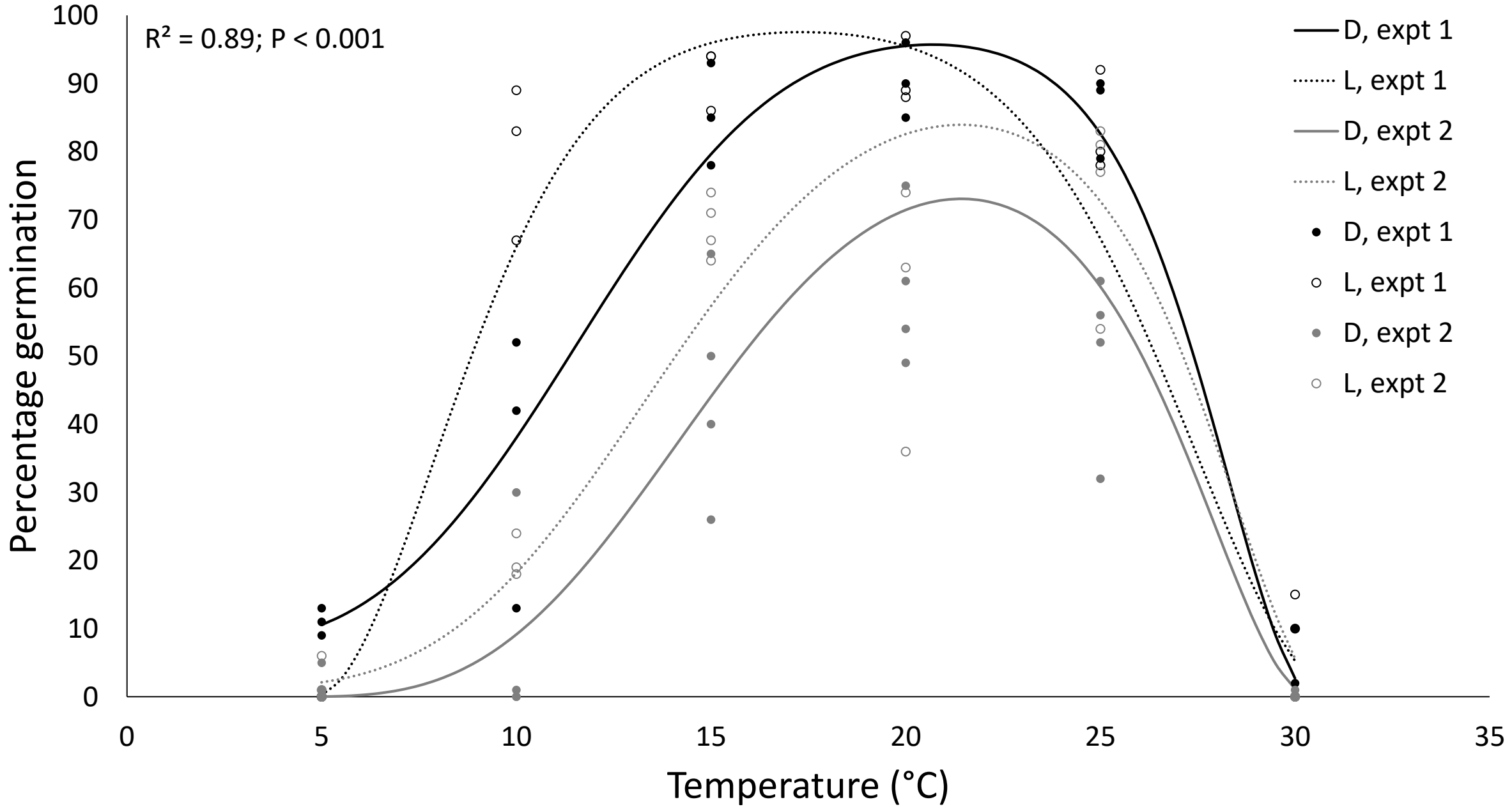

R² = 0.89; P < 0.001
Percentage germination
Temperature (°C)
0
10
20
30
40
50
60
70
80
90
100
0
5
10
15
20
25
30
35
D, expt 1
L, expt 1
D, expt 2
L, expt 2
D, expt 1
L, expt 1
D, expt 2
L, expt 2

Figure 1B

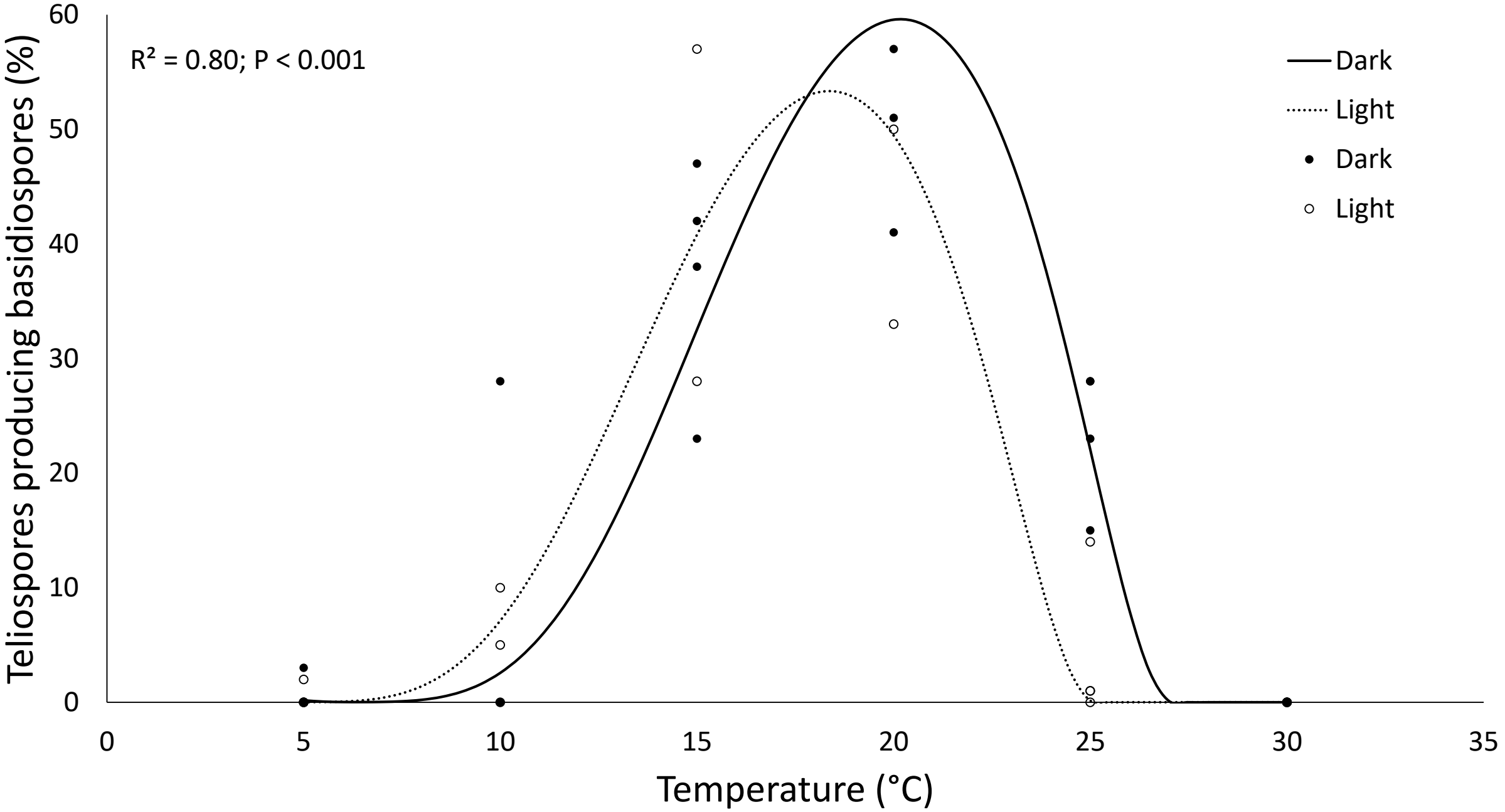
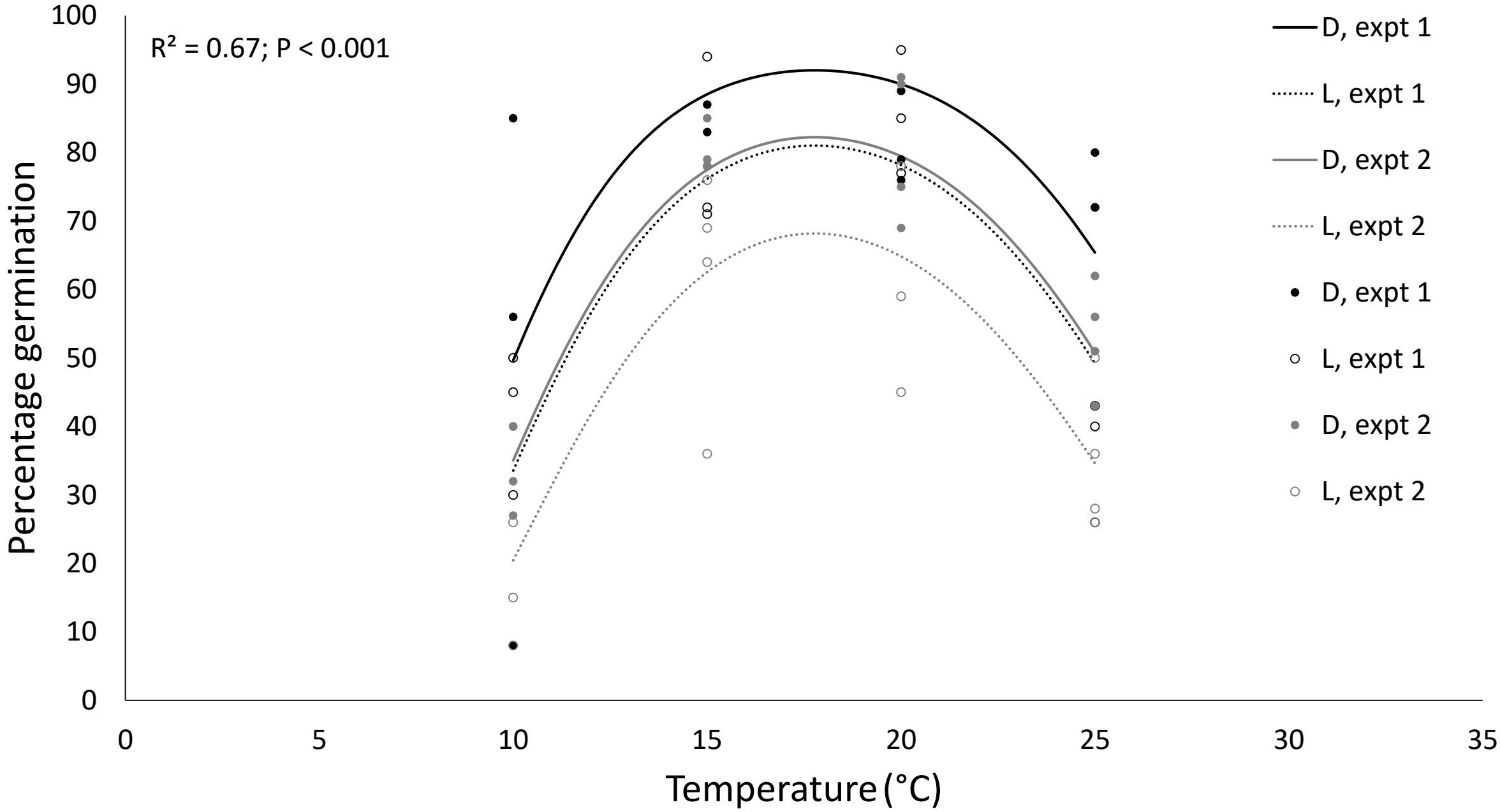
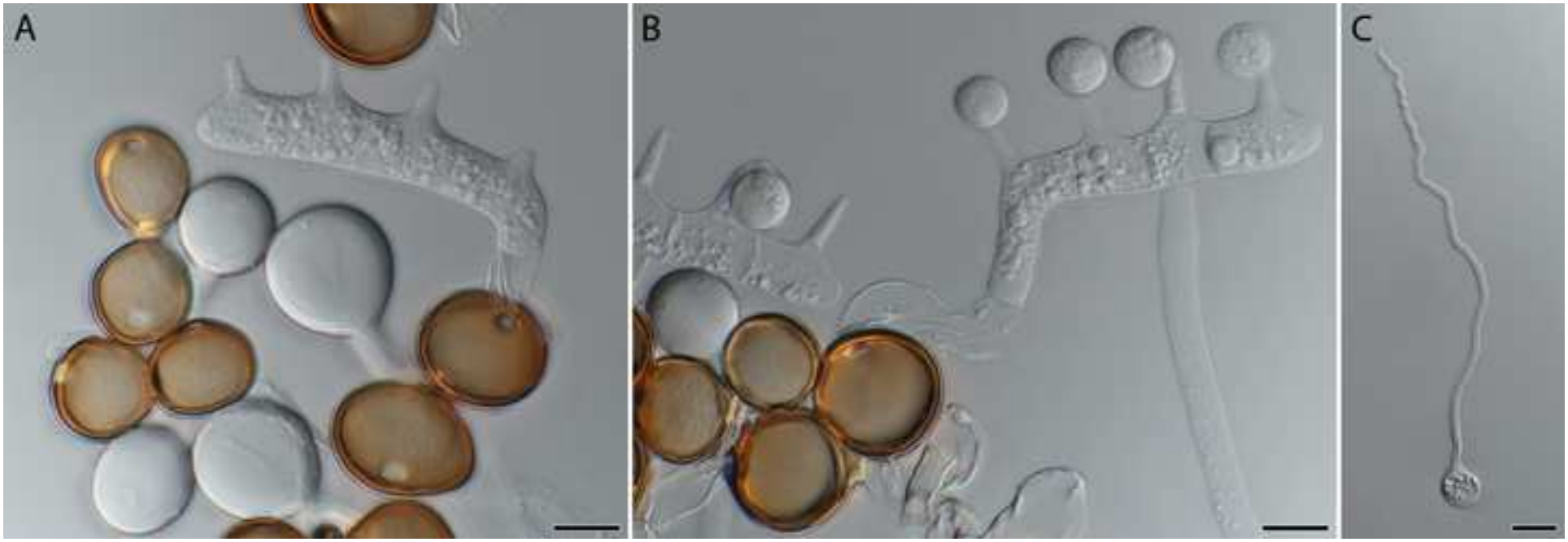
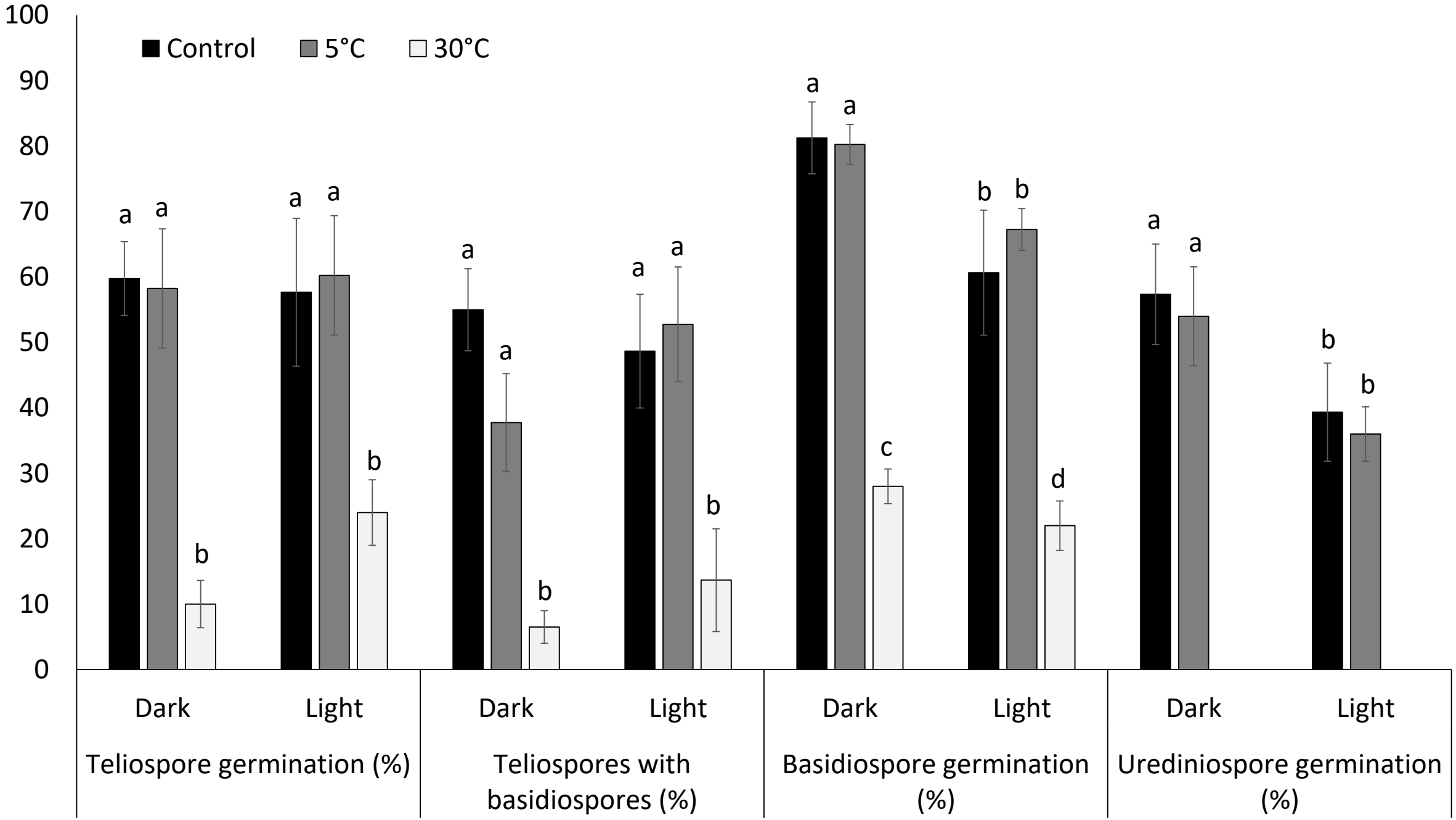

R² = 0.80; P < 0.001
Dark
Light
Dark
Light
Teliospores producing basidiospores (%)
Temperature (°C)
0
10
20
30
40
50
60
0
5
10
15
20
25
30
35

Figure 1C


R² = 0.67; P < 0.001
Percentage germination
Temperature (°C)
0
10
20
30
40
50
60
70
80
90
100
0
5
10
15
20
25
30
35
D, expt 1
L, expt 1
D, expt 2
L, expt 2
D, expt 1
L, expt 1
D, expt 2
L, expt 2


A
B
C


100
90
80
70
60
50
40
30
20
10
0
Control
5°C
30°C
a a b
a a b
a a b
a a b
a a c
b b d
a a b
Dark
Light
Teliospore germination (%)
Teliospores with basidiospores (%)
Basidiospore germination (%)
Urediniospore germination (%)

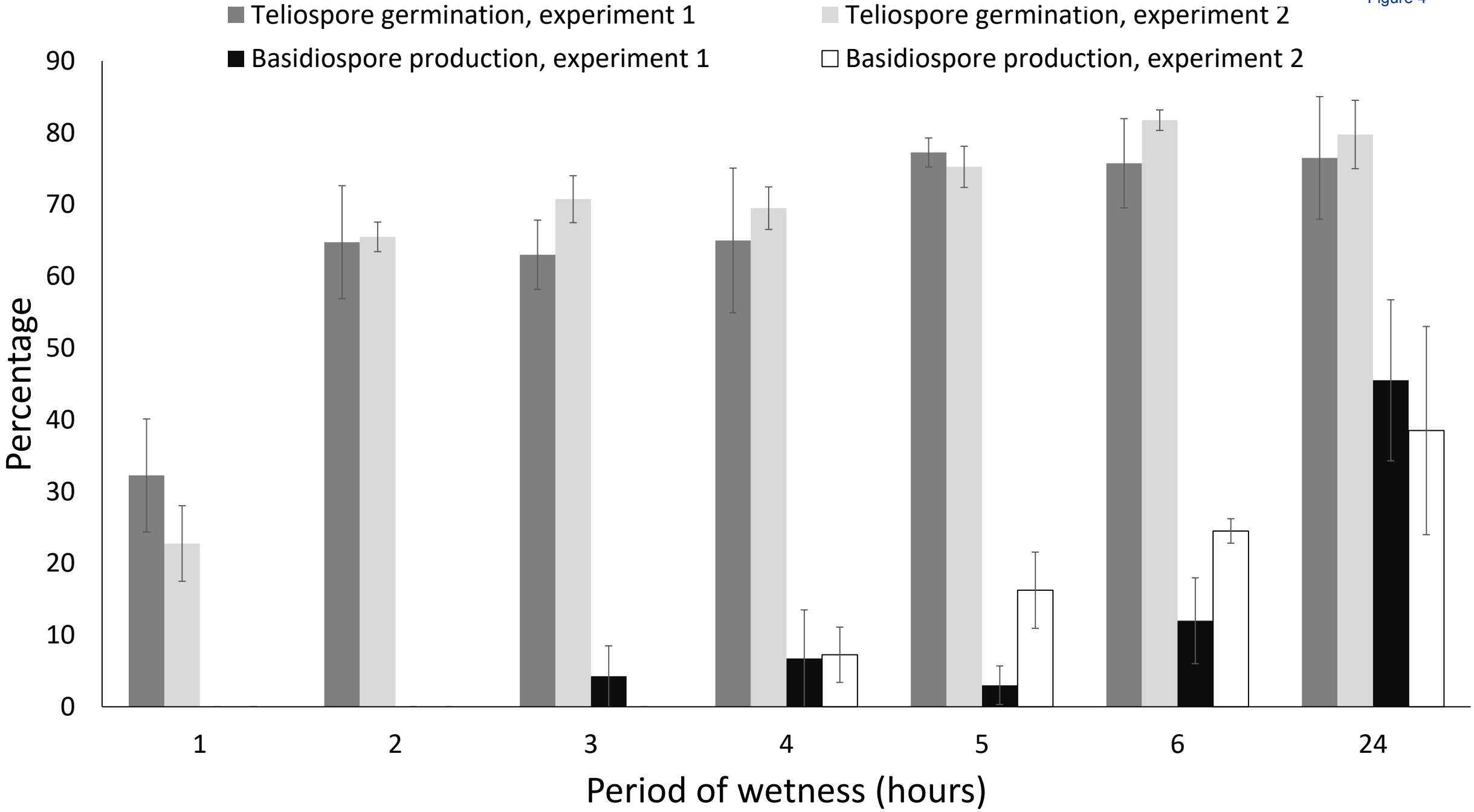

Teliospore germination, experiment 1
Teliospore germination, experiment 2
Basidiospore production, experiment 1
Basidiospore production, experiment 2
Percentage
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
24
Period of wetness (hours)

Figure 5A

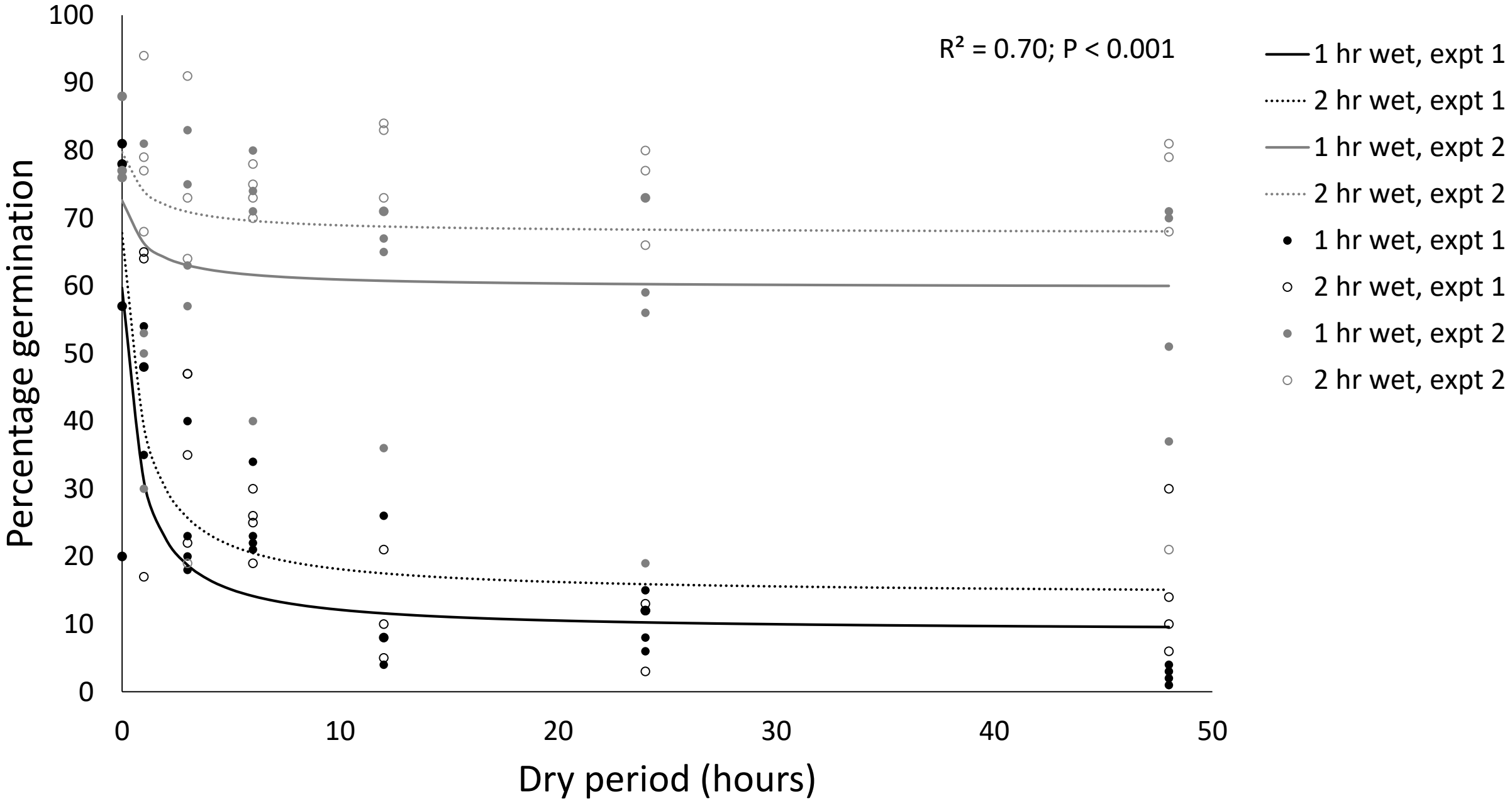

R² = 0.70; P < 0.001
Percentage germination
Dry period (hours)
0
10
20
30
40
50
60
70
80
90
100
1 hr wet, expt 1
2 hr wet, expt 1
1 hr wet, expt 2
2 hr wet, expt 2
1 hr wet, expt 1
2 hr wet, expt 1
1 hr wet, expt 2
2 hr wet, expt 2

Figure 5B

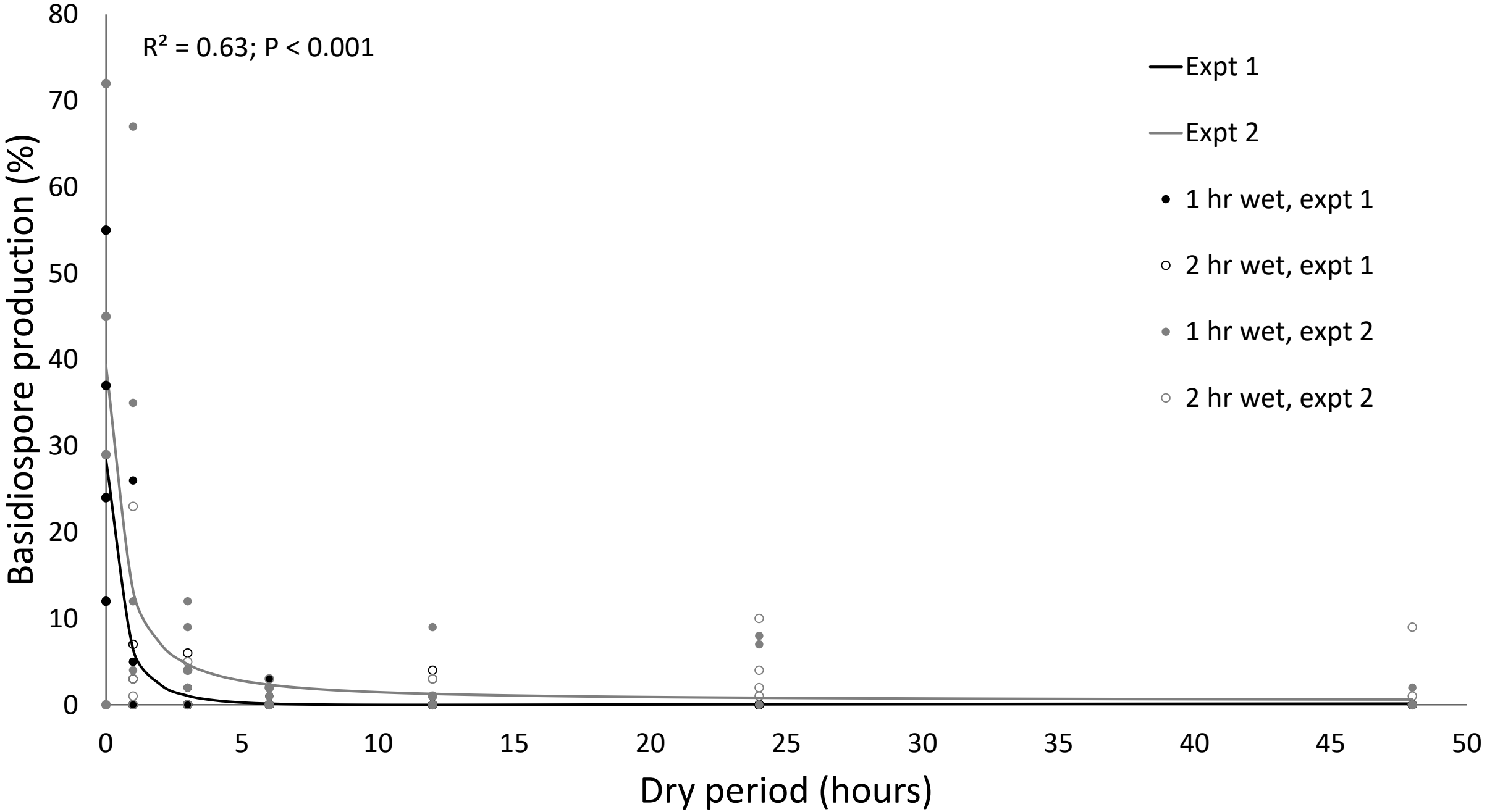
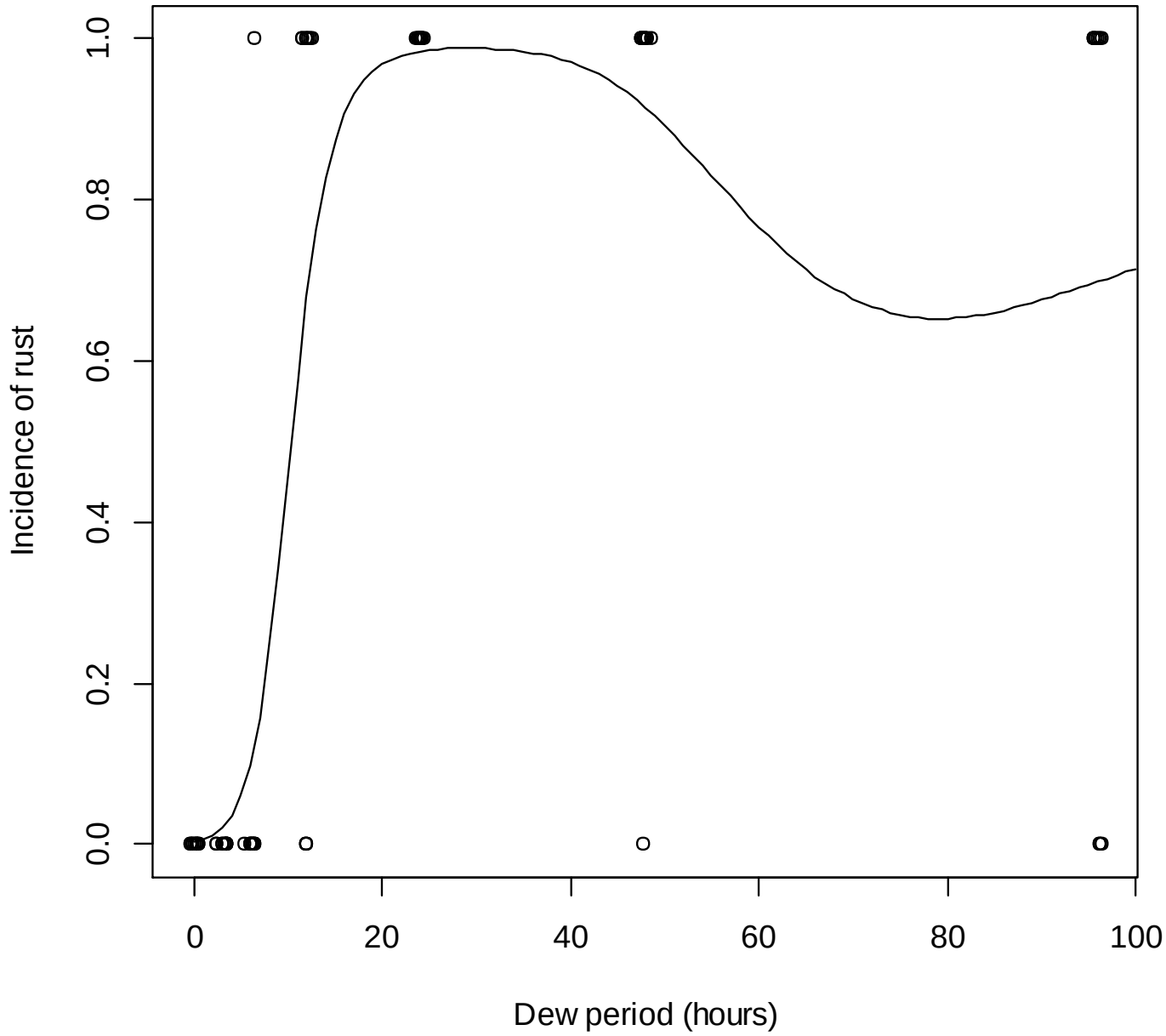
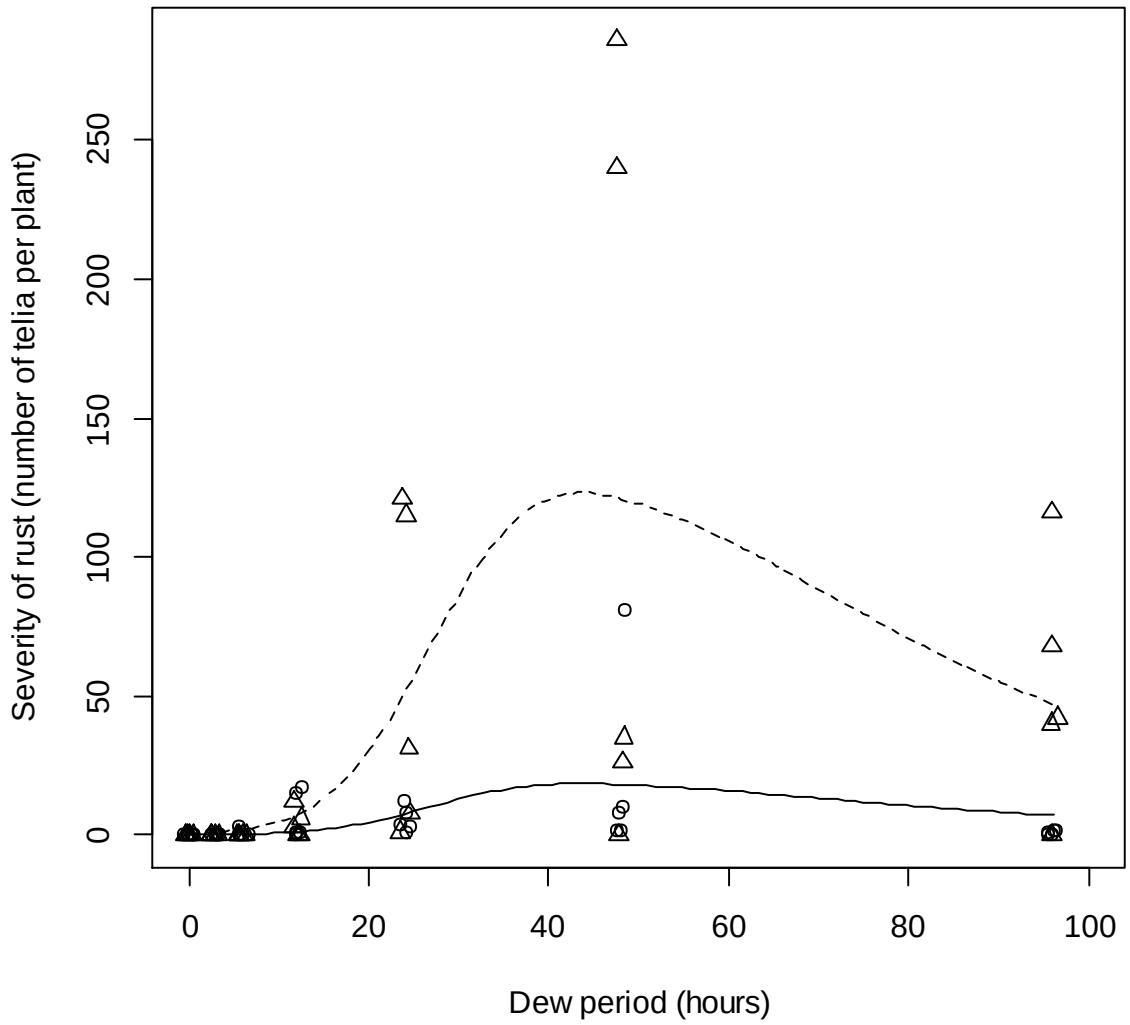
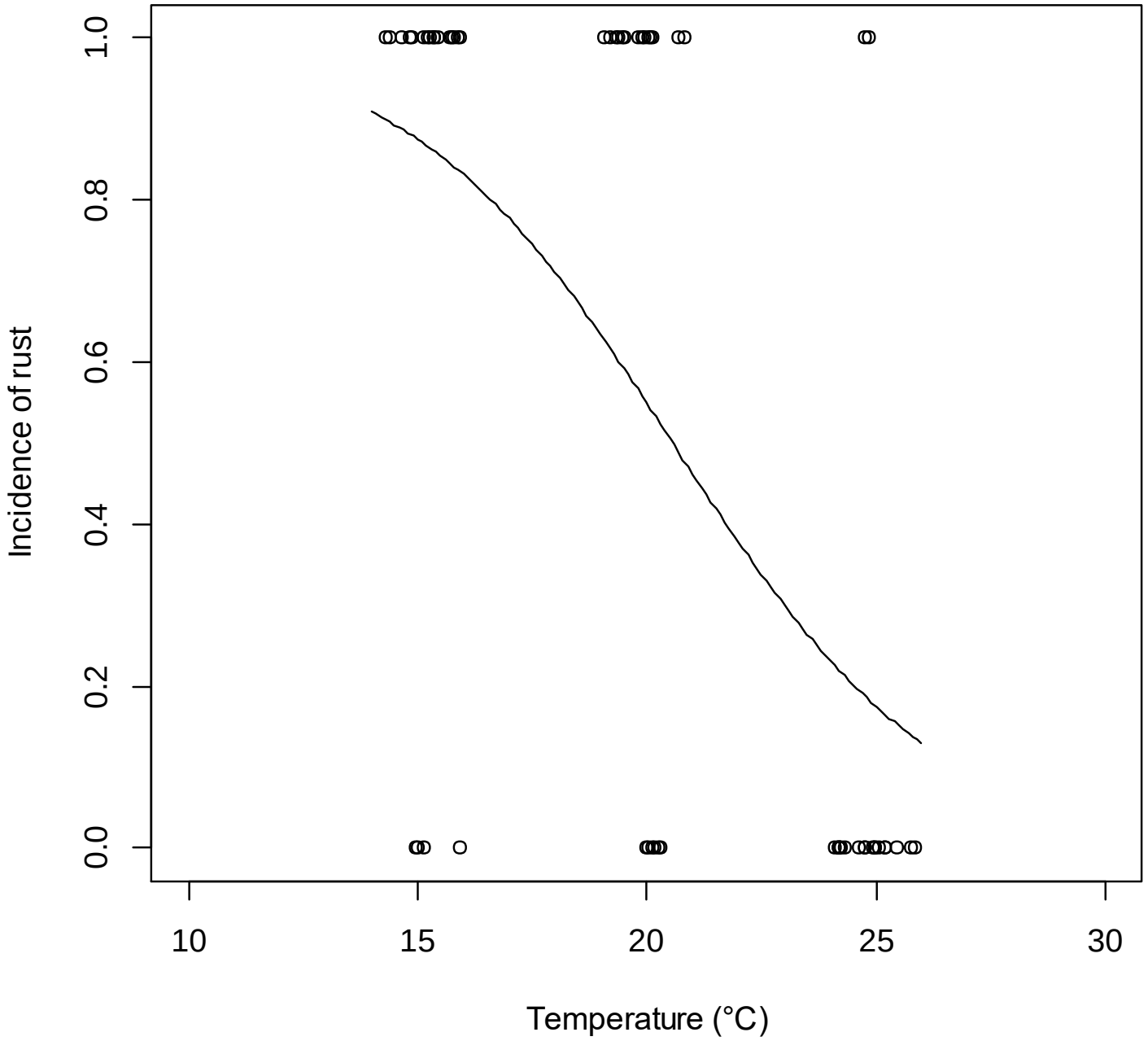
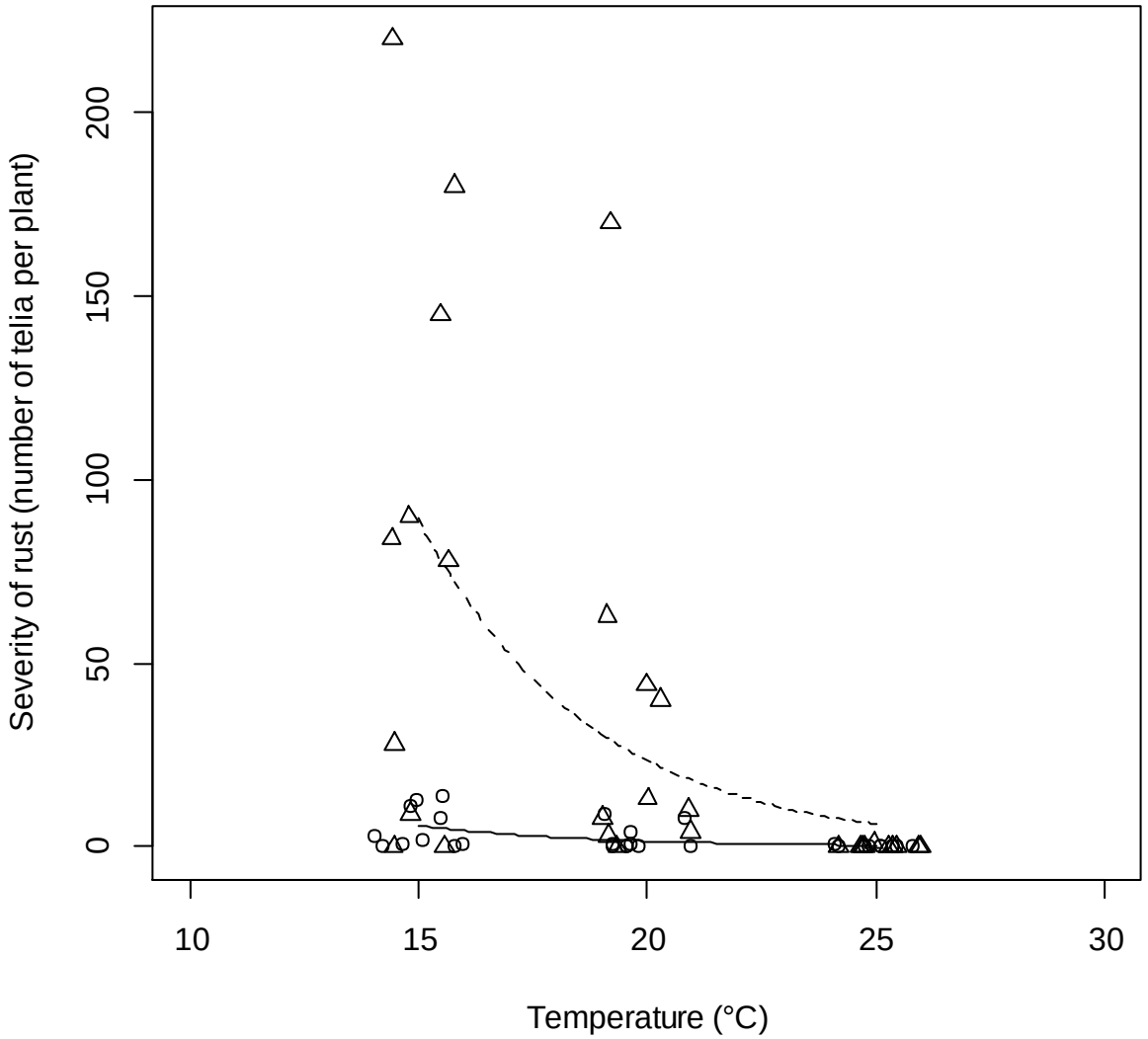

R² = 0.63; P < 0.001
Expt 1
Expt 2
1 hr wet, expt 1
2 hr wet, expt 1
1 hr wet, expt 2
2 hr wet, expt 2
Basidiospore production (%)
0
10
20
30
40
50
60
70
80
0
5
10
15
20
25
30
35
40
45
50
Dry period (hours)

Figure 6A


Incidence of rust
0.0
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80
100
Dew period (hours)


Severity of rust (number of telia per plant)
0
50
100
150
200
250
0
20
40
60
80
100
Dew period (hours)


Incidence of rust
0.0
0.2
0.4
0.6
0.8
1.0
10
15
20
25
30
Temperature (°C)


Severity of rust (number of telia per plant)
0
50
100
150
200
10
15
20
25
30
Temperature (°C)