

# Effects of *Melampyrum* Extracts on the Growth of Axenic Cultures of *Cronartium flaccidum* and *Peridermium pini*

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For 3–6 months, mycelial colonies cultured from 5 isolates of each of two pine stem rusts (*Cronartium flaccidum* and *Peridermium pini*) were grown on nutrient-rich agar supplemented with *Melampyrum* extracts. Non-autoclaved extracts of *M. pratense* significantly reduced the growth of *P. pini*. The growth of *C. flaccidum* isolates was slightly stimulated after the second month of incubation but after that was inhibited during incubation months 4–6. We observed considerable variation in colony growth, a significant component of which was explained by incubation time, isolate, growth medium and their interaction. Rust species (*C. flaccidum* or *P. pini*) was not an important factor in growth variation. While sterilized extracts of *M. pratense*, *M. sylvaticum* and *M. nemorosum* did not significantly affect growth, colonies of *C. flaccidum* were slightly stimulated, whereas colonies of *P. pini* were slightly inhibited. Generally, isolates of *P. pini* grew better and showed a slower rate of degeneration than *C. flaccidum* on all media.

**Keywords** alternate host, anti-fungal phytochemicals, fungal growth regulation, pine stem rust, Scots pine

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## 1 Introduction

Over the past 20–40 years, *Pinus* spp. in Europe have been suffering an epidemic of pine stem rust caused by *Cronartium flaccidum* (Alb. & Schwein.) G. Winter and *Peridermium pini* (Pers.)

Lév. (Diamandis and deKam 1986, Kaitera 2000). At the end of the 20th century, severe rust epidemics were sporadically detected in young stands of Scots pine (*Pinus sylvestris* L.) in northern Fennoscandia (Hantula et al. 1998, Kaitera et al. 1999, Holmberg 2004, Krekula et al. 2005).

Since then, epidemics have expanded in range and showed no sign of restraint (Barklund 2006, Sveriges lantbruksuniversitet 2008).

In northern Finland, pine stem rust incidence correlates with the frequency of *C. flaccidum* in the hemiparasite plant genus *Melampyrum* (family Orobanchaceae), an alternate host (Kaitera and Hantula 1998, Kaitera 2000, Kaitera et al. 2005). While *C. flaccidum* is capable of infecting and sporulating on *Melampyrum* species in natural forests (Kaitera et al. 2005), *M. sylvaticum* is highly susceptible, but *M. pratense* is somewhat resistant (Kaitera and Nuorteva 2003a, b). The factors that influence susceptibility among *Melampyrum* spp. to pine rust are unknown, but interspecific differences in leaf chemistry may be involved.

In order to explore the idea that differences in host preference are due to phytochemical variation among host plant species, we tested the effect of leaf extracts obtained from *M. sylvaticum*, *M. nemorosum* and *M. pratense* on the *in vitro* growth of *C. flaccidum* and *P. pini*.

## 2 Materials and Methods

### 2.1 Rust Isolates

In 2004–2005, aeciospores of *C. flaccidum* and *P. pini* were collected fresh from unopened aecia on Scots pine. Aeciospores were grown initially in 1.5% water agar prior to inoculation of a nutrient-rich agar medium (Lemco) developed specifically for rust fungi (Pei and Pawsey 1990). Spores were taken from isolates identified in earlier studies or harvested from pine inoculations in 1994–2005 (Hantula et al. 1998, Kaitera et al. 1999, Kaitera 2003, Kaitera and Nuorteva 2003a, b 2008). Unfortunately, most of the spores had lost their viability during storage and single- and multi-spore inoculations were necessary to produce mycelial cultures from the remaining viable material. The single-spores typically formed a single germ tube of variable length and showed negligible growth at 24 °C. Colonies resulting from multi-spore inoculations grew more vigorously. Only the multi-spore derived cultures with a large mycelial mass were selected and used as

inoculum. The cultures of both rusts used in this experiment originated from northern Finland.

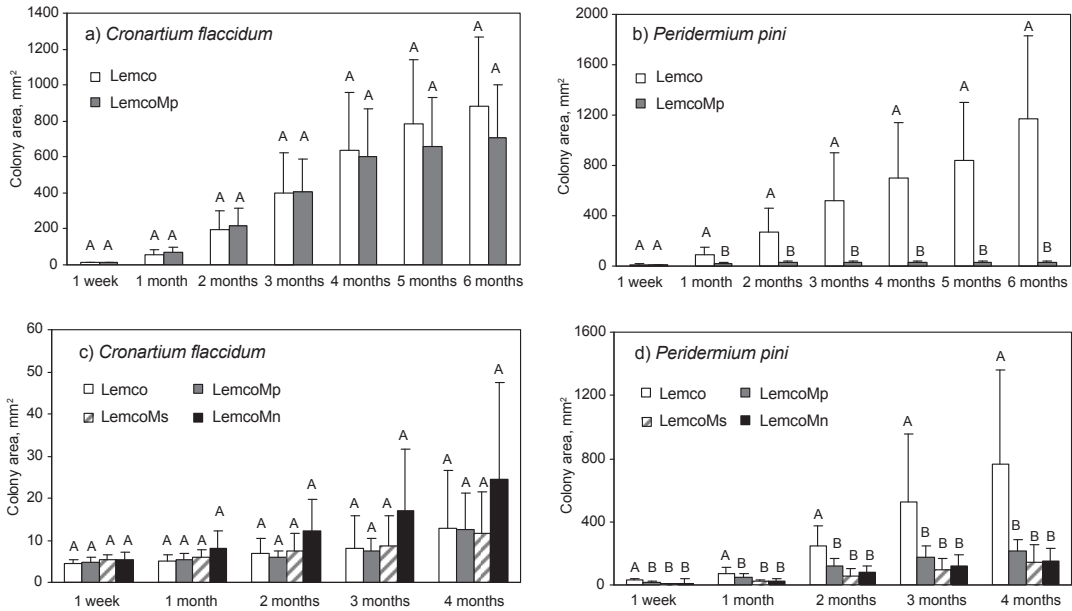
### 2.2 Experiment 1: Effects of Non-Autoclaved Extract-Agar Media on Growth of *C. flaccidum* and *P. pini*

Young *M. pratense* and *M. sylvaticum* were collected from Scots pine stands growing naturally in northern Finland in late June 2005. Initially, whole plants were stored at 7 °C for a few days, after which green and healthy leaves were removed (22 g of *M. sylvaticum*; 9 g of *M. pratense*), homogenized in 100 ml deionized water in liquid nitrogen with a Moulinex homogenizer, and then filtered through double gauze with a mesh diameter of approximately 0.1 mm. The supernatant was then vacuum-filtered through 0.45- $\mu$ m filter paper, added directly to 2 L (*M. sylvaticum*; LemcoMs) or 1.5 L (*M. pratense*; LemcoMp) of agar suspension at 50 °C (Lemco), and poured into Petri dishes in a laminar flow-hood.

Unfortunately, agar plates containing extract of *M. sylvaticum* (LemcoMs) were discarded prior to inoculation due to contamination with fungal endophytes and bacteria. Plates containing extract of *M. pratense* (LemcoMp) were free of contaminants and five colonies of uniform diameter (1 mm) with fluffy, actively growing mycelia of both *C. flaccidum* and *P. pini* were selected for inoculation using sterile forceps. Five replicates of each isolate were inoculated on Lemco and LemcoMp and incubated at 18 °C in the dark for a total of 6 months. The area of the rust colony was measured a week after inoculation and thereafter monthly with a stereomicroscope and 1 mm<sup>2</sup> paper.

### 2.3 Experiment 2: Effects of Autoclaved Extract-Agar Media on Growth of *C. flaccidum* and *P. pini*

In the second experiment, 60 g of fresh, green leaves of *M. nemorosum* were collected in southern Finland and an extract agar was prepared as in experiment 1 (added to 1.5 L agar suspension; LemcoMn). Extract agar of LemcoMp and LemcoMs were prepared similarly with similar fresh weights (60 g) of *M. pratense* and *M. sylvaticum*.



**Fig. 1.** Colony growth of isolates of *C. flaccidum* and *P. pini* on Lemco and LemcoMp (1a, b) in experiment 1, and on Lemco and LemcoMp, LemcoMs and LemcoMn (1c, d) after 4–6 months of incubation. Vertical bars indicate the  $0.5 \times \text{SD}$  of the mean of 25 replicates of five isolates. Means with the same letter are not significantly different according to Tukey's test ( $p < 0.05$ ).

As an additional step, all extract-agar suspensions were autoclaved prior to pouring. In experiment 2, the same isolates as in experiment 1 were inoculated on control and extract-agar media. Colony area was measured as in experiment 1: one week after inoculation and then monthly for 4 months. Incubation and measurement of growth ended when the fastest colony reached the Petri plate wall.

## 2.4 Statistical Analysis

The mean colony area (growth rate) of each isolate was compared among isolates and extract-agar media with the *t*-test and Tukey's test using SAS (SAS/STAT User's Guide 1989). Variation in colony area (growth rate) was modelled using incubation time, isolate, rust species, extract-agar media, and their interaction with the GLM-procedure of SAS.

## 3 Results

### 3.1 Experiment 1: Effects of Non-Autoclaved Extract-Agar Media on Growth of *C. flaccidum* and *P. pini*

Colonies of *P. pini* had a mucilaginous surface with a defined margin, while *C. flaccidum* had a fluffy surface with an ill-defined margin. After the first week of incubation, the growth of both rusts on LemcoMp was not significantly different from that on Lemco (control) media (Figs. 1a, b). After that, the growth of *P. pini* on LemcoMp was significantly slower (*t*-test; 1 month  $p < 0.01$ ; 2 months  $p < 0.01$ ; 3 months  $p < 0.005$ ; 4–6 months  $p < 0.001$ ) than on Lemco. The growth of *C. flaccidum* on LemcoMp was slightly but not significantly stimulated during incubation weeks 1–8 and slightly reduced after 4 months compared to Lemco. As an exception, the growth of one *C. flaccidum* isolate was stimulated on LemcoMp (not shown).

When modelling colony growth, the best model explained 31% of the variation with significant variables of medium ( $F=46.87$ ,  $p<0.001$ ), time ( $F=27.15$ ,  $p<0.001$ ), rust species ( $F=11.28$ ,  $p<0.001$ ), and interactions between either time and medium or rust species and medium. When rust species was replaced by isolate, the best model explained 77% of this variation, and included time, isolate, medium and their interactions as significant variables ( $p<0.001$ ). When modelling growth at each monthly measurement event, isolate was a significant variable ( $p<0.01$ ) but medium and the interaction between medium and isolate were initially non-significant (first week) before becoming significant variables ( $p<0.01$ ) thereafter. These models explained 36–84% of the variation in colony growth at each measurement event. Models including rust species, medium and their interaction explained only 4–21% of the variation in colony growth between each measurement event.

### 3.2 Experiment 2: Effects of Autoclaved Extract-Agar Media on Growth of *C. flaccidum* and *P. pini*

Autoclaved agar media containing extracts of *Melampyrum* spp. had no significant effect on the growth of either *P. pini* or *C. flaccidum*, when isolates were pooled. Generally, growth was slightly but not significantly reduced on extract-agar media with colonies on LemcoMs being the smallest and those on LemcoMp the largest. Between the two rusts, the growth of *C. flaccidum* was slightly but not significantly stimulated on all extract-agar media, while the growth of *P. pini* was significantly depressed on all extract-agar media (Figs. 1c, d). Generally, isolates of *C. flaccidum* had a lower growth rate and degraded more quickly than *P. pini* isolates during both experiments. Some isolates of *P. pini* showed a significant response on some extract-agar media.

In the best model ( $R^2=0.58$ ) including time, isolate, medium and their interactions, all variables accounted for significant variation in growth ( $p<0.001$ ). Although rust species was a significant variable with time, medium, and their interaction in another model, these variables explained

only 24% of the variation. At each measurement event, isolate was the dominant factor accounting for variation in growth although medium and the interaction between medium and isolate were also significant ( $p<0.001$ ;  $R^2=0.74-0.95$ ). In the best model, substituting rust species for isolate, less of the variation in growth was explained ( $R^2=0.22-0.38$ ) despite all variables being significant ( $p<0.01$ ).

When colonies were combined, the growth of *P. pini* was significantly greater than that of *C. flaccidum* at each measurement event (*t*-test,  $p<0.05$ ) on all media including Lemco. One colony of *C. flaccidum* showed increased growth on extract-agar media compared to the control and all isolates of *P. pini* showed variable responses after 2-months of incubation.

## 4 Discussion

In this study using Finnish samples, *in vitro* mycelial colonies of *C. flaccidum* and *P. pini* were cultured only by using multiple aeciospores as inocula. In contrast single spores of *P. pini* from southern England (East Anglia) were capable of forming *in vitro* cultures, while spores of this rust from northeast Scotland (Morayshire) could not be cultured singly (Pei and Gibbs 1992). In an earlier study, Pei and Gibbs (1991) emphasized the distinctively different morphology of colonies cultivated from English and Scottish *P. pini*. Similarly, distinct colony morphologies and growth sectors have been reported for isolates cultured from Italian (Moricca and Ragazzi 1996) and Finnish (Kaitera et al. 1999) spores, but such sectors were not seen in either rust during this study.

Non-autoclaved extracts of *M. pratense* strongly inhibited the growth of the autoecious rust, *P. pini*. They had little effect on *C. flaccidum* other than to slightly stimulate the growth of several colonies, which emphasizes the variable and individual response that different isolates can have to a given phytochemical substances. The variation in response was surprisingly high, especially considering that these rusts have been considered different forms of a single species (Hantula et al. 2002). The slightly enhancing or neutral

effect of *Melampyrum* extracts on the growth of *C. flaccidum* may partly explain the association between *C. flaccidum* and *Melampyrum* in natural forests.

Autoclaved extracts of *Melampyrum* spp. had negligible or neutral effects on the growth of either *C. flaccidum* or *P. pini* when isolates were pooled. Between rusts, growth of *C. flaccidum* was slightly stimulated by the extracts while *P. pini* was inhibited. This also indicates response variation among isolates to extracts of *Melampyrum*. This was also reflected in modelling colony growth, where rust isolate explained more of the response to extract than did rust species. Additionally, colonies of *C. flaccidum* degenerated more rapidly than *P. pini* during the investigation, which suggests that the former had a greater nutrient requirement for its growth and maintenance. It is also possible that autoclaving had altered the chemical structure of leaf extracts e.g. through partial hydrolysis of sucrose (George et al. 2008), which partially may have affected the fungal-promoting or inhibiting properties of *Melampyrum* extracts.

In conclusion, considerable variation was observed among rust isolates with respect to their *in vitro* growth response to *Melampyrum* extracts. This echoes the variation noted in virulence (Kaitera and Nuorteva 2008) and alternate host range of these rusts (Kaitera and Nuorteva 2003a, b). Chemical differences among *Melampyrum* species may be based on the physiological factors or linked to the relationship between the host plant and their ectomycorrhizal fungi (Salonen et al. 2000, Phoenix and Press 2005, Schädler et al. 2005, Walter 2005). Future work in this system should identify the phytochemicals produced by *M. sylvaticum* and *M. pratense* that are involved in their variable susceptibility to pine stem rusts.

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