



Synergy and interference in community interactions of fungal endophytes in the liverwort *Marchantia polymorpha*

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Abstract

Plant microbiomes are diverse communities of many microbial species. Growing evidence suggests that a complex network of interactions between members of the microbiome is necessary to maintain the health of host plants. Overall microbiome impacts can be difficult to predict and there is still much unknown about how taxonomic diversity links to function. All research on such dynamics so far has been limited to vascular plants, but the non-vascular bryophytes also have rich microbiomes and understanding these is a necessary component for understanding the evolution and ecology of plant microbiomes. This study takes advantage of the existing fungal endophyte culture collection for the model liverwort *Marchantia polymorpha* L. to begin expanding experiments on microbiome community interactions into bryophytes. This series of experiments reveals examples of synergy, interference, and priority effects in how fungal endophyte combinations impact *M. polymorpha*. These results constitute a foundation for the development of more complex synthetic community studies similar to those already existing for vascular plant models.

Keywords Endophyte · Fungi · *Marchantia* · Microbiome · Priority effect · Synthetic community

1 Introduction

Plants harbor diverse communities of microbes on their surfaces and within their tissues which are key to plant health and success. These microbial communities, called microbiomes, are composed of fungi, bacteria, archaea, and other eukaryotic organisms (Trivedi et al. 2020). Relationships with microbial symbionts can contribute to plant nutrient uptake, immunity, competitive ability, and adaptation (Fitzpatrick et al. 2019; Trivedi et al. 2020). Interactions between members of plant microbiomes can significantly impact the outcome for the host overall, for example when certain symbionts can protect the plant from a pathogen (Latz et al. 2018) or block the colonization of a mutualist (Mack and Rudgers 2008; Engelmoer et al. 2014). The final outcome for a plant host depends on the whole microbiome and its interactions with abiotic factors (Trivedi et al. 2020).

These dynamics complicate efforts to apply microbiome knowledge to improve agriculture, for example, when symbionts that are beneficial in experiments turn out to be unable to establish in field settings (Mitter et al. 2019). In fact, the majority of knowledge regarding plant microbiomes is still from diversity surveys, which are impossible to apply for practical use without experimental data. Therefore, it is critical to continue improving our understanding of the nuanced dependencies and interactions in plant microbiomes. Untangling such dynamics and their impacts on hosts requires a variety of study types, from network analyses of environmental microbiome sequencing data, to controlled laboratory experiments (Fitzpatrick et al. 2020; Song et al. 2020). For the latter type of study, systems using sterile plants and known microbiome members are necessary (Vorholt et al. 2017). Such gnotobiotic experimental systems will ideally use model plant hosts so the molecular mechanisms behind the host outcomes can also be studied based on the mutant lines and genetic studies that already exist. This work has already begun with synthetic communities of bacteria, fungi, and oomycetes from the *Arabidopsis thaliana* microbiome (Durán et al. 2018; Carlström et al. 2019; Voges et al. 2019).

From studies on *A. thaliana*, *Medicago*, and various crop plants, it is clear that microbiome impacts are difficult to

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predict. Comparisons of single and dual inoculations reveal a wide variety of interactions, with the isolates that best enhance growth alone not resulting in the best growth in combination (Hori et al. 2021). Order of arrival has been shown to cause additional variation across a wide range of fungal and bacterial endophyte interactions (Werner and Kiers 2015; Carlström et al. 2019; Ridout et al. 2019; Boyle et al. 2021), including even changing a microbe's impact between the two opposing functions of blocking and facilitating infection by a pathogen (Adame-Álvarez et al. 2014). Furthermore, microbiome members appear to have the potential to evolve in response to competition in ways that do not fit existing ecological hypotheses (Debray et al. 2023). Despite this apparent unpredictability, it has been demonstrated that it is possible to design synthetic bacterial communities to enhance particular plant functions in *A. thaliana* (Herrera Paredes et al. 2018). Inoculation with synthetic communities with dozens of members on *A. thaliana* revealed that more complex communities tend to result in better host outcomes, and that interactions between prokaryotic and eukaryotic microbiome members are critical for maintaining plant health (Durán et al. 2018).

What has already been learned from model angiosperm studies reveals a complex world of microbiome interactions and emergent properties. To better understand these dynamics and how they relate to plant evolution, it is necessary to expand such work to models from other clades of plants. Both from their evolutionary position and their ubiquity in ecosystems (Lindo and Gonzalez 2010), bryophytes (mosses, liverworts, and hornworts) are an important target for this kind of microbiome research. Given bryophytes' differences from vascular plants in anatomy and contact with their substrate, they provide an intriguing comparison to the existing work which has mostly focused on the rhizosphere. Investigations of individual mutualists and pathogens in bryophyte models are accumulating, exploring how liverworts and hornworts engage in mycorrhiza-like symbioses with fungi and how bryophyte immune responses compare to those in vascular plants (Pressel et al. 2021; Chen and Nelson 2022; Jeong et al. 2023). There exist some culture collections of microbiome diversity for mosses (Opelt et al. 2007; U'Ren et al. 2010, 2012; Chen et al. 2022) and liverworts (Davis and Shaw 2008; Nelson and Shaw 2019) and microbiome transfer experiments have been conducted for *Sphagnum* (Carrell et al. 2022). However, to the authors' knowledge, only one study has investigated co-inoculation of multiple defined microbes in a model bryophyte (Nelson et al. 2018), and none yet have used defined synthetic communities with more than two members.

For this study, we continue work with the model liverwort, *Marchantia polymorpha*. This bryophyte species has gained increasing popularity as a molecular model in the past few decades (Bowman et al. 2022) and is gaining

traction as a model for plant-microbial interactions (Poveda 2020a) despite its lack of mycorrhiza-like associations. Fungal endophytes have been isolated from *M. polymorpha* to study their diversity, impacts on the host plant, and chemical compounds (Nelson et al. 2018; Nelson and Shaw 2019; Stelmasiewicz et al. 2023). Fungal pathogens have also been isolated from *M. polymorpha* to build an experimental system to enhance understanding of land plant immunity (Matsui et al. 2020). In addition, various studies have tested the impacts of fungi and oomycetes isolated from other plants on *M. polymorpha*, developing our understanding of its immunity and comparing its molecular responses to those of vascular plants (Carella et al. 2018; Poveda 2020b; Redkar et al. 2022; Poveda et al. 2023). These studies demonstrate pathogenesis from known pathogens and some beneficial effects from known plant growth promoting species, but also pathogenic effects with known mutualists, such as arbuscular mycorrhizal fungi.

This study presents a series of experiments using sterile cultures of *M. polymorpha* and an existing library of its wild-collected fungal endophytes to investigate principles of microbiome community interactions. The experiments address (1) pairwise interactions between fungi in the host, (2) relatedness among interacting endophytes, (3) priority effects when two fungi are inoculated at different times, and (4) interactions between groups of four or five endophytes.

2 Methods

2.1 Plants and fungi

One sterile clone of *Marchantia polymorpha* subsp. *polymorpha* Bischler & Boisselier was used for all experiments. This culture was originally isolated from a plant collected in Vermont, USA in June 2015 (WRG: Winooski River Gorge 44.48071, -73.11571). The fungal cultures were isolated from individuals of *M. polymorpha* L. collected in North Carolina and Vermont (Table 1), as previously described (Nelson et al. 2018).

Identifications for fungal cultures were checked by phylogenetic analysis of the previously published ITS (internal transcribed spacer) and partial LSU (ribosomal large subunit) amplicons for the isolates (Nelson and Shaw 2019). Reference sequences for closely related fungi were obtained by downloading the top 250 NCBI GenBank accessions matching each isolate in question. Sequences were aligned with Muscle v5.3 (Edgar 2021) and trees generated with FastTree (Price et al. 2009).

Table 1 Fungal strain details. WRG: Winooski River Gorge (44.48071, −73.11571), CHR: Chatooga River Gorge (35.01564, −83.12671), NCB: North Carolina Botanical Garden (35.89900, −79.034346), Exp: indicates in which of the four experiments each strain was used

Strain	Identification	Authority	Family	GenBank	Location	Host subsp.	Exp
390	<i>Coprotus sp.</i>	Korf & Kimbr.	<i>Incertae sedis</i>	MG004784.1	WRG: VT	<i>polymorpha</i>	1,4
420	<i>Coprotus sp.</i>	Korf & Kimbr.	<i>Incertae sedis</i>	MK359687.1	WRG: VT	<i>polymorpha</i>	4
408	<i>Phoma herbarum</i>	Westend.	Didymellaceae	MG004796.1	WRG: VT	<i>polymorpha</i>	1,3
319	<i>Colletotrichum sp.</i>	Corda	Glomerellaceae	MG004755.1	CHR: NC	<i>ruderalis</i>	2
385	<i>Colletotrichum truncatum</i>	(Schwein.) Andrus & W.D. Moore	Glomerellaceae	MG004780.1	WRG: VT	<i>polymorpha</i>	1,2,3
391	<i>Colletotrichum sp.</i>	Corda	Glomerellaceae	MG004785.1	WRG: VT	<i>polymorpha</i>	1,4
387	<i>Colletotrichum acutatum</i>	J.H. Simmonds	Glomerellaceae	MG004782.1	WRG: VT	<i>polymorpha</i>	1,2,3,4
384	<i>Hypomontagnella submonticulosa</i>	(Y.M. Ju & J.D. Rogers) Sir, L. Wendt & C. Lambert	Hypoxylaceae	MG004779.1	WRG: VT	<i>polymorpha</i>	1
386	<i>Hypomontagnella submonticulosa</i>		Hypoxylaceae	MG004781.1	WRG: VT	<i>polymorpha</i>	1,4
246	<i>Nemania sp.</i>	Gray	Xylariaceae	MG004727.1	NCB: NC	<i>ruderalis</i>	2
253	<i>Nemania serpens</i>	(Pers.) Gray	Xylariaceae	MG004734.1	NCB: NC	<i>ruderalis</i>	2

2.2 General experimental procedures

Before the experiments, plant replicates were established on Hatcher's agar (Hatcher 1965) with 0.15% added malt extract. Each replicate was one plant clone in a separate Petri dish. Plants were inoculated with fungi grown on 1.5% malt extract agar for at least one month. All inoculum cultures used for the same experiment were started at the same time. To make the inoculum, 5 mm diameter circles of the fungal plate cultures were punched out from the edges of the fungal colonies. Each plant received half of one of these circles. In treatments that used mixtures of fungi, smaller fragments of the circles were used to add up to half a circle total. This method ensured that the overall dosage of fungal inoculum was similar between replicates, but did not ensure exactly equal dosages between all fungi in the same replicate. For controls, half circles of sterile malt extract agar were used. Each treatment had 10 biological replicates i.e. Petri dishes. Experimental plates were kept in a climate control chamber with 12 hours of light at 20°C and 12 hours of dark at 18°C.

Plant growth was tracked for four to five weeks after inoculation by photographing the experimental plates from above with a Lumix DMC G80 camera. The area of green plant tissue visible in each image was measured with a custom macro in ImageJ (Nelson et al. 2018), with parameters adjusted to the lighting and white balance of each set of photos. All measurements were checked and any disrupted by condensation on dishes or dark colored fungi were remeasured by drawing an outline around the green tissue manually in ImageJ.

Since plant growth usually followed an approximately linear trajectory, growth was analyzed based on initial and final size. For each plant replicate, relative growth was calculated as: (final size - initial size)/initial size. With this

metric, a plant that did not grow or shrink has a value of 0, so all positive values indicate growth and negative values indicate loss of green area. The lowest possible value is −1, the case in which all starting tissue has died. The relative growth data were analyzed with ANOVA in Rstudio running R version 4.1.2 (R Core Team 2024) and each treatment's relative growth trajectories over all weeks of each experiment visualized with ggplot2 (Wickham 2016).

2.3 Features of the four experiments

Experiment 1 investigated pairwise interactions between seven different fungi originally isolated from the same plant population of *M. polymorpha* subsp. *polymorpha* in Vermont (WRG). These fungi belonged to 6 species (Table 1). The observation that some fungi interfered with each other's individual effects in this experiment led to the following three experiments; for example, some fungi could prevent the beneficial or harmful effects of another fungus when co-inoculated.

Experiment 2 sought to check whether interference between pairs of fungi corresponded to how closely related those fungi are. This used three fungi from the genus *Colletotrichum* and two from the genus *Nemania* (Table 1). This experiment was set up the same as Experiment 1, with all possible single and double fungal treatments.

Experiment 3 built on Experiment 1 to investigate whether priority effects may impact the interference effects observed between pairs of fungi. The treatments included three fungi alone, all possible pairs inoculated simultaneously, and all possible pairs with one of the fungi inoculated one week before the other.

Experiment 4 investigated the effects of more than two fungi together since natural endophyte communities of *M.*

polymorpha are more complex and can contain dozens of taxa (Nelson and Shaw 2019). Five fungi all isolated from the same host plant in the wild (WRG population) were used, four of which were included in Experiment 1. Treatments included each fungus alone, all fungi together, and all possible combinations of four fungi.

2.4 Statistical analysis

Any plates which became visibly contaminated or had missing data photographs during the experiment were excluded from analysis. There were no more than three plates removed from any of the experiments. This resulted in ten replicates for all treatments, except for the following which had only nine: Experiment 1 386, 386+387, and 387+390; Experiment 2 319+246, 319+253, and control; and Experiment 3 385+387 and 408+385. Treatments that consistently resulted in complete death of the plant host were excluded when running the statistical tests since their variance was so much lower than the other treatments. This allowed the assumptions of the tests to be met and did not impair comparisons since plant death is a very clear effect distinct from all other possible interactions. The remaining treatments in each experiment were analyzed with ANOVA. The data were checked for homogeneity of variances and normality. If necessary, the data were transformed to fit the normality assumption. In experiment 2, the transformation was $\sqrt{x+1}$; in experiment 3, $\sqrt{x+2}$; and in experiment 4, $\log(x)$. The Tukey HSD *post hoc* test was used to determine p-values for pairwise comparisons after a significant p-value was obtained for an ANOVA.

3 Results

3.1 Experiment 1: Endophytes can interfere with each other's effects on the host

When inoculated alone, fungus JN0408 (*Phoma herbarum*) enhanced *M. polymorpha* growth (8.40 ± 4.89 relative growth vs. control 2.51 ± 0.95). Hereafter, similar numerical comparisons are reported with only the treatment names and average relative growth values with their standard deviations. JN0387 (*Colletotrichum acutatum*) and JN0391 (*Colletotrichum* sp.) killed the plant and the other four fungi did not significantly change host growth (Fig. 1). The beneficial effect of JN0408 was lost in combination with all other fungi except JN0390 (*Coprotus* sp., Fig. 1). The negative effect of JN0391 always overrode other fungi when in combination. For JN0387, combination with JN0385 (*Colletotrichum truncatum*) improved the outcome for the host (JN0387 -0.92 ± 0.22 , JN0387+JN0385 0.99 ± 1.46 , Fig. 1). The combination of two fungi that alone had no effect in two cases

resulted in worse outcomes, when JN0390 was combined with JN0384 and JN386 (both *Hypomontagnella submonticulosa*, Fig. 1).

Some fungi and combinations were less consistent in their effects than others. The beneficial fungus, JN0408, and the two strains of *H. submonticulosa*, JN384 and JN386, had a wider spread of growth outcomes than the other treatments (Fig. 1). While for JN0408, almost all outcomes were above the control range, JN384 and JN386 showed a mix of outcomes within, above, and below the control range. This spread of benefit and detriment compressed to a spread similar to the controls when these fungi were combined with JN0385 but was maintained and even slightly enhanced in combination with JN0408. Most treatments with negative effects were very consistent, but JN0391 and the combination of JN390 and JN0384 had some replicates which recovered after some initial tissue loss.

3.2 Experiment 2: Colletotrichum but not Nemanian strains show congeneric synergistic effects

The two *Nemanian* isolates both enhanced host growth (JN0246 6.27 ± 2.64 , JN0253 4.62 ± 2.01 , control 1.86 ± 0.93) but this effect was not additive, with the amount of growth not being statistically different between the combined treatment and either individual treatment ($p=1.000$, $p=0.836$). The *Colletotrichum* strain JN0387 was lethal to the plant. This was the case in combination with all other tested fungi except with JN0385, which protected the plant as observed in Experiment 1. The other two *Colletotrichum* strains, JN0319 and JN0385, were neutral alone, but JN0319 in combination with JN0385 or either *Nemanian* showed statistically higher growth (JN0319+JN0385 5.19 ± 2.29 , JN0319+JN0246 4.75 ± 2.34 , JN0319+JN0253 5.05 ± 1.18) compared to the control (with 385 $p=0.001$, with 246 $p=0.013$, with 253 $p=0.002$, Fig. 2), but not compared to the individual *Nemanian* treatments ($p>0.8$).

3.3 Experiment 3: Marchantia endophytes display priority effects

As in the previous experiments, JN0408 enhanced growth alone (JN0408 7.39 ± 2.32 , control 2.87 ± 0.97), JN0387 was lethal alone, and JN0385 was neutral alone. JN0385 protected the plant from JN0387 as shown before with simultaneous inoculation. This protection was lost if JN0387 was inoculated first and was less effective in simultaneous inoculation than if JN0385 was inoculated first (Fig. 3). Giving JN0408 a head start did not prevent JN0387 from killing the plant once it was added (Fig. 3). When JN0408 had priority in combination with JN0385, the plants had slight growth enhancement (PJN0408+JN0385 5.53 ± 2.35) but not nearly as much as when JN0408 is alone (Fig. 3).

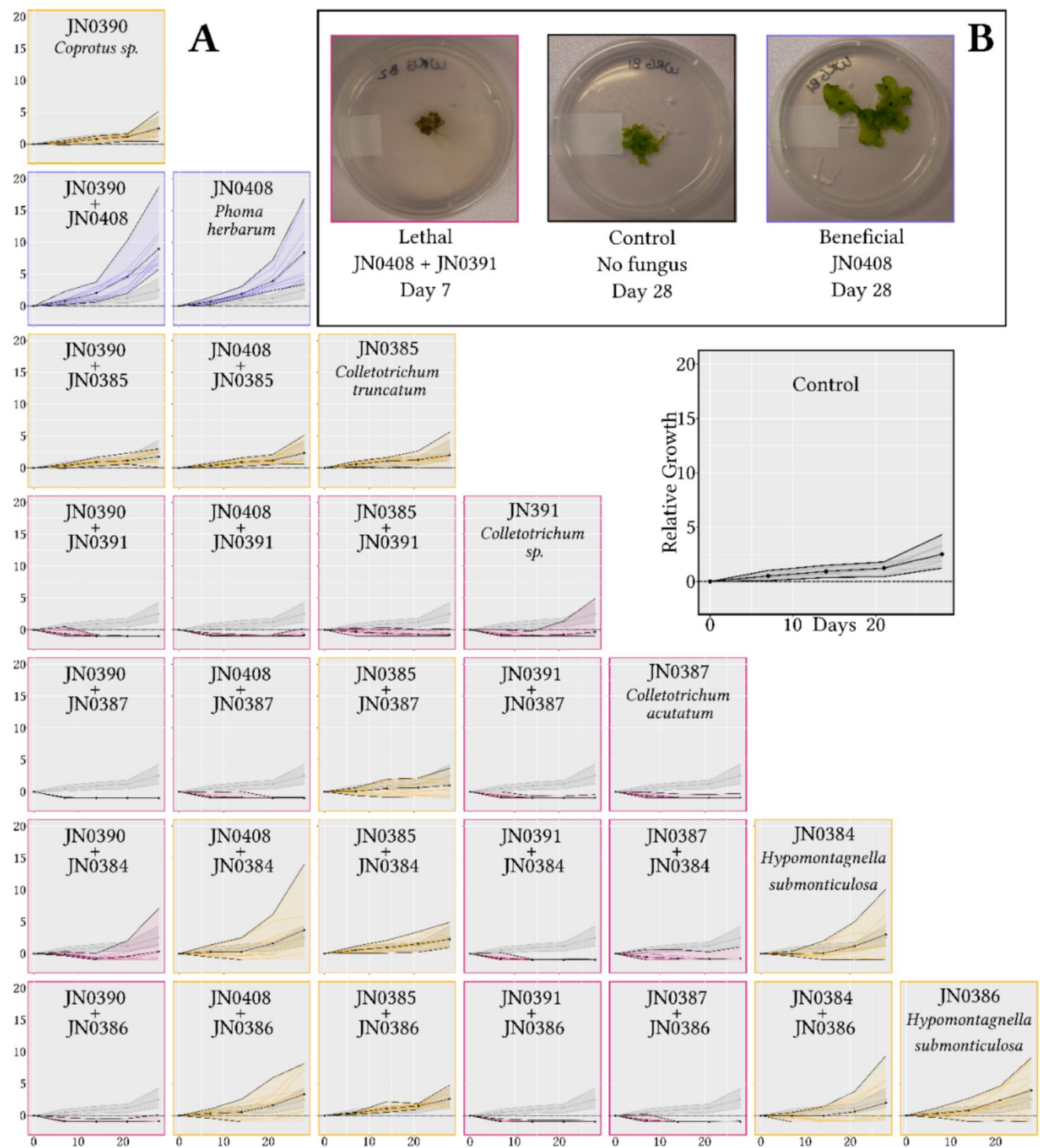


Fig. 1 Experiment 1, pairwise effects of seven isolates. **A** Individual inoculation treatments are shown on the diagonal, creating rows and columns showing all possible pairwise inoculations. The control plants are shown in gray on each treatment graph for comparison. Treatments that statistically significantly enhanced plant growth have their data shown in blue, treatments that killed part or all of the plants are shown in magenta, and treatments that did not differ statistically

significantly from the controls are shown in yellow. All axes are the same and aligned across panels. The x-axes show time in days and the y-axes show relative growth i.e. the difference between the size of the plant on that day and its starting size, divided by the starting size. **B** Examples of effects are shown on the upper right, including a treatment that killed the host, a control plant, and a treatment that promoted growth

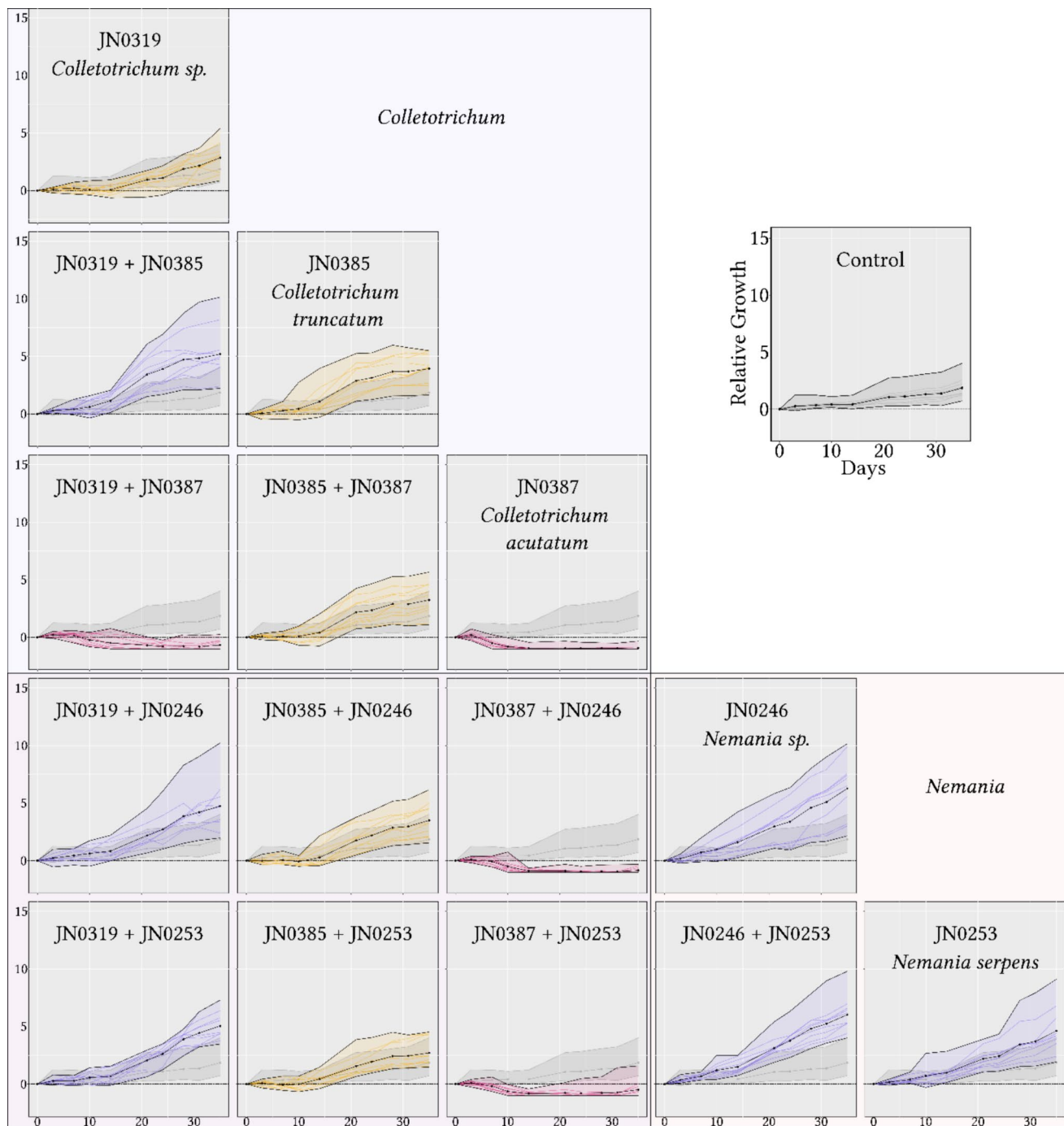


Fig. 2 Experiment 2, pairwise effects of *Colletotrichum* and *Nemanias* isolates. Growth of all treatments is shown with the same coding as in Fig. 1.

3.4 Experiment 4: Negative effects dominate in more complex mixes of endophytes

Individually, fungi JN0420 and JN0390 resulted in healthy

plants but their growth did not differ significantly from the controls (ANOVA $p=0.0602$). All other treatments

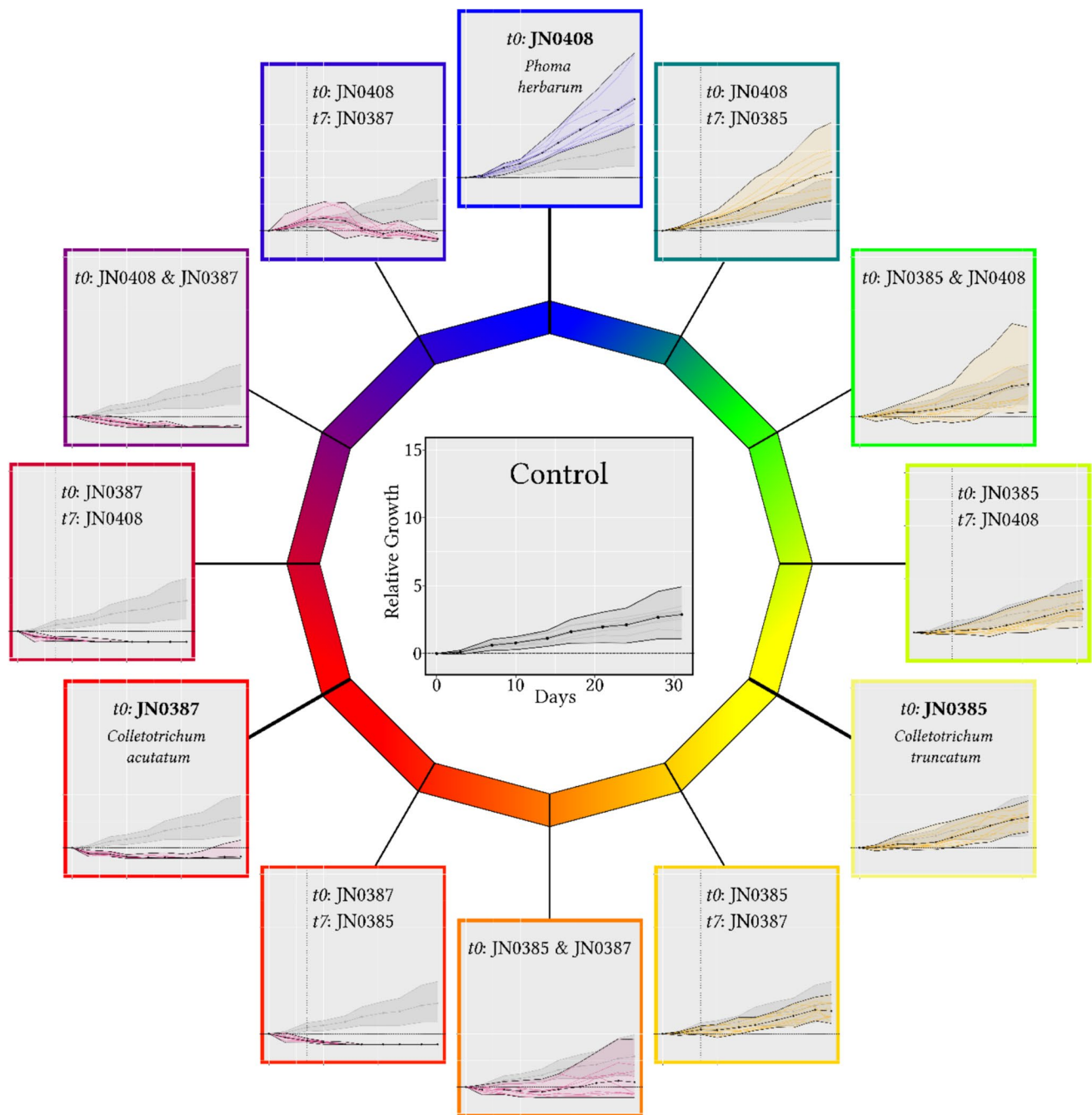


Fig. 3 Experiment 3, priority effects. Individual graphs follow the same format used in figures 1 and 2 but arranged around a color wheel to illustrate the different types of combination treatments. Individual inoculations are shown in primary colors (blue, yellow, red) with bold titles, simultaneous co-inoculations are indicated with secondary colors (green, orange, purple), and priority inoculations

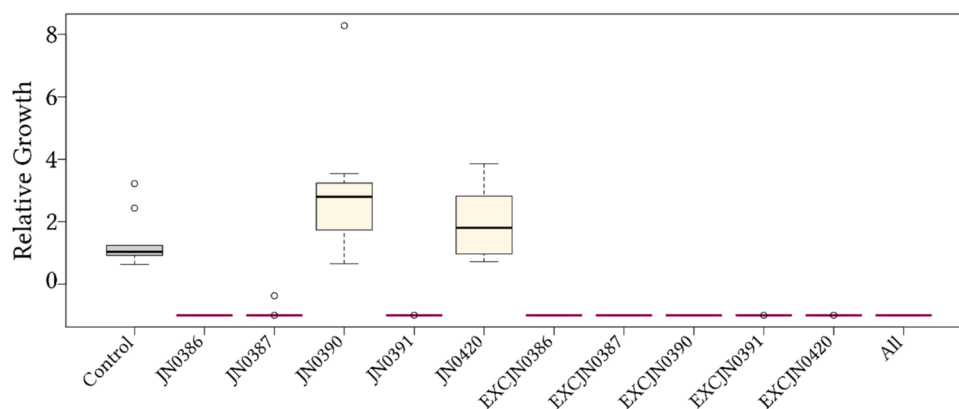
are shown in tertiary colors with titles indicating which fungus was added at the beginning of the experiment (t_0) and which one week later (t_7). The priority treatments are displayed closest to the individual treatment of the fungus inoculated first. The time t_7 is indicated on priority effect treatment graphs by a vertical dotted line.

consistently resulted in plant death (Fig. 4). This was unexpected in one case: JN0386, which had shown a neutral, if more variable, effect in experiment 1.

4 Discussion

Together these experiments show that the complexity in microbiome interactions found in angiosperm rhizosphere and phyllosphere communities also applies to bryophyte

Fig. 4 Experiment 4, larger synthetic communities. The treatments labeled with only numbers were inoculated with a single fungus, the treatments starting with “EXC” have four fungi excluding the strain corresponding to the number shown, and “ALL” included all five fungi together



microbiomes, with interference and synergy appearing apparently unpredictably. A broader panel of isolates, ideally with genomic information, would be required to reveal reliable predictors of interaction outcomes, should they exist, but the data presented here begin the process of defining what is possible and what can be tested further.

4.1 Lethality of endophytes from healthy plants

All the fungi used in these experiments were isolated from healthy host plants. In the laboratory experiments, however, many of them killed the plants (Figs. 1, 2). This is a common result for gnotobiotic systems using fungi isolated from healthy plant tissues (Junker et al. 2012; Keim et al. 2014; Durán et al. 2018) and reflects discussions of the symbiotic continuum in plant-fungal interactions (Schulz and Boyle 2005). The discrepancy between endophyte behavior in the wild and pathogenicity in the laboratory may be influenced both by the artificial abiotic conditions of agar plates and by the simplified biotic conditions which lack the diverse microbiome of wild plants. Experiment 4 (Fig. 4) clearly indicates that just making the community slightly more complex does not immediately solve this problem. However, more such experiments would be needed to narrow down explanations of this result.

The current data suggest either that the laboratory conditions are to blame, or that the organisms that would balance the effects are unculturable or from different groups e.g. bacteria, viruses, protists, invertebrates. Previous work with the *A. thaliana* microbiome has indicated that bacteria affect the ability of fungi to grow, but not *vice versa* and that this effect can often be attributed to direct antifungal effects (Durán et al. 2018). This lends support to the hypothesis that some fungi may be controlled by being in a diverse microbiome and emphasizes that future development of the *M. polymorpha* system should include building more diverse synthetic communities, including a bacterial endophyte culture library. Previous investigations of bacteriomes in several *Marchantia* species already indicate diverse communities

which respond to environmental conditions (Alcaraz et al. 2018; Marks et al. 2018). Future experiments with abiotic experimental conditions more similar to natural soil and different inoculation methods (e.g. spore suspensions) would also be helpful to narrow down the possible causes and build a model more indicative of natural microbiome dynamics.

Interestingly, one isolate, JN0386 *H. submonticulosa*, changed from neutral to negative between experiments (Figs. 1, 4). This could be due to contamination during inoculation (which cannot be checked after the fact), but it is also possible that this represents a real change in the laboratory strain. Previous experiments confirmed an opposite switch of a *Xylaria* isolate from negative to neutral (Nelson et al. 2018). Further testing would be needed to confirm the JN0386 switch, but it highlights another potential difficulty with synthetic community studies, namely the necessity of repeated culture in a laboratory can introduce changes in the behavior of isolates. Keeping voucher stocks can help reduce the problem, but even culture collections where these may be deposited need to grow the isolates sometimes to maintain them.

4.2 Priority effects

Part of the conundrum of lethal endophytes may be solved by the timing of colonization of the host. Experiment 3 clearly demonstrates priority effects, but these are also variable, with some fungi unable to dominate the outcome for the host even when given a head start (Fig. 3). The amount of time between inoculation in priority effect studies varies widely, from two days to several weeks, depending on the organisms being studied (Kinkel and Lindow 1993; Peay et al. 2012; Werner and Kiers 2015; Carlström et al. 2019). It is possible that waiting, for example, for two weeks instead of one between inoculations in the *M. polymorpha* system could allow the fungus with priority to establish more fully and block the latecomer. Future experiments could track the colonization rate of fungi in the *M. polymorpha* system to pinpoint times after inoculation for testing introduction of

competing strains. For arbuscular mycorrhizal fungi, it has been shown that a longer time (four weeks as compared to two) before a challenge with an invader gives a stronger advantage to the resident fungus (Werner and Kiers 2015). One study investigating priority effects with complex communities found that they were very robust to disruption by new taxa introduced three weeks after the initial inoculation (Carlström et al. 2019). It is possible that this stability has to do with timing, but it could instead come from the complexity of the mock communities. In the current experiment, it is unlikely that niches were saturated with so few fungi relative to the diversity commonly found in wild plants, so any stability from complex communities would not be observed.

4.3 Interference and interaction of endophyte effects

In Experiment 1, we observed that the fungus which could alleviate the impacts of a pathogenic fungus was a member of the same fungal genus, *Colletotrichum* (Fig. 1). Other studies have found a significant role for phylogenetic relatedness in plant-associated microbial community interactions. For example, in their study of nectar yeasts in *Mimulus*, Peay et al. (2012) found that more closely related yeasts competed more strongly and demonstrated stronger priority effects.

The observation of interference between *Colletotrichum* strains in Experiment 1 led to Experiment 2, in which we investigated combinations of fungi from this and one other genus, checking whether relatives within a genus seemed to be more able to interfere with each other. This experiment did not reveal new interferences. The additional *Colletotrichum* strain was unable to block the negative impacts of JN0387. It did however show another interaction: the *Colletotrichum* strains JN0319 and JN0385, which were neutral alone, produced positive effects when inoculated together (Fig. 2). However, the *Nemania* strains did not show a synergy to boost their already positive effects when inoculated together. This is similar to “offset effects” observed by Hori et al. (2021) when testing pairwise combinations of 13 fungi on the host *Brassica rapa* var. *perviridis*. In fact, they found that the greatest growth enhancement in their experiments did not come from individual isolates but from pairs of isolates that had been much less beneficial alone. Such synergistic effects of multiple endophytes have been detected with some regularity, but mechanisms behind these results have so far rarely been studied. These cases are therefore particularly interesting to follow up in future experiments with *M. polymorpha*, starting with transcriptome studies to build hypotheses and continuing with experiments targeting particular genes, signaling pathways, or hormones.

In our Experiment 3, the interaction between two *Colletotrichum* species had the strongest priority effect (Fig. 3). This of course is only detected indirectly through the impact

on the host plant and investigation of the colonization success of the fungal strains would be required to further clarify this. Throughout our experiments, relatedness of strains did not clearly predict the outcomes for the host plants, but the phylogenetic aspect warrants further attention in future experiments.

These four experiments indicate an intriguing variety of interactions and an important next step would be to investigate mechanisms. This could involve transcriptomics, quantitative PCR or microscopy to check if some fungi prevent others from colonizing, and investigations of whether fungi inhibit each other *in vitro* chemically, mycoparasitically, or otherwise. This could also include analysis of the strains' metabolite production and tests of the effects of fungal culture filtrates on *M. polymorpha* to assay plant response to fungal metabolites.

4.4 Similar fungi in other hosts

As noted when the effects of these fungal isolates were first reported (Nelson et al. 2018), some of these taxa have different effects in *M. polymorpha* than they are typically known for in vascular plants. The genus *Phoma* includes many pathogens of important crop plants, including *P. herbarum* (Deb et al. 2020), but this species has also sometimes been reported as a plant growth promoting fungus (Gupta et al. 2024). The strain of *P. herbarum* in our study was consistently growth-promoting, but with very little ability to maintain this effect in the presence of other fungi. Given that reports of its growth promotion in crops are tested on sterilized seeds, our results could suggest this is unlikely to be a fungus which can maintain its beneficial effects in field conditions.

Colletotrichum contains species with a wide variety of lifestyles and plant interactions, from severe crop diseases (De Silva et al. 2017), to the mycorrhiza-like nutritional symbiosis of *C. tofelfiae* with *A. thaliana* (Hiruma et al. 2016). Similarly, our *Colletotrichum* isolates showed a variety of effects and interactions, reinforcing that this as an interesting genus for studying the evolution of various relationships with plant hosts and their congeneric microbiome neighbors. This also highlights the danger with methods that attempt to assign ecological roles to fungi based on what is known of related taxa.

Coprotus is a genus of dung-inhabiting fungi (Kušan et al. 2018) but has been detected as endolichenic (Healy et al. 2022). Our experiments show that the *Coprotus* isolates are generally neutral but have some interesting interactions in combination with other fungi. Strain JN0390 was the only isolate that did not block the benefits of *P. herbarum*, but it also caused plant death when combined with the otherwise neutral *H. submonticulosa* strains. This genus has not

received much attention in plant interaction experiments yet and seems worth investigating further.

Hypomontagnella submonticulosa has been isolated as an endophyte from other hosts (Franco et al. 2022) and belongs to a family usually associated with wood or living plant tissues (Wibberg et al. 2021). Similarly, *Nemania* species are often found associated with wood or as endophytes, but they have also garnered some attention for their potentially useful chemical compounds (Tibpromma et al. 2021). The species *N. serpens* has been reported as a chemically interesting endolichenic (Lagarde et al. 2016) and endophytic fungus (Ibrahim et al. 2017). Our results suggest that some *Nemania* strains may also be growth promoting for their hosts, giving another reason for continuing research.

These connections between microbiomes of vascular plants, bryophytes, and lichens point to the complexity of ecosystem microbiomes and the importance of studying symbionts in many host contexts. Understanding the life cycles and ecological significance of these fungi will require studying many hosts using a variety of methods capturing the large-scale patterns of diversity in real communities but also the details of functions and interaction mechanisms.

4.5 Concluding remarks

These experiments, while they do represent the only exploration of such microbiome dynamics in bryophytes so far, are only an initial proof of concept. Even in small plants, microbiomes can be complex and their outcomes for their hosts hard to predict in reductionist experiments. This foundation should be built upon by investigating mechanisms behind the variable endophyte interactions and building more complex synthetic communities which include taxa beyond fungi. It is clear from previous work in angiosperms that prokaryotic and eukaryotic microbes have different roles and any understanding of a plant microbiome is incomplete without including both. It would also be beneficial to build collaborations to directly compare evolutionarily distant plant hosts, for example, by using the same synthetic communities for different hosts. Such investigations would be a significant contribution to understanding the evolution and ecological significance of plant microbiomes.

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Data availability Data, original photographs, and R scripts are available on Dryad (<https://doi.org/10.5061/dryad.dr7sqvb80>). Plant and fungal cultures used in this research are available by request from the corresponding author.

Declarations

Competing interests All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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