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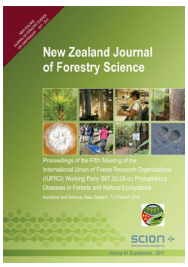

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Comparing virulence of *Phytophthora plurivora* and *P. pseudosyringae* towards *Fagus sylvatica* seedlings using a method ensuring equal growth of both pathogens†

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Abstract

Phytophthora plurivora T.Jung & T.I.Burgess and *P. pseudosyringae* T.Jung & Delatour exhibit different potential to colonise host plants. In order to clarify whether *P. plurivora* is more aggressive than *P. pseudosyringae* simply because of its faster growth and sporulation, a method for root infection was developed ensuring equal growth and sporulation for both pathogens during infection of *F. sylvatica* L. seedlings. Infection with *P. plurivora* strongly reduced CO_2 uptake of seedlings and five out of eight seedlings died by the end of the experiment. In contrast, *P. pseudosyringae* did not alter physiology of infected plants and no mortality was recorded. The DNA contents of roots infected by either pathogen were similar at the end of the experiment, which indicated that a similar amount of fungal material was present for each species. This indicates that the greater aggressiveness of *P. plurivora* in comparison to *P. pseudosyringae* cannot be explained by its faster growth compared to *P. pseudosyringae* at a given temperature.

Keywords: *Fagus sylvatica*; photosynthesis; *Phytophthora plurivora*; *P. pseudosyringae*; qPCR; root colonisation.

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Introduction

Diseases caused by *Phytophthora* species are a threat for many crops and forests world-wide. Approximately one hundred species are already identified and the majority of them are able to attack woody plants (Brasier, 2009).

The physiology and reproduction of these species are distinct and the responses to the environment can vary considerably. For example, Jung et al. (2003) showed that the effect of temperature on the growth rates of five *Phytophthora* species differed. This temperature-dependent behaviour can confound experimental results if infection experiments comparing the aggressiveness of different species are carried out under a single temperature regime. For instance, *in vitro* at 20 °C, *P. plurivora* T.Jung and T.I.Burgess cultures grow faster than those of *P. pseudosyringae* T. Jung and Delatour (6.3 mm d^{-1} for *P. plurivora* compared

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with a mean of 4.6 mm d^{-1} for *P. pseudosyringae*; (Jung & Burgess (2009) and Jung et al. (2003), respectively)

In vivo, both *Phytophthora plurivora*[1] and *P. pseudosyringae*[2] caused root rot and severely decreased fine root length as well as the number of root tips of *F. sylvatica* L. (Fleischmann et al., 2002, 2004; Jung et al., 2003). However, *P. plurivora* caused high mortality of infected plants, whereas no mortality was recorded for those plants infected with *P. pseudosyringae* (Fleischmann et al., 2002, 2004; Jung et al., 2003). Thus, *P. plurivora* is more aggressive than *P. pseudosyringae* towards *F. sylvatica.* The experiments described by these authors on different species were carried out at under the same temperature regimes. This raises the question of whether the measured differences in aggressiveness are due to different growth rates of the pathogens at a given temperature rather than inherent differences in the aggressiveness of the species. In order to clarify this possible effect, we analysed the growth and sporulation rates of *P. plurivora* and *P. pseudosyringae* at different temperatures *in vitro* and *in vivo*. We did this, *in vivo*, by developing a root-incubation system that allows us to adjust and to control root temperature during the whole experiment, while the aboveground organs of the plants are growing at a constant temperature. This device allows us to guarantee comparable growth rates of pathogens at different temperatures and to analyze plant-pathogen interactions more accurately in order to give clear conclusions on pathogen aggressiveness.

Materials and Methods

In vitro growth and zoospore formation of *Phytophthora plurivora* and *P. pseudosyringae* isolates

Growth assay: *P. plurivora*, isolate CIT55, and *P. pseudosyringae*, isolate PSEU1, (isolated from declining beech trees in a deciduous forest in Bavaria, held in the culture collection in the section Pathology of Trees, at the TUM-Freising, Germany), were grown in Petri dishes on V8 juice agar (V8 fruit juice, $CaCO_3$, distilled water and agar) at 20 °C in the dark until the mycelial colony had reached 2 cm diameter. Subsequently, agar plugs were taken and five replicates per isolate were prepared and incubated at 10, 14, 17, 20 and 24 °C in the dark. Radial growth was recorded daily over one week.

Sporulation assay: Petri dishes containing V8 juice agar and the two isolates (CIT55 and PSEU1) were grown at 20 °C in the dark until reaching 80% of the plate's surface. Subsequently, these plates were distributed to the following temperatures: 10, 14, 17, 20 and 24 °C and periodically washed with sterile water to induce sporangia formation. After seven days, in order to induce zoospores release, the plates were incubated for one hour at 4 °C. Zoospore concentration on plates was quantified for each temperature tested (10, 14, 17, 20 & 24 °C) using a Thoma counting chamber.

Design of the temperature controlling root boxes (TCR boxes)

Figure 1 shows a detailed diagram of the TCR boxes. Two plastic (PVC) boxes (height 13 cm, length 37 cm, width 17 cm) were coated with Styrofoam (size 3.5 cm) on each side, including the lids. Twenty-eight holes, each with a diameter of 3 cm were drilled into the lid to fit plastic tubes with screw-top lids (Falcon; 50 mL). Inside the boxes a flexible PVC-tube (19/25 mm inside/outside diameter, 1.7 m in length) was installed in curves onto the bottom and connected to an external integrated cooling device (Lauda, UKT 350), circulating tempered water through the tube. The inside of each box was filled with deionised water. In order to avoid temperature gradients, an aquarium pump (Compact 1000, Eheim, Germany) was fixed at the bottom of each box. The water temperature in each box was adjusted to either 14 or 20 °C. The

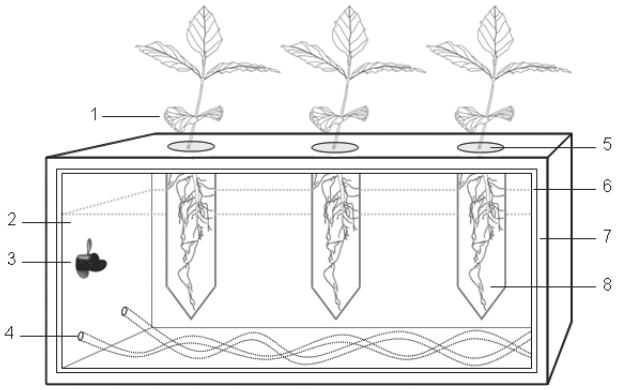

FIGURE 1: Technical diagram of TCR boxes (not in scale).
1: *Fagus sylvatica* seedlings in 50 mL plastic test tubes;
2: inside of the box filled with water;
3: aquarium pump;
4: tube connected with an integrated cooling device;
5: Para film sealing holes between shoot and root system;
6: PVC layer;
7: Styrofoam insulation; and
8: water inside the falcon tubes in contact with the root system.

[1] As *Phytophthora citricola sensu lato* in Fleischmann et al., 2002, 2004.

[2] As *Phytophthora syringae* in Fleischmann et al., 2002; see Jung et al. (2003) for clarification.

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temperature inside each plastic test tube was checked manually with a thermometer. Both root boxes were placed into phytotrons (York® International) which were set to long-day (16-hr light) conditions (20 °C, 65% relative humidity and photosynthetic photon flux density (PPFD) of 250 µmol m^{-2} sec^{-1}).

Infection of *Fagus sylvatica* seedlings

Fagus sylvatica seedlings were cultivated in trays with vermiculite (16 hours light and 20 °C) for three months (final shoot height approximately 15 cm and root length about 10 cm). Before inoculation seedlings were washed and placed into 50-mL plastic test tubes with 45 mL of distilled water. The tubes were transferred to the root boxes, adjusted to either 14 or 20 °C. After one day of acclimatization, eight seedlings per treatment were inoculated with 5 × 10^5 zoospores from a suspension of either *P. plurivora* or *P. pseudosyringae* at room temperature. Another eight plants were used as healthy controls.

Carbon dioxide exchange parameters were recorded using a LI-6400 portable photosynthesis system (Licor) at 250 µmol m^{-2} s^{-1} photosynthetic photon flux density and 400 ppm CO_2.

Seedlings were harvested eight days after inoculation and the roots examined visually for root rot. The amount of Phytophthora DNA present in the *Fagus sylvatica* root systems was quantified using real-time quantitative PCR-techniques. DNA was extracted from 20 mg of freeze-dried and milled root material using the Plant DNeasy Mini-kit (Qiagen, Hilden, Germany). Subsequently, DNA extracts were further purified by sodium acetate (3.0 M, pH 5.2)/ethanol (96 and 70%) precipitation. The amount of *P. plurivora* and *P. pseudosyringae* DNA within the DNA extracts was quantified on a SDS 7700 Sequence Detection System (Applied Biosystems, Frankfurt, Germany) using ABSOLUTE qPCR ROX chemicals (ABgene, Hamburg, Germany). The qPCR analysis was performed in three technical replicates. For both *P. plurivora* and *P. pseudosyringae* quantification, the primer sets and the fluorogenic probes of Böhm et al., (1999) and Tooley et al., (2006), respectively, were used. Dilution series of genomic DNA extracted of pure *P. plurivora* or *P. pseudosyringae* mycelium was used to calculate a linear standard curve of log [*Phytophthora* DNA] vs. CT-value.

Analyses of variance (ANOVA) were carried out and the Tukeys tests at 5% error probability were performed to test differences among mean values.

Results and Discussion

The growth rates of *Phytophthora plurivora* and *P. pseudosyringae* isolates have been studied *in vitro* on V8 medium previously (Jung & Burgess, 2009; Jung et al., 2003 respectively). Our results (Figure 2) support these earlier findings. Firstly, growth rates of *P. plurivora* were higher than those of *P. pseudosyringae* at all the temperatures tested here, Figure 2a. Secondly, in this study, the optimum growth rate of *P. pseudosyringae* at 20 °C was 3.6 ± 0.08 mm d^{-1}, which is within the range observed by Jung et al. (2003). However, the radial growth rate of 7.5 ± 0.24 mm d^{-1} measured here for *P. plurivora* at 20 °C was slightly higher than the rate of 6.3 ± 0.1 mm d^{-1} published by Jung and Burgess (2009).

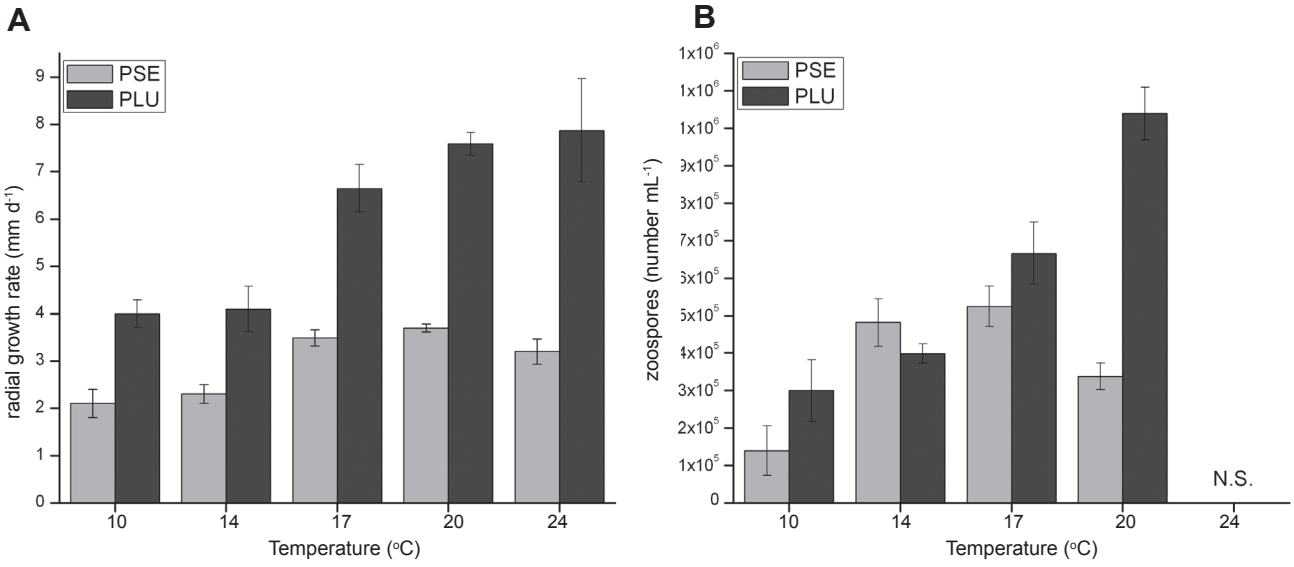

FIGURE 2: (A) Growth rate of *Phytophthora plurivora* (PLU) and *P. pseudosyringae* (PSE) on V8-agar plates at different temperatures; and (B) Zoospore release of *P. plurivora* (PLU) and *P. pseudosyringae* (PSE) at different temperatures. N.S.: No sporulation.

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TABLE 1: Net CO_2 assimilation rates (µmol m^{-2} s^{-1}) of infected and control *Fagus sylvatica* seedlings with roots held at 14 °C or 20 °C after 1, 4, 6, and 8 days post-inoculation.

dpi	CON 14 °C	CON 20 °C	PLU 14 °C	PLU 20 °C	PSE 14 °C	PSE 20 °C
1	1,101 a*	1,821 a	1,435 a	1,687 a	1,824 a	1,406 a
4	1,454 b	1,633 b	0,490 c	0,410 c	1,385 b	2,254 a
6	1,335 b	1,880 b	0,157 c	0,058 c	1,314 b	2,506 a
8	1,337 b	1,922 b	0,544 c	-0,132 d	1,458 b	2,519 a

*The mean values labelled with different letters differed significantly among the treatments at each time point (Tukey test, $p < 0.05$). CON = control; PLU = *Phytophthora plurivora*; PSE = *P. pseudosyringae*; dpi = days post inoculation.

Both isolates released spores at all temperatures tested up to 20 °C but sporulation ceased at 24 °C for both isolates. Maximum zoospore formation occurred at 17 °C for *P. pseudosyringae* and at 20 °C for *P. plurivora* (Figure 2b).

Eight days after inoculation, seedlings were harvested and the extent of root rot was examined. A higher extent of root rot in *Phytophthora plurivora* infected plants was visible in comparison with roots infected with *P. pseudosyringae*.

A decrease in root incubation temperature from 20 to 14 °C did not significantly change the photosynthetic rate of control seedlings (i.e. non-infected) (Table 1). These data are in accordance with results of Lyr and Garbe (1995), who did not find any significant difference regarding the growth of one-year-old *Fagus sylvatica* saplings, when the temperature of the root system was set to 15 or 20 °C. However, decreasing the temperature of the root system is known to influence whole plant metabolism, in particular leaf-gas exchange, of other species (Mangat, 1982; Delucia, 1986).

Compared to the control, photosynthesis (measured as net CO_2 assimilation rate) of seedlings infected with *Phytophthora plurivora* was significantly reduced four days after inoculation (at either 14 or 20 °C) (Table 1). Fleischmann et al. (2002) also found decreasing of the net CO_2 assimilation rate of four-month-old *Fagus sylvatica* seedlings 2 – 4 days after infection with *P. plurivora* (*as P. citricola*[1]) and *P. cambivora* at 20 °C, as well as high damage of roots and mortality of the seedlings when compared with the control plants.

Seedlings infected with *Phytophthora pseudosyringae* showed significantly higher photosynthetic rates at 20 °C but not at 14 °C, (Table 1) when compared to control seedlings. Similar results at 20 °C for this species (as *P. syringae*[2]) and with *P. undulata* were reported by Fleischmann et al., (2002) in infection experiments with *F. sylvatica* seedlings.

In the infection experiment, eight days after inoculation, five seedlings infected with *Phytophthora plurivora* at 14 °C exhibited severe wilting of leaves and died. At 20 °C, seven out of eight seedlings died. In contrast, none of the seedlings infected with *P. pseudosyringae* showed visible symptoms and all survived at either 14 or 20 °C.

The effect of temperature on the growth of these two *Phytophthora* species in *Fagus sylvatica* roots is illustratrated in Figure 3. For both species, quantitative PCR analysis indicated that the amount of DNA was higher at 20 °C than at 14 °C, indicating a temperature effect on the growth of both pathogens. However, the amount of *P. plurivora* DNA in roots of *F. sylvatica* seedlings was higher than that of *P. pseudosyringae* DNA at both the temperatures tested here. In fact, the amount of *P. plurivora* DNA in roots of *F. sylvatica* seedlings held at 14 °C was similar to the amount of *P. pseudosyringae* DNA in roots of *F. sylvatica* seedlings held at 20 °C.

No correlation was found between the amount of *Phytophthora* DNA on roots of *Fagus sylvatica* seedlings and leaf photosynthesis or mortality of plants tested. Since the amount of *Phytophthora* infection of roots does not explain the observed differences regarding

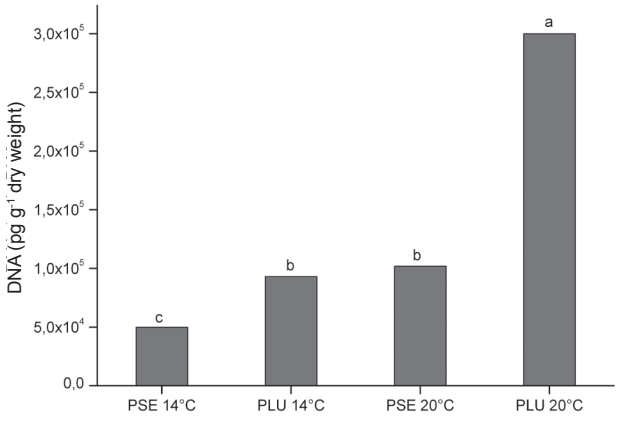

FIGURE 3: Quantitative PCR of *Fagus sylvatica* roots infected with *Phytophthora plurivora* (PLU) and *P. pseudosyringae* (PSE) at 14 and 20 °C. Bars with the same letter are not significantly different.

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seedling health, other still unknown mechanisms must be involved. Experiments are still ongoing to elucidate whether different infection strategies of the pathogens and/or different defence mechanisms of the host can explain the observed differences of *F. sylvatica* seedlings towards the two pathogens.

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