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Tolerance Against Co-Infection of Two Partitiviruses and Ourmia-Likevirus Is Common Among *Heterobasidion annosum* Strains on Artificial Media and in Dead Wood

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ABSTRACT

Some mycoviruses cause hypovirulence in fungi, but the effects often vary among different host strains. *Heterobasidion* partitiviruses 13-an1 and 15-pa1 (HetPV13-an1 and HetPV15-pa1) have been associated with strain-specific and variable hypovirulence of *Heterobasidion annosum*, but variation in phenotypic effects of HetPV15-pa1 or the coinfection of these viruses on different host strains has not been studied previously. In this investigation, the effects of single and double partitivirus infections were first studied using six Finnish *H. annosum* strains on malt agar plates (MEA). Secondly, the effects of single and double partitivirus infections on the growth rate of four *H. annosum* strains were tested outdoors using Scots pine billets as a natural substrate. Against our expectations, on MEA plates, the single or double partitivirus infections of HetPV13-an1 and HetPV15-pa1 did not have significant effects on three of the fungal strains studied and they slightly accelerated the growth rate of three host strains. In the billet experiment, the double partitivirus-infected strains were more often assorted to the fastest growing group than virus-free controls. Based on these results, HetPV13-an1 and HetPV15-pa1 do not debilitate the tested *H. annosum* strains on agar plates or dead wood but may even slightly increase the growth rate of the mycelium on artificial medium and in non-competitive growth conditions in dead pine wood.

1 | Introduction

Heterobasidion annosum (Fr.) Bref. sensu stricto is a forest pathogen that causes damage to various conifer species and can also infect many deciduous trees (Korhonen and Stenlid 1998). Potentially hypovirulent mycoviruses could be used to rehabilitate forest sites where *H. annosum* infections have already been established. *Heterobasidion* species act as hosts to a diverse and partially overlapping community of viruses (Vainio et al. 2017), including at least members of the *Partitiviridae*, *Curvulaviridae*, *Narnaviridae*, *Botourmiaviridae*, *Fusariviridae* and newly classified phylum *Ambiviricota* (Dálya et al. 2024; Kuhn et al. 2024; Sutela et al. 2021; Vainio et al. 2011, 2012, 2015).

Mycoviruses are also commonly observed in other fungi (Hantula and Vainio 2021), but normally infections do not result in any visible symptoms on the host fungus, although there are many exceptions when negative effects have occurred. Such hypovirulence has been found in numerous mycovirus families, including *Alphaflexiviridae*, *Botourmiaviridae*, *Closteroviridae*, *Chrysoviridae*, *Deltaflexiviridae*, *Endornaviridae*, *Fusariviridae*, *Genomoviridae*, *Hypoviridae*, *Megabirnaviridae*, *Mitoviridae*, *Mymonaviridae*, *Narnaviridae*, *Partitiviridae*, *Polymycoviridae*, *Reoviridae*, *Rhabdoviridae*, *Solemoviridae*, *Totiviridae* and *Virgaviridae* (Dálya et al. 2024; García-Pedrajas et al. 2019; Hao et al. 2018; Khan et al. 2023; Zhai et al. 2016). Slow growth, disturbed reproduction,

abnormal morphology and changes in pigmentation are common symptoms caused by hypovirulent mycoviruses (García-Pedrajas et al. 2019). Some mycoviruses with biocontrol potential also represent other beneficial features. *Pestalotiopsis theae* chrysovirus-1 (PtCV1) has been associated with an endophytic lifestyle of an otherwise virulent strain of *P. theae*, a pathogen of *Camellia sinensis* (Zhou et al. 2021). The PtCV1 infected strain also conferred resistance against upcoming infections of virulent strains. Hyder et al. (2013) observed that the mycoviruses HetRV3-ec1 and HetRV6-ab6 were sometimes associated with altered competitiveness of *Heterobasidion* spp. against other fungi.

Probably the best-known hypovirulent mycovirus is *Cryphonecria* hypovirus 1 (CHV1) that reduces growth and conidia formation of *Cryphonectria parasitica* and has been successfully used as a biocontrol agent in Europe for decades (Heiniger and Rigling 1994; Milgroom and Cortesi 2004; Rigling and Prospero 2018). A couple of partitiviruses that cause hypovirulence have been discovered. *Colletotrichum alienum* partitivirus 1 (CaPV1) decreases growth and virulence of four *Colletotrichum* spp. by disturbing endocytosis in vesicle transportation (Zhu et al. 2024). *Talaromyces pinophilus* partitivirus 1 (TpPV-1) reduces growth and affects the pigmentation of its host (Hassan et al. 2024). *Rhizoctonia solani* partitivirus 2 (RsPV2) reduces the growth and virulence of strain GD-118, but effects in other *R. solani* strains have not been tested (Zheng et al. 2014). *Sclerotinia sclerotiorum* partitivirus 1 (SsPV1/WF-1) reduces growth and virulence of *S. sclerotiorum*, *S. nivalis*, *S. minor* and *Botrytis cinerea* and has been associated with abnormal development of conidia in *B. cinerea* (Xiao et al. 2014). *Botrytis cinerea* partitivirus 2 (BcPV2) impedes conidia and sclerotia formation in all four fungal strains tested, but the virus-derived growth reduction has been observed only in one strain (Kamaruzzaman et al. 2019). Mycovirus infection can also result in an increased growth rate of fungi (Kotta-Loizou and Coutts 2017; Shah et al. 2020).

Most partitiviruses infecting the genus *Heterobasidion* do not cause dramatic effects on fungal phenotype. For example, the mycelial growth rate or appearance of the fungus is not usually affected on artificial growth media. However, two exceptions of hypovirulence causing mycoviruses are known and considered promising candidates for disease control: HetPV13-an1 and *Heterobasidion* partitivirus 15-pa1 (HetPV15-pa1) (Kashif et al. 2019; Vainio et al. 2018). Vainio et al. (2018) have discovered that two out of the six tested *H. annosum* strains have been debilitated by the virus HetPV13-an1, whereas the growth reduction by HetPV13-an1 and HetPV15-pa1 in *H. parviporum* has been more common among different fungal strains (Hantula et al. 2020; Kashif et al. 2024; Vainio et al. 2018). Recently, Piri et al. (2023) noticed that stump inoculation of HetPV13-an1 enhances the effect of *Phlebiopsis gigantea* in the protection of seedlings around the diseased stump.

HetPV13-an1 induces considerable changes in gene expression in the host strain 94233 (Vainio et al. 2018), including downregulation of many genes related to carbohydrate degradation and RNA silencing, as well as changes in redox-, detoxification-, cell wall and membrane-, cell cycle- and metabolism-related gene expression. The observed changes in carbohydrate metabolism might indicate that the effects of viruses could depend on the nutrition source and differ between artificial medium and wood.

HetPV15-pa1 also debilitates the growth of *H. annosum* strain 94233 (Kashif et al. 2019), but the tolerance among *H. annosum* strains and phenotypic effects of the double virus infection of HetPV13-an1 and HetPV15-pa1 are unknown. In addition, some *Heterobasidion* partitiviruses may affect the growth rate of non-native hosts in at least some temperatures, and this effect is associated with the accumulation of viral transcripts and depends on other co-infecting viruses (Hantula et al. 2020; Jurvansuu et al. 2014; Kashif et al. 2019). HetPV15-pa1 has been originally found in *H. parviporum*, and hence we hypothesised that it could harm other, less adapted *Heterobasidion* species, such as *H. annosum* strains.

The aim of this study was to examine how the virus HetPV15-pa1, in co-infection with HetPV13-an1 and HetOlV4-an1, affects the growth of several *H. annosum* strains on artificial medium or in Scots pine billets.

2 | Materials and Methods

2.1 | Growth Experiment on Malt Extract Agar Plates

Virus-infected strains of six Finnish *H. annosum* strains (Table 1) were previously constructed (Roininen et al. 2024). In addition, double partitivirus-infected strains of 06066, KA 401-10-58 and KA 11.42A were produced by growing two single virus-infected strains on the same 2% malt extract agar (MEA) plate. Subcultures were taken after two weeks' incubation at 20°C and virus infections were tested in RT-PCR. The presence of viruses in all experimental strains was tested by RT-PCR immediately before the growth experiment to confirm the presence of viruses. Primer pairs (PV15midFor2 5′-TTGGGGATTCGAAACAATTC-3′ & PV15midR 5′-CGAGCGATGTGATCGAAGTA-3′) and (PV13-midFor2 5′-TGCTCCTCTCCCGACTCTAC-3′ & PV13midR 5′-CTTGGGTAGCCATTGTGGTT-3′) and (SPOuConF2 5′-CC CRCGTAAAGTACCAGTGAAA-3′ & SPOuConRev2 5′-TCC CGGCACGAATACCA-3′) were used to detect HetPV15-pa1, HetPV13-an1 and HetOlV4-an1, respectively (Kashif et al. 2015; Roininen et al. 2024). For each fungal strain, one virus-free control, three independently constructed strains hosting HetPV15-pa1 and HetOlV4-an1, three independent strains hosting HetPV13-an1 and HetOlV4-an1, as well as three independent strains hosting both partitiviruses HetPV13-an1 and HetPV15-pa1 and HetOlV4-an1 were randomly chosen to perform the growth experiment. Random selection of strains that were successfully infected in a previous study (Roininen et al. 2024) was done by raffling three strains out of the infected ones. The control strain KA 401–10-58 and all its triplicate derivatives were infected by the additional virus HetAlV17-an2 (Roininen et al. 2024).

Each original fungal isolate is here considered as a strain, for example, *H. annosum* 06066. The strains hosting different viruses or virus combinations are called virotypes, for example, 06066-PV15 (strain 06066 hosting HetPV15-pa1). Transmission

TABLE 1 | Origin, year of collection and collector of *H. annosum* strains.

Strain[a]	Collection site	Year	Collector	References
06066	Finland, Alajärvi	2006	A. Pajula	Vainio et al. (2018)
KA 401–10-58	Finland, Karkkila	2007	T. Piri	Piri et al. (2020)[b]
KA 11.42A	Finland, Karkkila	2007	T. Piri	—
Kortesjärvi 2.3.31	Finland, Kauhava	2018	T. Piri	Piri et al. (2021)[b]
Kortesjävi 1.1.65	Finland, Kauhava	2018	T. Piri	—
Köyliö 6.21	Finland, Säkylä	2018	T. Piri	—
94,233	Poland, Krucz	1994	P. Lakomy	Kashif et al. (2015)

[a]Long term storage of all strains as agar pieces in cryotubes at +4°C to 5°C.
[b]The strain was collected during the cited study but was not explicitly mentioned by name in the text.

event refers to the individual virus-infected replicate that was produced in Roininen et al. (2024) or in the current study.

To manage workload we used stepwise start, with similar preculture actions. All strains were precultured at +15°C for a minimum of 5 days before the experiment, which was started within one day with all inoculations of the strains 06066, KA104-10-58 and Kortesjärvi 1.1.65 and on the subsequent day with all inoculations of the strains KA11.42A, Kortesjärvi 2.3.31, Köyliö 6.21 and 94233. Previously investigated (Vainio et al. 2018; Kashif et al. 2019) strain 94233 was included as control of experimental setup and growth conditions. A stepwise start was necessary to make the workload of a total of 520 plate inoculations and measurements manageable. The inoculant consisted of a 0.5 cm piece of actively growing mycelium collected at 0.5–1 cm from the edge of the mycelium with a sterilised Pasteur-pipette and placed approximately in the middle of the MEA plate. The eight replicates of all fungal strains were made at once using the same preculture plate and devices. The plates were incubated in 15°C in Termaks KB8400 climate cabinet (Rieger, Industrievertretungen GmbH, Wien, Austria). The growth measurements started on the third day after inoculation and were repeated every second day until the plate was fully covered with mycelium. The slow growing inoculates of strain 94233 did not show any measurable growth until the fifth day.

The plates were scanned with Canon imageRUNNER ADVANCE C5235i (Canon, Helsinki, Finland), and the areas of mycelial colonies were measured using ImageJ Fiji software (version 1.53c) (Schindelin et al. 2012). Growth was measured as the mean increase in colony radius and calculated based on colony area, assuming circular form. For analyses, we used growth between days 5 and 7 as the response variable, as the growth rate during this time was at the exponential phase and thus most stable.

The continuous variable growth was modelled using linear mixed-effects models under the assumption of a normal distribution. The analysis was conducted in R (version 4.4.2; R Core Team 2024) using the lme4 package (version 1.1-35.5; Bates et al. 2015). The model included strain, virus and their interaction (strain × virus) as fixed effects. First, we fitted the linear mixed effects model including transmission event and starting day as a random intercept with the lmer() function. Neither of the tested random terms was significant when tested with ranova() of the lmerTest package (version 3.1–3; Kuznetsova et al. 2017). Hence, we chose to use the simpler lm() function of the R Stats Package (version 4.4.1; R Core Team 2024) to fit the model (strain × virus). Model assumptions of normality and homoscedasticity were evaluated using residual plots and quantile-quantile (Q-Q) plots. Statistical significance of the fixed effects was determined at $p < 0.05$. p-values were calculated using Satterthwaite's Type III Analysis of Variance. Differences in growth between virus treatments within each fungal strain were tested by comparing estimated marginal means of all pairs using the emmeans package (version 1.10.1; Lenth 2024), and post hoc analyses were conducted using Bonferroni correction for multiple comparisons. Predicted mean and 95% confidence interval were computed with 1000 bootstrap replicates.

2.2 | Growth Experiment in Pine Billets

Ten healthy looking pine trees were cut down in a circa 45-year-old pine stand in southern Finland, about 50 m around the location 60.612583° N 24.409191° E. The root system of the stand was slightly infected by *H. annosum* and hence the billets were sawn at least 3 m from the base of pine to avoid possible hidden infections. Four billets were taken from each of ten trees. One billet from each tree was assigned to each fungal strain, giving a total of 40 billets for inoculation. The billets were 45–55 cm long and 15–25 cm in diameter and branches were avoided. Fungal strains used in the billet experiment were 06066, Kortesjärvi 1.6.65, Kortesjärvi 2.3.31 and Köyliö 6.21 which were selected to represent growth rates equal to or faster than the controls, based on a plate experiment.

Fungal mycelia were grown in liquid culture for two weeks at 20°C as described by Vainio et al. (2013), except that the total volume was adapted to 100 mL. A series of inoculations, consisting of four virotypes of specific strain (Figure 1.), was made on each billet and the billets were randomised to five blocks. Two-week-old cultures were homogenised with Ultra-Turrex Typ.TP18/10 (Janke & Kunkel IKA-Werke, Staufen, Germany) and 0.5 mL of the liquid culture was pipetted into a drilling hole of 1 cm in diameter and 2.5 cm in depth (Figure 1). The inoculations were covered with Neko garden wax (Neko, Hämeenlinna, Finland), and thereafter the fungi were allowed to grow for

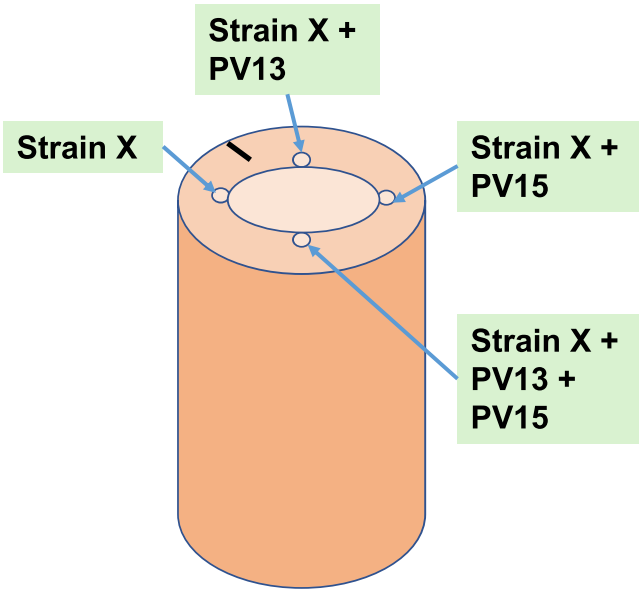

FIGURE 1 | Drilled holes of 2.5×1 cm were made at the border section of sapwood and heartwood and a series of four inoculations was inoculated to each billet. HetOlV4-an1 was accompanying the partitiviruses in all the virus hosting strains.

54 days (8.9.2021–1.11.2021). The billets were placed upright in blocks on sand area about 5×7 m and the sand was kept moist during the experiment. Information of weather conditions in the nearest available monitoring station (Nurmijärvi-Röykkä) is presented in the Figure S1.

At the end of the experiment, a 4.5 cm piece from the top of each billet was removed and thereafter 12 circa 1 cm discs were cut and collected for further analysis. The discs were peeled, rinsed and stored at −20°C to manage workload and to ensure a uniform handling process and incubation period for all samples, allowing reliable conidiophore detection. The discs were taken to room temperature to allow the mycelial growth and conidiophore development, which were used to identify the presence/absence of *Heterobasidion* mycelia under a microscope. The thickness of the discs was somewhat unequal and specific ruler measurements were done at the side of the disc pile for each of the four inoculations to enable as accurate growth measurement as possible. Measurements of the growth distance were made from the bottom of the drilling hole to the last disc surface where conidiophores were detected.

Due to technical limitations in the precise measurements of growth, an ordinal model was used to categorise the results. The dependent variable, growth distance, was divided into three categories: below 10, 10–12 and over 12 cm so that the classes were approximately equal in size. Measurements under 12 cm that had grown to the last wood disc were excluded from the analysis to prevent misclassification. Growth beyond the measurable discs was extensive in the Köyliö 6.21 strain, so this strain was excluded from the statistical analysis.

To assess whether the virus or strain was associated with specific growth distance categories, we employed a Cumulative Link Mixed Model (CLMM) fitted with the Laplace approximation. The model was implemented using the ordinal package (version 2023.12–4.1; Christensen 2023) in R via the clmm() function. The interaction term (virus×strain) was first tested, but it was not significant. Hence, we continued with virus and strain as fixed effects, while tree and billet were nested and included as random effects to account for variability among trees and billets. To check that test field circumstances were the same for all blocks, we also tested adding the random factor 'block,' but based on the likelihood ratio test, the models with and without block did not differ and it was not included in the final model.

The proportional odds assumption (i.e., consistency of effects across response thresholds) was evaluated by fitting virus and strain separately as nominal effects with a simplified random effect structure (billet only) using the clmm2() function. These nominal effect models, which do not assume proportional odds by allowing effects to vary between response thresholds, were compared to the selected model using likelihood ratio tests for cumulative link models. As the nominal models did not differ significantly ($p > 0.10$) from the selected model, we concluded that deviations from the proportional odds assumption were unlikely.

3 | Results

3.1 | Growth Experiment on MEA Plates

We used a linear mixed-effects model to evaluate the effects of strain, virus and their interaction on growth. The transmission event was included as a random intercept to account for variability between separately formed virotypes.

The main effect of the virus was significant ($F(3, 455) = 33.02$, $p < 0.001$), indicating that growth varied significantly across virus levels. Similarly, strain had a significant main effect on growth ($F(5, 455) = 14.77$, $p < 0.001$). Additionally, the interaction between virus and strain was significant ($F(15, 455) = 6.43$, $p < 0.001$), suggesting that the effect of the virus on growth depends on the specific strain being considered. The residual standard deviation was 0.4521.

Strain specific predicted values of mycelial growth in response to virus effects and 95% bootstrap confidence intervals are presented in Figure 2 (see Table S1 for full pairwise test statistics). In three of the strains (Kortesjärvi 1.1.65, Kortesjärvi 2.2.31, Köyliö 6.21) the virus infected strains grew faster than the control, but in three strains (KA 11.42A, KA401-10-58, 06066) the growth was similar in virus infected strains compared to virus-free control. Mean growth increasement compared to corresponding control strain varied between 7% in Kortesjärvi_1.1.65-PV13 and 19% in Köyliö_6.21-PV15.

3.2 | Growth Experiment in Pine Logs

We used a cumulative link mixed model to examine how predictors (virus infection status and fungal strain) relate to the ordinal response variable (growth distance class), while accounting for random variability across trees and billets nested within trees (Tree/Billet). The analysis showed that double partitivirus infection (HetPV13-an1 and HetPV15-pa1) significantly affected the response (estimate = 1.63, SE = 0.81,

z = 2.007, $p < 0.05$) (see Table S2 for full model summary). Categorisation of single partitivirus infected strains did not differ significantly from the virus-free strains. Specifically, double virus infection increased the likelihood of higher growth distance classes compared to virus-free cases. Figure 3A illustrates the overall categorisation into growth classes by virus infection status, showing that the number of samples in the fastest growth class (> 12) is also highest for the double virus-infected strains. Categorisation into growth classes by strain and virus infection status is shown in Figure 3B, where more samples assorted into class > 12 for the double virus-infected strains 06066 and Kortesjärvi 1.1.65 compared to the corresponding virus-free strains. Random intercept variances were 2.966 for trees and 7.599 for billets nested within trees, indicating considerable variability among groups. The model's thresholds were $\tau 1 = -1.645$ and $\tau 2 = 2.576$.

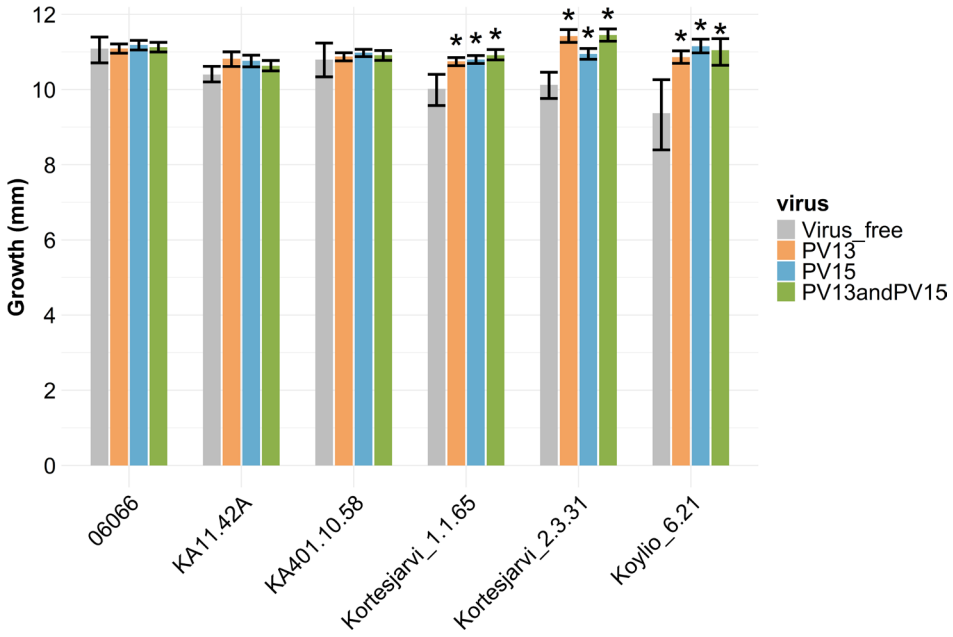

FIGURE 2 | Growth of *H. annosum* strains on MEA plates with and without viruses. Error bars represent 95% confidence intervals based on 1000 bootstrap replicates. Asterisks above bars indicate statistically significant differences compared to the corresponding control fungal strain. HetOlV4-an1 was accompanying the partitiviruses in all the virus-hosting strains.

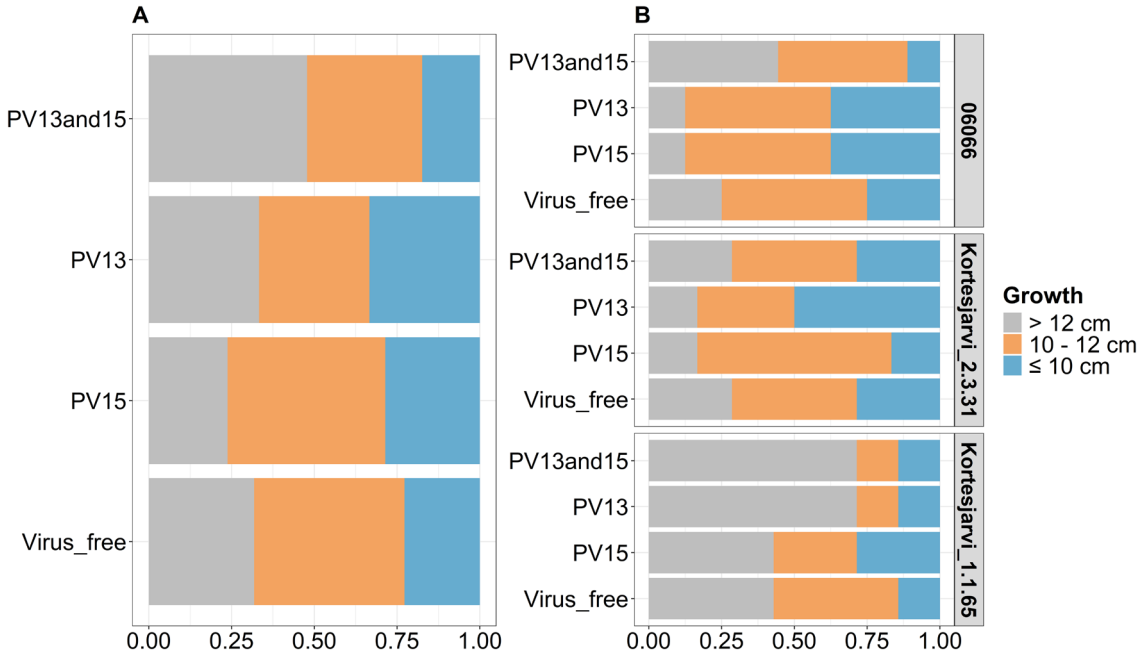

FIGURE 3 | (A). Share of the samples belonging to each of the three growth classes > 12, 10–12 and ≤ 10 cm by the virus infection status in the growth experiment in logs. (B) The count of samples classified into the three same growth classes as in (A), but considering the virus infection status and the fungal strain. HetOlV4-an1 was accompanying the partitiviruses in all the virus hosting strains.

4 | Discussion

Kashif et al. (2019) demonstrated that the viruses HetPV15-pa1 and HetPV13-an1 caused growth reduction in the host strain 94233 on MEA, both in single and double partitivirus infections, as part of a multiple-virus complex (Roininen et al. 2024; Vainio 2019). These partitiviruses have also been promising in controlling the growth of *H. parviporum* (Kashif et al. 2024; Vainio et al. 2018). Since the ecological niches of *H. annosum* and *H. parviporum* partially overlap, further investigation of their effects on *H. annosum* is warranted.

In this study, we evaluated the impact of HetPV13-an1 and HetPV15-pa1 (accompanied by HetOlV4-an1) and their coinfection on six different *H. annosum* strains. Contrary to expectations, the viruses increased the growth of some strains on MEA plates, but the observed acceleration was 7%–19%. A similar trend was observed in a two-month-long log experiment, where the relative growth increase was small but statistically significant. Importantly, all tested *H. annosum* strains were tolerant to HetPV15-pa1, originally identified in *H. parviporum*. The tolerance, seen as the lack of growth reduction, does not support the hypothesis of increased virulence in a new host species with this virus.

Our results align with prior findings, where partitiviruses occasionally caused accelerated growth on plates and displayed variable effects at different growth temperatures (Hantula et al. 2020; Jurvansuu et al. 2014; Vainio et al. 2018). This study concurs with the findings of Vainio et al. (2018), who reported that HetPV13-an1 reduced growth in only two out of six tested *H. annosum* strains on MEA. Similarly, Kamaruzzaman et al. (2019) observed that BcPV2 decreased the growth rate in only one of four strains tested. In the present study, the phenotypic effects observed in isolate 94233 (Kashif et al. 2019) were not seen among the tested *H. annosum* strains, which were not slowed down by HetPV15-pa1 or its coinfection with HetPV13-an1.

Previous studies have also reported an increase in fungal pathogenicity. Perhaps the best-known example of such an effect is the L1 virus of *Nectria radicidola* (NrV-L1), which increases the severity of ginseng root rot when present in fungal mycelium (Ahn and Lee 2001). Growth enhancement of host fungus has been reported with some mycoviruses like *Leptosphaeria biglobosa* quadrivirus 1 (Shah et al. 2020) and *Beauveria bassiana* polymycovirus 1 (BbPmV1) (Kotta-Loizou and Coutts 2017). Okada et al. (2018) have observed that a higher titre of *Alternaria alternata* chrysovirus 1 (AaCV1) in the host fungus is associated with larger lesions in pear, while its growth on potato dextrose agar (PDA) has deteriorated. *Fusarium circinatum* mitoviruses 1 and 2–2 have been associated with increased laccase activity and hypervirulence in vivo (Muñoz-Adalia et al. 2016). However, no hypervirulence was observed in an in vivo growth experiment with the same viruses later (Flores-Pacheco et al. 2017). *Rhizoctonia solani* rhabdovirus 1 (RsRhV1) and RsRhV2 have been associated with the hypervirulence of the fungal host in the experiment on rice leaves, even though the growth rate was not significantly altered by these viruses (Li et al. 2022). Pathogenicity can also be enhanced by increasing sporulation, as seen in NrV-L1 (Ahn and Lee 2001), BbPmV1 (Kotta-Loizou and Coutts 2017) and *Phytophthora infestans* RNA virus 2 (PiRV-2) (Cai et al. 2019).

Sometimes the combination of infecting viruses affects the outcome: for example, growth reduction of *Rosellinia necatrix* has been observed in the double virus infection of *Rosellinia necatrix* megabirnavirus 2 (RnMBV2) and *Rosellinia necatrix* partitivirus 1 (RnPV1), but not in strains infected by only one of these viruses (Sasaki et al. 2016). Also, other studies report growth reduction in multiple virus infections, like three endornaviruses in *Ceratobasidium* sp. (Cao et al. 2019) and a hypovirus with a mycoreovirus in *Valsa mali* (Yang et al. 2021). On the other hand, an opposite outcome was observed in multiple virus infections of *H. parviporum*, when the debilitating effects of *Heterobasidion* partitivirus 13-an1 (HetPV13-an1) faded away in the presence of other viruses (HetPV2-pa1, HetRV6-pa15, HetPV7-pa1, HetPV9-pa1) on malt agar (Hantula et al. 2020). The growth rate increasement in multiple virus infections compared to single virus infections has also been found with other fungi (Chun et al. 2024; Tran et al. 2019).

Kashif et al. (2019) detected growth differences between the biological replicates of the strain 94233. In the present study, the effect of biological replicates, produced in Roininen et al. (2024), was also taken into account as a random effect. This random effect of separate transmission events was minimal, which indicates that possible immediate effects seen after the transmission event are not determining later growth. Single infection of HetOlV4-an1 was not tested separately from the two partitiviruses and its effects as a single infecting virus remain unknown. Studies of ourmiavirus effects on *H. annosum* are still sparse, but Dálya et al. (2024) reported growth reduction by HetOlV2 in the newly infected host strains.

We want to note that our experimental setup, featuring unlimited resources and an absence of competing fungi or tree defences, cannot reveal potential long-term or cumulative effects on growth, phenotype, or virulence. Therefore, our findings do not necessarily conflict with those of Piri et al. (2023), who observed that fewer young trees near a diseased stump were infected when HetPV13-an1 was inoculated into a naturally *H. annosum* infected stump alongside *P. gigantea* treatment, offering a higher protective effect from their combined use compared to *P. gigantea* alone. Similarly, field experiments by Kashif et al. (2024) showed significant growth reduction (~41%) in *H. parviporum* infected with HetPV13-an1 and HetPV15-pa1, contrasting with in vitro findings (Poimala unpublished).

5 | Conclusion

This study demonstrated that HetPV15-pa1, in coinfection with HetOlV4-an1 or HetOlV4-an1 and HetPV13-an1, did not significantly reduce the growth of Finnish *H. annosum* strains on artificial medium or in dead Scots pine wood. Our findings suggest that tolerance to these coinfections is common under conditions where resources are unlimited and competition is absent.

Although these results do not indicate promising potential for the combined use of HetPV13-an1 and HetPV15-pa1 (and HetOlV4) as a biocontrol agent under controlled conditions,

their applicability in natural forest systems remains uncertain. Further studies are needed to explore interactions of these viruses considering natural root systems, host competition and tree defences to assess the efficacy in mitigating forest damage caused by *H. annosum*. Additionally, this study raises questions about the reliability of artificial experimental setups for evaluating the biocontrol potential of fungal viruses, emphasising the need for investigations in more ecologically relevant contexts.

Author Contributions

I certify that all authors have read and approved the final version of the manuscript and contributed significantly to the work. The manuscript has not been published previously and is not being considered for publication elsewhere.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Weather conditions at nearest available weather station (Nurmijärvi-Röykkä) during the billet experiment (open data by Finnish Meteorological Institute). **Figure S2:** Q-Q plot of the residuals based on the model used plate growth experiment. **Table S1:** Full pairwise test statistics of the growth experiment on MEA plates. **Table S2:** Summary of test statistics of the growth experiment in the billets.