Assessing the interaction between *Ophiostoma quercus* and *Heterobasidion* spp.: An *in vitro* study

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Swedish University of Agricultural Sciences, SLU
Faculty of Forest Science
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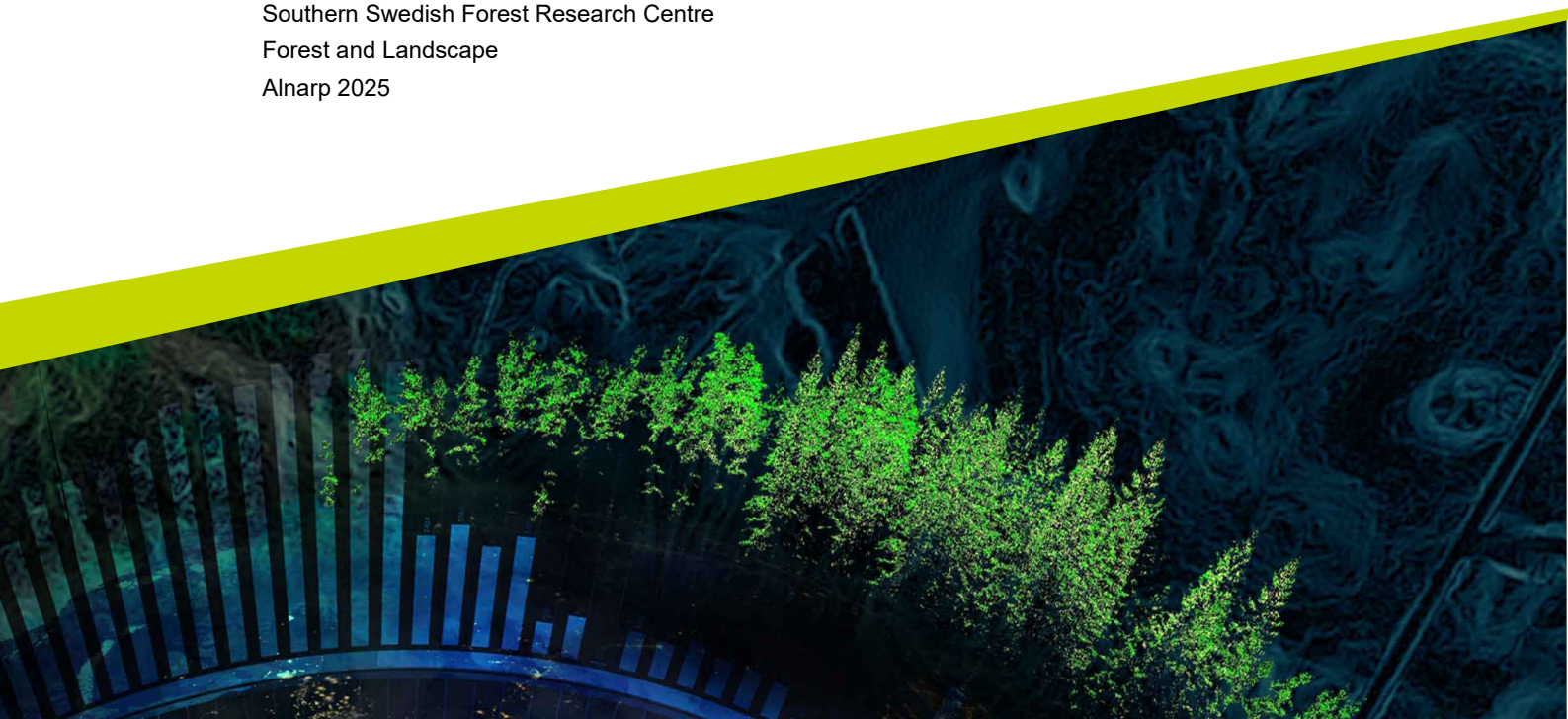

Assessing the interaction between *Ophiostoma quercus* and *Heterobasidion* spp: An *in vitro* study

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Abstract

The effects of climate change pose growing challenges for forestry, and the need for climate change-adapted management in the sector is growing. One of these changes is the need to increase the number of deciduous trees to improve forest resilience. The most dominant deciduous tree in Sweden is *Betula* spp (birch), and it will play an increasingly important role in adapting Swedish forests to climate change in the future. It is therefore important to consider potential challenges that may arise when planting more birch.

Heterobasidion root rot is a pathogenic fungus that infects several hosts, causes tree decay and leads to high economic losses all over Europe. While *Heterobasidion* primarily affects conifers, it can also infect broadleaf trees. Although some research indicates that birch can become infected with *Heterobasidion* when planted on previously infected sites, the degree to which it will become infected following thinning, in the absence of biological treatment, remains poorly understood. Therefore, a study (unpublished) was conducted to investigate the infection of *Heterobasidion* on birch stumps. The study revealed unexpectedly low indices of the pathogenic fungus *Heterobasidion* on the stumps, in contrast to the high prevalence of the non-pathogenic fungus *Ophiostoma quercus*. This raised the question of whether *Ophiostoma* is the cause of the low occurrence of *Heterobasidion*.

To investigate this, an *in vitro* pairing experiment was performed to determine if an interaction occurs between the two fungi. Contrary to the initial assumption that *O. quercus* would inhibit the growth of *Heterobasidion*, it appeared to not affect or in some cases increase its growth. Other interesting growth trends have been observed, however, which need to be investigated further.

Keywords: Heterobasidion, *Ophiostoma quercus,* Pairing, Interaction

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Abbreviations

Abbreviation	Description
DNA	Deoxyribonucleic acid
HA	*Heterobasidion annosum*
HP	*Heterobasidion parviporum*
ITS	Internal transcribed spacer
MEA	Malt extract agar
OP	*Ophiostoma*
PCR	Polymerase chain reaction
SLU	Swedish University of Agricultural Sciences

1. Introduction

Sweden has one of the largest areas of forest among EU countries with 28 million hectares of forest covering 70% of the total land area (Felton *et al.* 2022). Additionally, as one of the largest global exporters for timber, pulp and paper, forestry plays an important role for the national economy (Nielsen 2024). The forests are intensively managed by rotational clearcutting of even-aged stands, followed by soil scarification, removal of competition and planting seedlings (Petersson *et al.* 2021). Swedish forests are predominantly coniferous, consisting of about 41 % spruce (*Picea abies*), and 39,1 % pine (*Pinus sylvestris*). Broadleaves are an important component consisting of about, 12.1 % birch (*Betula pendula* and *Betula pubescens*) and about 7,9 % other tree species (Black-Samuelsson *et al.* 2020).

In the last few decades, the effects of climate change on forestry have become more prominent which has led to an increase in disturbances, such as the storm "Gudrun" in 2005 which damaged 70 million m^3 of production forest (Guillen & Felton 2025), and an unprecedented outbreak of spruce bark beetle (Jaime *et al.* 2024). Furthermore, the increase of intensive forestry and spruce monocultures has led to potential losses in biodiversity and forest resilience (Felton *et al.* 2010). These growing challenges are causing the sector to re-evaluate the traditional model of intensive forestry and instead work towards adopting climate change adaptation strategies into the current management. One proposed solution is to increase tree species diversity by creating mixed stands and planting more broadleaf species to improve forest resilience and safeguard biodiversity (Felton *et al.* 2024).

Birch is one of the most predominant broadleaved tree species in Sweden, which is becoming increasingly valued for biodiversity as well as resilience and is expected to play an important role in Sweden's efforts to diversify and adapt forests to climate change (SLU Riksskogstaxeringens 2025).

Birch (*Betula* spp.) is a fast-growing, light-demanding early successional species (Beck *et al.* 2016). Two species of birch are found in Sweden, *Betula pubescens* (downy birch) and *Betula pendula* (silver birch), with downy birch occurring predominantly in northern Sweden and silver birch in southern Sweden (Lidman *et al.* 2024). These two species make up around 12 % of the country's forest area (Black-Samuelsson *et al.* 2020). While birch is primarily used in the paper and pulp industry (Dahlgren Lidman 2022), it also promotes biodiversity by hosting a large number of species (810) (Black-Samuelsson *et al.* 2020) of which 369 are red listed species (Tingstad *et al.* 2018) and increasing its usage can contribute significantly to the diversification of forests.

However, with the growing interest in birch for use within forestry, it is also important to consider other potential challenges that may arise from planting more birch, particularly the risk of diseases.

One pathogen that causes significant losses to forestry in Sweden is root rot caused by *Heterobasidion* spp. If birch is to be promoted and more intensively managed in mixtures, the pathogen could become more problematic for birch.

Heterobasidion spp. is a pathogenic fungus that causes significant economic losses by causing decay inside the tree trunk which can lead to tree mortality. *Heterobasidion* root rot is one of the most economically significant forest pathogens in the northern hemisphere, and it causes losses of up to 1 billion Euros per year in Europe (Kovalchuk *et al.* 2022). Several recognized species exist within the *Heterobasidion* genus, including *H. annosum* (S-type) and *H. parviporum* (P-type), which are presenting Sweden and will be the focus of this experiment. These species have been categorized and named according to their preferred host: spruce (S = spruce) and pine (P = pine) (Kovalchuk *et al.* 2022).

In Sweden, *H. annosum* is present across southern and central Sweden but absent in the north, while *H. parviporum* can be found throughout the entire country (Berglund 2005; Wang *et al.* 2014).

Spores of *Heterobasidion* spp. infect the trees primarily through the fresh stumps of cut stems but trees can also get infected through secondary infection via root-to-root contact (Kovalchuk *et al.* 2022). There are biological control agents used for post-harvest treatment of conifer stumps, where the freshly cut stump is treated with an agent such as Rotstop®S gel, which contains the saprophytic fungus *Phlebiopsis gigantea* that can prevent infection (Blomquist *et al.* 2023). However, there is currently no method that provides complete protection against *Heterobasidion* (Kovalchuk *et al*. 2022).

Due to the importance of *Heterobasidion* spp. for Sweden, it is important to better understand the threat it poses to birch as well and if that risk warrants future protection by stump treatment agents after thinning, as is the case for other conifers. In an ongoing study (unpublished), the incidence and severity of *Heterobasidion* infection was assessed on *B. pendula* stumps following pre-commercially thinning to determine the risk to birch and whether biological control agents could be effective at limiting the infection. During this experiment however, it was discovered that only a small number of *Heterobasidion* spp. infections occurred on birch stumps. At the same time however, another fungus *Ophiostoma quercus* appeared to be quite prevalent, as indicated by the presence of conidiophores in a high percentage of stump disc samples collected from trees.

O. quercus is a widespread fungus found on a wide range of deciduous and coniferous trees. The fungus is characterized as saprotrophic and non-pathogenic (Taerum *et al.* 2018). However, a recent study found that *Ophiostoma quercus* is aggressive to mature *Quercus robur* (English oak) trees, contrary to the assumption that it is not pathogenic to *Quercus robur* (Milenković *et al.* 2025). Furthermore, the fungus has a high genetic diversity and is closely related to pathogenic fungus *Ophiostoma ulmi*, the cause of Dutch elm disease, which is why it is important to

recognise this fungus (Taerum et al., 2018). *O. quercus* is vectored by many insects, especially bark beetles, but also occasionally by *Scolytus ratzeburgi*, a beetle that frequently infests birch (Linnakoski *et al.* 2009; Taerum *et al.* 2018).

The low incidence of *Heterobasidion* spp. observed in birch stumps in conjunction with the high incidence of *O. quercus* raised the question of whether the presence of *O. quercus* might deter *Heterobasidion* spp. infections from establishing on cut stumps after thinning. To investigate whether such an effect is occurring, the interaction between these two fungi was studied *in vitro* i.e., under controlled laboratory conditions. I sought to answer the question: How does the presence of *O. quercus* affect the growth and development of *Heterobasidion annosum* and *Heterobasidion parviporum*.? I hypothesized that *the presence of O. quercus will negatively influence the growth and development of Heterobasidion spp.* If an interaction between *O. quercus* and *Heterobasidion* spp. could be demonstrated based on pairing experiments of the two fungi in culture, it may potentially explain the reduced incidence of *Heterobasidion* spp. infections on birch stumps in nature.

2. Methods

2.1 Background of the study

In the original study investigating the infection of *Heterobasidion* spp. on birch after pre-commercial thinning and the effectiveness of different biological control agents, six birch stands were selected for field experiments in the regions of Skåne, Blekinge and Småland in southern Sweden.

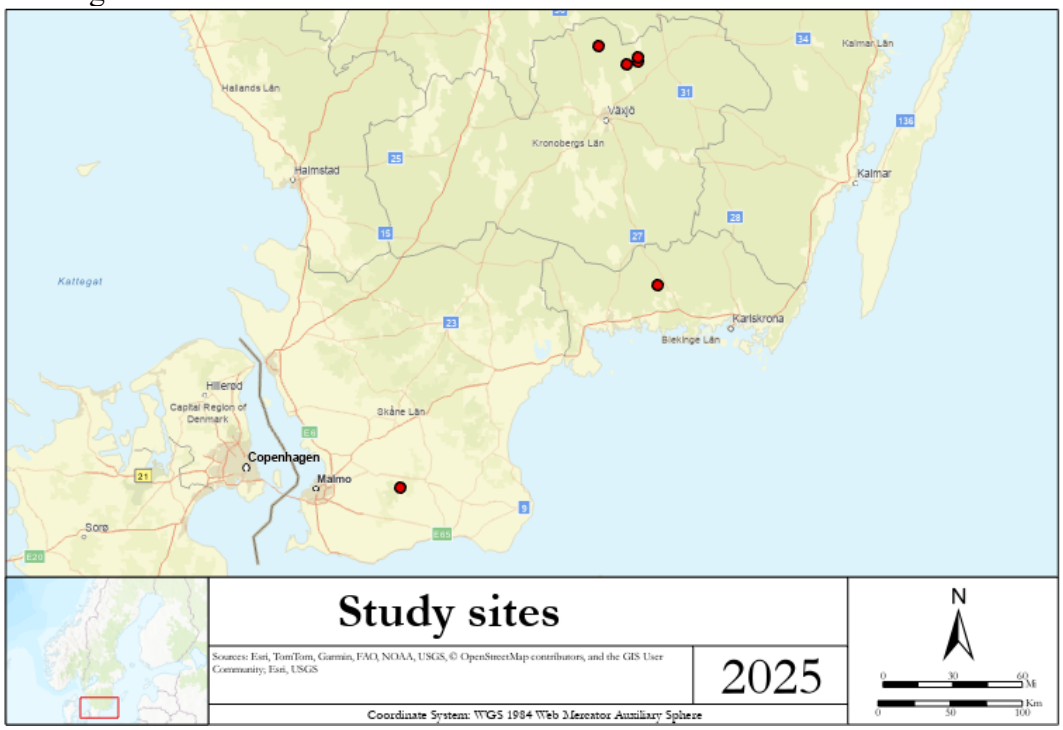

Figure 1: Map of the location of the study sites

At each site, 99 trees were divided among three treatments: the biological stump treatments Rotstop and Basinox, and control treatment, with 33 stumps assigned to each group. These treatments were randomly distributed within each site to ensure that the treatment was spread as evenly as possible across the site to avoid edge effects. Eight weeks after thinning and stump treatment, discs were taken from the stumps by first removing 1 cm of the top layer of the stump and then cutting a 5 cm cross-section disc. These discs were incubated at the laboratory at room temperature for 10 days for examination of *Heterobasidion* colonies under a stereo microscope (Nikon SMZ 645, Nikon Corporation, Tokyo, Japan). Since all discs could not be checked at once, some discs were stored at 4°C until incubation at room temperature. When fungal colonies were detected, they were quantified and isolated on media, either on Hagem or malt extract agar (MEA). Species-specific polymerase chain reaction (PCR) was then used to identify the *Heterobasidion* species present (Hantula & Vainio 2003). However, during the examination of the

discs a high prevalence of conidiophores of *Ophiostoma* spp. were found (Figure 2). The species was confirmed as *Ophiostoma quercus* with internal transcribed spacer (ITS) sequencing.

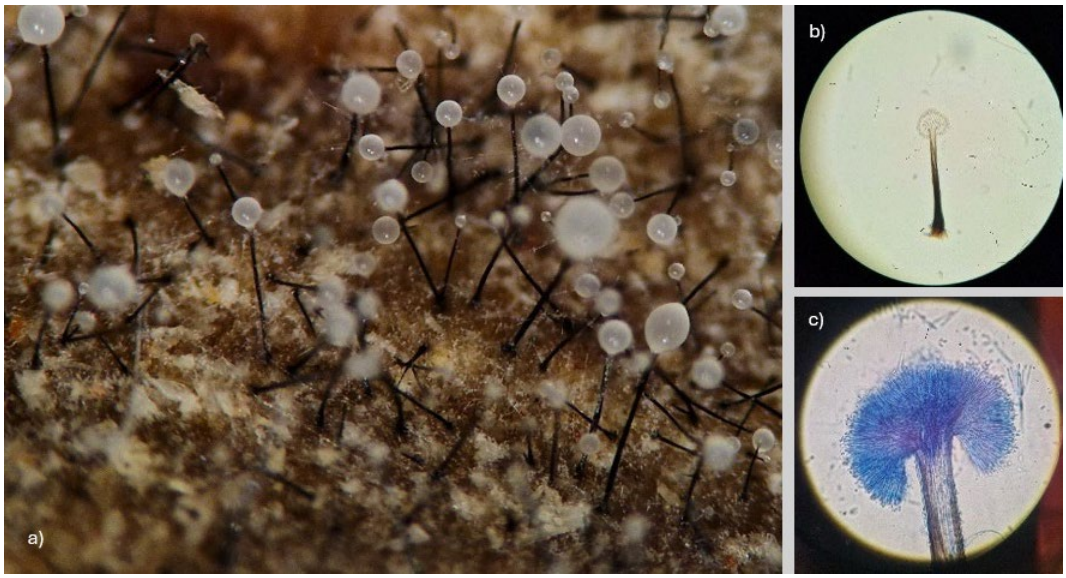

Figure 2: Conidiophores of O. quercus: a) showing several conidiophores on the disc, b/ c) give a close-up picture of the conidiophores (Source: Dusan Sadikovic and Sezer Olivia Kaya)

The conidiophores were isolated on MEA to obtain pure isolates of the fungi, and these isolates were used in the experiment described below.

2.2 Methods for the *in vitro* experiment

To analyse the potential interactions between *Heterobasidion* spp. and *O. quercus*, an *in vitro* pairing experiment was performed at three different temperatures (5°C, 15°C and 20°C).

The pairing experiment included two isolates of each *Heterobasidion* spp., *H. annosum* (HA1 and HA2) and *H. parviporum* (HP1 and HP2), and two isolates of *O. quercus* (OP1 and OP2).

The isolates of *O. quercus* were obtained from wood of birch trees in Åsa Experimental Forest, Småland and in Trehörna, Blekinge. The HA1 isolate were collected from *Betula pendula* in Russia (98040/2) and provided by R. Avramenko. HA2 originated from Norway Spruce in Italy (99080/1) supplied by K. Korhonen. The HP1 isolate was obtained from Norway Spruce in Russia (99049/3) by M. Lindgren and HP2 was collected from Norway Spruce in Italy (99077/1) by K. Korhonen.

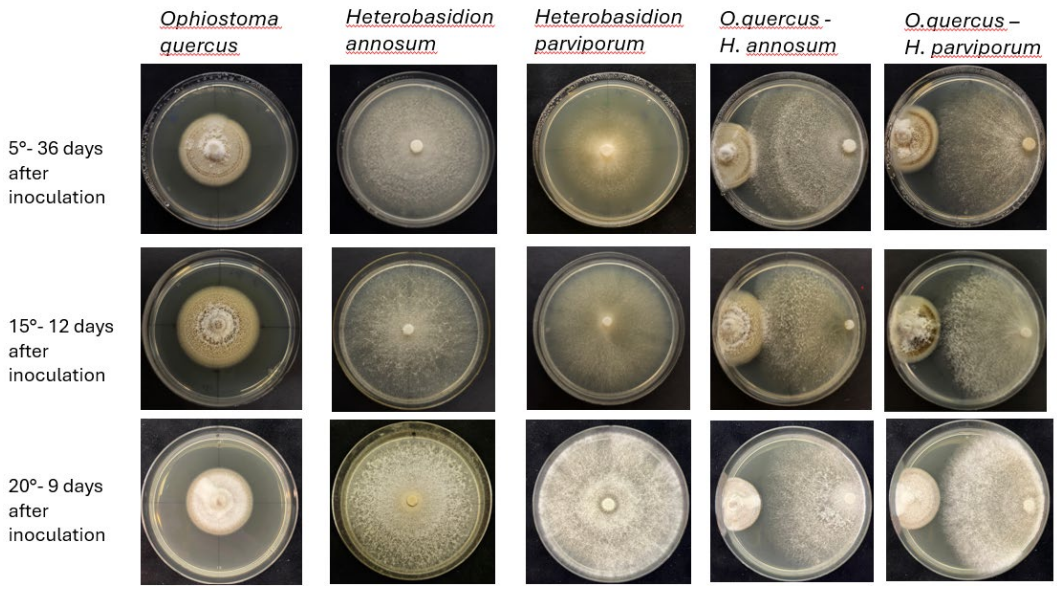

Figure 3: Growth of different treatments at three temperatures on the day the two fungi met

Control plates were prepared for each species in addition to the pairings for each combination of species interactions (Figure 3).

2.3 Execution of the experiment

To begin the experiment, Petri dishes were labelled accordingly, and reference lines were drawn on the bottom of each dish to facilitate the growth monitoring later on (Figure 4).

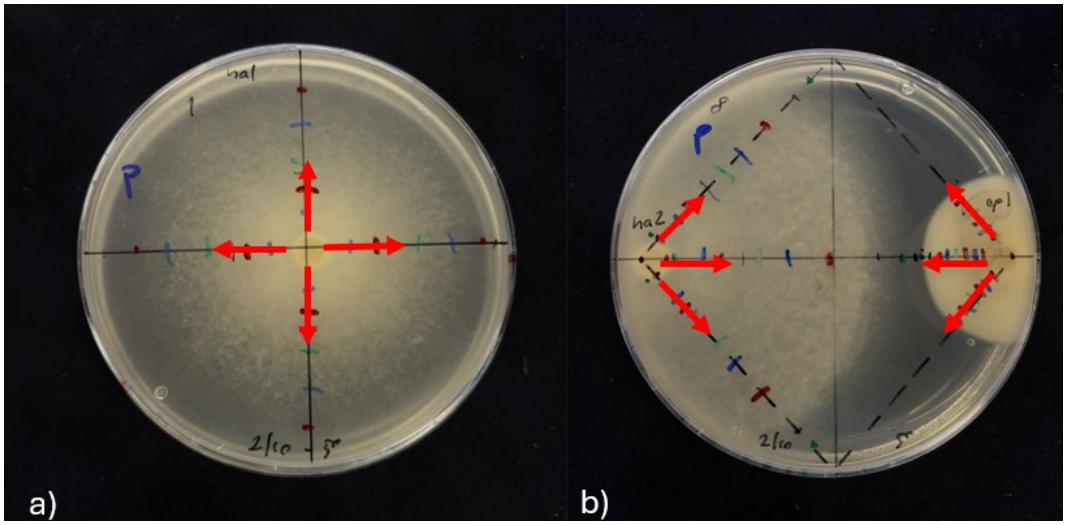

Figure 4: Drawings on the plates to facilitate growth measurement. a) shows the control plate with the arrows indicating the four growth measurements, from which the mean of these four measurements was calculated for data analysis. b) shows the pairing plate with the arrows indicating the three growth measurements for each fungus, from which the mean was calculated for data analysis.

After the drawings on plates were done, malt extract agar (MEA) was prepared according to the manufacturer´s instructions (VWR International AB) and poured halfway into the plates, which were then left open for sterilisation under UV light. Next, the inoculation process started under aseptic conditions in a laminar flow cabinet. The tools, including a cork borer No. 2 (6.25 mm) and scalpels, were sterilised using a Steri 350 instrument steriliser and were allowed to cool down. Fungal plugs were taken using the cork borer from the actively growing edges of each fungal colony, which were between 2-4 weeks old at the time of the transfer. These plugs were then transferred to the Petri dishes using the tip of a sterilised scalpel and placed according to the experimental design.

The control treatment had the fungal plug of each of the four isolates placed in the middle of the plate. For the paired plates, a plug of *O. quercus* was placed on the left side, at 0,5 cm from the edge, and a plug of *Heterobasidion* spp. was placed on the right side, at also 0,5 cm from the edge. For each control and pairing treatment, 8 replicates were prepared.

After all plugs were successfully transferred, Petri dishes were sealed with parafilm and stored at either 5°C; 15°C or 20°C. These specific temperatures were chosen for the dual test because 20°C is close to the optimal temperature range for both fungi, 15°C was chosen to represent the cooler climate of Sweden where the experiment is conducted, and 5°C was included because this is the lowest temperature at which fungi can still grow. The growth of the fungi was measured daily at 20°C for up to nine days until the growth plateau was reached. Growth was measured only every second day at 15°C for 12 to 18 days and at 5 °C for 24 to 67 days, as growth was significantly slower at the lower temperature. Fungal growth was measured by marking the furthest point of radial expansion along pre-drawn reference lines on the bottom of each plate. Measurements were then taken using a caliper and recorded for each observation.

2.4 Analysis

After obtaining the growth measurements, the data was compiled and cleaned in Excel. Data were analyzed using R (version 4.3.2) (R Core Team 2025) and R studio (Posit team 2023), with the aid of several packages including `tidyverse` (Wickham *et al.* 2019) `lme4` (Bates *et al.* 2015) `emmeans` (Lenth 2025), car (Weisberg 2019) and `ggplot2` (Wickham 2016). The growth rate for each combination of treatment and replicate was calculated using the following formula:

Growth Rate = (Maximum Length - Minimum Length) / Elapsed Time

In this formula, elapsed time refers to the number of days between the first and the last measurement within the defined growth period.

A linear model (`lm()`) was used to analyse the effect of treatment on fungal growth rate, with treatment included as a categorical fixed effect. Pairwise post-hoc comparisons between treatments were performed using estimated marginal means (`emmeans`), with Tukey adjustment used to consider multiple comparisons.

To assess the normality and homogeneity of variance of the data, the Shapiro-Wilk normality test and Levene's test were conducted, respectively. When the assumptions of normality and homogeneity were not met, a log transformation was applied to the growth rate data to attempt to improve the distribution. For all visualizations, the original (untransformed) data were used to maintain interpretability.

3. Results

The statistical analysis was performed to determine if there was an antagonistic interaction between *Heterobasidion* spp. and *Ophiostoma quercus* at one of the three temperatures tested (5°C, 15°C and 20°C).

Each treatment was assigned a label to facilitate statistical analysis.

Table 1: Treatment labelling

	Abbreviation	Treatment
Control groups	OP1	*O. quercus* isolate 1
	OP2	*O. quercus* isolate 2
	HA1	*H. annosum* isolate 1
	HA2	*H. annosum* isolate 2
	HP1	*H. parviporum* isolate 1
	HP2	*H. parviporum* isolate 2
Pairing groups	HA1xOP1	*H. annosum* isolate 1 X *O. quercus* isolate 1
	HA2xOP1	*H. annosum* isolate 2 X *O. quercus* isolate 1
	HP1xOP1	*H. parviporum* isolate 1 X *O. quercus* isolate 1
	HP2xOP1	*H. parviporum* isolate 2 X *O. quercus* isolate 1
	HA1xOP2	*H. annosum* isolate 1 X *O. quercus* isolate 2
	HA2xOP2	*H. annosum* isolate 2 X *O. quercus* isolate 2
	HP1xOP2	*H. parviporum* isolate 1 X *O. quercus* isolate 2
	HP2xOP2	*H. parviporum* isolate 2 X *O. quercus* isolate 2

3.1 Results at 5°C

3.1.1 *Heterobasidion annosum* at 5°C

The growth of *H. annosum* (HA) was analysed across six treatment groups, involving two isolates: HA1 and HA2.

Pairwise comparisons of growth rates between treatment groups revealed significant differences ($p<0.05$), summarized in Table 2. The growth rate of *H. annosum* was significantly lower in treatment HA2 x OP1 and HA2 than all other treatment groups. Treatment HA1 x OP2 had a significantly higher growth of *H. annosum* compared to all isolates of HA2 across all treatments and isolate HA1.

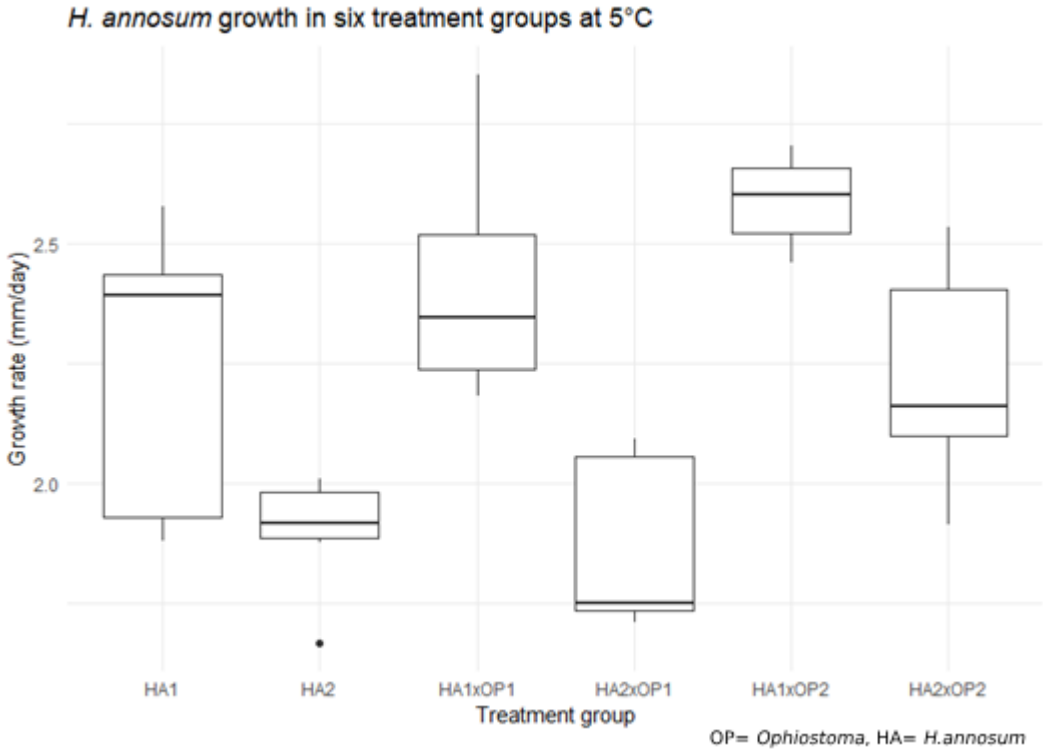

Figure 5: Growth rate of H. annosum in six treatment groups at 5°C

Table 2: Mean daily growth rates (mm/day) of H. annosum in six treatment groups at 5°C, with letters indicating significant differences based on Tukey's HSD post-hoc test ($p < 0.05$)

Treatment	Growth rate/day (mm)	Significance letters
HA2 x OP1	1.88	a
HA2	1.91	a
HA2 x OP2	2.23	b
HA1	2.25	b
HA1 x OP1	2.42	bc
HA1 x OP2	2.59	c

3.1.2 *Heterobasidion parviporum* at 5°C

The growth of *H. parviporum* (HP) was analysed across six treatment groups, involving two isolates: HP1 and HP2.

Pairwise comparisons of growth rates between treatment groups revealed significant differences ($p<0.05$), summarized in Table 3. The growth rate of isolate HP2 was significantly higher than that of isolate HP1 across all treatment groups, including the control and paired treatments.

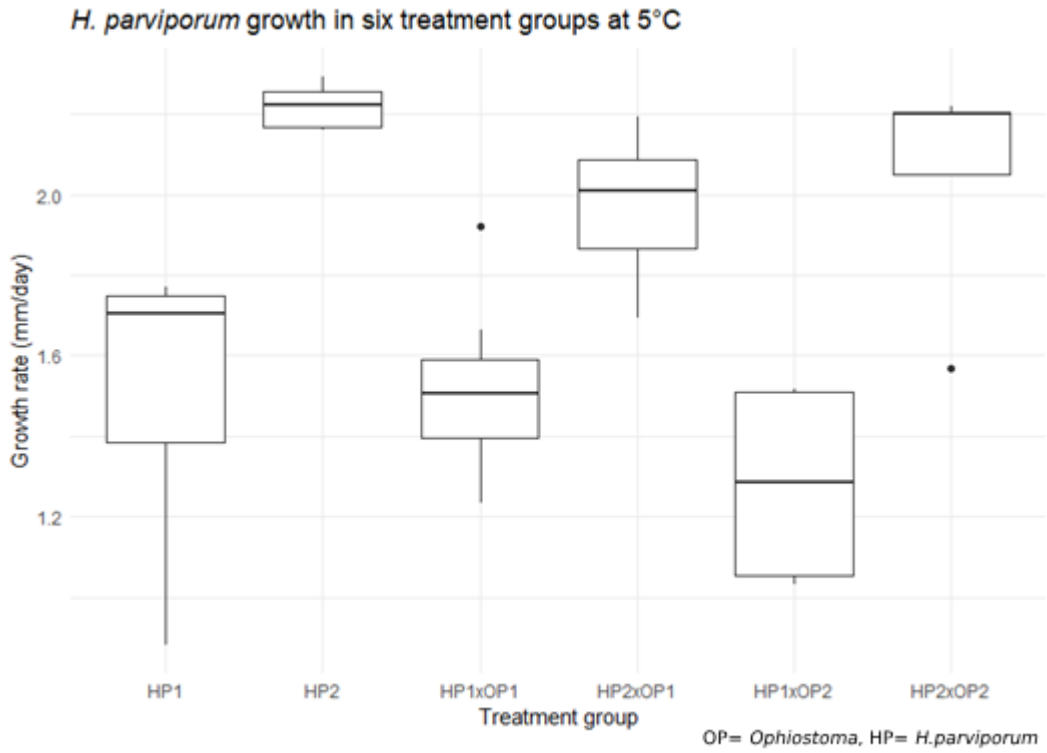

Figure 6: Growth rate of H. parviporum in six treatment groups at 5°C

Table 3: Mean daily growth rates (mm/day) of H. parviporum in six treatment groups at 5°C, with letters indicating significant differences based on Tukey's HSD post-hoc test ($p < 0.05$)

Treatment	Growth rate/day (mm)	Significance letter
HP1 x OP2	1.28	a
HP1 x OP1	1.52	a
HP1	1.54	a
HP2 x OP1	1.97	b
HP2 x OP2	2.07	b
HP2	2.22	b

In six out of eight replicates, *O. quercus* in the HP2 x OP1 treatment developed a darker pigmentation at the interaction zone with *H. parviporum*, as well as an elevated growth of HP2 when the two fungi met.

Similarly, *O. quercus* in treatment HP2 x OP2 showed a darker pigmentation in 6 out of 8 replicates at the edge where it met with *H. parviporum* and an elevated mycelial growth of HP2, when the two fungi met.

3.1.3 *Ophiostoma quercus* at 5°C

The growth of *O. quercus* (OP) was analysed across ten treatment groups, including two isolates: OP1 and OP2.

Pairwise comparisons of growth rates between treatment groups revealed significant differences ($p<0.05$), summarized in Table 4. The growth rate of *O. quercus* in the control treatment OP1 was significantly higher than the growth rate in paired treatments HA1 x OP1 and HA1x OP2. In addition, the growth rate of *O. quercus* in control treatment OP2 was significantly higher than in the paired treatment HA1 x OP2. The highest growth rate of *O. quercus* was observed in paired treatment HP2xOP2 (0.726 mm/day). This was significantly higher than growth rate in control treatment OP1 and paired treatments HA1xOP1 and HA1xOP2.

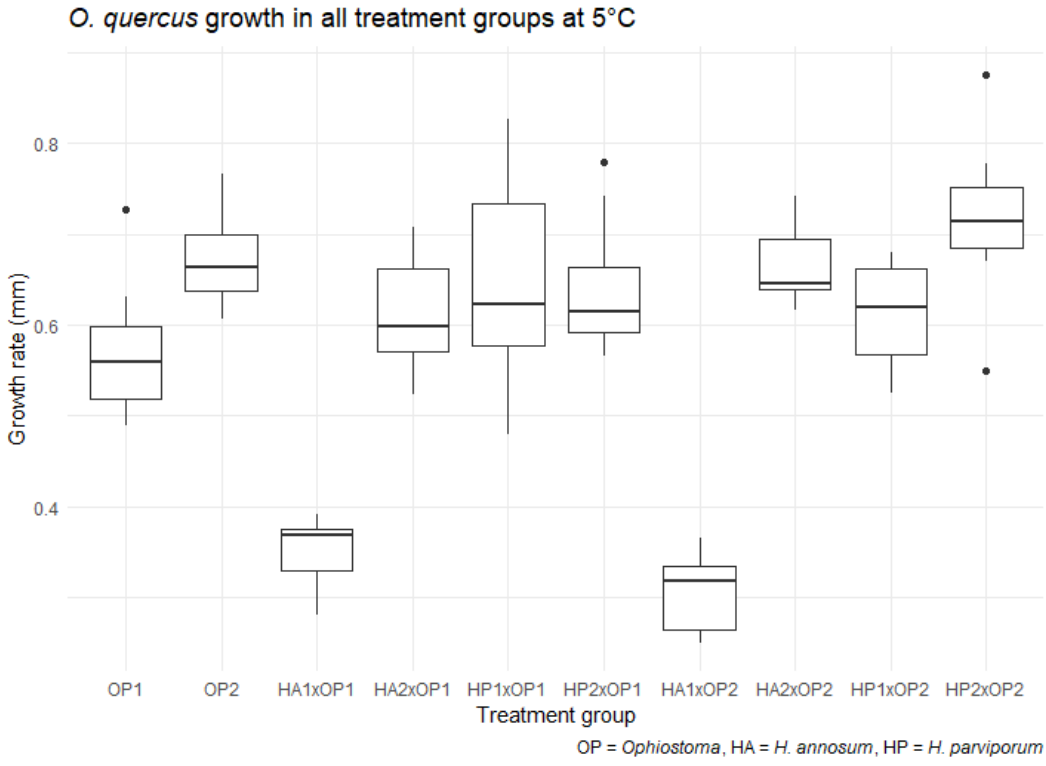

Figure 7:Growth rate of O.quercus in ten treatment groups at 5°C

Table 4: Mean daily growth rates (mm/day) of O. quercus in ten treatment groups at 5°C, with letters indicating significant differences based on Tukey's HSD post-hoc test ($p < 0.05$)

Treatment	Growth rate/day (mm)	Significance letter
HA1 x OP2	0.305	a
HA1 x OP1	0.351	a
OP1	0.571	a
HP1 x OP2	0.611	bc
HA2 x OP1	0.614	bc
HP2 x OP1	0.642	bc
HP1 x OP1	0.649	bc
HA2 x OP2	0.667	bc
OP2	0.672	bc
HP2 x OP2	0.726	c

3.2 Results at 15°C

3.2.1 *Heterobasidion annosum* at 15°C

The growth of *H. annosum* (HA) was analysed across six treatment groups, involving two isolates: HA1 and HA2.

Pairwise comparisons of growth rates between treatment groups revealed significant differences ($p<0.05$), summarized in Table 5. The growth rate of isolate HA1 was significantly higher than that of isolate HA2 across all treatment groups, including the control and paired treatments. In addition, growth rate of *H. annosum* was significantly lower in control isolate HA2 than in paired treatment HA2xOP1.

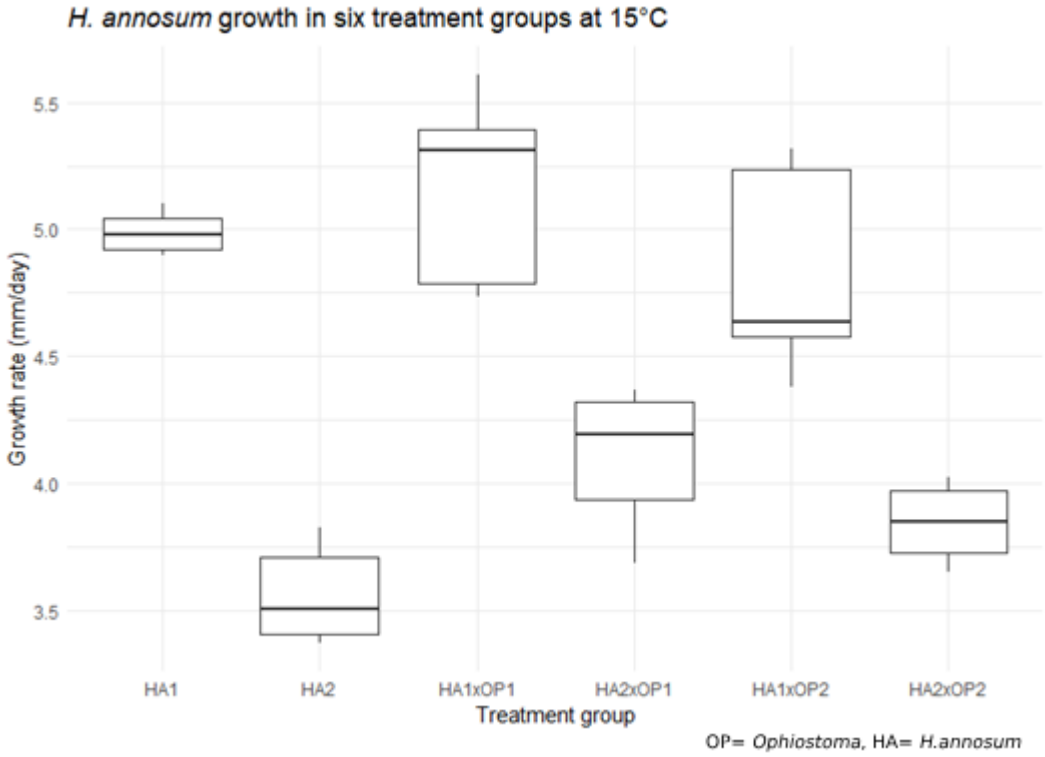

Figure 8: Growth rate of H. annosum in six treatment groups at 15°C

Table 5: Mean daily growth rates (mm/day) of H. annosum in six treatment groups at 15°C, with letters indicating significant differences based on Tukey's HSD post-hoc test ($p < 0.05$)

Treatment	Growth rate/day (mm)	Significance letter
HA2	3.56	a
HA2 x OP2	3.85	ab
HA2 x OP1	4.12	b
HA1 x OP2	4.83	c
HA1	4.99	c
HA1 x OP1	5.17	c

3.2.2 *Heterobasidion parviporum* at 15°C

The growth of *H. parviporum* (HP) was analysed across six treatment groups, involving two isolates: HP1 and HP2.

Pairwise comparisons of growth rates between treatment groups revealed significant differences ($p<0.05$), summarized in Table 6. The growth rate of isolate *H. parviporum* in the paired treatment HP1xOP1 was the highest with 4.20 mm/day and was significantly higher than the growth rates *H. parviporum* in the control group HP2 and in the HP2xOP2 pairing.

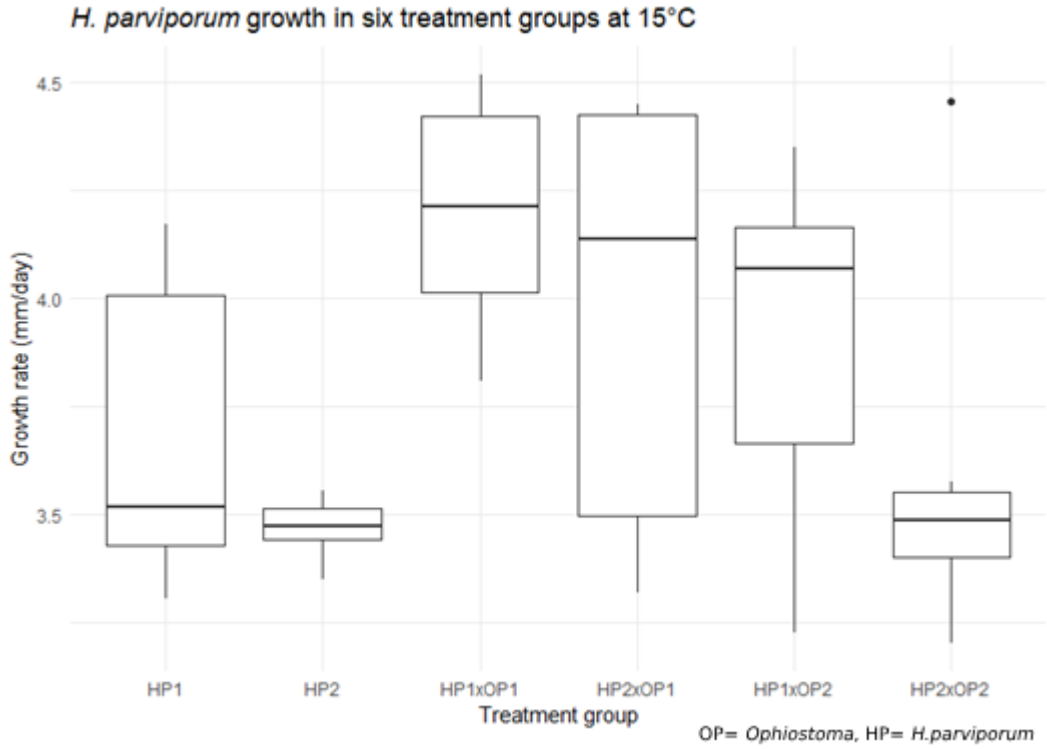

Figure 9: Growth rate of H. parviporum in six treatment groups at 15°C

Table 6: Mean daily growth rates (mm/day) of H. parviporum in six treatment groups at 15°C, with letters indicating significant differences based on Tukey's HSD post-hoc test ($p < 0.05$)

Treatment	Growth rate/day (mm)	Significance letter
HP2	3.47	a
HP2 x OP2	3.56	a
HP1	3.67	ab
HP1 x OP2	3.90	ab
HP2 x OP1	3.98	ab
HP1 x OP1	4.20	b

All replicates of HP2 x OP1 showed hyaline appearance of *H. parviporum* near the inoculation point, which changed to an orange discolouration and then turned white. In addition, a darker pigmentation of *O. quercus* was observed when the two fungi met.

This colour transition in *H. parviporum* was also observed in treatment HP2 x OP2. In three of the eight HP1 × OP2 replicates, HP1 showed strong mycelial growth near the inoculation point, followed by a zone of sparse growth, which then transitioned back to denser growth. Additionally, a darker pigmentation of *O. quercus* was observed when the two fungi met.

In four out of eight replicates in the treatment HP1 x OP1 a dark pigmentation was observed at the edge of *O. quercus.*

3.2.3 *Ophiostoma quercus* at 15°C

The growth of *O. quercus* (OP) was analysed across ten treatment groups, involving two isolates: OP1 and OP2.

Pairwise comparisons of growth rates between treatment groups revealed significant differences ($p<0.05$), summarized in Table 7. The growth of *O. quercus* was significantly higher in the control isolate OP2 than in the HA1x OP2 treatment, in addition, the control treatment OP1 had significantly higher growth of *O. quercus* than HA1xOP1.

HA1xOP1 and HA1xOP2 showed the lowest growth rate of *O.quercus*.

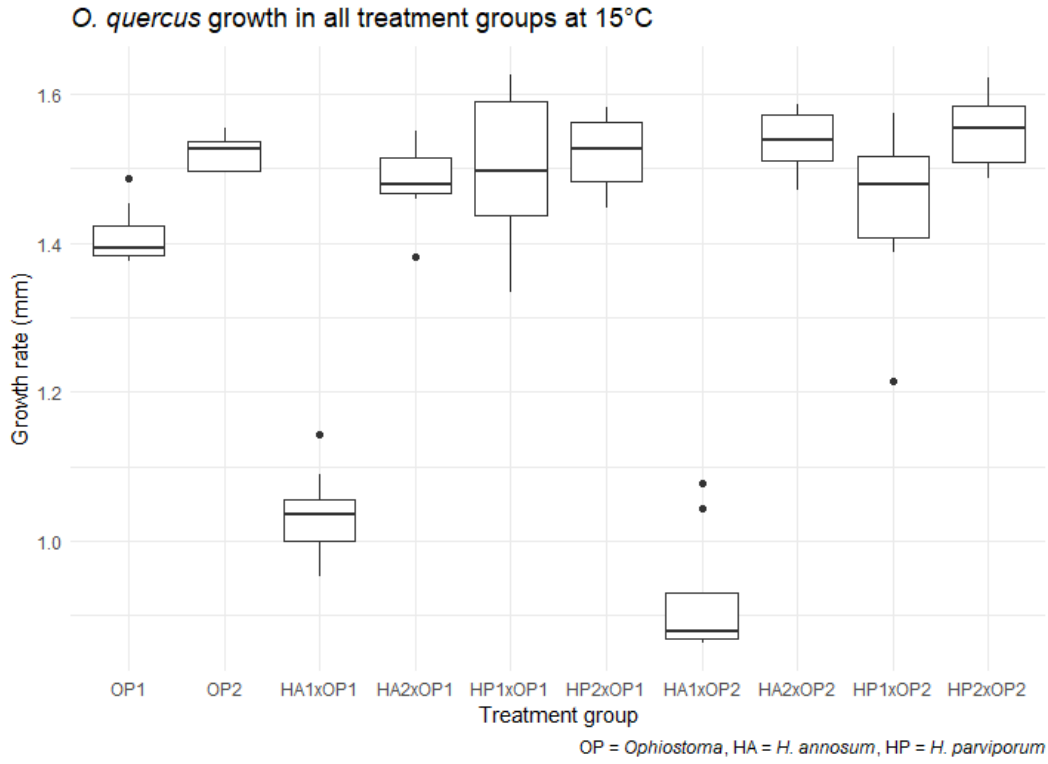

Figure 10: Growth rate of O. quercus in ten treatment groups at 15°C

Table 7: Mean daily growth rates (mm/day) of O. quercus in ten treatment groups at 15°C, with letters indicating significant differences based on Tukey's HSD post-hoc test ($p < 0.05$)

Treatment	Growth rate/day (mm)	Significance letter
HA1 x OP2	0.921	a
HA1 x OP1	1.036	b
OP1	1.410	c
HP1 x OP2	1.446	cd
HA2 x OP1	1.481	cd
HP1 x OP1	1.497	cd
HP2 x OP1	1.522	cd
OP2	1.522	cd
HA2 x OP2	1.537	d
HP2 x OP2	1.551	d

3.3 Results at 20°C

3.3.1 *Heterobasidion annosum* at 20°C

The growth of *H. annosum* (HA) was analysed across six treatment groups, involving two isolates: HA1 and HA2.

Pairwise comparisons of growth rates between treatment groups revealed no significant differences, summarized in Table 8. Treatment HA2xOP1 showed the highest growth rate of HA with 6.79 cm, but there was no significant difference between the treatment groups ($p>0.05$).

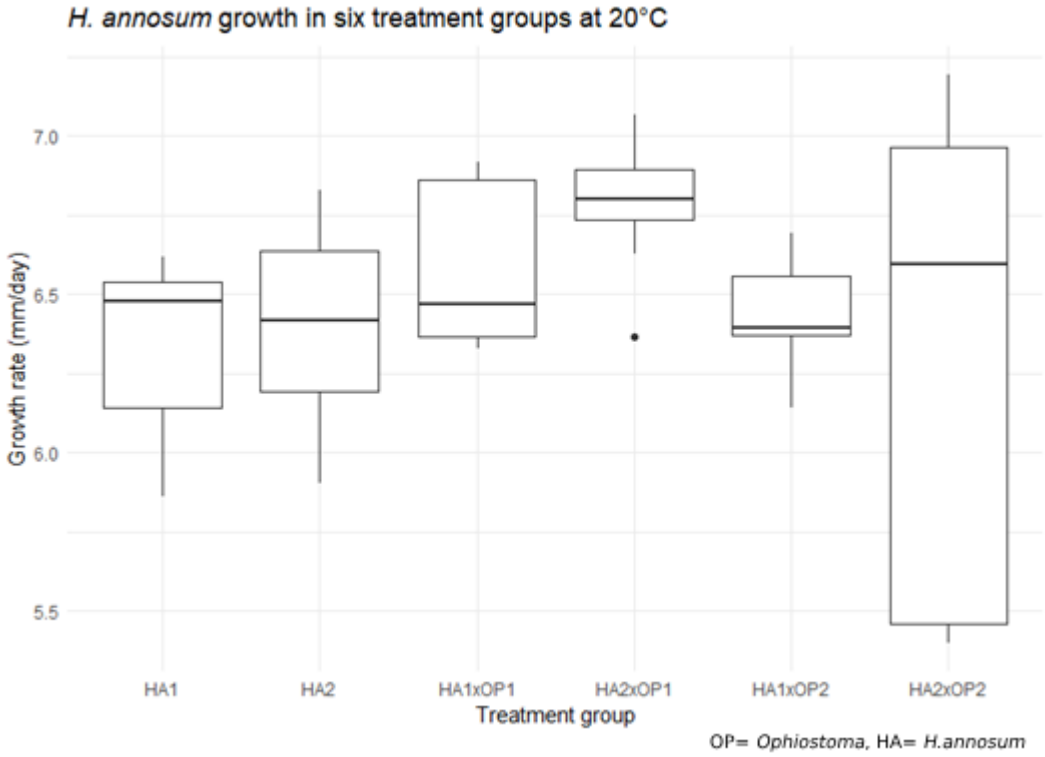

Figure 11: Growth rate of H. annosum in six treatment groups at 20°C

Table 8: Mean daily growth rates (mm/day) of H. annosum in six treatment groups at 20°C, with letters indicating significant differences based on Tukey's HSD post-hoc test (p > 0.05)

Treatment	Growth rate/day (mm)	Significance letter
HA2 x OP2	6.33	a
HA1	6.34	a
HA2	6.40	a
HA1 x OP2	6.44	a
HA1 x OP1	6.58	a
HA2 x OP1	6.79	a

3.3.2 *Heterobasidion parviporum* at 20°C

The growth of *H. parviporum* (HP) was analysed across six treatment groups, involving two isolates: HP1 and HP2

Pairwise comparisons of growth rates between treatment groups revealed significant differences ($p<0.05$), summarized in Table 9. The HP1xOP1 treatment had the highest growth rate of *H. parviporum* and was significantly higher than the control isolate of HP1 and HP2 x OP1. HP2 x OP1 was significantly lower than the treatments HP2 x OP2, HP1 x OP2, HP1 x OP1.

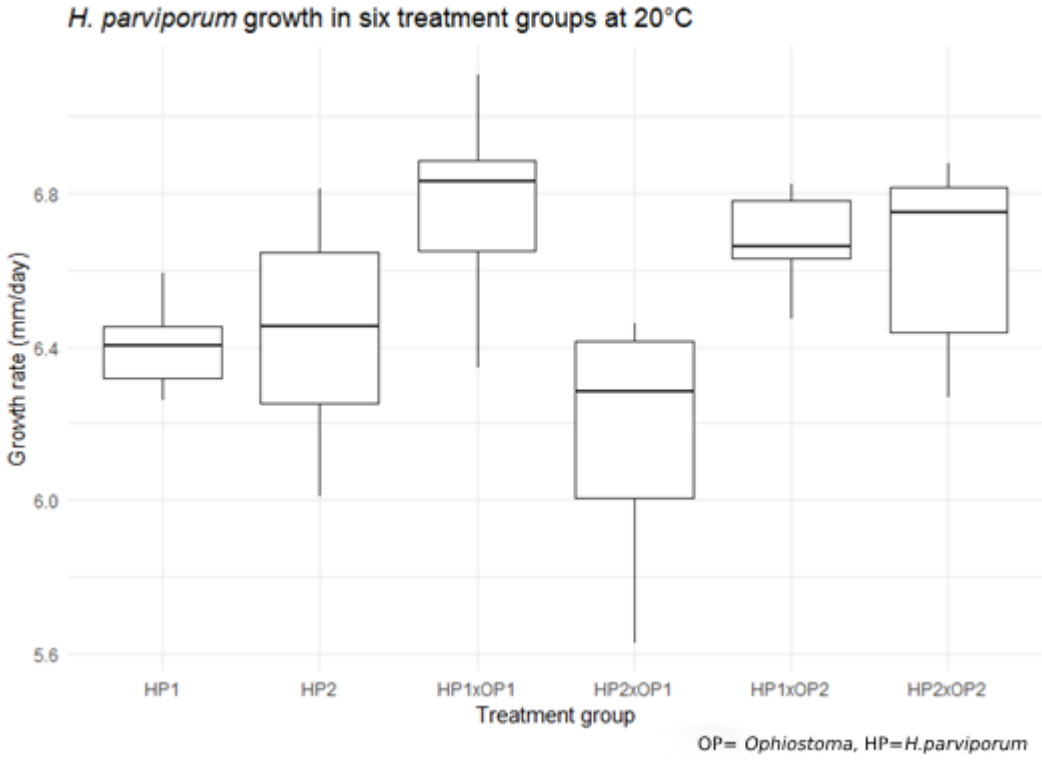

Figure 12: Growth rate of H. parviporum in six treatment groups at 20°C

Table 9: Mean daily growth rates (mm/day) of H. parviporum in six treatment groups at 20°C, with letters indicating significant differences based on Tukey's HSD post-hoc test ($p < 0.05$)

Treatment	Growth rate/day (mm)	Significance letter
HP2 x OP1	6.17	a
HP1	6.41	ab
HP2	6.43	abc
HP2 x OP2	6.63	bc
HP1 x OP2	6.67	bc
HP1 x OP1	6.77	c

3.3.3 *Ophiostoma quercus* at 20°C

The growth of *O. quercus* (OP) was analysed across ten treatment groups, involving two isolates: OP1 and OP2.

Pairwise comparisons of growth rates between treatment groups revealed significant differences ($p<0.05$), summarized in Table 13. The growth of *O. quercus* was significantly higher in the control isolate OP2 than in the treatments HA1 x OP2, HP1 x OP2 and HP2 x OP2. In addition, the growth rate of *O. quercus* in control isolate OP1 was significantly higher than HA1 x OP1 and HP1 x OP1. The lowest growth of *O. quercus* was observed at treatment HA1 x OP2 and HA1 x OP1.

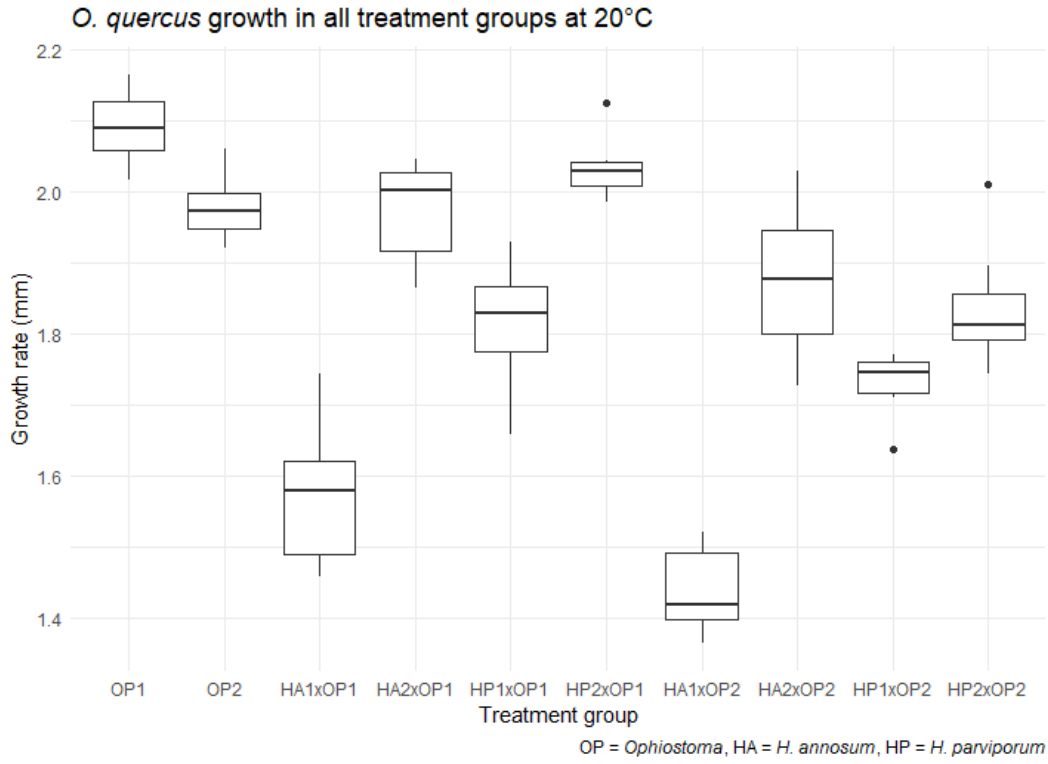

Figure 13: Growth rate of O. quercus in ten treatment groups at 20°C

Table 10: Mean daily growth rates (mm/day) of O.quercus in ten treatment groups at 20°C, with letters indicating significant differences based on Tukey's HSD post-hoc test ($p < 0.05$)

Treatment	Growth rate/day (mm)	Significance letter
HA1 x OP2	1.44	a
HA1 xOP1	1.57	b
HP1 x OP2	1.73	c
HP1 x OP1	1.82	cd
HP2 x OP2	1.84	cd
HA2 x OP2	1.88	de
HA2 x OP1	1.98	ef
OP2	1.98	ef
HP2 x OP1	2.04	f
OP1	2.09	f

3.4 Growth rate of *Heterobasidion* spp in all temperatures

The growth rate of *H. annosum* in all temperatures showed that 5°C had the lowest growth rate and 15°C and 20°C had a higher growth rate, showing similar growth pattern.

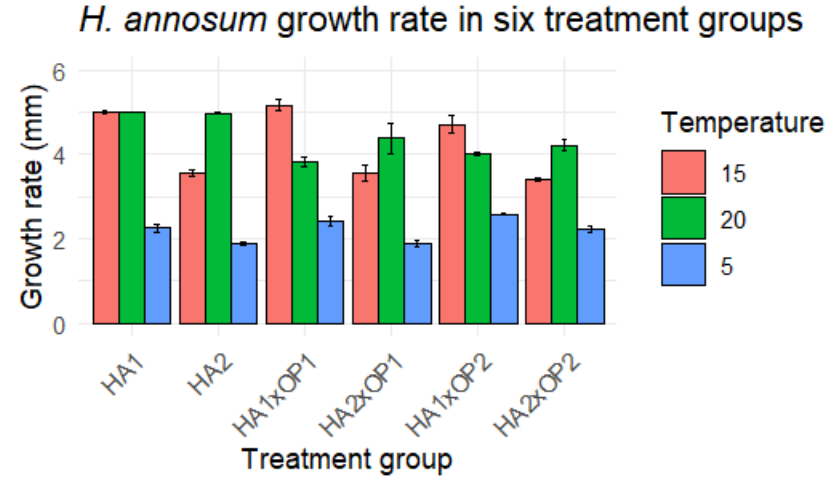

Figure 14: H. annosum growth rate per day across all treatments in all temperature groups

H. parviporum revealed the lowest growth rate for 5°C and the highest growth rate in 20°C. The growth rate of *H. parviporum* at 20°C in the paired treatment was higher than the growth in the control groups.

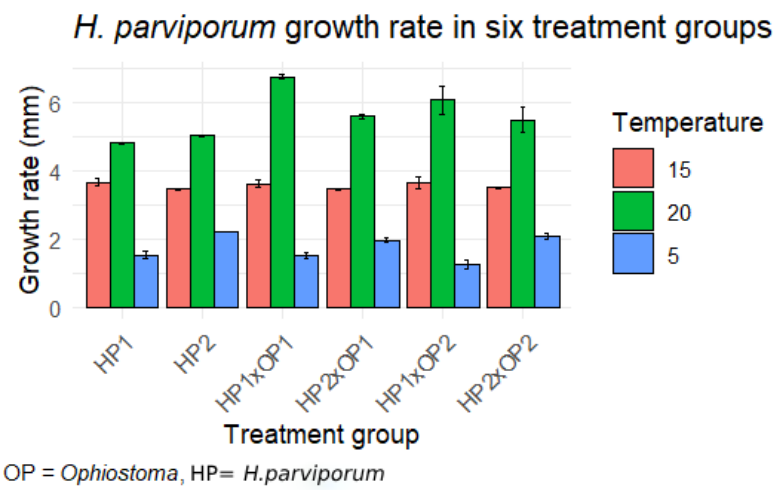

Figure 15: H. parviporum growth rate per day across all treatments in all temperature groups

4. Discussion

Growth rate analysis showed a similar growth pattern at15°C and 20°C.

H. parviporum grew more in most of the paired treatments in contrast to the control treatments at all three temperatures. Similarly, many paired treatments showed a higher growth of *H. annosum* compared to control treatments, suggesting that the presence of *Ophiostoma quercus* increased the growth of both *Heterobasidion* species. In addition, varying growth rate of isolates was observed at 5°C and 15°C, with HP1 growing slower than HP2 at 5°C and HA1 growing faster than HA2 at 15°C, demonstrating the high intraspecific variability. Meaning that there is a wide range of differences between individuals even though they belong to the same species.

The growth rate of *O. quercus* was similar across all three temperatures, with control isolates growing faster than the paired treatments on some occasions (for example at 20°C), which is the opposite of the effect observed in *Heterobasidion* and thus supports the result that *Heterobasidion* growth increased when paired with *O. quercus*.

Several trends emerged in paired treatments. Both at 15°C and 20°C, *O. quercus* showed the least growth when paired with HA1. However, when *O. quercus* was alone, it grew faster. One explanation for this is the strong growth of *H. annosum* on the paired plates slowing down the growth of *O. quercus*.

In addition, when *O. quercus* is paired with *H. parviporum*, the appearance of *H. parviporum* and occasionally of *O. quercus* has changed. Pigmentation of other fungus species in paired treatments was observed by Ujor *et al.* (2012), in which he mentioned that the interspecific mycelial combat is characterised by certain alterations, such as the change in pigmentation and the formation of barrage (elevated mycelium) observed in this study. This is a fungal response that enables them to recognize and react to foreign mycelia, helping them defend their colonized substrate and resources.

Statistical analysis did not show an effect of *O. quercus* on *Heterobasidion* spp. At none of the three temperatures (5°C, 15°C and 20°C) the growth rate of *Heterobasidion* spp. in paired treatment was significantly reduced compared to the control plates, suggesting that *O. quercus* does not decrease the development of *Heterobasidion*. This result therefore does not support the hypothesis proposed initially which suggested that *the presence of O. quercus negatively influence the growth and development of Heterobasidion spp.*

However contrary to expectations, in some cases *Heterobasidion* spp. grew more slowly on the control plates (inoculated alone) than in the paired treatments with *O. quercus*. This rather contradictory pattern suggests that under certain conditions the presence of *O. quercus* promotes rather than restricts the growth of *Heterobasidion.*

Several studies have shown that *Heterobasidion*, when paired with another organism (bacteria or fungi, such as *Phlebiopsis gigantea*), exhibits reduced mycelial growth and decreased colonisation on trees, when paired with an organism that is antagonistic to *Heterobasidion* spp. (Łakomy *et al.* 1998; Napierala-Filipiak & Werner 2000; Roy *et al.* 2001).

The variations in growth between isolates which can be clearly observed in 15°C for *H. annosum*, and 5°C for *H. parviporum* highlights the high intraspecific variability in growth of *Heterobasidion*. It shows that different traits of fungi such as life strategies can vary greatly within a species. These may include resource utilisation and different substrate use, and this may explain the differences in growth. This high intraspecific genetic variability in fungi has also been highlighted in a study by Koch *et al.* (2004), where numerous variations among isolates of the same species have been noted in arbuscular mycorrhizal fungi.

Variation among different isolates of *O. quercus*, *H. parviporum* and *H. annsoum* is also likely due to intraspecific genetic differences, as both genera are heterothallic and reproduce sexually, which promotes genetic recombination and diversity (Dyer & Kück 2017). Previous studies have shown that even within a species, fungal pathogens can differ in growth rate, morphology, and competitive ability due to their genetic background and ecological origin (Angeli *et al.* 2012). The findings support the idea that intraspecific variability can play a significant role in fungal interactions, with potential implications for understanding their behaviour in natural forest ecosystems or in biocontrol contexts.

Some of the observed differences may also reflect local adaptation or ecological specialization, as previously shown in other studies on fungal pathogens with broad host and habitat range (Bazzicalupo 2022).

The effect of temperature observed during the study was that at warmer temperatures (15°C and 20°C) similar growth patterns emerged, while 5°C, in contrast, showed very slow growth and different growth trends, can be linked to the temperature requirements of the fungi growth.

Heterobasidion has a wide temperature range in which it can grow. However, at warmer temperatures, the growth rate of the fungus increases, with the optimum temperature for *Hetereobasidion* growth varying between 24°C and 28°C according to Schwantes *et al.* (1976) and Negrutsky (1994). According to Taubert (2008) optimum temperature was between 17-22°C for *H. annosum* and 27°C for *H. parviporum*. The different optimal temperatures for *Heterobasidion* spp. show the intraspecific variation of growth rate. The temperatures used in our study, 15°C and 20°C are closer to these optimum temperatures and may reflect optimal growth. This temperature-dependent growth of *Heterobasidion* has important implications as the effects of climate change becomes more severe. As temperatures rise, conditions become more favourable to pathogens such as *Heterobasidion*. Warmer weather could help the fungus to expand its global range and increase its ability to

compete as well as the way in which the host and pathogen interact with each other (Coakley *et al.* 1999; Shen *et al.* 2024).

The lack of interaction observed in the study has also raised the question of why the infection rate of *Heterobasidion* spp. on the birch stumps at the study sites was very low. This could be due to several reasons, one of which is that the birch is not susceptible to get infected with *Heterobasidion* by primary infection (with spores), rather by secondary infection (root-to-root contact), as previous studies have shown that birch is affected by *Heterobasidion* infection when planted on previously infected sites (Lygis *et al.* 2004).

Direct studies on birch are limited, but it has been demonstrated that different tree species have different susceptibilities to primary infection, with some studies showing a low infection rate of birch with *Heterobasidion* spp. by primary infection (Gunulf *et al.* 2012; Brūna *et al.* 2021). This is likely because birch stumps provide only a brief window of suitable conditions, as they decay quickly and do not support successful colonization by *Heterobasidion* spores.

4.1 Limitations of study and future studies

The study had certain limitations that need to be considered. Since the study is an *in vitro* study, it only shows interactions that occurs in a controlled laboratory conditions and does not fully represent real-life situations where additional factors, such as changing environmental conditions and other organisms, play a role. Furthermore, this experiment was only conducted in one type of media (MEA). Different media may have different effects on fungi and could therefore lead to different growth trends, which was observed in a study by Olsson (2021). Finally, the study included only two isolates per species. A trend of different growth rates was seen for the different isolates, so a larger number of isolates could help to understand the growth and interaction patterns better.

Further studies need to be conducted to investigate the different growth trends that emerged in this study, in particular whether *O. quercus* affects the growth of *Heterobasidion in vivo.* To build on these results, future research should incorporate a greater number of isolates and test them across various types of media.

In addition, it is important to further investigate how problematic *Heterobasidion* can become for birch. Given birch's high potential as a species suitable for climate-adapted forest management, increased planting is expected. It is therefore essential to identify and address the challenges related to birch. (Lutter *et al.* 2021).

5. Conclusion

The aim of this study was to investigate the interaction between the two fungi *O. quercus* and *Heterobasidion* spp. and to determine whether *O. quercus* affects the growth of *Heterobasidion* spp. which could explain the low *Heterobasidion* incidence found on birch stumps. The results of the pairing experiment showed that there is an indication of an interaction between the two fungi in some cases, however contrary to expectations *O. quercus* appears to promote rather than restrict the growth of *Heterobasidion*.

Over the course of the study, other trends have emerged, such as different growth rates between isolates, an effect of temperature on growth and changes in visual appearance.

Additional studies need to be conducted to analyse these trends, and future *in vitro* studies should include more isolates as well as testing on different media.

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Appendix

Tabell 1: Pairwise comparison results for treatments including Heterobasidion annosum at 20°.Significant p-values are those < 0.05

PAIRWISE COMPARISONS OF TREATMENTS

20C HA

contrast	estimate	SE	df	t.ratio	p.value
HA1 - HA2	-0.06544	0.201	41	-0.326	0.9995
HA1 - HA1xOP1	-0.24592	0.201	41	-1.225	0.8221
HA1 - HA2xOP1	-0.45250	0.201	41	-2.254	0.2362
HA1 - HA1xOP2	-0.10375	0.208	41	-0.499	0.9959
HA1 - HA2xOP2	0.00367	0.201	41	0.018	1.0000
HA2 - HA1xOP1	-0.18048	0.201	41	-0.899	0.9445
HA2 - HA2xOP1	-0.38706	0.201	41	-1.928	0.4003
HA2 - HA1xOP2	-0.03831	0.208	41	-0.184	1.0000
HA2 - HA2xOP2	0.06910	0.201	41	0.344	0.9993
HA1xOP1 - HA2xOP1	-0.20658	0.201	41	-1.029	0.9054
HA1xOP1 - HA1xOP2	0.14217	0.208	41	0.684	0.9827
HA1xOP1 - HA2xOP2	0.24958	0.201	41	1.243	0.8130
HA2xOP1 - HA1xOP2	0.34875	0.208	41	1.678	0.5533
HA2xOP1 - HA2xOP2	0.45617	0.201	41	2.272	0.2286
HA1xOP2 - HA2xOP2	0.10742	0.208	41	0.517	0.9952

Tabell 2: Pairwise comparison results for treatments including Heterobasidion parviporum at 20°.Significant p-values are those < 0.05

20C HP

contrast	estimate	SE	df	t.ratio	p.value
HP1 - HP2	-0.0222	0.117	37	-0.190	1.0000
HP1 - HP1xOP1	-0.3649	0.117	37	-3.113	0.0386
HP1 - HP2xOP1	0.2358	0.127	37	1.862	0.4407
HP1 - HP1xOP2	-0.2679	0.127	37	-2.115	0.3019
HP1 - HP2xOP2	-0.2200	0.121	37	-1.813	0.4703
HP2 - HP1xOP1	-0.3427	0.117	37	-2.923	0.0605
HP2 - HP2xOP1	0.2580	0.127	37	2.038	0.3416
HP2 - HP1xOP2	-0.2456	0.127	37	-1.940	0.3954
HP2 - HP2xOP2	-0.1978	0.121	37	-1.629	0.5850
HP1xOP1 - HP2xOP1	0.6007	0.127	37	4.743	0.0004
HP1xOP1 - HP1xOP2	0.0971	0.127	37	0.766	0.9715
HP1xOP1 - HP2xOP2	0.1449	0.121	37	1.194	0.8368
HP2xOP1 - HP1xOP2	-0.5037	0.135	37	-3.720	0.0080
HP2xOP1 - HP2xOP2	-0.4558	0.130	37	-3.494	0.0147
HP1xOP2 - HP2xOP2	0.0479	0.130	37	0.367	0.9991

Tabell 3: Pairwise comparison results for treatments including Heterobasidion annosum at 15°.Significant p-values are those < 0.05

15C HA

contrast	estimate	SE	df	lower.CL	upper.CL	t.ratio	p.value
HA1 - HA2	1.429	0.140	40	1.0115	1.847	10.234	<.0001
HA1 - HA1xOP1	-0.178	0.129	40	-0.5646	0.209	-1.374	0.7419
HA1 - HA2xOP1	0.869	0.129	40	0.4821	1.256	6.720	<.0001
HA1 - HA1xOP2	0.165	0.129	40	-0.2220	0.552	1.275	0.7964
HA1 - HA2xOP2	1.142	0.129	40	0.7554	1.529	8.833	<.0001
HA2 - HA1xOP1	-1.607	0.140	40	-2.0251	-1.189	-11.506	<.0001
HA2 - HA2xOP1	-0.560	0.140	40	-0.9784	-0.142	-4.012	0.0033
HA2 - HA1xOP2	-1.265	0.140	40	-1.6825	-0.847	-9.053	<.0001
HA2 - HA2xOP2	-0.287	0.140	40	-0.7051	0.131	-2.056	0.3304
HA1xOP1 - HA2xOP1	1.047	0.129	40	0.6598	1.434	8.095	<.0001
HA1xOP1 - HA1xOP2	0.343	0.129	40	-0.0443	0.730	2.649	0.1089
HA1xOP1 - HA2xOP2	1.320	0.129	40	0.9330	1.707	10.207	<.0001
HA2xOP1 - HA1xOP2	-0.704	0.129	40	-1.0911	-0.317	-5.445	<.0001
HA2xOP1 - HA2xOP2	0.273	0.129	40	-0.1137	0.660	2.113	0.3016
HA1xOP2 - HA2xOP2	0.977	0.129	40	0.5904	1.364	7.558	<.0001

Tabell 4: Pairwise comparison results for treatments including Heterobasidion parviporum at 15°.Significant p-values are those < 0.05

15C HP -

contrast	estimate	SE	df	lower.CL	upper.CL	t.ratio	p.value
HP1 - HP2	0.2044	0.175	41	-0.320	0.728557	1.165	0.8504
HP1 - HP1xOP1	-0.5248	0.175	41	-1.049	-0.000666	-2.992	0.0495
HP1 - HP2xOP1	-0.3066	0.175	41	-0.831	0.217554	-1.748	0.5092
HP1 - HP1xOP2	-0.2268	0.182	41	-0.769	0.315794	-1.249	0.8100
HP1 - HP2xOP2	0.1087	0.175	41	-0.415	0.632895	0.620	0.9889
HP2 - HP1xOP1	-0.7292	0.175	41	-1.253	-0.205054	-4.158	0.0021
HP2 - HP2xOP1	-0.5110	0.175	41	-1.035	0.013166	-2.914	0.0598
HP2 - HP1xOP2	-0.4312	0.182	41	-0.974	0.111407	-2.375	0.1890
HP2 - HP2xOP2	-0.0957	0.175	41	-0.620	0.428507	-0.545	0.9938
HP1xOP1 - HP2xOP1	0.2182	0.175	41	-0.306	0.742390	1.244	0.8125
HP1xOP1 - HP1xOP2	0.2981	0.182	41	-0.245	0.840630	1.642	0.5766
HP1xOP1 - HP2xOP2	0.6336	0.175	41	0.109	1.157730	3.612	0.0099
HP2xOP1 - HP1xOP2	0.0798	0.182	41	-0.463	0.622410	0.440	0.9978
HP2xOP1 - HP2xOP2	0.4153	0.175	41	-0.109	0.939511	2.368	0.1914
HP1xOP2 - HP2xOP2	0.3355	0.182	41	-0.207	0.878065	1.848	0.4477

Tabell 5: Pairwise comparison results of transformed data for treatments including Heterobasidion parviporum at 15°.Significant p-values are those < 0.05

15C HP – transformed data

contrast	estimate	SE	df	lower.CL	upper.CL	t.ratio	p.value
HP1 - HP2	0.0538	0.0455	41	-0.0822	0.189812	1.182	0.8426
HP1 - HP1xOP1	-0.1355	0.0455	41	-0.2715	0.000474	-2.978	0.0513
HP1 - HP2xOP1	-0.0766	0.0455	41	-0.2126	0.059420	-1.683	0.5505
HP1 - HP1xOP2	-0.0588	0.0471	41	-0.1996	0.081942	-1.249	0.8101
HP1 - HP2xOP2	0.0309	0.0455	41	-0.1051	0.166869	0.678	0.9834
HP2 - HP1xOP1	-0.1893	0.0455	41	-0.3253	-0.053334	-4.161	0.0021
HP2 - HP2xOP1	-0.1304	0.0455	41	-0.2664	0.005612	-2.865	0.0669
HP2 - HP1xOP2	-0.1126	0.0471	41	-0.2534	0.028134	-2.391	0.1831
HP2 - HP2xOP2	-0.0229	0.0455	41	-0.1589	0.113061	-0.504	0.9957
HP1xOP1 - HP2xOP1	0.0589	0.0455	41	-0.0771	0.194950	1.295	0.7858
HP1xOP1 - HP1xOP2	0.0767	0.0471	41	-0.0641	0.217472	1.628	0.5853
HP1xOP1 - HP2xOP2	0.1664	0.0455	41	0.0304	0.302400	3.656	0.0088
HP2xOP1 - HP1xOP2	0.0177	0.0471	41	-0.1230	0.158526	0.377	0.9989
HP2xOP1 - HP2xOP2	0.1074	0.0455	41	-0.0286	0.243453	2.361	0.1940
HP1xOP2 - HP2xOP2	0.0897	0.0471	41	-0.0511	0.230478	1.904	0.4142

Tabell 6: Pairwise comparison results for treatments including Heterobasidion annosum at 5°.Significant p-values are those < 0.05

5C HA

contrast	estimate	SE	df	lower.CL	upper.CL	t.ratio	p.value
HA1 - HA2	0.3471	0.101	38	0.0453	0.6489	3.450	0.0162
HA1 - HA1xOP1	-0.1649	0.109	38	-0.4909	0.1611	-1.517	0.6555
HA1 - HA2xOP1	0.3766	0.104	38	0.0642	0.6890	3.617	0.0104
HA1 - HA1xOP2	-0.3360	0.101	38	-0.6378	-0.0342	-3.340	0.0215
HA1 - HA2xOP2	0.0215	0.104	38	-0.2909	0.3339	0.207	0.9999
HA2 - HA1xOP1	-0.5120	0.109	38	-0.8380	-0.1860	-4.712	0.0004
HA2 - HA2xOP1	0.0295	0.104	38	-0.2829	0.3419	0.283	0.9997
HA2 - HA1xOP2	-0.6831	0.101	38	-0.9849	-0.3813	-6.790	<.0001
HA2 - HA2xOP2	-0.3256	0.104	38	-0.6380	-0.0132	-3.127	0.0368
HA1xOP1 - HA2xOP1	0.5415	0.112	38	0.2057	0.8773	4.837	0.0003
HA1xOP1 - HA1xOP2	-0.1711	0.109	38	-0.4971	0.1548	-1.575	0.6194
HA1xOP1 - HA2xOP2	0.1864	0.112	38	-0.1494	0.5222	1.665	0.5623
HA2xOP1 - HA1xOP2	-0.7126	0.104	38	-1.0250	-0.4002	-6.844	<.0001
HA2xOP1 - HA2xOP2	-0.3551	0.108	38	-0.6777	-0.0325	-3.302	0.0238
HA1xOP2 - HA2xOP2	0.3575	0.104	38	0.0451	0.6699	3.433	0.0169

Tabell 7: Pairwise comparison results for treatments including Heterobasidion parviporum at 5°.Significant p-values are those < 0.05

5c HP

contrast	estimate	SE	df	lower.CL	upper.CL	t.ratio	p.value
HP1 - HP2	-0.6773	0.110	37	-1.0083	-0.3463	-6.147	<.0001
HP1 - HP1xOP1	0.0265	0.110	37	-0.3044	0.3575	0.241	0.9999
HP1 - HP2xOP1	-0.4284	0.110	37	-0.7594	-0.0975	-3.889	0.0050
HP1 - HP1xOP2	0.2630	0.135	37	-0.1424	0.6684	1.949	0.3901
HP1 - HP2xOP2	-0.5293	0.114	37	-0.8719	-0.1866	-4.641	0.0006
HP2 - HP1xOP1	0.7038	0.110	37	0.3728	1.0348	6.388	<.0001
HP2 - HP2xOP1	0.2488	0.110	37	-0.0821	0.5798	2.259	0.2367
HP2 - HP1xOP2	0.9403	0.135	37	0.5349	1.3457	6.968	<.0001
HP2 - HP2xOP2	0.1480	0.114	37	-0.1946	0.4907	1.298	0.7842
HP1xOP1 - HP2xOP1	-0.4550	0.110	37	-0.7860	-0.1240	-4.130	0.0025
HP1xOP1 - HP1xOP2	0.2364	0.135	37	-0.1689	0.6418	1.752	0.5077
HP1xOP1 - HP2xOP2	-0.5558	0.114	37	-0.8984	-0.2132	-4.873	0.0003
HP2xOP1 - HP1xOP2	0.6914	0.135	37	0.2861	1.0968	5.124	0.0001
HP2xOP1 - HP2xOP2	-0.1008	0.114	37	-0.4434	0.2418	-0.884	0.9480
HP1xOP2 - HP2xOP2	-0.7922	0.138	37	-1.2072	-0.3773	-5.736	<.0001

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