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Effects of Temperature and *Heterobasidion* Species on the Biological Control Efficacy of *Phlebiopsis gigantea*

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Front pictures: the upper left picture was taken by Kristine Kenigsvalde from Latvian State Forest Research Institute; the other three pictures were taken by Anan Zhao.

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Contents

Abstract

Heterobasidion annosum sensu stricto is a damaging forest pathogen causing large economic losses to European forestry. The biological control agent Rotstop® made of *Phlebiopsis gigantea* oidia spores is effective on protecting freshly cut stump surfaces from *H. annosum s.s.* airborne infection. However, as a biological control method, Rotstop® may be sensitive to environmental factors. In this study, the effects from temperature and different *Heterobasidion* species on Rotstop® efficacy and *Heterobasidion* infection were studied both in controlled conditions (growth chamber) and in field conditions. In both situations, Rotstop® F and Rotstop® S were inoculated on freshly cut Norway spruce (*Picea abies*) surfaces to compete *H. annosum* and *H. parviporum*. Temperatures of the growing chambers were set at 5°C, 10°C, 15°C, 20°C and 25°C, while temperature in field condition was monitored. Colonization rate was measured and calculated on total surface, sapwood and heartwood. The identity of the originally inoculated strains was checked by somatic compatibility. The study results showed that temperature affected colonization of *P. gigantea* on all parts of slice; while it could affect colonization of *Heterobasidion* species on sapwood. *P. gigantea* was more sensitive to temperature shifts than *Heterobasidion* species.. *H. parviporum* was better at colonizing on Norway spruce than *H. annosum* in all parts of slice. Capacity of *P.gigantea* differed with *Heterobasidion* species. Both Rotstop® F and Rotstop® S could effectively reduce the colonization of *Heterobasidion* species, but they might have problems of protecting stump surface in cooler seasons, protecting heartwood area and competing with *H. parviporum*.

Keywords: heartwood, Heterobasidion species*, Norway spruce (Picea abies), Phlebiopsis gigantea, Rotstop, sapwood, temperature.*

1. Introduction

Heterobasidion annosum sensu lato (Fr.) Bref. is the most damaging pathogen in temperate conifer forests of the Northern Hemisphere. In 1998, the economic losses of 800 million euros per year were estimated attributed to this destructive fungus in Europe (Asiegbu *et al.*, 2005). The fungus infects the surface of freshly-cut stumps with airborne spores, and then spreads to the trees nearby with vegetative mycelia through root connections. Infected trees show wood decay, retarded growth and wood discoloration symptoms, hence the wood quality is reduced. Due to the root rot caused by *Heterobasidion annosum sensu stricto* (Fr.) Bref., the risk of wind throw is also increased (Daniel *et al.*, 1998; Rönnberg *et al.*, 2006; Oliva *et al.*, 2008). Norway spruce (*Picea abies* (L.) Karst.) is sensitive to *H. annosum s.s.* among the conifer species.

At present, methods used for decreasing *H. annosum s.s.* infection include: (i) biological control by applying competitor fungal species such as *Phlebiopsis gigantea* (Fr.) Jül., *Trichoderma species* or *Fomitopsis pinicola* on newly cut surface (Asiegbu *et al.*, 2005; Berglund *et al.*, 2005), (ii) chemical control by applying urea solution or disodium octaborate tetrahydrate (DOT) on the surface (Brandtberg *et.al.*, 1996; Thor and Stenlid, 2005; Oliva *et al.*, 2008), (iii) silvicultural control such as operating thinning and clear-cutting during cold seasons (Yde-Anderson, 1962; Oliva *et al.*, 2010), growing host trees and non-host trees together (Lygis *et al.*, 2004; Asiegbu *et al.,* 2005), having stump removal after thinning or clear-cutting (Asiegbu *et al.*, 2005; Cleary *et al.*, 2013) or regenerating with other tree species on previous infected stands (Piri, 1996).

Among all the methods, biological control with *P. gigantea* is the most frequently used one (Nicolotti and Gonthier, 2005) and it is considered more environmental-friendly than chemical stump treatment. Rotstop® is the commercial preparation of oidia spores from *P. gigantea*, a saprotrophic basidiomycete causing wood decay (Vasiliauskas *et al.*, 2004). Rotstop® was firstly applied on stumps as commercial product in Finland in 1994 (Korhonen *et al.*, 1994). Other available formulations of *P.gigantea* include PG Suspension in UK (Tubby *et al.*, 2008), PG IBL in Poland (Pratt *et al.*, 2000) and Rotstop® S in Sweden (Rönnberg *et al.*, 2006). Currently about 35 000 ha Norway spruce are treated after with Rotstop® thinning annually in Sweden (Berglund and Rönnberg, 2004).

However, Rotstop® seems to rely on a good coverage upon cutting surfaces to get full protection according to Korhonen (2003). This result was further proved by Berglund and Rönnberg (2004), Rönnberg *et al.* (2006) and Oliva *et al.* (2010). Other factors have also been considered that might restrict the capacity of Rotstop®, i.e. temperature (Yde-Anderson, 1962; Gooding *et al.*, 1966; Kasanen *et al.*, 2011), different *Heterobasidion* species (Daniel *et al.*, 1998; Vasiliauskas and Stenlid, 1998; Oliva *et al.*, 2011; Gunulf *et al.*, 2012); wood moisture (Redfern, 1993; Bendz-Hellgren and Stenlid, 1998), and the amount of *Heterobasidion* spores (Berglund *et al.*, 2005; Nicolotti and Gonthier, 2005; Sun *et al.*, 2009). Amongst these factors, temperature could be the most potentially threatening since increasingly warming conditions due to climate change could expand the current range of *H. annosum s.s.* northwards (Witzell *et al*., 2010). In this case,

studies are also needed to assess the capacity of Rotstop® to control different *Heterobasidion* species at different temperature levels especially since *H. annosum s.s.* might be more invasive at higher temperature while *Heterobasidion parviporum* Niemela & Korhonen could withstand lower temperature.

The aim of this study was to investigate how the capacity of Rotstop® could be affected by different temperatures and different species of *Heterobasidion*. Specifically, this study was to answer the following questions: On Norway spruce (*Picea abies*),

(i) do different temperatures affect the colonization area of *P. gigantea*, *H. annosum s.s.* and *H. parviporum*?
(ii) does *P. gigantea* from Rotstop F and Rotstop S have different colonization area, especially when against *H. annosum* and *H. parviporum*?
(iii) do *H. annosum* and *H. parviporum* have different colonization area, especially when against *P. gigantea*?
(iv) do the three species show any preference at different parts of wood?

2. Materials & Methods

2.1 Study sites and inoculum source

This experiment was made up by two parts: chamber and field. Sample log segments for the chamber experiment were taken from western Lunsen forest between Uppsala and Knivsta on May 3rd and 17th, 2012. Inoculation of the field experiment took place in the vicinity of Tierp on May 14th, 22nd and 25th, 2012.

In this study both *Heterobasidion annosum s.s.* (former P type) and *H. parviporum* (former S type) were included. 3 isolates for each species were chosen: Rb200, Mj65, Mj20 for *H. annosum s.s.*, and Sä182, Sä150, Sä30 for *H. parviporum*. Two formulations of Rotstop (*Phlebiopsis gigantea* oidia spores' production) were used: Rotstop F, where F stands for Finland, and Rotstop S, where S stands for Sweden.

2.2 Preparation of spore suspension for inoculation

Suspension of *P. gigantea* oidia spores was made by adding Rotstop gel product into sterile water. According to the product description, the concentration was 1gr/l. Hence 1 ml/10 cm^2 of suspension was applied over stump surfaces. When applying this amount of product with a syringe, there was a lot of product spilling through the sides of the stumps. Therefore, the concentration was increased to 2gr/L and half of the amount of water was used instead.

Suspension of *Heterobasidion* conidia spores was made by collecting spores from pure isolate cultures. Several petri dishes were used for each isolate. Aprox. 3-5ml sterile water was added into each petri dish and the agar surface was scratched, and then the suspension was collected into a tube. A hemocytometer was used to count the number of spores per ml. It was aimed to apply 3000 spores/cm^2 upon stump surfaces.

2.3 Sampling and inoculation

For chamber experiment, seven Norway spruce logs with diameters of 12-16 cm were sawn into 30cm long segments. The heartwood area was marked immediately after tree felling. Diameters in two different directions were measured and the average diameter was counted. In total 120 segments were taken to the lab and randomly sorted in 5 groups. Each group was inoculated as Table 1. The volume of suspension was corrected according to the average diameter. Then the 5 groups were separately stored under 5, 10, 15, 20, and 25°C in darkness for 8 weeks.

For field experiment, three sites were settled and 24 Norway spruces of 10-16cm in diameter were sawn into stumps of 30 cm high in each site. Sawn surfaces were thoroughly cleaned, and the heartwood areas were marked when diameter were measured. Then they were inoculated as Table 1 on the spot. After inoculation, all stumps were left undisturbed in the forest for 9 weeks. A temperature monitor Tinytag was settled to record the temperature.

2.4 Fungal colonization

Eight/nine weeks after inoculation, the top 3 cm thick wood was discarded from each segment or stump and then a 2 cm thick wood slice was cut (Fig. 1a). The bark of slices was removed completely and afterwards the slices were cleaned with distilled water. After the extra water was dried with tissue paper, the slices were stored in opaque plastic bags at 20°C in the dark. After another 4 weeks, each slice's upper surface was checked under microscope and marked if there was fungal colonization (Fig. 1b). The area of total surface, sapwood, heartwood and the colonization by *Heterobasidion* species and/or *P. gigantea* was calculated by pixels counts with Photoshop CS6®.

Table 1. Inoculation design and numbers. W means sterile water. 'RF' means Rotstop F. 'RS' means Rotstop S.

***Heterobasidion* species and isolates**	**With RF**	**With RS**	**Controls**	
			Only *Heterobasidion*	**Only Rotstop**
***H. annosum* Rb 200**	Rb 200+RF	Rb 200+RS	Rb 200+W	RF+W
***H. annosum* Mj 65**	Mj 65+RF	Mj 65+RS	Mj 65+W	RF+W
***H. annosum* Mj 20**	Mj 20+RF	Mj 30+RS	Mj 30+W	RF+W
***H. parviporum* Sä 182**	Sä 182+RF	Sä 182+RS	Sä 182+W	RS+W
***H. parviporum* Sä 150**	Sä 150+RF	Sä 150+RS	Sä 150+W	RS+W
***H. parviporum* Sä 30**	Sä 30+RF	Sä 30+RS	Sä 30+W	RS+W
Numbers	**6**	**6**	**6**	**6**
Total	24			
Total of 5 temperatures in chamber	24 × 5 = 120			
Total of 3 sites in field	24 × 3 = 72			

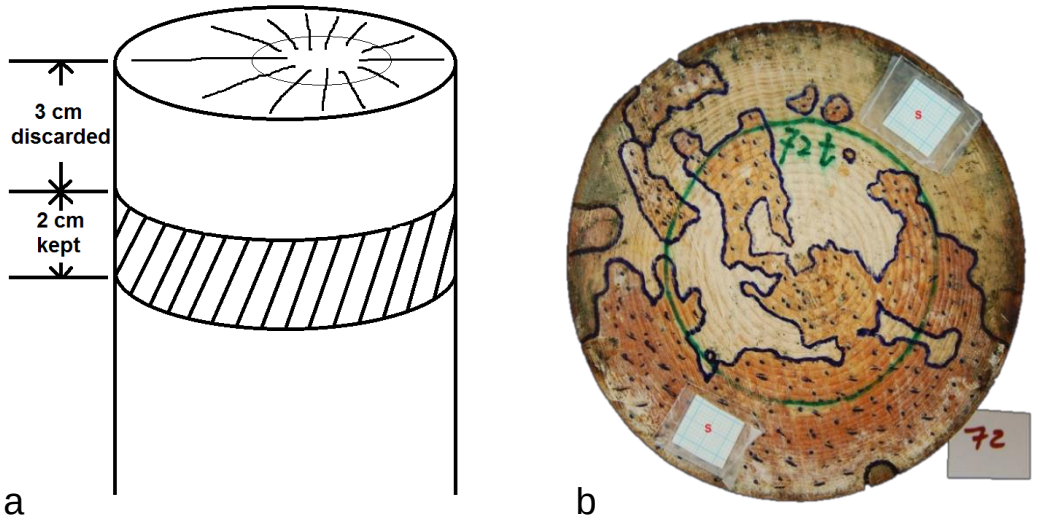

Figure 1. (a) shows how a wood slice (area marked with slashes) was cut from a log segment or a stump. (b) shows a slice with *Phlebiopsis gigantea* colonization. Heartwood area is marked in green. *P. gigantea* colonization area is marked with blue borders and dots. Red Ss show the 1 cm^2 squares which were used for counting the area.

2.5 Fungal isolation and somatic compatibility

In order to prepare isolates from each colonized wood slice, mycelia within the colonized areas was picked with a fine needle under microscope and cultured in agar amended with benlate (20g malt extract, 15g agar, 0.2g chloramphenicol, 1L water, and an addition of 10mg Benlate after autoclaved). Cultures were transferred into new benlate petri dishes within 3-4 days and the isolates were retrieved. It was aimed to have 4 isolates per slice randomly taken from different places within the colonized area. If isolation failed it was attempted once more.

Somatic compatibility between the original isolates and the retrieved isolates on the same slice was crossing-tested according to Stenlid (1985) in order to check whether the retrieved mycelia corresponded with the originally inoculated isolates. Mycelia of each isolate was cultured in a hagem agar petri dish alone in order to get fast-growing mycelia. If the mycelia was not growing fast enough, it was cultured first in water agar and then plated again into hagem agar. When both the original and the retrieved mycelia were displaying fast growth, they were paired in one hagem agar petri dish 1 cm apart each other. The crossings were checked every 2-3 days to ensure fast and even growth of both mycelia. After 4-5 weeks, a barrier or a clear zone between them was interpreted as a sign of somatic incompatibility between the two isolates (Fig. 2). Then the data of somatic compatibility was used for correcting the data of colonization. In this study only colonization of *Heterobasidion* species was corrected with somatic compatibility data (Table. 3). Corrected *Heterobasidion* species colonization in the chamber experiment was not included in the results since all the factors showed no significant effect. Uncorrected and corrected *Heterobasidion* species colonization data in the field experiment were presented together.

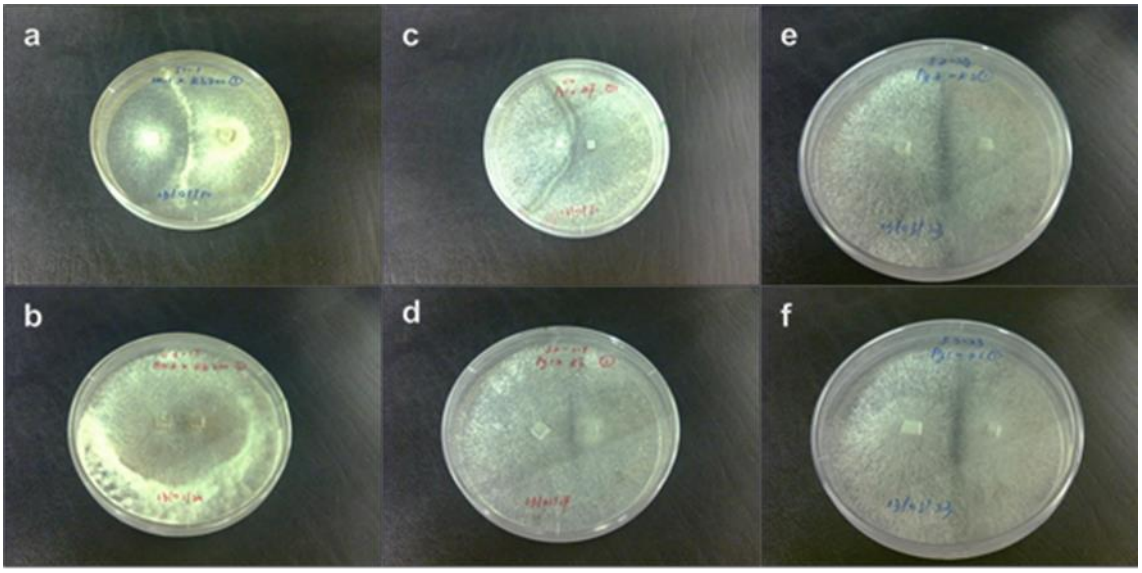

Figure 2. Petri dishes for somatic compatibility checking. (a) and (b): somatic incompatibility and compatibility between an original *Heterobasidion* isolate (Rb 200) and retrieved *Heterobasidion* isolate; (c) and (d): somatic incompatibility and compatibility between an original *Phlebiopsis gigantea* isolate (Rotstop F) and retrieved *P.gigantea* isolate; (e) and (f): both somatic incompatibility between original *P. gigantea* isolate (Rotstop S) and retrieved *P. gigantea* isolate.

2.6 Statistical analysis

Colonization data was analyzed with General Linear Model of ANOVA (GLMA) using Minitab®16.2.3. Residual plots were examined to see if the data fit a normal distribution. We used a significance level of p=0.05 to reject the null hypothesis. Means were also compared by Tukey method. The factors and response variables are shown in Table 2. Analysis focused on temperature and site respectively for the chamber and field experiment, *Heterobasidion* species, Rotstop treatment, as well as their interactions. In order to distinguish colonization caused by the treatments from colonization caused by natural infection, somatic compatibility data was used to correct the colonization rate as in Table 3.

The mean values of colonization rate from GLMA analysis are used in the results. Some of the mean values were negative due to analytical reason, and they were replaced by average values of colonization rate which were counted in EXCEL.

Table 2. Factors and response variables in analysis. Levels are shown in brackets next to each factor.

Factors	Chamber	**Temperature (5, 10, 15, 20 and 25°C)** ***Heterobasidion species* (*H. annosum*, *H. parviporum*, None)** Rotstop treatment (Rotstop F, Rotstop S, None) *Heterobasidion* isolates (*H. annosum*: Rb200, Mj65, Mj20; *H. parviporum*: Sä182, Sä150, Sä30; None) Trees (1,2,3,4,5,6,7)
	Field	**Site (Site1, Site2, Site3)** ***Heterobasidion species* (*H. annosum*, *H. parviporum*, None)** Rotstop treatment (Rotstop F, Rotstop S, None) *Heterobasidion* isolates (*H. annosum*: Rb200, Mj65, Mj20; *H. parviporum*: Sä182, Sä150, Sä30; None)
Response variables	*Heterobasidion* (uncorrected and corrected) and *Phlebiopsis gigantea* colonization rate on: Whole slice surface, sapwood, heartwood.	

Table 3. Example of using somatic compatibility data to correct colonization rate.

Segment or stump no.	Somatic compatibility data	Colonization rate	Corrected colonization area
1	80%	100%	(80% × 100%) = 80%
2	100%	60%	(100% × 60%) = 60%
3	0%	75%	(0% × 75%) = 0%

3. Results

3.1 Somatic compatibility

In general, *Phlebiopsis gigantea* showed a higher frequency of colonization than both *Heterobasidion annosum s.s.* and *H. parviporum* (Table 4). *P. gigantea* was more efficient at colonizing in both chamber and field conditions than *Heterobasidion* species; average colonization rate of *Heterobasidion* species is lower in chamber than in field condition, while average colonization rate of *P. gigantea* in chamber is slightly higher than in field condition. (chamber: *Heterobasidion* species: 1.7%, *P.gigantea*: 40.7%, $p<0.001$; field: *Heterobasidion* species: 10.5%, *P.gigantea*: 38.6%, $p<0.001$) Most colonized slices were inoculated and isolated successfully (Table 4). The reason for failed *Heterobasidion* inoculation, which was 17% of the cases, was unknown; while 26% of the cases unknown for failed *P. gigantea* inoculation. The main reason for failed re-isolation was contamination by bacteria or moulds. Crossings showed somatic compatibility on most of *Heterobasidion* species, which meant in most of the cases the same isolate that was originally inoculated colonized successfully in the wood. For *P. gigantea*, crossings of Rotstop F presented positive results for both chamber and field experiments (chamber: 94%, field: 100%). But all isolates from Rotstop S showed somatic incompatibility toward their original isolates potentially due to self-mating incompatibility between homokaryons.

3.2 Chamber experiment

3.2.1 Effect of Temperature on Phlebiopsis gigantea and Heterobasidion colonization

Lower temperature significantly reduced colonization of *P. gigantea* on the total slice surface ($p<0.001$) and on different parts of the slice (sapwood: $p<0.001$; heartwood: p=0.001) (Table 5). Colonization rate of *P.gigantea* was higher on sapwood than on total surface and heartwood. In both sapwood and total surface colonization was lowest at 5°C, while no significant differences occurred between 10°C to 25°C (Fig. 3a). In the case of heartwood colonization, the pattern was less clear since no differences between colonization at 5°C, 15°C and 25°C were observed (Table 5, Fig. 3a). There was no significant difference between the colonization rate of the two Rotstop strains at any

Table 4. Numbers of sample slices from segments (chamber) and stumps (field). Values in parentheses present percentages of the slices that showed 100% somatic compatibility out of cross-tested slices. 'Tested with the original isolate' column shows the numbers of slices from which the fungi were successfully isolated and cross-tested.

Experiment	Species	Inoculated	Colonized	Tested with the original isolate	100% Somatic compatibility
Chamber	*Heterobasidion species*	90	12	10	8(80%)
	Phlebiopsis gigantea	90	73	36	34(94%)
Field	*Heterobasidion species*	54	31	28	22(79%)
	Phlebiopsis gigantea	54	40	19	19(100%)

temperature level (Fig. 3b). For *Heterobasidion* species, temperature significantly affected sapwood colonization (p=0.020). Average sapwood colonization rate of *Heterobasidion* species reached the top at 10°C and bottom at 5°C. Although temperature had no effect on heartwood colonization (p=0.084), colonization rate at 15°C was significantly higher than at 5°C according to Tukey method. Colonization rate was higher on heartwood than on total surface and sapwood (Fig. 3c). Under the same temperature, average colonization rates of *Heterobasidion* species were much lower than of *P. gigantea* (Fig. 3d).

Table 5. Significance (p-values) of the factors and interactions among factors effects on the responds in the growth chamber. Bold values indicate significant effects at p<0.05. Temp=temperature. Ha=*Heterobasidion* species Rotstop=Rotstop treatment.

	Factors							
	Trees	**Temp**	**Ha**	**Rotstop**	**Temp × Ha**	**Temp ×Rotstop**	**Ha ×Rotstop**	**Temp × Ha × Rotstop**
H.spp-Total	0.191	0.110	**0.043**	**<0.001**	0.093	0.298	0.265	**0.039**
H.spp-Sapwood	**0.018**	**0.020**	0.195	**<0.001**	0.802	0.902	0.963	0.322
H.spp-Heartwood	0.471	0.084	**0.013**	**<0.001**	**0.015**	0.083	**0.044**	**0.003**
P.gigantea-Total	**0.009**	**<0.001**	**0.003**	0.523	**0.009**	0.412	0.208	**0.017**
P.gigantea-Sapwood	**0.001**	**<0.001**	**0.004**	0.726	**0.001**	0.465	0.254	**0.007**
P.gigantea-Heartwood	0.156	**0.001**	**0.021**	0.433	0.200	0.422	0.351	0.247

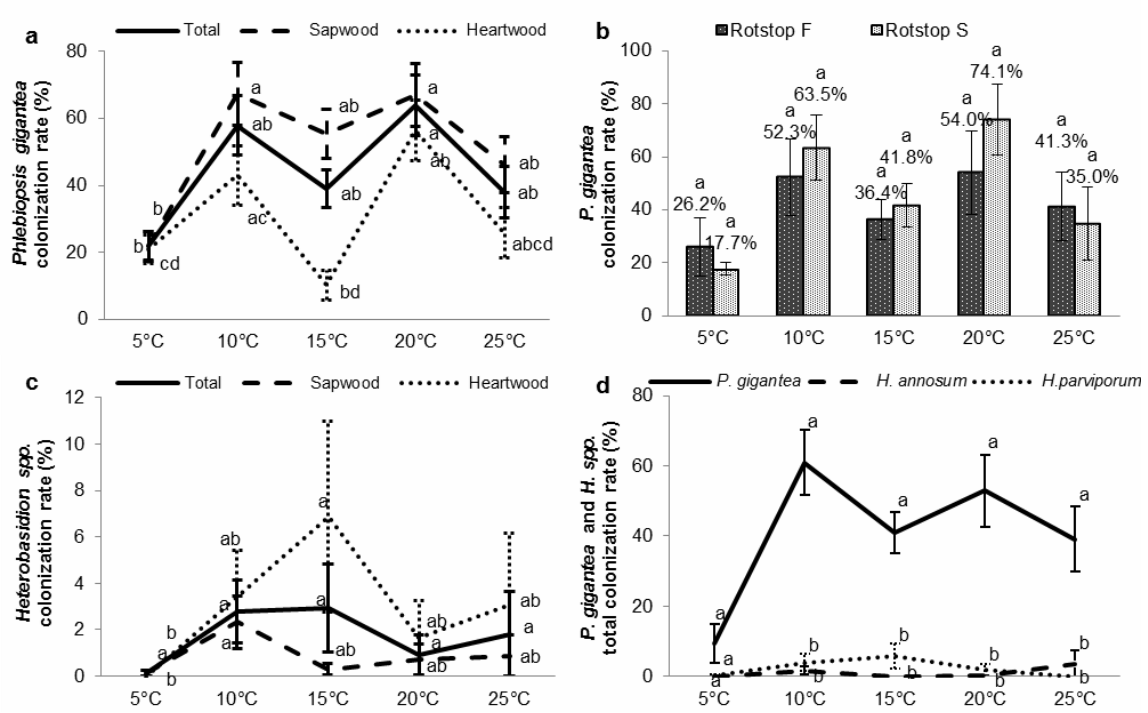

Figure 3. Colonization rate of manually inoculated *Phlebiopsis gigantea* (Rotstop) and *Heterobasidion* species on Norway spruce segments incubated under different temperature in growth chamber 12 weeks after inoculation. (a) shows the colonization rate of *P. gigantea* on all parts of slice while (b) presents the rate of Rotstop F and Rotstop S on total surface. (c) shows the average colonization rate of *Heterobasidion*. species on all parts of slice. (d) displays the average colonization rate of all fungi on total surface. In (a), (c) and (d), small letters indicate significant differences among different temperatures by Tukey method. In (b) small letters indicate significant differences between the two Rotstop strains within each temperature by Tukey method. Sample number is 18 of each temperature in (a) and (c), 9 in (b) for each type at each temperature. In (d) sample number is 18 for *P. gigantea* and 9 for each *Heterobasidion* species at each temperature. Error bars presents standard errors.

3.2.2 Colonization preferences between Heterobasidion species

There were significant colonization differences between *H. annosum* s.s. and *H. parviporum* on heartwood (p=0.013) and on the whole slice (p=0.043). On all parts of the slice, *H. parviporum* had a higher colonization rate than *H. annosum s.s*.. While colonization rate of *H. annosum s.s* was almost the same on different parts of the slice, *H. parviporum* had a distinctly higher colonization rate on heartwood than sapwood (Fig.4a). Surprisingly, co-inoculation of *P. gigantea* with *H. annosum s.s.* increased the colonization rate of *P. gigantea* on all part of slice (total: p=0.003; sapwood: p=0.004; heartwood: p=0.021) (Fig.4b). The same effect was not seen when co-inoculation was done with *H. parviporum*.

3.2.3 Effect of Rotstop treatment on Heterobasidion colonization

Rotstop treatment had a highly significant effect on colonization of *Heterobasidion* on both total (p<0.001) and individual parts (sapwood and heartwood: p<0.001), since virtually no *Heterobasidion* species colonization was found on the slices when co-inoculated with Rotstop. For the slices without Rotstop inoculation, *Heterobasidion* species had higher colonization rate on heartwood (9.0%) than on sapwood (2.6%) (Fig.5a). No significant difference was found on *P. gigantea*'s colonization between the two Rotstop strains (Table 5, Fig.5b).

3.2.4 Effect from other factors

There were significant differences amongst different trees used for the inoculation experiment. The colonization rate of *Heterobasidion* species on sapwood (p=0.018) and colonization rates of *P. gigantea* on total (p=0.009) and sapwood (p=0.001) were affected by different trees (Table 5). No significant difference was found among the colonization rates of different *Heterobasidion* isolates.

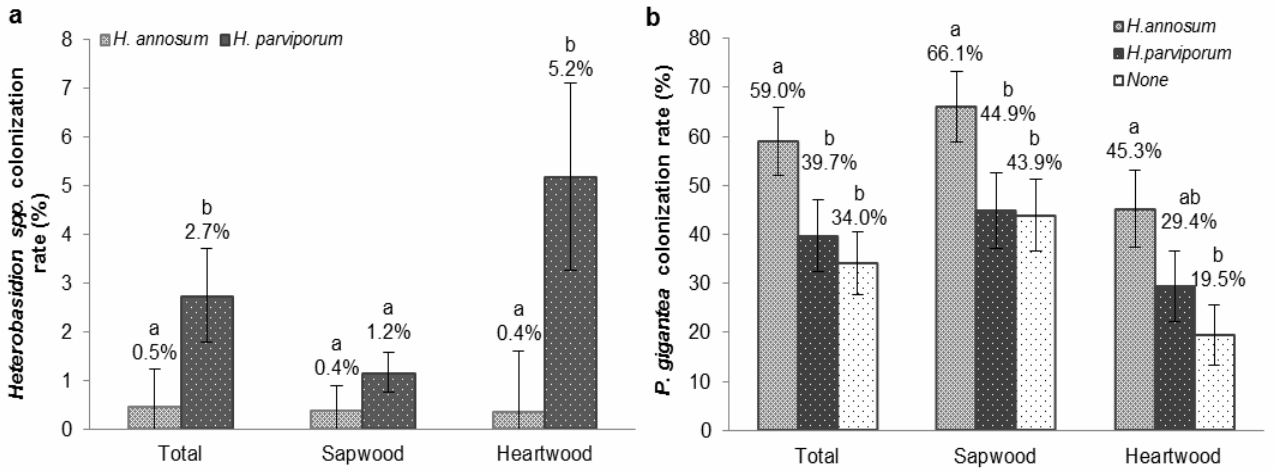

Figure 4. Colonization rate of manually inoculated *Heterobasidion* species (*H. annosum* and *H. parviporum*) and *Phlebiopsis gigantea* (Rotstop) on Norway spruce segments in a growth chamber. Colonization rate of *Hterobasidion* species is shown in (a) and *P.gigantea* colonization rate under different *Heterobasidion* treatment (*H. annosum. s.s*., *H. parviporum*, None) is show in (b) on total surface, sapwood and heartwood 12 weeks after inoculation. Bars present the standard error. Sample number is 45 for each column in (a) and 30 in (b). Small letters indicate significant difference among the colonization rate of different *Heterobasidion* treatment by Tukey method.

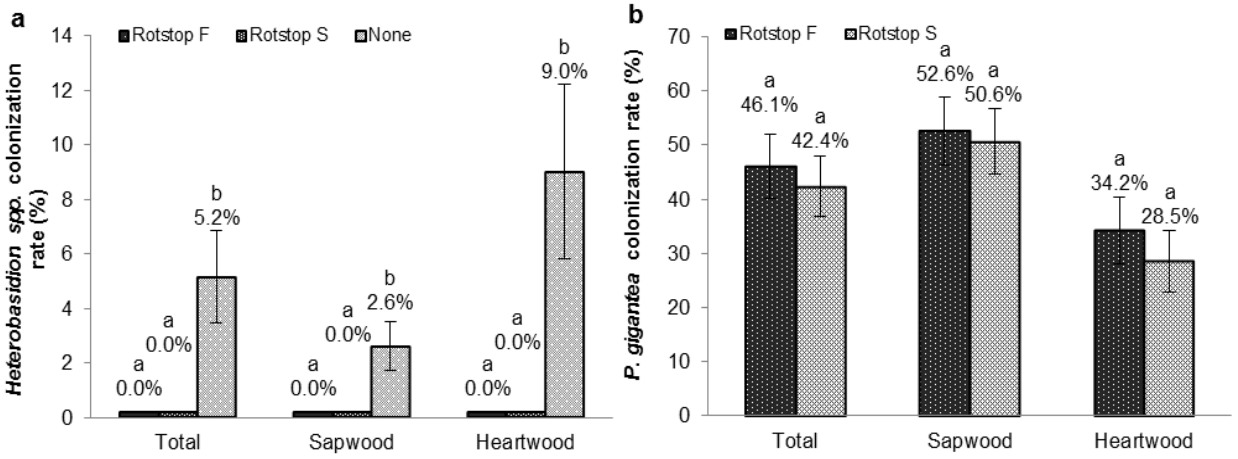

Figure 5. Colonization rate of manually inoculated *Heterobasidion* species (*H. annosum* and *H. parviporum*) and *Phlebiopsis gigantea* (Rotstop) on Norway spruce segments in a growth chamber. Colonization rate of *Heterobasidion* species under different Rotstop treatment (Rotstop F, Rotstop S, None) is shown in (a) while colonization rate of two Rotstop strains is shown in (b) on total surface, sapwood and heartwood 12 weeks after inoculation. Bars present the standard error. Sample number is 30 for each column in (a) and 45 in (b). Small letters indicate significant difference among the colonization rate of different Rotstop treatment by Tukey method.

3.3 Field experiments

3.3.1 Effect from Sites

Phlebiopsis gigantea colonization rates were different among different sites (total: p=0.024; sapwood: p=0.021; heartwood: p=0.034), and it was higher in Site 3, which had the highest sum of degree per hour (Fig.6a). Overall the colonization rates on sapwood were higher than on total surface and heartwood (Fig 6b). Colonization rates of *Heterobasidion* species had no difference among different sites with either uncorrected data or corrected data (Fig.6c). When only observing the stumps co-inoculated with both *P. gigantea* and *Heterobasidion* species, in Site1 and Site2, there was no significant difference among the colonization of *P. gigantea*, *H. annosum s.s.* and *H. parviporum*. In Site3, no difference was found among the three species by Tukey method, but the p-value in GLMA showed significant difference among them (p=0.022). Colonization rates of *P. gigantea* showed an increasing trend as temperature increased while no significant difference was found in the colonization rate of either *H. annosum s.s.* or *H. parviporum* as temperature rose.(Table 6, Fig.6d).

3.3.2 Effect from Heterobasidion species

With corrected data, there was no difference between the colonization rate of *H. parviporum* and *H. annosum s.s* on sapwood or heartwood (Fig. 7a). Overall, the colonization rate on heartwood was higher than on total surface and on sapwood, which was more distinct for *H. annosum s.s.* than for *H. parviporum*. With uncorrected data, significant difference was found on the entire slice surface and on sapwood (Table 6) between the two species. Contrarily to what was observed in the growth chamber, the colonization of *P.gigantea* was not affected by being co-inoculated with either of the two *Heterobasidion* species (Fig.7b).

Table 6. Significance (*p*-values) of the factors and interactions among factors effects on the responds in the growth chamber. Bold values indicate significant effects at $p<0.05$. Temp=temperature. Ha=*Heterobasidion species* Rotstop=Rotstop treatment. '-'=not included in analysis.

Responds	**Factors**						
	Sites	**Ha**	**Rotstop**	**Sites × Ha**	**Sites × Rotstop**	**Ha × Rotstop**	**Sites × Ha × Rotstop**
Corrected *H.spp*-Total	0.996	0.162	**0.028**	0.632	0.998	0.199	-
Corrected *H.spp*-Sapwood	0.985	0.146	**0.049**	0.836	0.999	0.109	-
Corrected *H.spp*-Heartwood	0.943	0.327	**0.020**	0.333	0.985	0.856	-
H.spp-Total	0.580	**0.010**	**<0.001**	0.288	0.884	**0.010**	0.735
H.spp-Sapwood *H.spp*-	0.537	**0.005**	**0.001**	0.450	0.865	**0.002**	0.813
Heartwood *P.gigantea*-	0.616	0.129	**<0.001**	0.157	0.935	0.458	0.837
Total *P.gigantea*-	**0.024**	0.673	0.946	0.439	0.345	0.753	0.597
Sapwood *P.gigantea*-	**0.021**	0.587	0.838	0.450	0.307	0.599	0.630
Heartwood	**0.034**	0.759	0.765	0.443	0.567	0.900	0.394

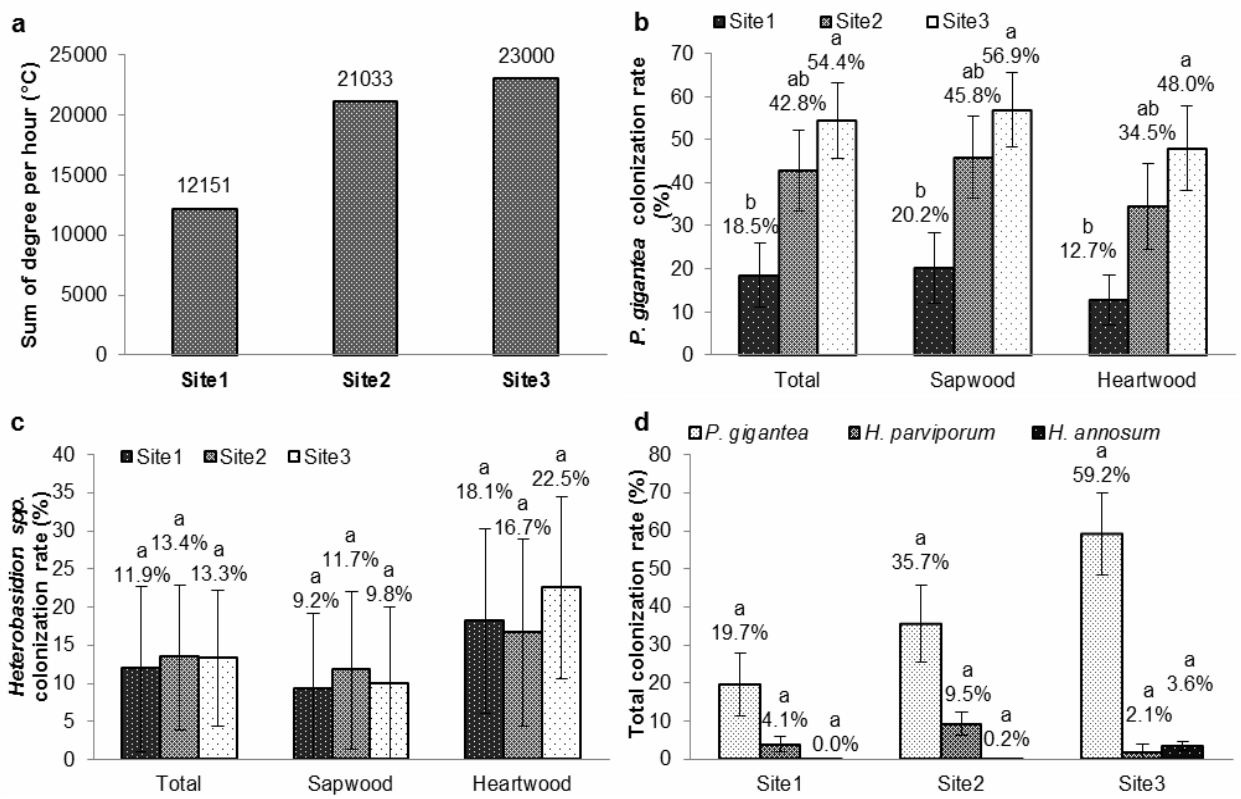

Figure 6. (a) shows the sum of degree per hour of each site during the first 9 weeks after inoculation in field condition. (b), (c) and (d) show the colonization rate of *Phlebiopsis gigantea* (Rotstop) and average colonization rate of *Heterobasidion* species (corrected) presented on total surface, sapwood and heartwood 13 weeks after inoculation. Sample number is 18 for each column in (b). In (c), sample number is 4 for site 1, 5 for site 2, 6 for site 3. (d) shows the comparison of average colonization rate of *P.gigantea* and *Heterobasidion* species when co-inoculated together on total surface. Sample number is 12 for each *P. gigantea* column. For *H. parviporum*, sample number is 3 for Site 1, 4 for Site 2, 2 for Site 3. For *H. annosum s.s.*, sample number is 1 for Site 1 and 2, 4 for Site 3. Bars present the standard error. Small letters are result by Tukey method, indicating significant difference among colonization rate in different sites in (b) and (c); in (d), small letters show significant difference among colonization rate of the three fungi in each site.

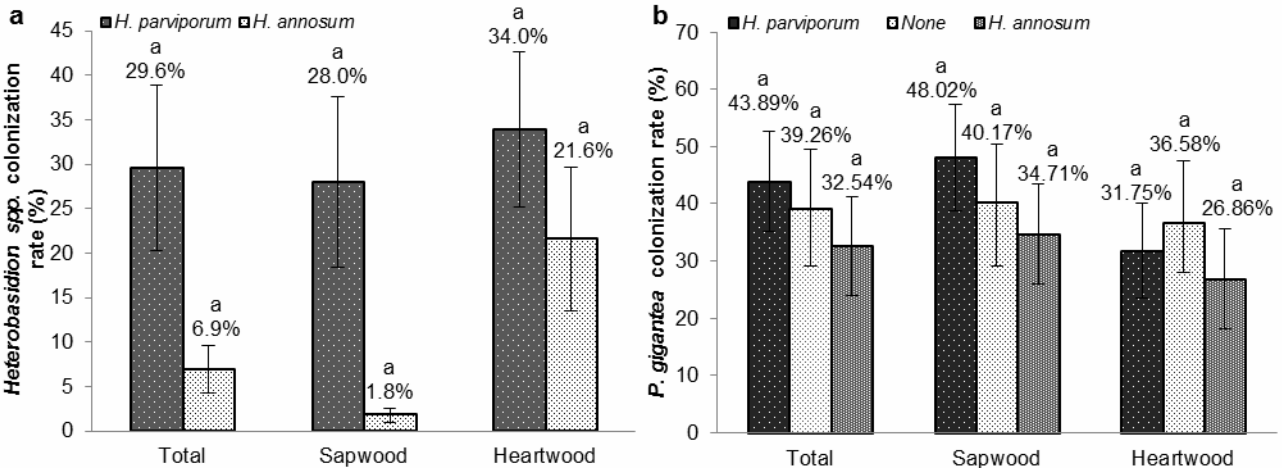

Figure 7. Corrected colonization rate of manually inoculated *Heterobasidion* species (*H. annosum* and *H. parviporum*) and *Phlebiopsis gigantea* (Rotstop) on Norway spruce stumps in field condition. Average values of colonization rate of *Heterobasidion* species are shown and mean colonization rate of *P.gigantea* under different *Heterobasidion* treatment are shown on total surface, sapwood and heartwood 13 weeks after inoculation. Bars show the standard error. In (a), sample number is 16 for *H. parviporum* and 12 for *H. annosum. s.s.* at each column on each part of slice. In (b), sample number is 18 for each column on each part of slice. Small letters indicate significant difference among the values of colonization rate within total, sapwood or heartwood by Tukey method.

3.3.3 Effect from Rotstop treatment

With both uncorrected and corrected data, Rotstop treatment showed significant effects on *Heterobasidion* species colonization for both total and the individual parts (corrected: total p=0.028; sapwood p=0.049; heartwood p=0.020). Average values of both *Heterobasidion* species colonization rate was much lower if Rotstop was also inoculated (Fig. 9a). There were no significant differences between *Heterobasidion* species colonization rate against Rotstop F and Rotstop S (Fig. 9c and 9d). Overall the colonization rate was higher on heartwood than on total and sapwood (Fig. 8a). No significant difference was found between the colonization rates of the two Rotstop strains (Fig. 9b).

3.3.4 Effect from other factors

There was no significant difference among the colonization of different *Heterobasidion* isolates with either uncorrected or corrected data in the field conditions.

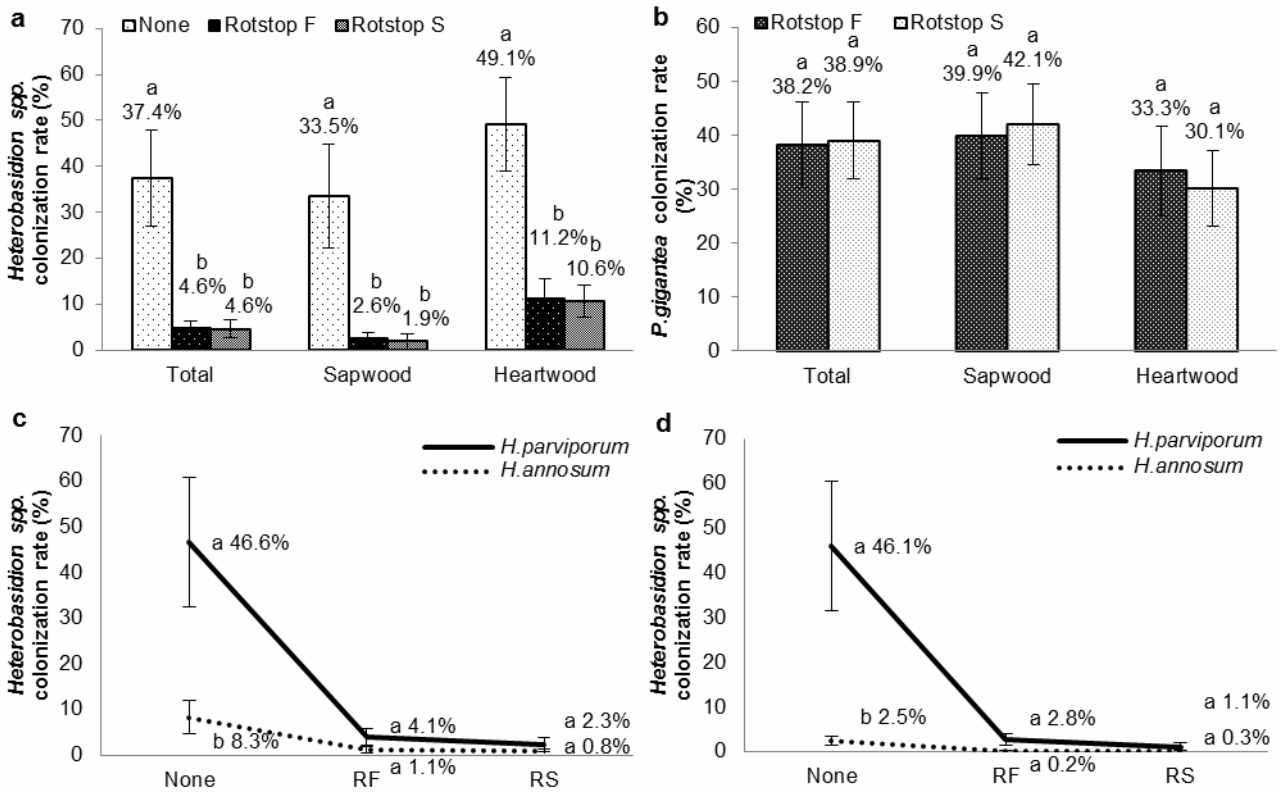

Figure 9. Colonization rate of manually inoculated *Heterobasidion* species (*H. annosum* and *H. parviporum*) and *Phlebiopsis gigantea* (Rotstop) on Norway spruce stumps in field condition. Corrected colonization rate of *Heterobasidion* species under different Rotstop treatment (Rotstop F, Rotstop S, None) is shown in (a) while colonization rate of two Rotstop strains is shown in (b) on total surface, sapwood and heartwood 13 weeks after inoculation. (c) and (d) present the uncorrected colonization rate of each *Heterobasidion* species under different Rotstop treatment on total surface (c) and sapwood (d). Sample number in (a) is 13 without Rotstop inoculation, 9 for against Rotstop F and 6 for against Rotstop S for each column in each part of slices; in (b) it is 27 for each column. In (c) and (d) the sample number is 9 for each species under each treatment. Small letters show results by Tukey method, indicate significant difference among the colonization rate of different Rotstop treatment in (a) and (b), while in (c) and (d) they show significant difference between the two species under different Rotstop treatment. Bars present the standard error.

4. Discussion

4.1 Temperature

This study showed that temperature significantly affected colonization of *Phlebiopsis gigantea* in both chamber and field conditions. In the growth chamber, *P. gigantea* colonization was decreased when temperature was under 5°C. In field conditions, *P. gigantea* colonization reached the highest at the largest sum of degree per hour and the lowest value appeared at the smallest sum of degree per hour. Similar results were also shown in a study by Swanwick (2007) where *P.gigantea* growth rate in both malt extract agar and wood agar reduced significantly at a decreasing incubation temperature from 25°C to 20°C and 15°C.

The colonization of *Heterobasidion* species was not affected by temperature except on sapwood in chamber. However, in chamber conditions the colonization rates of *Heterobasidion* species were quite low, from which it seems hard to tell if the observed pattern has any biological meaning. In several studies, it was shown or indicated that the infection incidence of *Heterobasidion* was higher if thinning was taken during summer (June-August) and lower if the thinning was in winter (November-Febrary) (Yde-Anderson, 1962; Gooding, 1966; Brandtberg, 1996; Piri, 2007). The optimum temperature for hyphae growth of *H. annosum s.s.* is 22-28°C, and at 12°C the growth minimized to 50% (Kasanen, 2011). A comparison of linear growth of *H. annosum s.s.* in stem sections showed an obvious decline at 30°C and no growth at 35°C (Gooding, 1966). Despite of no statistically significant difference between colonization of *H. annosum s.s.* and *H. parviporum* under different temperatures in current study, the trend was that *H. annosum s.s.* might have a higher colonization rate with higher temperature while *H. parviporum* could withstand a cooler environment for better colonization (Fig. 3d, Fig. 6d).

In both chamber and field conditions *P. gigantea* appeared to be much more sensitive to changes in temperature than *Heterobasidion.* In chamber condition, the segments were not fully randomized as expected since the amount of work would be huge, and this might be one reason why the colonization of different temperatures showed fluctuations. In field conditions, while *P. gigantea*. colonization raised as the increase of sum of degree per hour, no such effect could be seen in the case of *Heterobasidion* (Fig. 6d). Therefore, this study suggests that *P.gigantea* might benefit from warmer temperatures being able of outcompete more efficiently both *H. parviporum* and *H*. *annosum s.s.*. However, it is possible that *P. gigantea* might need more time to adapt temperature shifts than *Heterobasidion* species, which might cause a less efficacy of Rotstop in the warming condition by climate change.

4.2 *Heterobasidion* species

Different *Heterobasidion* species had different colonization rates. *H. parviporum* behaved more capable than *H. annosum s.s.* at colonizing in both chamber and field conditions. Study results of Piri (1996) also showed a higher infection rate of *H. parviporum* (26.3%) than of *H. annosum s.s.* (0.3%) on a previous *Heterobasidion*-

infected Norway spruce stands. According to Vasiliauskas (1998), *H. parviporum* was better at growing in Norway spruce stumps than *H. annosum s.s.*. Gunulf (2012) found that *H. parviporum* had an overwhelming advantage than *H. annosum s.s.* on colonizing speed and spatial growth in the beginning of colonization. Oliva (2011) observed that *H. parviporum* could spread to the neighboring trees more frequently than *H. annosum s.s.*. However, Daniel (1998) claimed that compared with *H. parviporum*, *H. annosum s.s.* was better at wood-degrading than the former. Overall *H. parviporum* showed stronger infection ability than *H. annosum s.s.* on Norway spruce.

The capacity of *P. gigantea* was also affected by different *Heterobasidion* species in chamber. Although the affecting pattern was intricate and different between chamber and field condition, *P. gigantea* seemed to have a higher colonization rate while co-inoculated with *Heterobasidion* species. It is not the first time that the capacity of *P. gigantea* tended to be enhanced by the presence of another fungus. Hamberg (2012) found that the efficacy of *P. gigantea* was improved when mixed with *Chondrostereum purpureum* since it could generate more hemicellulose and benefited the growth of *P. gigantea*. Another potential reason might be that the competitiveness of *P. gigantea* would be stimulated when co-inoculated with other fungus.

4.3 Rotstop treatment

Both Rotstop F and Rotstop S showed an equally strong effect on colonization of *Heterobasidion* species. In growth chamber, there was no *Heterobasidion* species colonization if Rotstop was inoculated. In field conditions, the colonization rate of *Heterobasidion* species was also quite low. Both Rotstop F and Rotstop S had the same capability at colonizing and neither of the two *Heterobasidion* species colonization showed differences at competing against any of the two Rotstop types. Rönnberg (2006) did not find significant difference between the *Heterobasidion*-reduction ability of Rotstop F and Rotstop S, either. In a study by Sun (2009), however, Rotstop F showed only moderately on growth in wood and control efficacy while Rotstop S showed poorly on both among all tested *P.gigantea* strains. One of the reasons for being less competitive could be due to the homokaryotic nature of the Rotstop S strain. Homokaryons may display lower growth and could elicit mating incompatibility amongst the different colonies in the stump. Current study suggest that indeed Rotstop S seems to grow unevenly in petri dishes and would form a barrier when being crossed with itself. However, despite these observations, no differences in stump coverage and protection against *Heterobasidion* could be detected between the heterokaryotic and the homokaryotic formulations.

4.4 Preference on different parts of wood

The current study showed that *P. gigantea* was good at colonizing on sapwood while *Heterobasidion* species preferred heartwood. Consequently, when considering the control efficacy i.e. the amount of reduced infection area, *P. gigantea* control efficacy was higher in sapwood (86% reduction) than in heartwood (67% reduction). These results indicated that *P. gigantea* might not be good at protecting heartwood area of the cutting surface from *Heterobasidion* species. This fact may be even more relevant with

regards to *H. parviporum* that was proved in this study to be more capable at colonizing on heartwood than *H. annosum* s.s., which may increase the difficulty of competing *H .parviporum* for Rotstop. Oliva (2013) also found that both *H. annosum s.s.* and *H. parviporum* preferred heartwood, and *H. parviporum* was even better at colonizing at heartwood than *H. annosum s.s.*. Berglund (2004) suggested that heartwood of Norway spruce was susceptive to *Heterobasidion*. Oliva (2011) also hypothesized a possible relationship between the ability of *H. parviporum*'s spreading frequency and the amount of heartwood in host trees, which enhanced the importance of heartwood protection and required Rotstop a better capacity. Compared with the large amount of study results showing *Heterobasidion* species' preference on heartwood, few study had discribed the preference on sapwood by *P. gigantea*. Therefore, more studies are necessary on whether it is the instinct of *P. gigantea* or it is forced to colonize on sapwood by *Heterobasidion* species.

5. Conclusions

Phlebiopsis gigantea was more sensitive to temperature shifts than *Heterobasidion* species, indicating that warming climate conditions could benefit the biocontrol capacity of Rotstop against the pathogen. *Heterobasidion parviporum* is better at colonizing than *H. annosum s.s.* and both species prefer heartwood, while *P. gigantea* is good at sapwood colonizing. Rotstop® F and Rotstop® S were equally effective in reducing the colonization of *Heterobasidion* species, although both preparations may have limitations when applied at lower temperature, and when protecting heartwood area especially against *H. parviporum*.

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