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Growth Characteristics and Freezing Tolerance of Ectomycorrhizal and Saprotrophic Fungi: Responses to Normal and Freezing Temperatures

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Abstract: In boreal and temperate forests, symbiotroph and saprotroph soil fungi must survive months of low temperatures or freezing during winter. In the temperate biome, this is particularly the case for high-elevation mountain forests. Soil freezing is thus an important stress factor in these forests. The objective of this study was to assess how temperature and freezing conditions affect the growth and survival of symbiotic and saprotrophic fungi. To assess the cold and freezing tolerance of ectomycorrhizal (EM) and saprotrophic (SAP) fungi, we conducted a study from 2021 to 2023, using isolates from forests located at lower and high-elevation mountain sites, as well as from forests in Mongolia, at altitudes ranging from 525 m to 1800 m. The isolates were grown in vitro at temperatures of 22, 15, and 4 °C and exposed to freezing conditions at −4 or −18 °C. The response to temperature and freezing was determined based on radial growth. Triphenyltetrazolium chloride (TTC) reduction was used to measure relative metabolic activity and viability. Fungi that originated from higher-elevation mountain sites, and thus colder climate conditions, tended to have a lower response to temperature and a higher tolerance to freezing. We could find no evidence of a higher freezing tolerance among different exploration types of ectomycorrhizal fungi. Sensitivity to low temperatures appears to be taxa-specific rather than exploration-type-specific.

Keywords: ectomycorrhizal fungi; saprotrophic fungi; freezing tolerance; viability; growth rate; density; ergosterol

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1. Introduction

Fungi are fundamental to the health and function of forest ecosystems, where they drive processes such as nutrient cycling and organic matter decomposition [1]. Two key groups of fungi, ectomycorrhizal (EM) fungi and saprotrophic (SAP) fungi, dominate these ecosystems, each playing distinct but interconnected roles in sustaining forest productivity and ecological balance [2]. EM fungi form mutualistic associations with tree roots, enhancing plant nutrient acquisition, while SAP fungi specialize in breaking down complex organic matter and recycling carbon and other essential elements back into the soil [3,4]. Ectomycorrhizal-associated fungi, including some helotialean species identified as EM

fungi, likely play significant roles in forest ecosystems by influencing the growth and regeneration of host trees, though their precise ecological functions remain unclear [5]. Root endophytic fungi have been reported to enhance the tolerance of trees to extreme temperatures by maintaining root functionality through hormonal regulation and osmoprotectant production, offering critical support under climate-induced temperature fluctuations [6]. The extent to which ectomycorrhizas form an extramatrical mycelium is used to divide them into functional groups [7,8]. Agerer [7] defined five main exploration types, ranging from contact (C) exploration types with smooth mycorrhizal tips having only a few short emanating hyphae via short- (SD) and medium-distance (MD) exploration types, to long-distance (LD) exploration types with highly differentiated rhizomorphs [9]. Recently, Jörgensen et al. [10] suggested that EM that produce extensive extramatrical mycelium (MD and LD) do not have higher proliferation rates in soils, and thus higher growth rates, but may have a longer lifespan in soils. Longer lifespans are associated with more conservative and stress-resistant lifestyles, including freezing stress [11].

In temperate and boreal regions, soil temperatures can be below 0 °C during winter [12,13]. Prolonged freezing conditions can have a significant impact on whether EM and SAP fungi can survive or successfully reproduce [14]. Cold tolerance is thus a crucial adaptive trait for fungi inhabiting cold ecosystems [15]. The ability of fungi to withstand freezing and thawing cycles is essential not only for their survival but also for maintaining ecosystem functions during and after winter [13,16]. Strategies to avoid cold or freezing injury can involve both avoidance of freezing temperatures and cellular protection against chilling or freezing injury [17]. The cellular freezing tolerance of ectomycorrhizal fungi has been studied mostly in in vitro investigations using agar plates or liquid cultures. In many of the studies, tolerance to freezing has been estimated by measuring the resumption of growth after freezing. In one of the earliest studies, Moser [18] found that ectomycorrhizal fungi have the ability to survive long periods (up to 3 months) of freezing temperatures (−5 °C). Clear differences could be shown between different species, for example, whereas *Amanita muscaria* (MD) was very sensitive to freezing, *Suillus* species (LD), particularly *Suillus grevillei*, were resistant to freezing. Some other species, for example, *Paxillus involutus*, also appeared to be more resistant to freezing when the culture originated from a high-elevation source compared to a low-elevation source. This has led to the suggestion that fungi originating from colder environments are better adapted to freezing temperatures [12]. Lehto et al. [19] studied the survival and regrowth rate of four types of ectomycorrhizal fungi after freezing. They found that all isolates survived −8 °C, and a small percentage of the samples from the species *Laccaria laccata* and *Suillus variegatus* survived temperatures down to −48 °C. Ma et al. [20] exposed four different isolates of ectomycorrhizal fungi (*Cortinarius multiformis*, *Russula densifolia*, *Suillus granulatus*, and *Lactarius deliciosus*) to a series of freeze–thaw events at temperatures between +4 and −40 °C and measured their survival. They showed that under laboratory conditions, the lethal temperature for 50% of samples was between −7.6 and −13.7 °C. In SAP fungi, a threshold of about −17 °C was also shown as the point at which the metabolic activity of the fungi was lost after several weeks of freezing [21]. To the best of our knowledge, no investigations of freezing tolerance in relation to exploration type have been carried out; however, soil warming has been shown to influence the abundance of exploration types in forests. Mucha et al. [22] found that soil warming reduced C and MD exploration types, such as the *Russula* genus, *Tomentella* genus, and *Clavulina* genus. Similarly, Deslippe et al. [23] and Jarvis et al. [24] also reported a negative effect of warming on C, SD, and MD exploration types. These studies indicate that temperature sensitivity may differ between exploration types, although effects via the host tree may also be a factor.

One of the challenges in assessing fungal viability under freezing conditions is the lack of methods to evaluate both survival and metabolic damage or recovery. To assess the effects of freezing on root tissues, the methods of relative electrolyte leakage (REL) [25] and reduction of triphenyltetrazolium chloride (TTC) to triphenylformazan (TF) [26] have been used. The method of REL provides information on freezing-induced membrane damage but not metabolic activity, whereas the reduction of triphenyltetrazolium chloride (TTC) is directly related to the activity of the mitochondrial respiratory chain [26,27]. Ruf and Brunner [26] used TTC reduction to measure the vitality of fine roots of trees and showed that decreasing root vitality by adding Cd resulted in lower levels of TTC reduction. Recently, Reiter et al. [28] used TTC reduction to assess the viability of orchid seeds. TTC reduction has also been used to measure microbial metabolic activity [29] and to determine the activity of fungal dehydrogenase enzymes in soil [30].

A common method to measure fungal biomass is the determination of membrane lipid ergosterol [31,32]. For example, Sterkenburg et al. [33] and Hydbom et al. [34] used ergosterol as a marker to assess fungal biomass in forest soils. Ergosterol is considered to be unstable and in soils to degrade quickly and is thus used as a marker of living fungal biomass. However, there is evidence to suggest that when associated with dead fungal materials, ergosterol remains stable [35].

In this work, we assessed the growth of both EM and SAP fungi isolated from different forests. These include colder high-elevation forests to warmer low-elevation forests. We subjected these fungi to different temperatures to assess the growth response to low temperatures and survival at freezing temperatures. We tested Hypothesis 1, which states that fungi originating from colder environments would show greater tolerance to low and freezing temperatures than fungi originating from warmer environments. Based on the assumption that hyphae of LD and MD exploration types have a longer lifespan, we tested Hypothesis 2, which states that for EM, a greater tolerance to freezing would be shown in LD and MD exploration types compared to exploration types that form smaller amounts of extramatrical hyphae such as contact (C) exploration types. The objective of this study was to assess how temperature and freezing conditions affect the growth and survival of symbiotic and saprotrophic fungi. With this work, we attempted to gain insight into how winter conditions affect forest fungi.

2. Materials and Methods

2.1. Sources of Fungi

The ectomycorrhizal and saprotrophic fungi assessed in this study, conducted from 2021 to 2023, originated from various sources (Table 1). All of the ectomycorrhizal and wood-degrading saprotroph isolates were from mountain regions in Austria, Mongolia, or Slovenia. The other saprotrophs were spontaneous isolates from unsuccessful isolation attempts using ectomycorrhizal sporocarps from Austria or Mongolia. The sites were selected to provide temperature differences due to elevation and sites in Mongolia were selected due to extreme winter conditions. The collection sites in Austria, Stoderzinken, and Zirbitzkogel are at an elevation of 1800 m and have a forest cover dominated by Swiss stone pine (*Pinus cembra* L.). The annual mean temperature is about 4.4 °C and the annual mean precipitation ranges between 1247–1788 mm with approximately 30% falling as snow. Long-term measurements at a site close to Zirbitzkogel have shown that soil temperatures are around 0 °C in winter due to the isolating effect of the deep snow cover (Wang and Godbold, unpublished). The mean winter temperature is approximately 5.0 °C at the Stoderzinken [36]. The Nukht site at Bogd Khan protected area in Mongolia is at an elevation of 1500 m and the forest cover is dominated by Siberian pine (*Pinus sibirica* Du Tour) and Scots pine (*Pinus sylvestris* L.) [13].

Table 1. Description of the source sites of the fungal taxa or strains.

Source	Geographic Coordinates	Fungal Taxa	Guild	Dominant Tree Species	Soil
Zirbitzkogel, Austria, 1800 m	47°4′40″ N, 14°35′1″ E	*Cortinarius semisanguineus* (Fr.) Gillet.	EM	*Pinus cembra*	Histic Leptosol
		Inocybe cincinnata (Fr.) Quél.	EM		
		Lactarius porninsis Rolland	EM		
		Russula decolorans Fr.	EM		
		Suillus placidus (Bonord.) Singer	EM		
		Thelephoraceae Ehrh. ex Willd.	EM		
		Helotiales sp. *2 OV-2018* Nannf. ex Korf & Lizon	EM-A		
		Helotiales sp. Nannf. ex Korf & Lizon	EM-A		
		Eleutheromyces subulatus (Tode) Fuckel	SAP		
		Thysanophora penicillioides (Roum.) W.B. Kendr.	SAP		
		Trichoderma spirale 1 Bissett	SAP		
		Trichoderma spirale 2 Bissett	SAP		
		Trichoderma spirale 3 Bissett	SAP		
		Leptodontidium irregulare (de Hoog) de Hoog	ENDO		
		Cladosporium cladosporioides Fresen.	ENDO		
Stoderzinken Austria, 1700 m	47°27′33″ N, 13°49′17″ E	*Boletus edulis* Bull.	EM	*Pinus cembra*	Calcic Leptosol
		Cortinarius caperatus (Pers.) Fr.	EM		
		Valsa nivea Fabre	SAP/pathogen		
		Fomes fomentarius (L.) Fr.	Xylo-SAP		
		Gloeophyllum trabeum (Pers.) Murrill	Xylo-SAP		
		Trametes hirsuta (Wulfen) Pilat	Xylo-SAP		
Nukht, Bogd Khan protected area, Mongolia, 1500 m	47°52′19″ N, 106°55′58″ E	*Absidia* cf. *psychrophilia* Hesselt. & J.J. Ellis	SAP	*Pinus sibirica*, *Pinus sylvestris*	Cambisol
		Exophiala sp. J.W.Carmich.	SAP		
Postojna, Slovenia, 755 m	45°45′59″ N, 14°13′58″ E	*Melanogaster broomeanus* Berk.	EM	*Fagus sylvatica*, *Pinus sylvestris*	Shallow organic layer over clay soil on limestone
Gozd Martuljek, Slovenia, 755 m	46°16′39″ N, 13°59′29″ E	*Rhizopogon roseolus* (Corda) Th. Fr.	EM	*Picea abies*	Histic Fluvisol
Velike Lašče, Slovenia, 525 m	45°50′35″ N, 14°35′15″ E	*Amanita muscaria* (L.) Lam.	EM	*Pinus sylvestris*, *Quercus petraea*, *Fagus sylvatica*	Deep organic acidic soils on silicate sandstone
		Amanita rubescens (Pers. ex Fr.) Gray	EM		
		Boletus subtomentosus (L.) Quél.	EM		
		Laccaria bicolor (Maire) P.D. Orton	EM		
		Suillus granulatus (L.) Roussel	EM	*Quercus petraea* (Matt.) Liebl., *Carpinus betulus* L., *Corylus avellana* L., *Picea abies*	Shallow clay neutral soil with little organic matter on limestone
Eichenhain Nature Park, Austria, 500 m	48°16′57″ N, 16°13′34″ E	*Lactarius aurantiacus* (Pers.: Fr.) Gray	EM	*Fagus sylvatica*, *Quercus robur* L., *Pinus sylvestris*	Eutric Cambisols
		Hymenopellis aff. radicata R.H.Petersen	SAP		
		Penicillium eben-bitarianum Baghdadi	SAP		
		Hypholoma fasciculare (Huds.) P. Kumm.	SAP		
		Talaromyces variabilis (Sopp) Samson, Yilmaz, Frisvad & Seifert	SAP		

EM, ectomycorrhizal; EM-A, ectomycorrhizal associated; SAP, saprotrophic; Xylo-SAP, wood-associated saprotroph; and ENDO, endophytic.

The soils at Nukht Bogd Khan experience strong winter freezing down to −11.0 °C [13]. In lower-altitude areas (500–755 m), sporocarps were collected at Eichenhain Nature Park in the Viennese forest around Vienna, Austria. The forest is dominated by oak (*Quercus petrea* L.) and beech (*Fagus sylvatica* L.). The isolates from Slovenia originate from a collection of the Slovenia Forestry Institute. The original fungi were collected as sporocarps from Velike Lašče, Velike Lašče–Kamen Vrh, Postojna, and Gozd Martuljek in Slovenia. These lower-elevation (525–755 m) forests are dominated by oak (*Quercus petrea*), beech (*Fagus sylvatica*), Norway spruce (*Picea abies* L. Karst.), or Scots pine (*Pinus sylvestris*). In Slovenia,

the average minimum soil temperature is 3.5 °C during the winter months [37,38], and the sites sampled are characterized by high snow cover during winter.

2.2. Sample Preparation and Fungal Identification

The Austrian isolates were obtained from sporocarps collected in the forests investigated. The sporocarps collected were transported in a cool box and isolated within 24 h of collection. For isolation, 3–5 mm blocks of fungal tissue were taken from the cap and stipe, and the surfaces were sterilized in hydrogen peroxide (3%) for 10 s and placed on modified Melin–Norkrans (MMN) agar culture medium in Petri dishes (9 cm diameter). The MMN medium contained 10 g L^{-1} glucose, 3 g L^{-1} malt extract, 1.9 mM $(NH4)_2SO_4$, 3.7 mM KH_2PO_4, 0.6 mM $MgSO_4$, 0.5 mM $CaCl_2$, 0.4 mM NaCl, 6.0 mM $FeCl_3$, and 10 µg L^{-1} thiamine [29]. The medium was set using 2% agar and was adjusted to pH 5.6 before sterilization. The medium was sterilized at 121 °C for 15 min. $FeCl_3$ and thiamine were sterile-filtered and added to the medium after it cooled to 70 °C. The cultures were grown for 15 days at 22 °C and the resulting fungal isolates were transferred to fresh MMN agar plates.

The sporocarps were identified initially based on morphology and the isolates were further identified using DNA sequences. Details of the identification are provided in Supplementary Materials.

2.3. Determination of Radial Growth Rate

From the stock cultures, a plug of hyphal tissue (6 mm in diameter) was placed on a fresh MMN plate and grown for 15 days at 22 °C. After this period, from the growing edge of the hyphal mat, a 6 mm hyphal plug was taken and transferred to new MMN agar plates. The fungi were then grown at 22, 15, or 4 °C for up to 100 days. For the freezing temperatures, the fungi were first grown at 22 °C to a point at which the fungi were actively growing and then placed in a freezer at either −4 or −18 °C for a duration of two months. Five replicates were used for each fungal taxa and temperature.

For the 22, 15, or 4 °C treatments, the radius of the colonies was measured every 5 days until the entire surface of the MMN agar plates was completely covered by the mycelium mat. For the fungi exposed to −4 and −18 °C, the colony radius was measured before and after freezing. The radial growth rate (cm day^{-1}) was calculated from the slope of the radius increase over time. The fungal biomass was determined by taking 6 mm diameter plugs from the fungal mat with the agar attached, and a further plug of agar only, using 3 replicates. The plugs were placed on a cellulose acetate membrane and dried at 105 °C for 24 h and then weighed. The fungal biomass was calculated as the difference between the means of the fungi plus agar and agar-only plugs. The density of the fungal mat was calculated by dividing the area of the plug by the dry weight.

2.4. Determination of the Hyphal Mat Thickness

To determine the thickness of the hyphal mat, plugs were taken from *Boletus subtomentosus* and *Amanita muscaria*, both of which form a solid hyphal mat, and the thickness was measured using an AxioCam ERc5s microscope (ZEISS AG, Oberkochen Germany) equipped with an eyepiece graticule. Observations were made under 10x magnification.

2.5. Triphenyltetrazolium Chloride (TTC) Reduction

For the TTC test [26], from three of the five replicates, two 6 mm diameter plugs were taken from each plate and transferred into microtubes (2 mL) using sterile transfer pipettes. In each tube, 1.5 mL of a solution consisting of 0.6% TTC and 0.05% Tween 20 in 0.1 M potassium phosphate buffer (pH 7.0) was added, followed by subjecting the samples to vacuum for 10 min to eliminate air from intercellular spaces and facilitate the infiltration of

the TTC solution into the tissue. The samples were then incubated for 24 h at 30 °C. After incubation, the TTC solution was removed. After the addition of 1.5 mL of 90% ethanol and 2 g of glass beads (2 mm in diameter), the samples were ground at 6.0 m s^{-1} speed for 1 min in a FastPrep-24™ 5G bead beating grinder (MP Biomedicals, Santa Ana, CA, USA). Following cell disruption, the reaction tubes were centrifuged at 10,000× *g* for a period of 2 min, 0.3 mL of the supernatant was removed, and absorbance at a wavelength of 520 nm was determined in an xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). Blanks were prepared by placing a set of tubes with fungi into boiling water for 20 min, and then TTC reduction was determined as above.

The reduction of TTC was calculated from the absorption of triphenylformazan (TF) produced per gram of dry weight, and also per cm^{-2} of surface area of the fungal mat. To convert the absorbance values to TF, the absorbance values were multiplied by a factor of 0.1024 and the blank was subtracted. The conversion factor was derived from a linear calibration curve of TF used by Ruf and Brunner [26]. The values obtained are in mM TF cm^{-2} or mM TF g dwt^{-1}.

As the dry weight and thickness of the hyphal mat were found to increase with temperature, this increase was partially corrected by calculation of the ratio between the measured TTC value and an expected value. The expected values were calculated from the TTC reduction value at 22 °C and multiplied by the quotient of the dry weight of the hyphal mat at the target temperature divided by the dry weight at 22 °C.

2.6. Determination of Ergosterol

The total amount of ergosterol was measured after culture at 22 °C and −18 °C. From two of the five replicates, four 6 mm diameter plugs were taken from each plate and placed into 2 mL microtubes using sterile transfer pipettes. After the addition of 1 mL of 10% (*w*/*v*) KOH dissolved in methanol and 2 g of glass beads (2 mm in diameter), the samples were ground at 6.0 m s^{-1} speed for 1 min using a FastPrep-24™ 5G bead beater. After cell disruption, the contents were transferred to new glass tubes, where an additional 4 mL of 10% KOH in methanol was added. The samples underwent 15 min of sonication and were vortexed for 5 s. The samples were heated for 60 min at 70 °C. After the samples had cooled to room temperature, 1 mL of deionized water and 2 mL of cyclohexane were added. The samples were centrifuged at 3000 rpm for 5 min, and the hydrophobic phase was transferred to clean tubes. The samples were washed with 2 mL cyclohexane, and the two cyclohexane phases were combined and evaporated under nitrogen at 40 °C. The residues were dissolved in 400 µL of methanol, filtered through a 0.45 µm syringe filter, and subsequently analyzed using a Pharmacia HPLC system equipped with UV detection for the conjugated double bond [31,39]. The agar media used were also analyzed and were found to not contain ergosterol. The ergosterol contents were expressed based on dry weight and surface hyphal mat surface area.

2.7. Statistical Analyses

To investigate the radial growth rate of fungi at different temperatures, linear regression analysis was conducted with the days of fungal cultivation as the explanatory variable. The analysis was carried out using the SigmaPlot software, version 14.0. Tests and correlations were performed using the SPSS statistical software package (Version 22.0, SPPS Inc., Chicago, IL, USA). For the TTC test and density, significant differences between the treatments for each fungus were tested by one-way analysis of variance (ANOVA, Duncan's test, $p < 0.05$). The Pearson correlation and Spearman rank correlation test were computed to assess the relationships between radial growth rate, TTC test density, and different temperatures of fungal cultivation ($p < 0.05$, $p < 0.01$, and $p < 0.001$).

3. Results

3.1. Fungal Radial Growth Rate at 22 °C

A total of 35 fungal taxa strains were screened, of which 16 were ectomycorrhizal (EM) fungi and 15 were saprotrophic (SAP) fungi, of which three were wood-degrading saprotrophs, two were ectomycorrhizal-associated (EM-A) fungi, and two were endophytic (ENDO) fungi (Table 1). Ectomycorrhizal fungi were further classified into exploration types [40], of which five were contact, one was short-distance, three were medium-distance, and six were long-distance types. Two of the fungi screened belong to *Helotiales*, some of which have been reported to form EM fungi [41,42]. In this work, we placed *Helotiales* in the ectomycorrhizal-associated (EM-A) group.

Within the SAP, *Talaromyces variabilis* had the fastest observed radial growth rate at 22 °C (1.20 cm day^{-1}), followed by *Hymenopellis aff. radicata* (0.65 cm day^{-1}). In contrast, in the EM group, the fastest growth rate was shown by *Amanita muscaria*, but was only 0.55 cm day^{-1} (Figure 1). Within the exploration types, considerable variation was shown. Between the two groups' contact and long-distance exploration types, the mean maximum growth rate for all taxa did not differ significantly. In the endophytic group, the maximum growth rate was less than 0.15 cm day^{-1}.

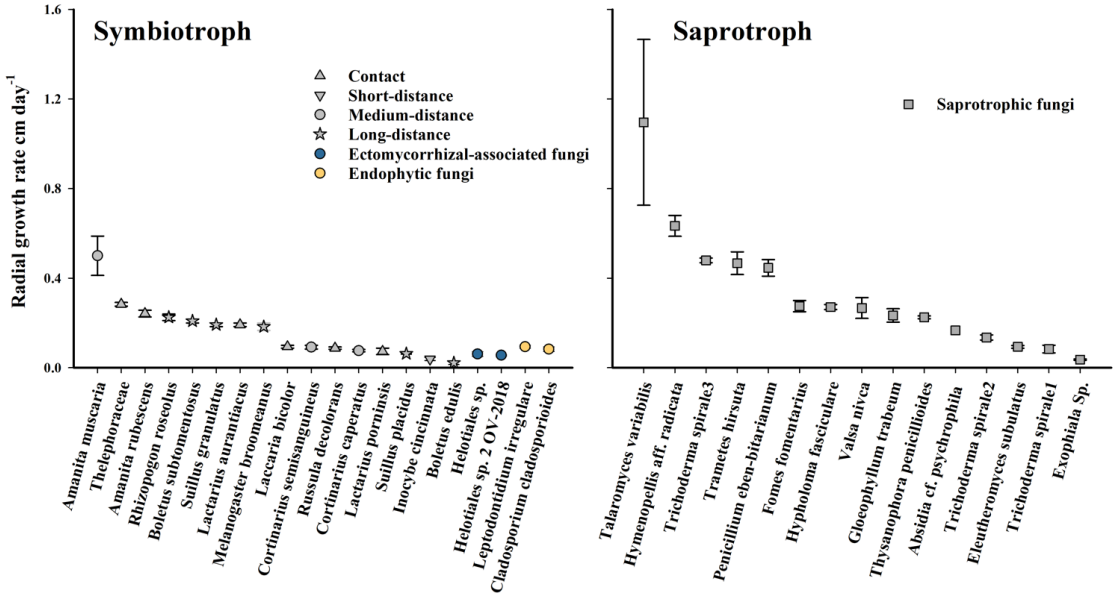

Figure 1. Maximum values of radial growth rate (cm day^{-1}) at 22 °C for ectomycorrhizal fungi (including contact, short-distance, medium-distance, and long-distance exploration types), ectomycorrhizal-associated fungi, endophyte fungi, and saprotrophic fungi. Bars show the standard error.

In the ectomycorrhizal fungi, the taxa with the fastest growth rates (*Amanita muscaria* to *Melanogaster broomeanus*) were primarily collected from the lower-elevation sites in Slovenia (Table 1), whereas those with the slower growth rates (*Russula decolorans* to *Boletus edulis*) were primarily from the high-elevation sites, and often associated with *Pinus cembra*. For the saprotrophic fungi, again, three of the five fastest-growing fungi at 22 °C (*Talaromyces variabilis*, *Hymenopellis aff. Radicata*, *Penicillium eben-bitarianum*) were from the lower-elevation Eichenhain Nature Park site. The slowest-growing taxa, *Exophilia* sp. originated from Mongolia.

3.2. Effect of Lower Temperatures on Radial Growth Rate

To determine the effect of the temperature on the radial growth rate, individually for each fungal isolate, a linear regression analysis was performed (Table S2). Generally, the

radial growth expansion decreased with time, except for the two taxa with the highest growth rates *Amanita muscaria* and *Talaromyces variabilis*, which showed a constant increase in growth rate over time, until the edge of the Petri dish was reached. The equation of linear regression models generally indicated a negative correlation between radial growth rate and incubation time at 22, 15, and 4 °C.

The EM taxa (Figure 2a) with the highest growth rates at 22 °C showed the greatest temperature dependency. *Amanita muscaria*, *Boletus edulis*, *Thelephoraceae*, *Amanita rubescens*, *Lactarius aurantiacus*, *Boletus subtomentosus*, *Melanogaster broomeanus*, *Rhizopogon roseolus*, and *Suillus granulatus* all showed a lower growth rate at 15 and 4 °C. In contrast to all the other EM fungi, *Helotiales* sp. and *Cortinarius semisanguineus* from the higher-elevation Zirbitzkogel site exhibited the fastest growth rate at 15 °C (Figure 2a). The two taxa from the genus *Cortinarius* did not grow at 4 °C, whereas the remainder of the EM taxa grew at 4 °C. *Amanita rubescens*, *Laccaria bicolor*, *Lactarius aurantiacus*, *Russula decolorans*, *Boletus subtomentosus*, *Melanogaster broomeanus*, *Rhizopogon roseolus*, *Suillus granulatus*, and *Suillus placidus* continued to grow at 4 °C at a slow, but constant, growth rate for periods exceeding 60 days. In ENDO taxa, the radial growth rate of *Cladosporium cladosporioides* showed no temperature dependency over an 80-day growth period.

A similar growth pattern at the different temperatures was shown for the SAP taxa investigated (Figure 2b). *Trametes hirsute*, *Hymenopellis aff. Radicata*, *Talaromyces variabilis*, and *Eleutheromyces subulatus* all had rapid growth rates at 22 °C. There were, however, some clear differences in the effect of temperature. The growth of *Absidia* cf. *psychrophila* and *Exophiala* sp. was less affected by temperature compared to the other taxa and had similar growth rates at 22 and 15 °C, but also grew at 4 °C. Both of these fungi were isolates collected in Mongolia. The growth of the taxa *Valsa nivea* and *Gloeophyllum trabeum* was completely suppressed at 4 °C. In contrast, unlike all other taxa, *Talaromyces variabilis* and *Hymenopellis aff. radicata* showed at 4 °C an increase in radial growth rate with increasing incubation time. Neither for ectomycorrhizal fungi nor saprotrophic fungi did any radial growth occur at −4 and −18 °C.

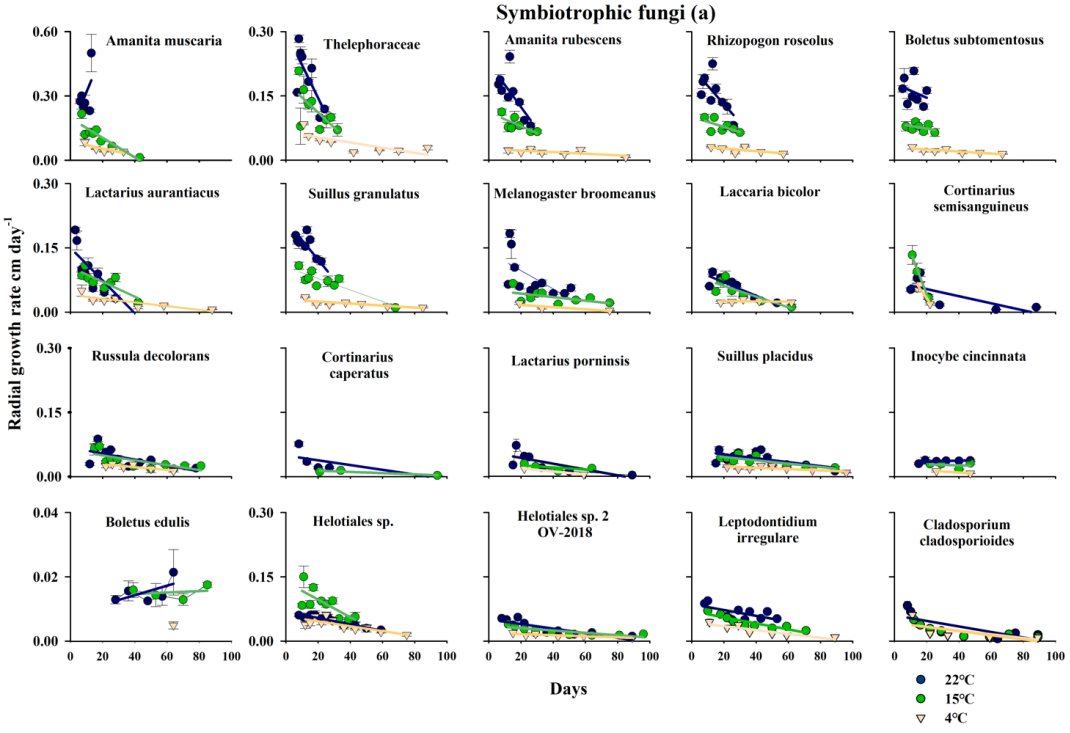

Figure 2. *Cont.*

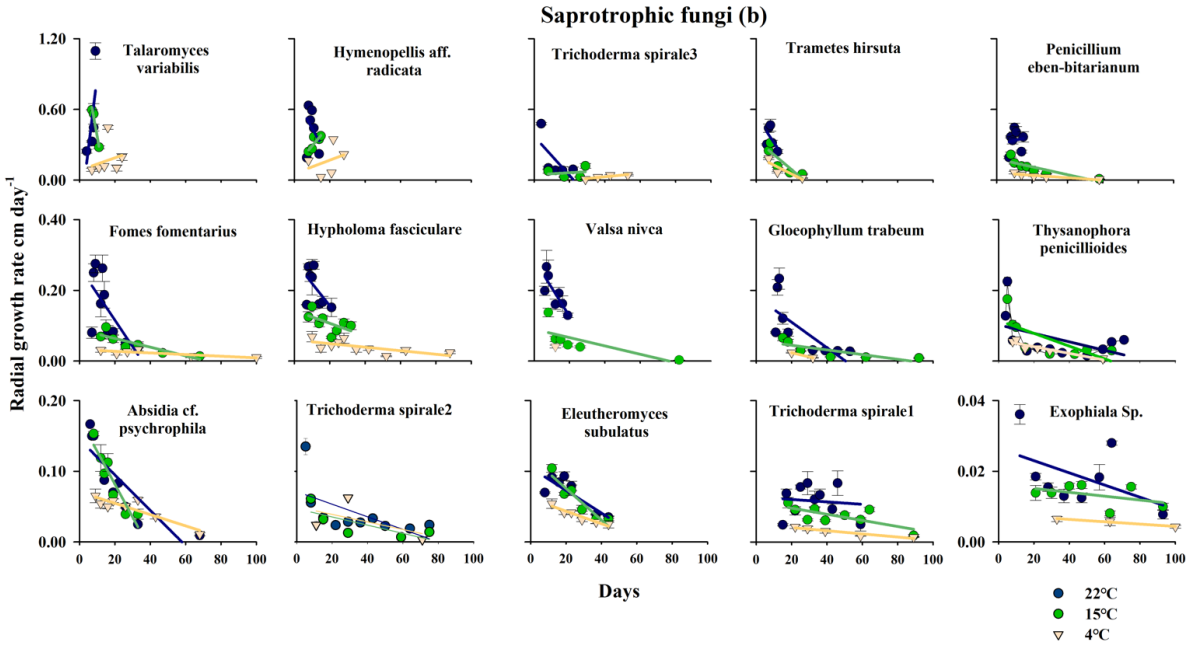

Figure 2. Linear relationships between the radial growth rate (cm day^{-1}) of symbiotrophic fungi (**a**) and saprotrophic fungi (**b**) and the duration of exposure to distinct temperatures over a time span of up to 100 days are depicted by solid lines. The error bars represent the standard errors of the mean values of radial growth rate (cm day^{-1}).

3.3. Effect of Temperature on Density and Thickness of the Hyphal Mat

For all fungi tested, there was a trend that the weight of the hyphal mat growing on the agar measured as weight per surface area (mg cm^{-2}) increased as the growth temperature decreased (Table 2), even at −4 and −18 °C. For some fungi, there was a highly significant correlation between the density of the hyphal mat and temperature over the temperature range of 22 to 4 °C. This was shown in the ectomycorrhizas for both *Amanita* taxa, *Boletus subtomentosa*, *Inocybe cincinnata*, *Laccaria bicolor*, *Melanogaster broomeanus*, and one *Helotiales* sp. *2 OV-2018* taxa. In the saprotrophs, this was seen for *Hymenopellis aff. Radicata*, *Penicillium eben-bitarianum*, and *Exophilia* sp., and the wood-degrading fungi *Trametes hirsute* and *Fomes fomentarius*. For these fungi, the hyphal mat density was significantly different between each temperature treatment. The fungi with a significant increase in density with temperature tended to be the fungi with a low density at 22 °C and thus an initially thinner hyphal mat. This phenomenon was rarely shown in the fungi with a higher density at 22 °C, and thus a thicker hyphal mat.

Table 2. The density (mg cm^{-2}) of symbiotrophic fungi (a) and saprotrophic fungi (b) is shown as the mean ± standard error at different temperatures. CDGR represents Spearman's rank correlation coefficient between density and radial growth rate (cm day^{-1}) for each fungal taxon for 22 to 4 °C. The significance levels for Spearman correlation analysis were $p < 0.05$ * and $p < 0.01$ **, respectively.

(a) Symbiotrophic Fungi	22 °C mg cm^{-2}	15 °C mg cm^{-2}	4 °C mg cm^{-2}	−4 °C mg cm^{-2}	−18 °C mg cm^{-2}	CDGR
Amanita muscaria	0.30 ± 0.14	1.67 ± 0.14	2.84 ± 0.05	7.05 ± 0.13	8.50 ± 0.11	−0.91 **
Thelephoraceae	0.37 ± 0.14	0.64 ± 0.23	0.73 ± 0.30	6.74 ± 1.71	8.52 ± 0.73	−0.32
Amanita rubescens	0.36 ± 0.31	1.34 ± 0.24	2.96 ± 0.42	5.96 ± 0.63	10.22 ± 0.37	−0.88 **
Rhizopogon roseolus	0.76 ± 0.26	0.86 ± 0.29	2.73 ± 0.19	7.40 ± 0.42	10.11 ± 0.67	−0.52
Boletus subtomentosus	0.43 ± 0.36	2.04 ± 0.28	3.33 ± 1.04	6.28 ± 0.43	8.92 ± 0.09	−0.81 **
Lactarius aurantiacus	2.45 ± 0.16	2.49 ± 0.73	2.88 ± 0.41	8.85 ± 0.71	51.93 ± 14.95	−0.24
Suillus granulatus	1.70 ± 0.66	2.39 ± 0.28	3.29 ± 0.34	6.75 ± 0.56	10.46 ± 0.77	−0.47
Melanogaster broomeanus	0.84 ± 0.26	1.27 ± 0.07	3.78 ± 0.19	5.74 ± 0.19	8.81 ± 0.67	−0.82 **

Table 2. *Cont.*

Laccaria bicolor	0.38 ± 0.00	1.72 ± 0.85	1.68 ± 0.49	6.83 ± 0.51	10.32 ± 0.27	−0.77 *
Cortinarius semisanguineus	1.42 ± 0.37	2.39 ± 0.21	2.60 ± 0.99	10.05 ± 0.39	11.95 ± 0.60	0.32
Russula decolorans	1.15 ± 0.15	1.16 ± 0.00	1.74 ± 0.32	8.64 ± 0.88	10.70 ± 0.32	−0.24
Cortinarius caperatus	1.30 ± 0.00	4.79 ± 0.00	6.16 ± 0.00	9.44 ± 0.91	8.46 ± 0.00	0.57
Lactarius porninsis	3.52 ± 0.00	3.65 ± 0.33	6.02 ± 0.00	8.13 ± 0.33	11.04 ± 0.18	−0.52 *
Suillus placidus	1.36 ± 0.80	1.31 ± 0.48	2.04 ± 0.65	9.42 ± 0.20	10.73 ± 0.35	−0.30
Inocybe cincinnata	0.98 ± 0.03	2.50 ± 0.58	2.54 ± 0.52	5.82 ± 0.80	7.79 ± 0.53	−0.68 *
Boletus edulis	3.13 ± 0.68	3.41 ± 0.63	3.94 ± 1.90	6.87 ± 0.68	9.47 ± 0.59	0.25
Helotiales sp.	0.80 ± 0.28	1.21 ± 0.10	1.27 ± 0.06	6.13 ± 0.19	8.86 ± 0.54	−0.12
Helotiales sp. *2 OV-2018*	1.14 ± 0.00	2.75 ± 0.00	3.28 ± 0.14	12.22 ± 0.70	13.86 ± 0.78	−0.89 **
Cladosporium cladosporioides	0.70 ± 0.22	0.88 ± 0.48	0.90 ± 0.15	10.08 ± 0.16	15.79 ± 1.08	−0.50 *
Leptodontidium irregulare	0.61 ± 0.34	0.71 ± 0.03	0.73 ± 0.09	5.98 ± 0.38	11.27 ± 0.15	−0.53 *
(b) Saprotrophic Fungi	**22 °C mg cm^{-2}**	**15 °C mg cm^{-2}**	**4 °C mg cm^{-2}**	**−4 °C mg cm^{-2}**	**−18 °C mg cm^{-2}**	**CDGR**
Talaromyces variabilis	0.38 ± 0.03	3.02 ± 0.00	4.89 ± 1.32	9.44 ± 0.92	12.00 ± 0.67	−0.55
Hymenopellis aff. radicata	0.20 ± 0.18	0.56 ± 0.00	1.35 ± 0.16	9.34 ± 0.67	9.44 ± 0.23	−0.82 **
Trichoderma spirale3	0.15 ± 0.02	0.31 ± 0.06	0.33 ± 0.14	4.57 ± 0.63	8.74 ± 0.22	−0.04
Trametes hirsuta	0.24 ± 0.03	1.37 ± 0.01	1.45 ± 0.06	8.90 ± 0.50	7.13 ± 0.70	−0.76 *
Penicillium eben-bitarianum	0.13 ± 0.05	0.48 ± 0.11	0.85 ± 0.31	10.39 ± 0.00	7.61 ± 0.00	−0.70 *
Fomes fomentarius	0.46 ± 0.26	0.78 ± 0.25	3.75 ± 0.54	5.46 ± 0.41	8.48 ± 0.39	−0.75 *
Hypholoma fasciculare	1.33 ± 0.19	1.38 ± 0.06	2.61 ± 0.00	7.02 ± 0.12	6.48 ± 0.36	−0.53
Valsa nivca	0.28 ± 0.17	1.40 ± 0.01	3.36 ± 0.00	5.84 ± 0.92	8.84 ± 0.16	−0.49
Gloeophyllum trabeum	1.62 ± 0.65	1.68 ± 0.42	1.80 ± 0.00	5.76 ± 0.45	38.67 ± 29.41	0.02
Thysanophora penicillioides	1.17 ± 0.04	1.22 ± 0.29	1.89 ± 0.57	5.98 ± 2.96	7.14 ± 0.30	−0.66 *
Absidia cf. *psychrophila*	0.46 ± 0.00	0.77 ± 0.00	0.99 ± 0.30	14.84 ± 0.97	9.69 ± 0.29	−0.17
Trichoderma spirale2	1.88 ± 0.20	2.77 ± 0.40	4.88 ± 0.39	7.89 ± 0.44	8.66 ± 0.71	−0.72 **
Eleutheromyces subulatus	2.11 ± 0.87	2.57 ± 0.77	2.83 ± 0.46	8.64 ± 0.42	12.37 ± 0.18	0.30
Trichoderma spirale1	1.96 ± 0.18	3.77 ± 0.15	4.29 ± 0.35	6.71 ± 0.20	9.66 ± 0.56	−0.86 **
Exophiala sp.	3.00 ± 1.11	3.89 ± 1.29	10.65 ± 0.30	7.74 ± 0.40	14.56 ± 1.26	−0.75 *

3.4. Effect of Temperature on TTC Reduction

After correction for the changes in dry weight using the observed and expected ratio of TTC reduction (Figure 3a), in most of the symbiotrophic taxa the TTC reduction was not significantly different over the temperature range 22 to 4 °C, with the exceptions of *Amanita muscaria*, *Amanita rubescens*, *Thelephoraceae*, and *Boletus subtomentosa*. In *Amanita muscaria*, *Amanita rubescens*, and *Boletus subtomentosa* taxa, TTC reduction was lower at 15 and 4 °C than at 22 °C. In *Thelephoraceae*, TTC reduction increased as temperature increased. All taxa showed low TTC reduction at −4 and −18 °C, with the exception of *Lactarius porninsis*.

A generally similar pattern was shown in the saprotrophic fungi (Figure 3b), where for most of the taxa, the TTC reduction was not significantly different over the temperature range 22 to 4 °C. Exceptions were *Talaromyces variabilis*, *Hymenopellis aff. radicata*, and *Valsa nivea*, which showed lower TTC reduction at 15 and 4 °C than at 22 °C; *Fomes formentarius* and *Exophilia* spp., which showed lower TTC reduction only at 4 °C; and *Gloeophyllum trabeum*, which had higher TTC reduction at 4 °C. All saprotrophic taxa showed low TTC reduction at −4 and −18 °C.

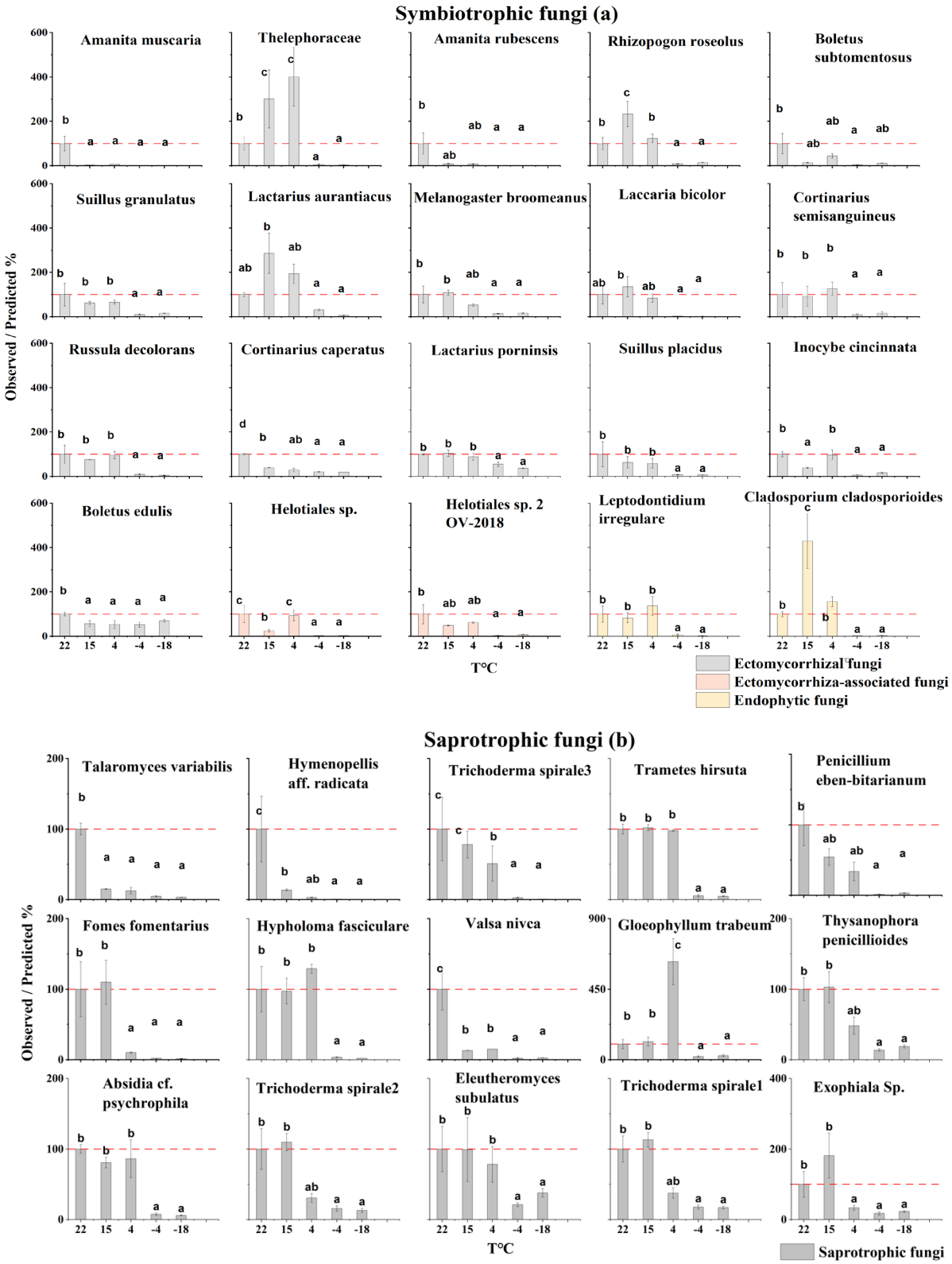

Figure 3. Observed/predicted percentage (%) of triphenyltetrazolium chloride (TTC) reduction by symbiotrophic fungi (**a**) and saprotrophic fungi (**b**) after exposure to different temperatures. Different letters represent a statistically significant difference ($p < 0.05$). The error bars represent the standard errors of the mean values. The dashed red line shows 100%.

3.5. Effects of Fungal Sources on Density, Radial Growth Rate, and TTC Reduction

In all plots, PC1 explained a significant proportion of the variance, ranging from 74.4% to 85.4%, while PC2 accounts for smaller but relevant variability, ranging from 13.0% to 17.3%. Symbiotrophic fungi (Figure 4a,b) showed tighter clustering at lower temperatures (4 °C and −18 °C), whereas saprotrophic fungi (Figure 4c,d) exhibited more dispersed patterns, suggesting a greater variation in response to temperature. Similarly, the different locations exhibited variations in patterns of response to temperature. Tighter clustering was shown for Stoderzinken and Eichenhain, but no clear separation was shown for any of the source sites.

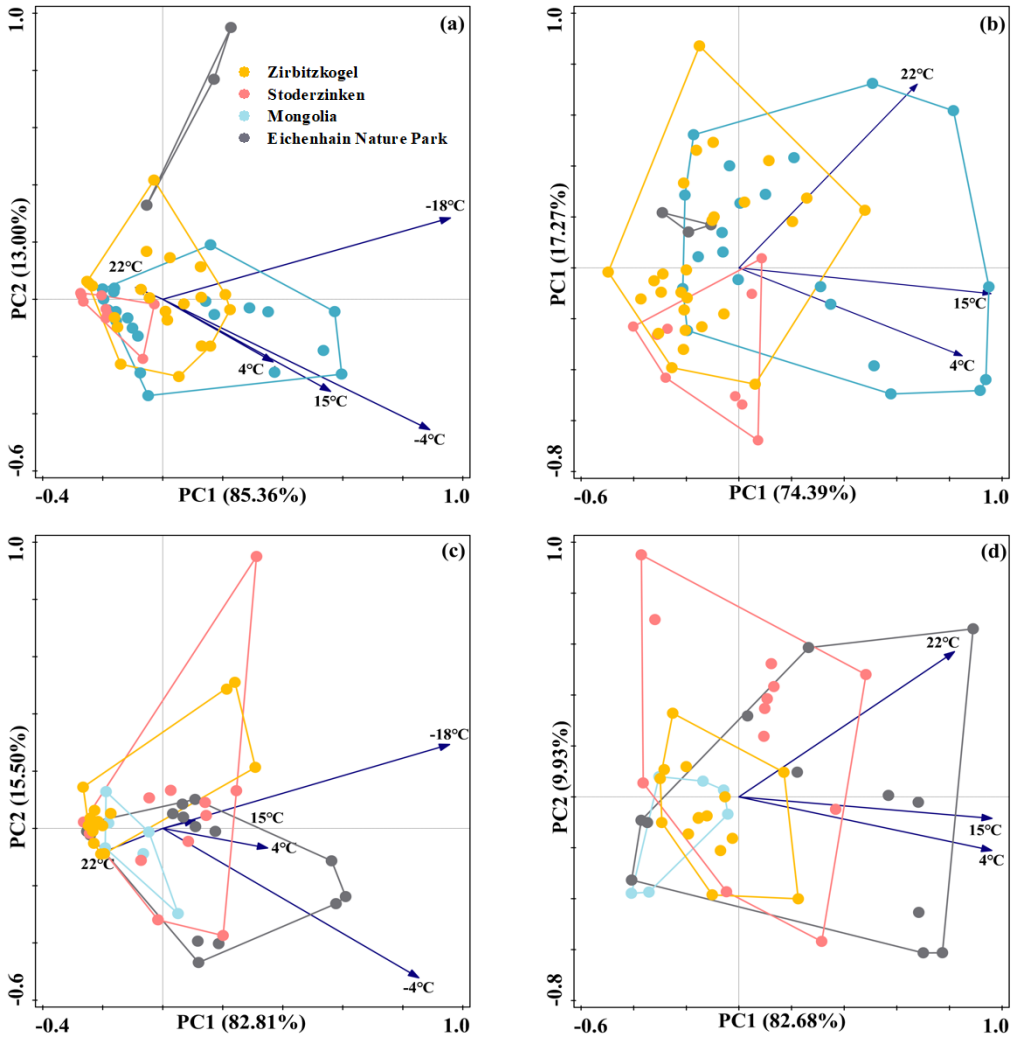

Figure 4. Principal component analysis (PCA) of the ratio of density to observed/predicted percentage (**a**,**c**) and the ratio of radial growth rate to observed/predicted percentage (**b**,**d**) for symbiotrophic fungi (**a**,**b**) and saprotrophic fungi (**c**,**d**) at different temperatures, with fungal sources from Zirbitzkogel, Stoderzinken, Slovenia, Mongolia, and Eichenhain Nature Park.

3.6. Ergosterol Concentration and Thickness of EM Fungal Mycelium

The ergosterol concentrations, measured as mg g^{-1} (per unit of biomass) and mg cm^{-2} (per unit of colony area), revealed significant temperature-dependent variations across fungal taxa (Table 3). The taxa were divided into two groups: those with relatively high ergosterol concentration and those with a relatively low ergosterol concentration. At 22 °C, the highest ergosterol concentrations based on dry weight (mg g^{-1}) or surface area (mg cm^{-2}) were determined in *Rhizopogon roseolus*, *Melanogaster broomeanus*, *Suillus granulatus*, *Amanita muscaria*, and *Helotiales*. All the other taxa had a 10-fold lower

ergosterol concentration. In the taxa with a high ergosterol concentration, at −18 °C, most species demonstrated a reduced ergosterol concentration on a dry weight basis (mg g^{-1}), but an increased ergosterol concentration expressed on an area basis (mg cm^{-2}). An exception was *Suillus granulatus*, where using both measures, the ergosterol concentration was lower at −18 °C.

Table 3. Ergosterol concentration expressed on a hyphal dry weight basis (mg g^{-1}) or a hyphal mat area basis (mg cm^{-2}) of ectomycorrhizal taxa growth at 22 °C or −18 °C. The means of two replicates are shown.

Fungal Taxa	Ergosterol mg g^{-1}		Ergosterol mg cm^{-2}	
	22 °C	**−18 °C**	**22 °C**	**−18 °C**
Amanita muscaria	8.75	0.87	6.25	7.36
Boletus edulis	0.96	0.11	0.51	1.07
Cortinarius semisanguineus	0.08	0.72	0.21	11.41
Helotiales sp.	7.64	0.82	6.14	7.28
Inocybe cincinnata	0.69	1.75	0.67	13.64
Lactarius aurantiacus	0.11	0.11	0.25	5.85
Melanogaster broomeanus	14.92	1.08	6.57	9.56
Rhizopogon roseolus	31.38	2.25	15.31	22.74
Russula decolorans	0.11	1.16	0.12	12.36
Suillus granulatus	12.79	0.41	11.42	4.25
Suillus placidus	0.53	0.93	0.28	10.02

For fungal mycelium thickness measurements, we examined *Amanita muscaria* and *Boletus subtomentosus*. The mycelium thickness of *Amanita muscaria* increased from 0.19 cm at 22 °C to 0.25 cm at −18 °C, and in *Boletus subtomentosus* it increased from 0.14 cm at 22 °C to 0.33 cm at −18 °C.

4. Discussion

On the MMN medium, the radial growth of the saprotrophs was faster than that of the EM fungi, despite the fact that the MMN medium is composed of the culture of EM fungi [43]. Within the different groups of fungi investigated, there were considerable differences in radial growth rate, but with no clear patterns by division or lifestyle. Among the saprotrophic fungi, there were both fast- and slow-growing ascomycetes or basidiomycetes. Among the Penicillium fungi, there were also both fast- (*Talaromyces variabilis*) and slower-growing (*Thysanophora penicillioides*) taxa.

Ectomycorrhizal fungi are commonly grouped using exploration type as a trait [7]. Among the exploration types, particularly the medium fringe and long-distance exploration types have been associated with the ability to access organic substrates [40], and thus attributed to saprotrophic properties. However, between contact and long-distance fungi, there were no differences in growth rate, with both faster- and slower-growing taxa within these groups. Saprotrophic properties determined as the ability to express enzymes involved in the degradation of complex organic substrates have been shown for a range of ectomycorrhizal fungal taxa including *Laccaria bicolor* and some *Cortinarius* species [44]. However, as the primary C source in MMN medium is glucose and maltose, the growth rates of both the ectomycorrhizal and saprotrophic fungi probably reflect the ability to utilize these substrates, rather than a measure of saprotrophy. For example, the genus *Amanita* [45] does not have the genes associated with enzymes for cell wall degradation, but the genes for enzymes such as glucosidase, are involved in cleavage of maltose. *Laccaria bicolor* lacks carbohydrate-active enzymes required for breaking down plant cell walls and retains the capacity to degrade simple polysaccharides [46,47].

Between the ectomycorrhizal and saprotrophic fungi, there were differences in response to temperature. The faster-growing ectomycorrhizal fungi showed clear differentiation in growth rates between the temperatures, this was not shown in the slower-growing taxa. Ectomycorrhizal fungi have been suggested to be highly sensitive to temperature [48,49], and even small changes may affect metabolic activity. TTC reduction as a measure of metabolic activity was shown in most taxa with the range 22 to 4 °C; however, the considerable loss of metabolic activity was shown in the faster-growing taxa *Amanita muscaria*, *Amanita rubescens*, and *Boletus subtomentosa* at 15 and 4 °C. This was also shown in the faster-growing saprotrophic fungi *Talaromyces variabilis*, *Hymenopellis aff. radicata*, and *Valsa nivea*. The radial growth of all these taxa also greatly decreased at lower temperatures. The slower-growing ectomycorrhizal taxa grew continually at 4 °C but did not increase growth rates at higher temperatures, and the metabolic activity measured by TTC reduction was unchanged. There was, however, not such a clear pattern in the saprotrophic fungi.

Although based on PCA there was no complete separation of the fungi by origin, fungi originating from the higher-elevation sites tended to show a slower growth rate. Based on the regrowth of fungi after freezing, Moser showed a higher tolerance to freezing in fungi originating from high-elevation sites (1800–2100 m) in the Austrian Alps [18]. The fungi originating from the Zirbitzkogel and Stoderzinken sites were, based on both growth and TTC reduction, the most unresponsive to temperate and also freezing-tolerant. Only two taxa (*Helotiales sp.* and *Cortinarius semisanguineus*) showed the highest growth rate at 15 °C and could be classed as psychrophilic fungi [15,16]. Both of these fungi originated from Zirbitzkogel. In both of these fungi, the dry weight also did not greatly increase with temperature. Only two taxa showed TTC reduction after freezing at −4 and −18 °C, *Lactarius porninsis* and *Boletus edulis*, both of which originated from the higher-elevation sites. Thus, in summary, these results support our first hypothesis that origin may be an important factor in response to temperature, both in terms of growth rate and also the occurrence of low-temperature tolerant taxa. Across genera, *Amanita* was the most sensitive to temperature and freezing based on TTC reduction, particularly *Amanita muscaria*. Again, Moser [18] showed a similar temperature and freezing sensitivity of *Amanita muscaria*. Although evidence of psychrophily was found in the ectomycorrhizal fungi, none of the saprotrophic fungi showed faster growth at low temperatures, even those originating from Mongolia. Although *Absidia* cf. *psychrophila* showed only a low response in growth to temperature.

Using the vitality marker relative to electrolyte leakage to estimate freezing tolerance, Lehto et al. [19] found that this marker did not correspond to estimates using radial growth. Similarly, in our study, TTC reduction also did not always correspond to radial growth. A complicating factor for the interpretation of the TTC reduction changes was the increase in dry weight and hyphal mat thickness of the fungi during temperature treatments. For many of the fungi, dry weight increased even during freezing at −4 °C and −18 °C. The increase in dry weight per area was in part due to an increase in the thickness of the hyphal mat. In *Boletus subtomentosa* and *Amanita muscaria,* after freezing at −18 °C, the thickness of the hyphal mat was approximately double, but this was accompanied by a much greater increase in dry weight per unit area, suggesting an accumulation of intra or extracellular materials. The measurement of ergosterol supports the idea of an accumulation of intra or extracellular materials and an increase in hyphal mat thickness. If ergosterol is assumed to be a measure of active cells [35], then the decrease in ergosterol per g of dry weight at −18 °C suggests an increase in non-membrane materials, similarly the increase in ergosterol per cm^{-2} area suggests that the hyphal mat became thicker, in agreement with the direct measurements. These changes occurred as a response to lower non-freezing temperatures but also developed while the fungi were frozen. There is considerable evidence to show

that fungi are active at freezing temperatures. Lehto et al. [19] estimated lethal freezing temperatures to be −8.3 °C and −13.5 °C but found regrowth of the fungi even after exposure to temperatures of −48 °C [19,50]. Fungi in the ascomycete genus *Geomyces* have been shown to grow continuously at temperatures down to −17 °C [21,51]. In Arctic and Antarctic soil fungi, low-temperature-activated enzymes are well documented and are thought to maintain metabolic activity at temperatures below 0 °C [52,53]. Accumulation of solutes under low temperatures has been shown in many fungi [15,16]. Tibbet et al. [16] showed an accumulation of trehalose, mannitol, and arabitol as cryoprotectants even at 4 °C. Accumulation of high levels of cryoprotectants has been suggested to be a carbon drain, which lowers growth rates in slow-growing artic fungi [16,53]. In our investigation, the greatest increase in dry weight was shown in fungi that initially had the thinnest hyphal mat at 22 °C. These fungi showed high rates of radial growth and thus high rates of exploratory growth leading to thinner hyphal mats. Particularly, this exploratory growth of the hyphae appears to be temperature sensitive, leading to an upward or reiterative growth within the hyphal colony rather than radial expansion. As a consequence, we observed higher ergosterol per unit area.

We hypothesized that LD and MD exploration types of ectomycorrhizal fungi would have a greater tolerance to freezing temperatures than other exploration types, as these fungi have been suggested to have a greater lifespan in soils [10] and would be expected to need to survive low soil temperatures in winter. One of the taxa with the greatest freezing tolerance was an LD exploration type (*Boletus edulis*); however, the contact exploration type *Lactarius porninsis* was also tolerant to freezing, and the most sensitive taxa to low temperatures was a medium-distance exploration type (*Amanita muscaria*). In contrast to our second hypothesis, our results suggest that both temperature response and tolerance to freezing are taxa-specific, rather than trait-specific. We could find no evidence that a higher freezing tolerance is a factor linked to postulated greater longevity of hyphae of the MD and LD exploration types. However, all our isolates originate from sites with a high insulating snow cover in winter, and as a consequence are less exposed to deep soil freezing.

5. Conclusions

Differences could be shown in the growth rates and responses to temperature of ectomycorrhizal and saprotrophic fungi. Fungi that originated from higher-elevation mountain sites, and thus colder climate conditions, tended to have a lower response to temperature and a higher tolerance to freezing. We could find no evidence of a higher freezing tolerance of ectomycorrhizal fungi with LD or MD exploration types compared to other exploration types that produce lower amounts of extramatrical mycelium. Sensitivity to low temperatures appears to be taxa-specific rather than exploration-type-specific. Thus, it appears that LD or MD exploration types with overwintering hyphal biomass are not greatly more freezing-tolerant than other exploration types. This may be due to the insulating effect of winter snow cover, which prevents soil freezing, and thus reduces the need for greater freezing tolerance. In the context of climate change with decreasing winter snow cover, such fungi are potentially at risk in high-elevation mountain forests.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/f16020191/s1: Table S1: Primer details; Table S2: The equation of linear regression models between radial growth rate (cm day^{-1}) of symbiotrophic fungi (a) and saprotrophic fungi (b) and incubation time at different temperatures [37].

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