Bioprotection - enhancing growth and health Research Programme

Bio-Protection
Bioprotection science for New Zealand

Colonisation and persistence of *Pinus radiata* cuttings with selected *Trichoderma* treatments

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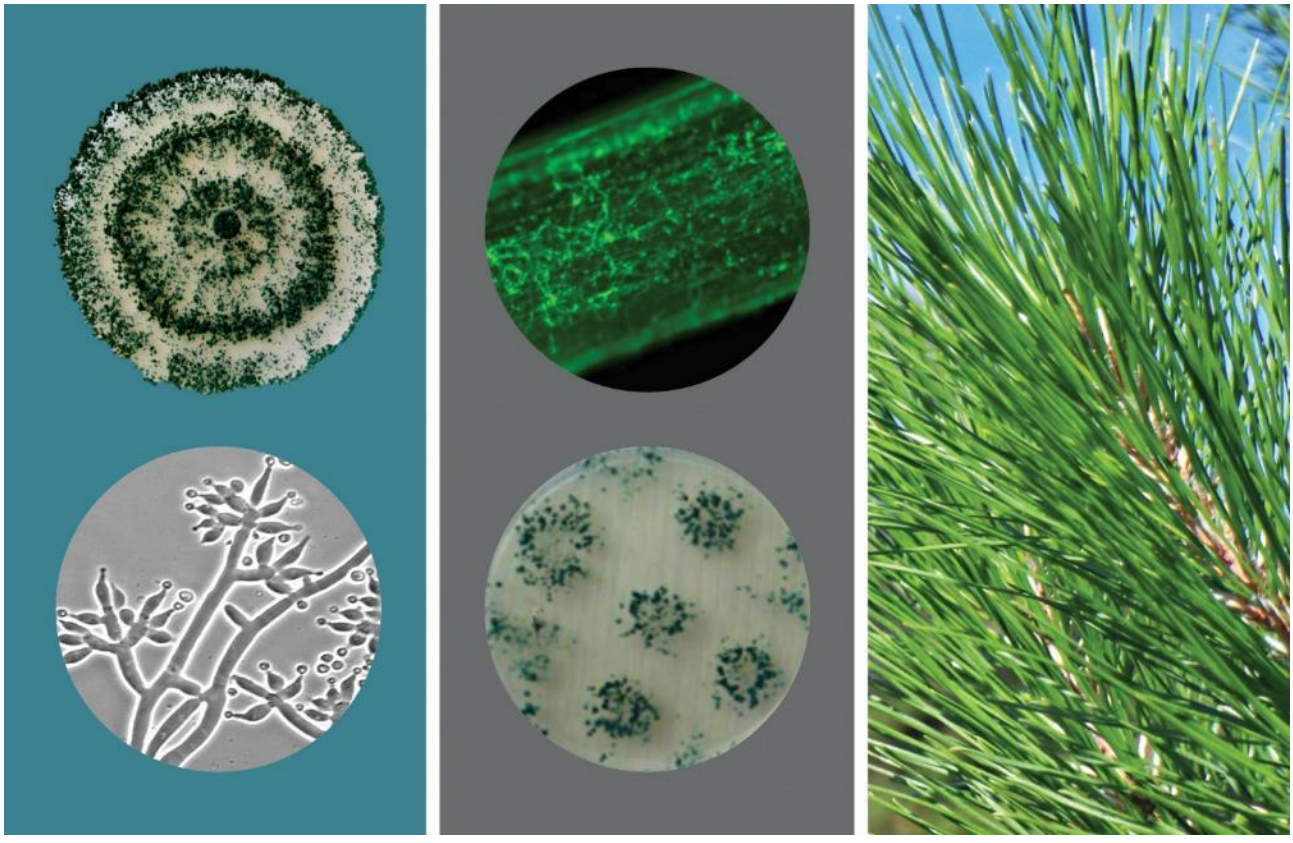

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FOREST GROWERS RESEARCH

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EXECUTIVE SUMMARY

Specific endophytic isolates of *Trichoderma* species have been used recently in *Pinus radiata* forestry bioprotection trials in New Zealand. An important question about deployment of these *Trichoderma* isolates is whether they are able to colonise and persist in the roots of *P. radiata* plants, potentially providing long-term protection in the forest. Previous studies have determined that these selected *Trichoderma* isolates are fast, abundant and persistent colonisers of containerised seedlings grown under nursery conditions. However, no information is available on the colonisation and persistence of *Trichoderma* in cuttings.

This report concludes a series of studies examining colonisation and persistence of a set of core *Trichoderma* isolates used in our forestry bioprotection research in *P. radiata* plants grown in nursery conditions. Colonisation and persistence were determined by direct re-isolation of *Trichoderma* cultures on agar plates and by fluorescent visualisation of fungal hyphae using confocal microscopy, in different surface-sterilised tissue types of *P. radiata* cuttings over a seven-month period.

Key Results

- Containerised *P. radiata* cuttings, grown in nursery conditions, were colonised by *Trichoderma* for at least the first seven months of growth. *Trichoderma* was found in three tissue types (callus, roots and needles towards the top and bottom of the cutting) at five and six months after setting and inoculation, with the highest levels (at least 80% of pieces colonised) in callus tissue.

- At seven months after setting and inoculation, colonisation levels had declined in callus and needle tissue, while levels were maintained or increased in roots, compared to earlier measurement dates.

- All isolates were found to have colonised the callus and roots, with LU633, FCC327, FCC13 and FCC14 being strong colonisers during the study (20–48% of root pieces were colonised, based on direct plating data). A lower level of colonisation was found in LU753, compared to the other *Trichoderma* isolates.

- The fluorescent labelling of fungal chitin and confocal microscopy allowed extremely detailed visualisation of fungal hyphae and their endophytic development in cutting callus and root tissues.

INTRODUCTION

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* programme is to induce systemic resistance against foliar diseases through the use of beneficial endophytes and elicitors. As part of this project, a number of forest trials have been established at sites around New Zealand to examine the effects of specific endophytic *Trichoderma* isolates on *Pinus radiata* growth and disease resistance. An important question about deployment of these *Trichoderma* isolates is whether they are able to colonise and persist in the roots of *P. radiata* plants, potentially providing long-term protection in the forest.

In these trials, *P. radiata* seeds were coated with the selected isolates of *Trichoderma* and the seedlings grown in containerised nursery conditions until deployment. Previous work (Hill *et al.,* 2016, Whelan and Hill, 2017) has shown *Trichoderma* capable of effectively establishing in seedling roots grown in nursery conditions. Colonisation of roots occurred during early growth, at or before the seedlings emerged from the soil (Hill *et al.,* 2016). During seedling development, abundant and persistent colonisation was found up to eight months after planting and inoculation (Whelan and Hill, 2017). In *P. radiata* nurseries, containerised cuttings are a common production stock; however, no information is available on the colonisation and persistence of *Trichoderma* in cuttings.

Two methods were used to successfully measure colonisation during early root growth in the Hill *et al*. (2016) and Whelan and Hill (2017) studies: plating of surface-sterilised root tissue on *Trichoderma* isolation agar, and examination of fluorescently labelled root tissue with fluorescence microscopy. The fluorescent labelling technique enabled detailed visualisation of *Trichoderma* endophytic colonisation in the roots. The dye reacted with the fungal chitin and its bright green fluorescent signature was easily seen in *Trichoderma*-treated roots once colonised. Confocal microscopy can overcome limitations of traditional fluorescence microscopy by using point illumination to improve optical resolution (Pawley, 1991), and was used in this study to improve image generation.

This report follows on from work described by Hill *et al.* (2016) and Whelan and Hill (2017) with the aim to determine the colonisation and persistence of selected *Trichoderma* isolates in *P. radiata* cuttings over a 7-month period.

METHODS

3.1 Seedling establishment and *Trichoderma* inoculation

P. radiata cuttings (harvested from plants grown from unrated seed and of 50 to 70 mm height) were set into plastic trays (BCC Sweden) with each tray containing 81 cells with a volume of 100 mL per cell (39 mm diameter and 85 mm depth) on 8 June 2018 (Figure 1). Each tray contained unsterilised potting mix (30 L composted bark, 15 L peat, 15 L perlite medium grade, 300 g Osmocote Exact (16-3.9-9.1, 12-14 months), 240 g gypsum, 90 g dolomite and 60 g Hydroflo wetting agent). Each treatment was applied to one tray and trays were placed into a shade house at Lincoln University Nursery.

Figure 1: Cuttings at setting on 8 June 2018.

Experimental treatments included five *Trichoderma* isolates (LU633, FCC327, FCC13, FCC14 and LU753) and a control (sterile water) treatment. Isolates were grown on malt yeast extract agar (MYE, Appendix A) at ambient temperature in natural light for 15 days. Spore suspensions were produced by flooding the plates with sterile 0.01% Tween 80, scraping the plates with a sterile scraper, and filtering the suspension through miracloth. Suspensions were diluted to a concentration of 1×10^6 conidia/mL based on haemocytometer counts. Spore germination was checked by mixing a diluted spore suspension with potato dextrose broth, transferring to microscope slides for 20 hours and counting germinated conidia with a compound microscope. Conidia were considered to have germinated if the germ tube length was greater than the diameter of the conidia. Conidial viability was at least 95% for all *Trichoderma* treatments.

Five mL of spore suspension was applied directly onto the potting mix in each cell using a pipette at cutting setting. Control trays received 5 mL of sterile distilled water. Trays were hand-watered lightly every second day to maintain media moisture levels. No chemicals were applied to control lichen growth (to avoid possible chemical interactions with the *Trichoderma*) and weeds were removed by hand.

3.2 Root colonisation and persistence measurements

Colonisation and persistence of *Trichoderma* isolates was determined using two techniques:

1. Re-isolation - incubation of surface-sterilised root pieces on *Trichoderma* isolation agar Malt Yeast Extract with Rose Bengal, (MRB; Appendix B)
2. Microscopic visualisation of surface-sterilised root pieces treated with fluorescent Wheat Germ Agglutinin - Alexa Fluor® 488 (WGA-AF488).

3.2.1 Re-isolation by direct plating

Eight seedlings were randomly sampled from each treatment at five, six and seven months after setting and *Trichoderma* inoculation. Cuttings were thoroughly washed with tap water and dissected into three tissue types: callus, root and needles (between 15 and 30 mm from the top of the cutting (top needles) and needles at the bottom of the cutting (bottom needles; Figure 2)). Tissues were dissected into 2-3 mm, 5-10 mm and 15 mm lengths for callus, root and needles respectively. Roots were only sampled at the six- and seven-month stage when they first became present. Tissue pieces were placed into a petri dish, soaked in Virkon (1% w/v) for 10 min for surface sterilisation and then rinsed in sterile distilled water. For direct plating, approximately 14, 20, 50 and 50 pieces were randomly sampled for callus, root, top and bottom needles in each treatment, respectively. Remainder pieces were used for fluorescent labelling.

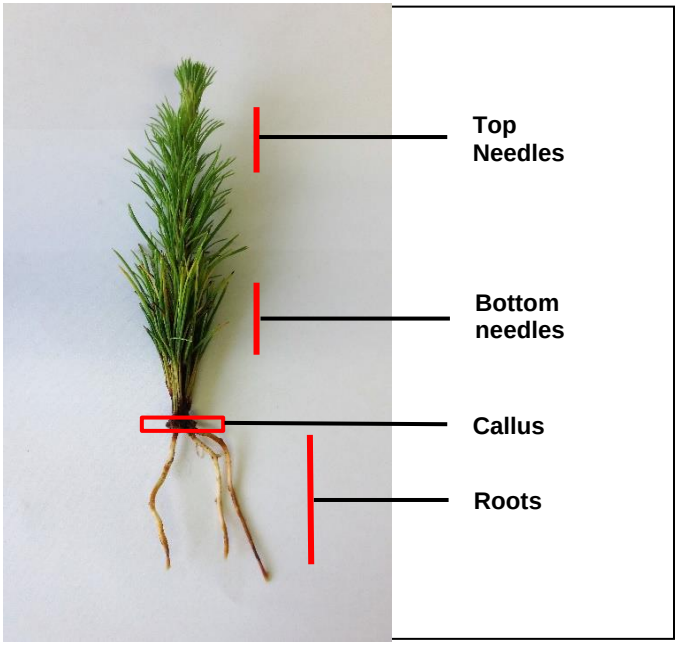

Figure 2: Tissue types sampled for direct plating.

For direct plating, tissue pieces were aseptically transferred onto replicate plates of MRB and incubated on a laboratory bench top with ambient light and temperature conditions for 14 days.

Following incubation, plates were visually assessed and the total number of *Trichoderma* colonies were counted. Percentage of pieces colonised for each tissue type were calculated by dividing colony number by total number of pieces in each treatment at each sampling date.

3.2.2 Confocal microscopy imaging

Two fluorescent dyes were used to visualise fungal and plant material:

1. WGA-AF488, which binds to fungal chitin (a component of fungal cell walls) and allows a visual contrast between fungal and plant cell walls (Vierheilig *et al.,* 2005), and
2. Direct Red 80, which binds to plant chitin (a component of plant cell walls) and allows visualisation of the plant cells.

Surface-sterilised tissue pieces (see section 3.2.1) were placed in small beakers and treated with 90°C potassium hydroxide (10% w/v) for 5 minutes to remove ('clear') the cell contents and cell wall pigments, then washed in tap water. Pieces were then bleached with 90°C hydrogen peroxide (3% w/v) for five minutes and washed in tap water.

Fifteen pieces of root and callus tissue in each treatment were placed in 2 mL Eppendorf vials with a mixture of approximately 100 µl of WGA-Alexa Fluor™ 488 (10 µg/mL w/v; Botium, Inc., https://biotium.com) and 100 µl Direct Red 80 (10 µg/mL w/v; Sigma-Aldrich, https://www.sigmaaldrich.com). Samples were infiltrated with the staining solution using a vacuum pump (Labconco CentriVap Concentrator) for ten minutes. The roots were de-stained of unbound fluorescent dye by placing in tap water for one to six hours. Roots were drained and then placed on a microscope slide. Confocal microscopy was performed using a LSM510 Meta instrument (Zeiss, Jena, Germany). Fluorescence was excited with an argon laser at 561 nm and detected at wavelengths of 505-550 nm (WGA-AF488) or 575 nm (Direct Red 80). Images were processed and arranged using Zen 2009 software (Zeiss, Jena, Germany).

RESULTS

In this study, the interaction between *P. radiata* cuttings and *Trichoderma* by plating of plant tissues on MRB agar and fluorescence confocal microscopy was observed.

4.1 Re-isolation by direct plating

Colonisation of cutting tissues, measured as colony growths on MRB agar, was found in most *Trichoderma* treatments at five months after setting and inoculation (Figure 3). However, colonisation levels changed in each tissue type as the cuttings grew.

Callus tissue was highly susceptible to colonisation and appeared to be the main entry point into the cutting for *Trichoderma* applied as a potting mix drench. Isolates LU633, FCC13, FCC327 and FCC14 were strong colonisers of callus tissue, with greater than 80% of pieces colonised at five and six months after setting and inoculation. At the same measurement dates, lower levels of inoculation (20% and 40%) were found in LU753 treatment. There were no obvious signs of necrosis in the callus tissue suggesting that *Trichoderma* can co-inhabit in *P. radiata* callus tissue without causing harm, even at very high levels of colonisation. At seven months after setting, colonisation levels in callus tissue had reduced in *Trichoderma* treatments by approximately 17 to 84%, compared to levels at six months.

Trichoderma in callus tissue may have aided in colonisation of roots due to the close proximity of the callus to the roots as they developed from the cambium. Colonisation of roots in *Trichoderma* treatments was at lower levels (ranged between 12 and 40%; Figure 3) at six months after setting and inoculation, compared to callus tissue, possibly because the roots were formed and colonised later and/or were less susceptible to *Trichoderma* than the callus tissue. At seven months after setting and inoculation, root colonisation levels were similar to, or had increased in all *Trichoderma* treatments (to between 19% and 48%), compared to the levels at six months. Isolates LU633, FCC327 and FCC14 were strong colonisers of root tissue, while no *Trichoderma* was present in the cuttings treated with isolate LU753 seven months after setting and inoculation.

The needles at the bottom of the cuttings were in close proximity to the inoculated potting mix and had *Trichoderma* colonisation levels of between 13% and 60% (Figure 3). However, levels dropped rapidly to approximately 3% at six and seven months after setting and inoculation. Two treatments, LU633 and FCC13, appeared to have the ability to colonise needles towards the top of the cutting at five months after setting and inoculation (when cuttings ranged between approximately 50 and 120 mm in height). However, at six and seven months after setting and inoculation, when cuttings ranged between approximately 120 and 200 mm in height, no *Trichoderma* were found in the top needles. While needles, both at the top and bottom of the cuttings, were initially susceptible to colonisation by *Trichoderma*, callus and root tissue became preferred habitats over time.

Untreated control cuttings had minimal or no *Trichoderma* present, indicating low levels of environmental *Trichoderma* in the cuttings or nursery, and therefore did not have an impact on the results.

Colonisation of cutting roots, measured as colony growths on MRB agar, was similar to that found in seedling roots at the same root age (Whelan and Hill, 2017), although isolates FCC327 and LU753 had lower levels in this study.

It was observed that the inoculated cuttings appeared to have more callus development and cutting shoot extension at each sampling date (Figure 4), particularly isolates FCC327 and FCC14. Survival at seven months after setting also appeared to be higher in the inoculated cuttings compared to the untreated control cuttings (Figure 5).

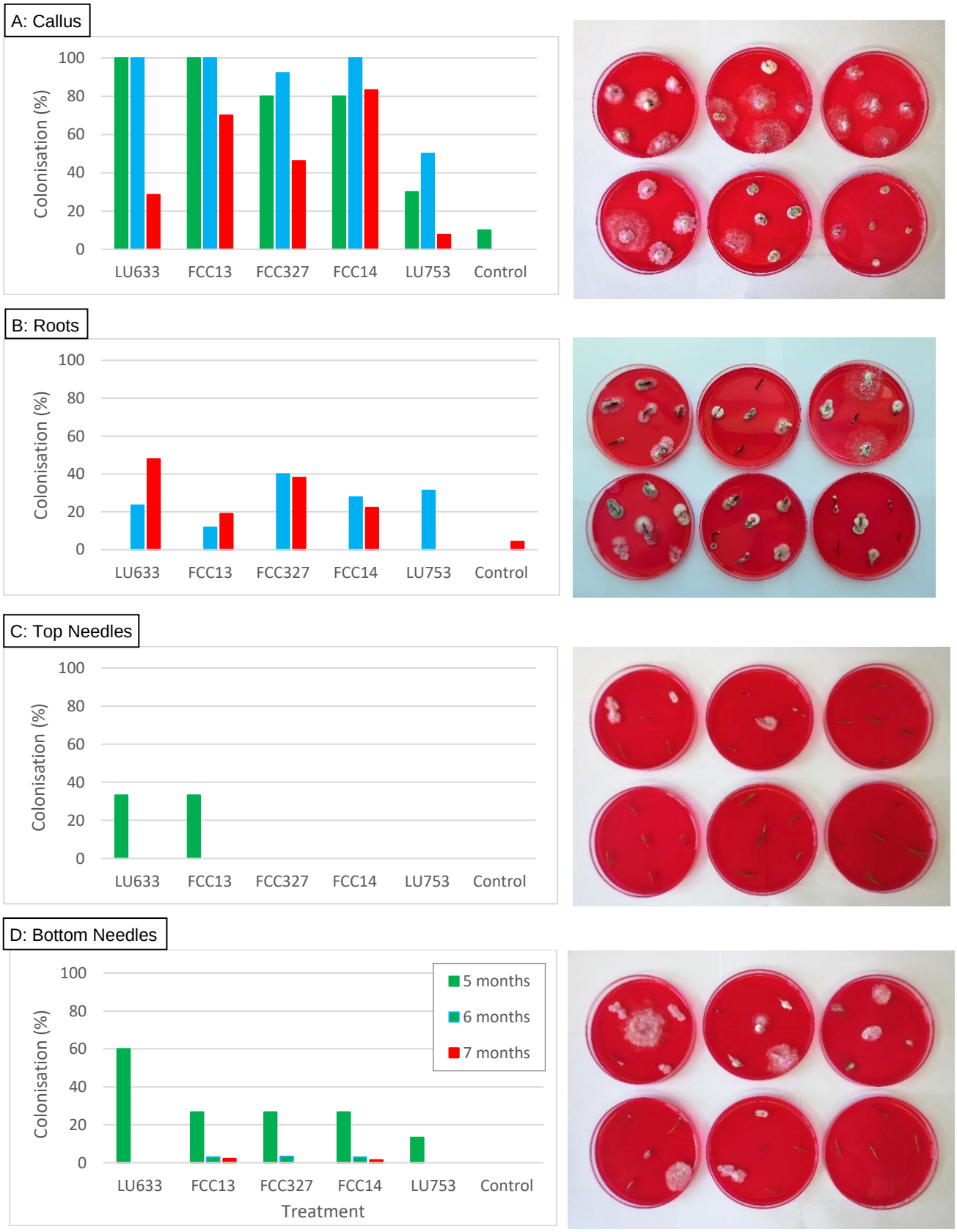

Figure 3: Colonisation (%) of *Trichoderma* isolates in *P. radiata* cutting (a) callus, (b) root, (c) top needles and (d) bottom needles, 5 (green bars), 6 (blue bars) and 7 (red bars) months after setting and *Trichoderma* inoculation, based on MRB plating data. Colonisation (%) of roots was only measured at 6 and 7 months after setting and *Trichoderma* inoculation. Right images: colony growth on MRB agar of callus, top and bottom needles sampled 5 months, and of roots, sampled 7 months, after setting and *Trichoderma* inoculation. Isolates from top left to bottom right: LU633, FCC13, FCC327, FCC14, LU753 and Control. Non-*Trichoderma* colonies were generally *Penicillium* species.

Figure 4: Growth of (a) FCC 327 inoculated (left) and untreated (Control; right) treated cuttings five months after setting and *Trichoderma* application. Note the (b) highly, and (c) poorly developed callus tissue in the FCC327 and Control cuttings respectively.

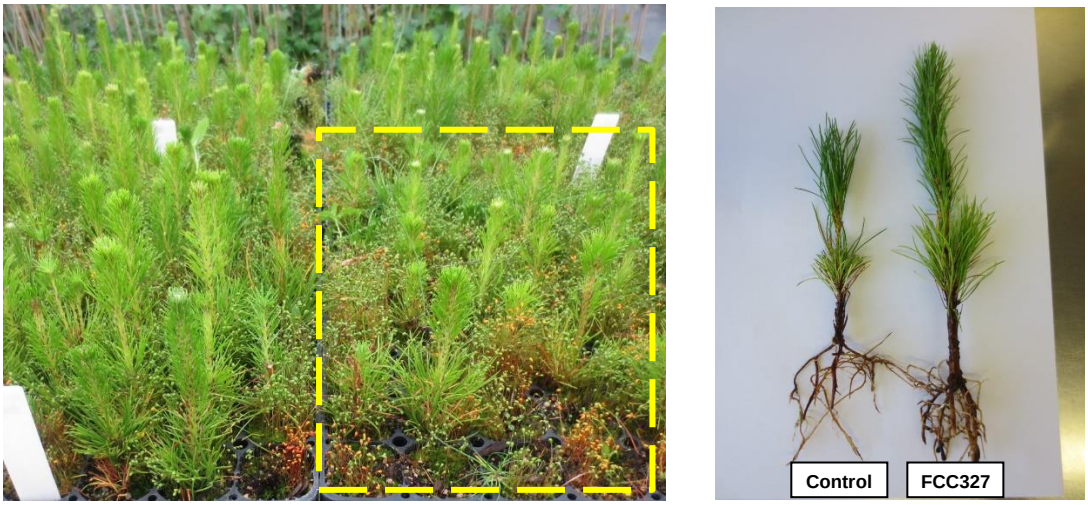

Figure 5: Cutting growth seven months after setting and *Trichoderma* inoculation in the FCC327 treatment (left) and untreated Control (yellow zone) trays. Right image is individual cutting growth in Control and FCC327 treatment.

4.2 Confocal microscopy imaging

Visualisation of fungal colonisation in *P. radiata* cuttings was achieved by examining root and callus tissue labelled with fluorescent WGA-AF488 using a confocal microscope. Hyphae exhibited a bright green fluorescent image which was in contrast to the plant cell walls stained with Direct Red 80.

The following features were observed:

1. A very close and abundant interaction of *Trichoderma* hyphae and individual root cells (Figure 6) with hyphae generally growing between the cells in the intercellular and middle lamella spaces (Figure 7). A few examples of intracellular hyphal development were found and were similar in number to that found in the Whelan and Hill (2017) seedling study. Hyphae may find the path between the plant cell walls to be of least physical resistance and/or not have the ability to successfully colonise inside the cell on a frequent basis. There appeared to be no obvious signs of physical constriction to the growth of the plant cells caused by the development of fungal hyphae.
2. Callus cells were highly colonised with hyphae (Figure 8) but there was no obvious growth pattern of the hyphae, probably due to the callus parenchyma cells being unstructured.
3. Root cells were initially colonised by hyphae growing parallel along the main axis of the root (Figure 9) with minimal branching. As the root cells aged, hyphae were observed growing in parallel along, and in transverse to, the main axis of the root and without any particular orientation (Figure 10). In addition, hyphae were often highly branched.
4. A few root cells were highly colonised with hyphae (Figure 10) but no localised dense mats of hyphae were observed between five and seven months after setting and inoculation. There appeared to be no sign of necrosis in the root cells.
5. At seven months after setting and inoculation, hyphae had colonised the rhizodermal, sub-epidermal and cortex cells of the roots, but not the immature xylem vessels (Figure 7).

Fungal endophytic colonisation patterns in roots of cuttings, visualised using confocal microscopy, were similar to those found in roots of seedlings (Hill *et al*., 2016, Whelan and Hill, 2017), although hyphal development was less advanced in this study (i.e. no dense hyphal mats were observed and hyphae were not found in the xylem vessels) due to the shorter time for root development.

In this series of studies, both traditional fluorescence and confocal microscopy were equally useful at generating imagery of the interaction between root cells and *Trichoderma.* Confocal microscopy may be more useful than fluorescence microscopy for generation of extremely high resolution images (x 400 or 630), but this was not attempted in this study.

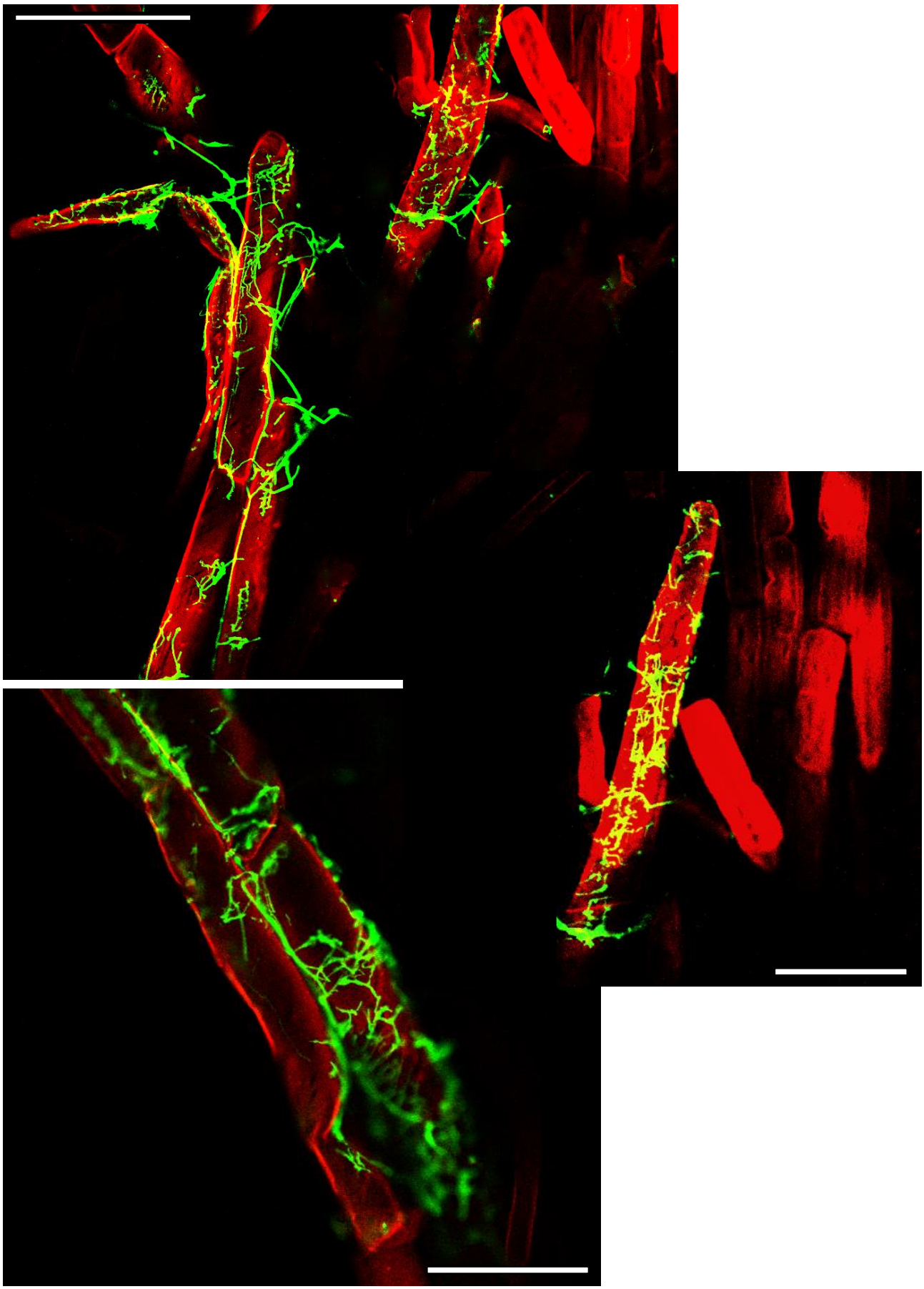

Figure 6: *Trichoderma* hyphae (green) of isolate FCC327 closely associated with individual *P. radiata* cutting root cells (red). Bars = 50 μm.

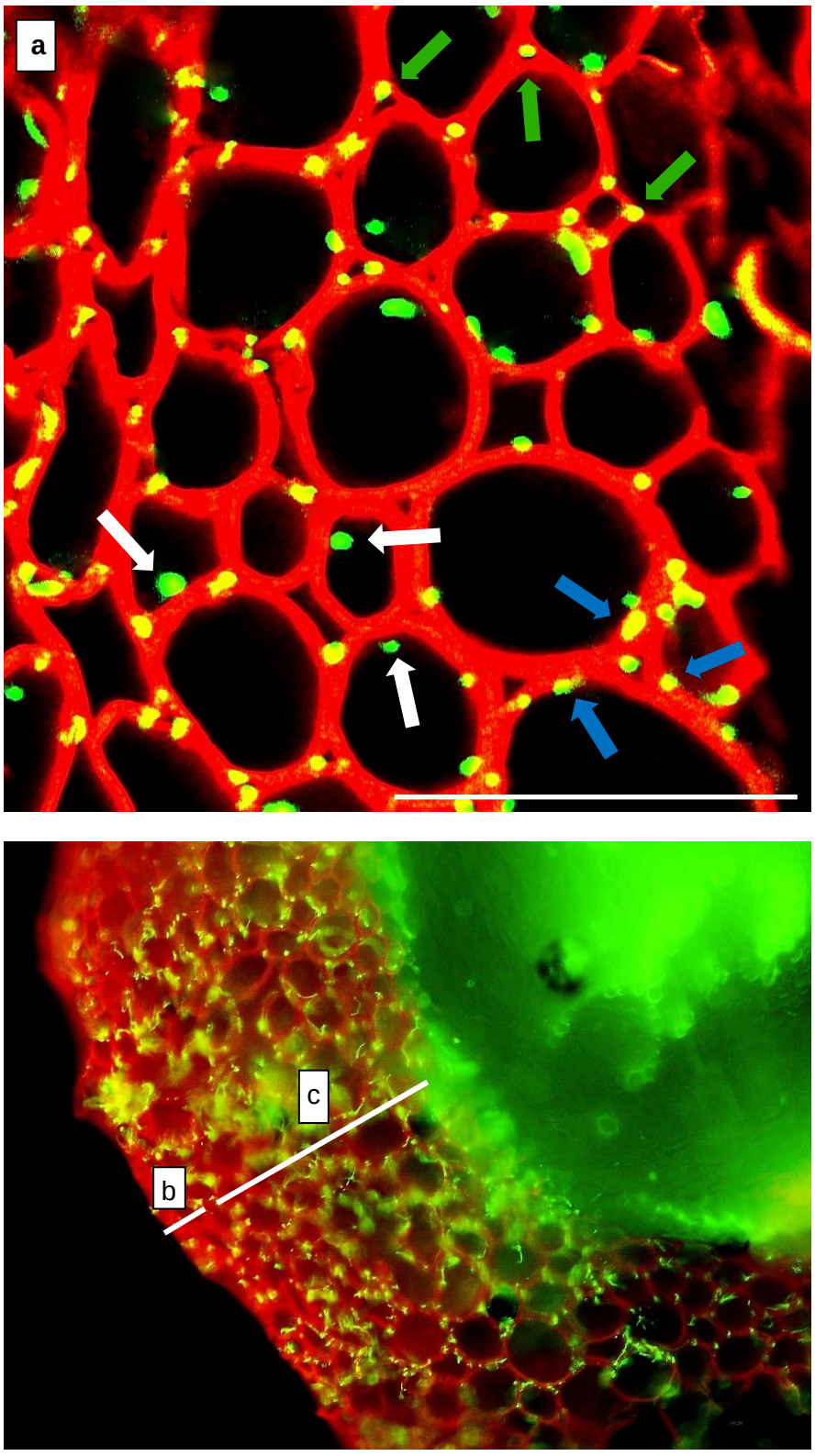

Figure 7: *Trichoderma* hyphae of isolate LU633 found in the (a) intercellular (blue arrows), intracellular (white arrows) and middle lamella spaces (green arrows) in the (b) rhizodermal and sub-epidermal and (c) cortex cells of cross-sectional pieces of *P. radiata* roots seven months after setting of cuttings and *Trichoderma* inoculation. Bars = 100 μm.

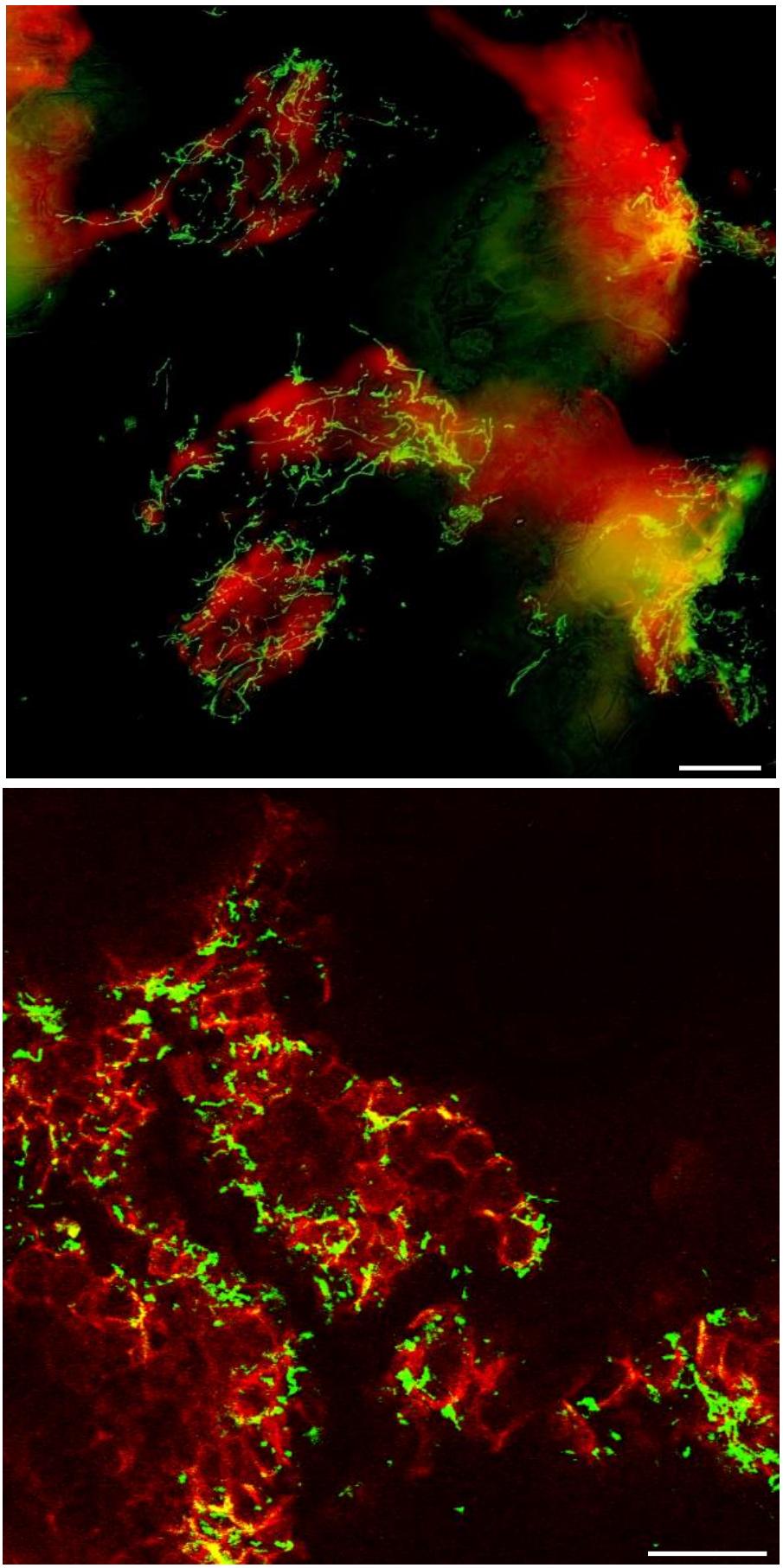

Figure 8: Hyphae of *Trichoderma* isolate FCC327 in callus pieces of *P. radiata* cuttings six months after setting of cuttings and *Trichoderma* inoculation. Bars = 100 *µ*m.

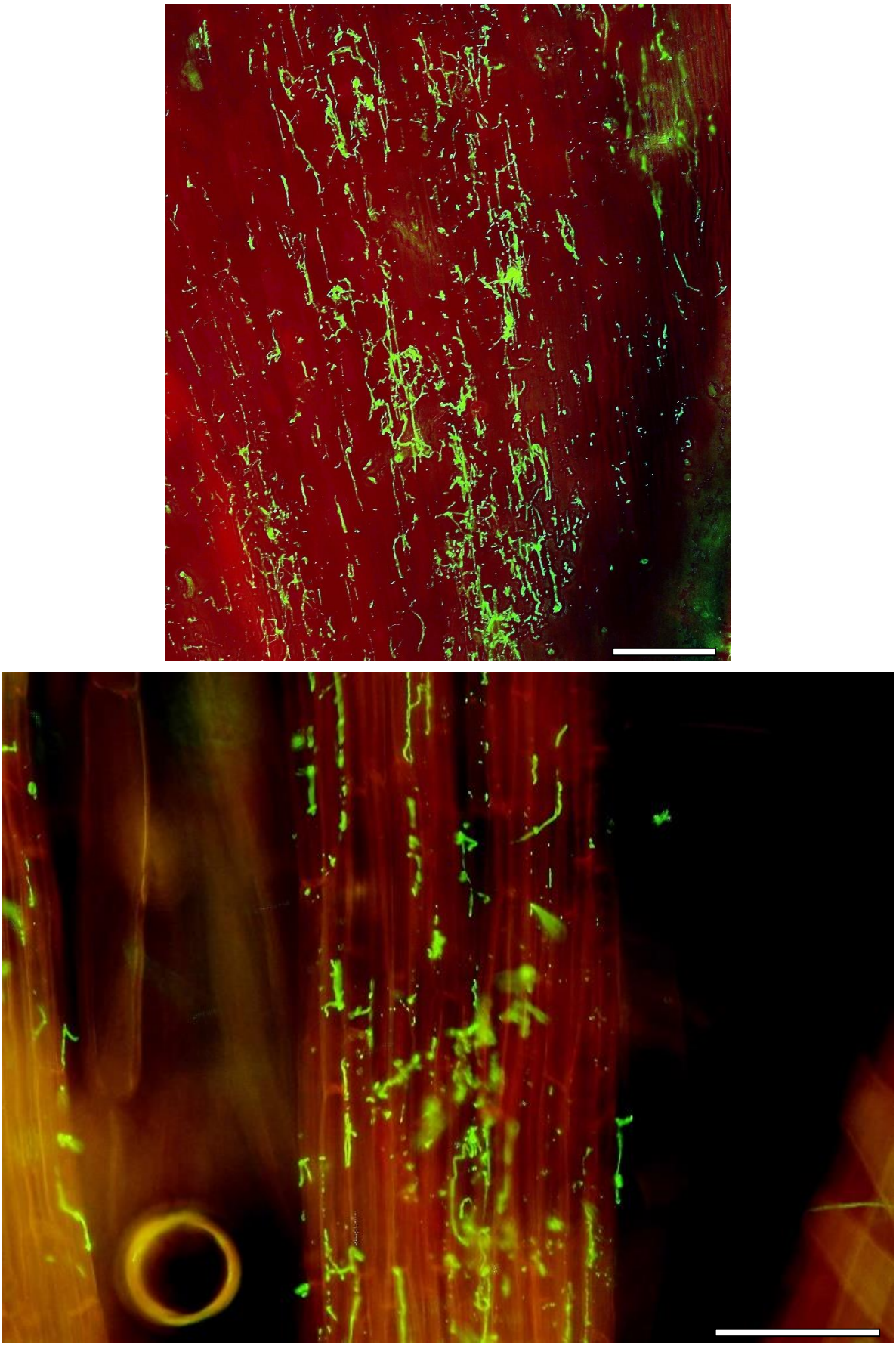

Figure 9: Early hyphal development of *Trichoderma* isolate FCC14 in *P. radiata* root pieces at five months after setting of cuttings and *Trichoderma* inoculation. Bars = 100 *µ*m.

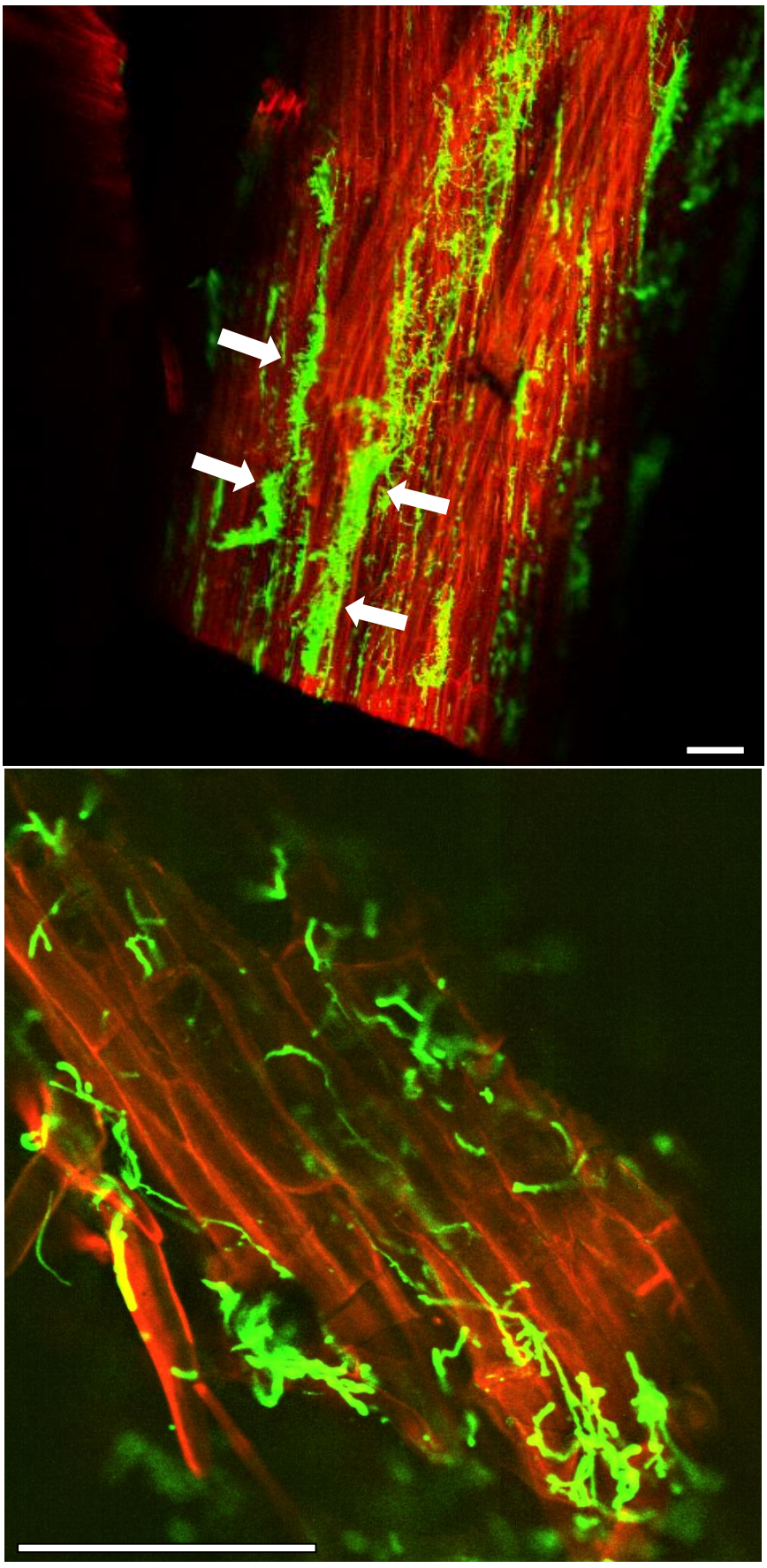

Figure 10: Extensive hyphal development of *Trichoderma* isolate FCC327 growing parallel along and in transverse to the main axis and without any particular orientation, in *P. radiata* roots seven months after setting of cuttings and *Trichoderma* inoculation. Plant cells fully colonised by hyphae are indicated with arrows. Bars = 100 μm.

CONCLUSION

In this study a selection of *Trichoderma* isolates, used in New Zealand forestry trials, were found to colonise and persist in nursery cuttings for at least seven months after setting and inoculation. *Trichoderma* was found in callus, roots and needles at the top and bottom of the cuttings at five and six months after setting and inoculation, with the highest levels (between 80% and 100%) in callus tissue. After seven months of growth, the preferred habitat for *Trichoderma* was the callus and roots, with isolates LU633, FCC327, FCC13 and FCC14 being strong colonisers.

This study showed that growing conditions similar to those found in *P. radiata* nurseries were conducive to *Trichoderma* colonisation but further work is required to determine the persistence of *Trichoderma* isolates in cuttings over longer periods, particularly once planted in the forest.

The fluorescent labelling technique and confocal microscopy enabled extremely detailed visualisation of *Trichoderma* endophytic activity in the cutting roots, including:

- a very close and abundant interaction of *Trichoderma* hyphae with individual callus and root cells
- growth of hyphae between root cells often in the intercellular and middle lamella spaces and occasionally inside the cells
- hyphae colonisation of the rhizodermal, sub-epidermal and cortex cells
- hyphae development in all directions within the callus and root with the hyphae often being highly branched.

ACKNOWLEDGEMENTS

Patrick Milne, Southern Cypresses, Kaiapoi, Canterbury, is greatly thanked for providing guidance and cutting material.

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APPENDICES

Appendix A: Malt Yeast Extract Agar (MYE) Recipe

Malt extract	10 g
Yeast extract	1 g
Agar	20 g

Make up to 1 L with distilled water.

Appendix B: 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.