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Fluctuations in Rhizoctonia Solani Efficacy under Different Interspecific Plant Interaction and Fungal Communities

Joliese Teunissen [1,2], Alejandra Gallo-Sandoval [3,4], Alyssa van Cleef [1], Klaas Bouwmeester [3], Anne Kupczok [2,*] and Stefan Geisen [1,*]

[1] Laboratory of Nematology, Plant Sciences Group, Wageningen University & Research, 6708 PB Wageningen, The Netherlands
[2] Bioinformatics Group, Plant Sciences Group, Wageningen University & Research, 6708 PB Wageningen, The Netherlands
[3] Biosystematics Group, Plant Sciences Group, Wageningen University & Research, 6708 PB Wageningen, The Netherlands
[4] Plant Breeding, Plant Sciences Group, Wageningen University & Research, 6708 PB Wageningen, The Netherlands
* Correspondence: anne.kupczok@wur.nl (A.K.); stefan.geisen@wur.nl (S.G.)

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Abstract: Modern agriculture is vulnerable to soil-borne pathogen outbreaks, largely caused by the uniform field cultivation of single crop species. Sustainable alternatives such as intercropping may enhance natural antagonistic properties of the soil and boost pathogen suppression. Here, we investigated how both a non-host plant neighbour and soil fungal communities influence *Rhizoctonia solani* infection in the host crop *Brassica oleracea*. In a controlled greenhouse experiment, *B. oleracea* seedlings inoculated with *R. solani* were grown either with an intraspecific neighbour, a non-host neighbour (*Allium fistulosum*), or without neighbour. Seedlings were additionally subjected to soil fungal communities of native soils, soil feedback and sterilized soils. The presence of a non-host neighbour strongly reduced disease severity in *B. oleracea* and lowered persistence of pathogen DNA in the soil. Pathogen suppression was reduced in soils with low fungal diversity as well as in highly diverse soils, under the presence of other potential fungal pathogens. Neighbour effects changed under different soil mycobiomes, revealing an interaction between plant identity and soil fungal communities. Our findings highlight that intercropping with a non-host species can limit pathogen impact and that pathogen suppression is not dependent on the taxonomic diversity of the mycobiome. These insights advance the development of sustainable alternatives to manage soil-borne diseases.

Keywords: intercropping; soil-borne pathogen; soil mycobiome; ITS metabarcoding

1. Introduction

Uniformity is a hallmark of the efficiency and efficacy of modern agricultural methods. Through uniform crop production, harvest efficiency is increased and yield is maximized. Advancements in selective crop breeding and agronomic technology have optimized the cultivation of a selection of high-yielding elite cultivars. For example, maize, one of the five most cultivated staple crops and the world's leading cereal, achieved a production of 1.2 billion tonnes in 2020 representing 12% of global primary crop output [1,2]. However, despite a rapid increase in output in the past 50 years, global yields are approaching a plateau. One of the main reasons for this plateau, and consequently one of the biggest threats to food security, is the occurrence of pathogen outbreaks [3].

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The physiological, genetic, and field-wide uniformity of crops that facilitates high yields also lends itself to rapid pathogen buildup and disease spread [3]. Pathogens cause an estimated yield loss between 20-30% globally [3,4].

Pathogen outbreaks and disease incidence are additionally linked to shifts in microbial communities belowground [5,6]. In particular, soil fungi, collectively referred to as the mycobiome, provide various ecosystem processes that are important for maintaining plant health. Fungal epi- and endophytes, saprotrophs and other symbionts facilitate nutrient acquisition by plants, soil nutrient cycling and interplant communication [7,8]. The mycobiome also includes fungal plant-pathogens such as *Fusarium, Verticillium* and *Rhizoctonia*, which are amongst the most pervasive and aggressive microbial pathogens [2,9]. Beneficial fungi can reduce pathogen efficacy through inducing plant resistance, secreting anti-pathogenic compounds, or through resource competition [10]. For example, various *Trichoderma* spp. are known antagonists and biological agents of pathogenic fungi like *Fusarium oxysporum, Alternaria alternata* and *Sclerotinia sclerotiorum* [11,12]. In addition, plants capable of symbiosis with arbuscular mycorrhizal fungi (AMF) show increased pathogen resistance through enhanced nutrient availability and defence priming [13,14]. Understanding shifts in the mycobiome is thus crucial for managing overall plant health, specifically the incidence of pathogens.

The soil mycobiome is shaped partially by plant species. Through distinct morphology and root exudation profiles, plants uniquely shape the soil environment, and thus also shape the soil mycobiome [15]. Introducing belowground heterogeneity through plant diversification could offer a sustainable approach to increasing beneficial fungal-plant interactions. In the context of pathogen suppression, diversifying the distribution of roots can inhibit pathogen spread both physically, with root barriers, as well as biogeochemically, by creating rhizospheres that are inhospitable for pathogen establishment [16,17]. The presence of a non-host neighbouring crop can actively inhibit pathogen spread from an infected host crop by limiting dispersion and establishment. In addition, the interspecific interaction between plants (i.e. interaction between different plant species) can induce shifts in root exudation and gene expression in host-plants, facilitating the recruitment of beneficial pathogen antagonists [18,19]. Furthermore, the plant-specific influence on soil fungi reaches beyond the individual rhizosphere, and shapes the surrounding soil mycobiome. This plant-specific shaping has lasting effects, which can also influence subsequent crop generations, in what is known as soil-feedback. Soil-feedback is one of the main drivers of pathogen build-up in monoculture systems, but conversely can be utilized to modulate a more favourable soil mycobiome through crop rotation or cover cropping [20,21]. For example, Hannula et al. [22] showed that promoting AMF through soil-feedback had beneficial effects against root pathogens. Zhou et al. [23] revealed that key microbial species, which were present in crop rotation but not in monocropped peanut fields, participated in the suppression of fungal pathogens via synergistic interactions. Comparisons between the effects of microbial shifts on pathogen efficacy under plant diversification and soil-feedback have not been made directly before, but are crucial in elucidating plant-pathogen-microbe dynamics in the soil.

One notable example of a soil pathogen is *Rhizoctonia solani,* an economically important root-infecting fungus that poses a serious threat to global food production [24,25]. *R. solani* is capable of causing yield losses ranging between 20-50% in over 200 different crop species, including cabbage [26,27]. In one case, Zhang et al. [28] reported an outbreak of *R. solani* cabbage head rot, which reduced yield by approximately 50% across 2,500 ha. Due to the persistence of soil-borne sclerotia, i.e., dense hyphal survival structures, infection can reoccur for many years. As such, crop rotation and the use of fungicide seed treatments have varying degrees of success [29]. For example, Nemadodzi et al. [30] reported no reduction in *R. solani* densities when using legumes as rotation crops following maize. An alternative sustainable approach to pathogen suppression is the concurrent cultivation of a susceptible host crop with a non-host neighbour or companion crop. Several studies demonstrated that this approach can effectively reduce disease incidence across various crop-pathogen combinations. For example, Fu et al. [31] demonstrated that using onion as a companion crop to tomato reduced the incidence and severity of *Verticillium dahlia* tomato infection by 27%. In addition, incidence of the soil-borne fungal pathogen *Fusarium* spp. was reduced in faba bean-wheat intercropping with 42% [32], in wheat-watermelon intercropping with 20% [33], and in faba bean-maize intercropping with 7% [34]. Crop diversification as an alternative strategy to combat *R. solani* has rarely been explored, and the role of non-host neighbouring plants in particular remains unknown. Elucidating the mechanisms by which non-host neighbours alter *R. solani* progression are necessary to advance the development of sustainable alternatives to current management practices [35].

In the present study, we aimed to uncover the effect of a non-host neighbour on pathogen efficacy, and to decipher the importance of the mycobiome in pathogen resistance. To do so, we performed a greenhouse experiment using the interspecific combination of broccoli (*Brassica oleracea*) with spring onion (*Allium fistulosum*) as a model set-up. *B. oleracea*, in all its various morphotypes, is an economically important cash crop, which suffers from damping-off and wirestem disease under *R. solani* infection [36]. The use of *Allium spp.* as a companion crop with *Brassica spp.* has been employed before, to effectively reduce aphid infestations [37] and

other insect pests [38]. The effect of *Allium spp.* as neighbour crop on a belowground pathogen is lesser known. We monitored *B. oleracea* seedling performance under *R. solani* infection, and quantified *R. solani* DNA amounts using qPCR when under the presence of an interspecific neighbour (*A. fistulosum*), intraspecific neighbour (*B. oleracea*), or the absence of a neighbouring plant. We additionally used metabarcoding of the internal transcribed spacer (ITS) region to study the role of the soil mycobiome in *R. solani* efficacy by manipulating the native soil microbiome of agricultural field soil. We hypothesize that (1) plant neighbour identity alters *R. solani* disease severity, with a decreased seedling mortality and *R. solani* abundance in the presence of an interspecific neighbour; (2) altering the mycobiome through soil sterilization increases *R. solani* disease severity; (3) the effect of soil-feedback on *R. solani* abundance is crop-species specific, with lower disease incidence in non-host conditioned soils.

2. Materials and Methods

2.1. Plant and Pathogen Selection

The efficacy and persistence of *Rhizoctonia solani* strain M00011 (anastomosis group AG2-2; [36]) was evaluated when combined with a host, a non-host, or in the absence of a neighbouring plant. We used *Brassica oleracea var. italica* 'Cheng Chi no. 4' (broccoli) as a susceptible host, and *Allium fistulosum* 'Ishikura Long White' (spring onion) as a non-host neighbour. This specific combination of pathogen strain and plant species was selected based on a prior pilot study, testing multiple candidate *R. solani* strains and plant species based on susceptibility and resistance. *R. solani* strains were maintained on 1:5 V8 juice agar (1.5% *w*/*v* agar No. 1 (Agar bacteriological, Thermo Fisher Scientific, Waltham, MA, USA) and 3 g/l calcium carbonate) at 20 °C in the dark. *B. oleracea* seeds were sourced from the Centre for Genetic Resources, the Netherlands. *A. fistulosum* seeds were obtained from Van der Wal Zaadhandel, the Netherlands.

2.2. Soil Origin and Treatment

In addition to the effect of plant neighbour, we also investigated the role of the soil mycobiome on pathogen efficacy. The soil used in this assay was a sandy loam soil, collected from a strip-intercropping field at Wageningen University and Research, the Netherlands (51°59′27.5″ N, 5°39′34.4″ E). No *Brassica* or *Allium* species were grown on the soil in the past two years. Soils were treated in different ways to develop three distinct mycobiome types. (1) As a baseline, field soil with a native microbiome was used (Field Soil). (2) To evaluate the role of the soil mycobiome, microbial diversity was reduced through two-fold autoclaving of the field soil (Sterilized Soil). Soil was autoclaved at 121 °C, 15 psi, 30 min each cycle. The sealed autoclaved bags were cooled for 48 h between cycles to allow growth of resistant microbial survival structures. (3) To investigate the effect of soil-feedback, field soil was conditioned by growing either broccoli plants (Soil Feedback Broccoli) or spring onion plants (Soil Feedback Spring Onion) for 9 weeks. After the growth period, conditioned soils were sieved, homogenized and mixed in a 1:9 ratio with autoclaved field soil prior to being used in the assay. To improve aeration and structure, soil of the different mycobiome types was mixed with commercial potting soil containing perlite (Lensli B.V., the Netherlands) in a 6:4 ratio of field soil to potting soil. The pathogen assay was performed in trays without drainage to prevent cross-contamination, with dimensions of 30 × 11.5 × 8.5 cm (L × W × H). Soil was maintained at a 40% moisture level throughout the assay.

2.3. Pathogen Assay

To equilibrate seedling biomass, we used two weeks old spring onion seedlings and one week old broccoli seedlings, which were transplanted in five different configurations as shown in Figure 1. For every broccoli seedling, three spring onion seedlings were planted in a row. Our seedling pairings consisted of a host to host, and non-host to non-host, configuration (Intraspecific Broccoli and Intraspecific Spring Onion), the host to non-host configuration (Interspecific) and a configuration that lacked a direct plant neighbour (No Neighbour). Each configuration had one pathogen and one control treatment, with six replicates each. We inoculated the first plant in the configuration using the agar plug method, as described in Ampt et al. [35]. The pathogen-inoculated trays received agar plugs containing ten day old *R. solani* culture with actively growing mycelium, while the control treatment received pathogen-free agar plugs. Two 5 mm agar plugs were placed in such a manner that the mycelium made direct contact with the seedling roots. The planted and inoculated trays were arranged in a randomized block design and grown for four weeks in a greenhouse chamber with a 23/18 °C day/night temperature cycle of and 75% humidity to achieve close to optimal circumstances for pathogen growth [39–41]. To maintain 40% soil moisture, trays were watered every two days based on weight.

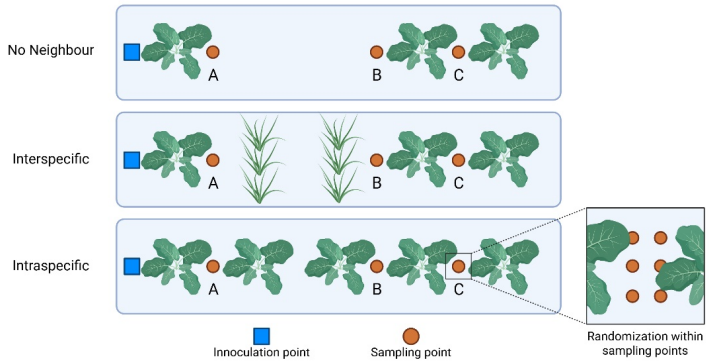

Figure 1. Overview of plant configurations with inoculation location. 30 × 11.5 × 8.5 cm trays were filled with soil and planted with one of the following configurations; Intraspecific Broccoli, Intraspecific Spring Onion, No Neighbour broccoli, No Neighbour spring onion, and Interspecific. The Intraspecific Spring Onion and No Neighbour Spring Onion configurations are not shown. All configurations had a treatment tray (inoculated with *R. solani*) and a control tray (inoculated without *R. solani*). Point of inoculation is indicated with the blue square.

2.4. Tracking Pathogen Efficacy and Presence

Pathogen efficacy was tracked by scoring disease symptoms every two days post-inoculation. Monitoring involved visually assessing the following disease symptoms: stunted plant growth, rotting of the hypocotyl, wilting or collapse, as similarly described in Ampt et al. [35]. To further quantify pathogen presence, soil samples were taken for qPCR analysis. Three locations in the tray were sampled every five days (Figure 1). To prevent continuously disturbing the same area, samples were taken in a randomized grid pattern (Figure 1). Soil samples were taken as soil cores of 1 cm diameter and 10 cm depth, which were freeze-dried for 72 h after collection. Total soil DNA was extracted following an in-house protocol, as described by Harkes et al. [42]. After DNA extraction, qPCR was performed, using the *R. solani* M00011 strain-specific primer pair AG2-2IIIB.F (5'-GGTTGTAGCTGGCTCCATTAG-3') and AG2-2IIIB.R (5'-GTTYTCCCATCCATGTCTCTGC-3'), as described by Schulze et al. [43]. qPCR on soil samples, along with a positive control (pure *R. solani* DNA) and a negative control, were prepared in triplicate using 10 µL iQ SYBR Green Supermix (Bio-Rad Laboratories B.V., Hercules, CA, USA), 5µM of each primer, 3 µL DNA (10× dilution) until a final volume of 20 µL per sample. The triplicate samples were randomized over plate runs to reduce potential differences across runs. qPCR was performed using a CFX Opus 96 Real-Time PCR system (Bio-Rad Laboratories B.V., Hercules, CA, USA) with the following program: denaturation at 95 °C for 3 min followed by 50 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s. To quantify *R. solani* in the soil, DNA was additionally extracted from pure *R. solani* M00011 cultures. A dilution range (from 0 to 100-fold dilution) in triplicate was made with the extracted DNA, and subsequently amplified in qPCR with the aforementioned primers and PCR program. A standard curve was generated from the resulting Ct values and the log10-transformed DNA concentrations, as determined with High Sensitivity Qubit (Thermo Fisher Scientific Inc., Waltham, MA, USA). As no seedling damage or *R. solani* presence in the soil was detected past the first seedling and soil sampling point A, we limited the analysis to only the first seedling and the shared rhizosphere between the first and second seedling.

2.5. Soil Mycobiome Diversity and Composition

To assess the impact of mycobiome manipulation, and to investigate the effect of *R. solani* inoculation and interspecific plant interactions on fungal community dynamics, a subset of 84 DNA samples collected at 26 dpi was selected for ITS metabarcoding, using the MinION Mk1C sequencing platform (Oxford Nanopore Technologies, Oxford, UK). The subset consisted of all mycobiome types, but was limited in soil samples to three configurations (Intraspecific Broccoli and Spring Onion, Interspecific), excluding the 'No Neighbour' configuration. In addition, the control inoculation treatment was only sequenced for all three configurations in field soil, and fully for the Intraspecific Broccoli configuration, see Table 1. The sequencing library was prepared by amplification of the Internal Transcribed Spacer (ITS) region using the primer pair ITS9munngs (5'-TACACACCGCCCGTCG-3') and ITS4ngs (5'-TTCCTSCGCTTATTGATATGC-3' [44,45]. Equimolar pools of DNA were prepared with individual inputs of 80 ng DNA, as determined with broad-range Qubit. This pooled sample was subsequently purified using Nucleomag NGS Clean-up beads at a 1:1 beads-to-sample ratio

(Macherey-Nagel, Düren, Germany). A total of 200 fmol of the purified pool was used for library preparation following the SQL-LSK114 nanopore kit instructions (Oxford Nanopore Technologies, Oxford, UK).

Table 1. Number of biological replicates selected for ITS metabarcoding.

	Field Soil		Sterilized Soil		Broccoli Soil Feedback		Spring Onion Soil Feedback	
Inoculation	***R. solani***	**Control**	***R. solani***	**Control**	***R. solani***	**Control**	***R. solani***	**Control**
Intraspecific Broccoli	5	4	5	4	5	4	5	4
Intraspecific Spring Onion	5	4	5	-	5	-	5	-
Interspecific	5	4	5	-	5	-	5	-

2.6. Bioinformatic Processing

The raw data produced after ITS nanopore sequencing (POD5 format) was basecalled with the super-accuracy basecalling model and demultiplexed using Dorado (v.1.0.2) [46]. The reads were filtered on >Q17 quality and min 600 max 1200 bp length using Nanofilt within the EZpore pipeline [47]. Within the pipeline, ITS regions were extracted and reads were clustered into Operational Taxonomic Units (OTUs) with 97% identity threshold, followed by OTU classification, using vsearch [48]. Non-fungal OTUs and OTUs without at least Phylum-level information were removed. OTUs with fewer than 10 reads and singletons were additionally filtered out. After filtering, 2346 OTUs were discarded, and 1326 OTUs were used for further analysis. The soil samples on average contained 35,304 ± 9036 (avg. ± stdev.) reads.

2.7. Statistical Analysis

To investigate the effect of *R. solani* on plant performance across both broccoli and spring onion, the days until seedling death were noted for each individual replicate. A multilevel Bayesian survival model with the variables ‘mycobiome type’ and ‘plant configuration’ in interaction was used with a Weibull distribution. The resulting survival curves were plotted and the posterior estimates (Est.) with 95% credible intervals (CI) of the contrasts were used to estimate differences from the baseline reference (field soil and intraspecific broccoli configuration). If the 95% CI of the contrast did not cross zero, we interpreted this result as a directional effect. Estimated marginal means were further used to assess differences between combinations of microbiome type and plant configuration.

To quantify the presence of *R. solani* in the soil, the log10 of the calculated DNA concentrations were analysed using a linear mixed effects model (LME), with the interaction between dpi, configuration type and mycobiome type as fixed effects. Both the randomized block design of the pots and the plate ID of the qPCR runs were taken as random effects in a crossed design. Validation graphs were plotted to confirm normality and homogeneity of residuals. Post-hoc pairwise comparisons with Benjamini-Hochberg correction were made with estimated marginal means.

The effect of our experimental treatments (*R. solani* inoculation, mycobiome manipulation, plant configuration) on the soil fungal communities was assessed. Fungal alpha diversity was calculated using observed OTU counts rarefied to the minimum library size. Statistical differences in OTU count measures were analysed using LME. A basic linear regression model was first constructed including all variables, to assess violation of normality and heterogeneity. Interactions between variables were stepwise reduced to achieve the best model fit, based on AIC model fit and statistical significance. The final LME model contained the variables ‘mycobiome type’, ‘inoculation’ and ‘configuration’, as fixed effects without interaction. The configuration variable contained both the type of configuration (inter- or intraspecific) and the plant identity (broccoli, spring onion or interspecific combination). Block was taken as a random effect. Post-hoc pairwise comparisons with Benjamini-Hochberg correction were made with estimated marginal means. Fungal community composition was assessed using cumulative sum scaling (CSS) normalized abundances data to calculate Bray-Curtis distance measures. Distance measures were visualized using Principal Coordinates Analysis (PCoA). To assess the effect of our experimental treatments on community composition, a PERMANOVA and pairwise PERMANOVA with Benjamini-Hochberg correction were used. The PERMANOVA was performed using 999 permutations, and the model included the factors ‘mycobiome type’, ‘inoculation’ and ‘configuration’. The permutations were restricted across blocks. In addition, the homogeneity of dispersion was tested prior to PERMANOVA analysis. Differentially abundant fungi were identified using Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC), with Benjamini-Hochberg correction [49]. The differential effects of mycobiome type, plant configuration and inoculation were tested. As a reference baseline, sterilized soil, intraspecific broccoli, and control inoculation were used, respectively. Block effects were accounted for as a covariable in the formula. Fungal ecological guilds were

assigned using FUNGuild (v.1.1). To assess which fungal ecological guilds characterise our different experimental treatments, a linear discriminant analysis of effect size (LDA-LEfSe) was used [50].

All analyses were performed in the R environment (v. 4.4.1) [51]. The following packages were used; *vegan* (v. 2.6-10) [52], *ggplot2* (v. 3.5.2) [53], *nlme* (v. 3.1-168) [54] *lme4* (v. 1.1-37) [55], *emmeans* (v. 1.11.0) [56], *phyloseq* (v. 1.50.0) [57], *microeco* (v. 1.14.0) [58], *brms* (v. 2.23.0) [59], *ANCOMBC* (v.2.8.1) [49] and *metagenomeSeq* (v. 1.48.1) [60].

3. Results

3.1. Influence of Plant Neighbour and Mycobiome Type on Seedling Survival

We found that our Intraspecific Spring Onion pairings showed no seedling death or detectable *R. solani* presence after inoculation, demonstrating the potential non-host status of Spring Onion to *R. solani* strain M00011. The Intraspecific Spring configuration was not included in the analysis of seedling survival rate and progression of *R.solani* amounts due to a lack of change across the days post inoculation. In all remaining combinations of mycobiome types and plant configurations, seedling death occurred after 5 dpi (Figure 2). Overall, survival of broccoli seedlings was elevated in the presence of spring onion (Interspecific) with an average increase in survival of 25%, when compared to the Intraspecific Broccoli pairing (Est. = 0.57, CI = [0.058, 1.13]). The absence of a neighbour increased seedling survival overall as well, when compared to the Intraspecific Broccoli pairing (Est. = 0.47, CI = [−0.05, 0.98]). Though Interspecific and No Neighbour pairings responded differently to the Spring Onion Soil Feedback mycobiome in particular, the overall response across mycobiome types was comparable (Est. = 0.1, CI = [−0.66, 0.49], Supplementary Material Figure S1). Manipulation of the mycobiome through soil sterilisation overall did not affect seedling survival in comparison to our baseline Field Soil (Est. = 0.01, CI = [−0.52, 0.57]). Soil feedback did increase seedling survival across all plant configurations, both in Broccoli Soil Feedback (Est. = 0.89, CI = [0.26, 1.56]) and Spring Onion Soil Feedback (Est. = 0.64, CI = [0.05, 1.27]) compared to Field Soil. In more detail, with an Intraspecific Broccoli pairing and Broccoli Soil Feedback, seedling survival was 39% higher compared to Field Soil, and 24% higher compared to Sterilized Soil. Under Spring Onion Soil Feedback, the survival of intraspecific seedlings was 50% higher compared to Field Soil and 38% higher compared to Sterilized Soils. The effects of soil feedback did not differ between broccoli and spring onion conditioned soils (Est. = 0.25, CI = [−0.99, 0.43]).

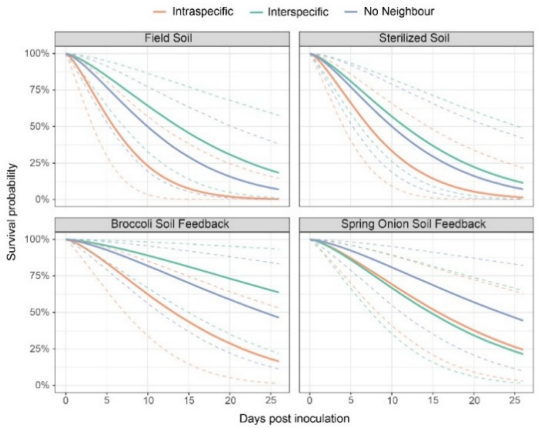

Figure 2. Survival probability of broccoli seedlings under *R. solani* inoculation. Plotted lines show the survival probability (%) as modelled with Weibull distribution survival analysis. Dashed lines indicate the 95% credible intervals. Each panel corresponds to a different mycobiome type (Baseline Field Soil, Sterilized Soil, Broccoli Soil Feedback and Spring Onion Soil Feedback). Line colours indicate different plant configurations (Intraspecific broccoli, Interspecific, No Neighbour).

3.2. Rhizoctonia Solani Presence under Different Plant Neighbours and Mycobiome Types

The concentration of *R. solani* DNA detected in our soils increased in the days post inoculation (dpi) (lme; F = 14.7, $p = 7.7 \times 10^{-7}$). This progression across time differed both based on mycobiome type (lme; F = 2.7, $p = 0.014$), and plant configuration (lme; F = 2.25, $p = 0.024$). In addition, mycobiome type influenced overall *R. solani* presence not only directly (lme; F = 12, $p = 6.7 \times 10^{-7}$), but also modulated the effect of planting configuration (lme; F = 2.9, $p = 6.8 \times 10^{-4}$). Specifically, we found that soil lacking a neighbour plant was more sensitive to mycobiome changes, compared to soil with intraspecific—or interspecific neighbour plants. The No Neighbour configuration showed

strong differences in *R. solani* DNA concentrations between mycobiome types ($p < 0.015$), whereas inter- and intraspecific configurations did not. Overall, planting configuration showed discernible individual patterns in pathogen progression across dpi and soil mycobiome types (lme; F = 7.13, $p = 1.66 \times 10^{-5}$).

To visualize *R. solani* progression over time, we calculated mean log2 fold change in *R. solani* DNA concentration (log10 of ng), relative to concentrations at 2 dpi (Figure 3). In general, *R. solani* presence in the soil declined past 16 dpi with an average decrease of 68% when in configuration with spring onion (Interspecific) or No Neighbour. Though both configurations showed a declining or neutral trend from 16 to 26 dpi in all mycobiomes, the Interspecific configuration showed the most consistent trend, with an average decline in *R. solani* presence of 84%. This declining pattern was not consistently visible across the different mycobiomes for the Intraspecific Broccoli configuration, where *R. solani* on average instead increased with 34% from 16 to 26 dpi (Figure 3).

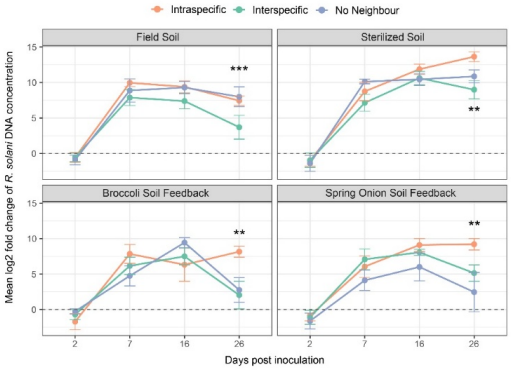

Figure 3. Mean fold change (relative to 2 dpi) of *R. solani* DNA concentrations in soil across 26 days post inoculation. Each panel corresponds to a different mycobiome type (Baseline Field Soil, Sterilized Soil, Broccoli Soil Feedback and Spring Onion Soil Feedback). Line colours indicate different plant configurations (Intraspecific broccoli, Interspecific, No Neighbour). Standard errors are indicated with error bars. Differences in DNA concentration between plant configurations were tested per dpi and mycobiome type, using linear mixed effects models with Benjamini-Hochberg correction. Significance is indicated with asterisks ($p < 0.01$ = **, $p < 0.001$ = ***).

When looking at all configurations at 26 dpi, we find that *R. solani* presence differed depending on the plant configuration (lme; F = 8.24, $p = 1.8e{-}5$), as well as mycobiome type (lme; F = 5.20, $p = 0.015$). Soil conditioned by Broccoli Soil Feedback overall showed the largest decrease in *R. solani* presence ($p = 0.0355$) in comparison to the other mycobiome types. Differences in *R. solani* DNA concentration between plant configurations were, however, most apparent in sterilized soils (Figure 4).

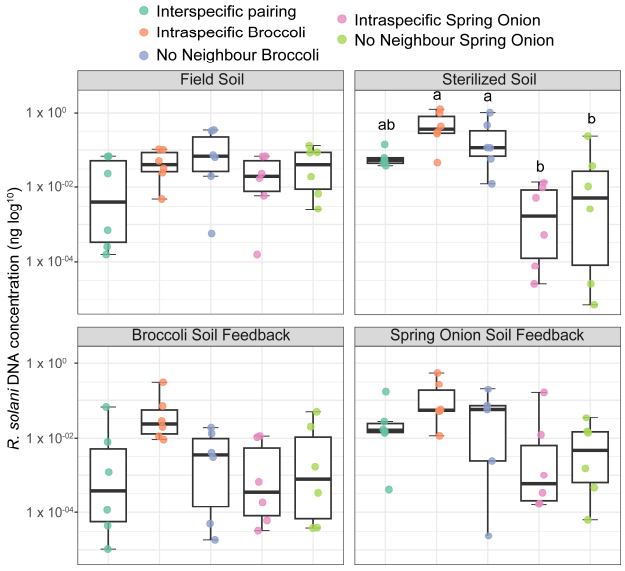

Figure 4. Log10 of *R. solani* DNA concentrations at 26 dpi. Data is separated by mycobiome type, and plant configurations are indicated with colours. Differences in DNA concentration (log10) between plant configurations was tested per mycobiome type, using linear mixed effects models with Benjamini-Hochberg correction. Significant ($p < 0.05$) differences between plant configurations are indicated with lowercase-letters.

3.3. Soil Fungal Community Shifts under R. solani, Plant Neighbour and Mycobiome Type

Inoculation of the soil with *R. solani* did not affect the observed fungal OTU count (lme; F = 2.70, $p = 0.105$). However, planting configuration overall did alter OTU count (lme; F = 56.5, $p < 0.0001$), as did mycobiome manipulation (lme; F = 947.6, $p < 0.0001$). This pronounced effect of mycobiome manipulation on fungal alpha diversity was evident with OTU counts decreasing by an average of 66% when comparing baseline Field Soil to Sterilized Soil (Figure 5A). In addition, the mycobiome of Soil Feedback soil was on average 30% lower in fungal OTU count compared to the baseline Field Soil. Finally, the effect of plant configuration on OTU count did not shift based on mycobiome type (lme; F = 2.08, $p = 0.067$; Figure 5A). Manipulation of the soil mycobiome also strongly modulated fungal community composition (PERMANOVA; F = 47.46, $p = 0.001$). It should be noted that the dispersion of fungal communities also differed across mycobiome types (betadisper; F = 35.6, $p < 0.0001$). Nevertheless, differences in mycobiome types were not only determined by differing dispersions, as clearly shown through ordination (Figure 5B). The inoculation of *R. solani*, or the presence of an inter- or intraspecific neighbouring plant did not significantly alter fungal community composition ($p > 0.2$).

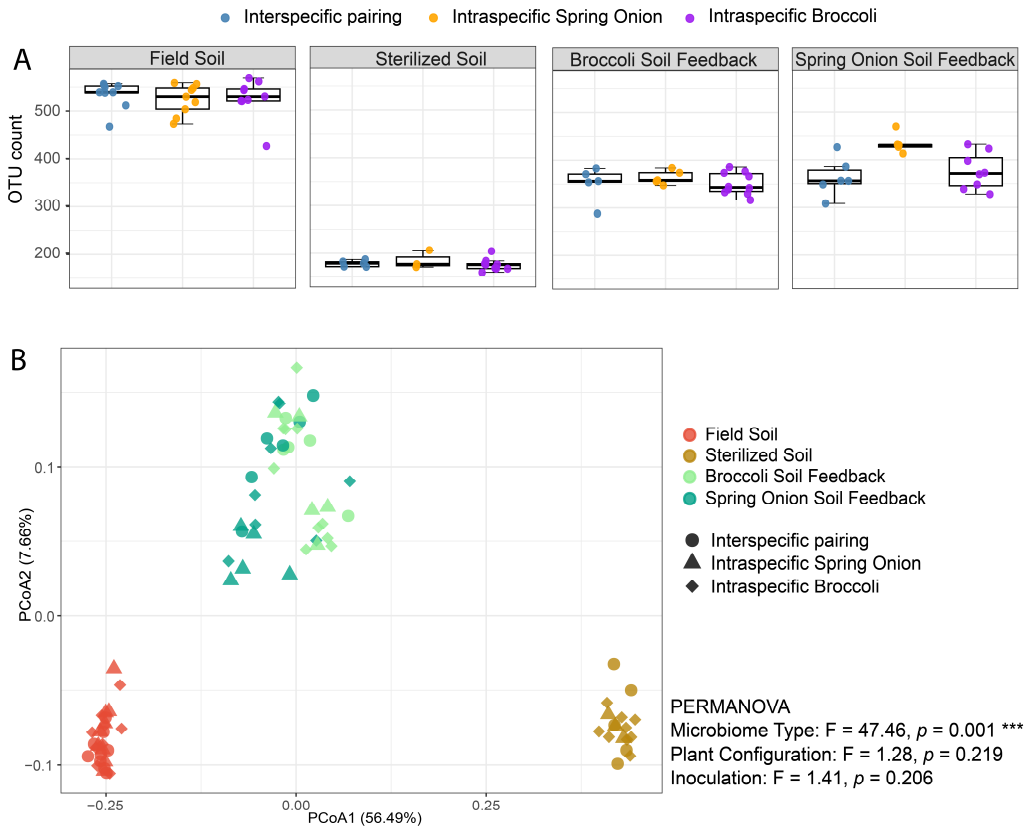

Figure 5. Alpha diversity and composition of the mycobiome types and plant configurations. (**A**) Distribution of observed OTU counts across plant configurations. Each panel corresponds to a different mycobiome type (Baseline Field Soil, Sterilized Soil, Broccoli Soil Feedback and Spring Onion Soil Feedback). Line colours indicate different plant configurations (Interspecific, Intraspecific Spring Onion, Intraspecific Broccoli). Differences in OTU count between plant configurations were tested per mycobiome type, using linear mixed effects models with Benjamini-Hochberg correction. (**B**) Dissimilarity in fungal community composition, represented by Principal Coordinate Ordination on Bray-Curtis distance measures. Colours indicate mycobiome type (Baseline Field Soil, Sterilized Soil, Broccoli Soil Feedback and Spring Onion Soil Feedback), shapes indicate plant configurations (Interspecific, Intraspecific Spring Onion, Intraspecific Broccoli) Significance is indicated with asterisk ($p < 0.001$ = ***).

3.4. Differential Effects of Plant Neighbour and Mycobiome Type on Fungal Taxa and Function

Compared to the soil of Intraspecific Broccoli plants, Intraspecific Spring Onion soils were differentially enriched in various fungal taxa (Supplementary Material Figure S2A). Most notably, *Cladosporium halotolerans* and various taxa of the AMF genus *Funneliformis* were enriched. The inoculation of soil with *R. solani* resulted in only minor shifts (lfc <0.5; Supplementary Material Figure S2B) in taxa, besides the detected enrichment of Ceratobasidiaceae (lfc = 2.95, $p < 0.05$) and *Rhizoctonia solani* (lfc = 1.79, $p < 0.05$) as a direct result of inoculation. Manipulation of the soil mycobiome additionally resulted in differentially abundant taxa (Figure 6A).

Our baseline Field Soil was naturally enriched in various fungi, compared to Sterilized Soil. Notably, various *Fusarium, Mortierella* and *Gibellulopsis* species were enriched in our Field Soil. Excluding Sterilized Soil from the analysis and comparing Field soil to both Soil Feedback soils showed an enrichment of taxa dominated by saprotrophic fungi (Supplementary Material Figure S3). Assigning function to taxa with FUNGuild suggests that the relative abundance of especially animal pathogens, symbiotrophs, pathotrophs and plant pathogens were enriched in Field Soil (LDA > 3; Figure 6B). Sterilized Soils were predominantly characterized mainly by various saprotrophic ecological guilds (LDA > 4; Figure 6B). In contrast, both Soil Feedback soils were not strongly characterized by differential ecological guilds. Similarly, both plant configuration and inoculation had little impact on the relative abundance of ecological guilds.

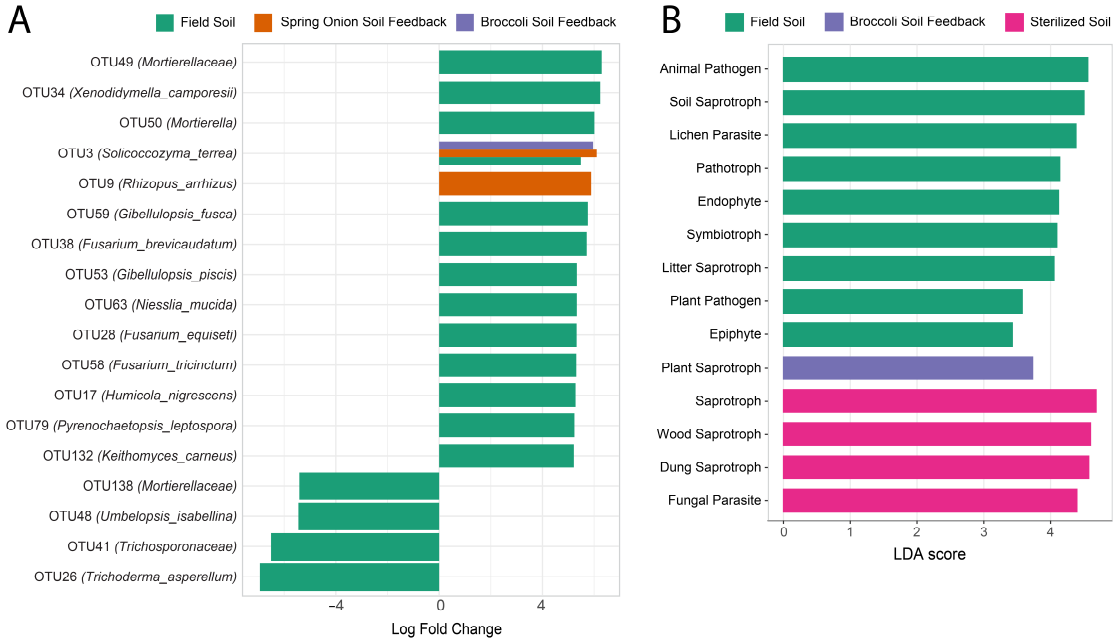

Figure 6. Enriched OTUs and fungal functional groups by mycobiome type. (**A**) Differentially abundant fungal OTUs, as determined with ANCOMBC analysis. Top 20 differentially abundant OTUs are given, ordered by Log Fold Change magnitude. Sterilized soil was used as the reference level. (**B**) Linear discriminant analysis (LDA) scores of fungal ecological guilds significantly enriched across mycobiome types, as determined by LEfSe. Mycobiome type is indicated by colour. Only guilds with LDA scores ≥ 3.0 are shown.

4. Discussion

4.1. Non-Host Neighbour Decreases B. oleracea Mortality and R. solani Persistence

In the present study, we investigated the effect of pairing a susceptible host crop with a non-host neighbour on pathogen suppression. We hypothesized that disease severity of *R. solani* would be reduced when pairing the host crop broccoli with the non-host crop spring onion. Our hypothesis was confirmed with a markedly improved broccoli seedling survival under *R. solani* inoculation in the presence of the non-host spring onion (interspecific), compared to when broccoli was paired with itself (intraspecific). Our findings align with those of Ampt et al. [35] and Abdel-Monaim et al. [61], who both reported a reduction of *R. solani* disease severity when host plants were paired with other non-host plant species. We additionally detected a reduction of *R. solani* DNA concentrations in the soil past 16 dpi, indicating that *R. solani* is also less persistent when faced with a non-host plant in the shared rhizosphere. This finding demonstrates that the influence of a non-host neighbour can reach beyond active suppression of plant damage, towards the potential reduction of pathogen build-up. This effect of reduced *R. solani* DNA persistence has not been demonstrated previously in the context of plant-plant interactions.

Beyond the suppressive effect of an interspecific neighbour, we found that the absence of a neighbour similarly reduced *R. solani* disease severity. This is in line with the findings of Dey et al. [62], who demonstrated that plant density is correlated with *R. solani* incidence in rice plants. By reducing (intraspecific) competition for resources and space, baseline stress at pathogen introduction is likely reduced and resistance is enhanced [63]. Nonetheless, reducing plant density in a field setting alone is not sufficient to combat *R. solani*, due to the spread and persistence of both sclerotia and infected crop and weed residues [64]. While plant density is a key component of disease severity, it is also crucial for achieving optimal yield in a cost-efficient manner [65,66]. Here, we

demonstrate the potential utilisation of spring onion as a beneficial neighbour to reduce *R. solani* damage in broccoli, without compromising (plant density-dependent) yield.

4.2. Fungal Diversity Does Not Drive Pathogen Efficacy, but Does Influence Pathogen Persistence

We further sought to study the role of soil fungal communities in pathogen suppression. We hypothesized that a reduction of fungal diversity and disruption of community composition through sterilisation would reduce pathogen suppressive capacities of the soil, compared to an intact soil mycobiome in our baseline field soil. We additionally expected to find a crop-species specific effect of soil feedback on pathogen efficacy. Through sterilisation of the soil, we achieved a strong reduction in fungal diversity and shift in community composition when compared to our baseline field soil. Against our expectations, this stark difference in fungal communities was not reflected in a difference in seedling mortality, or *R. solani* presence between our baseline field soil and our sterilized soil. Sterilization of the soil alters not only the mycobiome, but can also alter soil nutrients, other microbial groups, and physicochemical properties. We cannot exclude that these altered properties had an influence on seedling survival under *R. solani* inoculation beyond reduced fungal diversity. Our soil conditioned through both broccoli and spring onion soil-feedback, however, did reduce seedling mortality, whilst averaging fungal diversity between the baseline and sterilized soils. Previous studies have assigned high soil diversity as a key factor influencing pathogen suppression [67,68]. Here, we show that the relationship between fungal diversity and pathogen suppression might be less linear and more nuanced than the previously proposed trend. Our findings are in line with the recently developed narrative, that not diversity per se, but the composition of key species drives functionality [69]. Through qPCR, we confirmed that our baseline soil was free of naturally occurring *R. solani* strains of various anastomosis groups (AG2-1, AG2-2, AG4 and AG5). However, with analysis of our ITS sequencing data we found that our baseline field soil was enriched in OTUs of other potential plant pathogens, such as numerous *Fusarium* spp. and *Gibellulopsis* spp. [70,71]. Radujković et al. [72] similarly reported increased fungal pathogen abundance in soils with high levels of soil biodiversity. We thus speculate that the higher degree of pathogen suppression found in both our soil-feedback soils might be a result of dilution of these naturally occurring pathogens, either through our method of soil mixing, or through soil conditioning. Additional experiments are needed to validate the relationship between the diversity of viable fungal pathogens and pathogen suppression. Previous studies have highlighted the plant species-specific effects of soil feedback on plant performance and pathogen resistance [21]. In our study, we found an overall lower concentration of *R. solani* DNA 26 days post inoculation in soils conditioned by broccoli plants. In addition, fungal community composition in conditioned soils was plant species-specific. However, these findings were not reflected in strongly differing seedling survival rates between host conditioned and non-host conditioned soil. The unpredictability of soil feedback effects has been elaborated upon previously [73]. Hannula et al. [22] attribute positive soil feedback effects under pathogen infection to the presence or promotion of AMF. *Brassica* spp. are characterised by their lack of AMF association [74,75], whilst a strong AMF symbiosis with *Allium* spp. has been shown previously [76]. However, using FUNGuild and ANCOM-BC, we found that mycorrhizal fungi were not enriched in our two soil feedbacks, nor in our baseline field soil. It should be noted that the prediction of taxonomic and functional shifts based on ITS sequencing data is not without uncertainties, and might not have revealed the full scope of differential functional shifts in our soils. Additional exploration of pathogenic and mutualistic fungal colonisation is needed to gain a better understanding of fungal dynamics under soil-feedback and interspecific plant interactions. Furthermore, exploration of the bacterial communities would yield additional insights, as the antagonistic interaction between bacteria and pathogenic fungi plays a key role in disease suppression [77,78].

4.3. Mycobiome Modulates Plant Neighbour Effects

Through manipulation of the soil mycobiome, the effect of different plant neighbours on seedling survival and *R. solani* presence was modulated. Most notably, we found that the presence of *R. solani* in the absence of a neighbour plant was most sensitive to manipulation of the mycobiome. The absence of a neighbouring plant could have lessened active modulation of the soil mycobiome or root exudation by the plant, increasing reliance on the present soil microbes for a defensive response to pathogen introduction [19]. However, due to the exclusion of the 'No neighbour' treatment from sequencing analysis, we cannot infer if the response to mycobiome manipulation is a reflection of fungal community changes or other plant or soil variables. The interactive effect between the soil mycobiome and active plant-specific pathogen suppression was reinforced by the difference we found in *R. solani* quantities at 26 dpi when comparing Intraspecific Broccoli plant configurations with Intraspecific Spring Onion configurations in sterilized soils. In the absence of a potentially pathogen suppressive mycobiome, plant neighbour identity modulated pathogen presence most strongly. In comparison, *R. solani* presence was similarly reduced or

elevated regardless of plant neighbour in favourable mycobiomes (Broccoli Soil Feedback) or unfavourable mycobiomes (Field Soil), respectively. These results highlight the importance of a dual approach in pathogen suppression, by both increasing soil health and plant diversity.

In conclusion, our study highlights the potential of pairing a susceptible crop with a non-host crop to reduce the impact of *R. solani,* as broccoli seedling survival increased when grown alongside spring onion, and pathogen persistence in soil was reduced. We speculate that this plant neighbour effect is partially dependent on the soil mycobiome functional composition, rather than fungal diversity itself, through a weakened pathogen suppression under unfavourable, pathogen-rich mycobiomes. These interactive effects should be considered when further exploring the plant-plant dynamics in pathogen suppression efforts. Additional research under field conditions and varying mycobiomes is needed to reveal if this beneficial crop combination of host to non-host can be expanded beyond our controlled greenhouse study, and if these findings additionally extend to other crop combinations. At present, our research cannot establish whether the observed effects are driven by shifts in the mycobiome alone, or by changes in the overall microbial communities. As such, incorporating all members of the microbiome, including bacteria, viruses, protists and nematodes, in pathogen suppression research is essential in disentangling the relative contributions of individual species, as well as inter-microbial interactions that can drive the suppressive capacities of the soil.

Supplementary Materials

The additional data and information can be downloaded at: https://media.sciltp.com/articles/others/2611181436557836/SE-26070151-Supplementary-Materials.pdf. Figure S1. Effect of mycobiome and plant configuration on broccoli seedling survival probability under R. solani inoculation. Survival probability (%) as modelled with Weibull distribution survival analysis. 95% credible intervals are indicated with dashed lines. Data is separated by plant configuration, mycobiome types are indicated with colours; Figure S2. Top 20 Enriched fungal OTUs. (a) Differentially abundant fungal OTUs by plant configuration, as determined with ANCOMBC analysis. Top 20 differentially abundant OTUs are given, ordered by Log Fold Change magnitude. Intraspecific broccoli configuration was used as the reference level. (b) Differentially abundant fungal OTUs under R. solani inoculation, as determined with ANCOMBC analysis. Top 20 differentially abundant OTUs are given, ordered by Log Fold Change magnitude. No inoculation control was used as the reference level; Figure S3. Top 20 Enriched fungal OTUs. Differentially abundant fungal OTUs by microbiome type, as determined with ANCOMBC analysis. Top 20 differentially abundant OTUs are given, ordered by Log Fold Change magnitude. Sterilized soil was excluded from the analysis, and Field soil was used as the reference level.

Author Contributions

J.T.: Conceptualization, Methodology, Investigation, Visualization, Formal analysis, Data curation, Writing—original draft, Writing—review & editing,. A.G.-S.: Conceptualization, Methodology, Investigation, Writing—review & editing. A.v.C.: Investigation, Writing—review & editing. K.B.: Conceptualization, Resources, Writing—review & editing. A.K.: Conceptualization, Methodology, Supervision, Writing—review & editing. S.G.: Conceptualization, Methodology, Supervision, Writing—review & editing. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The sequence data has been deposited in the NCBI Sequence Read Archive (SRA) database and can be accessed under the BioProject ID PRJNA1403222.

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Conflicts of Interest

The authors declare no conflict of interest. Given the role as Editorial Board Member, Stefan Geisen had no involvement in the peer review of this paper and had no access to information regarding its peer-review process. Full responsibility for the editorial process of this paper was delegated to another editor of the journal.

Use of AI and AI-Assisted Technologies

No AI tools were utilized for this paper.

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