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Low Water Availability Increases Necrosis in *Picea abies* after Artificial Inoculation with Fungal Root Rot Pathogens *Heterobasidion parviporum* and *Heterobasidion annosum*

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Abstract: Research Highlights: Dedicated experiments to investigate how disturbances will affect *Heterobasidion* sp.—Norway spruce pathosystems are important, in order to develop different strategies to limit the spread of *Heterobasidion annosum* s.l. under the predicted climate change. Here, we report on a greenhouse experiment to evaluate the effects of water availability on the infection severity of *Heterobasidion parviporum* or *Heterobasidion annosum*, respectively, on *Picea abies* saplings. Background and Objectives: Changes in climatic conditions and intense logging will continue to promote *H. annosum* s.l. in conifer forests, increasing annual economic losses. Thus, our aim was to test if disease severity in Norway spruce was greater after infection with *H. parviporum* or *H. annosum* in low water availability conditions, compared to seedlings with high water availability. Materials and Methods: We performed inoculation studies of three-year-old saplings in a greenhouse. Saplings were treated as high (+) or low (−) water groups: High water group received double the water amount than the low water group. The necrosis observed after pathogen inoculation was measured and analyzed. Results: The seedling growth was negatively influenced in the lower water group. In addition, the water availability enhanced the necrosis length of *H. parviporum* in phloem and sapwood (vertical length) in the low water group. *H. annosum* benefited only in horizontal length in the phloem. Conclusions: Disturbances related to water availability, especially low water conditions, can have negative effects on the tree host and benefit the infection ability of the pathogens in the host.

Keywords: *Heterobasidion parviporum*; *Heterobasidion annosum*; Norway spruce; disturbance; water availability; pathogen; infection

1. Introduction

Projected climate change will increase the disturbances related to water availability in forests e.g., drought, wind and snow [1]. In addition to their direct negative effects on trees, such as timber quality losses, the weakening of the tree's immune system or early decay or death, the impacts of pests and pathogens in changing environments represent some of the most important threats to global forest health [1]. To mitigate these climate change induced disturbances, such as drought, it is important to

understand the factors that contribute to the development of forest tree disease epidemics and host susceptibility [2].

Norway spruce (*Picea abies* (L.) H. Karst.), together with Scots pine (*Pinus sylvestris* L.), forms the basis for raw materials of the forest sector in Europe, contributing to several billion EUR net income annually. Currently, Norway spruce covers 25% of the forest area in Germany [3]. Thus, interest in the biology and ecology of plant pathogenic fungi of conifers in forest ecosystems, with special emphasis on sustainable management strategies for forestry, is high. The main fungal pathogens causing devastating losses of wood quality in conifers are the root rot pathogens of the species complex *Heterobasidion annosum sensu lato* (s.l.) [4,5]. Consequently, *H. annosum* s.l. is considered the most dangerous and economically most significant root rot pathogen in coniferous forests [4,5]. *Heterobasidion* species induce root and stem rot and bark necrosis. Resin flow on the stem is one of the visible symptoms with which *H. annosum* s.l. can be detected in living trees (Figure 1). In the above-average warm years (2012 and 2018), when precipitation was low (average precipitation in Oerrel, Germany 2012: 201−250 mm; 2017: 301−350 mm) [6], these symptoms could be observed more regularly in Germany (Figure 1) [7]. The water balance as well was lower in this area in the year 2012 (0−49 mm) compared to the year 2017 (51−100 mm) [6]. The anamorph stage *Spiniger meineckellus* (A.J. Olson) Stalpers could be observed, proving infestation of roots, stems and other host tissues with *H. annosum* s.l. (Figure 1) [8,9].

Figure 1. Resin flow on the stem is one of the symptoms that indicates the possible infection of *H. annosum* s.l. Living Norway spruce, confirmed to be infected by *Heterobasidion annosum* s.l.; (**a**) resinating stem necrosis; (**b**) stem necrosis with removed bark. These symptoms were observed more in the year 2012 in a forest stand close to Oerrel, Germany.

Extensive logging of cultivated conifer forests has created a habitat favourable for this pathogen, which has dramatically increased the occurrence of infections. Primary infections by *Heterobasidion*

species are established when airborne basidiospores land and germinate on freshly cut stump surfaces and wounds [10–12]. Spore deposition is followed by rapid colonisation of the wood material. In Europe, three species of native *Heterobasidion* exist: *H. abietinum* Niemelä and Korhonen, *H. annosum* (Fr.) Bref. and *H. parviporum* Niemelä and Korhonen. All *Heterobasidion* species have different, but overlapping, host preferences, mainly associated with fir (*H. abietinum*), pine (*H. annosum*), and spruce (*H. parviporum*) [5,13,14]. In North-West Germany, only *H. annosum* and *H. parviporum* are present [9,15,16]. Differentiation between these two species can be performed with species-specific primers developed for boreal species of *H. parviporum* and *H. annosum* ([17], this study). Silvicultural control of *Heterobasidion* species is difficult because they cause secondary infections by spreading through root contacts to neighbouring trees [18,19]. This is the main pathway for infections to spread inside stands [9,16,20]. Furthermore, *Heterobasidion* species can remain viable and infective in stumps for decades [21–23], resulting in an inoculum source for new tree generations [23,24].

Changes in climatic conditions and intense logging will continue to promote *H. annosum* s.l. in conifer forests [1,25,26], increasing the economic losses on a yearly basis. Linnakoski et al. [2] could show already, that the fungal strain of *Endoconidiophora polonica* (Siemaszko) Z.W. de Beer, T.A. Duong and M.J. Wingf. caused greater disease severity in *P. abies* seedlings with low water availability compared to those with high water availability. Similarly, Gori et al. [27] found indications that *H. parviporum* infection makes *P. abies* trees more susceptible to drought stress in the field. Thus, dedicated experiments to investigate how disturbances will affect the *Heterobasidion* sp.—Norway spruce pathosystems are important, in order to develop different strategies to limit the spread of *H. annosum* s.l. under the predicted climate change.

Here, we report an in vivo experiment, conducted in a greenhouse in Germany, to evaluate the effects of water availability on the infection severity of *H. parviporum* or *H. annosum* in *P. abies* saplings during the growing season of 2018. Dimitri and Schumann [28] found that the infection incidence did not change with the age of inoculated ramets among clones of *P. abies*. This indicates that results from inoculation experiments (for at least some tree species) with seedlings may be applicable to trees. The aim of this research was to test if the low tolerance of Norway spruce to water scarcity will lead to greater disease severity caused by *H. parviporum* and *H. annosum*, when compared to seedlings with optimal water availability. Drought disturbances in European forests are predicted to increase [1]. Based on our observations in the field in the year 2012 (Figure 1), we hypothesize that *H. annosum* s.l. can cause greater necrosis in *P. abies* saplings under low water availability.

2. Materials and Methods

2.1. Plant and Fungal Material

Plant material consisted of 355 three-year-old, apparently healthy and vital, Norway spruce saplings purchased from the nursery Schlegel and Co Gartenprodukte (provenience of the saplings: No. 840 11, Thüringer Wald and Frankenwald, montane zone 600 m). Seedlings were potted onto 3-liter plastic pots filled with fertilized peat (Flora gard, TKS®2 Instant Plus, Hermann Meyer KG, Rellingen, Germany). Pots were placed into tables covered with plastic sheets where excess water could accumulate and be absorbed later. The potted saplings were acclimatized to the greenhouse conditions for 16-days prior to the water experiment, during which time they received tap water, as required, to maintain moist soil. No additional fertilization was given during the experiment. Before and after the experiment, the sapling height was measured to the nearest 0.1 cm.

2.2. Pre-Experiment Detection of Heterobasidion Species

Three of the saplings were transferred to North West German Forest Research Institute (Göttingen) in order to examine the pre-colonization in the internal tissues for pathogens and endophytes. Two different methods were used to detect *Heterobasidion* species: (1) Incubation method after Langer and Bressem [16]; (2) isolation of fungi on artificial agar media. Firstly, stem-disks (10 mm) cut from saplings (Figure 2) and

two randomly chosen root segments were pre-incubated in a refrigerator for four weeks and subsequently wrapped in moist newspaper. After incubation at room temperature for 14 days, the samples were analyzed under a binocular microscope for the presence of *S. meineckellus* [6,16]. Secondly, surface sterilized pieces of the trunk were placed on petri-dishes with Malt-Yeast-Peptone (MYP-agar, after Langer [29]). After 7 and 14 days they were checked for the presence of *H. annosum* s.l. conidiophores and conidiospores.

Figure 2. Pre-experiment detection of *Heterobasidion* species from Norway spruce saplings. The disks from spruce trunk were cut 3.5 cm above and 3.5 cm underneath the root neck, which is shown in the figure as a red line. The topmost 1 cm and the lowermost 1 cm of the trunk were stored in the refrigerator for pre-incubation.

The remaining trunk parts and root segments were surface sterilized (1 min in 70% ethanol/5 min 4% sodium hypochlorite/1 min 70% ethanol) and cut into units (0.5 cm). Three units each were plated on a petri dish filled with 1.5% MYP-agar. The presence for *H. annosum* s.l. and other fungi were checked after 7 and 14 days.

2.3. Fungal Material

Heterobasidion sp. strains were received from the North-West German Forest Research Institute, both collected by G. Langer and colleagues. *H. annosum* (strain NW-FVA 3492) was isolated from *P. abies* in 2016 (Germany, Lower Saxony, forest department Oerrel, Karrenbusch). *H. parviporum* (strain NW-FVA 0459) was isolated from *P. abies* in 2010 (Germany, Lower Saxony, forest department Oerrel, Bobenwald). Before inoculations, the fungal strains used in this study were tested for their specificity with species-specific primers developed for *H. parviporum* and *H. annosum* [17]. Similarly, to make sure the pathogen cultures were not contaminated, the DNA of both cultures were extracted and PCR was performed with ITS1-F and ITS4 primers and sequenced.

2.4. DNA Extractions, PCR and Sequencing

DNA of *H. parviporum* and *H. annosum* was extracted from 150 mg of the homogenized mycelium sample using the "innuPREP Plant DNA Kit" (Analytik Jena AG, Jena, Germany), according to the manufacturer's instructions. Species-specific primers (*H. annosum*, MJ-F and MJ-R [17]; *H. parviporum* KJ-F and KJ-R [17]) were used to confirm *Heterobasidion* species [17]. In brief, DNA template (100 ng), buffer (KCl extra buffer, 1×), 1.5 mM MgCl2, primers (each concentration of 0.5 µM), a dNTP-mix (each deoxynucleotide in a concentration of 200 µM) and 20 U/mL of DNA-polymerase (VWR) was adjusted to 25 µL reaction with autoclaved MQ H_2O. The cycling conditions used were: Initial denaturation 10 min at 95 °C, followed by 3 step cycling: Denaturation 30 s 95 °C; annealing 35 s 67 °C; extension 1 min 72 °C, number of cycles 40; final extension 7 min in 72 °C. Both species were run with both primer pairs. Taq DNA polymerase (Qiagen) was used for PCR amplification of ITS regions with the primer

pair, ITS1-F and ITS4 [30,31]. Briefly, the PCR protocol was as follows: 1X CoralLoad PCR Buffer, 200 µM dNTP, 0.5 µM primer 1, 0.5 µM primer 2, 100ng template DNA, 0.2 U/µL DNA polymerase; the reaction was adjusted to 25 µL with autoclaved MQ H_2O. The PCR conditions used were 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min, and 72 °C for 10 min. Possible contaminations were determined with a negative control using sterile water as a template in both PCR protocols. StainIN™ RED Nucleic Acid Stain was used to confirm DNA amplicons on a 2% agarose gel and the visual detection was made by ultraviolet transillumination. ITS region PCR products were purified and sequenced using the ITS4 primer at Microsynth SEQLAB (Göttingen, Germany). The FASTA files thus obtained were checked with BioEdit [32] to confirm that the pathogen was not contaminated with other fungi.

2.5. Experimental Design

The experiment was conducted at the Forest Botany and Tree Physiology greenhouses, Göttingen, Germany (51°33′28.4″ N 9°57′30.5″ E) from early April until early August 2018. The 352 seedlings were randomly block-assigned to waterproof tables with either high water (high water group = +group) or low water (low water group = −group) availability treatments [2], and experimentally inoculated with *H. parviporum* (66 per treatment group), *H. annosum* (66 per treatment group), mock-inoculated controls (25 per treatment group) or left entirely untreated (19 per treatment group). The water treatment experiment was running for 35 days before the inoculations were performed. Fungal isolates were plated on 2% Malt Extract Agar (MEA) and grown at 21 °C for two weeks prior to the experimental inoculations. Inoculations were made approximately mid-way up the stem of a first-year shoot (distance of the inoculation from the stem base was ∼5 cm). A sterile 5mm cork borer was used to punch through the bark to reach the sapwood surface. Equal sized plugs from pure culture of *Heterobasidion* sp. or control (2% MEA) were placed onto the exposed surface and sealed with Parafilm® [2]. The inoculation experiment was run for 70 days.

Since seedling water intake varied with the ambient temperature, it was necessary to continuously monitor and adjust the watering amounts, in order to maintain the essential level in high water availability treatment (moist soil). The water amounts needed to be continuously adjusted to the increasing temperatures during the growing season 2018. Overall, high water availability seedlings received double the water of low water availability seedlings. This was considered the suitable level of drought in low water treatment [2]. At the beginning of the experiment, each high water availability seedling was given 60 mL of water three times per week (Monday, Wednesday and Friday), and each low water availability seedling was given 30 mL of water three times per week. After two-weeks, the watering regimes were modified to 120 mL × 3 (+group), and 60 mL × 3 (−group). After five weeks, the water level was modified again to 180 mL × 3 (+group) and 90 mL × 3 (−group). Water quantities were increased in July (384 mL × 3 (+group), and 192 mL × 3 (−group)), and maintained at that level until August. Seedlings were rotated on tables monthly, in order to minimize potential variation in water requirements, due to positioning in the greenhouse. Throughout the experiment, temperatures were measured inside the greenhouse every watering day using digital thermometers. The average daily temperatures during the experiment period are presented in Table 1.

Table 1. The average, minimum and maximum temperatures in each month measured inside a greenhouse.

Month	Average, °C	Min, °C	Max, °C
April	17.9	13.3	20.7
May	21.8	15.6	25.7
June	24.8	20.4	31.1
July	24.2	17.4	30.9
August	32.5	32.5	32.8

2.6. Data Collection and Post-Experiment Detection of Heterobasidion Species

Seedling height was measured at the beginning and the end of the experiment, in order to determine seedling growth ($height_{end} - height_{start}$) during the experiment. At the end of the experiment, the lesion lengths (nearest 0.001 mm) were measured with a stereomicroscope (Stemi 508, Zeiss, Omnilab-laborzentrum GmbH & Co.KG, Gehrden, Germany) with an attached camera (Axiocam ERc5s, Zeiss, Omnilab-laborzentrum GmbH & Co.KG, Gehrden, Germany) by using the freely available software Labscope (Carl Zeiss Microscopy GmbH, Jena, Germany). First, the bark was gently peeled to expose the necrosis in phloem (Figure 3a), measured, and then further sapwood (Figure 3b) was exposed and measured. The lesion length was measured in horizontal and vertical directions. After measurements, DNA was extracted directly from the inoculated sapwood (Figure 3b,c) (100 mg) using the "innuPREP Plant DNA Kit" (Analytik Jena AG, Jena, Germany), according to the manufacturer's instructions. From each treatment, 10 randomly selected inoculated saplings (in total 60 saplings) was selected. To confirm infection, species-specific primers were used as described above to detect *Heterobasidion* in inoculated stems [17]. Additionally, five inoculated stems (Figure 3b,c) from each treatment (30 samples), were placed inside a plastic bag and incubated in darkness for five days. The presence of *H. annosum* s.l. was identified based on conidiophores and conidiospores under stereomicroscopy.

Figure 3. Measuring the necrosis in phloem and sapwood of Norway spruce saplings. (**a**) Necrosis in phloem after inoculation with *H. parviporum*; (**b**) necrosis in sapwood after inoculation with *H. parviporum*; (**c**) no sign of necrosis in the sapwood of mock-inoculated control.

2.7. Data Analysis

Data were analyzed using the SPSS version 25.0 (IBM Corporation, New York, NY, USA). A generalized linear model was constructed to evaluate the fixed effects of necrosis length (mock-inoculated-control, *H. annosum*, *H. parviporum*) and water availability treatments on sapling growth ($height_{end} - height_{start}$). Initial fixed explanatory variables in the seedling growth model included water treatment group, inoculation group, lesion length (sapwood/phloem; vertical/horizontal), and all their interactions. Table number was set as a random factor in the model. In addition, non-treated saplings were included in this model. A generalized linear model was also constructed to evaluate the fixed effects of inoculation method (mock-inoculated-control, *H. annosum*, *H. parviporum*) under different water treatment (high, low) on necrosis length in phloem and sapwood (vertical/horizontal). Initial fixed explanatory variables in the necrosis length model included inoculation method in the water treatment group, sapling growth and sapling start height. Non-treated saplings were not included in this model. Table number was set as a random factor in the model. The length of necrosis caused by *H. parviporum* and *H. annosum* (horizontal/vertical) in phloem and sapwood per water treatment was further assessed by ONE-WAY-ANOVA and pairwise differences were found using Dunnett's C tests. Differences were considered statistically significant if the p-value was equal or below the threshold of 0.01. Pearson's correlation analysis was used to determine correlations of seedling growth ($heigth_{start}$ and growth) with the sizes of necrotic lesions (phloem/sapwood and vertical/horizontal) caused by *Heterobasidion* species. Similarly, necrosis length (vertical/horizontal) in sapwood and phloem were analyzed with

Pearson's correlation. Correlations were considered as statistically significant if the *p*-value was below the threshold of 0.05.

3. Results

3.1. Necrosis Length

The extention growth of lesions and necroses occuring in pathogen inoculated stems was significantly higher than in mock-inoculated controls in sapwood (vertical and horizontal) (Figure 4a,c) and phloem (vertical and horizontal) (Figure 4b,d), except for the horizontal extension of *H. annosum* in phloem, which did not differ from control (Figure 4d). *H. parviporum* outcompeted *H. annosum* in vertical necrosis length in sapwood (Figure 4a) and phloem (Figure 4b), and in horizontal necrosis in phloem (Figure 4d). The damage caused by *H. parviporum* was statistically greater in the low water group (−group) in the vertical direction in sapwood (Figure 4a) and phloem (Figure 4b). Necroses, due to *H. annosum* were statistically higher only for the low water group in the horizontal direction in phloem (Figure 4d). Even though both pathogens were affected by the low water availibility to the seedlings, *H. parviporum* was able to cause greater vertical necrosis. The vertical necrosis in sapwood (Figure 4a) is also presented as necrosis length (mm) as a porportion of seedling growth ($height_{end} - height_{start}$, mm) during the experiment (Figure 5). In the untreated saplings there were no lesions and necroses observed.

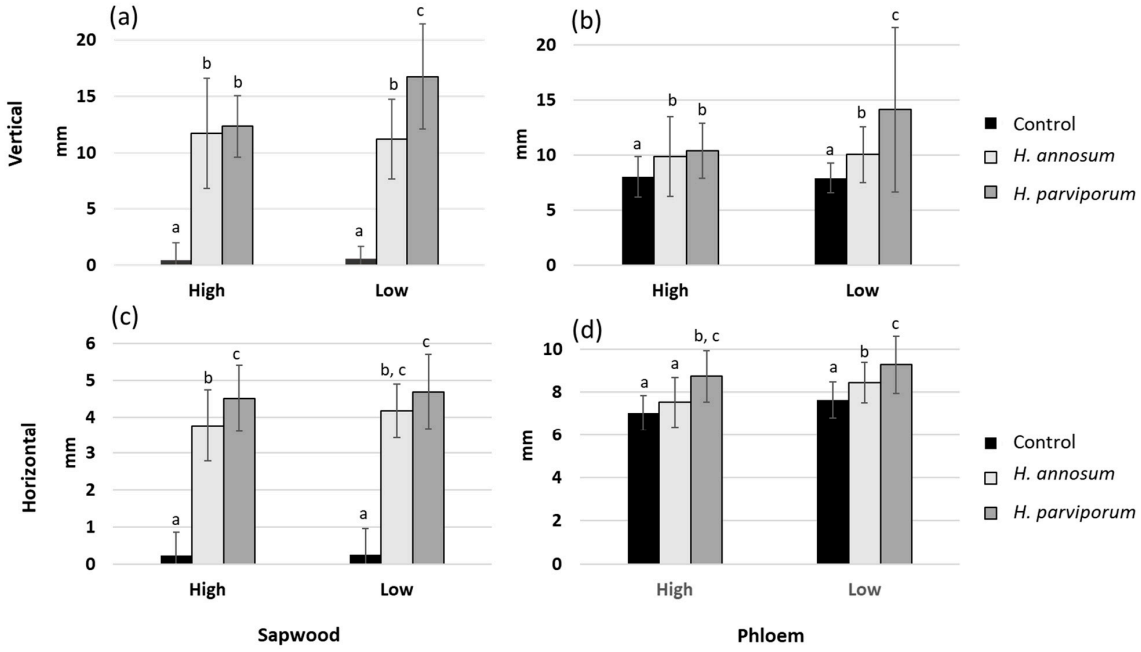

Figure 4. Length of necrosis (mm) observed after inoculation experiment with mock-inoculated control, *H. annosum* and *H. parviporum* in Norway spruce saplings in different water availability groups. Averages of vertical (**a**,**b**) and horizontal (**c**,**d**) length (mm) of necrosis in sapwood (**a**,**c**) and phloem (**b**,**c**) measured to the nearest 0.001 mm.

The necrosis length model for pathogens (GLM) showed that seedling starting height ($p < 0.01$) affected horizontal necrosis length in phloem and sapwood (Table 2). Based on Pearson correlation coefficients (r), there were significant positive correlations between the seedling height and horizontal pathogen necrosis in phloem ($p < 0.01$, $r = 0.3$) and sapwood ($p < 0.05$, $r = 0.1$) (Figure 6). Higher seedling height indicated higher horizontal necrosis length (Figure 6). However, the strength of these associations was low ($0.1 < r < 0.3$). Statistically significant ($p < 0.01$) and strong positive correlations were found between sapwood vertical and horizontal necrosis length ($r = 0.8$), as well as between vertical phloem and sapwood necrosis length ($r = 0.6$) (Figure 7a). When vertical necrosis in sapwood

increased, the necrosis in horizontal direction increased (Figure 7a). Similarly, when necrosis in phloem increased, it also increased in sapwood (Figure 7a). Significant positive correlations ($p < 0.01$) were found also between horizontal phloem and sapwood necrosis length ($r = 0.5$) (Figure 7b) and between horizontal and vertical necrosis in phloem ($r = 0.4$) (Figure 7b). The strength of these associations was moderate ($0.4 < r < 0.5$).

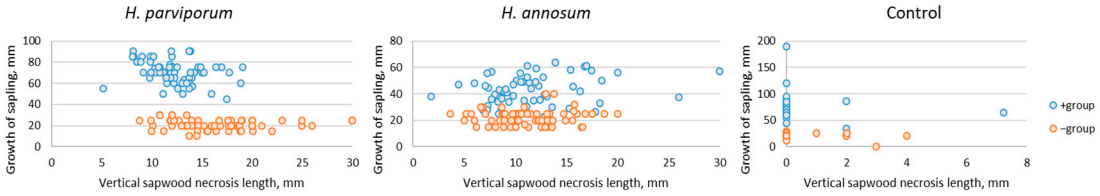

Figure 5. Vertical necrosis (mm) observed in sapwood after inoculation with *H. parviporum*, *H. annosum* and mock-inoculated control in high (+group) and low (−group) water groups as proportions of the sapling growth (mm). The Norway spruce saplings in low water group (−group) in each treatment grew less during the experiment than in high water group (+group). *H. parviporum* caused higher necrosis in the low water group (−group).

Table 2. Estimates from the generalized linear models (GML), statistically significant effect ($p < 0.01$) are bolded.

Variable	Fixed Effects	Std. Error	F	Sig.
Seedling growth	Water treatment	0.281	341.586	**<0.001**
	Inoculation treatment	0.529	0.751	0.603
	Phloem, vertical	0.027	1.032	0.311
	Phloem, horizontal	0.084	5.004	0.026
	Sapwood, vertical	0.032	3.866	0.05
	Sapwood, horizontal	0.114	2.156	0.143
Sapwood, vertical	Inoculation × water treatment	1.117	106.57	**<0.001**
	$Height_{start}$	0.028	1.802	0.181
	Seedling growth	0.151	2.236	0.136
Sapwood, horizontal	Inoculation × water treatment	0.36	186.452	**<0.001**
	$Height_{start}$	0.007	13.592	**<0.001**
	Seedling growth	0.035	0.009	0.926
Phloem, vertical	Inoculation × water treatment	1.56	11.542	**<0.001**
	$Height_{start}$	0.032	0.569	0.451
	Seedling growth	0.172	0.03	0.863
Phloem, horizontal	Inoculation × water treatment	0.426	15.252	**<0.001**
	$Height_{start}$	0.008	11.164	**<0.001**
	Seedling growth	0.044	2.012	0.157

3.2. Saplings Growth

Low water availability negatively affected the growth of Norway spruce saplings during the experiment. Sapling growth ($height_{end} - heigth_{start}$, +group = 7.2 ± 2.0 cm; −group = 2.2 ± 0.6 cm) was statistically ($p < 0.01$) affected by water availability, (Figure 5, Table 2), but was not affected by the fungal infections or necrosis length (horizontal/vertical) of the phloem or sapwood, or by seedling starting height (Table 2). In total, 11 seedlings died during the experiment; one seedling in *H. parviporum* (+group), one in *H. annosum* (−group), six in *H. annosum* (+group) groups and three in non-treated (−group). The death of seedlings was assumed to be random, and they were removed from data analysis.

3.3. Plant and Fungal Material

H. annosum s.l. could not be detected in the Norway spruce saplings sampled and investigated by the Northwest German Forest Research Institute before the experimet started. The endophyte-community was isolated from the roots, which included species of *Diaporthe* sp., *Epicoccum nigrum* Link, *Sydowia polyspora* (Bref. and Tavel) E. Müll. and *Alternaria* sp. (data not shown). ITS region sequencing indicated no contamination in the cultures of *Heterobasidion* species. The species-specific primers [17] also showed species speficity and worked only for the target species. The PCR run from inoculated sapwood samples (altogether 60 samples) with species-specific primers detected the *H. parviporum* and *H. annosum* from 70% (14/20: Ten from +group; four from −group) and 70% (14/20: Ten from +group; 4 from −group), respectively. In mock-inoculated controls (20 samples) no *Heterobasion* was detected (data not shown). From inoculated sapwood (30 stems of saplings) incubated in darkness, *H. parviporum* could be detected from nine samples, (four from +group; five from −group) and *H. annosum* from seven samples (three from +group; four from −group). From mock-inoculated controls (10) no *Heterobasidion* was detected.

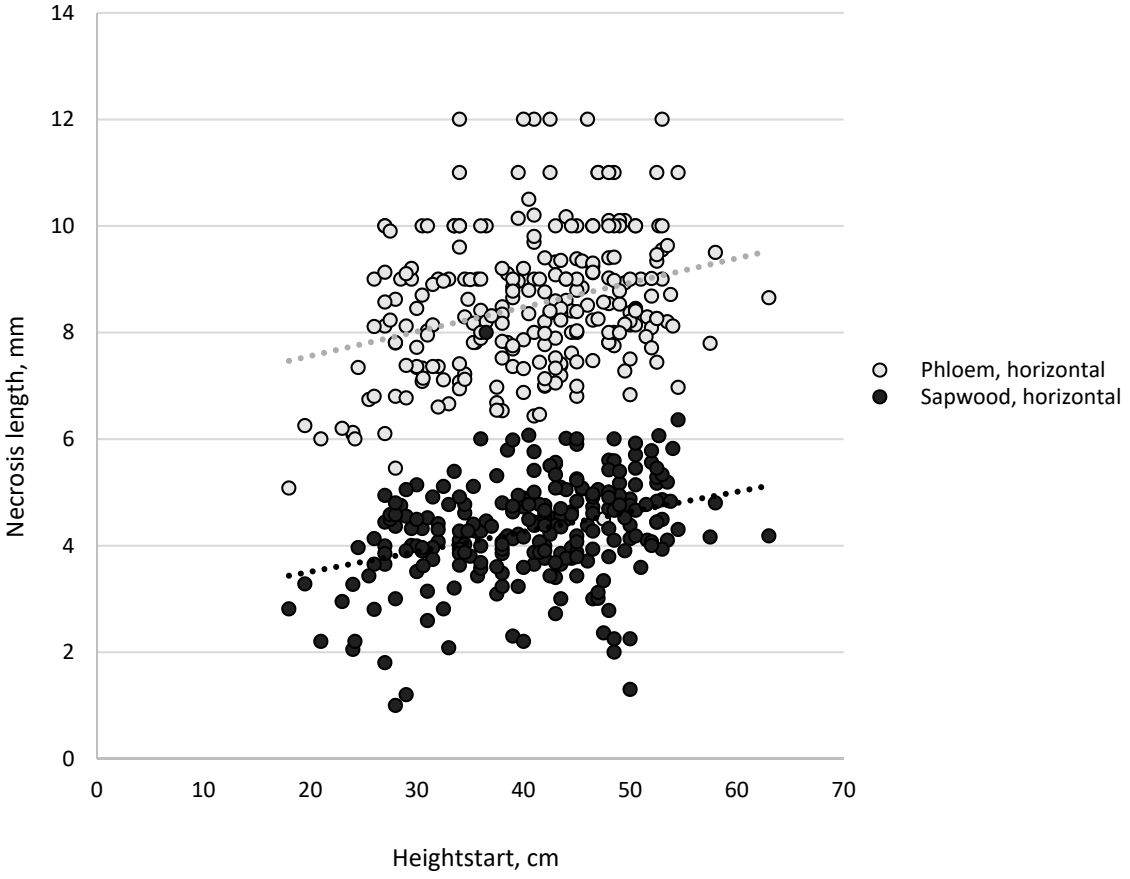

Figure 6. Combined horizontal necrosis length of *H. parviporum* and *H. annosum* correlated positively with seedling height at the start of the experiment. Higher seedling height indicates higher horizontal necrosis length.

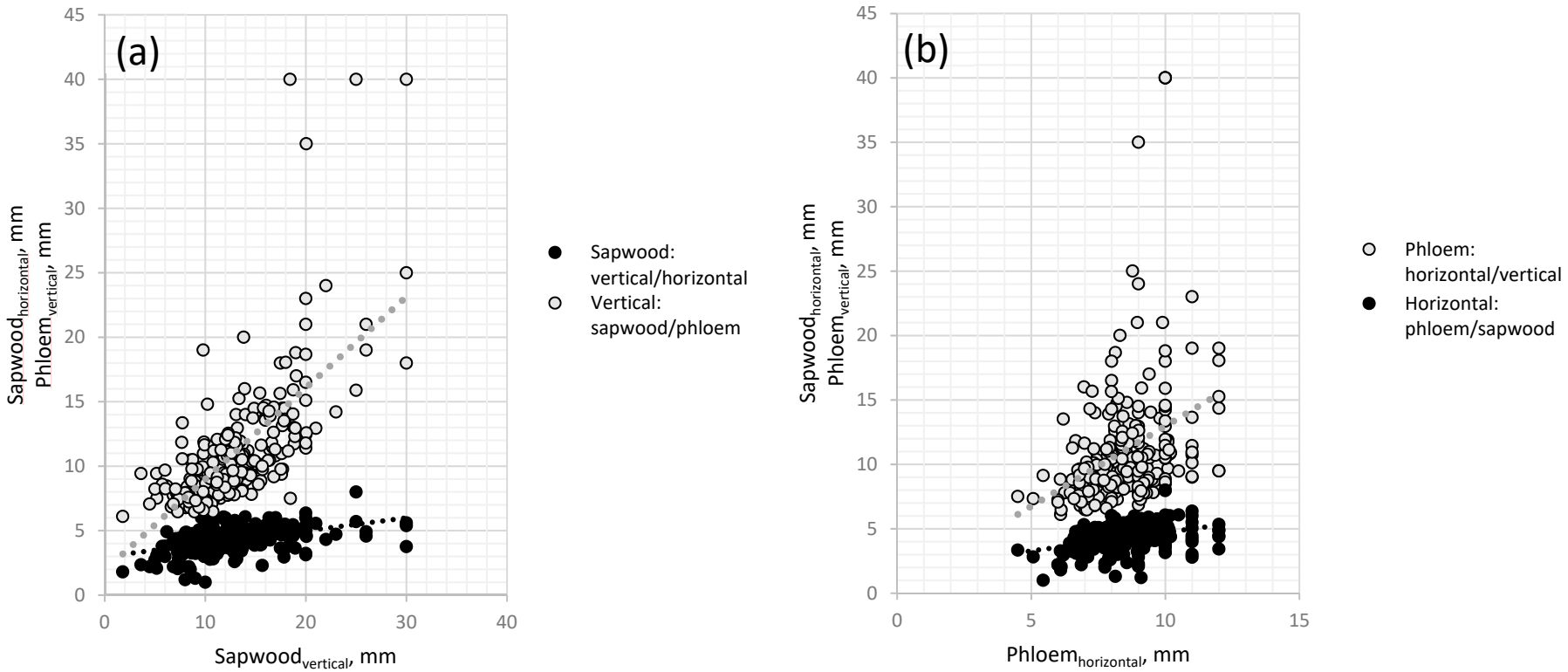

Figure 7. (**a**) Statistically significant ($p < 0.01$) positive correlations between vertical and horizontal necrosis length in sapwood (black), and vertical necrosis length between sapwood and phloem (grey); (**b**) Statistically significant ($p < 0.01$) positive correlations between vertical and horizontal necrosis length in phloem (grey), and horizontal necrosis length between sapwood and phloem (black). Positive correlations indicate that when one variable increases the other variable simultaneously increases.

4. Discussion

Pathogen growth, here expressed by necrosis length (vertical/horizontal) in phloem and sapwood, was shown to increase under abiotic disturbance (drought) in *P. abies* saplings. This result is consistent with Linnakoski et al. [2], who found that blue stain fungi *E. polonica* caused greater necrosis and mortality in *P. abies* seedlings with low water availability compared to those with high water availability. Madmony et al. [33] inoculated *H. parviporum* into two-year-old branches of two different Norway spruce clonal ramets (4-year old) and subjected them to well-watered and drought environments. They observed increased pathogen growth in well-watered seedlings [33], which is in contrast to our results. However, the duration of water availability treatment (22 days) in Madmony et al. [33] was much shorter than in this study (105 days). These results highlight the need for well-established inoculation studies for this pathosystem, when studying effects of abiotic disturbances. In our study, the necrosis length did not affect the growth of the saplings, but horizontal necrosis had a significant correlation with the sapling height ($height_{start}$) measured at the start of the experiment (Figure 6). A similar observation was made in an inoculation study of three-year-old Norway spruce ramets with *H. parviporum* [34]. However, this is in contrast to other studies, where Norway spruce saplings' height were negatively correlated with the necrosis length caused by *H. parviporum* [35]. Based on these results, saplings with lower values of growth parameters (in this case height) are not automatically considerably more sensitive to fungal inoculation, rather the necrosis development most likely involves multiple, interacting factors, e.g., different experimental settings and origins of pathogens and trees. However, the possibility that the duration of pathogen exposure to the host and the damage caused by increasing necrosis has an influence on the vitality of the host cannot be ruled out. The latter could have affected height growth increment (Table 2).

In nature, disturbances caused by water limitations leads to reduced establishment of aboveground tissues [2,36]. A similar phenomenon was observed in this study, as Norway spruce growth was significantly lower under the low water availability treatment than high. Necrotrophic fungal infections activate defensive responses in trees that aim to stop infection [37]. As previously mentioned, the necrosis length did not affect the growth of the seedlings during the experiment, indicating that the defense activated in saplings did not lead to a trade-off between resources allocated to growth. Neilson et al. [38] opined that calculations of the costs of the trade-off between growth and chemical defense related secondary metabolites can be overestimated and should be investigated in parallel with the identification of new supplemental functions. In this study, the sapling growth was not influenced by lesion length indicating that the infection with fungal pathogen did not require more resources compared to a mock-inoculated wound [2]. Pairwise comparison between non-treated saplings and inoculated saplings indicated that water treatment was the only parameter affecting growth (data not shown). These results suggest that low water availability (drought) affected the growth of Norway spruce saplings, making them more susceptible to root rot pathogens (Figures 4 and 5).

The adaptation of a tree species to changes in local climate may be too slow to successfully respond to the present rapid rate of climate change [1,39]. Further uncertainty in the climate change predictions is the possibility of change in tree resistance to fungal pathogens at abiotic disturbances, e.g., drought and higher temperatures [1]. *Heterobasidion* species growth is optimum at temperatures above 20 °C and below 30 °C [40–43]. Wood moisture content is not critical for *H. annosum* stump infection, whereas the further colonization of sapwood is decreased significantly with increasing stump moisture [44]. Müller et al. [43] stated that after having invaded living woody tissue, *Heterobasidion* species could expect to have sufficient moisture to continue growing as long as the tree is alive [43]. In this sense, the growth of *Heterobasidion* may not be affected by decreased water availability [43,44], in contrast to the host [36,45]. The probability of longer summer drought periods [46] may decrease the defense ability of trees towards increasing activity of root rot fungi [27,36,45]. Our results support this hypothesis, as the growth of Norway spruce saplings was decreased in the lower water group, leading to higher necrosis length (Figures 4 and 5).

Linares et al. [45] found that infected *Abies pinsapo* Boiss. trees have decreased ability to withstand drought stress, and root rot infections (*H. abietinum*) act as predisposing factors of forest decline and mortality. Gori et al. [27] found that *H. parviporum* infection made *P. abies* more susceptible to drought stress at a low elevation site. The artificial fungal inoculation of seedlings and saplings of trees has been widely practiced under controlled greenhouse conditions, in order to investigate hosts susceptibility to *Heterobasidion* infection [34,35,47,48]. For the current experiment, three-year-old saplings were used instead of mature trees. This enabled us to employ larger sample numbers with manipulation treatments, all within a relatively short time. While previous inoculation studies indicate that young saplings and seedlings are an effective model for larger tree health in *Heterobasidion*—Norway spruce pathosystems [28], we highlight the importance of research on mature trees, which will be the most accurate reflection of *Heterobasidion* effects on spruce trees in nature. Fine roots response negatively by decreasing their biomass during drought [49]. This effects the vitality of Norway spruce and causes more environmental stress. As *Heterobasidion* species are not dependent on the fine roots we suggest that these pathogens can benefit from the stress of their host trees caused by drought. Consequently, combined effects to the host root system by drought and *Heterobasidion* in more detail remains to be investigated.

5. Conclusions

This study found significant increase in the necrosis length after inoculation of *H. parviporum* (vertical necrosis in phloem and sapwood) and *H. annosum* (horizontal necrosis in phloem) in *P. abies* saplings that were stressed, due to lower water availability. This highlights the fact that root rot pathogens in the genus *Heterobasidion* can benefit from disturbances caused by projected climate change. The number of long drought periods in summer is expected to increase in Europe in the next decades [1,39,46,50], highlighting the need for the development of new adaptation strategies in forestry [50,51]. Here we provide experimental evidence that reduced water availability can enhance necrosis length in Norway spruce saplings after inoculation with *H. parviporum* or *H. annosum*. This study contributes to experimental research on interactions between biotic and abiotic disturbances in forest trees. Further empirical and theoretical research on mature trees under these disturbance conditions (drought and root rot) are required to better understand the genetic measures of host resistance and pathogen virulence, which can ultimately lead to different control strategies via resistance breeding.

In the course of global climate change, the next generation of Norway spruce forests in Germany are exposed to several risks besides *H. annosum* s.l. Norway spruce is the most vulnerable to wind damage, which is predicted to increase due to climate change [1], of all tree species in Germany [52]. This highlights the need for more applied studies of how different abiotic (wind and drought) disturbances can benefit the biotic (*H. annosum* s.l.) disturbance in Norway spruce dominated forests. In future, in German lowlands with limited water supply, *P. abies* will be a high-risk tree species with respect to forest protection and abiotic or biotic risk factors [53–55].

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