Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

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Vera Claessens

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Environmental Health Sciences

SUPERVISOR :

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The effect of heatwaves and thermal acclimation on the growth and intraspecific competition of a common soil saprotrophic fungus*

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*Running title: The effects of heatwaves on soil decomposer fungi

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ABSTRACT

According to the IPCC, human-induced climate change is responsible for an increase in extreme weather events such as heatwaves. Besides the adverse effects of heatwaves on humans, heatwaves also negatively affect natural systems. It impacts all types of ecosystems and their trophic levels, including the smallest microorganisms. Such tiny and essential microorganisms are saprotrophic soil fungi; they play a crucial role in ecosystem functioning. They are the dominant decomposers of soil organic matter and release CO_2 into the atmosphere, mediating carbon exchanges between the biosphere and atmosphere. Increased fungal activity due to heatwaves could lead to more significant soil carbon losses and increase positive climate feedback. However, it is thought that thermal acclimation (physiological down-regulation) could mitigate the potential adverse effects of elevated temperatures. Nonetheless, little is known about how heatwaves and thermal acclimation influence density-driven population regulation through intraspecific competition. Therefore, we tested the hypotheses that (1) heatwave temperatures increase the growth rate and intraspecific competition and (2) thermal acclimation to heatwave temperatures will result in slowed growth rates and a shift in intraspecific interactions. Our results proved our first hypothesis that under heatwave temperatures, there is a significant increase in growth rate and severity of intraspecific competition, leading to a lowered carrying capacity and, thus, a lower sustainable population size. However, unlike the second hypothesis, no effects of thermal acclimation on intrinsic growth rate, intraspecific competition and carrying capacity were measured. Addressing these ecological impacts of acclimation in a changing environment is crucial for more accurate ecological predictions

INTRODUCTION

Human-induced climate change is responsible for an increase in extreme weather events such as heavy rainfall, temperature extremes, droughts, and floods, as well as compound events such as heatwaves (1,2). According to the IPCC (Intergovernmental Panel on Climate Change), it is virtually certain that the intensity and frequency of heatwaves will continue to increase, even if global warming will be stabilised at 1.5ºC (2,3). By 2040 the land areas affected by heatwaves are thought to be quadrupled (4). This could come with various problems as heatwaves lead to multiple adverse events, such as impacts on human health, causing increased mortality (5). Additionally, it could impact agriculture, leading to an increased risk of crop failure (6). Besides affecting us humans, it is also widely known that heatwaves cause negative consequences for natural systems, where it impacts all types of ecosystems and their trophic levels, from large animals to the smallest soil microorganisms (7,8).

These soil microorganisms play a crucial role in ecosystem functioning. Most events visible on the above-ground level are driven by the below-

ground processes and interactions of soil microbes (9). Efforts have been made to classify soil microbes into three broad functional groups. The first group (mainly represented by bacteria and soil fungi) are the chemical engineers responsible for nutrient cycling and decomposition of organic matter (9). A second group (mainly represented by microfauna as well as soil fungi) are the biological regulators (also known as bio controllers) who are responsible for the regulation of population dynamics and pests (9,10). The third group (consisting of microfauna, soil fungi, and bacteria) are the ecosystem engineers responsible for regulating the soil structure and simultaneously the availability of resources to other species (9,10). According to this classification, we can see that soil fungi belong to all three functional groups and are therefore considered key players in ecosystem functioning. Additionally, soil fungi are the dominant members of many soil communities (11). It is, therefore, essential to understand how soil fungi are affected by climate change events such as heatwaves.

This study focuses on a specific group of soil fungi, namely the cord-forming saprotrophic fungi (also known as decomposer fungi). In the plant litter zone, these soil saprotrophs are the dominant decomposers of soil organic matter (SOM) (12). Complex SOM compounds get broken down and converted into smaller inorganic nutrients, which are released into the environment or used for their own growth, making them the primary regulators of soil nutrients and carbon cycling (11,12). However, during the decomposition of SOM, there is a respiratory release of CO_2, making soil saprotrophs key players in soil carbon fluxes between the biosphere and atmosphere and hence in climate feedback (11–13). An increasing temperature within the optimum growth range of most saprotrophic soil fungi (25 ºC – 30 ºC) accelerates activity and hence growth rates (14,15). Such an increasing fungal activity could potentially have negative consequences for climate feedback by an increased respiratory release of CO_2 into the atmosphere, enhancing global warming (11,13). Despite the fact that an increase in temperature will enhance fungal activity, it is also known that soil saprotrophs

possess a high level of phenotypic plasticity (10,16). This phenotypic plasticity can be described as the ability to respond to different environmental conditions with a wide range of physiological and morphological changes (16). Acclimation is a specific type of phenotypic plasticity, also known as a reversible non-genetic change in response to a specific environmental stressor (17,18). When this stressor is temperature, this phenomenon is called thermal acclimation (13,17,18). The ability to undergo thermal acclimation has been proven for many organisms, such as plants, cyanobacteria, and mycorrhizal fungi (12,18–20). Even more, In 2013, Crowther and Bradford studied the ability of soil saprotrophs to undergo thermal acclimation in agar plate experiments (13). These thermal acclimation experiments showed that, in most cases, warm-acclimated individuals had a lower growth and respiration rate than cold-acclimated individuals (13). These results suggest that thermal acclimation can mitigate the increased soil carbon efflux expected during heatwave events and consequently mitigate the positive climate feedback (13,22,23). Nonetheless, the relevance of testing in more realistic settings than petri dishes has been demonstrated by Klingen *et al.,* who in 2001 tested the inhibitory effect of isothiocyanate on insect pathogenic fungi and showed that in petri dishes, the fungi were inhibited, while in microcosms, there was no inhibition at all (24). However, empirical data on the effects of thermal acclimation on soil saprotrophs in more realistic experimental settings, like a microcosm, remain scarce.

Nevertheless, in a recent study conducted by Alster *et al.* in 2021, the phenotypic plasticity of soil fungi in response to temperature and moisture was tested using sterile litter microcosms. The study results suggest that fungal biomass and extracellular enzyme activity varied in different moisture and temperature environments. Furthermore, the response also varied between different fungal isolates. These results thus indicate that soil fungi studied in a more complex setting exhibit a plastic response to temperature and moisture (16).

Besides the effect of temperature and thermal acclimation on physiological responses such as

growth, very little is known about how these factors will influence soil fungal populations and communities. Both populations and communities are shaped by two factors, namely, environmental parameters (e.g. resources, temperature, water availability, space) and interactions (i.e. intra- and interspecific competition) (25,26). To study the effects of heatwaves on soil fungal populations/communities, it is besides understanding how single environmental factors affect physiological responses such as growth, also essential to understand how interactions are affected (27). As already stated, there are two types of interactions. The first type is intraspecific competition which represents the competition for limited resources between members of the same population and is known to induce plastic changes in growth rates (28). The second type is interspecific competition, representing the competition for limited resources between members of different populations (28). The ratio between intraspecific and interspecific competition within a community is mainly caused by the niche overlap between the present species (29). However, in communities with coexisting species, the force of intraspecific competition is

stronger than that of interspecific competition (12,30). In other words, population growth within a (healthy) community is mainly controlled by self-limitation rather than species limiting the population growth of their competitors (30). These factors make intra- and interspecific competition the drivers of population and community responses. Therefore, they are interesting parameters to study in relation to environmental stressors caused by human-induced climate change (27). Theoretical studies on the effect of temperature on intraspecific competition in ectotherms show that intraspecific competition is positively correlated with temperature (31,32). This hypothesis is mainly expected when due to a higher temperature, activity levels increase, consequently leading to increased resource requirements (31,33). Despite the importance of understanding how intraspecific competition is affected by temperature and thermal acclimation and how soil fungal population dynamics are affected, this topic remains understudied. So far, studies mainly focused on plants and insects (34).

Therefore, this study focuses on determining the effect of heatwaves and thermal acclimation
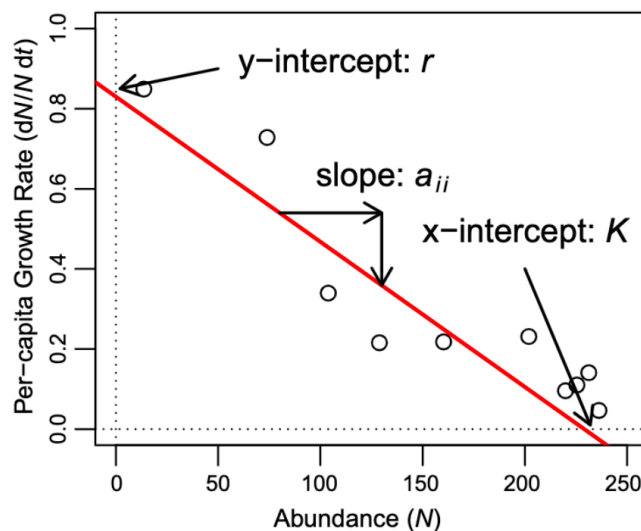

Fig. 1 – Modified Lotka-Volterra regression model: The regression model compares the per-capita growth rate (Y-axis) calculated as $\ln(N_f/N_i)/\Delta t$ and species biomass (X-axis) when grown in monoculture. The Lotka-Volterra demographic population parameters can be distracted from this model, the Y-intercept represents the intrinsic growth rate (r), the slope of the model represents the sign and intensity of intraspecific competition (a_{ii}), and the X-intercept represents carrying capacity (K). N_f: final abundance; N_i: initial abundance; Δt: difference in time. (Figure derived from Mühlbauer et al. 2020) (39).

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on the growth and intraspecific competition of the common soil saprotroph *Trichoderma harzianum* (phylum: Ascomycota). This study compares the mycelial biomass of ambient- and warm-acclimated (environmental history) *T. harzianum* when grown in ambient and heatwave temperatures (environmental conditions) in sterilised soil microcosms. Soil microcosms were chosen because they are more ecologically realistic regarding physicochemical properties (e.g., nutrient availability, 3D growth matrix, water-holding capacity) than simple agar plate experiments (24). Mycelial biomass was chosen as a measure of mycelial growth because it will tell us something about the capacity of the fungus to encounter and decompose SOM and thus provide essential knowledge of the effects of temperature on fungal functioning (13). The DNA-based technique digital droplet PCR (dd-PCR) was used to measure mycelial biomass. dd-PCR shows high accuracy and sensitivity compared to other DNA-based techniques, such as qPCR (35–37). High accuracy and sensitivity are essential when working with microcosms because they hold very small fungal biomasses. However, dd-PCR is mainly used in clinical studies, leading to protocols that deliver suboptimal results when used on environmental samples with a rather complex chemical and biological origin (36). Therefore, in this study, a newly designed and optimised dd-PCR protocol for soil samples containing mycelium was prepared.

Ultimately, measurements of mycelial biomass in response to environmental history (EH) and environmental conditions (EC) were used to prepare modified Lotka-Volterra regression models. These models describe the change in species abundance over time, also known as Per-capita Growth Rate (PCGR). Furthermore, these models are used to obtain demographic population parameters such as intrinsic growth rate (r), intraspecific competition (a_{ii}) and carrying capacity (K) (38,39) (Fig 1). The intrinsic growth rate is a measure of the maximal per capita growth rate of a species within a population at low density and in the absence of other species (39). The intensity of the intraspecific competition parameter shows how a species will affect its own growth (31,39). The

parameter carrying capacity is used as a measure of the maximal sustainable abundance of a species in a given environment (39). Altogether, the use of modified Lotka-Volterra models to study the effects of EC and EH on the common soil saprotroph *T. harzianum* will gain us valuable insights into how population dynamics of this strain will respond to temperature stress as well as thermal acclimation in controlled laboratory settings when grown in monoculture in soil microcosms.

We hypothesise that (1) exposure to different temperatures (EC) will influence the growth and intraspecific competition of *T. harzianum* when grown in sterile soil microcosms. We predict that at heatwave conditions of 25ºC, there is an increased growth rate and stronger intraspecific competition. (2) Thermal acclimation (EH) will result in slowed growth rates when warm-acclimated and accelerated growth rates when ambient-acclimated, leading to a shift in intraspecific interactions.

EXPERIMENTAL PROCEDURES
Origin of soil and fungal strain – The used soil originated from a meadow in South-West Germany (Rheinland-Pfalz/Leimersheim) and was not exposed to pesticides, biocidal fertilisers, or organic manure at least five years before sampling. The soil was taken at a 0-20 cm depth and sieved with a 2mm screen by the supplier (Lufa-Speyer). The fungal strain of *Trichoderma harzianum* (MUCL 29707) used for the experiments originated from an unspecified soil type in Flemish-Brabant, Belgium, provided by the Belgian coordinated collections of microorganisms. Strain stocks were prepared on Minimum Merlin Norkans (MMN) agar (40). Plates were sealed with Parafilm®, and mycelium was grown at 25ºC in an incubator for approximately 2 days and stored in a cold room (5ºC).

Strain incubation, microcosm preparation, and mycelium processing – The strain was cultured for 7d at 25ºC/18ºC on sterilised MMN-agar in 9-cm diameter Petri dishes (n=25 per temperature) containing sterilised cellophane sheets or accurately collecting mycelium without collecting agar. The defined heatwave

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temperature of 25ºC was based on previous experiments in the Ecotron facility of Hasselt University. The ambient temperature of 18ºC functions as a reference condition. Petri dishes were sealed with Parafilm® to reduce risks of contamination. Per dish, one fungal plug (∅: 7,50 mm) was used. Experiments took place in microcosms containing 15-gram sterilised soil. Soil sterilisation was done by a three-time autoclaving cycle at 121ºC for 20 minutes. After every cycle, the soil was left to rest for 48 hours to ensure the growth of remaining resistant spores. After sterilisation, the soil was replaced into sterile 50mL falcon tubes. Mycelium that was grown on MMN plates was collected and transferred into 150mL 1/6 diluted Fries medium (41) and mixed in a blender (3x 20s) to obtain mycelium pieces of ~2mm. Based on the mycelial growth standard curves (see supplement S2, Fig S2) and the mycelial dry biomass approximation (see supplement S1, Fig S1), dilutions were made to obtain 4 different relevant initial mycelial abundances and a negative control that were inoculated and mixed as a 5mL solution in sterilised 15-gram soil microcosms (0,0 mg/ g soil; 0,04 mg/ g soil; 0,16 mg/ g soil; 0,64 mg/ g soil; and 1,9 mg/g soil) all initial abundances are ecological relevant as they are within the range of measured natural occurring fungal biomasses (~0.013 – 5.2 mg dry weight / g soil) (42,43).

Pre-experiment: growth dynamic of T. harzianum – To determine the maximum possible duration of a heatwave experiment, it was necessary to investigate the growth dynamics of *T. harzianum* in soil microcosms. The maximum possible duration of the heatwave experiment depended on the length of the exponential growth phase of the strain, which means that for accurate predictions of final abundances, the heatwave should last no longer than the length of this growth phase. Mycelium was grown at 25ºC and processed as described in the "strain incubation", "microcosm preparation", and "mycelium processing" section. All microcosms were exposed to 25ºC for 0,2,4 and 7 days. At every time point, one microcosm for every initial abundance was sacrificed. Ultimately, this resulted in 20 microcosms containing (4 initial abundances + negative control) * 4-time points.

The experiments were terminated by freezing the soil at -80ºC. All soil samples were homogenised using a mortar, pestle, and liquid nitrogen. Soil samples of ~ 0.25g were taken from all microcosms, and phenol: chloroform DNA extractions took place. DNA extracts were further analysed with dd-PCR to obtain the growth dynamics of *T. harzianum*.

Heatwave experiment experimental set-up– To determine the effects of thermal acclimation on the growth response of *T. harzianum* during a heatwave and ambient conditions, *T. harzianum* was acclimated for seven days to ambient conditions of 18ºC (n=25) and heatwave conditions of 25ºC (n=25), according to the "strain incubation" section. After acclimation, the mycelium of each temperature treatment was processed as previously described in the "mycelium processing" section. The obtained initial abundances and negative control were inoculated into the soil microcosms. In total, 80 microcosms were used in this experiment, comprising four experiments for all the initial abundances (+ negative control), all in quadruplicate (1) mycelium acclimated to 25 ºC exposed to a heatwave of 25ºC; (2) mycelium acclimated to 25 exposed to an ambient condition of 18ºC; (3) mycelium acclimated 18ºC exposed to a heatwave of 25ºC; (4) mycelium acclimated 18ºC exposed to an ambient condition of 18ºC (see Fig. 2 for a schematic overview of the experimental design). After 3 days, the experiments were terminated by freezing the microcosms at -80ºC. All soil samples were homogenised using a mortar, pestle, and liquid nitrogen. Finally, soil samples of ~0.25g were taken from all microcosms and phenol: chloroform DNA extractions took place. DNA extracts were further analysed with dd-PCR, and data output was used to fit modified Lotka-Volterra regression models.

DNA extraction and quality control - Up to 0.25g of homogenised soil of each microcosm was collected in Eppendorf's, followed by adding 0.7 mm Garnet beads, 600 μL Bead solution (181 mM NaPO₄, 121 mM guanidinium isothiocyanate, pH 7.5), and 60 μL SR1 solution (150 mM NaCl, 4% SDS, 0.5 M tris, pH 10) to

disperse cells and soil particles, aid cell lysis and break down fatty acids and lipids of cell membranes. 600 µL phenol:chloroform:isoamyl alcohol (pH 8) was added for optimal lysis efficiency by separating the lysed cell components and proteins from the nucleic acids. Cell lysis was obtained by mixing samples for 10 min at 25Hz in a Retsch mixer. The tubes were centrifuged at 13,400 rpm for 2 min to obtain phase separation of the mixture. The upper aqueous phase containing total nucleic acids was transferred to clean tubes and centrifugated at max speed for 2 min to pellet the proteins and humic acids. Up to 450µL supernatants were transferred to a 2mL deep well block and mixed with 25µL NucleoMag B-beads (Machery Nagel) and 475µL MWA2 binding buffer (95-100% isopropanol). A suitable DNA extraction protocol was run on the MagMAX™ Express magnetic particle processors (Thermo Fisher Scientific,

inc., U.S.). DNA samples were washed in 850µL MWA3 solution (3.2M Guanidine hydrochloride, 30 mM tris, 30% ETOH, pH 6.8) and 850µL MWA4 solution (10 mM Tris, 100 mM NaCl, 50% EtOH, pH 7.5). Finally, the DNA extracts were eluted in 100µL Rnase-free H_2O. After DNA extraction, quality control with Nanodrop was done to determine the purity of the DNA extract, and Qubit 2.0 Fluorometer (Thermo Fisher Scientific, inc., U.S.) was used to determine the DNA concentration present in samples. DNA samples were stored at -20 °C.

dd-PCR procedure – DNA samples were diluted (1/200) with RNase-free water to overcome PCR inhibition by humic acids in the soil samples. dd-PCR reaction mix (per reaction) contained 10µL 2x QX200™ ddPCR™ EvaGreen® Supermix (Bio-Rad), 1µL Forward primer (ITS1S: 5′-TACAACTCCCAA ACCCAATGTGA-3'), 1µL
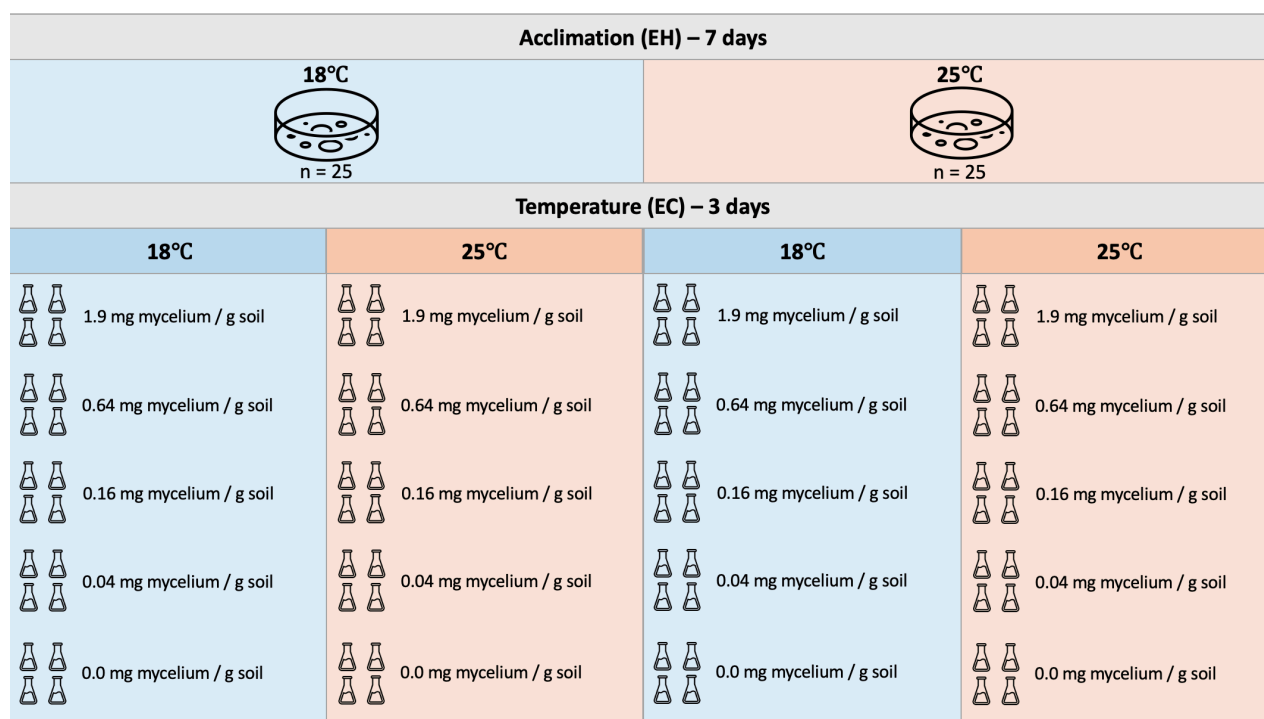

Fig. 2 – Experimental design heatwave experiment: Mycelium of *T. harzianum* was acclimated (EH) to 18ºC and 25ºC for 7 days on MMN-agar (n=25 for each EH). Acclimated mycelium was collected, and 4 different initial abundances and a negative control were inoculated into sterilised soil microcosms. Each initial abundance of acclimated mycelium (EH:25ºC/18ºC) was exposed to 18ºC or 25ºC (EC) for 3 days. Ultimately final abundances were measured by using dd-PCR and modified transformed Lotka-Volterra regression were prepared to study the effect of EH and EC on the growth and intraspecific competition of *T. harzianum* monocultures in sterilised soil microcosms. A total of n=80 microcosms was used in this experiment.

Reverse primer (ITS1R: 5′-CCGTTGTTGAAAGTTTTGATTC ATTT-3′); both primers were designed by Lopez-Mondejar *et al.* (44). ITS (Internal Transcribed Spacer) regions (Fig. S3) were used because they show a high probability of successful identification for a broad range of fungi and are the most used barcode for fungal identification at the species level (45,46). For the reduction of mid-amplitude droplets (rain) in the dd-PCR output, the protocol was optimised by adding 6μL of 0.4μg/μL BSA dissolved in RNase-free water (see S4 - dd-PCR optimisation experiments, Fig. S4) (47). 18μL reaction mix and 2μL of diluted DNA were loaded into the DG8™ Cartridge for the QX200™/QX100™ droplet generator, followed by the addition of 70μL oil for EvaGreen®. Droplets were generated using the QX200™ Droplet Generator (Bio-Rad). After droplet generation, 40μL of droplets (per sample) were carefully loaded into a 96-wells plate (Bio-Rad) and heat sealed at 180°C for 5 sec using the PX1™ PCR Plate Sealer (Bio-Rad). DNA amplification was obtained by using C1000 Touch™ Thermal cycler (Bio-Rad), with the following cycling conditions: enzyme activation at 95°C for 5 min, denaturation at 95°C for 30 sec, annealing/extension (40 cycles) at 59,5°C for 1 min, signal stabilisation at 4 °C for 5 min followed by 90°C for 5 min, hold at 4 °C for infinity. A ramp rate of 2°C/sec was used during all thermal cycling steps. Optimal annealing/extension temperatures were determined by using a temperature gradient (see S5 - dd-PCR temperature gradient, Fig. S5)

Statistical analysis – dd-PCR output (ITS copies/μL) of the heatwave experiment in combination with the standard curve giving the relationship between absolute fungal biomass (mg mycelium/g soil) – DNA content (ITS copies/μL) (see supplement S1, Fig S1) was used to calculate absolute fungal biomass. This data was then used to prepare transformed modified Lotka-Volterra linear regression models by first calculating the dependent variable Per-capita growth rate (PCGT) according to the following formula (PCGR = $\frac{\ln (N_f/N_i)}{\Delta t}$), with N_f being the final abundance, N_i being the initial abundance,

and Δt being the length of the experiment. PCGR was then plotted against the natural logarithm of the independent variable N_i. The model was used to calculate different population parameters, such as intrinsic growth rate (r_i), sign and intensity of intraspecific competition between individuals of the same species (a_{ii}) and the carrying capacity (K). Following this, an unpaired two-sample T-test analysis was conducted to compare the means of the dependent variables intraspecific competition, intrinsic growth rate and carrying capacity, the independent variables environmental history (EH) (18ºC/25ºC), environmental conditions (EC) (18ºC / 25ºC). Additionally, a two-way ANOVA was conducted to test the effect of EH and EC on the PCGR of the lowest initial abundance in the experimental design (0.04 mg mycelium/ g soil). For this test, independent variables were EH (18ºC / 25ºC), EC (18ºC / 25ºC), and the dependent variable was PCGR. Before the statistical tests were conducted, assumptions of normality and homogeneity of variances were tested using Shapiro-Wilk and Bartlett's tests. Non-parametric alternatives (Wilcoxon-test for t-test / aligned ranks transformation ANOVA for Two-way ANOVA) were used when assumptions were not met. All statistical analyses were conducted in R-studio (version: 2023.03.0+386) and R (version: 4.3.0) (48).

RESULTS

Growth dynamics of T. harzianum – Initial abundances 1.9 and 0.04 mg mycelium/g soil reached the exponential growth phase around 3 days of growth in sterile soil microcosm at 25ºC (Fig S6: dark blue and orange lines). An initial abundance of 0.16 mg mycelium/g soil reached exponential growth at ~5 days in sterile soil microcosms at 25ºC (Fig S6: yellow line). No real growth was measured for the initial abundance of 0.64 mg mycelium/g soil (Fig S6: light blue line). The negative control showed no growth of *T. harzianum* (Fig S6: red line), indicating that no *T. harzianum* is present in the sterilised soil. Based on the exponential growth phase of *T. harzianum,* which reaches a plateau phase at a biomass of >5.4 mg/mycelium (see results of log-regression growth standard curve – supplement S2 and Fig

S2), the maximal duration of the heatwave experiment was set to 3 days.

Effect of temperature (EC) on the intrinsic growth rate, intraspecific competition and carrying capacity – Transformed Lotka-Volterra regression models for EC (Fig 3A) show the PCGR of *T. harzianum* in monoculture under different EC (18ºC and 25ºC). Slopes (a_{ii}) of the lines represent the intensity of intraspecific competition of *T. harzianum* in a soil microcosm. Due to the model transformation ($\ln(N_i)$), the intrinsic growth rate (the theoretical maximal increase of a population per individual at very low density) could not be readily based on the Y-intercept as proposed in Fig 1. In this transformed modified Lotka-Volterra regression model, the Y-intercepts represent an initial abundance of 1 mg mycelium/g soil ($\ln N_i = 0$ equals $N_i = 1$). Therefore, the intrinsic growth rate (r_i) at a lower N_i ($\sim$ 0 mg mycelium/ g soil) was calculated using the estimate of $\ln N_i = -200$ mg mycelium/g soil (not visible on the graph). X-intercepts represent the population carrying capacity (maximum sustainable population density). Our results show that EC significantly affected the intrinsic growth
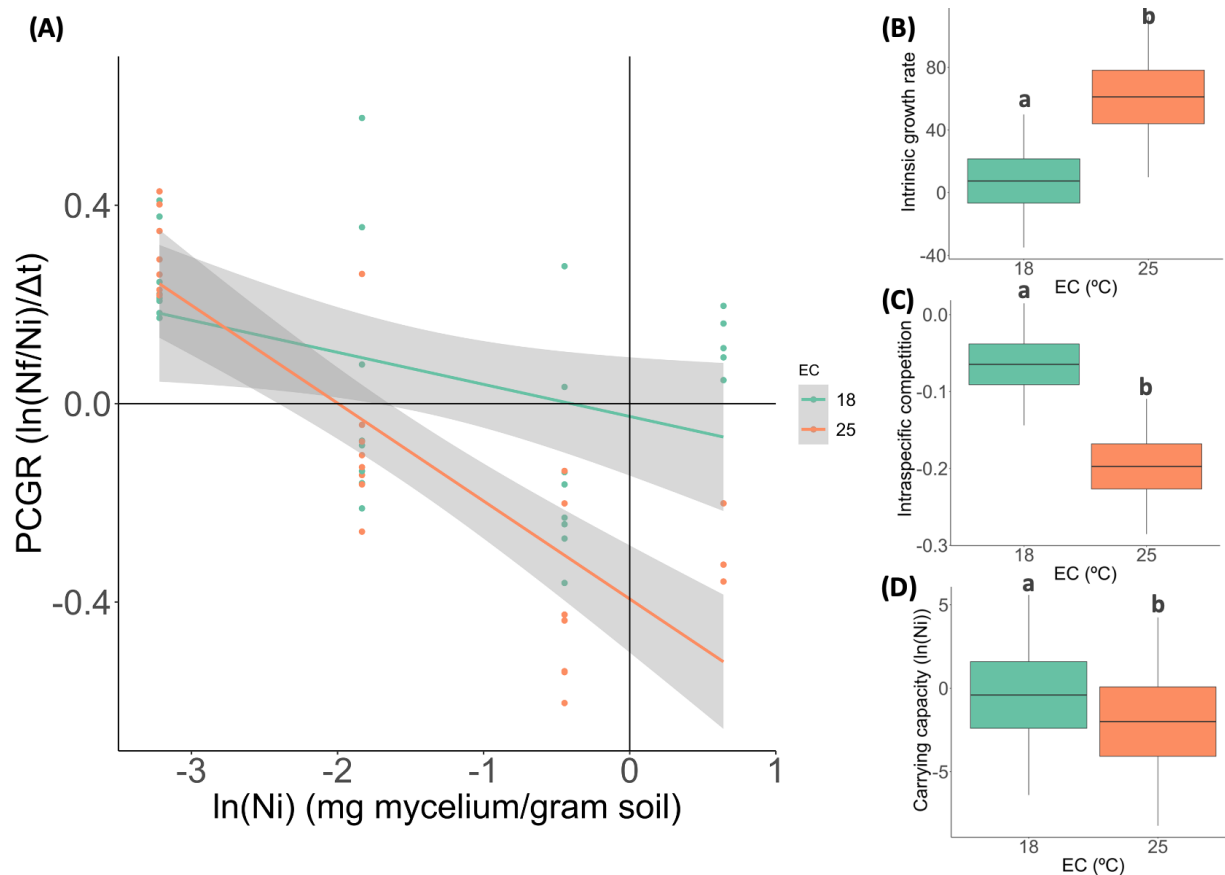

Fig. 3 – Transformed Lotka-Volterra regression model showing the effect of environmental conditions (EC) on the intrinsic growth rate, intraspecific competition and carrying capacity of *T. harzianum*: **(A)** Y-axis: represents the Per Capita Growth Rate calculated as $\ln(N_f/N_i)/\Delta t$, X-axis: represents the natural logarithm of the N_i in mg mycelium/ gram soil, green line represents *T. harzianum* exposed to EC 18ºC ($R^2 = 0.1519$) , orange line represents *T. harzianum* exposed to EC 25ºC ($R^2 = 0.7194$) ; shaded regions are the 95% CI of the regression lines. **(B-D)** Boxplots show mean ± SE for each Lotka-Volterra regression parameter; **(B)** The effect of EC on intrinsic growth rate (EC 18ºC: 7.453±14.139; EC 25ºC: 61.078±17.059); **(C)** The effect of EC on intraspecific competition (EC 18ºC: -0.065±0.026; EC 25ºC: -0.197±0.029); **(D)** The effect of EC on carrying capacity (EC 18ºC: -0.4±1.2; EC 25ºC: -1.938±2.079). Boxplots with different letters indicate a significant difference (p <0.05)

rate (p-value = < 2.2 *10^{-16}, Fig 3B). At EC 25ºC, the mean intrinsic growth rate was ~8.2x higher than at EC 18ºC (r_i 18ºC: 7.453 vs r_i 25ºC: 61.078). EC also significantly affected intraspecific competition intensity (p-value = < 2.2 *10^{-16}, Fig 3C). At 25ºC, the intraspecific competition was ~3x more intense than at 18ºC (a_{ii} 18ºC: -0.065 vs a_{ii} 25ºC: -0.197). Ultimately, EC also significantly affected the carrying capacity of the strain grown in sterile soil microcosms in monoculture (p-value = 0.0002, Fig 3D). At 25ºC, the carrying capacity was ~5x lower than at 18ºC (K 18ºC: -0.4 vs K 25ºC: -1.938).

The effect of acclimation (EH) on the intrinsic growth rate, intraspecific competition and carrying capacity – Transformed Lotka-Volterra regression models representing the effect of EH on PCGR (Fig 4A) were prepared the same way as the models representing the effect of EC. Regression parameters (intrinsic growth rate, intraspecific competition, and carrying capacity) were also calculated similarly. EH had no significant effect on the intrinsic growth rate (p-value = 0.5563, Fig 4B), nor on the intensity of intraspecific competition (p-value = 0.1259, Fig 4C), and the carrying capacity (p-value = 0.05417, Fig 4 D).
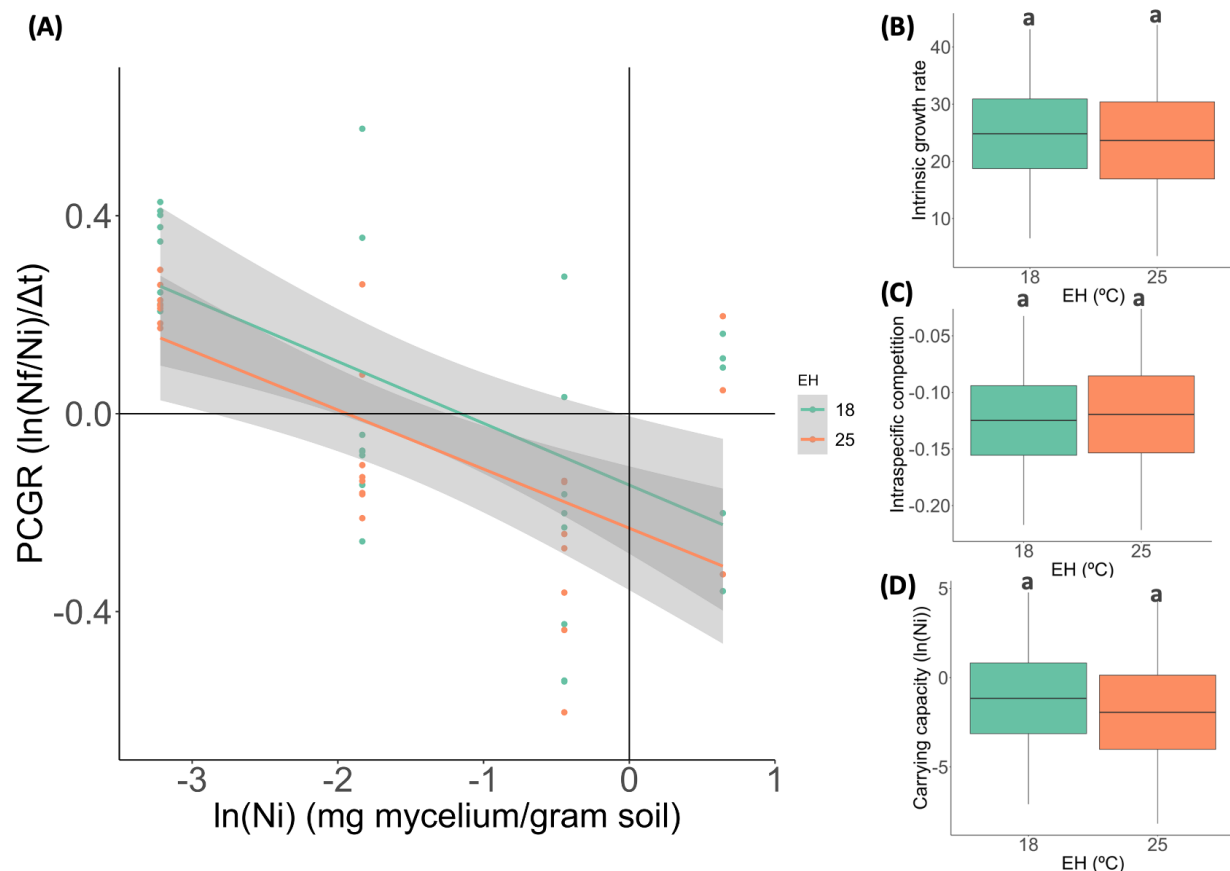

Fig. 4 – Transformed Lotka-Volterra regression model showing the effect of environmental history (EH) on the intrinsic growth rate, intraspecific competition and carrying capacity of T. harzianum: **(A)** Y-axis: represents the Per Capita Growth Rate calculated as $\ln(N_f/N_i)/\Delta t$, X-axis: represents the natural logarithm of the N_i in mg mycelium/ gram soil, green line represents *T. harzianum* acclimated to EH 18ºC (R^2 = 0.3306), orange line represents *T. harzianum* acclimated to EC 25ºC (R^2 = 0.4116); shaded regions are the 95% CI of the regression lines. **(B-D)** Boxplots show mean ± SE for each Lotka-Volterra regression parameter; **(B)** The effect of EH on intrinsic growth rate (EH 18ºC: 24.82 ±6.089; EH 25ºC: 23.66 ±6.735); **(C)** The effect of EH on intraspecific competition (EH 18ºC: -0.125 ±0.03075; EH 25ºC: -0.119 ±0.034); **(D)** The effect of EH on carrying capacity (EH 18ºC: -1.156 ±1.977; EH 25ºC: -1.938 ±2.079). Boxplots with different letters indicate a significant difference (p <0.05)

Effects of EC and EH on the PCGR of T. harzianum with an initial abundance of 0.04 mg/g soil – An additional statistical analysis on the effect of EC and EH on the PCGR at an initial abundance of 0.04 mg mycelium/g soil showed that the PCGR was not significantly affected by EC (p-value = 0.21111, Fig 5). However, EH significantly affected PCGR (p-value = 0.02367, Fig 5). At an EH of 25ºC, the PCGR was ~1.5x lower than at an EH of 18ºC. No interaction between EH and EC was found. The PCGR of *T. harzianum* acclimated to EH 18ºC (standard deviation EC 18ºC: 0.0986; EC 25ºC: 0.0920) showed higher variance than when acclimated to 25ºC (standard deviation EC 18ºC: 0.0228; EC 25ºC: 0.0326).

DISCUSSION

Heatwave temperatures enhance growth rate and intraspecific competition – Our findings show that the intrinsic growth rate, as well as the intraspecific competition, was higher when *T. harzianum* was exposed to heatwave temperatures of 25ºC. These findings prove our first hypothesis stating that exposure to different temperatures (EC) will influence the growth and intraspecific competition of *T. harzianum* grown in sterile soil microcosms. Additionally, we found that at 25ºC, the carrying capacity of the tested *T. harzianum* strain was significantly lower than at 18ºC. This finding suggests that during a 25ºC heatwave event lasting three days, the maximum sustainable population density is lower than when
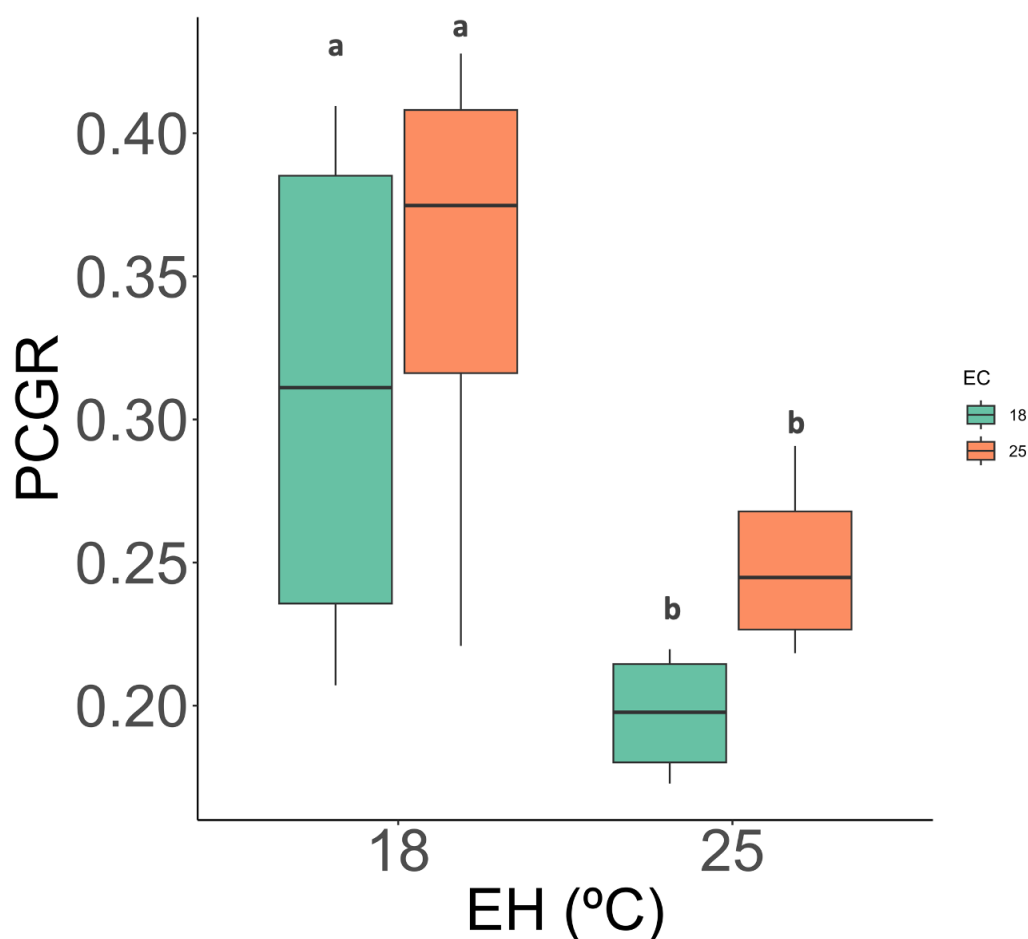

Fig. 5 – Boxplots representing the effects of environmental conditions (EC), and environmental history (EH) on the PCGR of *T. harzianum* at an initial abundance of 0.04 mg mycelium/ gram soil: boxplots show the median ± standard deviation for each PCGR of each EC and EH(EH18:EC18: 0.31±0.0986; EH18EC25: 0.37±0.0920; EH25:EC18: 0.2 ± 0.0228; EH25:EC25: 0.24±0.0326). Boxplots with different letters indicate a significant difference (p<0.05).

exposed to 18ºC. There are multiple possible explanations for the strain's higher intrinsic growth rates at 25ºC.

The first explanation could be that at 25ºC, the metabolic activity of the strain is higher, as 25ºC is closer to the T_{opt} of *T. harzianum* (T_{opt} *Trichoderma* spp. 27-30ºC) (49). According to the metabolic theory of ecology (MTE), the metabolic rates of organisms are temperature dependent (50). This indicates that at a temperature closer to the optimum, the energy taken up is more likely to be metabolised towards growth (51). At 18ºC, the metabolic rates are lower, which could simply lead to lower growth rates (50,51). The MTE could also explain the observed lowered carrying capacity at 25ºC. It has been proven that a negative relationship exists between temperature and carrying capacity, mediated by an increased per capita metabolic resource demand driven by an increased respiration rate (52,53).

Another possible explanation which elaborates on the first explanation could be to look at the lifestyle strategies for microbes, also known as the Y-A-S framework (originally proposed by Malik *et al.,* 2020) (54). The Y-A-S framework is a modification of Grime's C-S-R (competitor- stress tolerator – ruderals) framework designed for plants (55). Y-A-S is a classification for three microbial life history strategies that are placed along two main axes of environmental variation (resource availability and abiotic stress). The first strategy is the high yield (Y) strategy, which maximises resource uptake of fractions allocated towards the central metabolism and assimilatory pathways for building cellular components. The high-yield strategy is favoured in circumstances with resource abundance and absence of abiotic stress (54,56). Important to note is that high yield does not perse reflect growth rate, as the growth rate depends on both growth yield and resource acquisition (54). Resource acquisition (A), the second strategy, is the production of extracellular enzymes needed to break down the complex resources in the environment (54,57). It is proposed that this strategy prevails in environments with low resources leading to a decline in growth yield (54). The last strategy is stress tolerance (S) which prevails when microbes

are exposed to suboptimal abiotic conditions, which may lead to investing in traits that are linked to stress tolerance (molecular as well as phenotypical traits) (54). Soil fungi are known for their high plasticity and ability to adapt to the environment they live in (10). Also, soil fungi cannot be classified into one life history strategy by itself, but they consist of different combinations of traits belonging to different strategies (58). We can thus assume that within their preferred life-history strategies, they also consist of a certain degree of plasticity. As discussed, the strategies are based on trait trade-offs, which were not measured during this experiment. However, if we place our results of lower intrinsic growth rate at 18ºC in this framework, we can hypothesise that the mycelium grown at 18ºC in the soil microcosm was exposed to stressful abiotic conditions and resource limitation. According to the previously discussed Y-A-S framework, this would mean that *T. harzianum* exposed to 18ºC mainly invests in stress tolerance (S) and resource acquisition (A), which will go at the expense of growth yield (Y). When growth yield and growth rate are positively correlated, as first suggested by Ng H. in 1969, this will lead to a decrease in growth rate (54,57,59). When we place *T. harzianum* that was grown at 25ºC in a soil microcosm with limited resource conditions in this framework, we can assume that in this condition, there will be more investment in resource acquisition (A), this will also go in the expense of growth yield (Y) and ultimately growth rate. However, more energy is still available for growth as there is no investment in stress tolerance (S) needed. Other research done by Alster *et al.* in 2021 on Californian grassland field experiments of decomposer fungi in response to abiotic stress (drought) proposes a modification of the Y-A-S framework. This new framework combines the Y and A strategies (60). When placing our findings in this framework, we could assume that at 18ºC, there is more investment in stress tolerance at the expense of growth yield and resource acquisition. While at 25ºC, there is more investment in growth yield and resource acquisition, ultimately leading to a higher growth rate. By following this framework, the uncertainty of explaining the effect of the different strategies on the growth rate is solved, as

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here growth rate depends on both the Y and A strategies (57). Additional trait measurements would be needed to prove whether trait trade-offs exist between the proposed microbial life history strategies in the current experimental design.

Connecting these observations with potential outcomes for carbon efflux, we suggest that the increased activity of *T. harzianum* when temperatures rise towards heatwave conditions, in the first instance, could lead to increased carbon efflux into the atmosphere and thus accelerate positive climate feedback. This is expected because theoretical and experimental studies have proven that under soil warming conditions, the carbon use efficiency of soil microorganisms is expected to drop, even when temperatures have not reached the optimal growth temperature (52,53,61). However, we also detected that at 25ºC, the strain has a significantly lowered carrying capacity and increased mortality. If the pattern of higher mortality at higher temperatures is general for soil fungi, this could ultimately lead to increased carbon sequestration in the soil during long-term soil warming events; however, this depends highly on the recalcitrance of fungal biomass in the soil (13,62).

No effect of thermal acclimation of T. harzianum – No significant effect of EH (acclimation) was seen on the intrinsic growth rates nor the intraspecific competition. These findings disprove our second hypothesis, stating that thermal acclimation (EH) will result in slowed growth rates when warm-acclimated and accelerated growth rates when ambient-acclimated, leading to a shift in intraspecific interactions. This result can also be explained in multiple ways.

The first possible explanation for no thermal acclimation effect is that the duration of acclimation we choose (7 days) is not long enough. When looking at the acclimation period of symbiotic fungi, Malcom *et al.* showed in 2008 that seven days of acclimation could be enough for some ectomycorrhizal strains but definitely not all (19). However, for most members of the soil microbial community, it is thought that thermal acclimation will manifest over several weeks (18). Hence, previous research done by Crowther *et al.* in 2013 focused on the thermal

acclimation effect in cord-forming basidiomycete fungi used an acclimation period of 9 weeks. Results indicated as one of the first studies in this field that saprotrophs could undergo thermal acclimation. However, the acclimation period was highly species-specific (13). Nonetheless, the choice of the 7-days acclimation period for this experiment is more realistic in terms of an actual heatwave duration compared to more extended periods of up to 9 weeks.

For a second possible explanation, we can again involve the Y-A-S framework for microbial lifestyle strategies. As previously discussed, we can assume that soil fungi possess a certain degree of plasticity within their preferred life history strategy, making them able to have the ability to contain characteristics of multiple strategies under different environmental conditions (54,58,60). The fact that we acclimated the fungal strain on MMN agar in petri dishes but exposed them to the environmental conditions in soil microcosms could lead to no observable thermal acclimation effect. During the acclimation on agar plates, the mycelium grows in an environment with resource abundance (19). Hence, when exposed to the tested temperatures in soil microcosms, the environment changes at many levels (e.g., nutrients, 3d matrix, water content), leading to a resource-limited environment. As already discussed, the Y-A-S framework is a classification for three microbial life history strategies that are placed along two main axes of environmental variation (resource availability and abiotic stress) (54). We suggest that during the acclimation in a resource-abundant environment, the strain invests in acclimating certain traits (i.e., Y and A traits). However, it could be that those specific traits do not come into play in a resource-limited environment. In our case, this could be seen as follows. When the mycelium is acclimated at 25ºC, the strain may invest primarily in central metabolism and assimilation for building cellular components (i.e., Y-strategy traits). While at 18ºC, there could already be an investment in stress tolerance traits. When the mycelium is then placed in the soil microcosms with resource limitation, the mycelium still must invest in other traits (i.e., A-strategy traits), meaning that they have not been acclimated to the right combination of characteristics of different

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strategies. Ultimately, this could lead to no detected thermal acclimation. The possible effect of low humidity on the fact that we did not detect thermal acclimation can be ruled out, as the soil of the microcosms stayed wet during the 3-day incubation. Nonetheless, one way to still be able to measure a possible but smaller effect of acclimation could be to add an intermediate acclimation temperature to which the current EH are compared.

Another important point to consider is that based on the PCGR of the measured initial abundances, we see that above 0.16 mg mycelium/gram soil mortality is already observed (negative PCGR), which means three of the four initial abundances we measured exceeded carrying capacity. To obtain more robust modified Lotka-Volterra regression models, it is necessary and recommended to repeat this same experiment but shift the scope to smaller initial abundances (N_i < 0.16 mg mycelium/gram soil). By doing this, the obtained regression model will be less biased by mortality, and hence, a better linear relationship could be observed without the need for model transformations. Hence, a possible effect of acclimation might be observed, as already suggested by the results of acclimation on the PCGR of the lowest initial abundance. Shifting the scope to lower initial abundances will still be ecologically relevant, as observed fungal biomass in real-life ecosystems is estimated between (~0.013 – 5.2 mg dry weight / g soil) (42,43).

Nonetheless, we can conclude that these results thus show that this *T. harzianum* strain in the currently used experimental set-up does not thermally acclimate; ultimately, this will mean that there is most likely no mitigating effect on the expected carbon efflux and positive climate feedback (13).

Thermal acclimation influences the PCGR of T. harzianum – An additional statistical analysis was conducted on the effects of EC and EH on the PCGR of *T. harzianum* because of the appearance of mortality at N_i >0.16 mg mycelium/ gram soil. PCGR is a parameter that consists of the effects of intrinsic growth rate and intraspecific competition. Therefore, this result will not allow us to base conclusions on the effect of those two parameters separately. Nonetheless, we saw that PCGR was not significantly affected by EC. However, a significant effect of EH was noticed, showing a lower PCGR for *T. harzianum* that has been acclimated to 25ºC compared to when it has been acclimated to 18ºC. Our results and literature clearly show that the optimum growth temperature of this specific *T. harzianum* strain is ≥ 25ºC, meaning that at 18ºC, the strain will be exposed to cold stress (49). According to our second hypothesis, our results suggest that in this initial abundance, the growth rate is accelerated when acclimated to ambient conditions and slowed when acclimated to heatwave conditions.

A possible way of explaining this accelerated PCGR when acclimated to 18ºC could be based on the theory of hormesis. Hormesis is the effect that, at low stress, growth and activity can be stimulated (63). These hormetic responses have been found in all sorts of organisms, including fungi (63). In our case, 18ºC can be considered as mild cold stress, which could, for example, mean that the strain stores more energy for growth than when acclimated to more optimal conditions (25ºC). Ultimately this could lead to a higher fitness in terms of PCGR for the strain when acclimated to 18ºC (65).

Nonetheless, we also noticed higher variability (bigger standard deviation) in the PCGR when acclimated to 18ºC, meaning that the response of the strain acclimated to this condition is less predictable and uniform than when acclimated to more optimal temperatures. However, this analysis is based only on one initial abundance with only four replicates per EH:EC combination. As previously stated, to prove this acclimation effect, it is necessary to repeat this experiment with multiple initial abundances (< 0.16 mg mycelium/gram soil).

Hence, contradictory to the previous results, this result indicates that there might still be a possible effect of acclimation on the population parameters, which ultimately can have mitigating effects on carbon efflux and positive climate feedback. However, this effect might have gone unnoticed due to the high mortality measured in the regression models.

The use of dd-PCR in environmental samples – A newly optimised dd-PCR protocol was designed for this experiment. dd-PCR uses a partitioning technique in which DNA is divided over ~10.000 droplets, making this technique less vulnerable to PCR inhibition than qPCR, which is currently the golden standard DNA-based method in environmental research (36,66). According to previous research on the use of dd-PCR on soil fungal samples, the lowest detection for fungal (*Cadophora luteo-olivacea*) DNA derived from soil samples was around 25 fg DNA/ul. In contrast, the limit of detection of qPCR in this experiment was found to be 125 fg DNA/ul (67). The lowest initial abundance (0.04 mg mycelium/g soil) tested during our dd-PCR experiments was ~15 fg DNA/ul, even lower than the lowest DNA concentration measured in the previously mentioned experiment. Based on the standard curve (S2- mycelium growth standard curves, Fig S2), the estimated biomass was 0.064 mg mycelium/gram soil. The difference between the estimated value and the aimed initial abundance could be explained by the difficulty of preparing a mycelial dilution of exactly the wanted mycelial biomass. Difficulties in doing this include that the collected mycelium to prepare this initial abundance is estimated based on earlier dry-weight experiments. Thus, the amount of mycelium grown on a Petri dish can change from time to time, as growing conditions will never be completely the same (e.g., nutrients in agar or incubator conditions). Besides this, the accuracy of pipetting the exact right amounts of mycelium for making dilutions could be prone to pipetting errors as the volumes were rather large.

Nonetheless, the accuracy of the estimated biomass in these types of experiments is rather promising. We can thus conclude that dd-PCR, when correctly optimised for soil fungal samples, can be used way below the limit of detection of qPCR and is thus a useful alternative, especially when working with small DNA concentrations or in studies requiring high resolution (36). Additionally, in microcosm experiments like the one presented in this study, dd-PCR allows us to measure the effect of environmental stressors on the population parameters of multiple species when using species-specific primers (36). This approach is advantageous when working with multiple species in one microcosm and makes it possible to measure both intraspecific and interspecific competition.

Future research – For future research on the impact of heatwaves on soil microbial communities, more realistic circumstances are needed by using more complex experimental designs. In this study, only monocultures have been tested. However, in natural ecosystems, one fungal strain never appears alone. According to the coexistence theory, the outcome of species interactions (intra- and inter-specific competition) and growth are influenced by the diversity in response of different species to stressors (23). Recent research done by Lammel *et al.* in 2023 on the effect of recurring heatwave events on the growth and interactions of saprotrophic soil fungi proves this theory. The study showed that not only temperature can affect the growth of these soil fungi but also the co-occurrence between different strains (23). Besides adding multiple fungal (and bacterial) species, another important stressor to be included in future studies is drought. A heatwave is a compound event between high temperatures and extreme drought (2). Including drought in the experimental design would increase the ecological relevance of these kinds of studies; however, the complexity of disentangling the effects of drought and temperature separately, as well as their interaction, will be challenging.

Additionally, as already mentioned, measuring traits to determine trade-offs during heatwaves is necessary to show whether the fungi (and other soil microbes) use their plasticity to deal with heatwave events. Ultimately, testing trait trade-offs could further make it possible to use the Y-A-S framework with more certainty. Possible traits that could be valuable measures for the specific lifestyle strategies are biomass and respiration rate measures as a metric of growth yield (Y) (54). By measuring how much of the carbon (C) substrate is invested in biomass related to the C lost through respiration, the growth efficiency (and thus growth yield) could be measured (54,60). Resource acquisition (A) traits measurements could include siderophore assays; siderophores are small organic compounds that can chelate the essential but low bioavailable growth element ferric iron (Fe(III)) and solubilise

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it for uptake by the fungus (54,68). Additionally, extracellular enzyme assays giving a measure of the degradation of complex substrates such as cellulose and hemicellulose could be interesting (54,60). Simple measures for stress tolerance (S) traits that could be used are pigmentation and (conidial) spore count. Fungi that increase pigmentation could increase their thermotolerance, while sporulation increases the chances of the fungus surviving unfavourable conditions (2,69). An interesting extension of this type of research could be to study how possible changes in life-history traits due to environmental stress could affect interactions between different trophic levels (e.g., plants, bacteria, microfauna) and ultimately lead to phenological mismatches in soil ecosystems (15,25).

The current study design did only include a single heatwave event. However, heatwave–ambient temperature cycles are projected to increase in frequency (1–3). Therefore, the experimental design could be more realistic by including multiple heatwave-ambient events. This type of experiment could also reveal a possible lagged acclimation response.

Lastly, including higher heatwave temperatures could be an interesting addition, as the severity of heatwaves is also projected to increase (1–3). Even more, it could be considered that the current extremes we measure now could become the new-normal natural fluctuations and that more severe temperature stress should be considered in experimental designs (70).

CONCLUSION

In this study, we aimed to show the effects of heatwaves and thermal acclimation on the growth and intraspecific competition of the common soil saprotroph *T. harzianum*. Unlike what we hypothesised, we did not see an effect of thermal acclimation on the population parameters intrinsic growth rate, intraspecific competition and carrying capacity. However, the fact that there was no observable acclimation effect could be due to the experimental design, and there are indications that thermal acclimation of this *T. harzianum* strain in the tested conditions might still be possible.

Furthermore, our hypothesis stating that higher temperatures will lead to increased intraspecific competition and growth rates was proven. Ultimately, this led to a lower carrying capacity during heatwave conditions. These results suggest that heatwaves could lead to a decrease in the growth efficiency of *T. harzianum.* In the short term, this could lead to an increase in carbon efflux from the soil and lead to an increase in positive climate feedback.

However, further research using more complex experimental designs with more species (also including other soil microbes such as bacteria) is needed to predict the effect of heatwaves and thermal acclimation on soil microbial communities.

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SUPPLEMENTARY MATERIAL
S1 – MYCELIAL DRY BIOMASS APPROXIMATION
Trichoderma harzianum was incubated on sterilised Minimum Melin Norkans (MMN) agar plates (40) for 7 days to 18ºC (n= 5) and 25ºC (n=5). Afterwards, all mycelium per plate was collected in pinched Eppendorf tubes and frozen at -20ºC. Eppendorf's with mycelium were freeze-dried overnight in a lyophilisator. Finally, the dry weight of mycelium grown on MMN medium in different conditions was determined, and data was ultimately used for making dilutions in the heatwave experiment.
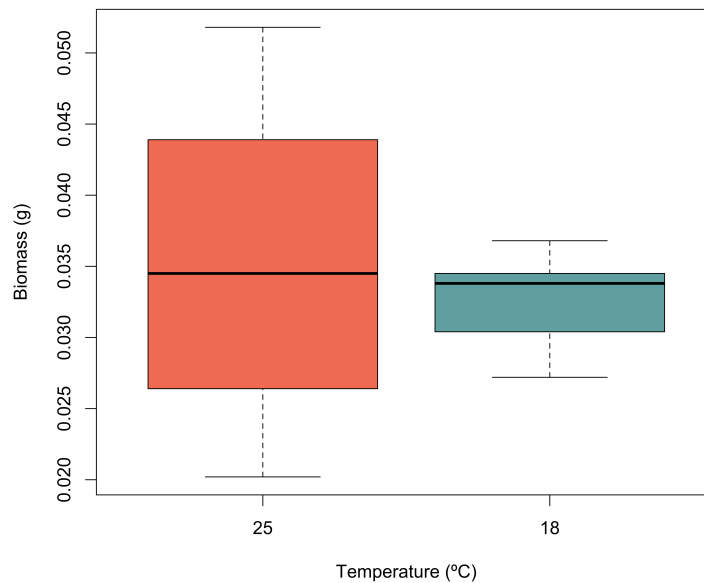

Fig S1 –boxplots of mycelial biomass of T. harzianum grown at 25ºC and 18ºC: boxplots representing temperature in ºC. Whiskers represent the minimum and maximum biomass (25ºC: 0.0202 – 0.05180; 18ºC: 0.02720 – 0.05770), borders of the box represent the first quartile (25ºC: 0.02610; 18ºC: 0.03397) and the third quartile (25ºC: 0.04325; 18ºC: 0.03857), the middle line of the box represents the median (25ºC:0.03195; 18ºC: 0.03725), t-test p-value: 0.4376.

S2- MYCELIUM GROWTH STANDARD CURVES

To obtain an approximation of the absolute abundance of mycelium in soil microcosms, 25 MMN plates were grown at 25ºC for 7 days. The mycelial biomass is based on the results of mycelial biomass approximation (S1, fig S1). All the mycelium of the remaining 25 plates (~0.86 grams) was collected in 150 mL 1/6 diluted Fries medium (41), and a serial dilution series was prepared (0; 0.04; 0.16; 0.64; 1.90; 5.4 mg mycelium/ g soil). The dilutions were then inoculated in sterilised 15-gram soil microcosms, followed by immediate soil homogenisation using a pestle and mortar. The soil was then frozen in 50 mL Falcon tubes at -80ºC. Ultimately, soil samples of ~ 250 mg were taken from all dilutions and phenol: chloroform: isoamyl alcohol DNA extractions took place. DNA extracts were further analysed with dd-PCR to obtain standard curves showing the relationship between biomass – DNA content. Data was fitted in log-regression models to find the highest possible R^2, and with this the maximum possible abundance of mycelium showing log-regression. The final standard curve is presented in Fig. S2.

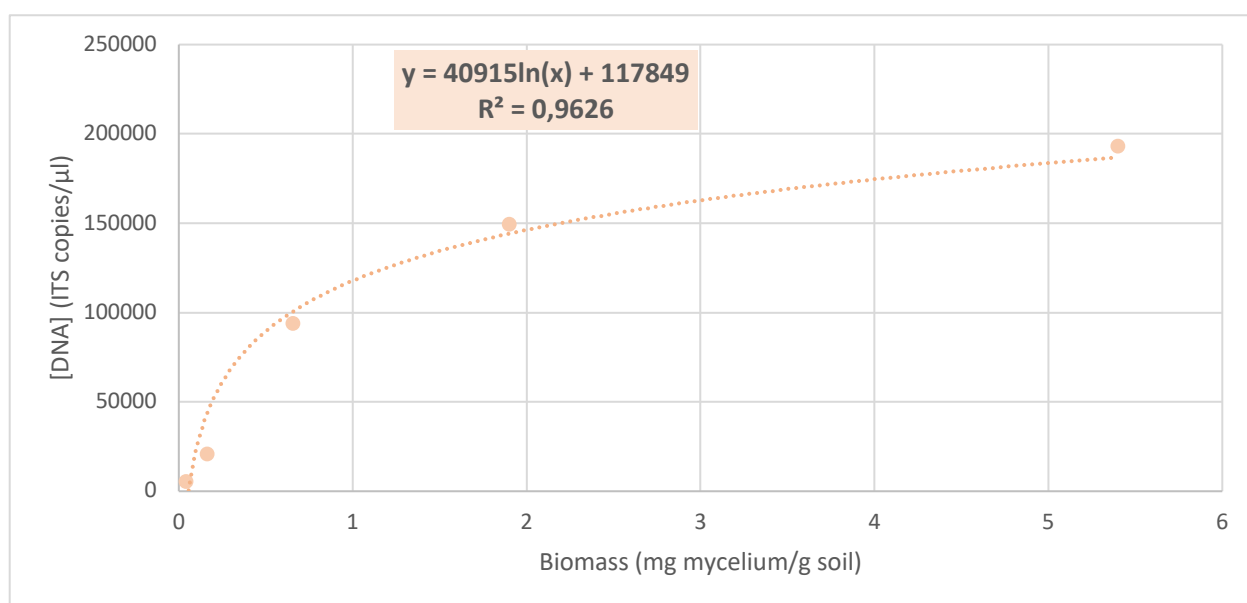$$y = 40915\ln(x) + 117849$$
$$R^2 = 0{,}9626$$

Fig S2 – Standard curve of mycelium growth of *T. harzianum* in 15-gram soil microcosms: A logarithmic relationship between biomass and DNA content was found. X-axis: biomass of *T. harzianum* mycelium in mg mycelium/ g soil. Y-axis: DNA content in ITS copies/μL. After fitting log-regression models, a maximum amount of mycelium showing a log-regression curve was set at 5,4 mg mycelium/g soil. Hence, for accurate biomass predictions in the experiments, the final abundance should not exceed this 5,4 mg mycelium/g soil threshold.

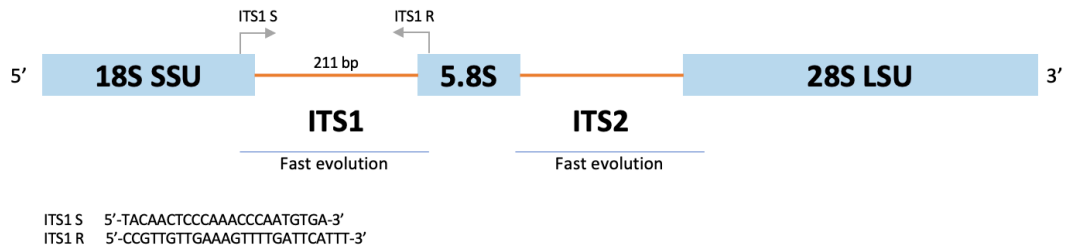**S3 – rDNA Internal Transcriber Spacer region (ITS)**

Fig S3 – Schematic representation of the rDNA Internal Transcribed Spacer region (ITS): For the experiment, the ITS1 region was used to identify *Trichoderma harzianum* using the forward primer ITS1 S and the reverse primer ITS1 R (44). The ITS regions of the rDNA are prone to fast evolution, making them suitable for identifying fungi at the species level. Moreover, ITS regions are present in tandem repeats, making them identifiable in samples with low DNA content (45,46). ITS1 region is flanked by the 18S small subunit (SSU) on the 5' end and by the 5.8S subunit downstream. The ITS2 region is flanked by the 28S large subunit (LSU) on the 3' end and by the 5.8S subunit upstream. The length of the ITS1 amplicons generated during the experiments was 211 bp (44) - figure made by myself.

S4 - dd-PCR optimisation experiments

Different approaches were taken to optimise the dd-PCR protocol for soil samples containing mycelium. Non-optimised dd-PCR output showed a high degree of so-called "rain", also known as mid-amplitude droplets. A large subset of these droplets might represent false positive signals and are most likely caused by the presence of inhibitors (e.g., humic acids) in the DNA-Extracts (36). Different DNA extract purification methods (addition of $CaCl_2$, BSA, DMSO) were tested to reduce the "rain" and increase the measurement accuracy. $CaCl_2$ consists of multivalent cations that are capable of eliminating inhibitors present in the soil during the extraction process (71). BSA increases PCR yield and binds to lipids and organic compounds such as humic acids during the PCR reaction (47,72,73). DMSO mainly reduces secondary structures in DNA samples (74). Results indicated that 0.4 μg/μL BSA was the best purification method as the rain was substantially reduced compared to other treatments or combinations of treatments (Fig S4A and B). Additionally, the detected concentration of DNA was not substantially reduced compared to dd-PCR measurements, where no treatments were used (Fig S4B: sample A02).
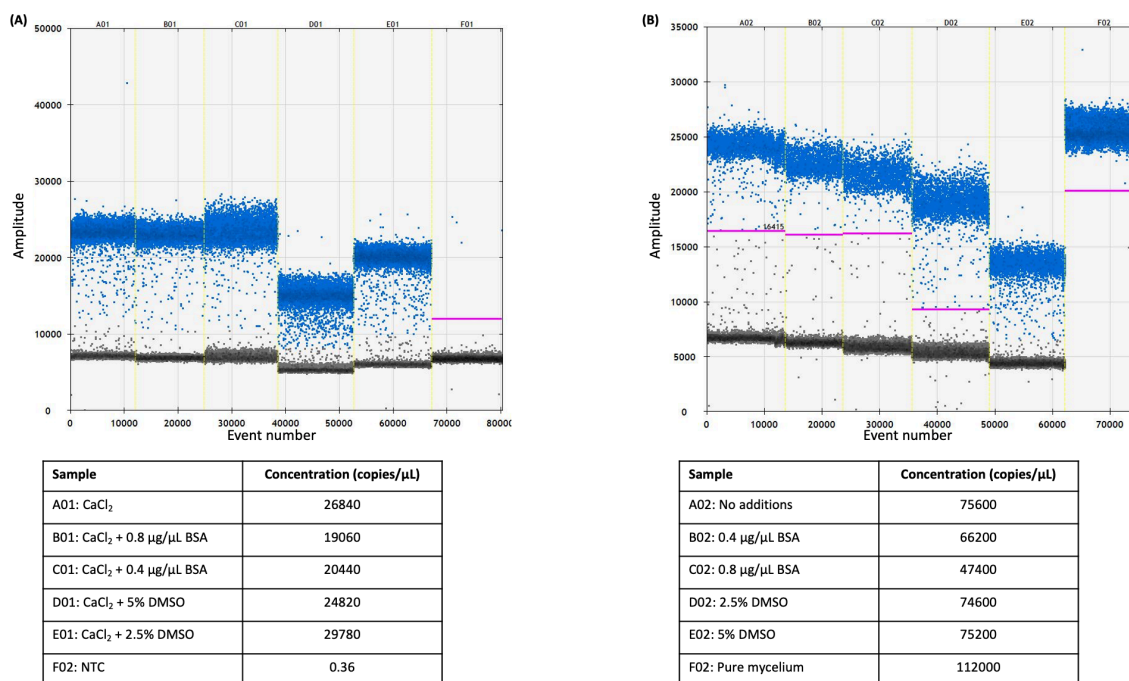

Sample	Concentration (copies/μL)
A01: $CaCl_2$	26840
B01: $CaCl_2$ + 0.8 μg/μL BSA	19060
C01: $CaCl_2$ + 0.4 μg/μL BSA	20440
D01: $CaCl_2$ + 5% DMSO	24820
E01: $CaCl_2$ + 2.5% DMSO	29780
F02: NTC	0.36

Sample	Concentration (copies/μL)
A02: No additions	75600
B02: 0.4 μg/μL BSA	66200
C02: 0.8 μg/μL BSA	47400
D02: 2.5% DMSO	74600
E02: 5% DMSO	75200
F02: Pure mycelium	112000

Fig S4 – dd-PCR output of different purification methods ($CaCl_2$, BSA and DMSO) for fungal DNA in soil samples to reduce PCR inhibition and mid-amplitude droplets (rain): $CaCl_2$ consists of multivalent cations that are capable of eliminating inhibitors present in the soil during the extraction process (71). BSA increases PCR yield and binds to lipids and organic compounds such as humic acids during the PCR reaction (47,72,73). DMSO is mostly used to reduce secondary structures in DNA samples (74). **(A)** dd-PCR output of DNA samples extracted with the addition of 0.150 mM $CaCl_2$ alone or with the addition of BSA (0.4 μg/μL / 0.8 μg/μL) or DMSO (2.5% / 5%). A01: $CaCl_2$; B01: $CaCl_2$ + 0.8 μg/μL BSA; C01: $CaCl_2$ + 0.4 μg/μL BSA; D01: $CaCl_2$ + 5% DMSO; E01: $CaCl_2$ + 2.5% DMSO; F01: NTC. **(B)** dd-PCR output of DNA samples extracted with the normal phenol:chloroform: isoamyl alcohol protocol alone or with the addition of BSA (0.4 μg/μL / 0.8 μg/μL) or DMSO (2.5% / 5%). A02: No additions; B02: 0.4 μg/μL BSA; C02: 0.8 μg/μL BSA; D02: 2.5% DMSO; E02: 5% DMSO; F02: pure mycelium. The DNA extraction with the addition of 0.4 μg/μL BSA (B02) shows the least rain. Comparing the DNA concentration between the different treatments, we see that adding $CaCl_2$ reduced DNA content by ~3x (A01 vs A02). The EvaGreen® dye fluorescence amplitude of the amplified droplets is represented on the Y-axis. Blue dots represent positive droplets (containing target DNA), and grey dots represent negative droplets (no target DNA). X-axis represents the number of droplets number (event number).

S5 - dd-PCR temperature gradient

Optimal annealing/extension conditions for the used primers were determined by using a temperature gradient. The most optimal temperature was determined based on the separation between positive droplets (blue dots) and negative droplets (grey dots) and was ultimately set to 59.5ºC (Fig. S5)
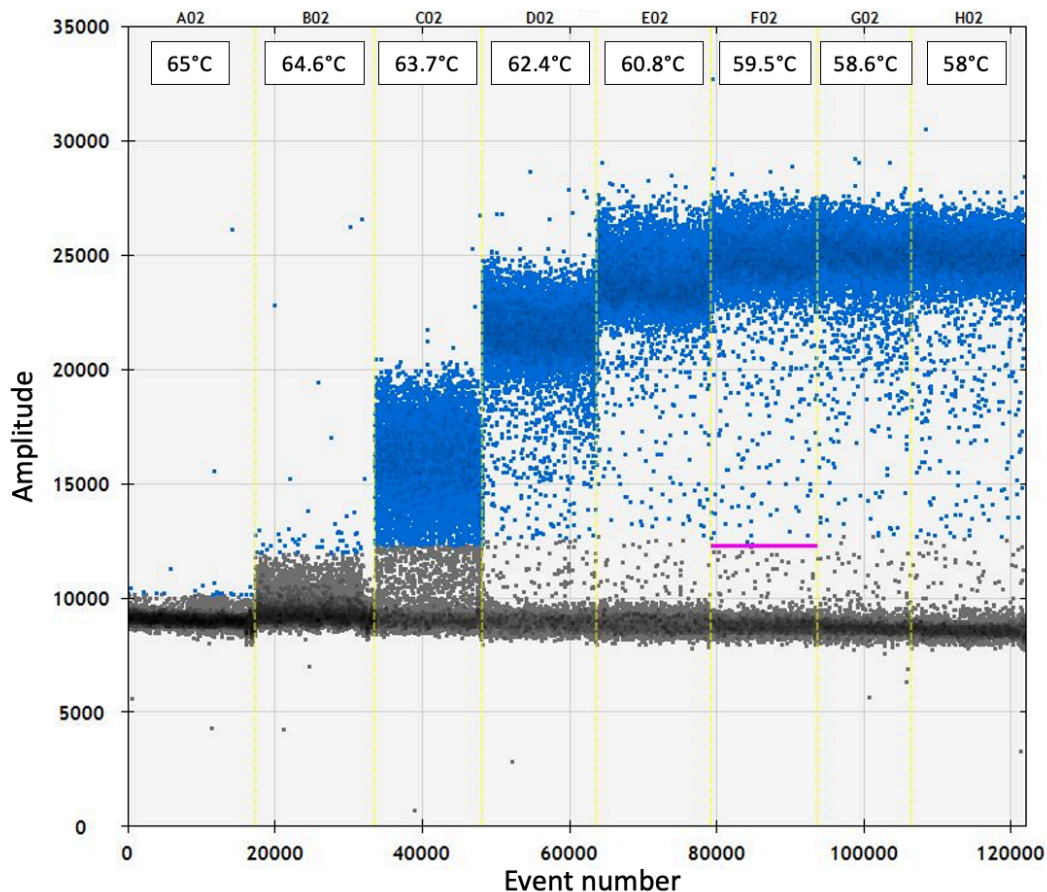

Fig S5 – dd-PCR output of the temperature gradient experiment (65°C - 58°C) on soil samples with *T. harzianum*: The cycling conditions used for this experiment were: enzyme activation at 95°C for 5 min, denaturation at 95°C for 30 sec, annealing/extension (40 cycles) at a gradient (65°C - 58°C) for 1 min, signal stabilisation at 4°C for 5 min, followed by 90°C for 5 min, hold at 4°C for infinity. For all cycling steps, a ramp rate of 2°C/sec was used. The EvaGreen® dye fluorescence amplitude of the amplified droplets is represented on the Y-axis. Blue dots represent positive droplets (containing target DNA), and grey dots represent negative droplets (no target DNA). X-axis represents the number of droplets number (event number). Annealing/extension temperature of 59.5°C (F02) resulted in the most optimal separation between negative and positive droplets and the least mid-amplitude droplets ("rain").
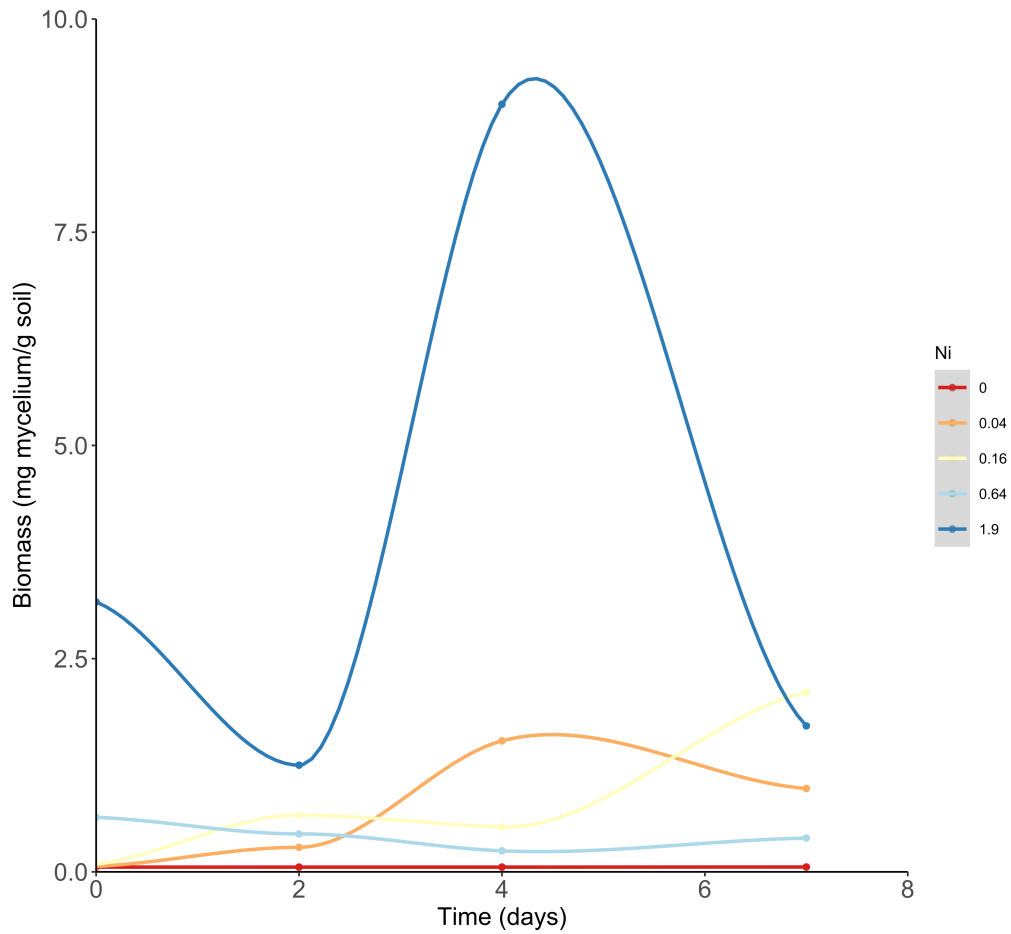
S6 – Growth dynamics of T. harzianum

Fig S6 – **Growth dynamics of *T. harzianum* when acclimated at 25°C for seven days on MMN-agar and exposed to 25°C in sterilised soil microcosms for seven days.** Y-axis: biomass in mg mycelium/g soil calculated using dd-PCR #ITS copies output. X-axis: time in days, points resemble the days at which the biomass was measured. The graph represents the results of 4 different initial abundances in mg mycelium/gram soil (Ni: 0.04; 0.16; 0.64; 1.9) and a negative control (Ni = 0).