Bioprotection - enhancing growth and health Research Programme

Bio-Protection
Bioprotection science for New Zealand

Isolation and characterisation of *Trichoderma* isolates from *Pinus radiata* roots in warm and cold regions

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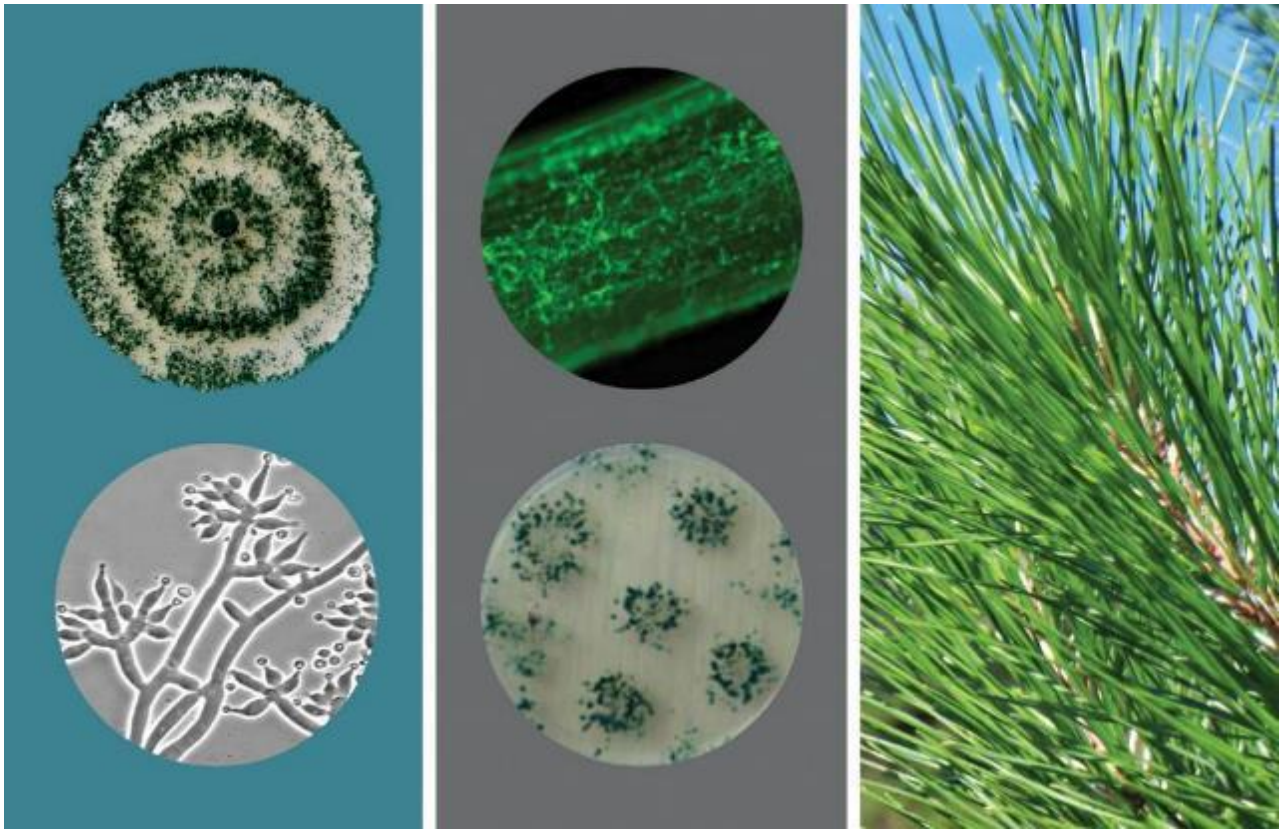

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Task 2.1

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TABLE OF CONTENTS

EXECUTIVE SUMMARY 1
INTRODUCTION 2
METHODS 3
Sample Collection and *Trichoderma* Isolation 3
DNA Extraction 6
Identification of Isolates using DNA Sequencing 7
RESULTS AND DISCUSSION 8
CONCLUSIONS AND RECOMMENDATIONS 16
ACKNOWLEDGEMENTS 17
REFERENCES 17
APPENDICES 18

Simons Hill Forest, Pukaki

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EXECUTIVE SUMMARY

The main goal of the *Bioprotection for foliar diseases and disorders of radiata pine* programme is to induce systemic resistance against foliar diseases using beneficial endophytes and elicitors. As part of this project, many plantation trials were established at sites around New Zealand to examine the effects of specific endophytic *Trichoderma* strains on *Pinus radiata* growth and disease resistance. A major question arising from this work is whether the applied strains can persist under field conditions to provide long-term growth benefits and protection from disease. Persistence of these strains may be influenced by the soil environment, in particular, the temperature. Isolation and characterisation of the background *Trichoderma* population in plantations with different soil temperatures will provide important prerequisite information for future examination of the effectiveness of the applied *Trichoderma* strains.

This report describes *Trichoderma* species isolated from roots of *P. radiata* collected from five warm (Northland) and four cold (South Canterbury and South Otago) forests. *Trichoderma* species were identified using sequence polymerase chain reaction (PCR) primers and direct sequencing of the *tef1*α barcoding region. The growth response of the isolates to temperature was characterised by measurement of colony mycelial growth on PDA agar plates. A selection of beneficial *Trichoderma* strains from the New Zealand *Trichoderma* plantation trials were also included in the temperature study for comparison with the regional isolates.

Key Results

- Fifteen *Trichoderma* species were identified through direct sequencing of *P. radiata* root samples collected in nine New Zealand forest plantations in warm and cold regions. Species included, *T austrokoningii, T. atroviride, T. caerulescens, T. composticola, T. crassum, T. fertile, T. hamatum, T. harzianum, T. koningii, T. polysporum, T. spirale, T. viride, T. viridescens* and two currently undescribed taxa, *T. sp. 273* and *T. sp. 787*.

- *Trichoderma* species appear to have adapted to the warm and cold environments in this study with only three species common to both regions (*T. atroviride, T. spirale* and *T. viridescens*). *T. sp. 787*, followed by *T. viridescens*, were the most dominant species in the cold region. In the warm region, three species were equally dominant *(T. atroviride, T. crassum and T. hamatum*).

- Although the diversity found in the two regions was relatively similar (eight and ten species for the cold and warm regions, respectively), the diversity of species within each forest was very different:
    - in the warm region, each forest had six *Trichoderma* species present
    - in the cold region, each forest frequently had only one or two species present.

- Isolates from each region and beneficial strains had very different mycelial growth rates at temperatures of 2, 7, 12, 22 and 27°C. Optimum temperature for mycelial growth was estimated to occur at approximately 22°C, between 22 and 27°C, and 27°C or higher for the cold region, warm region and beneficial strain groups, respectively. Mean growth rate of the three groups was not significantly different ($P<0.05$) at 17°C.

- Targeted deployment of current beneficial biocontrol mixtures to specific temperature zones may be required for improved performance and persistence, with PR6 mixture suitable for central temperate and sub-tropical regions and PR3a mixture suitable for cooler temperate regions of New Zealand.

INTRODUCTION

Endophytic *Trichoderma* fungi show great potential for improving health of forest trees (Hill and Whelan, 2017). The main goal of the *Bioprotection for foliar diseases and disorders of radiata pine* research programme in New Zealand is to induce systemic resistance against foliar diseases using beneficial endophytes and elicitors. A large number of *Trichoderma* strains have been isolated and screened for the ability to promote growth and suppress disease in laboratory and nursery trials. The most effective strains have been applied as nursery seed treatments and are currently being evaluated for field performance in forest plantation trial sites around the country.

An important question about deployment of these *Trichoderma* strains in the Bioprotection research programme is how effective they are in different abiotic and biotic environments, potentially affecting the long-term bioprotection in the plantation. In New Zealand, *P. radiata* has been established in most regions, ranging from sub-tropical zones in the north to more temperate zones further south. However, low temperatures in winter, or hot temperatures in summer, may cause problems for biological control by influencing the activity of the biocontrol agents.

Information on naturally occurring *Trichoderma* species in different environments gives an indication of the preference these species have to the conditions. In New Zealand, Cummings and Hill (2016) characterised background populations of *Trichoderma* species in four temperate regions, Nelson, Gisborne, Waikato/Bay of Plenty and Blenheim, but no information was included for warmer or colder regions (*e.g*. Northland and Canterbury/ Otago/Southland). If substantial differences are found in natural populations, this information may assist in targeting beneficial biocontrol *Trichoderma* strains to specific temperature zones.

The aim of Task 2.1 was to isolate and characterise *Trichoderma* cultures from surface-sterilised *P. radiata* tree roots in cold and warm region New Zealand forest plantations. DNA analyses of partial *tef1*α gene sequences of selected cultures was used for reliable, unambiguous identifications to species level for *Trichoderma*. Colony mycelial growth was measured at different temperatures and compared to those of selected beneficial *Trichoderma* strains previously used in plantation trials.

METHODS

Sample Collection and *Trichoderma* Isolation

Regions selected to represent warm and cold areas of New Zealand were defined as having the highest and lowest monthly mean temperatures 10 cm below ground level, respectively (Table 1), and where commercial forestry plantations were present. The regions selected to represent warm and cold temperature zones were Northland and South Canterbury/South Otago, respectively (Figures 1a and 1b).

Table 1: Mean monthly and annual 10 cm earth temperatures (°C) for the selected warm and cold locations for the 1981-2010 period.

Location	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Year
Warm:													
• Kaitaia	20.0	20.1	18.4	16.2	13.6	11.6	10.6	10.7	12.3	14.0	16.0	18.5	15.2
Cold:													
• Mt Cook	14.8	14.5	12.3	8.7	5.1	2.2	0.7	2.2	4.1	7.2	10.7	13.4	8.0
• Lake Tekapo	15.4	14.3	11.3	7.9	3.8	1.0	0.0	1.4	4.3	6.6	11.2	13.5	7.6
• Dunedin	15.7	15.2	13.2	10.2	7.5	5.2	4.1	5.0	7.5	9.8	12.3	14.4	10.0

(data from https://www.niwa.co.nz/education-and-training/schools/resources/climate/earthtemp)

Roots were collected in March to May 2018 from eight to twelve healthy *P. radiata* trees in each of five plantations located in both regions. Collection details are listed in Table 2. The roots sampled were generally 1 to 5 mm in diameter and harvested within the top 20 cm from ground level. Roots were moistened and stored in zip-locked bags at 4°C and processed within 5 days of harvest.

Root samples were processed at the Bio-Protection Research Centre (Lincoln University) using established protocols. Roots were thoroughly washed in running tap water and cut into pieces of approximately 10 mm lengths. Root pieces were surface-sterilised under aseptic conditions by placing in a petri dish and soaking in Virkon (1% w/v) for 10 minutes. Root pieces were then rinsed in sterile water, placed on sterile paper to dry for 10 minutes and then transferred onto *Trichoderma* selective MRB media plates (Appendix A). Plates with roots were incubated under laboratory conditions (20-25°C) with ambient light for 14 days. *Trichoderma* colonies were sub-cultured to plates of MYE (malt extract 1.0%, yeast extract 0.1%, agar 2%) and incubated under laboratory conditions (20-25°C) with ambient light for 7 days. Colonies selected for sub-culturing and DNA identification were chosen based on different visual morphological characteristics with the aim of capturing as much species diversity as possible. Non-sequenced isolates were identified based on similar colony morphology.

Figure 1a: Location of plantation forests in the region defined as warm.

Figure 1b: Location of plantation forests in the region defined as cold.

Table 2: Collection details for root samples and number of *Trichoderma* isolates recovered in this study.

Temperature Zone	Name of Forestry Company and Plantation Name	Location (GPS coordinates in brackets)	Age of trees sampled (years)	No. of root pieces plated on agar plates	No. of *Trichoderma* isolates cultured	No. of *Trichoderma* isolates identified using sequence data
Warm:	Rayonier Ltd, Topuni Forest	Kaiwaka (-36.21438, 174.44314)	4, 6, 8, 9	135	18	7
	Hancock Forest Management (NZ), Otaenga Forest	Tautoro (-35.59598, 173.81805)	3, 4	120	12	9
	Rayonier Ltd, Glenbervie	Glenbervie (-35.61890, 174.33400)	6, 20	165	11	6
	Hancock Forest Management (NZ), Tungrove Forest	Tautoro (-35.56709, 173.83155)	2, 11, 14, 14, 20	160	18	7
	Hancock Forest Management (NZ), Pipiwai Forest	Pipiwai (-35.57035 173.99810)	4	120	12	6
Total				700	71	35
Cold:	Tony Walls, Simons Hill Forest	Pukaki (-44.21906, 170.28439)	23	195	1	1
	MacKenzie District Council, Woodburn old plantation	Kimbell / Burkes Pass (-44.03195, 170.70876)	22	180	29	13
	MacKenzie District Council, Woodburn young plantation	Kimbell / Burkes Pass (-44.04706, 170.68330)	8	195	10	4
	Balmoral Station plantation	Tekapo (-44.03952, 170.41574)	15	195	3	2
	Rayonier Matariki Forests, Glendhu Forest	Lawrence (-45.87164, 169.87164)	6, 27	325	19	6
Total				1090	62	26

DNA Extraction

Genomic DNA was extracted from vegetative mycelium using a Chelex 100 buffer method. Approximately 50 mg of 7-day-old mycelium was aseptically harvested from pure cultures and placed into sterile 1.7 mL Eppendorf tubes. Chelex 100 (0.5 mL, 5%, Bio-Rad Laboratories) was added to each tube and mycelium was ground with a sterile pestle. Tubes were placed in boiling water for 12 minutes then centrifuged at 13000 rpm for 30 minutes. The supernatant containing the DNA extract was pipetted into new 1.7 mL Eppendorf tubes and stored at 4°C.

Identification of Isolates using DNA Sequencing

Sequence data from the translation elongation factor 1α (*tef1α)* gene is generally used for routine identification of *Trichoderma* species and can be used to differentiate all known *Trichoderma* species (Bissett *et al.,* 2015). For each *Trichoderma* isolate an approximately 900 base pair (bp) fragment from the 5' end of the *tef*1α gene was amplified using the primer pair tef85f (AGGACAAGACTCACATCAACG) and tef954R (AGTACCAGTGATCATGTTCTTG; Shoukouhi and Bissett, 2009).

All PCR amplifications were performed in a total reaction volume of 25 µL, including 2.5 µL of 10x reaction buffer + 2.5 mM $MgCl_2$, 200 µM dNTPs, 400 µM of each primer, 0.5 µL Purified BSA (BioLabs, Inc.), 2.5 µL FastStart Taq DNA polymerase (Roche Applied Science) and 2 µL undiluted template DNA. PCR reactions were initiated with a DNA denaturing step for 5 minutes at 95°C; followed by 40 cycles each of 45 seconds at 95°C, 45 seconds at 56°C and 2 minutes at 72°C; with a final extension step of 7 minutes for 72°C. A negative control (water used in PCR amplification) was included in each assay.

The quality of the DNA extracts was confirmed by conventional PCR of the *tef1*α region with primers tef85f and tef954R. PCR products were visualised by staining with RedSafe (iNtRON Biotechnology) following electrophoresis of 6 µL of each product in 1.0% agarose gel. PCR products were then purified using a commercial cleanup kit (Agencourt CleanSEQ) and sequenced in the forward direction using the internal sequencing primer tef85f in an Applied Biosystems 3103xl Genetic Analyzer at the Bio-Protection Research Centre DNA Sequencing Facility (Lincoln University). Consensus sequences were assembled from forward sequencing chromatograms using ChromasPro v. 1.7.6 (https://technelysium.com.au/wp/chromaspro). Sequences were then searched in BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify species affiliations.

Colony Growth Rates

The effect of temperature on colony mycelium growth was determined by measuring individual isolate colony radial growth on MYE agar plates. Nine *Trichoderma* isolates were selected to represent the range of species found in each region. Additional beneficial *Trichoderma* strains from the Bio-Protection Research Centre (BPRC) culture collection, used in the 2018 New Zealand plantation trials, were compared to the cold and warm isolates. Isolates selected were FCC55 (*T. harzianum),* FCC318 (*T. atrobrunneum),* FCC327 (*T. harzianum)* and FCC340 (*T. harzianum),* that consist the PR6 mixture, and FCC13 *(T. asperellum*), FCC14 *(T. atroviride),* FCC15 (*T. atroviride*) and FCC180 (*T. crassum*), that consist the PR3a mixture.

Agar plugs (6mm in diameter) were cut with a cork borer from five-day-old culture margins grown on MYE agar plates. Plugs were inverted and placed singularly onto the centre of 3 replicate PDA agar plates and placed in climate controlled growth cabinets ((Contherm Biocell 1000, Contherm Scientific Ltd, New Zealand) set to different temperatures (2, 7, 12, 17, 22 and 27°C) and 12 hours light (1350Lm) and dark conditions. Temperatures were chosen to represent the range of mean monthly 10cm earth temperatures experienced in Northland and South Canterbury/South Otago. Radius of colonies were measured after 46 to 50 hours, at 7 days and at 5 weeks, and radial mycelial growth rate per day was calculated allowing for the initial radius of the plug. Data were analysed for significance by analysis of variance (ANOVA) and least significant difference (LSD) tests (GenStat, v19).

RESULTS AND DISCUSSION

Trichoderma Species Diversity

This study describes the diversity of *Trichoderma* species isolated from plantation *P. radiata* root samples in warm and cold regions of New Zealand. In total, 112 trees were sampled, 1790 root pieces were tested, and 133 *Trichoderma* isolates were isolated and cultured. A greater proportion of isolates grew from the root pieces sampled in the warm (10.1%), compared to the cold region (5.7%). Approximately half of the isolates, selected for being morphologically distinct, were plated to fresh media (examples shown in Figure 2) and identified through direct sequencing. Isolates were distributed amongst 13 currently named species; *T. atroviride, T. austrokoningii, T. caerulescens, T. composticola, T. crassum, T. fertile, T. hamatum, T. harzianum, T. koningii, T. polysporum, T. spirale, T. viride* and *T. viridescens* and two undescribed taxa (designated as *T. sp. 273* and *T. sp. 787*).

This study has contributed to the knowledge of endophytic *Trichoderma* naturally occurring in New Zealand *P. radiata* forest plantations. Seven species (*T. caerulescens, T. composticola, T. fertile, T. koningii, T. viridescens, T. sp. 273 and T. sp. 787*) were found in addition to those of Cummings and Hill's 2016 *P. radiata* plantation study of four central New Zealand regions, Nelson, Gisborne, Waikato/Bay of Plenty and Blenheim. However, these species were documented in an extensive survey of *Trichoderma* species in New Zealand (Braithwaite *et al.,* 2017) and, therefore, are not considered to be novel species.

In both regions, most species were from the Viride Clade and subclade groups with a few species from the Polysporum, Semiobis, Green and Green/Harzianum groups (Table 3). Each region had different species present, with only three species, *T. atroviride, T. spirale and T. viridescens,* found in both regions. *T. sp. 787, followed by T. viridescens,* were the most dominant species in the cold region. In the warm region, three species were equally dominant *(T. atroviride, T. crassum and T. hamatum*). Adaptation of *Trichoderma* species to regions with different temperatures has also been found in other studies. Ghildiyal and Pandey (2008) isolated three cold-tolerant species, *T. aureoviride*, *T. harzianum* and *T. viride,* from the Indian Himalayan region, capable of growing on agar at 5°C. In North Carolina, Virginia and Washington State forests, *T. viride and T. polysporum* were largely restricted to cool temperate regions, whereas *T. harzianum* was dominant in warm climates and *T. koningii* and *T. hamatum* were widely distributed in areas of diverse climatic conditions (Danielson and Davey, 1973).

The diversity of *Trichoderma* species in each temperature zone was similar, with eight and nine species present in the cold and warm region, respectively (Table 3). However, within each cold region plantation, with the exception of Woodburn Old plantation, there was low species richness with only one or two species present. These specific *Trichoderma* species appear to have adapted to the local environment. Biodiversity in ecosystems is mainly determined by temperature, but is also impacted by large seasonal differences between summer and winter (Stevens, 1989). In this study, exposure to low winter and high summer temperatures and summer dryness may be driving the lack of species diversity in the cold region forests, particularly in the Balmoral Station and Simons Hill forests. In contrast, greater species richness was found in each of the warm region forests (six *Trichoderma* species present), possibly because of warmer monthly temperatures and less variation in temperature and water availability.

In New Zealand, three-quarters of plantation forests are planted in more temperate zones of Waikato/Bay of Plenty, Gisborne, Hawkes Bay, Southern North Island and

Nelson/Marlborough (MPI, 2017), compared to the sub-tropical (Northland) and southern-temperate (South Canterbury/South Otago) zones in this report. Cummings and Hill (2016) and Hill *et al.,* (2017) found similar richness and abundance of *Trichoderma* strains in New Zealand temperate plantations, with a dominance of Viride Clade and a presence of Green and Green/Harzianum Clade species. Many of the current BPRC *Trichoderma* biocontrol strains were sourced from the central temperate zone and identified as Viride, Green and Green/Harzianum species, therefore, will be suitable for deployment in central temperate regions of New Zealand. In Northland, performance of the current biocontrol strains, particularly those based on *T. harzianum* (eg PR6 mixture) should not be affected, or may be improved, by the warm temperatures.

The PR3a biocontrol mixture should be targeted for deployment to cooler temperate regions. PR3a mixture mainly consists of Viride Clade species, the most common species found in South Canterbury/South Otago region, and therefore, is more likely to have better effectiveness and persistence than *T. harzianum*-based mixtures.

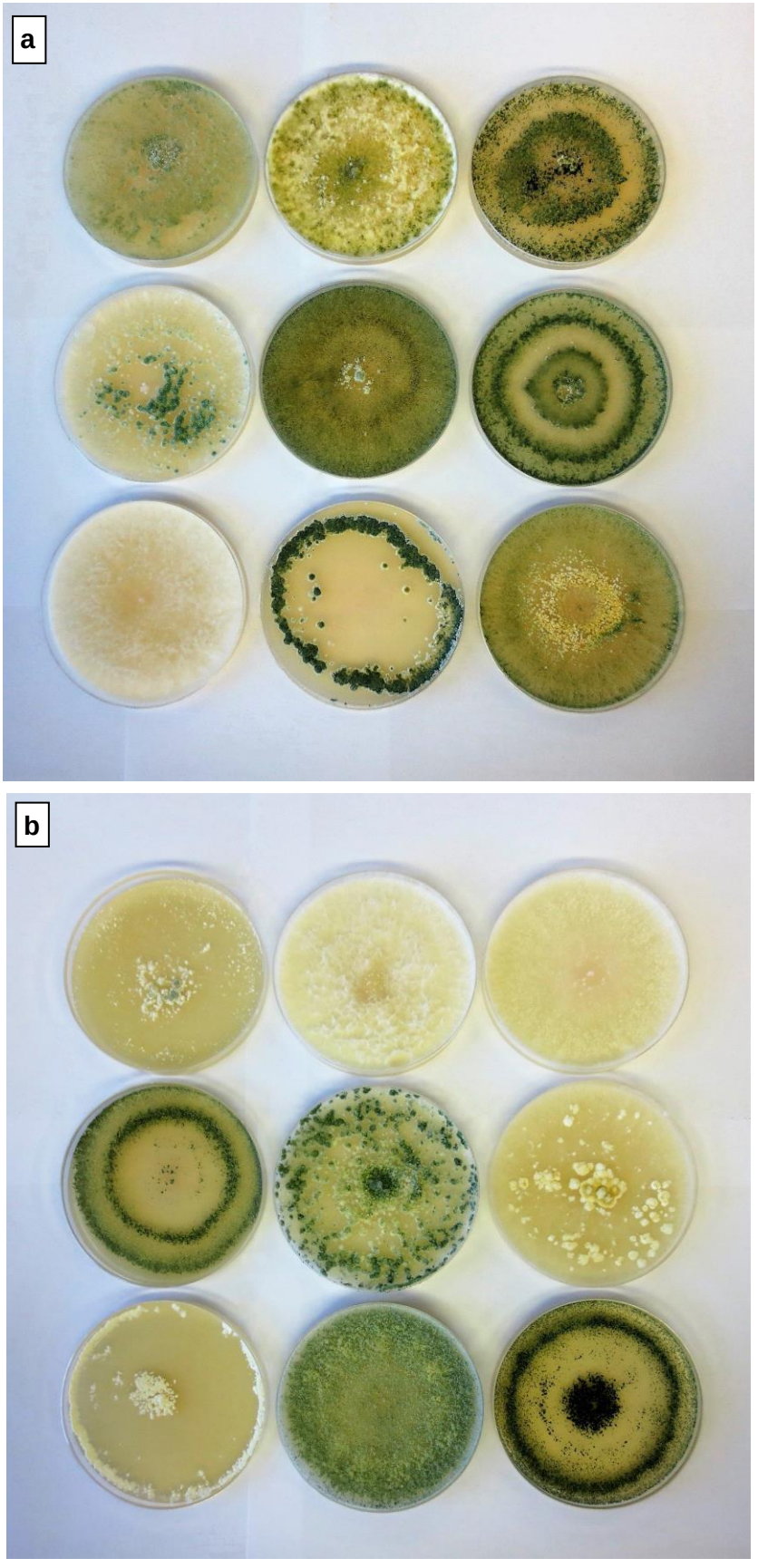

Figure 2: Fourteen-day old *Trichoderma* colonies grown on MYE agar, sub-cultured from surface-sterilised *P. radiata* root pieces, sampled from plantations in a) warm and b) cold regions of New Zealand. Species identified (from left to right and top to bottom) were a) *T. viride, T. atroviride, T. harzianum, T. koningii, T. crassum, T. spirale, T. caerulescens, T. hamatum* and *T. austrokoningii;* b) unidentified, *T. sp. 273, T. sp. 787, T. fertile, T. viridescens, T. atroviride, T. polysporum, T. compositicola* and *T. fertile.*

Table 3: *Trichoderma* species identified in warm and cold regions of New Zealand and their occurrence in the Clades and Subclades of *Trichoderma* described as being present in New Zealand, according to Braithwaite *et al.,* (2017).

Clade and Subclade [a]	Species Identified [b]	No. of isolates										Total Number
		Warm Region					Cold Region					
		Otaenga	Pipiwai	Tungrove	Glenbervie	Topuni	Glendhu	Simons Hill	Woodburn Old	Woodburn Young	Balmoral Station	
Viride and Viride/Viridescens	***T. atroviride***	3	1	6	2				2			14
	T. caerulescens				1	1						2
	T. composticola								1			1
	T. viridescens	1	1	1					11			14
	T. viride	2		2	2	1						7
	T. sp. 787						19		3	6		28
Viride/Koningii	*T. koningii*		1		1	5						7
Viride/Rogersonii	*T. austrokoningii*		3									3
Viride/Hamatum	*T. hamatum*		3	2	4	8						17
	T. sp. 273								2		3	5
Polysporum	*T. polysporum*									3		3
Semiorbis	*T. fertile*								4			4
Green	*T. crassum*	2	3	5	1	1						12
	T. spirale	2				1		1				4
Green/Harzianum	*T. harzianum*	1		2								3

a Informal clade/subclade names as described by Jaklitsch and Voglmayr (2015)

b Species identification based on analyses of *tef1*α DNA sequences; species in bold are present in both regions.

Response of Isolates to Temperature

The response of selected *Trichoderma* isolates to temperature, measured by mycelial radial growth on PDA media, was highly dependent on the temperature and the region the isolates were cultured from (Figures 3, 4 and 5). As temperature increased, the growth rates increased with approximately 40% of isolates reaching optimum growth at approximately 22°C, mainly in the cold group (Figure 5). Accurate estimation of optimum temperature for mycelial growth can be made by modelling the growth response to a large number, and range, of temperatures, but this was not attempted in this study. However, optimum temperature for mycelial growth was estimated to occur at approximately 22°C, between 22 and 27°C, and approximately 27°C or higher for the isolates in the cold and warm regions and beneficial strains, respectively.

Mycelial growth rates of isolates were most divergent at temperatures of 7, 22 and 27°C (Figure 4). At 7°C, the mean growth rate of isolates from the cold region was double and triple that of the warm and beneficial strains, respectively. In comparison, at warmer temperatures of 22 and 27°C, the isolates from the warm region and beneficial strains grew at a higher mean growth rate, compared to the isolates from the cold region. The growth rate of isolates from the three groups was not significantly different ($P<0.05$) at 17°C. At seven days incubation at 2°C, seven isolates collected from the cold region had mycelial development, with *T. polysporum* and *T. fertile* having the most growth (Table 4). No isolates from the warm or beneficial groups exhibited mycelial growth. At five weeks of incubation at 2°C all cold region isolates, except *T. spirale,* exhibited mycelial growth, compared to only four (*T. crassum, T. koningii, T. viride and T. viridescens*) in the warm isolate group. Heavy sporulation was observed in the cold region species *T. sp. 787* and *T. fertile,* with limited or no sporulation in the other isolates. Production of spores in *T. sp. 787* and *T. fertile* appear to be a strategy for survival in extreme cold environments, for example the South Canterbury region that may experience sub-zero temperatures.

Three isolates had different growth responses compared to the rest of the isolates in their grouping. *T. polysporum* had very low growth rates at 12, 17 and 22°C, compared to the rest of the cold region isolates (Figure 5a). This species also had a very low growth rate compared to the warm and beneficial isolates (Figure 5b and c) and suggests that *T. polysporum* may have adapted to cool temperate forest soils (Danielson and Dave, 1973). *T. sp. 273* also had a different response, compared to the rest of the cold region isolates, with a fast growth rate at 27°C (Figure 5a). *T. sp. 273* is closely related to *T. asperellum (*Braithwaite *et al.,* 2017*)* and both the *T sp. 273* isolate and beneficial *T. asperellum* FCC13 strain had similar response patterns to the temperatures tested. The *T. harzianum* isolate collected from the warm region, had a large increase in mycelial growth rate between 22 and 27°C, relative to the other warm region isolates. This pattern was similar to the three beneficial *T. harzianum* strains tested, particularly FCC327 (Figure 5c). *T. harzianum* has been shown to have a high optimum growth temperature (Danielson and Davey, 1973).

T. spirale and *T. viridescens,* two of the three species isolated from both regions, had similar mycelial growth rate patterns within species (Figure 5a and b). The third species that was found in both regions, *T. atroviride,* had more variation in the growth rate patterns which may indicate that the isolates cultured were more genetically variable.

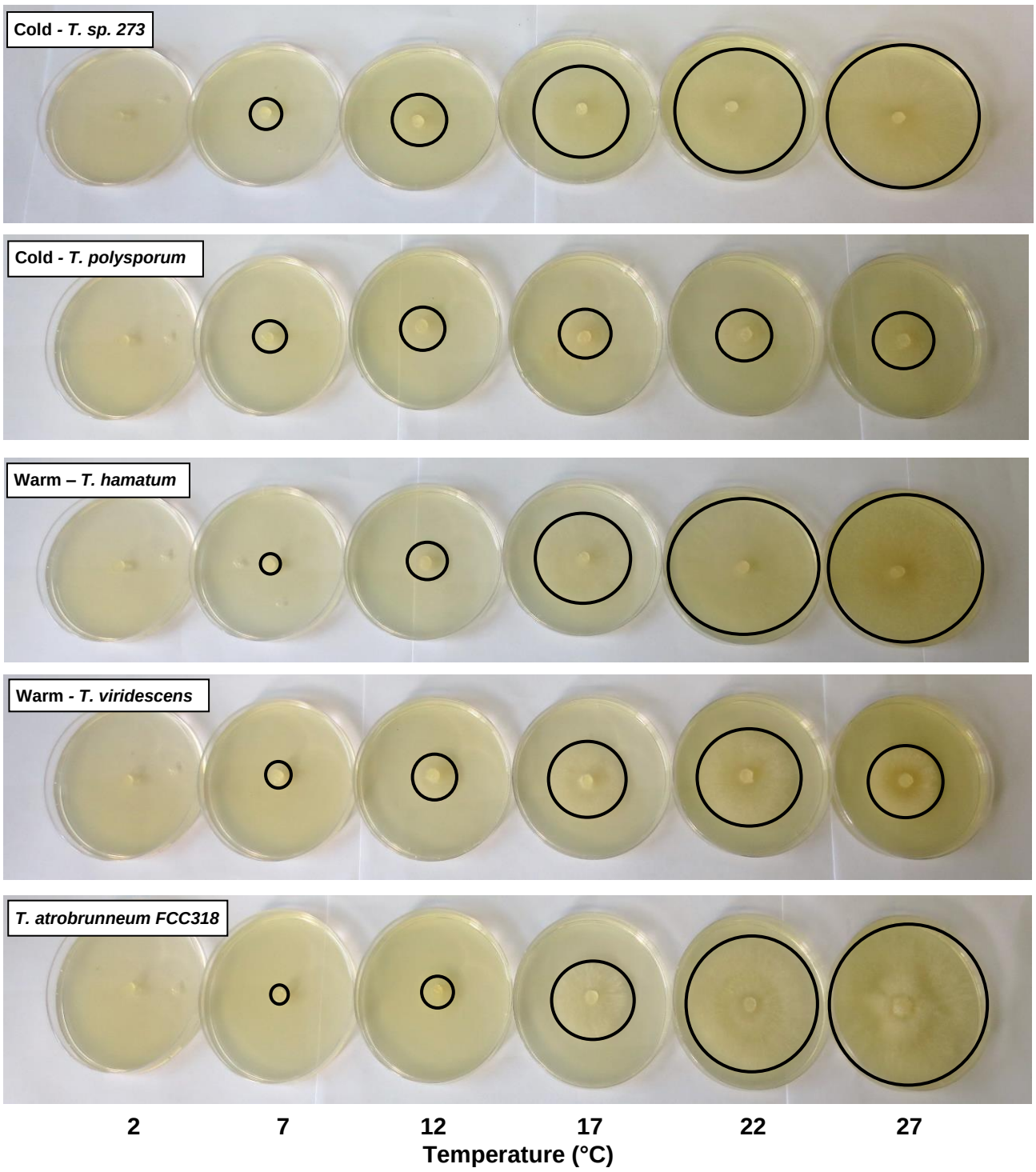

Figure 3: Examples of mycelial growth of selected cultures of *Trichoderma* isolated from *P. radiata* roots in cold and warm regions and *T. atrobrunneum FCC318* used in plantation trials on PDA plates at 2, 7, 12, 17, 22 and 27°C after 46 to 50 hours of incubation. Circles have been drawn to show colony edges.

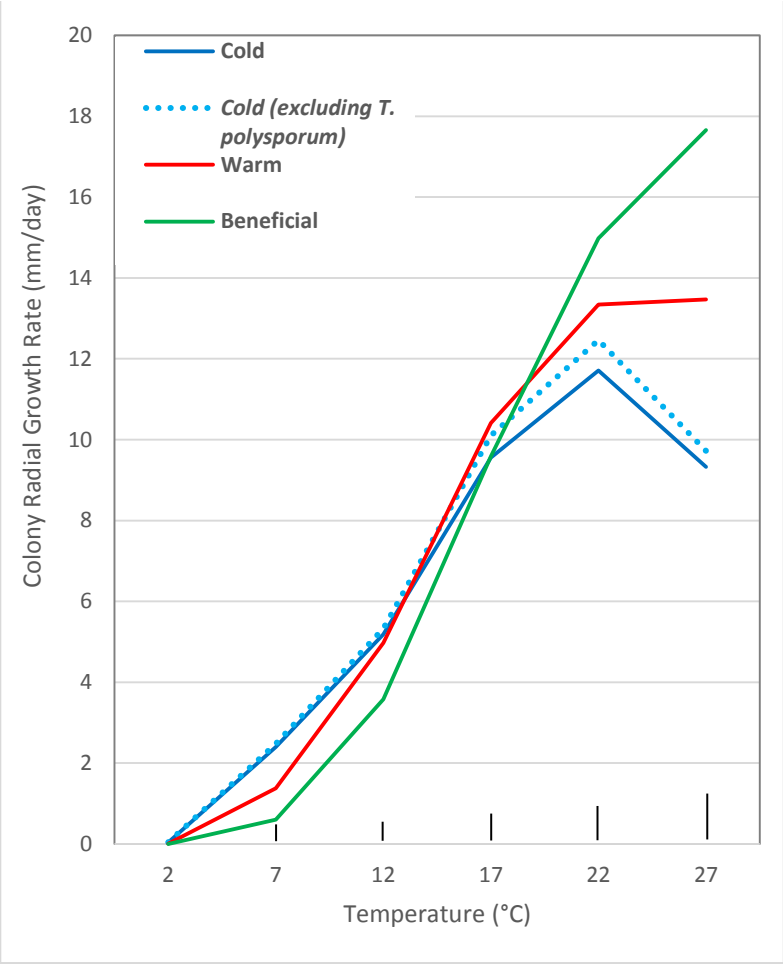

Figure 4: Radial growth rate (mm/day) of selected mycelial cultures of *Trichoderma* isolated from *P. radiata* roots in cold and warm regions and eight BPRC beneficial strains used in plantation trials with various temperatures. Growth data was calculated as mean for each region, the cold region excluding *T. polysporum* and beneficial strains. Vertical lines show LSD (5%).

Table 4: Radial growth rate (mm/day) of selected cultures of *Trichoderma* isolated from *P. radiata* roots in cold and warm regions that developed mycelial growth, after seven days and five weeks incubation at 2°C.

Isolate	**Mean Radial Growth Rate (mm/day)**	
	7 days incubation	**5 weeks incubation**
Cold Region:		
T. sp. 273	0.02	0.10
T. sp. 787 (1)	0.05	0.67
T. sp. 787 (2)	0.19	0.64
T. atroviride	0.04	0.13
T. composticola	0.00	0.17
T. fertile	0.49	1.30
T, polysporum	0.44	1.30
T. spirale	0.00	0.00
T. viridescens	0.01	0.20
Warm Region*:*		
T. crassum	0.00	0.02
T. koningii	0.00	0.03
T. viride	0.00	0.03
T. viridescens	0.00	0.11

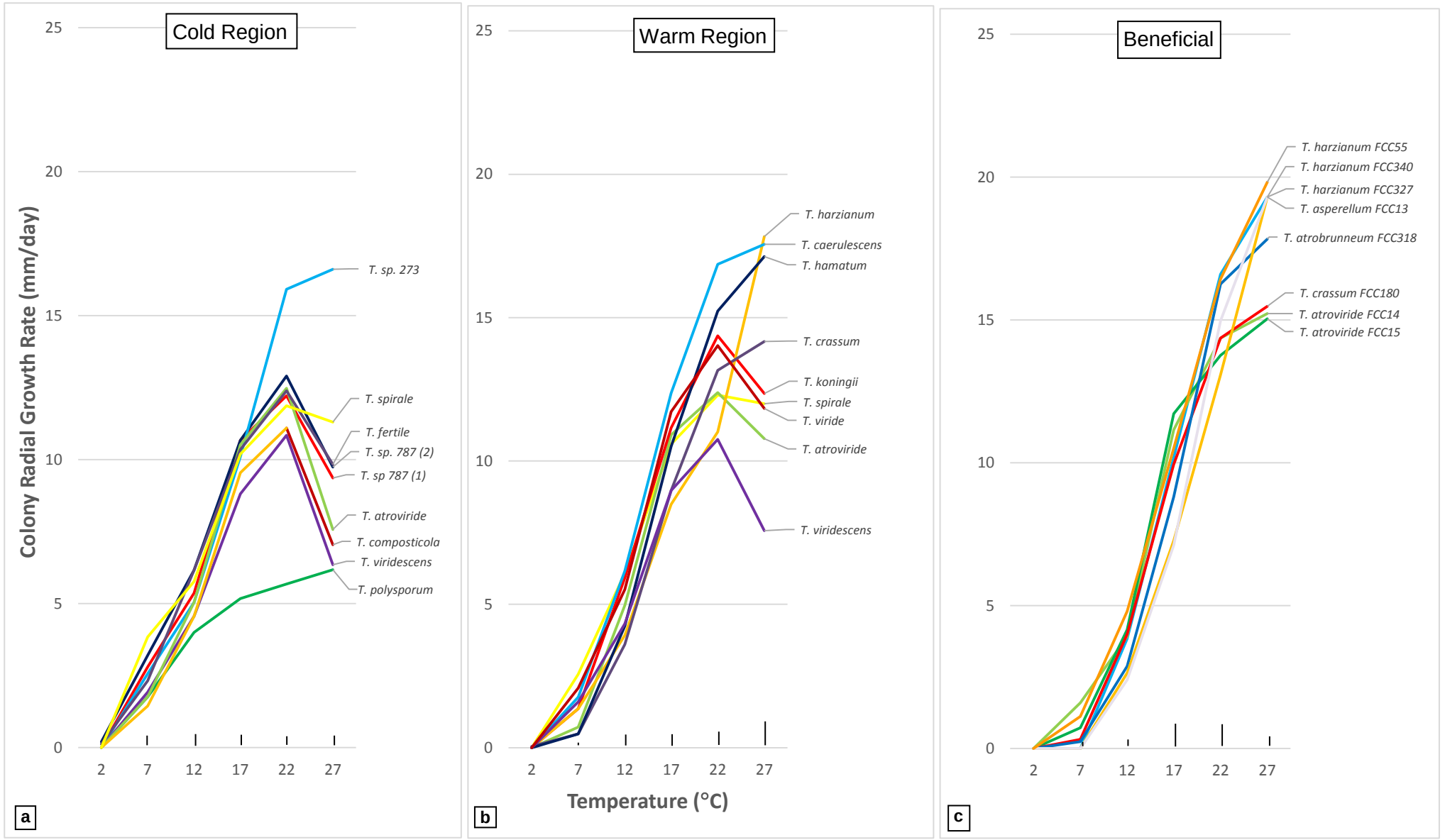

Figure 5: Radial growth rates (mm/day) of selected mycelial cultures of *Trichoderma* isolated from a) cold and b) warm regions and c) eight beneficial strains used in plantation trials with various temperatures**.** Species identification based on analyses of *tef1*α DNA sequences. Vertical lines show LSD (5%).

Climate change for New Zealand is predicted to make small but increasing mean temperature rises with generally fewer frosts, longer growing seasons, a greater number of hot days above 25°C, particularly at already warm northern locations, and changing rainfall patterns (IPCC Working Group II Fifth Assessment Report, 2014). Climate change is also expected to generate large temperature extremes, with the occurrence of both warmer and colder temperatures in some locations. High temperatures caused by climate change may also lead to increased infection and sporulation of existing foliar diseases (Harvell *et. al*., 2002), allow latent pathogens to establish as major disease agents and provide advantageous growing conditions for new pathogens to invade and establish (Sturrock *et al.,* 2011). If predicted climate change scenarios eventuate, the current *Trichoderma* strains used in biocontrol trials, particularly mixtures based on *T. harzianum* (*e.g.* PR6 mixture), are likely to continue to be beneficial for *P. radiata* growth and disease suppression. Biocontrol mixtures specifically based on *T. atroviride, T. sp. 787, T. fertile* and/or *T. viridescens* (*e.g.* PR3a mixture or new mixtures) may be necessary in regions with low eventual mean temperatures or where the occurrence or severity of frosts increases. These species may have better persistence than the *Trichoderma* species that prefer warmer conditions.

CONCLUSIONS AND RECOMMENDATIONS

Sequenced-based identifications revealed a diverse population of *Trichoderma* species in natural association with plantation *P. radiata* trees in cold and warm regions, including at least thirteen named, and two undescribed species. Knowledge of *Trichoderma* diversity in *P. radiata* will contribute to enhanced understanding of the role of these fungi.

Temperatures chosen in this study represented the range of mean monthly 10 cm earth temperatures (°C) experienced in the warm and cold regions. However, natural or deployed *Trichoderma* species may experience short-term or prolonged periods of temperatures outside this range. Experimentation with a wider range of temperatures, both below 2°C and above 27°C, would define the response of species and isolates to extreme temperatures.

Further work is required to determine the impact of other abiotic and biotic environmental factors on the effectiveness of the *Trichoderma* strains used in biocontrol of fungal disease in New Zealand. Some important parameters to be considered are the effects of water potential, pH and organic matter content, and the presence of pesticides, metal ions and antagonistic fungi (refer to Hill *et al.,* 2017) and bacteria in the soil. Once these relationships are better understood, there is potential for modification of the endophyte biocontrol mix to better suit the environmental zones in New Zealand.

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REFERENCES

Bissett, J., Gams, W., Jaklitsch, W. and Samuels G. 2015. Accepted *Trichoderma* names in the year 2015. IMA Fungus 6: 263-295.

Braithwaite, M., Johnston, P., Ball, S., Nourozi, F., Hay, A., Shoukouhi, P., Chomic, A., Lange, C., Ohkura, M., Nieto-Jacobo, M., Cummings, N., Bienkowski, D., Mendoza-Mendoza, A., Hill, R., McLean, K., Stewart, A., Steyaert, J. and Bissett, J. 2017. *Trichoderma* down under: species diversity and occurrence of *Trichoderma* in New Zealand. Australasian Plant Pathology 46: 11–30.

Cummings, N. and Hill, R. 2016. Persistence of *Trichoderma* in nursery and forest plantations. Technical report FOA-BIO-T011 to NZ Forest Owners Association, December 2016, 15p.

Danielson, R. and Davey, C. 1973. Nonnutritional factors affecting the growth of *Trichoderma* in culture. Soil Biological Biochemistry 5: 495-504.

Ghildiyal, A. and Pandey, A. 2008. Isolation of cold tolerant antifungal strains of *Trichoderma* sp. from glacial sites of Indian Himalayan region. Research Journal of Microbiology 3: 559-564.

Harvell, C., Mitchell, C., Ward, J., Altizer, S., Dobson, A., Otsfeld, R. and Samuel, M. 2002. Climate warming and disease risks for terrestrial and marine biota. *Science* 296: 2158–2162.

Hill, RA. and Whelan, HG. 2017. Bio Protection Successes. Forest Growers Research Conference, Christchurch. 17-18 October 2017. 13p.

Hill, R., Cummings, N. and Whelan, H. 2017. Isolation and characterisation of fungal partners in the *Pinus radiata* root microbiome. Technical report FOA-BIO-T016 to NZ Forest Owners Association, December 2017, 16p.

Jaklitsh, W. and Voglmayr, H. 2015. Diversity of *Trichoderma (Hypocreaceae*) in southern Europe and Macaronesia. Studies in Mycology 80: 1-87.

National Exotic Forest Description to 1 April 2017. Ministry for Primary Industries, Wellington. 68p.

Shoukouhi, P. and Bissett, J. 2009. Preferred primers for sequencing the 5' end of the translation elongation factor 1-alpha gene (eEF1a1). International subcommission on *Trichoderma* and *Hypocrea* taxonomy. http://wwwisthinfo/methods.

Stevens G. 1989. The latitudinal gradient in geographical range: how so many species coexist in the tropics. The American Naturalist 133: 240-256.

Sturrock, R, Frankel, S, Brown, A., Hennon, P., Kliejunas, J., Lewis, K., Worrall, J. and Woods, A. 2011. Climate change and forest diseases. Plant Pathology 60: 133-149.

WGII Report. Climate Change 2014: Impact, adaptation and vulnerably. Intergovernmental Panel on Climate Change. 1150p.

APPENDIX A:

Malt Yeast Extract Agar with Rose Bengal (MRB) Recipe

Malt extract	10 g
Yeast extract	1 g
Rose Bengal (50 mg/mL)	3 mL
Terrachlor 75WP	0.2 g
Agar	20 g
Chloramphenicol stock solution (100 mg/mL)	1 mL

Make up to 1 L with distilled water.

APPENDIX B:

Isolates identified in this study using DNA analysis of partial tef1α gene sequences

Isolate	Identification and Accession Numbers
Cold Region:	
C1	*Trichoderma sp. 787* JB-2014 KJ871273.1
C2	*Trichoderma sp. 787* JB-2014 KJ871273.1
C3	*Trichoderma sp. 787* JB-2014 KJ871273.1
C5	*Trichoderma sp. 787* JB-2014 KJ871273.1
C6	*Trichoderma sp. 787* JB-2014 KJ871273.1
C7	*Trichoderma sp. 787* JB-2014 KJ871273.1
C8	*Trichoderma sp. 787* JB-2014 KJ871273.1
C9	*Trichoderma sp. 787* JB-2014 KJ871273.1
B15	*Trichoderma sp. 273* KX098486.1
C11	*Trichoderma sp. 273* KX098486.1
C12	*Trichoderma viridescens* BMCC:LU1360 KJ871276.1
C13	*Trichoderma sp. 273* KX098486.1
C14	*Trichoderma sp. 273* KX098486.1
C15	*Trichoderma sp. 787* JB-2014 BMCC:LU787 KJ871273.1
C16	*Trichoderma sp. 787* JB-2014 BMCC:LU787 KJ871273.1
C17	*Trichoderma sp. 787* JB-2014 BMCC:LU787 KJ871273.1
C18	*Trichoderma polysporum* DAOM:216501 KJ871208.1
C21	*Trichoderma viridescens* JB G2005 KJ871277.1
C22	*Trichoderma viridescens* JB G2005 KJ871277.1
C23	*Trichoderma atroviride* CIB T93 EU279997.1
C24	*Trichoderma fertile* DAOM:167161 KJ871131.1
C25R	*Trichoderma composticola* DAOM:233833 KJ871257.1
C26	*Trichoderma fertile* DAOM:167161 KJ871131.1
C27	*Trichoderma viridescens* JB G2005 KJ871277.1
C28	*Trichoderma fertile* BMCC:LU1303 KJ871154.1
B9	*Trichoderma spirale* BMCC:LU811 KJ871152.1

Warm Region:

1	*Trichoderma hamatum* DAOM 167057 EU279965.1
2	*Trichoderma caerulescens* DAOM 229854 EU280000.1
3	*Trichoderma hamatum* DAOM 167057 EU279965.1
4	*Trichoderma viridescens* BMCC:LU1360 KJ871276.1
5	*Trichoderma viride* strain CBS 586.95 KJ871113.1
6	*Trichoderma koningii* BMCC:LU1310 KJ871254.1
7	*Trichoderma hamatum* strain DAOM 167057 FJ763170.1
8	*Trichoderma hamatum strain DAOM 237553 EU279966.1*
9	*Trichoderma hamatum* strain DAOM 167057 FJ763170.1
10	*Trichoderma hamatum* DAOM 237553 EU279966.1
11	*Trichoderma hamatum* DAOM 237553 EU279966.1
12	*Trichoderma atroviride* DAOM:231653 KJ871097.1
13	*Trichoderma crassum* BMCC:LU555 KJ871144.1
B8	*Trichoderma austrokoningii* CBS:119080 KJ871163.1
16	*Trichoderma hamatum* strain CIB T144 EU279959.1
17	*Trichoderma spirale* BMCC:LU811 KJ871152.1
18	*Trichoderma crassum* BMCC:LU555 KJ871144.1
19	*Trichoderma crassum* BMCC:LU555 KJ871144.1
20	*Trichoderma spirale* BMCC:LU811 KJ871152.1
22	*Trichoderma atroviride* strain Th035 AB568378.1
23	*Trichoderma atroviride* strain CIB T93 EU279997.1
24	*Trichoderma atroviride* strain Th035 AB568378.1
25	*Trichoderma atroviride* strain DAOM242940 KX463436.1
26	*Trichoderma hamatum* strain DAOM 167057 FJ763170.1
B10	*Trichoderma koningii* BMCC:LU1310 KJ871254.1
28	*Trichoderma atroviride* strain CIB T93 EU279997.1
29	*Trichoderma harzianum* BMCC:LU631 KJ871182.1
30	*Trichoderma crassum* BMCC:LU555 KJ871144.1
31	*Trichoderma harzianum* strain DAOM242937 KX463434.1
32	*Trichoderma crassum* BMCC:LU555 KJ871144.1
33	*Trichoderma hamatum strain* CIB T144 FJ763171.1