Biological Control 198 (2024) 105633

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

PaLAR3 genotype variability for enhanced resistance against *Heterobasidion parviporum* in Norway spruce: Insights into allelic frequencies, disease response, and the role of mycoviruses

Muhammad Kashif, Eeva Terhonen [*], Leena Hamberg, Tuula Piri, Matti Haapanen, Sirkku Pöykkö, Eeva J. Vainio, Jarkko Hantula

Natural Resources Institute Finland (Luke), Latokartanonkaari 9, FI-00790 Helsinki, Finland

HIGHLIGHTS

- Norway spruce had 64%, 30%, and 6% of PaLAR3 genotypes AA, AB, and BB, respectively.
- Two *H. parviporum* strains caused significantly variable necrosis responses.
- The two mycovirus strains tested significantly reduced lesion width in Norway spruce.
- The two mycovirus strains in *H. parviporum* reduced fungal growth in sapwood up to 41%

ARTICLE INFO

Keywords:
Heterobasidion
Norway spruce
Heterozygous
Homozygous
Resistance
Mycovirus

ABSTRACT

Heterobasidion parviporum is a destructive forest pathogen causing root and stem rot of Norway spruce (*Picea abies*) in northern hemisphere. Previous research has suggested that a gene form of PaLAR3 that encodes for the leucoanthocyanidin reductase enzyme, may increase resistance to *H. parviporum* in Norway spruce, whether heterozygous or homozygous. Our experiment was initially focused on testing this assertion with materials from the Finnish Norway spruce breeding program, comprising 52 full-sib families. Additionally, we investigated the influence of mycoviruses on the growth of *H. parviporum*. The frequencies of the three PaLAR3 genotypes AA, AB, and BB were 64%, 30%, and 6%, respectively. We found no significant differences in the necrotic response to *H. parviporum* between the homozygous (BB) and heterozygous (AB) genotypes vs. the homozygous (AA) genotype across the two fungal strains tested, suggesting the gene is rather ineffective as a marker for overall *H. parviporum* resistance. The two *H. parviporum* strains used in the study showed significantly different necrosis responses from the control. Mycovirus infection in *H. parviporum* strains led to a significant reduction in lesion width in phloem and sapwood. Furthermore, the growth of the fungus was notably restricted when hyphae hosted mycoviruses, indicating a high potential of mycoviruses to reduce fungal growth and necrosis development.

1. Introduction

Fungal pathogens of the genus *Heterobasidion* cause root and stem rot in conifers and are considered among the most devastating forest pathogens in the northern hemisphere. *Heterobasidion* spp. causes economic losses exceeding 50 million euros only in Finland (Hantula et al., 2023), and up to 1.2 billion euros in Europe (Woodward et al., 1998, inflation corrected). Within Europe, three distinct species of *Heterobasidion* exist: *H. annosum*, *H. parviporum*, and *H. abietinum*. These species exhibit host preferences but are not confined exclusively to pines, spruces, and firs, respectively (Niemelä & Korhonen, 1998; Gonthier & Garbelotto 2011).

Heterobasidion spp. are efficiently transmitted via basidiospores, that infect freshly cut stumps and wounds on trees. The fungal mycelium spreads thereafter to root systems and further on vegetatively to neighboring trees via root contacts (Stenlid & Redfern, 1998; Woodward et al., 1998; Korhonen 1978). Because of global warming, forest ecosystems are increasingly exposed to elevated risks due to forest

* Corresponding author.
E-mail address: eeva.terhonen@luke.fi (E. Terhonen).

https://doi.org/10.1016/j.biocontrol.2024.105633
Received 9 June 2024; Received in revised form 19 September 2024; Accepted 7 October 2024
Available online 12 October 2024

pathogens (Björkman et al., 2015; La Porta et al., 2008; Lindner et al., 2010). In Finland, this manifests most seriously as a heightened incidence of root rot, attributed to prolonged periods of warm weather (Müller et al., 2014). This phenomenon is exacerbated by a reduction in frost periods, compelling forest operations to shift to non-frost periods of the year. This change results in escalating harvest damage and a longer duration available for hyphal growth and spore production. Current stump treatments involving a competing fungus or urea effectively prevent new infections but do not impact existing *Heterobasidion* spp. within the spruce root systems. Also, forest management operations such as thinning result in a spread of previously established *Heterobasidion* infections to the remaining trees in the forest site (Korhonen et al., 1998; Piri & Korhonen, 2008). Consequently, the vegetative spread of *Heterobasidion* mycelium poses a challenge in rotational forests and an even greater risk in continuous cover forests, where the new generation of trees forms dense root networks conducive to *Heterobasidion* dispersal (Piri & Korhonen, 2001; Piri & Valkonen, 2013).

Enhancing the resistance of Norway spruce (*Picea abies*) reforestation materials could serve as a novel measure for controlling *H. parviporum*. Resistance or tolerance of Norway spruce against *H. parviporum* is considered to be controlled by many loci (QTL) (Lind et al., 2014). One of these QTL is defined as the PaLAR3 gene, which encodes leucoanthocyanidin reductase (LAR) (Lind et al., 2014) which increase production of catechin and through that, resistance against fungal pathogens (Hammerbacher et al., 2014; Fossdal et al., 2012). Nemesio-Gorriz et al. (2016) showed that there are two PaLAR3 allelic lineages (defined as PaLAR3A and PaLAR3B alleles from here on) in Norway spruce, of which genotypes with at least one allele, PaLAR3B, significantly restricts *H. parviporum* growth in sapwood in certain spruce families. However, the presence of this allele did not significantly reduce *H. parviporum* infections via root contacts between Norway spruce trees (Terhonen et al., 2022).

Mycoviruses, with double-stranded RNA (dsRNA) or single-stranded RNA (ssRNA) genomes, are commonly observed in fungi, but they rarely show any obvious symptoms, and instead remain cryptic. However, there are also some virus strains showing debilitating effects on their fungal hosts (Ahn & Lee, 2001; Osaki et al., 2002; Yu et al., 2010; Hyder et al., 2013; Ghabrial et al., 2015; García-Pedrajas et al., 2019; van Diepeningen, 2021; Vainio et al., 2024). Among these virus strains, CHV1 hosted by *Cryphonectria parasitica* has been most thoroughly studied for its hypovirulent effects (Anagnostakis & Day, 1979; Chen et al., 1996; Turina & Rostagno, 2007; Rigling and Prospero, 2018). *Heterobasidion* species are also known to host numerous dsRNA and ssRNA mycoviruses (Vainio & Hantula, 2016; Sutela et al., 2021). Two of them, the alphapartitiviruses HetPV13-an1 and HetPV15-pa1, are promising virocontrol agents with a major negative effect on the growth of their *Heterobasidion* hosts (Vainio et al., 2018; Kashif et al., 2019).

In this study we focused on testing the presence of PaLAR3 genotypes among the candidates for the next generation breeding material of the southern Finnish Norway spruce. Furthermore, in two experimental setups, we (i) tested if spruces with PaLAR3B allele in homozygous or heterozygous genotype could better resist infection (showing less necrosis/growth) by *H. parviporum* compared to those with homozygous PaLAR3AA genotype; and (ii) investigated the influence of mycoviruses on *H. parviporum* using the same spruce genotypes.

2. Materials and methods

2.1. Genotyping PaLAR3 alleles

The plant material consisted of 1862, 10-year-old Norway spruce young trees (52 full-sib families) in Haapastensyrjä field unit (Terhonen et al., 2022) originally produced by controlled crosses as part of the southern Finnish spruce breeding programme (Suppl. Table). Needles for PaLAR3 analyses were collected from three-year-old cuttings originating of these trees and stored at −20 °C. Trees for the inoculation experiment were randomly selected from the 1834 ten-year-old trees to include different families (at that moment the PaLAR3 genotypes had not yet been determined). Out of all 52 families, at least eight genotypes were initially genotyped (Suppl. Table). If all eight turned out to be the same homozygotic genotype (i.e., PaLAR3AA or PaLAR3BB), the remaining genotypes within a family were assigned as the same genotype.

Three methods were used to identify PaLAR3 alleles. At first, DNA was extracted directly from needles by Phire plant direct PCR kit using specific primers for PaLAR3A and PaLAR3B alleles (Nimesio-Gorriz et al., 2016). PCR program including 98 °C for 5 min, followed by 98 °C for 5 sec, 60 °C for 10 s, 72 °C for 10 sec for 40 cycles and extension with 72 °C for 60 sec. After noticing minor problems in reproducibility, we validated obtained results by re-isolating DNA using DNeasy Plant Pro Kit Plant (Qiagen) following manufacturer ́s instructions. The isolated DNA were analyzed for the concentration and quality by NanoDrop™ One/One^{c} Microvolume UV–Vis Spectrophotometer.

PCR was done by using a ThermoFisher Scientific™ DreamTaq Green DNA Polymerase (5U/μL) kit with primers designed for PaLAR3 locus (Edesi et al., 2021). DNA with a known PaLAR3 allele combination were used as positive controls and the PaLAR3A/PaLAR3B alleles were visually detected by UV transillumination of DNA amplicons on a 1.5 % agarose gel (Edesi et al., 2021; Terhonen et al., 2022).

2.2. Additional DNA isolation and qPCR based validation

The DNA extraction was further done following the method described by Pirttilä et al. (2001). The DNA templates were diluted to half of their volume before being used in quantitative PCR (qPCR) using EvaGreen dye (Solis BioDyne, Estonia) and allele-specific primers as described by Nemesio-Gorriz et al. (2016) on a Rotor-Gene device (Qiagen, USA).

2.3. Heterobasidion parviporum fungal and virus strains

In order to test if spruces with a homo- or heterozygous PaLAR3B allele can better resist the infection (necrosis/growth) of *H. parviporum,* we used two partitivirus-free *H. parviporum* strains (collected by Dr. Tuula Piri), SB2011 (Solböle, 2011) and SB2005 (Durodola et al., submitted), that were used to artificially inoculate 105 healthy 10-year-old spruce trees in 2018 (Table 1). Fungal isolates were grown for one week on wood plugs (0.5 cm) before being inoculated into the stems of spruce trees approximately 10 cm above the ground. Out of 105 inoculations, ten inoculated samples were not infected, or lost while processing, and discarded from the analysis.

In the second experiment, our aim was to examine how the presence of partitiviruses affects the performance of *H. parviporum* using the same spruce genotypes. The experiment was conducted using partitivirus-free isolates of *H. parviporum* SB2011 and SB9.3 and their isogenic counterparts that had been infected with two partitiviruses (HetPV13-an1 and HetPV15-pa1) using dual cultures (as described in Kashif et al., 2019). Virus-free controls were prepared by heat treatment and confirmed by RT-PCR as described earlier (Vainio et al., 2018). In 2021, two sets of inoculation experiments were carried out. In the initial set, 74 Norway spruce saplings were inoculated with either *H. parviporum* strain (strain SB2011 or strain SB9.3) hosting two partitiviruses (virus +) or the same *H. parviporum* strains without viruses (virus −). After three weeks, additional inoculations (65) with either *H. parviporum* (SB2011 and SB9.3) with or without partitiviruses were conducted, thereby augmenting the overall number of inoculations to 139 (Table 1). All the *Heterobasidion* sp. isolates were confirmed to be heterokaryotic based on the presence of clamp connections and exhibited normal growth rate and hyphal appearance on MAE plates.

Table 1
Artificial inoculations of *H. parviporum* strains with/without viruses into spruce.

Inoculation material used in 2018	No. of spruce trees with PaLAR3 alleles genotypes		
Fungal strains/controls	**AA**	**AB**	**BB**
SB2011	12	17	11
SB2005	12	17	14
Controls (without fungi)	10	6	6
Total no. of inoculations = 105	34	40	31
No. of successful obs. = 95			
Fungal growth in sapwood (FGS) (SB2011 + SB2005)	21	28	17
Total no. of obs. = 66			
Inoculation material used in 2021	No. of spruce trees with PaLAR3 alleles genotypes		
Fungal strains/controls	**AA**	**AB**	**BB**
SB2011 (virus +) (13an1 + 15pa1) = V1	16	10	6
SB9.3 (virus +) (13an1 + 15pa1) = V2	15	11	6
SB2011 (virus −) = C1	11	16	3
SB9.3 (virus −) = C2	8	13	3
Controls (without fungi)	9	10	2
Total no. of inoculations = 139	59	60	20
Obs. for Phloem (V1 + V2)	24	19	12
Obs. for Phloem (Controls)	21	27	5
Obs. for Xylem (V1 + V2)	24	19	12
Obs. for Xylem (Controls)	21	27	5
Xylem + Phloem (V1 + V2) = 110	90	92	34
Xylem + Phloem (C1 + C2) = 106	**Total no. of obs. = 216**		
Fungal growth in sapwood (FGS)	**Total no. of obs. = 108 (FGS)**		

Virus strains 13an1 = HetPV13-an1, 15pa1 = HetPV15-pa1, C = virus-free *H. parviporum* strain

2.4. Post-inoculation harvest and processing

Twelve weeks post-inoculation, inoculated trees were cut 20–30 cm above from the base (in 2018 and 2021), with the cut point positioned 10 cm above the inoculation site. In the first experiment (2018), the trunk up- and downwards of the inoculation point was sliced into 5 wood discs of each 0.5 cm thick. In the second experiment (2021), tree trunks were sliced up- and downwards from the inoculation point to five wood discs with thicknesses of 1 cm. All the wood discs were washed under running tap water and placed in plastic bags. The samples were incubated for 5–7 days at room temperature allowing the *H. parviporum* conidiophores to develop. The presence of the pathogen in each disc was confirmed under a stereo microscope (Vainio et al., 2015; Piri & Valkonen, 2013). The spread of the fungal infection was estimated based on the occurrence of conidiophores in wood discs. Conidiophores and associated mycelia were also transferred to 2 % MAE (Malt Agar) plates for the purpose of re-isolating the pathogen.

To analyze the susceptibility of host trees to the fungal infection, the sizes of the necrotic lesions were measured horizontally and vertically (length/width) in both phloem and sapwood according to previous studies (Mukrimin et al., 2018; Durodola et al., 2023). The distance of fungal growth upwards in the sapwood was determined by the presence of conidiophores indicating the presence of mycelium. The stability of the partitiviruses HetPV13-an1 and HetPV15-pa1 in re-isolated strains (pure cultures) was confirmed from 13 randomly chosen samples by RT-PCR with virus specific primers (Kashif et al., 2019).

2.5. Statistical analysis

The Hardy-Weinberg equilibrium among parental and offspring populations was tested using the Chi-square test.

The effects of the three PaLAR3 genotypes (AA, AB or BB) and the treatment (*H. parviporum* inoculum vs. control) on the lesion length (cm) and width (cm) were investigated using a linear mixed model with library *nlme* in the statistical program R (Pinheiro et al., 2019, R Core Team, 2019). In the models, we had lesion length or width (cm) as a response variable ($n = 95$). As mentioned above, differences in the lesion length and width were investigated using the following explanatory variables in the models: 1) the PaLAR3 genotypes (a factor with three levels: AA, AB and BB), 2) the treatment with or without fungal strain (a factor with three levels: control, *H. parviporum* strain SB2005, and *H. parviporum* strain SB2011), and 3) the diameter of a Norway spruce seedling (cm). As we expected that different genotypes may respond differently to the treatments, an interaction term was included in the models between the genotype and the treatment. The origin of Norway spruce saplings (tree family) was included as a random factor to the models to take into account the fact that trees within a family may respond more similarly than randomly chosen trees. Lesion width was log-transformed for the analysis to achieve normality. An interaction term was removed from the final model if it was not statistically significant.

The effects on the growth of *H. parviporum* hyphae from the inoculation point (in cm) was investigated as above by using a linear mixed effect model, but the control samples (without fungus) were removed from the model ($n = 66$).

For the second experiment, effects of virus free (−) and virus hosting (+) *H. parviporum* strains on lesion length and width (cm) in Norway spruce trees were investigated using linear mixed models as above. Models were estimated separately for virus free ($n = 106$) and virus hosting *H. parviporum* strains ($n = 110$) including responses from phloem and xylem. As a response variable we had lesion length or width (cm), and explanatory variables included in all models were: 1) *H. parviporum* strain (a factor with two levels: either virus free C1 and C2 or virus hosting strains V1 and V2, see Table 1), 2) the PaLAR genotype of Norway spruce (a factor with three levels: AA, AB and BB), 3) section of wood disc (a factor with two levels: phloem and xylem), and 4) tree diameter (cm). Tree family was included in the model as a random factor. Log transformation for the responses (lesion length and width) was used in all models except for lesion width model when virus hosting *H. parviporum* strains were used.

To see the general effect of virus free and virus hosting *H. parviporum* strains on lesion length and width, we ignored different fungal strains and genotypes and estimated common linear mixed models separately for lesion length and width. Lesion length and width (cm) were as response variables in the models ($n = 216$), and as explanatory variables we had: 1) an interaction term between treatment (a factor with two levels: virus-free and virus-infected *H. parviporum* strains) and section of wood disc (a factor with two levels: phloem and xylem), and 2) tree diameter (cm). Tree family was used as a random factor in the models. Models were log-transformed to achieve normality.

Differences in the growth of virus free and virus hosting *H. parviporum* strains in the sapwood were also investigated using linear mixed models ($n = 108$). As mentioned above, the same two explanatory variables we used and tree family was again included as a random factor to the model.

Result figures were drawn in R, and library *AICcmodavg* was utilized to calculate the predicted values based on the linear mixed models and library *ggplot2* to draw figures (Mazerolle, 2019,;R Core Team, 2019, 2023; Wickham, 2016).

3. Results

3.1. PaLAR3 allele frequencies

The analysis of PaLAR3 genotype of 1862 trees showed that 1189 trees (64 %) were homozygous for the PaLAR3A allele (genotype PaLAR3AA). Heterozygous PaLAR3AB genotype (PaLAR3AB) was observed in 565 trees (30 %). The homozygotic PaLAR3B genotype (PaLAR3BB) was found only in 108 trees (6 %) (Fig. 1). Therefore, the allele frequencies were 0.78 for PaLAR3A and 0.22 for PaLAR3B. The genotype frequencies did not differ significantly from the Hardy-Weinberg

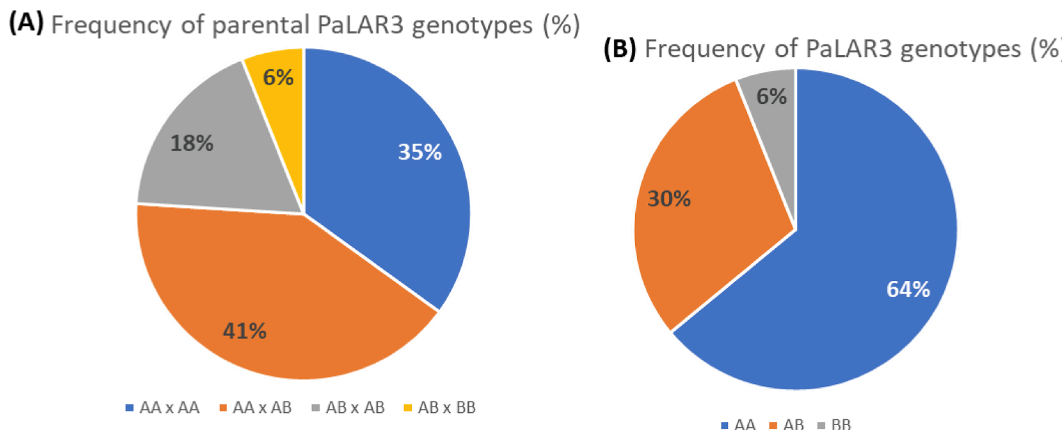

Fig. 1. (A) Frequencies of parental PaLAR3 genotype combinations among the 52 families of Norway spruce. (B) Frequency of the PaLAR3 genotypes among the trees of the 52 Norway spruce families.

equilibrium.

The study material was composed of 52 full-sib families with variable number of full siblings. All the three PaLAR3 genotypes were observed in nine families, whereas eighteen families had only the homozygous AA genotype. In addition, 21 families had both AA and AB genotypes and three families contained both AB and BB genotypes (Suppl. Table). Based on these observations, the allele frequencies in parental populations were 0.75 for PaLAR3A and 0.25 for PaLAR3B, with genotype frequencies in accordance with the Hardy-Weinberg equilibrium.

3.2. *Necrotic lesions and growth by H. parviporum strains between PaLAR3 genotypes*

In the first experiment, the lesion lengths caused by strains (SB2011 and SB2005) were compared to lengths caused by mock controls between the PaLAR3 genotypes (Table 2, Fig. 2a). The strain SB2011 exhibited varying responses to different PaLAR3 genotypes compared to the control, showing prolonged necrosis in AA genotypes. The lesions caused by the other strain (SB2005) were longer than in the control, but the trend across the genotypes was quite similar as lesions were shortest in genotype AA (Fig. 2a).

An increase in seedling diameter (cm) was associated with the decrease in lesion length ($p = 0.001$, Table 2). There were no differences in lesion width between the genotypes, but lesions were narrower in the control treatment (Table 2, Fig. 2b).

There was no significant difference in hyphae growth between the PaLAR3 genotypes (AA, AB, and BB) or between the two strains (SB2005 and SB2011) of *H. parviporum* in Norway spruce sapwood. Neither was the diameter of seedlings significantly related to the growth of the fungi.

3.3. *Impact of partitiviruses on necrotic lesions due to H. parviporum in PaLAR3 genotypes*

In the second experiment, the necrotic lesion length by *H. parviporum* without partitiviruses (virus −) showed no difference between genotypes PaLAR3AA and PaLAR3AB ($p = 0.513$). However, lesions were shorter in PaLAR3BB than in PaLAR3AA and PaLAR3AB genotypes ($p < 0.050$, Fig. 3A). In inoculations carried out with *H. parviporum* strains hosting partitiviruses HetPV13-an1 and HetPV15-pa1, the opposite trend was observed as the genotype PaLAR3AA exhibited less necrosis compared to other Norway spruce genotypes ($p < 0.050$, Fig. 3B) and tree diameter had no effect on the results ($p > 0.050$). There were no differences in lesion widths between the Norway spruce genotypes ($p > 0.050$, Figure not shown).

When all trees were combined, the lesion lengths had no difference between infections using virus free and virus hosting *H. parviporum* ($n = 261$, $p = 0.121$). However, lesions were statistically significantly wider in both phloem and xylem in infections by virus free (−) strains ($p = 0.002$, Fig. 4). Here, lesions were narrower in xylem than in phloem ($p < 0.001$), but tree diameter had no effect on the results ($p = 0.465$).

Table 2
Differences in lesion length and width (cm) in sapwood of Norway spruce (*Picea abies*) trees ($n = 95$) in different PaLAR3 genotypes (AA, AB and BB), and *Heterobasidion parviporum* treatments (fungal free control, SB2005, SB2011). Linear mixed models were used to analyze the data. Statistically significant ($p < 0.050$) coefficients (Coeff.), standard errors of means (SE) and *p*-values are denoted in bold.

	Model			
	Lesion length (cm)		Lesion width (cm) [h]	
Explanatory variables	Coeff. ± SE	*p*	Coeff. ± SE	*p*
Intercept	**3.079 ± 0.489**	**<0.001**	**0.380 ± 0.077**	**<0.001**
GenotypeAB [a]	0.904 ± 0.532	0.094	−0.031 ± 0.048	0.516
GenotypeBB [b]	0.844 ± 0.753	0.266	0.013 ± 0.061	0.825
H.parvi SB2005 [c]	**1.194 ± 0.468**	**0.013**	**0.104 ± 0.052**	**0.048**
H.parvi SB2011 [d]	**2.040 ± 0.485**	**<0.001**	**0.141 ± 0.055**	**0.012**
Seedling diameter (cm) [e]	**−0.316 ± 0.094**	**0.001**	−0.013 ± 0.016	0.429
GenotypeAB:H.parvi SB2005 [f]	−0.311 ± 0.691	0.654	–	–
GenotypeBB:H.parvi SB2005 [f]	0.185 ± 0.852	0.828	–	–
GenotypeAB:H.parvi SB2011 [g]	**−1.810 ± 0.700**	**0.012**	–	–
GenotypeBB:H.parvi SB2011 [g]	**−1.992 ± 0.962**	**0.042**	–	–

[a] Difference between genotypes AA and AB in the control.
[b] Difference between genotypes AA and BB in the control.
[c] Difference between the levels of curves of the control and the *H. parviporum* treatment (strain SB2005) in genotype AA.
[d] Difference between the levels of curves of the control and the *H. parviporum* treatment (strain SB2011) in genotype AA.
[e] Effect of seedling diameter on lesion length or width.
[f] Difference between the response curves of the control and the *H. parviporum* treatment (strain SB2005).
[g] Difference between the response curves of the control and the *H. parviporum* treatment (strain SB2011).
[h] Response was log-transformed for the analysis. Interaction was not statistically significant for lesion length, and therefore a simpler model is presented.

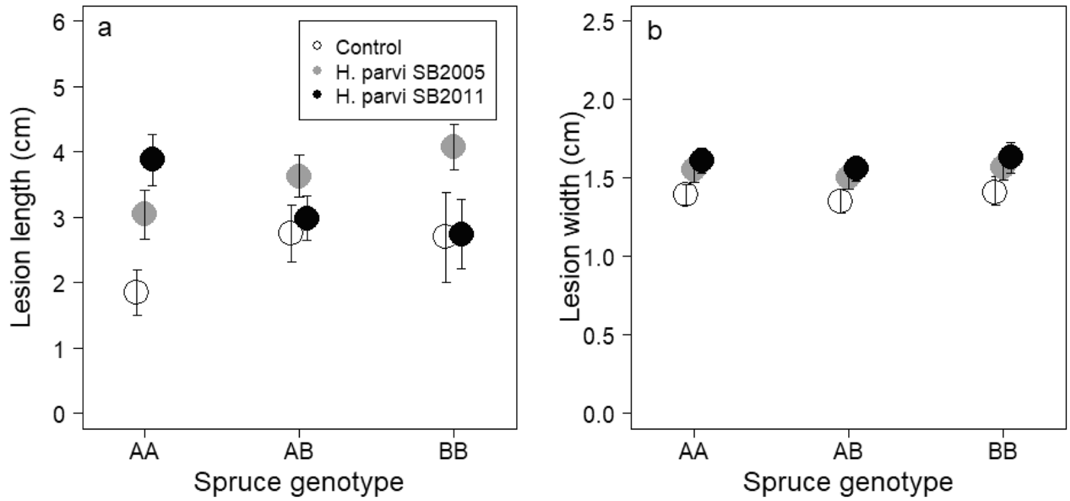

Fig. 2. Lesion length (a) and width (b) between Norway spruce PaLAR3 genotypes (AA, AB, BB) ($n = 95$) due to *Heterobasidion parviporum* strain SB2005 and SB2011, and the mock-control (fungal free). Predicted values based on the models with standard errors of means are presented.

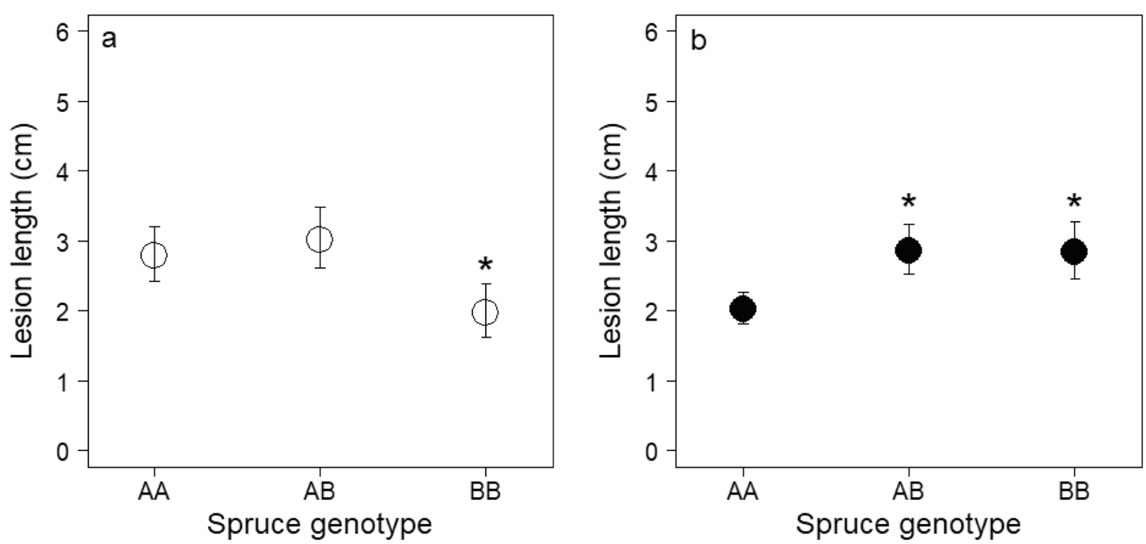

Fig. 3. Differences in lesion lengths (including measurements from phloem and xylem) in Norway spruce sapwood between PaLAR3AA and other PaLAR3 genotypes when (a) virus free *Heterobasidion parviporum* strains ($n = 106$) and (b) virus hosting *H. parviporum* strains were used ($n = 110$). Predicted values based on the models with standard errors of means are presented. Statistically significant differences ($p < 0.050$) between fungal treatments relative to PaLAR3AA and other PaLAR3 genotypes are indicated with an asterisk.

3.4. The effect of virus infection on the growth of H. parviporum in spruce sapwood

The overall effect of partitivirus infections on the fungal growth was clear and showed that the partitivirus strains HetPV13-an1 and HetPV15-pa1 reduced fungal growth up to 41 % as compared to isogenic virus free *H. parviporum* isolates ($n = 108$, $p < 0.001$, Fig. 5). Tree diameter did not affect the growth ($p = 0.990$).

4. Discussion

The overall results of this investigation show that the two alleles in PaLAR3 gene, previously shown to affect spruce tolerance against Heterobasidion root and butt rot, are present in the second-generation breeding materials of the Finnish Norway spruce breeding programme (Nemesio-Gorriz et al., 2016). The allele PaLAR3A is about three times as common as PaLAR3B, which is very close to the relative frequencies observed by Nemesio-Gorriz et al., 2016; Edesi et al., 2023; and Varis et al., 2023. Based on our observations, the allele frequencies in parental populations were 0.25 for PaLAR3B which supports the results of recent studies where the average frequency of this allele was reported to be 0.25 (Edesi et al., 2019; Simonen, 2023; Varis et al., 2023).

The mycelial growth of *Heterobasidion* isolates did differ among the three PaLAR3-genotypes. This agrees with root-mediated spread measurements of *H. parviporum* by Terhonen et al. (2022), but not Nemesio-Gorritz et al.'s (2016) study, which found slower *H. parviporum* growth in trees with allele PaLAR3B. The reasons for this inconsistency may relate to e.g. differences in the sizes of the inoculated trees or fungal isolates used in inoculation methods. Although the three PaLAR3 genotypes showed similar overall responses to the strains, one strain caused shorter lesion lengths in genotype AA and longer lesion lengths in genotype BB (i.e., AA < AB < BB). On the other hand, the other fungal isolate had the opposite effect, with the order AA > AB > BB. Similar variability of necrotic lesions and growth by different *H. parviporum* strains was recently reported by Simonen (2023). The necrotic lesions were considered as an indicator of susceptibility and the variability in their sizes are as in the previous studis (Durodola et al., 2023; Mukrimin et al. (2018). While the interaction was observed only for lesion length,

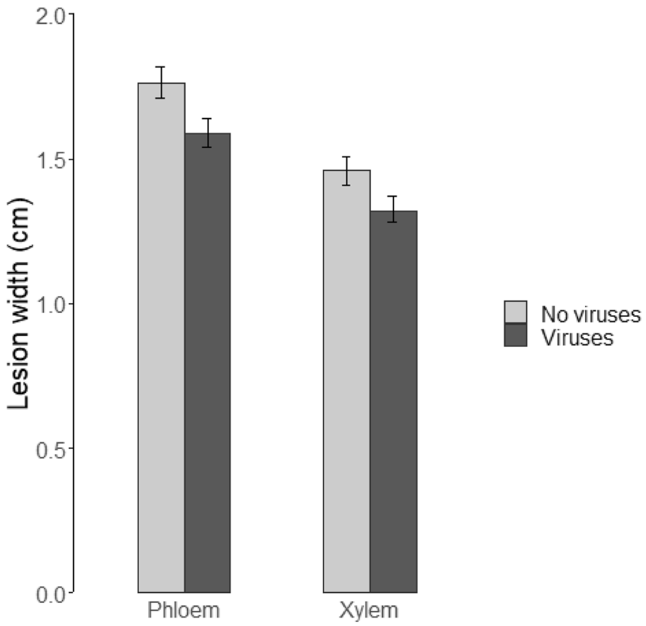

Fig. 4. Differences in lesion width in Norway spruce trees when they were infected by using partitivirus free and partitivirus hosting *Heterobasidion parviporum* strains ($n = 216$). Differences between phloem and xylem, and between virus and virus-free treatments were statistically significant ($p < 0.050$). Predicted values based on the models with standard errors of means are presented.

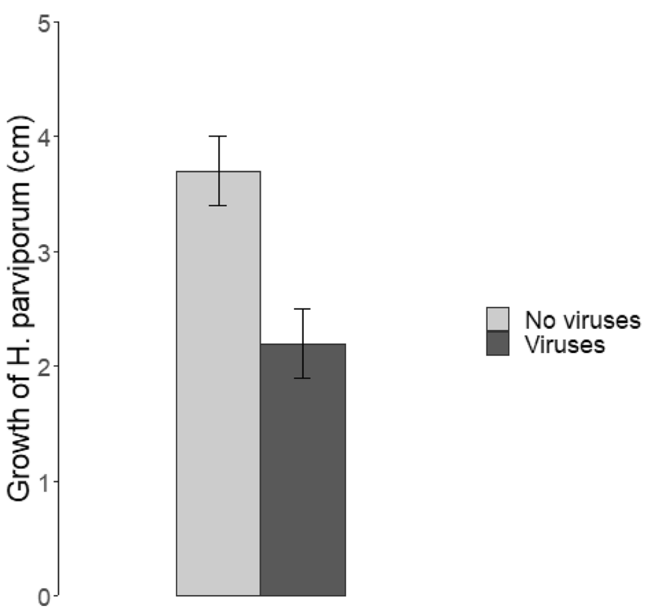

Fig. 5. Combined effect of viruses (HetPV13-an1 and HetPV15-pa1) on the growth of *Heterobasidion parviporum* in the sapwood of Norway spruce trees ($n = 108$). Difference in growth was statistically significant when virus-free and virus-hosting *H. parviporum* strains were used ($p < 0.001$). Predicted values based on the models with standard errors of means are presented.

and not for lesion width, the finding is still intriguing and supports the importance of using multiple isolates in pathogenicity tests, as suggested by Durodola et al. (2023).

It has previously been shown that the presence of HetPV13-an1 in the mycelium can reduce by 90 % the area of *H. parviporum* in the cross-section of the trunk taken 10 cm above the inoculation point (Vainio et al., 2018). It has also been shown that treatment of *Heterobasidion*-infected pine stumps with *H. annosum* strain hosting HetPV13-an1 significantly reduces the growth of the pathogen in root systems treated with *Phlebiopsis gigantea*, used commercially for a prophylactic control of *Heterobasidion* infection (Piri et al., 2023). In the current study, we tested the effect of a combined use of HetPV13-an1 and HetPV15pa1, as it was previously shown that both viruses reduce growth of *H. annosum* equally on malt agar plates (Kashif et al., 2019).

The effect of Heterobasidion partitiviruses HetPV13-an1 and HetPV15-pa1 on the development of necrosis and on the mycelial growth in the sapwood of spruce trees showed that the viral effect on necrosis was significant, although the viruses seemed to have somewhat different effects on lesion widths in xylem regions of trees with different PaLAR3 genotypes. However, the mycelial growth did not correlate with observations related to necrotic lesions. More importantly, since the growth of *Heterobasidion* mycelium in the sapwood was reduced on average by 41 %, the results strengthen the view that an efficient biocontrol application based on partitiviruses HetPV13-an1 and HetPV15-pa1 could be developed.

The virulence-related diversity of *H. parviporum* strains and its impact on fungal infection in different PaLAR3 genotypes deserve further study. Also, further resistance markers associated to infection caused by *H. parviporum*, such as recently found QTL based resistance markers like PaNAC04 and those related to the NAC family (Chaudhary et al., 2020) should be searched and verified prior to their possible inclusion in breeding. We also showed that a double infection by partitiviruses HetPV13-an1 and HetPV15-pa1 substantially reduces the growth of *Heterobasidion* mycelium in spruce sapwood, which supports previous findings (Vainio et al., 2018, Kashif et al., 2019; Hantula et al., 2021), and provides further support on the use of these two viruses as a promising alternative to control Heterobasidion root rot.

CRediT authorship contribution statement

Muhammad Kashif: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Eeva Terhonen:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Formal analysis. **Leena Hamberg:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Methodology, Investigation, Formal analysis. **Tuula Piri:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Matti Haapanen:** Writing – review & editing, Resources, Funding acquisition. **Sirkku Pöykkö:** Supervision, Resources. **Eeva J. Vainio:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Formal analysis, Conceptualization. **Jarkko Hantula:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Funding

The study was funded by the Kolli Foundation (Marjatta ja Eino Kollin Säätiön), Foundation for Forest Tree Breeding and Natural Resources Institute Finland (Luke).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We are grateful to Juha Puranen, Ari Rajala, Tuija Hytönen, Carita Karenius and Katri Leino for technical assistance.

authors' contributions

MK, EV, TP, ET and JH designed the research project. TP provided the fungal strains used in this study and MH and SP kindly provided the experimental trees and the field. MK, TP, SP and JH performed the experiments. LH, JH, ET and MK analyzed the data, and MK, LH, EV, TP, ET and JH wrote the manuscript followed by editing and revision by all authors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2024.105633.

References

Ahn, I.P., Lee, Y.H., 2001. A viral double-stranded RNA up regulates the fungal virulence of nectria radicicola. Mol. Plant. Microbe. in. 14 (4), 496–507. https://doi.org/10.1094/MPMI.2001.14.4.496.

Anagnostakis, S., Day, P., 1979. Hypovirulence conversion in Endothia parasitica. Phytopathology 69, 1226–1229.

Björkman, C., Bylund, H., Nilsson, U., Nordlander, G., Schroeder, L.M. (2015). Forest management to mitigate insect damage in a changing climate. Pp. 248-266 in: Björkman, C. & Niemelä, P. (eds.) Climate Change and Insect Pests. CABI, UK, ix + 266 pp. ISBN-13: 978 1 78064 378 6. https://www.cabidigitallibrary.org/doi/10.1079/9781780643786.0248.

Chaudhary, R., Lundén, K., Dalman, K., Dubey, M., Nemesio-Gorriz, M., Karlsson, B., Stenlid, J., Elfstrand, M., 2020. (2020) Combining transcriptomics and genetic linkage based information to identify candidate genes associated with Heterobasidion-resistance in Norway spruce. Sci. Rep. 10 (1), 12711. https://doi.org/10.1038/s41598-020-69386-0.

Chen, B., Gao, S., Choi, G.H., Nuss, D.L., 1996. Extensive alteration of fungal gene transcript accumulation and elevation of G-protein-regulated cAMP levels by a virulence-attenuating hypovirus. Proc. Natl. Acad. Sci. U.S.A 93, 7996–8000. https://doi.org/10.1073/pnas.93.15.7996.

Durodola, B., Blumenstein, K., Terhonen, E., 2023. Genetic variation of Picea abies in response to the artificial inoculation of *Heterobasidion parviporum*. Eur. J. Forest Res. 142, 443–453. https://doi.org/10.1007/s10342-023-01534-3.

Edesi, J., Tikkinen, M., Elfstrand, M., Olson, Å., Varis, S., Egertsdotter, U., Aronen, T., 2021. Root rot resistance locus palar3 is delivered by somatic embryogenesis (SE) pipeline in Norway spruce (picea abies (L.) karst.). Forests 12 (2). https://doi.org/10.3390/f12020193.

Fossdal, C.G., Nagy, N.E., Hietala, A.M., Kvaalen, H., Slimestad, R., Woodward, S., Solheim, H., 2012. Indications of heightened constitutive or primed host response affecting the lignin pathway transcripts and phenolics in mature Norway spruce clones. Tree Physiol 32, 1137–1147.

García-Pedrajas, M.D., Cañizares, M.C., Sarmiento-Villamil, J.L., Jacquat, A.G., Dambolena, J.S., 2019. Mycoviruses in biological control: from basic research to feld implementation. Phytopathology. 109 (11), 1828–1839.

Ghabrial, S.A., Castón, J.R., Jiang, D., Nibert, M.L., Suzuki, N., 2015. 50-plus years of fungal viruses. Virology. 479–480, 356–368. https://doi.org/10.1016/j.virol.2015.02.034.

Gonthier, P., Garbelotto, M., 2011. Amplified fragment length polymorphism and sequence analyses reveal massive gene introgression from the European fungal pathogen *Heterobasidion annosum* into its introduced congener H. irregulare. Mol. Ecol. 20, 2756–2770.

Hammerbacher, A., Paetz, C., Wright, L.P., Fischer, T.C., Bohlmann, J., Davis, A.J., Fenning, T.M., Gershenzon, J., Schmidt, A., 2014. Flavan-3-ols in Norway spruce: biosynthesis, accumulation, and function in response to attack by the bark beetle-associated fungus Ceratocystis polonica. Plant Physiol. 164, 2107–2122.

Hantula, J., Ahtikoski, A., Honkaniemi, J., Huitu, O., Härkönen, M., Kaitera, J., Koivula, M., Korhonen, K., Lindén, A., Lintunen, J., Luoranen, J., Matala, J., Melin, M., Nikula, A., Peltoniemi, M., Piri, T., Räsänen, T., Sorsa, JA., Strandström, M., Uusivuori, J & Ylioja, T. (2023). Metsätuhojen kokonaisvaltainen arviointi : METKOKA-hankkeen loppuraportti. http://urn.fi/URN:ISBN:978-952-380-688-7.

Hantula, J., Vainio, E., Kashif, M., 2021. Method for preventing wood decay, related biocontrol agent and uses. Patent. WO2020058580A1.

Hyder, R., Pennanen, T., Hamberg, L., Vainio, E.J., Piri, T., Hantula, J., 2013. Two viruses of Heterobasidion confer beneficial, cryptic or detrimental effects to their hosts in different situations. Fungal Ecol. 6, 387–396. https://doi.org/10.1016/j.funeco.2013.05.005.

Kashif, M., Jurvansuu, J., Vainio, E.J., Hantula, J., 2019. Alphapartitiviruses of Heterobasidion wood decay fungi affect each other's transmission and host growth. Front. Cellular Infect. Microbiol. 9 (MAR). https://doi.org/10.3389/fcimb.2019.00064.

Korhonen, K., 1978. Intersterility groups of *Heterobasidion annosum*. Commun. Inst. Forest. Fenn. 94, 1–25.

Korhonen, K., Delatour, C., Greig, B.J.W. & Schönhar, S. (1998). Silvicultural control. *Heterobasidion annosum*: Biology, ecology, impact and control (Woodward, S., Stenlid, J., Karjaleinen, R. & Hüttermann, A.). Wallingford, UK: CAB International. ISBN 0851992757.

La Porta, N., Capretti, P., Thomsen, I.M., Kasanen, R., Hietala, A.M., Von Weissenberg, K., 2008. Forest pathogens with higher damage potential due to climate change in Europe. Can. J. Plant Pathol. 30, 177–195. https://doi.org/10.1080/07060661.2008.10540534.

Lind, M., Källman, T., Chen, J., Ma, X.F., Bousquet, J., Morgante, M., Zaina, G., Karlsson, B., Elfstrand, M., Lascoux, M., Stenlid, J., 2014. A Picea abies linkage map based on SNP markers identifies QTLS for four aspects of resistance to *Heterobasidion parviporum* infection. PLoS ONE 9 (7). https://doi.org/10.1371/journal.pone.0101049.

Lindner, M., Maroschek, M., Netherer, S., Kremer, A., Barbati, A., Garcia-Gonzalo, J., Seidl, R., Delzon, S., Corona, P., Kolström, M., Lexer, M.J., Marchetti, M., 2010. Climate change impacts, adaptive capacity, and vulnerability of European forest ecosystems. For. Ecol. Manage. 259 (4), 698–709. https://doi.org/10.1016/j.foreco.2009.09.023.

Mazerolle, M. J. (2019) AICcmodavg: Model selection and multimodel inference based on (Q)AIC(c). R package version 2.2-2. https://cran.r-project.org/package=AICcmodavg.

Mukrimin, M., Kovalchuk, A., Neves, L.G., et al., 2018. Genome-wide exon-capture approach identifies genetic variants of Norway spruce genes associated with susceptibility to Heterobasidion parviporum infection. Front. Plant Sci. 9, 793. https://doi.org/10.3389/fpls.2018.00793.

Müller, M.M., Sievänen, R., Beuker, E., Meesenburg, H., Kuuskeri, J., Hamberg, L., Korhonen, K., 2014. Predicting the activity of *Heterobasidion parviporum* on Norway spruce in warming climate from its respiration rate at different temperatures. Forest Pathology 44 (4). https://doi.org/10.1111/efp.12104.

Nemesio-Gorriz, M., Hammerbacher, A., Ihrmark, K., Källman, T., Olson, Å., Lascoux, M., Stenlid, J., Gershenzon, J., Elfstrand, M., 2016. Different alleles of a gene encoding leucoanthocyanidin reductase (PaLAR3) influence resistance against the fungus *Heterobasidion parviporum* in *Picea abies*. Plant Physiology 171 (4). https://doi.org/10.1104/pp.16.00685.

Niemelä, T. & Korhonen, K. (1998). Taxonomy of the genus Heterobasidion. (In:) S. Woodward, J. Stenlid, R. Karjalainen, A. Hütterman (eds). *Heterobasidion annosum*: Biology, Ecology, Impact and Control. C.A.B. International, 27–33.

Osaki, H., Nomura, K., Iwanami, T., et al., 2002. Detection of a double stranded RNA virus from a strain of the violet root rot fungus Helicobasidium mompa Tanaka. Virus Genes 25, 139–145. https://doi.org/10.1023/A:1020105701017.

Pinheiro, J., Bates, D., DebRoy, S. & Sarkar, D. R Core Team (2019). nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-142, URL: https://CRAN.R-project.org/package=nlme.

Piri, T., Korhonen, K., 2001. Infection of advance regeneration of Norway spruce by *Heterobasidion parviporum*. Can. J. for. Res. 31, 937–942. https://doi.org/10.1139/x01-021.

Piri, T., Korhonen, K., 2008. The effect of winter thinning on the spread of *Heterobasidion parviporum* in Norway spruce stands. Canad. J. Forest Res. 38 (10), 2589–2595. https://doi.org/10.1139/X08-103.

Piri, T., Vainio, E.J., Hantula, J., 2023. Preventing mycelial spread of *Heterobasidion annosum* in young Scots pine stands using fungal and viral biocontrol agents. Biological Control 184. https://doi.org/10.1016/j.biocontrol.2023.105263.

Piri, T., Valkonen, S., 2013. Incidence and spread of Heterobasidion root rot in uneven-aged Norway spruce stands. Can. J. for. Res. 43, 872–877.

Pirttilä, A.M., Hirsikorpi, M., Kämäräinen, T., et al., 2001. DNA isolation methods for medicinal and aromatic plants. Plant Mol. Biol. Rep. 19, 273. https://doi.org/10.1007/BF02772901.

R Core Team, 2019. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria https://www.R-project.org/.

R Core Team, 2023. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria https://www.R-project.org/.

Rigling, D., Prospero, S., 2018. Cryphonectria parasitica, the causal agent of chestnut blight: invasion history, population biology and disease control. Mol. Plant Pathol. 19 (1), 7–20. https://doi.org/10.1111/mpp.12542.

Simonen, E. (2023): Root rot resistance detection in vegetatively propagated Norway spruce. Msc. Thesis. Department of Environmental and Biological Sciences, biology. University Of Eastern Finland (40 cp), 47pp.

Stenlid, J. & Redfern, D.B. (1998). Spread within the tree and stand. *Heterobasidion annosum*: Biology, ecology, impact and control (Woodward, S., Stenlid, J., Karjaleinen, R. & Hüttermann, A.). Wallingford, UK: CAB International. ISBN 0851992757.

Sutela, S., Piri, T., Vainio, E.J., 2021. Discovery and community dynamics of novel ssRNA mycoviruses in the conifer pathogen *Heterobasidion parviporum*. Front. Microbiol. 12, 770787.

Terhonen, E., Kashif, M., Piri, T., Haapanen, M., Hantula, J., 2022. Allele PaLAR3B in root rot resistance locus does not influence the infection pressure by *Heterobasidion parviporum* through root contacts. Forest Pathology 52, e12769.

Turina, M., Rostagno, L., 2007. Virus-induced hypovirulence in Cryphonectria Parasitica: still an unresolved conundrum. J. Plant Pathol. 89 (2), 165–178. http://www.jstor.org/stable/41998375.

Vainio, E.J., Hantula, J., 2016. Taxonomy, biogeography and importance of Heterobasidion viruses. Virus Research 219, 2–10. https://doi.org/10.1016/j.virusres.2015.10.014.

Vainio, E.J., Jurvansuu, J., Hyder, R., Kashif, M., Piri, T., Tuomivirta, T., Poimala, A., Xu, P., Mäkelä, S., Nitisa, D., Hantula, J., 2018. Heterobasidion Partitivirus 13 mediates severe growth debilitation and major alterations in the gene expression of a fungal forest pathogen. J. Virol. 92, e01744–e01817. https://doi.org/10.1128/JVI.01744-17.

Vainio, E., Müller, M., Korhonen, K., et al., 2015. Viruses accumulate in aging infection centers of a fungal forest pathogen. ISME J 9, 497–507. https://doi.org/10.1038/ismej.2014.145.

Vainio, E.J., Rumbou, A., Diez, J.J., Büttner, C., 2024. Forest tree virome as a source of tree diseases and biological control agents. Current Forest. Rep. 1–22.

van Diepeningen, A.D., 2021. Biocontrol via mycoviruses: a neglected option for bioprotection? In: Jürgen Köhl, J., Willem Ravensberg, W. (Eds.), Microbial Bioprotectants for Plant Disease Management. Burleigh Dodds Science Publishing Limited, pp. 541–584. https://doi.org/10.19103/AS.2021.0093.20.

Varis, S., Tikkinen, M., Edesi, J., Aronen, T., 2023. How to Capture Thousands of Genotypes Initiation of Somatic Embryogenesis in Norway Spruce. Forests 14–810. https://doi.org/10.3390/f14040810.

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York.

Woodward, S., Stenlid, J., Karjalainen, R., Hüttermann, A., 1998. *Heterobasidion annosum*: biology, ecology, impact and control. CAB International, Wallingford.

Yu, X., Li, B., Fu, Y., Jiang, D., Ghabrial, S.A., Li, G., Peng, Y., Xie, J., Cheng, J., Huang, J., Yi, X., 2010. A geminivirus-related DNA mycovirus that confers hypovirulence to a plant pathogenic fungus. Proc. Natl. Acad. Sci. U.S.A. 107, 8387–8392. https://doi.org/10.1073/pnas.0913535107.