Bioprotection - enhancing growth and health Research Programme

Bio-Protection
Bioprotection science for New Zealand

Colonisation and Persistence of *Pinus radiata* seedlings with selected *Trichoderma* treatments

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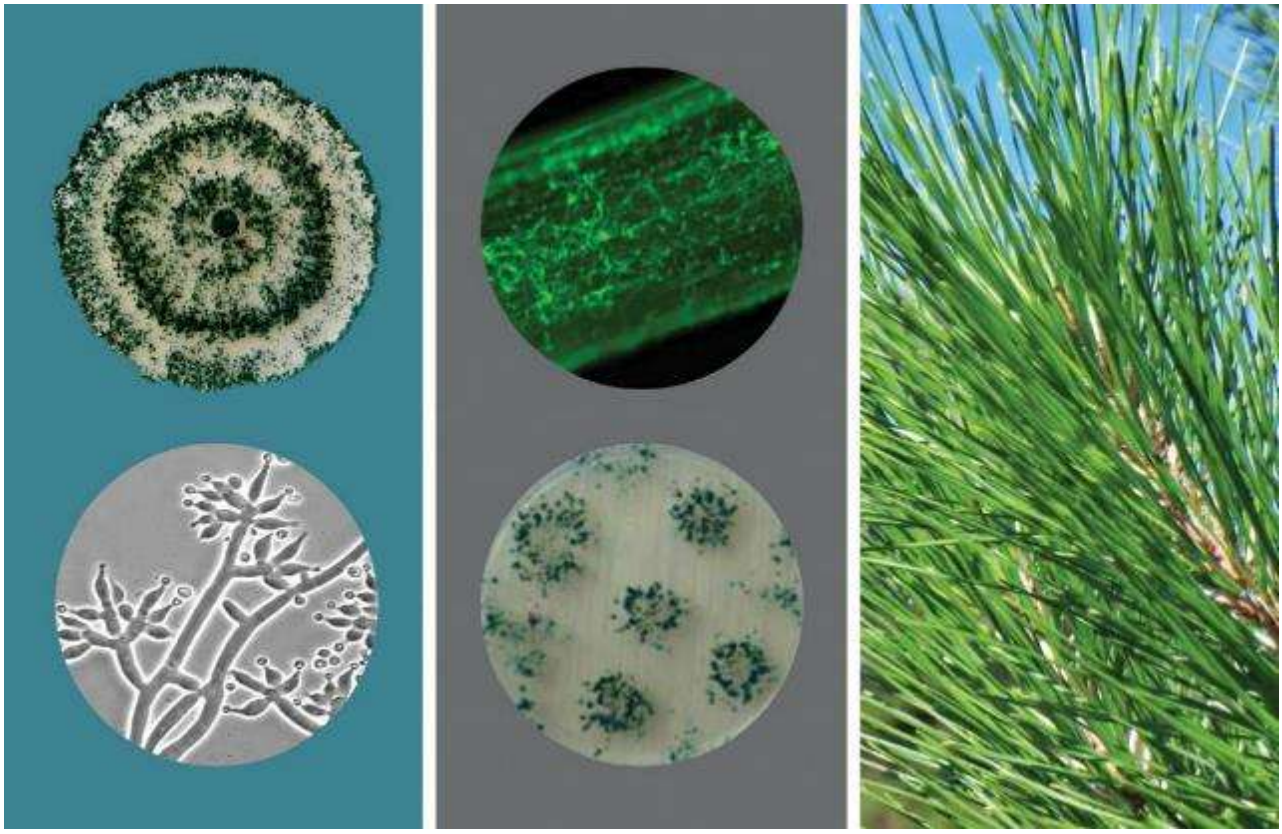

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TABLE OF CONTENTS

EXECUTIVE SUMMARY

Trichoderma fungi show great potential for improving health of forest trees. 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 NZ to examine the effects of specific endophytic *Trichoderma* strains on *Pinus radiata* growth and disease resistance. An important question about deployment of these *Trichoderma* strains is whether they are able to persist and become established in the roots of *P. radiata* plants, potentially providing long-term protection in the forest.

In this report we describe results from a glasshouse experiment examining the root colonisation and persistence of a set of core *Trichoderma* isolates used in our forestry bioprotection research in *P. radiata* seedlings. Colonisation and persistence were determined by direct re-isolation of *Trichoderma* cultures and by visualisation of fungal structures using fluorescence microscopy over an 8-month period. In addition, a selection of *Trichoderma* isolates cultured from seedlings with the LU633 isolate treatment were tested with a sensitive, strain-specific real-time polymerase chain reaction (PCR) assay to confirm the presence of the applied LU633 strain.

Key Results

- The fluorescent imagery system (which detects fluorescently-labelled fungal chitin) allowed extremely detailed visualisation and quantitation of *Trichoderma* endophytic colonisation of root tissue.

- *Trichoderma* re-isolation and fluorescent labelling techniques showed that all isolates had colonised the roots 2.5 months after planting and inoculation and these colonisation levels were generally maintained or increased at 8 months after planting.

- Eight months after planting, isolate FCC327 was found to be the strongest coloniser of *P. radiata* roots with 65% and 100% of root pieces infected in the re-isolation and fluorescent labelling techniques respectively.

- Lower levels of colonisation were found in the other isolates (LU633, FCC13, FCC14 and LU753) and ranged between 31-37% and 60-78% in the re-isolation and fluorescent labelling techniques respectively, at 8 months after planting.

- *Trichoderma* colonisation was greater in tap root, compared to lateral root tissue at each measurement time.

- Colonisation of seedling roots with the *Trichoderma atroviride* strain LU633 was verified using a strain-specific probe-based real time PCR assay.

INTRODUCTION

Specific strains of *Trichoderma* species have been used recently in bioprotection forestry trials in New Zealand, but it is not known how long these strains persist in the pine roots*. Trichoderma* strains have been found to colonise roots of *Pinus radiata* seedlings at an early stage of seedling growth, at or before seedlings emerge from the soil (Hill *et al.,* 2016). This report follows on from the work described by Hill *et al.,* (2016) with the primary aim of Task 2.2 being to determine the colonisation and persistence of selected *Trichoderma* strains in *P. radiata* seedling roots over an 8-month period.

In the Hill *et al*., (2016) study, two methods were used to determine colonisation: plating of surface-sterilised root tissue on *Trichoderma* isolation medium, and microscopic examination of root tissue after fluorescent labelling. The fluorescent labelling technique enabled detailed visualisation of *Trichoderma* endophytic activity in the roots. The dye reacted with the fungal chitin and its bright green fluorescent signature was easily seen in *Trichoderma*-treated roots once infected. Both methods were successful in measuring colonisation during early root growth but changes to the fluorescent labelling technique were suggested to improve image generation. The second aim of Task 2.2 was to further develop this technique and to apply it to *P. radiata* roots of older age.

METHODS

3.1 Seedling Establishment and Isolate Application

P. radiata seeds (sourced from PF Olsen Seeds, Seddon, New Zealand) were stratified by soaking overnight, placing on moist paper towels and storing at 4°C in the dark for 12 days. Seeds were sown on 31 March 2017 in plastic trays (BCC Sweden) with each tray containing 81 cells with a volume of 100mL per cell (39mm diameter and 85mm depth). One seed was sown in alternate cells, at a depth of 10mm, into unsterilised potting mix (30L composted bark, 15L peat, 15L perlite medium grade, 300g Osmocote Exact (16-3.9-9.1, 12-14 month), 240g gypsum, 90g dolomite and 60g Hydroflo wetting agent). Each treatment was applied to two trays and the trays were arranged in a completely randomised block design in a glasshouse at Lincoln University (Appendix A).

Experimental treatments included five *Trichoderma* isolates (LU633, LU753, FCC13, FCC14 and FCC327) and a control (sterile water) treatment. Isolates were grown on malt yeast extract agar (MYE, Appendix B) 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 97% for all *Trichoderma* treatments, apart from LU753 at 92%.

Five ml of spore suspension was applied to each cell in the seedling trays at planting. Control trays received 5ml of sterile distilled water. The potting mix was covered with a plastic sheet for the first week to maintain moisture levels, then hand-watered once a day.

Seedlings emerged 2 weeks after planting. The trays were transferred from the glasshouse to a shaded nursery area at 3 months of age then to an uncovered area at 5 months of age until final harvest.

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 medium Malt Yeast Extract with Rose Bengal, (MRB; Appendix C)
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

A total of 8 seedlings were randomly sampled from both trays in each treatment at 3 times:

1. 2.5 months after planting and inoculation
2. 5 months after planting and inoculation
3. 8 months after planting and inoculation

Roots were thoroughly washed with tap water, dissected into two types (tap and lateral roots) and sectioned into approximately 5 to 10mm lengths. The root pieces were placed into a petri dish and soaked in Virkon (1% w/v) for 10 min for surface sterilisation. After rinsing in sterile distilled water, the root pieces were used for either direct plating or root labelling (see section 3.2.2).

For direct plating, five or six pieces per treatment and root type were aseptically transferred onto one of each of five replicate plates of MRB. Agar plates with roots were 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 root pieces colonised for each root type were calculated by dividing colony number by total number of pieces in each treatment at each sampling date. At each sampling date, an overall percentage of root pieces colonised for each treatment was calculated by dividing the colony number of two tap root plates plus five lateral root plates by total number of pieces.

3.2.2 Root Microscopy

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

i) 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)

ii) Direct Red 80 which binds to plant chitin (a component of plant cell walls) and allows visualisation of the plant cells.

Surface sterilised root pieces (see section 3.2.1) were placed in small beakers and treated with 90°C potassium hydroxide (10% w/v) for 10 minutes to remove ('clear') the cell contents and cell wall pigments, then washed in tap water. A few additional seedlings were harvested but the roots were not treated with Virkon to allow observation of the fungal hyphae on the root surface.

Fifteen root pieces from each root type and *Trichoderma* treatment were placed in 2ml Eppendorf vials with a mixture of approximately 100µl of WGA-AF488 (10µg/ml w/v; Botium, Inc., https://biotium.com) and 100µl Direct Red 80 (10µg/ml w/v; Sigma-Aldrich, https://www.sigmaaldrich.com). The solution was vacuum infiltrated into the tissue using a vacuum pump (Labconco CentriVap Concentrator) for 10 minutes. The roots were de-stained of unbound fluorescent dye by placing in tap water for 1 hour to 1 week. Roots were drained and then placed on a microscope slide and viewed using an Olympus BX51 Compound Microscope with a U-RFL-T Burner. WGA-AF488 was excited at 495nm and detected at 519nm, while Direct Red 80 was excited at 546nm and detected at 590nm. Images were generated using the cell^F computer programme and where appropriate, the WGA-AF488 image was superimposed onto the Direct Red 80 image.

Root colonisation, based on fluorescent labelling data, was quantified using a modified frequency and intensity rating system of Trouvelot *et al.,* (1986).

COLONISATION FREQUENCY (C_F) (the level of fungal incidence in root pieces) was determined as:

$$C_F = N_e/N_t * 100$$

where N_e = number of root pieces colonised and N_t = total number of root pieces.

COLONISATION INTENSITY (C_I) (the level of fungal severity in root pieces), was based on visually rating the area colonised by hyphae using a rating from 0 to 5 (refer to Figure 2 of Hill *et al.,* 2016) where:

Rating 0 = no colonisation
1 = 0.1 – 1% colonisation
2 = 2 – 10% colonisation
3 = 11 – 50% colonisation
4 = 51 – 90% colonisation
5 = 91%+ colonisation

Colonisation Intensity was calculated as:

$$C_I = (95.n_5 + 70.n_4 + 30.n_3 + 6.n_2 + 0.5.n_1)/ N_t$$

where n_5, n_4, n_3, n_2 and n_1= number of root pieces which were given that rating and N_t = total number of root pieces measured.

3.2.3 Verification of *Trichoderma* Isolate LU633 Infection

Colonisation of roots with the *Trichoderma atroviride* strain LU633 were verified using a sensitive, species-specific probe-based real time PCR assay. Details of methods are described in Mellow *et al.,* (2015). Specific LU633 primers used were RM5ka and RM6ka. The LU633 specific fluorescent probe used was TrichKa with the 5'and 3' ends labelled with 6-carboxyfluorescein (FAM) and quencher dye BHQ-1 respectively. Real time assays were done using the StepOnePlus™ Real-Time PCR System (Applied Biosystems™).

Thirteen random *Trichoderma* colonies cultured from seedlings treated with isolate LU633 were tested. Cultures were selected from tap and lateral root samples taken at the first and second assessments.

RESULTS AND DISCUSSION

4.1 Root Colonisation and Persistence

4.1.1 Plating with *Trichoderma* Isolation MRB Agar

Colonisation of root pieces, measured as colony growths on MRB plates, was found in all *Trichoderma* treatments at the three measurement times (Figure 1). Isolate FCC327 was a very strong coloniser, with 78% of the root pieces colonised at 2.5 months after seed planting and inoculation. At the same measurement, lower levels of infection, ranging from 17% to 35%, were found in the other *Trichoderma* treatments. By 5 months after planting, levels of colonisation had reduced in each treatment except isolate LU753 which maintained 18% colonisation. However, at 8 months after planting, colonisation increased in all isolates, compared to the levels at 5 months, and ranged from 20% (isolate FCC14) to 65% (FCC327). At 8 months *Trichoderma* hyphae may have had more time to grow in the roots that were beginning to become restrained by the dimensions of the planter cells. There were no obvious signs of necrosis in either the tap or lateral roots infected with the *Trichoderma* strains. This would imply that they can co-inhabit in *P. radiata* tap roots for long periods of time without causing harm, even at high levels of infection.

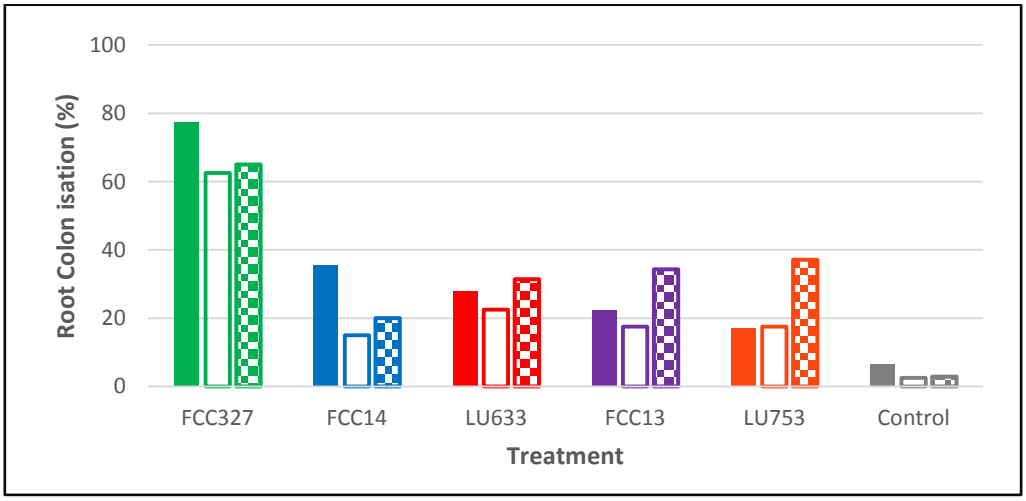

Figure 1: Colonisation (%) of *Trichoderma* isolates in *P. radiata* roots sampled at 2.5 (solid bars), 5 (open bars) and 8 (hatched bars) months after planting, based on MRB plating data. Mean of 5 lateral and 2 tap root plates.

The untreated control plants showed a small number of *Trichoderma* colonies (3 to 6%), probably from environmental *Trichoderma* strains (e.g. from the potting mix and/or airborne spores in the glasshouse).

During all stages of growth, higher levels of colonisation occurred in the tap root compared to the lateral root pieces, except for isolate LU753 where the amounts were generally similar (Figure 2). This was likely to be due to the longer period available for colonisation in the tap roots.

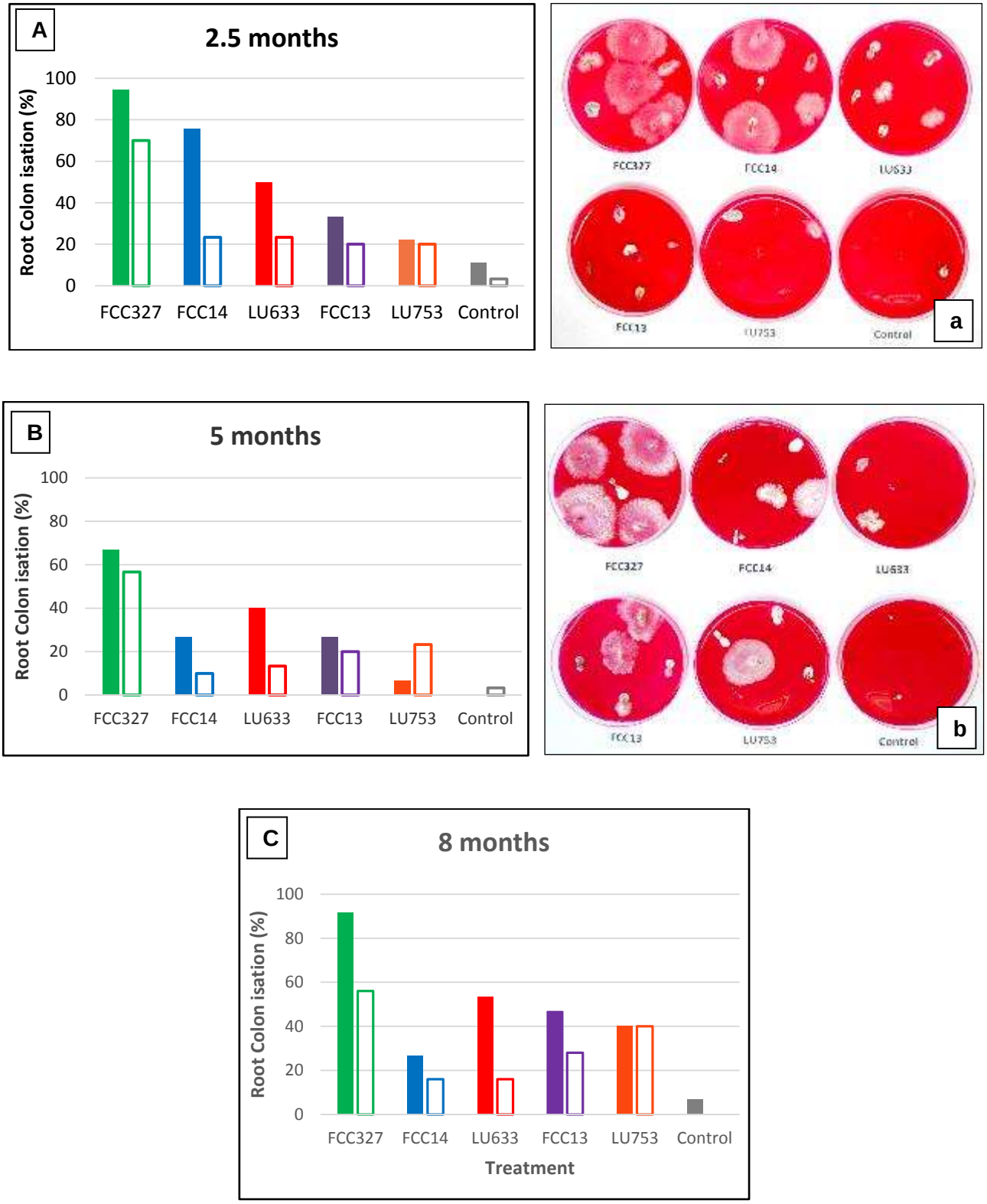

Figure 2: Colonisation (%) of *Trichoderma* isolates in *P. radiata* tap (solid bars) and lateral (open bars) roots sampled (A) 2.5 months, (B) 5 months and (C) 8 months after planting, based on MRB plating data. Colony growth on MRB plates at (a) 2.5 and (b) 5 months after emergence for tap root samples. Non-*Trichoderma* colony growths were generally *Penicillium* species.

4.1.2 Root Labelling with WGA-AF488

Fluorescent Imagery
Visualisation of fungal colonisation was achieved by examining root tissue labelled with fluorescent WGA-AF488. Hyphae exhibited a bright green fluorescent image which was often more intense than the surrounding plant cell walls.

Fungal endophytic colonisation patterns were generally similar to the fluorescent imagery study of Hill *et al.,* (2016) in *P. radiata* seedlings. However, improvements in labelling techniques, particularly the addition of Direct Red 80 to define plant cell walls, led to images with greater definition**.** The following features were observed:

1. A very close association of *Trichoderma* hyphae and individual plant cells (Figure 3) with hyphae generally growing between the plant cells often in the intercellular and middle lamella spaces (Figure 4). Hyphae may find this path to be of least physical resistance. Some examples of intracellular hyphal development were found (Figure 4) but were fewer in number compared to the 2016 study. 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. Some plant cells were highly colonised with hyphae (Figures 5 and 6) and appeared as localised dense mats of hyphae. There appeared to be no sign of plant cell necrosis in these cells.
3. Root hairs were a common entry point for colonisation by *Trichoderma* hyphae (Figures 5 and 6). Hyphal penetration of the root surface was also observed (Figure 7).
4. Hyphae colonised the rhizodermal, sub-epidermal, cortex and young xylem vessel cells (Figure 4 and 8). However, over time visualisation of the fungal hyphae in the centre of the root pieces became difficult due to the high natural fluorescence ("auto-fluorescence") of the primary and secondary xylem cell wall pigments (Figure 9). Changes to the methodology were not able to overcome the strong auto-fluorescence characters of these mature plant cells.
5. Hyphae were found 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 (Figure 6, 10 and 11).
6. Approximately half of the root tips observed at 2.5 and 5 months after planting were colonised with *Trichoderma* hyphae. Some roots were highly colonised at the tip end (Figure 11).

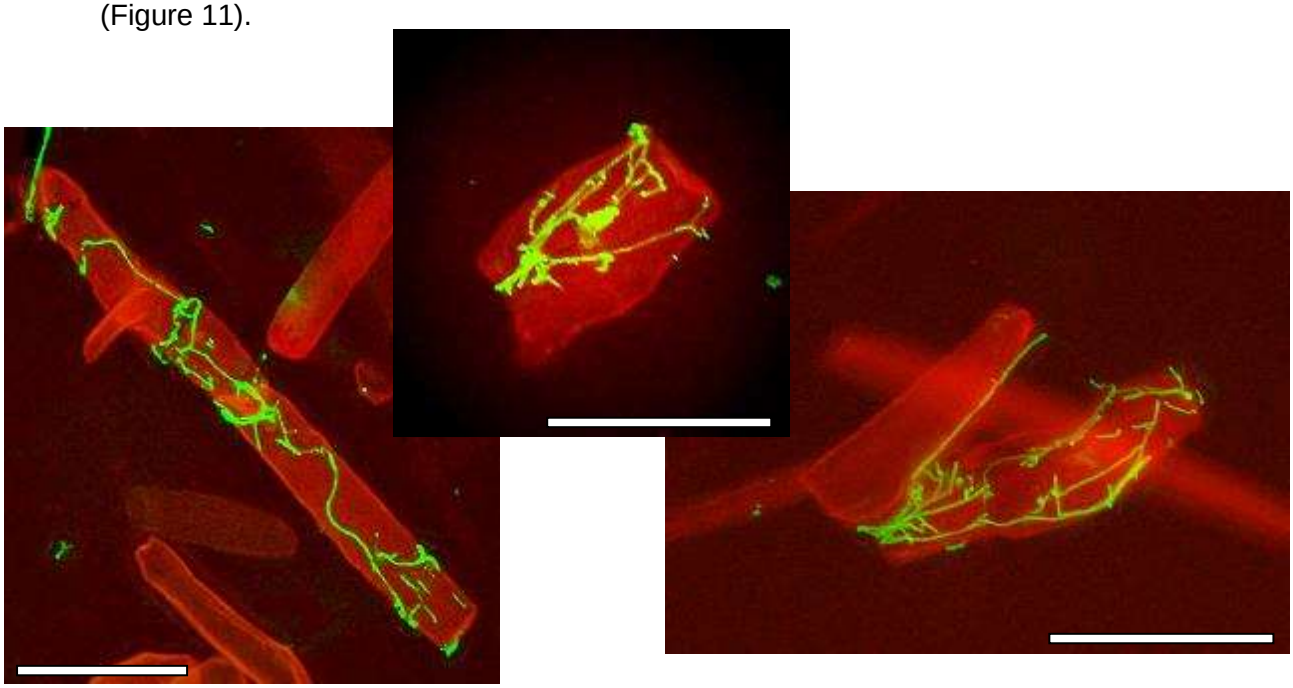

Figure 3: *Trichoderma* hyphae (isolate FCC327) highly associated with individual *P. radiata* cells. Bars = 50μm.

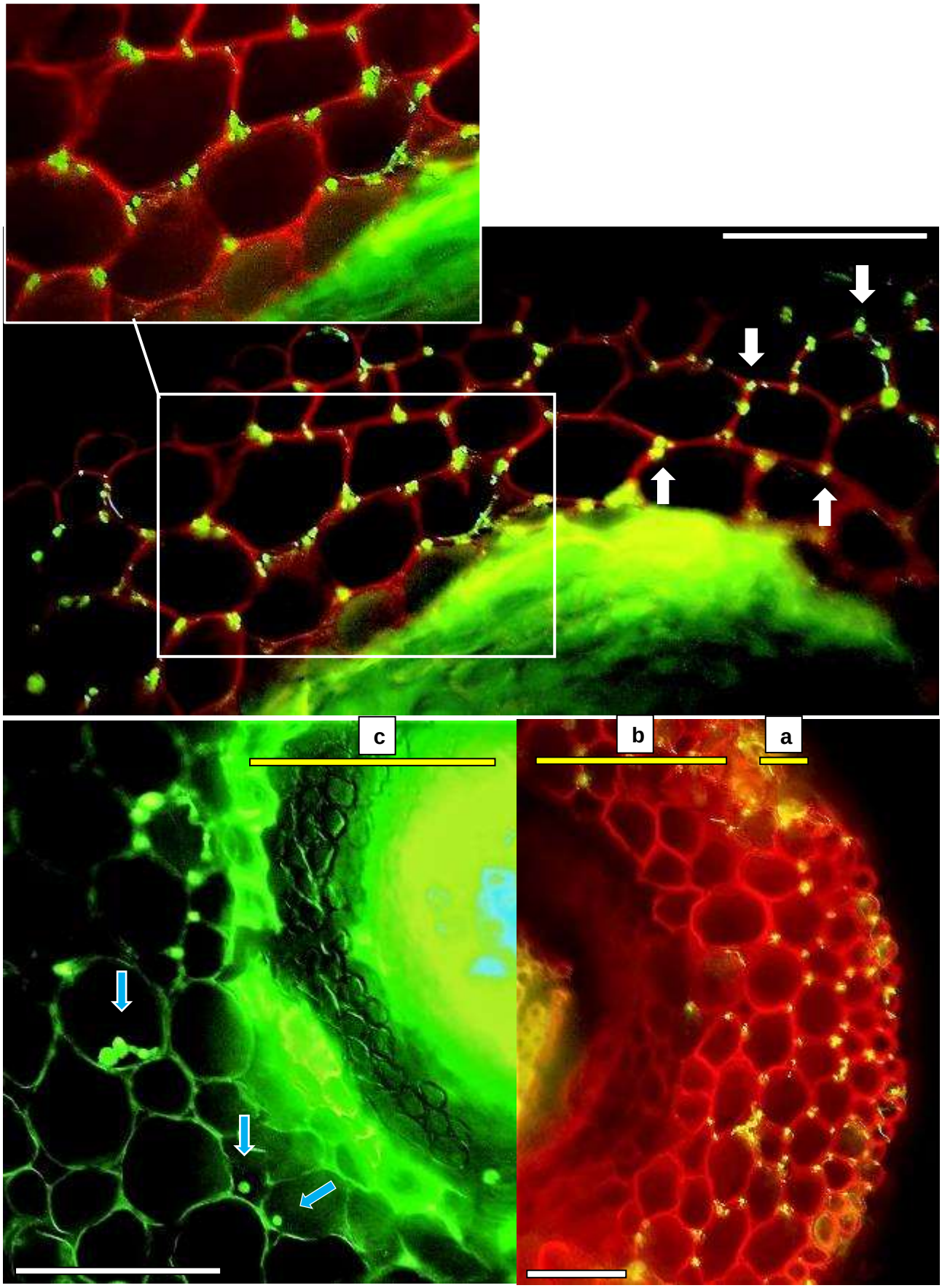

Figure 4: Hyphae (isolate FCC327 and LU633) in cross-section pieces of *P. radiata* tap roots 2.5 months after planting. Hyphae found in the (a) rhizodermal and sub-epidermal, (b) cortex and (c) young xylem vessel cells, mainly in the intercellular and middle lamella spaces (white arrows) but also in the intracellular spaces (blue arrows). White scale bar = 100μm.

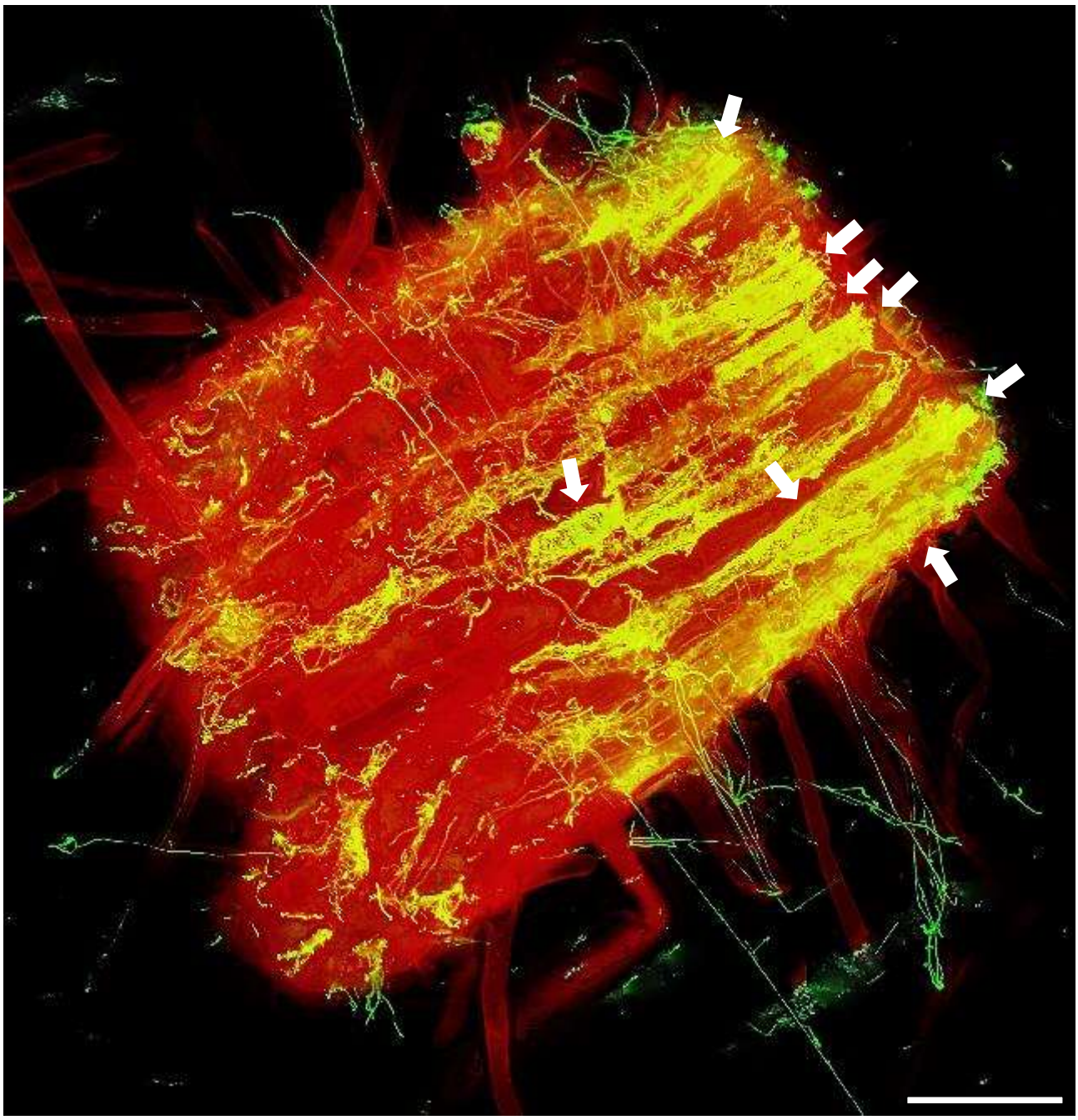

Figure 5: Extensive hyphal development of *Trichoderma* isolate FCC327 in a *P. radiata* root piece. Plant cells fully colonised with hyphae are indicated with arrows. Bar = 200*µ*m

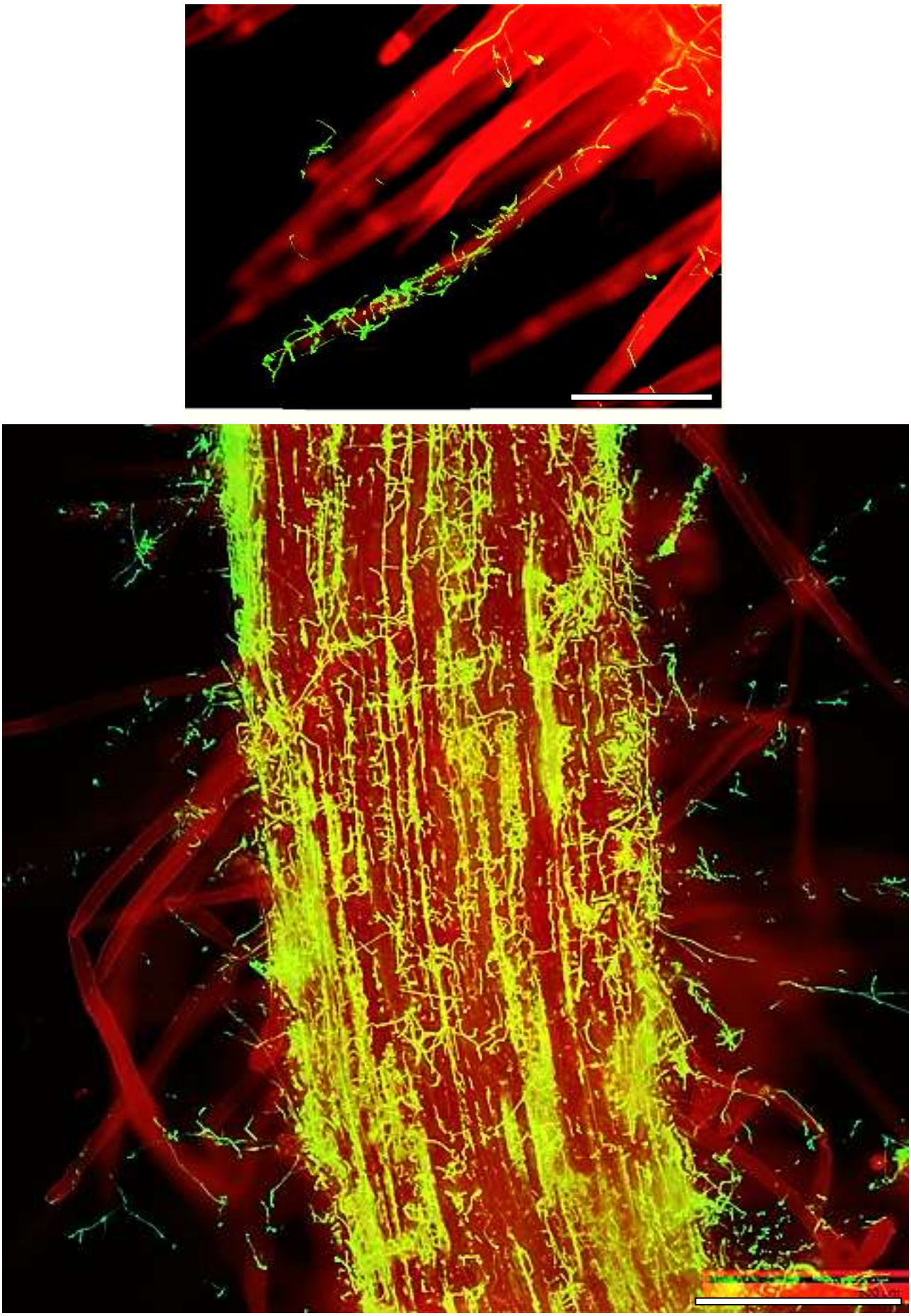

Figure 6: Extensive hyphal development of *Trichoderma* isolate FCC327 on an un-sterilised *P. radiata* root hair (top image, bar = 50 μm) and within a tap root piece (bottom image, bar = 500μm)

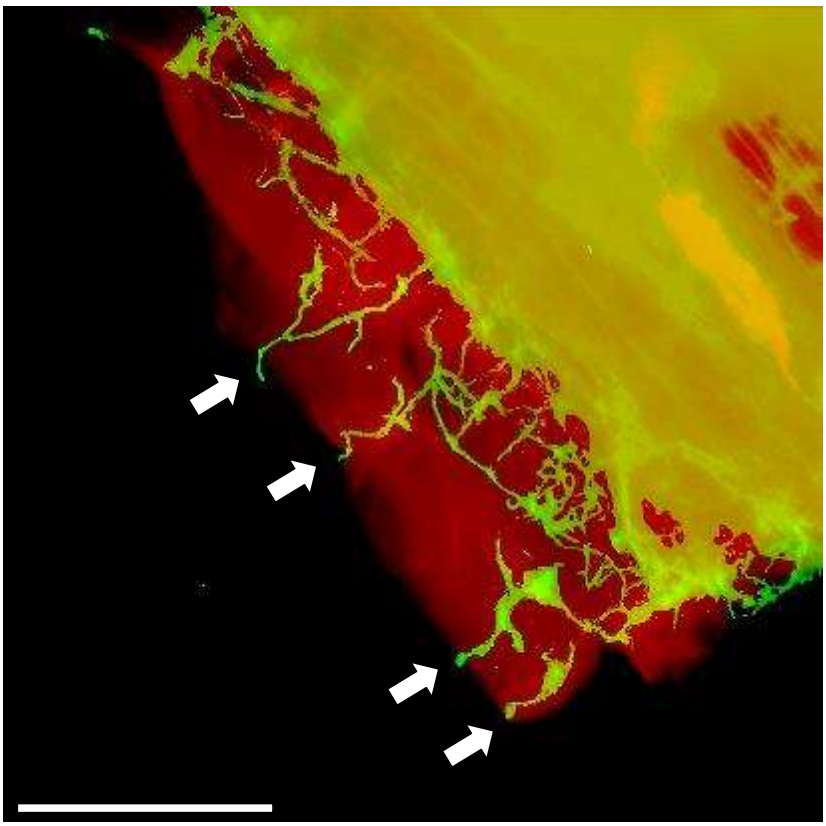

Figure 7: Penetration sites (arrows) of *P. radiata* root surface with *Trichoderma* hyphae (isolate FCC327). Bar = 200*µ*m.

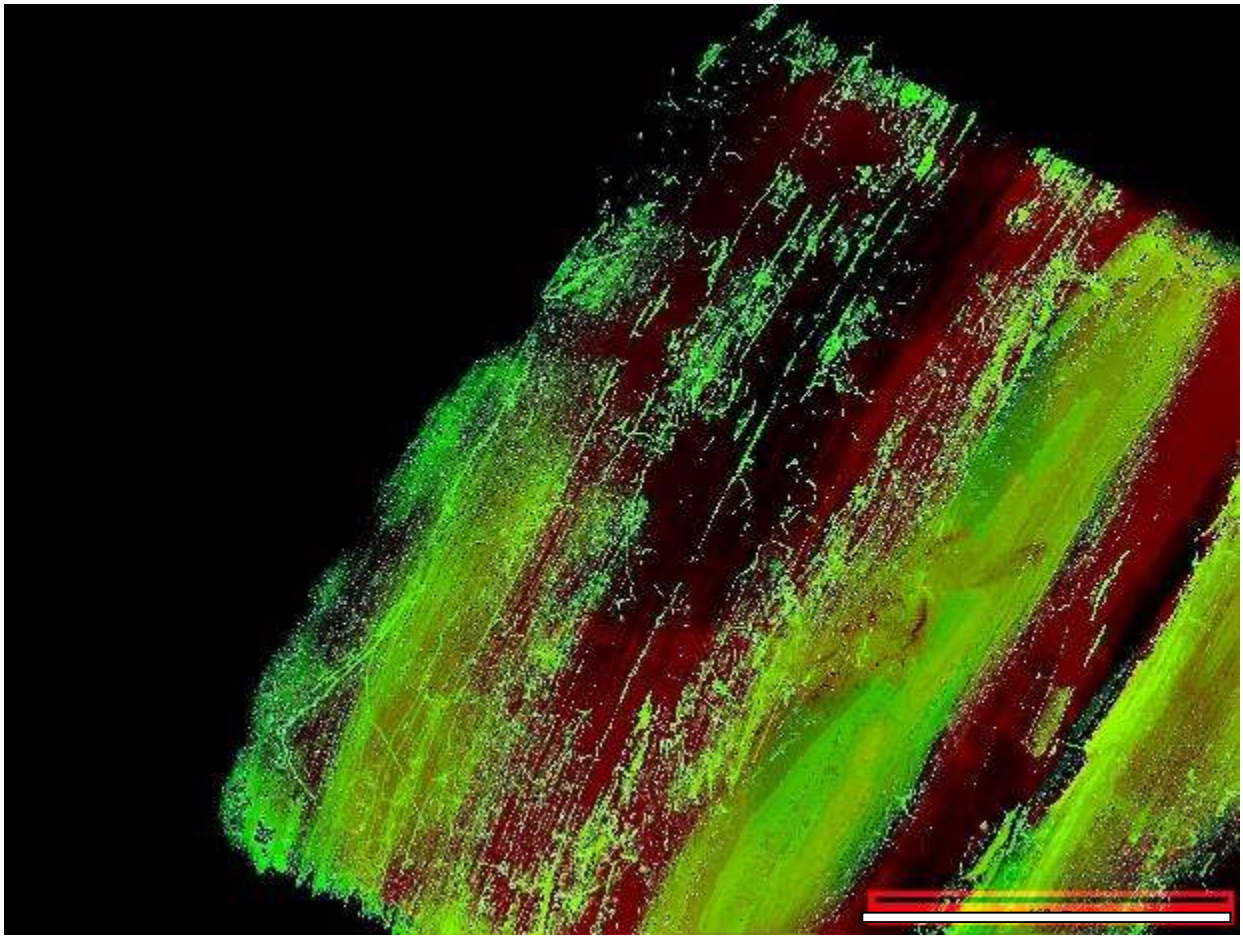

Figure 8: *Trichoderma* hyphae (isolate LU633) in a *Longitudinal* cross-section of a *P. radiata* tap root 2.5 months after planting. Bar = 500*µ*m.

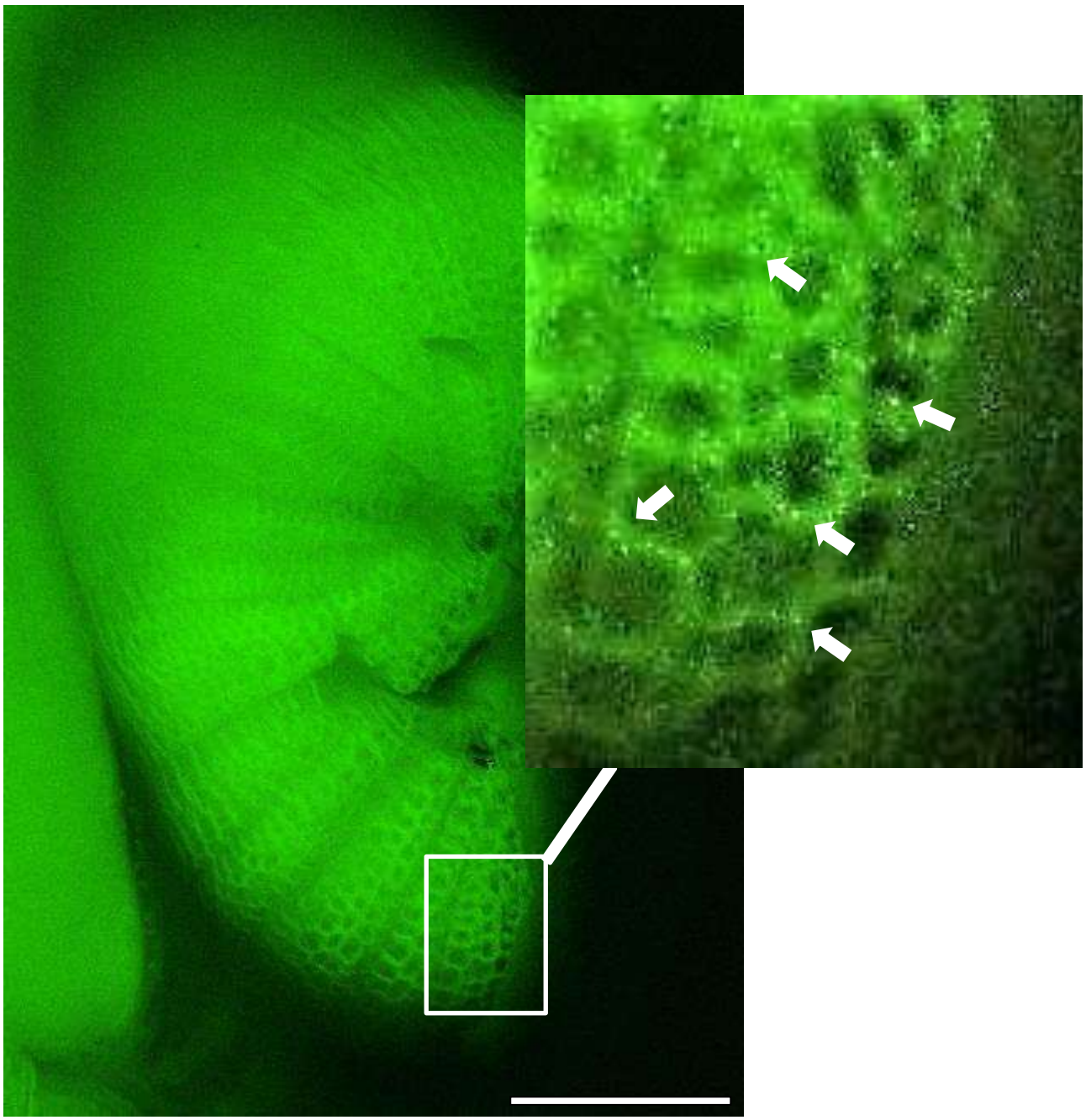

Figure 9: Cross-section image showing the strong auto-fluorescence signal of mature xylem tissue in a *P. radiata* tap root sampled 8 months after planting (Bar = 200μm). Insert image shows numerous *Trichoderma* hyphae (arrows) of isolate FCC327 generally in the intercellular and middle lamella spaces.

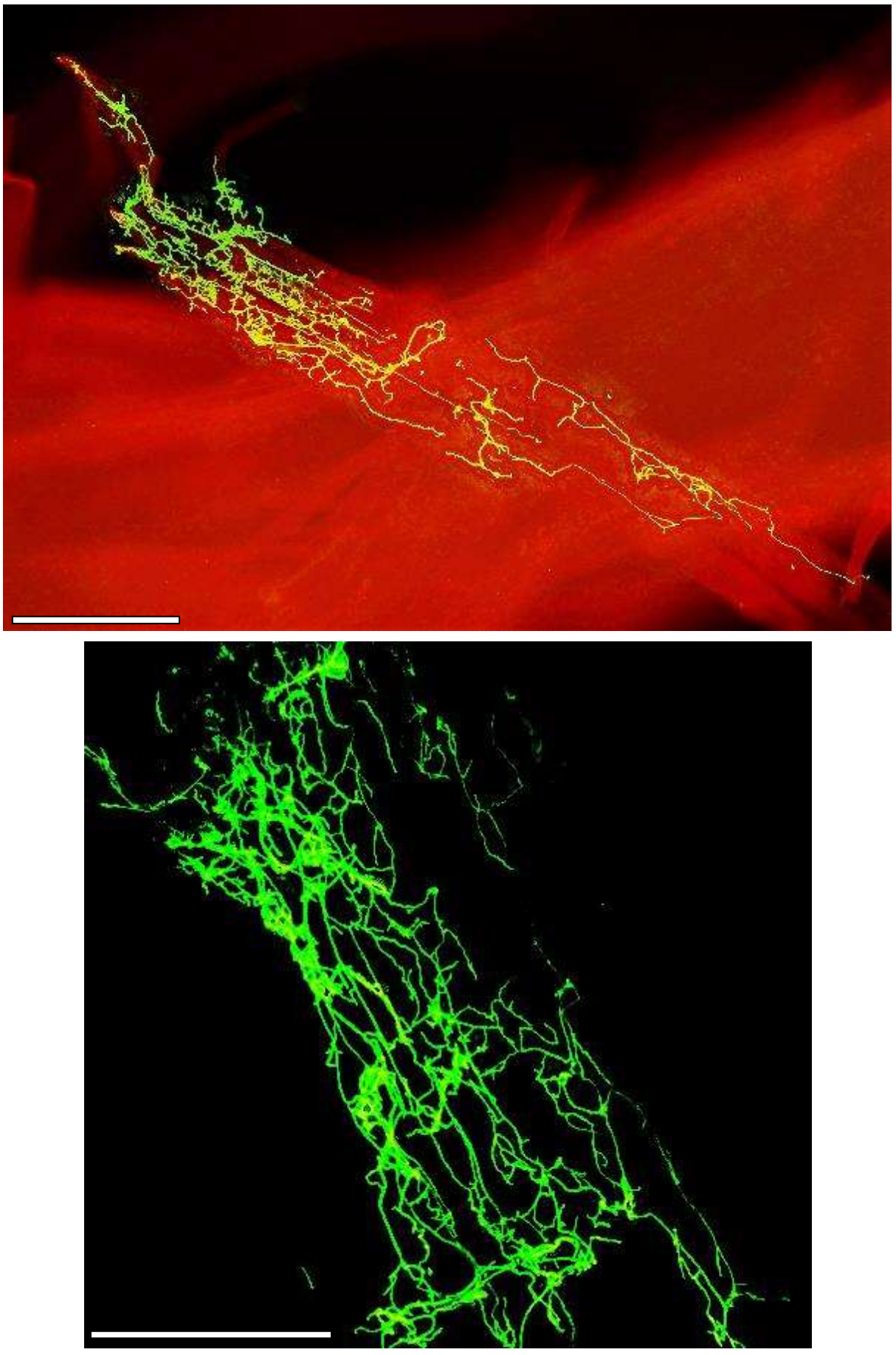

Figure 10: Hyphae of *Trichoderma* isolate FCC327 growing parallel along, and in transverse to the main axis of a *P. radiata* root and without any particular orientation. Note the highly branched nature of the hyphae. Bar = 100μm.

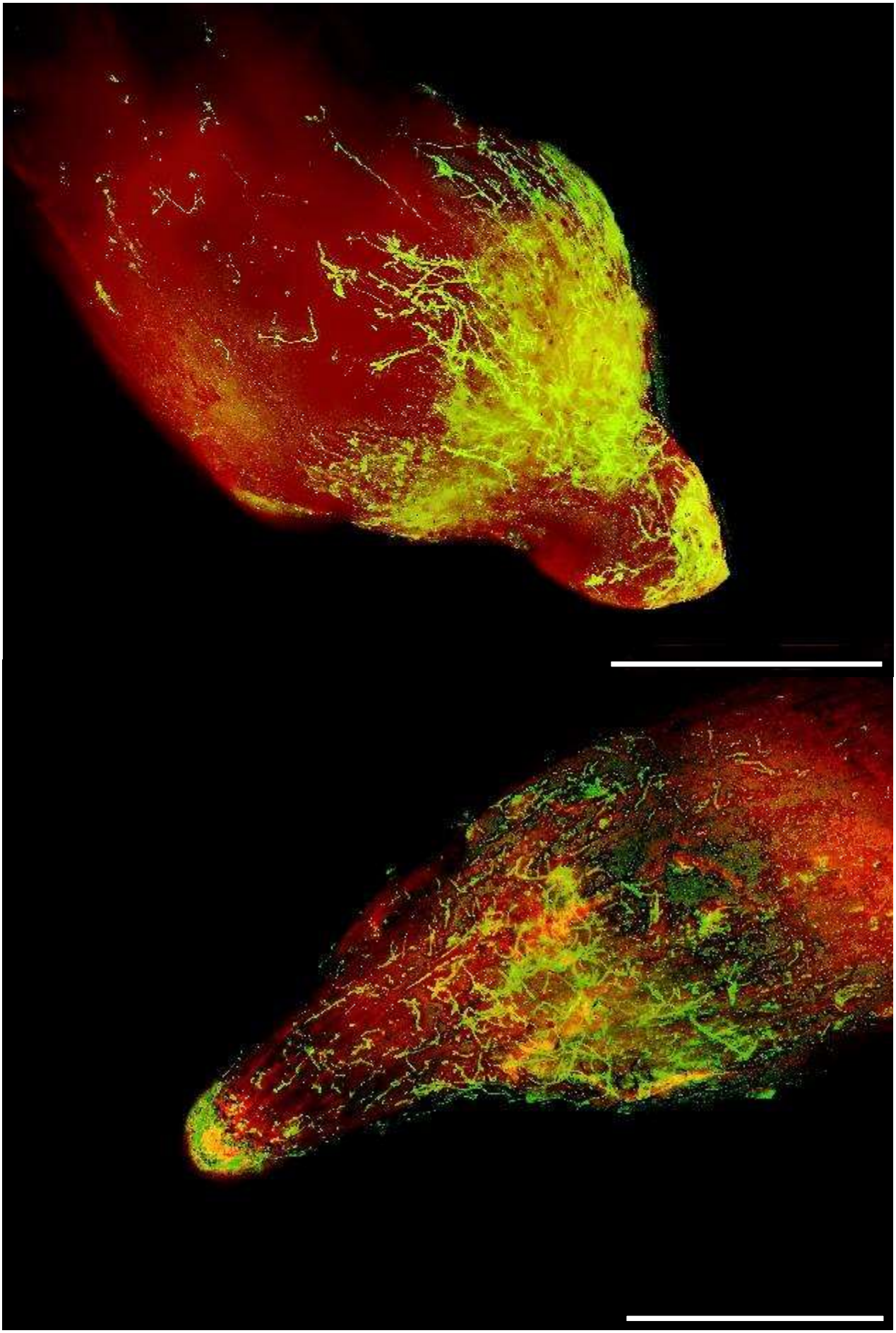

Figure 11: Extensive development of *Trichoderma* hyphae (isolate LU633) in surface-sterilised *P. radiata* root tips at 2.5 months after planting. Bars = 500*µ*m.

Colonisation Frequency and Intensity

Colonisation of root pieces, measured by the incidence of fluorescent hyphae, was found in all *Trichoderma* treatments at the three measurement times (Figure 12). Similarly, to the MRB plating method, isolate FCC327 was the strongest coloniser, with greater than 80% of root pieces colonised throughout the growth period. The other *Trichoderma* treatments had colonisation levels ranging from approximately 22% (LU753) to 78% (LU633). By 8 months after planting, levels of colonisation in all *Trichoderma* treatments were the highest and ranged from 60% (FCC14) to 100% (FCC327). Intensity of colonisation followed a similar pattern to colonisation frequency for each isolate but was considerably lower (between 0.4 and 11.1%; Figure 12).

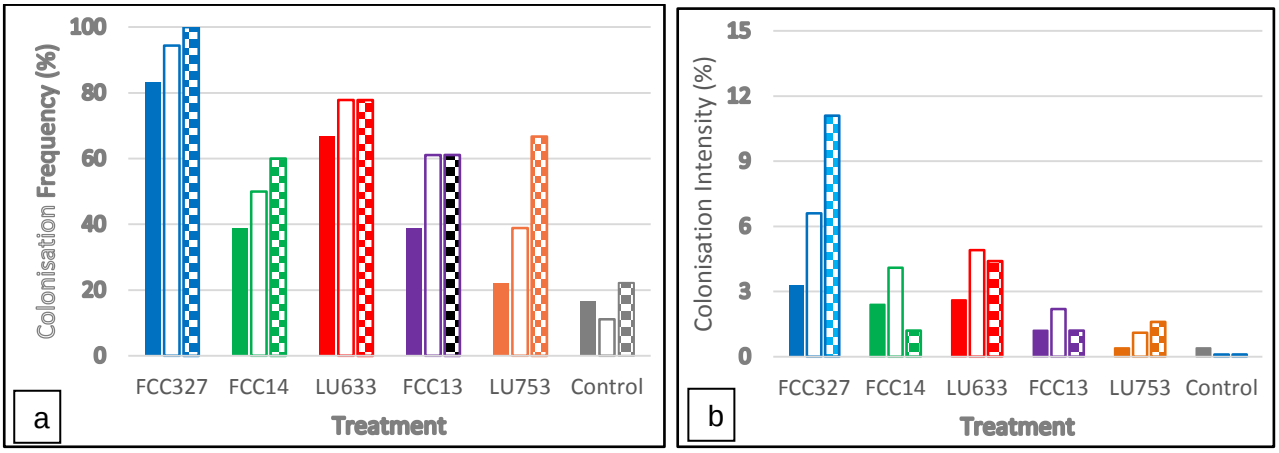

Figure 12: Colonisation (a) frequency and (b) intensity (%) of *Trichoderma* isolates in *P. radiata* roots sampled at 2.5 (solid bars), 5 (open bars) and 8 (hatched bars) months after planting, based on fluorescent data. Mean of 15 lateral and 3 tap root pieces.

Frequency of *Trichoderma* colonisation was about 10% higher in the tap, compared to the lateral roots, when averaged over the three measurement times (Figure 13). Intensity of colonisation followed a similar pattern to colonisation frequency for each isolate but was considerably lower (between 0.5 and 11.6%; Figure 13).

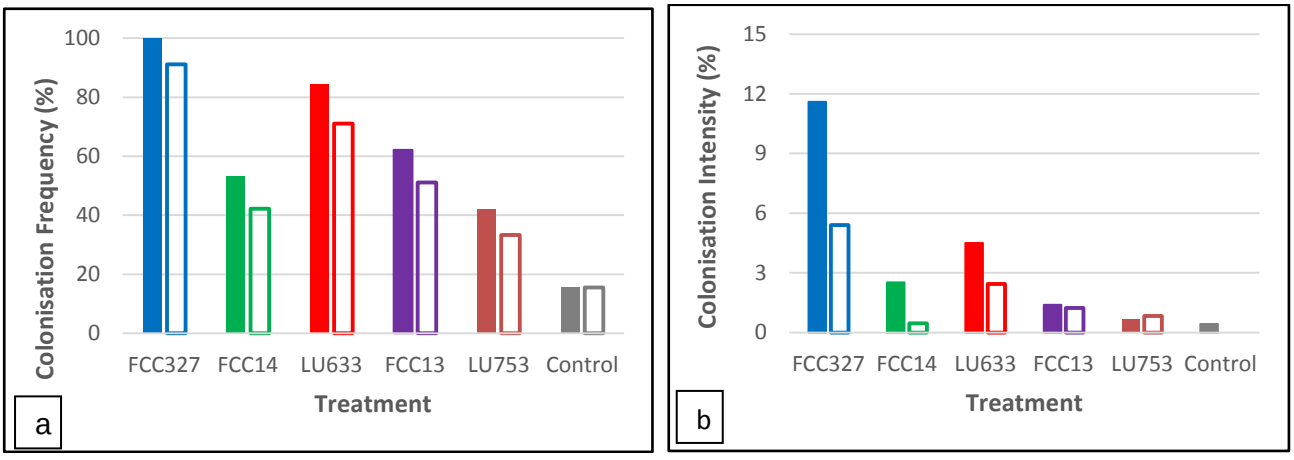

Figure 13: Colonisation (a) frequency and (b) intensity (%) of *Trichoderma* isolates in *P. radiata* tap (solid bars) and lateral (open bars) roots, based on fluorescent data. Colonisation frequency was the mean of data (15 lateral and 15 tap root samples) for the three measurement times. Colonisation intensity was the mean of data for the 2.5 and 5 month measurements only as there was not 15 tap root samples where hyphae could be visulatised for intensity at 8 months. Data for each measurement time is detailed in Appendix E.

Frequency of fungal colonisation at 8 months after planting, as measured by fluorescent labelling (Figure 12), was higher compared to colonisation determined by the number of colony growths on MRB plates (Figure 1). Both methods were useful for determining levels of colonisation in *P. radiata* roots, but different interpretations may need to be made. The number of colony growths on MRB plates do not distinguish how many hyphae have caused the growth of the colony so may underestimate the incidence of root hyphae. However, colony counts on MRB plates do indicate the potential viability of the inoculum in the root piece. The fluorescent imagery method visualised the majority of fungal hyphae present in the root pieces so provides a more precise estimate of inoculum incidence. Fluorescent labelling also enables quantification of the intensity of hyphal colonisation and can be used as a predictor of total inoculum in the root (Ayliffe *et al.,* 2013). A potential disadvantage with fluorescent labelling is WGA-AF488 labels all fungal cell walls and although the vast majority of hyphae observed in this experiment were of similar structure and size, hyphae of different fungal genera may have been observed.

Plating with MRB was a relatively cost-effective method. However, receipt of colonisation information was delayed by 14 days due to the time it takes for colony growth on the plates. Fluorescent labelling methodology required a specialised microscope and stains but once the equipment is established sample costs can be low (Ayliffe *et al.,* 2013) and colonisation information can be achieved within a few hours of samples being taken.

4.1.3 Verification of Trichoderma Isolate LU633

Twelve of the thirteen selected *Trichoderma* colonies isolated from the LU633-treated seedlings were verified as being LU633 with low cycle threshold (Ct) values in the real time PCR assay (Table 1). Isolate GH9 had a higher Ct value compared to the other isolates and may have been a mixture of LU633 and another *Trichoderma* species. It is likely that the *Trichoderma* colonies observed in other treatments were also the treatment isolates applied due to the high levels of inoculum that were applied at planting.

Table 1. Real-time PCR specificity test with LU633-strain specific primers and probes.

Sample Name	Sample Source	Ct values
Trichoderma virens (negative control)	Lincoln University	55
GH1	Tap root / plate 1, isolate 2 / 2.5 months after planting	17.0
GH2	Tap root / plate 3, isolate 3 / 2.5 months after planting	19.9
GH3	Tap root / plate 3, isolate 2 / 2.5 months after planting	17.2
GH4	Lateral root / plate 2, isolate 2 / 2.5 months after planting	16.7
GH5	Lateral root / plate 1, isolate 2 / 2.5 months after planting	19.4
GH6	Tap root / plate 2, isolate 1 / 2.5 months after planting	18.5
GH7	Tap root / plate 3, isolate 4 / 2.5 months after planting	18.5
GH8	Tap root / plate 1, isolate 1 / 2.5 months after planting	18.1
GH9	Tap root / plate 2, isolate 1 / 5 months after planting	28.3
GH10	Tap root / plate 2, isolate 2 / 5 months after planting	18.5
GH11	Lateral root / plate 1, isolate 4 / 5 months after planting	17.0
GH12	Lateral root / plate 1, isolate 3 / 5 months after planting	19.9
GH13	Lateral root / plate 1, isolate 5 / 5 months after planting	16.9
LU633 (positive control)	Lincoln University	18.2

Ct values indicates the PCR cycle number at which PCR cycle amplification became exponential. The amplification diagram is provided (Appendix D). Ct levels are inversely proportional to the amount of target nucleic acid in the sample.
Cts < 29 are strong positive reactions indicative of abundant target nucleic acid in the sample
Cts of 30-37 are positive reactions indicative of moderate amounts of target nucleic acid
Cts > 38 are weak reactions indicative of minimal amounts of target nucleic acid, which could represent an environmental contamination.
Values represent average of three replicates.

CONCLUSION

In this study a selection of *Trichoderma* strains, used in New Zealand forestry trials, were found to colonise and persist on roots for eight months after seed planting and inoculation. High levels of colonisation, as determined by plating and fluorescent imagery techniques were found in all strains, particularly strain FCC327. This study represented growing conditions similar to those found in *P. radiata* seedling nurseries for the first 8 month of seedling growth. Further work is required to determine the persistence of *Trichoderma* strains over longer periods, particularly once planted in the forest.

The fluorescent labelling technique enabled extremely detailed visualisation of *Trichoderma* endophytic activity in the roots. This technique allowed visualisation of:

- the very close association of *Trichoderma* hyphae with individual plant cells
- the abundant colonisation of many plant cells with hyphae, that appeared as localised dense mats of hyphae
- hyphae growing between the plant cells often in the intercellular and middle lamella spaces and hyphae growing inside plant cells
- frequent infection of root hairs by *Trichoderma* hyphae
- hyphae colonising the rhizodermal, sub-epidermal, cortex and young xylem vessel cells
- hyphae growing in all directions within the root and were highly branched
- the abundant colonisation of the root tip.

Further work is required to improve the visualisation of fungal hyphae in mature *P. radiata* tissue with thickened cell walls. The labelling method could potentially be refined by increasing the soaking time in KOH to improve the clearing of cell contents.

Colonisation of seedling roots with the *Trichoderma atroviride* strain LU633 was verified using a species-specific probe-based real-time PCR assay. In addition, the untreated control seedlings had low levels of *Trichoderma* colonisation. Researchers should have confidence that the *Trichoderma* isolated from treated seedlings is likely to be the same strain applied to that treatment, if applied under similar experimental conditions.

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APPENDICES

Appendix A: Layout of planting trays in glasshouse.

Appendix B: Malt Yeast Extract Agar (MYE) Recipe

Malt extract	10 g
Yeast extract	1 g
Agar	20 g

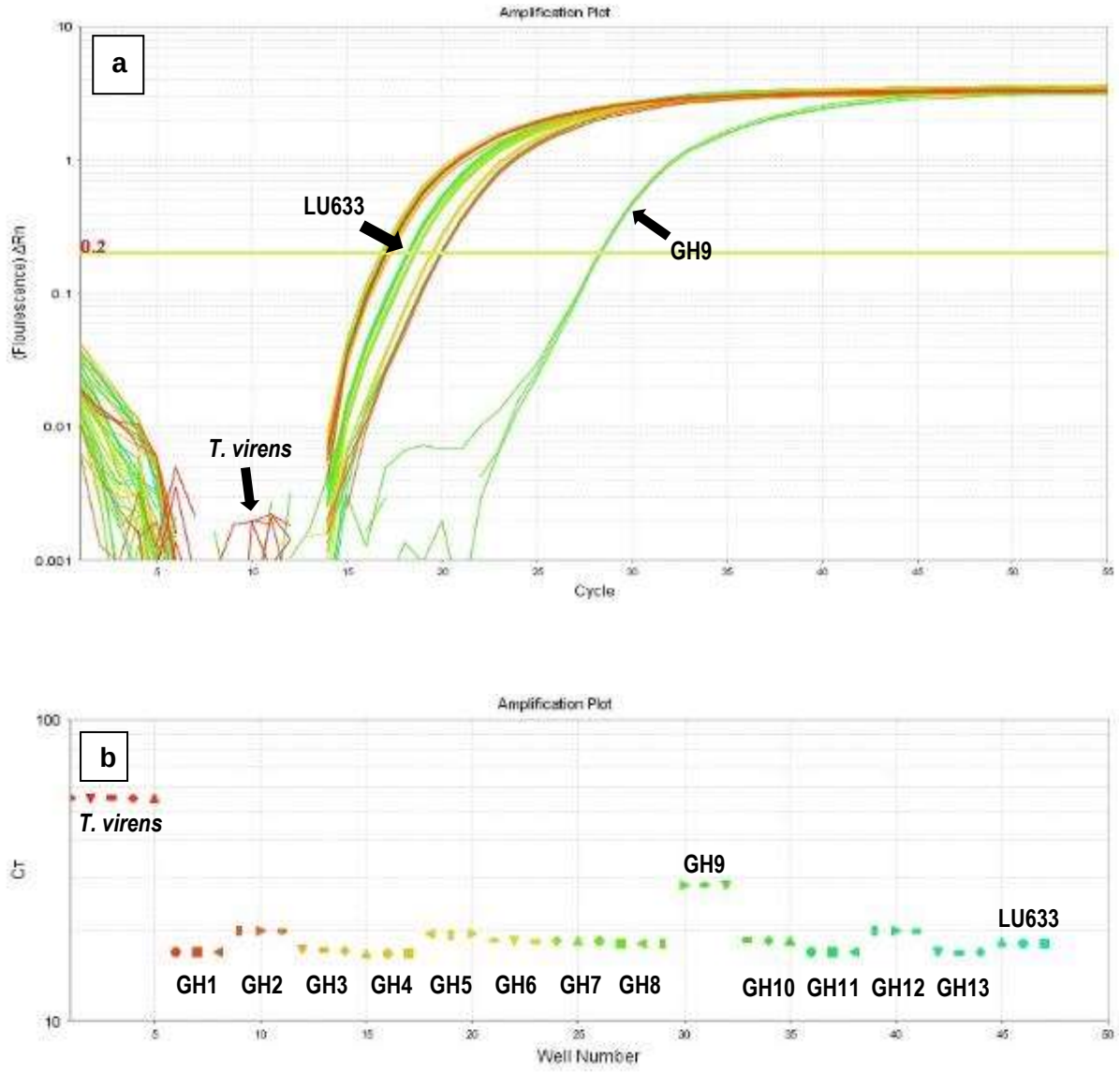
Make up to 1L with distilled water.

Appendix C: 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 D: Amplification plot used to calculate Ct values for glasshouse isolate and control samples (a); sample colours notated in diagram b.



Appendix E: Colonisation frequency (left graphs) and intensity (right graphs) of *Trichoderma* isolates in *P. radiata* tap (solid bars) and lateral (open bars) roots (A) 2.5, (B) 5 and (C) 8 months after planting, based on fluorescent labelling data. Mean of 15 root pieces per treatment per measurement time. [a] Colonisation intensity for the 15 tap root pieces at 8 months was not presented due to high levels of plant cell wall auto-fluorescence in many of the samples.