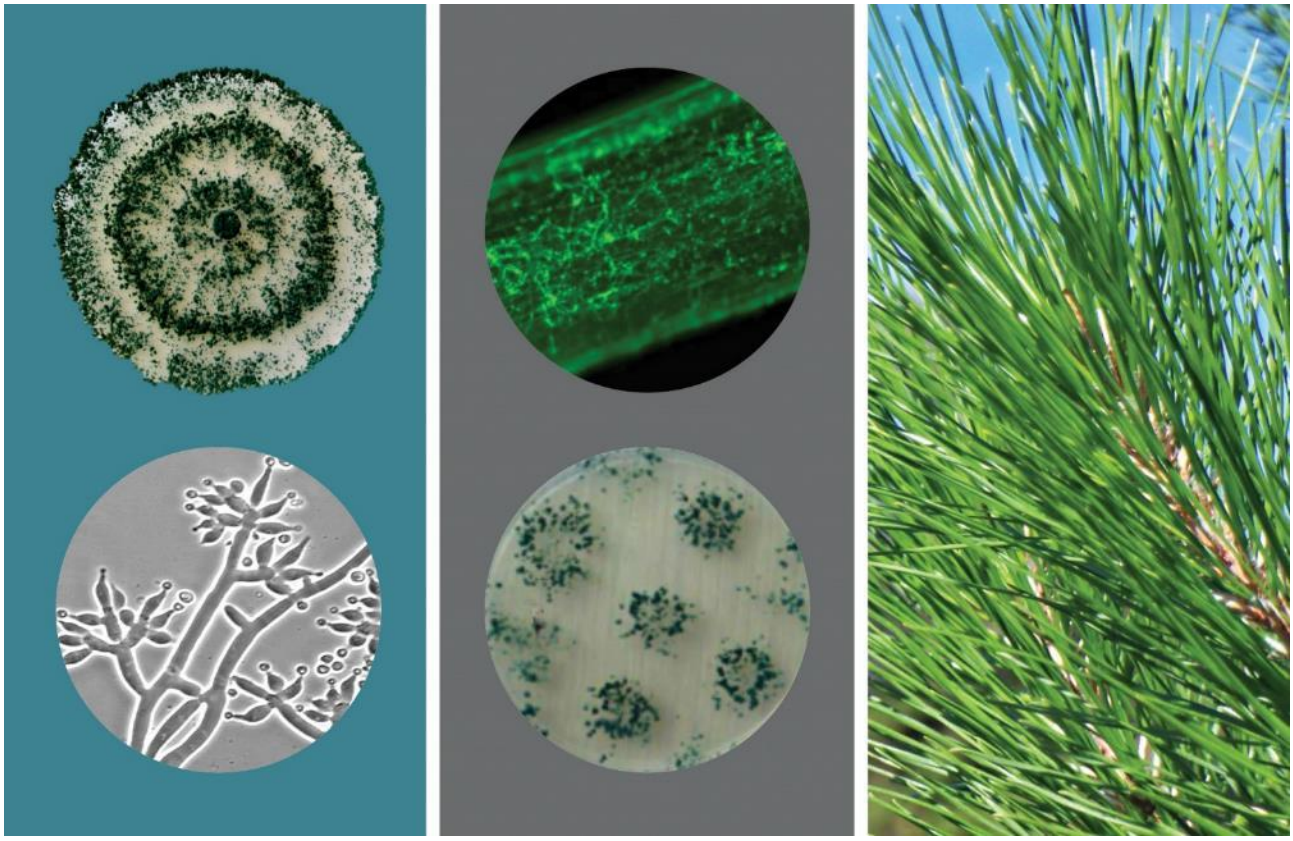
Bioprotection - enhancing growth and health Research Programme

Bio-Protection
Bioprotection science for New Zealand

Inoculation and colonisation of *Pinus radiata* seedlings with selected *Trichoderma* treatments

Authors:
Robert Hill, Helen Whelan and Nicholas Cummings
Bio-Protection Research Centre, Lincoln University



Date: December 2016

Confidential Report No: BIO-T012

Task: 3.10

supported by forestgrowers commodity levy

FOREST OWNERS ASSOCIATION

TABLE OF CONTENTS

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 through the use of beneficial endophytes and elicitors. As part of this project, a number of plantation trials have been established at sites around NZ to examine the effects of specific *Trichoderma* strains on *Pinus radiata* growth and disease resistance. The *Trichoderma* isolates in current trials originated from surface-sterilised roots of a variety of plant species and represent species able to colonise root surfaces and establish endophytic associations as internal colonists of root tissues.

In this report we describe results from glasshouse experiments examining the root colonisation of *P. radiata* seedlings by a set of core *Trichoderma* isolates used in our forestry bioprotection research. Colonisation was determined by direct re-isolation of *Trichoderma* cultures and by visualisation of fungal structures using florescence microscopy.

Key Results

- The fluorescent staining system (which detects fungal chitin) allowed detailed visualisation of *Trichoderma* growth on root surfaces and endophytic colonisation of root tissue.
- *Trichoderma* re-isolation and fluorescent staining techniques showed that all isolates had colonised the roots within 10 days after planting and inoculation.
- Isolates LU633, FCC327, FCC13 and FCC14 were found to be the strongest colonisers of *P. radiata* roots.

INTRODUCTION

Glasshouse experiments were conducted to examine colonisation of *Pinus radiata* seedlings by *Trichoderma* isolates LU132, LU140, LU297, LU584, LU633, LU668, LU753, FCC13, FCC14, FCC320, and FCC327.

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 staining. Both methods indicated that that *Trichoderma* strains colonised roots at an early stage of seedling growth. Roots were found to be colonised within 10 days of planting and inoculation and colonisation was confirmed 3 days before emergence when roots were between 10 and 20mm long.

All isolates had colonised *P. radiata* roots 10 days after planting, with some isolates (LU140, LU584 and LU753) having high levels of colonisation (at least 50% of the root pieces infected) at this stage. Isolates LU633, FCC327, FCC13 and FCC14 were the strongest colonisers of roots.

The fluorescence labelling technique enabled extremely detailed visualisation of *Trichoderma* endophytic activity in the roots. The stain interacted with the fungal chitin and its bright green fluorescent signature was easily seen in all *Trichoderma*-treated roots once infected. This technique allowed visualisation of:

- penetration of root tissue by hyphae
- hyphal growing tips
- inter- and intracellular hyphae
- hyphal growths in both localised and extensive zones
- hyphal 'mats' in mature root tissue

Fluorescent labelling also allowed quantification of the intensity of colonisation and could be used as a predictor of total inoculum in the root.

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 27 days.Seeds were sown (Appendix A) in plastic trays (BCC Sweden) with each tray containing 63 cells with a volume of 100mL per cell (39mm diameter and 85mm depth). One seed was sown in each cell, at a depth of 10mm, into unsterilised potting mix (50L composted bark, 25L peat, 25L perlite medium grade, 200g Osmocote Exact Mini (16-3.5-9.1), 400g gypsum, 150g dolomite and 100g Hydroflo wetting agent). Each treatment was applied to one tray at planting. The trays were arranged in a completely randomised design and placed onto a heat pad in a glasshouse at Lincoln University.

Experimental treatments included eleven *Trichoderma* isolates (LU132, LU140, LU287, LU584, LU633, LU668, LU753, FCC13, FCC14, FCC320 and FCC327) and a control (sterile water) treatment. Isolates were grown at ambient temperature in natural light for 20 days. Spore suspensions were produced by flooding the plates with 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.
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 twice a day.Spore germination was checked by mixing a diluted spore suspension with potato dextrose broth, transferring to microscope slides

for 29 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.

3.2 Root Colonisation Measurements

The growth stage at which T*richoderma* isolates colonised the root tissue was determined using two techniques:

1. Re-isolation - incubation of surface-sterilised root pieces on *Trichoderma* isolation medium (MRB)
2. Microscopic visualisation of root pieces treated with fluorescent Wheat Germ Agglutinin - Alexa Fluor® 488 (WGA-AF488)

3.2.1 Re-isolation by direct plating

Seven seedlings were randomly sampled from each treatment at three times (Figure 1; Appendix A):

1. three days before emergence
2. one day after emergence
3. seven days after emergence

Roots were thoroughly washed with tap water and dissected into one, two or three sections (depending on the growth stage of the seedling) of approximately 10mm length:

1. a section just below the potting mix surface (‘Top’)
2. a section in the middle (‘Middle’)
3. a section at the growing tip (‘Tip’)

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, seven pieces per treatment were transferred onto a plate of *Trichoderma* isolation medium (MRB; Appendix C). Agar plates with roots were incubated on a laboratory bench top with ambient light and temperature conditions for 10 days.

Following incubation, plates were visually assessed and the total number of *Trichoderma* colonies were counted. Percentage of root pieces colonised was calculated by dividing colony number by total number of pieces in each treatment at each sampling date. Percentage of colony growths on each root section, out of the total number of root pieces, was also calculated.

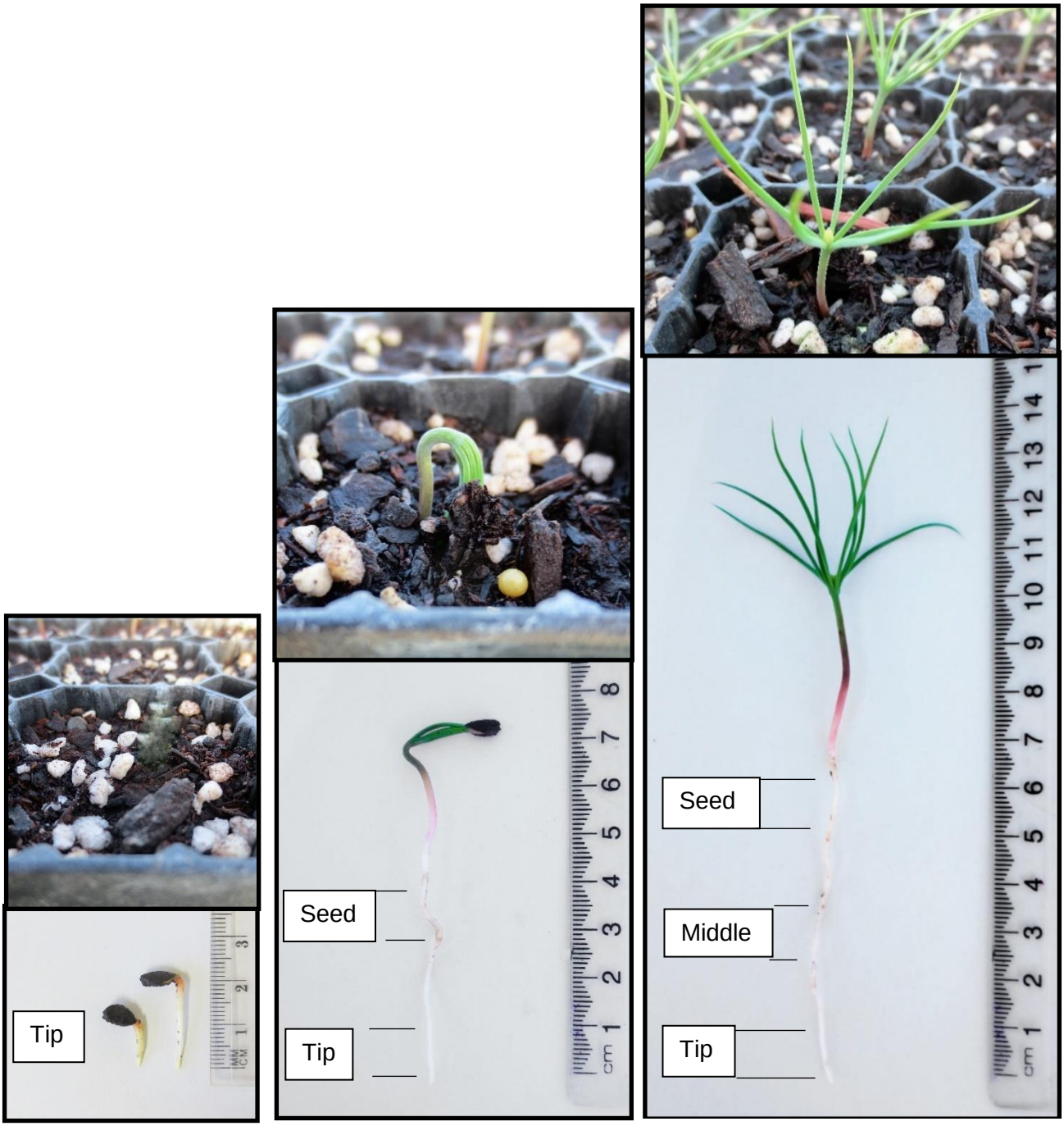


Figure 1: Sampling times and root sampling positions for *P. radiata* seedlings.

3.2.2 Root Microscopy

An additional seven seedlings were sampled for root labelling at each harvest date and the roots were dissected as above (see Appendix A). Root pieces were surface sterilised in Virkon (1% w/v) for 10 minutes, washed in distilled water and placed in small beakers. The pieces were then 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 distilled water. A few additional seedlings were harvested but the roots were not treated with Virkon to allow observation of the fugal hyphae on the root surface.

WGA-AF488 binds to fungal chitin (a component of fungal cell walls) and allows a visual contrast between fungal and plant material (Vierheilig *et al.,* 2005). Root pieces from each treatment were placed in 2ml Eppendorf vials with approximately 100µl of WGA-AF488 (10µg/ml w/v; Molecular Probes,

http://www.invitrogen.com). The solution was vacuum infiltrated into the tissue using a vacuum pump (Labconco CentriVap Concentrator) for 10 minutes. The roots were placed on a microscope slide and viewed using a BX51 Compound Microscope with U-RFL-T Burner with fluorescent excitation at 495nm and emission at 519nm. Bright light images were also taken at the same focusing distance and magnification. Where appropriate, the fluorescent image was superimposed onto the same view taken with bright light. Images were generated using the cell^F computer programme.

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) 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.

The data for the root sections (top/middle/tip and top/tip, respectively) was combined to generate an overall value of colonisation frequency for each seedling.

COLONISATION INTENSITY (C_I) (the level of fungal spread in root pieces), was based on visually rating the area colonised by hyphae using a rating from 0 to 5 (Figure 2) 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.

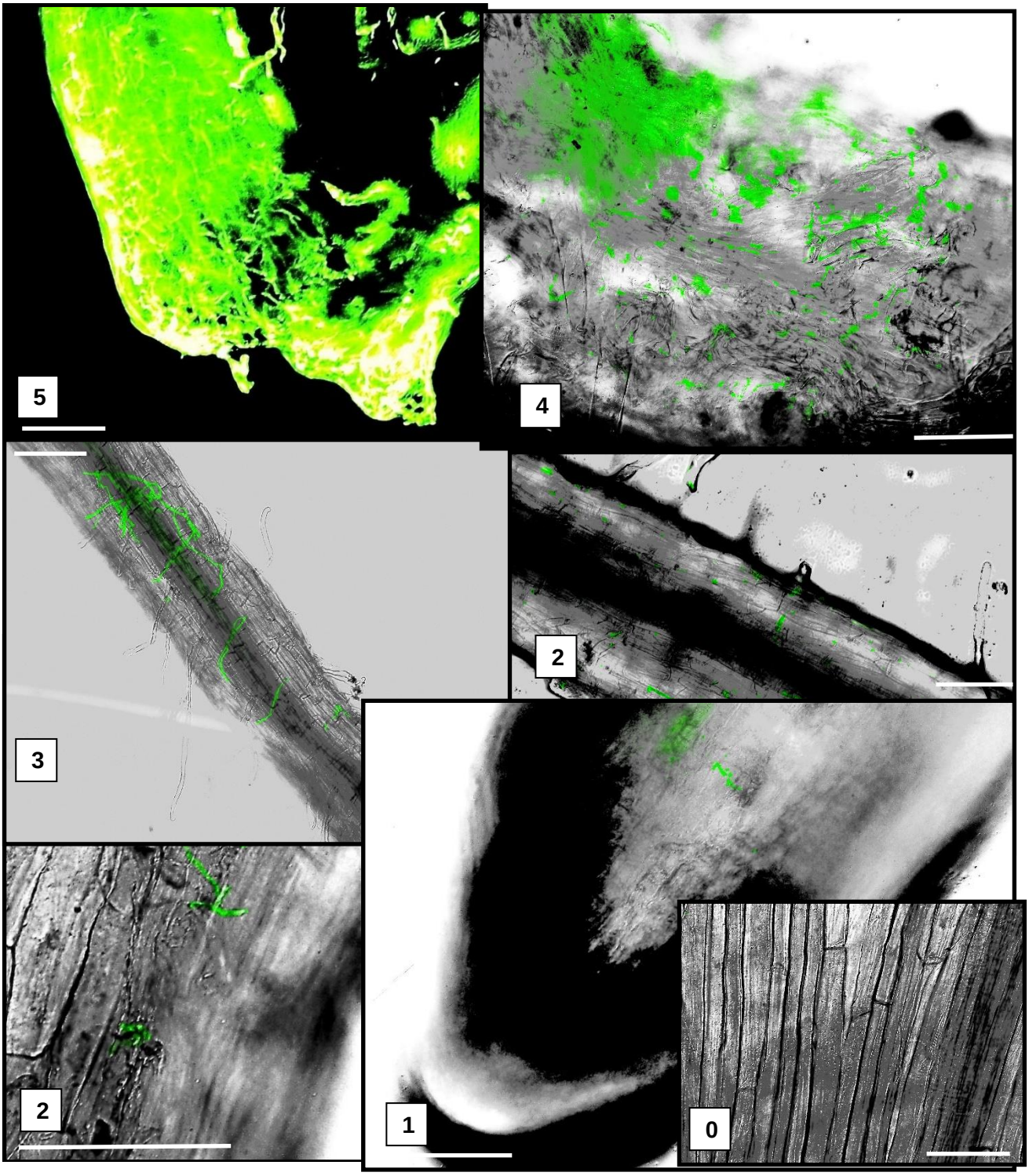


Figure 2: Quantification of *Trichoderma* colonisation using fluorescent labelling data. Rating System from 0 (no hyphae) to 5 (91%+ colonisation). Note: In this study, no ratings of 4 or 5 were found when applied over the whole piece of root. Bars = 100µm.

RESULTS

4.1 Root Colonisation

4.1.1 Plating with *Trichoderma* Selective MRB Agar

Colonisation of root pieces, measured as colony growths on MRB plates, was found in all *Trichoderma* treatments (Figures 3, 4 and 5). *Trichoderma* colonies that were observed are likely to be the treatment isolates due to the high levels of inoculum that were applied.

In *P. radiata*, all *Trichoderma* treatments produced colony growths in samples taken 3 days before emergence (Figure 3a). Isolates LU140, LU584 and LU753 were strong colonisers, with at least 50% of the root pieces colonised. Similar levels of infection were found at 1 day after emergence, (Figure 3a). By 7 days after emergence, high levels of colonisation, ranging from 65 to 79% were found in isolates LU132, FCC13 and LU753 (Figure 3a and c). Clearly, initial colonisation in *P. radiata* roots occurred before 10 days of planting and inoculation .

The untreated control plants showed a small number of *Trichoderma* colonies (5%) in the later sampling, probably from environmental *Trichoderma* strains (e.g. from the potting mix and/or airborne spores in the glasshouse).

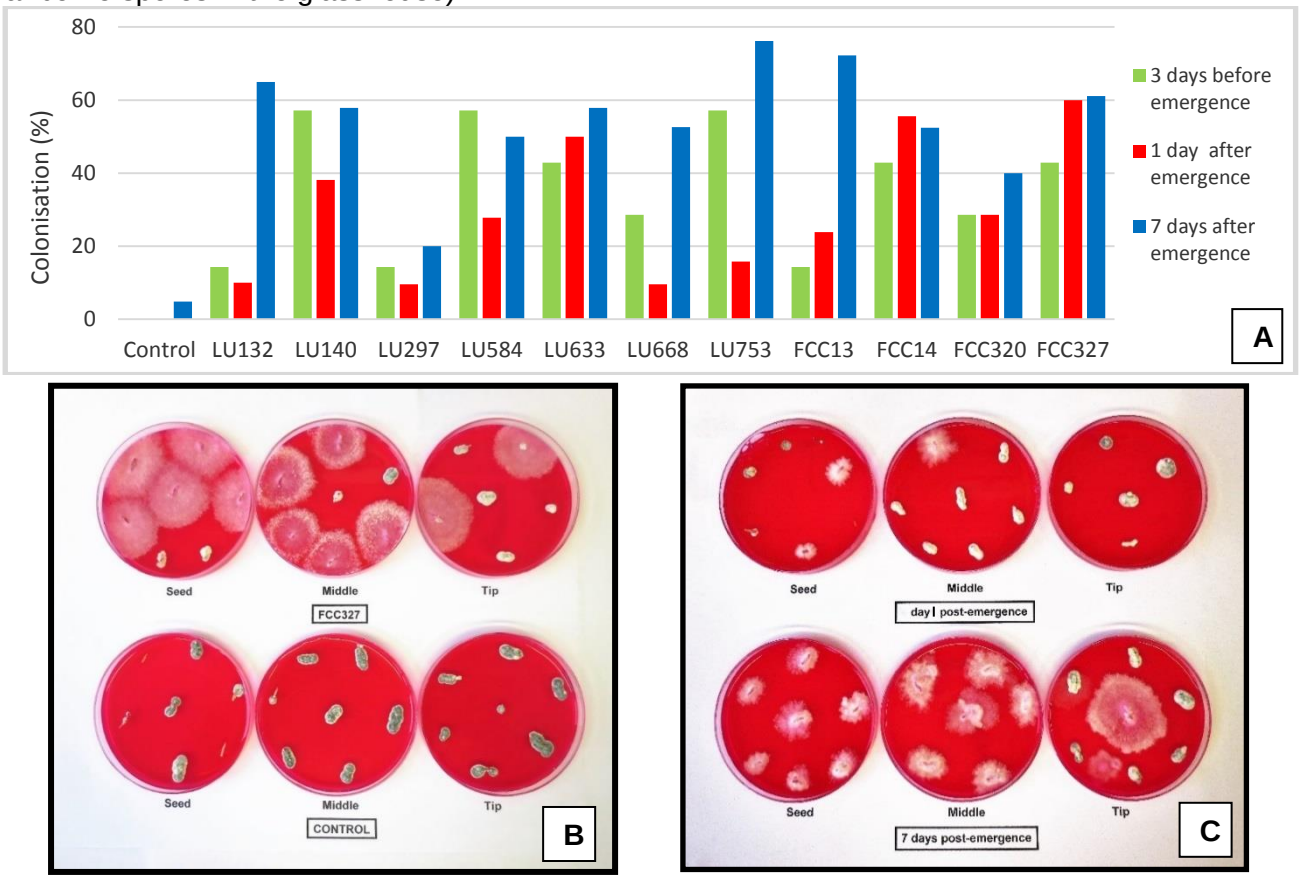


Figure 3: Colonisation (%) of *P. radiata* roots sampled 3 days before emergence and 1 day and 7 days after emergence of seedlings (A), based on MRB plating data. Colony growth on MRB plates of treated (FCC327) and untreated (Control) root pieces sampled 1 day after emergence (B). Colony growth *on* MRB plates of treated (isolate LU753) seedlings sampled 1 day and 7 days after emergence (C). Green colony growths were *Penicillium* species.

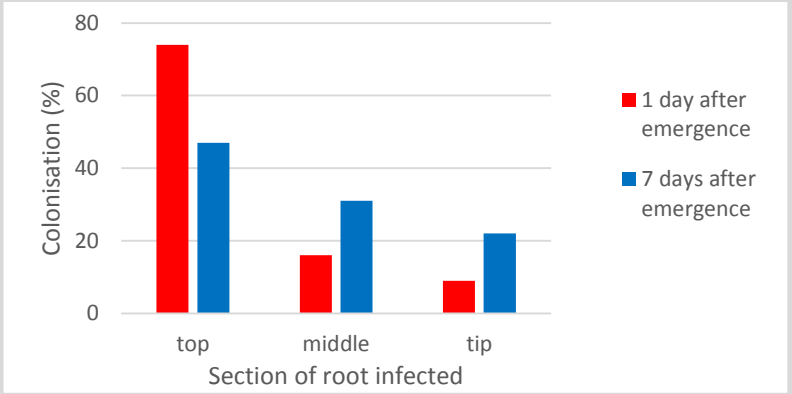


Figure 4*:* Colonisation (%) of different sections of the roots 1 day and 7 days after emergence in P. radiata seedlings, based on MRB plating data. Mean of all *Trichoderma* treatments.

During early root growth the majority (74%) of *Trichoderma* colonisation occurred in the root section closest to the potting mix surface ('top'; Figure 4) when averaged over all *Trichoderma* treatments. Few (9%) colonisations were observed at the tip of the root. Six days later, proportionately more tips were colonised compared to the top end of the root. Colonisation in each root section was variable for each isolate (Appendix D).

4.1.2 Root Labelling with WGA-AF488

Fluorescent Imagery

Visualisation of fungal colonisation was achieved by examining labelling of root tissue with stained with fluorescent WGA-AF488. Hyphae exhibited a bright green fluorescent image which was often more intense than the surrounding plant cell walls (Figures 5, 6, 7 and 9). The presence of fungi, most likely the applied *Trichoderma* strains, was confirmed in many samples by observing fungal septa (Figures 6a and b).

Visualisation of the fungal hyphae was difficult because of the higher natural fluorescence ('auto-fluorescence') of the cell walls throughout the root structure (Figures 5 and 7).

Fluorescent labelling allowed visualisation of fungal hyphae and their endophytic development in roots. Stages of hyphal development, similar to those found in *P. radiata* roots infected with *Fusarium circinatum* (Martín-Rodrigue *et al*., 2015) and in barley roots infected with *Piriformospora indica* (Deshmukh *et al*., 2006), were observed as follows:

1. germination of spores on the root surface, or growth of hyphae originating from soil particles, lead to fungal hyphae growing closely to the surface of rhizodermal cells (Figure 10), particularly in association with the root hairs. Images were often of numerous small fluorescent flecks at this stage (Figure 7)
2. intercellular penetration of the rhizodermal cells by hyphae and development of short hyphal fragments
3. hyphal branching at these sites leading to the formation of sub-epidermal intercellular networks. Hyphal growing tips illustrated in Figure 6c. Hyphae found growing in parallel along, and in transverse to (Figure 10), the main axis of the root and without any particular orientation (Figure 11). Hyphae found growing in localised or widespread areas in the young root pieces.
4. filling of young differentiated cells intracellularly with hyphae (Figures 8 and 11)
5. mature root tissue becoming occupied with a network of intracellular hyphae (Figures 9 and 10); localised or widespread in the root pieces.
6. further proliferation of hyphae finally leading to the development of dense 'mats' of hyphae which have very strong fluorescent signals.

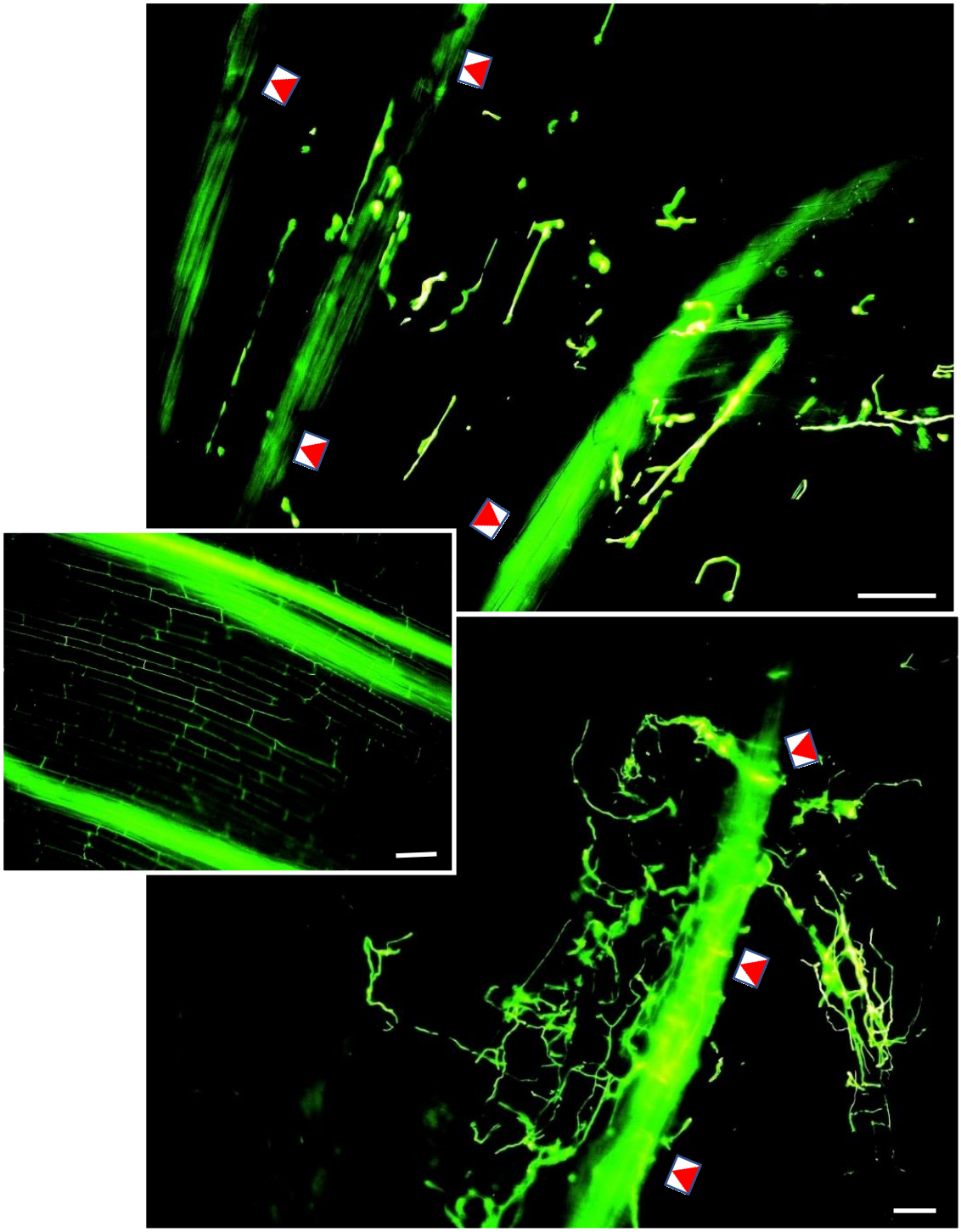


Figure 5: Fluorescent signature of root cells and *Trichoderma* hyphae in *P. radiata* seedlings 1 day after emergence. Auto-fluorescence of root cells indicated by arrows (including all fluorescence in inserted image). Other fluorescent structures are fungal hyphae (isolate LU633). Bars = 100µm.

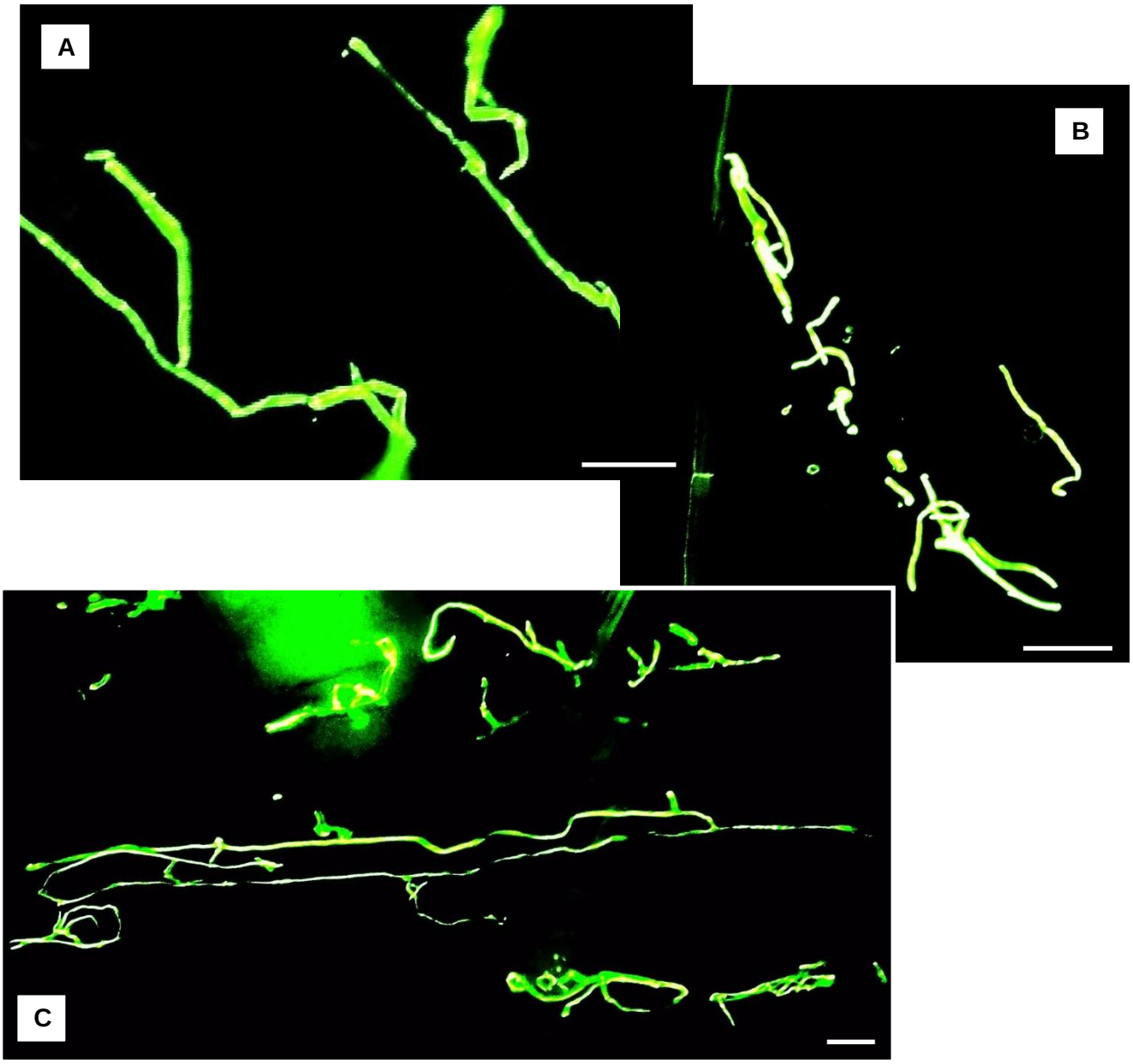


Figure 6: Hyphae with visible septa observed in *P. radiata* (A; isolate LU132) roots. Hyphae growing tips in P. radiata roots (B and C; isolate FCC13). Bars = 30µm.

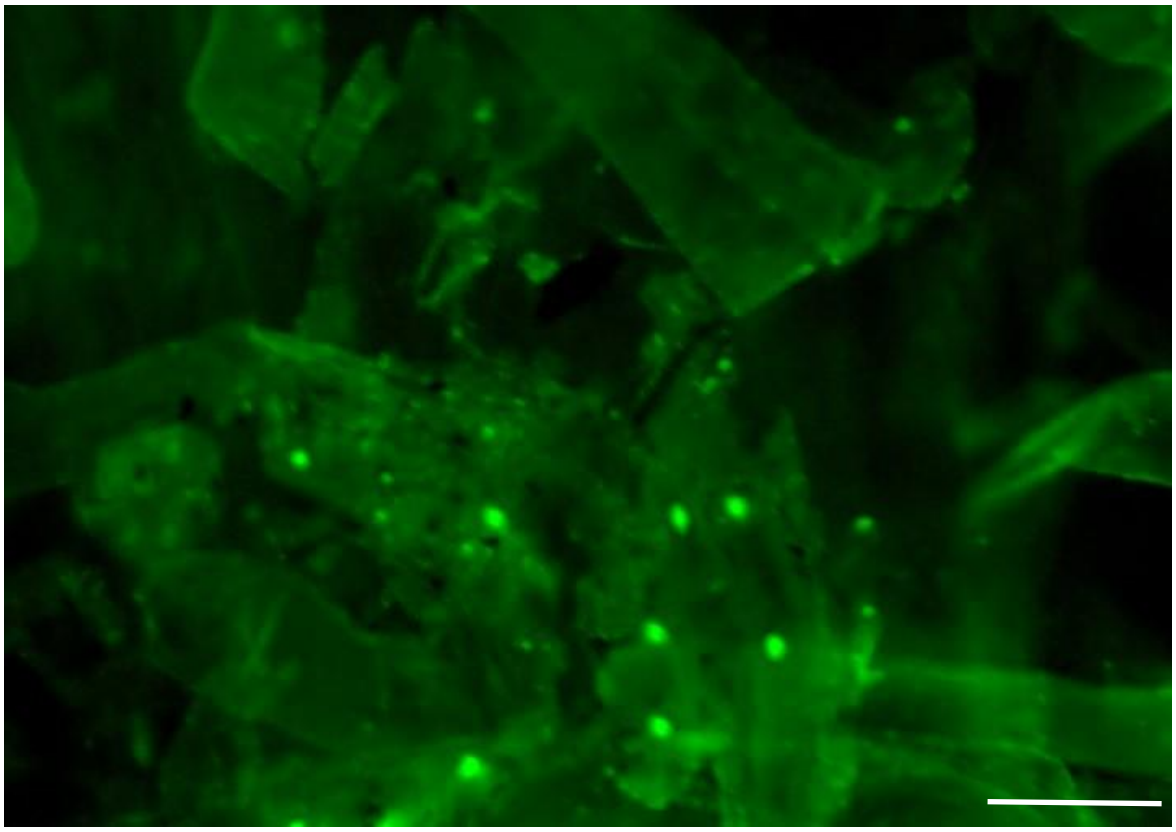


Figure 7: Early development of *Trichoderma* (spore germination) in *P. radiata* (isolate FCC14) 1 day after seedling emergence. Bar = 100µm.

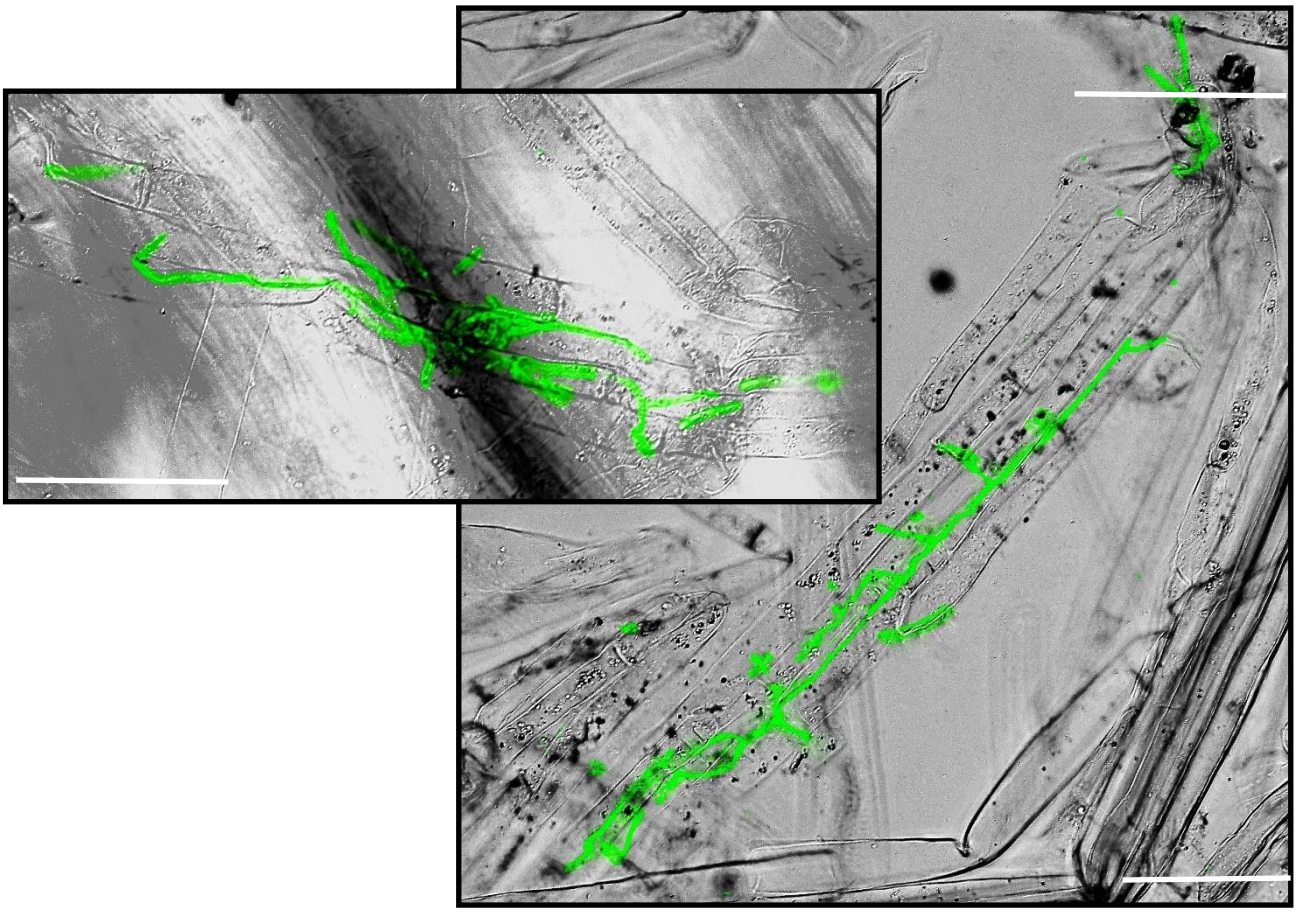


Figure 8: *P. radiata* root cells with intracellular fungal colonisation (isolates FCC13 and LU633). Fluorescent images of fungal hyphae were superimposed onto bright light images. Bars = 100µm.

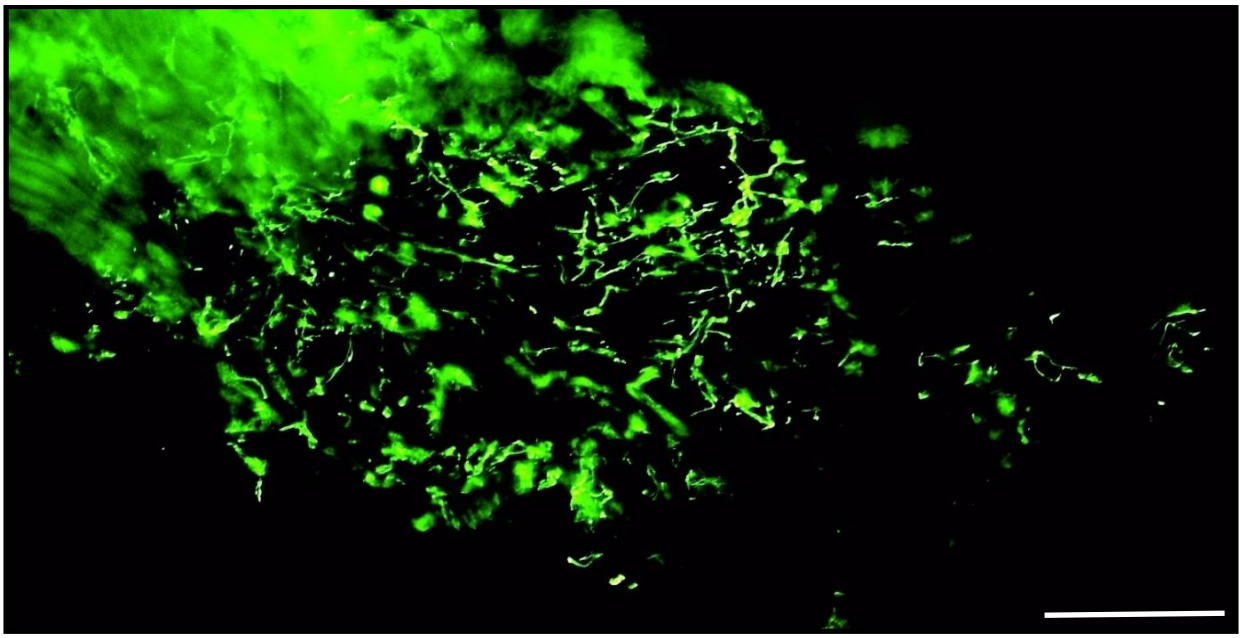


Figure 9: Development of *Trichoderma* (isolate LU132) in *P. radiata* roots 1 day after seedling emergence. Bar = 100µm.

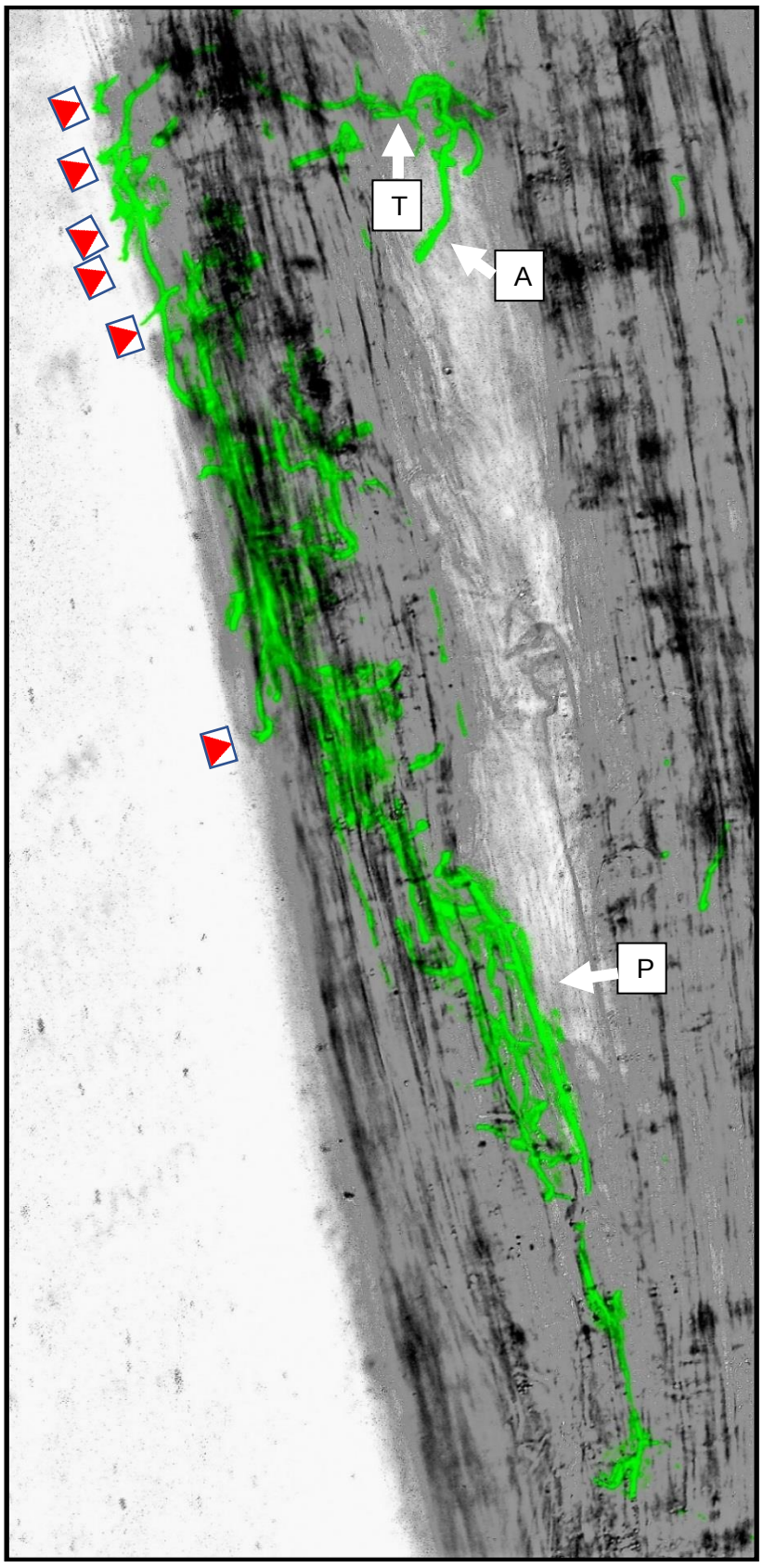


Figure 10: Hyphae of *Trichoderma* isolate FCC13 growing in parallel along (P), and transverse to (T), the main axis of the root and without any particular orientation (A) in a *P. radiata* seedling 1 day after seedling emergence. Arrows indicate *Trichoderma* root penetration sites. The fluorescent image of fungal hyphae was superimposed onto an image taken with bright light microscopy. Bar = 100µm.

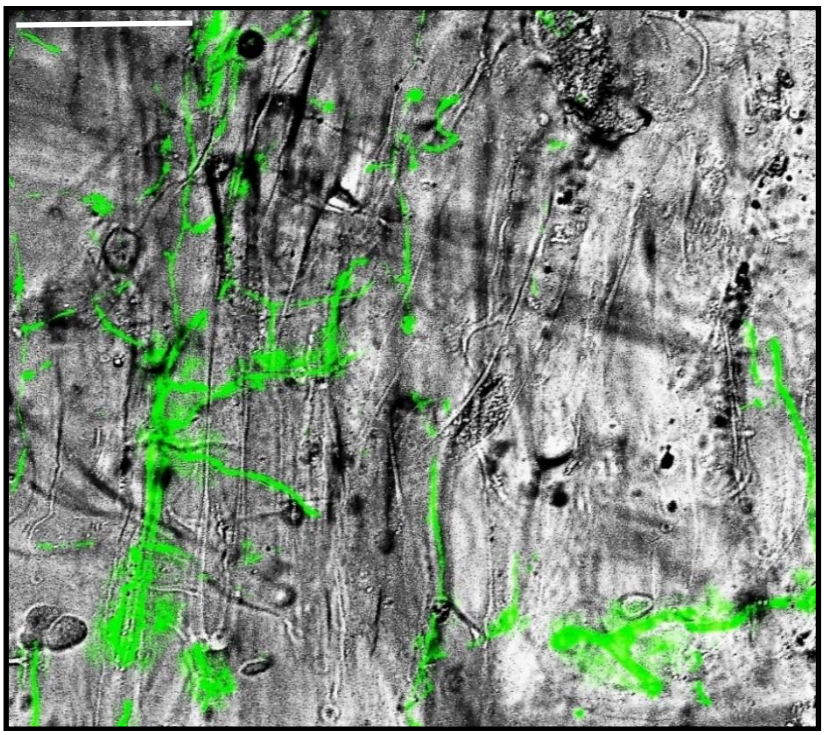


Figure 11: Intracellular development of *Trichoderma* hyphae (isolate LU663) in P. radiata roots 1 day after seedling emergence. The fluorescent image of fungal hyphae was superimposed onto an image taken with bright light microscopy. Bar = 50µm.

Colonisation Frequency and Intensity

In roots sampled 3 days before emergence, fungal hyphae were visualised as infrequent small fluorescent flecks (Figure 7) in most of the *Trichoderma* treatments (except LU297 and LU668; Figure 12a) but intensity of colonisation was very low (<1%). Between 3 days before, and 1 day after emergence, all isolate treatments had colonised the roots (Figure 12b) and hyphal development was rapid. Isolates LU633, FCC13, FCC14, LU132, FCC320 and FCC327 were found to be strong colonisers at greater than 67%, although colonization intensity was still relatively low (≤24%). No fluorescent labelling was done on the seedlings sampled 7 days after emergence because of the earlier positive result.

In the control treatment, no hyphae were observed in the roots 3 days before emergence (Figure 12a). A low level (≤20%) of hyphae were detected in the untreated (Control) roots of the seedlings at 1 day after emergence.

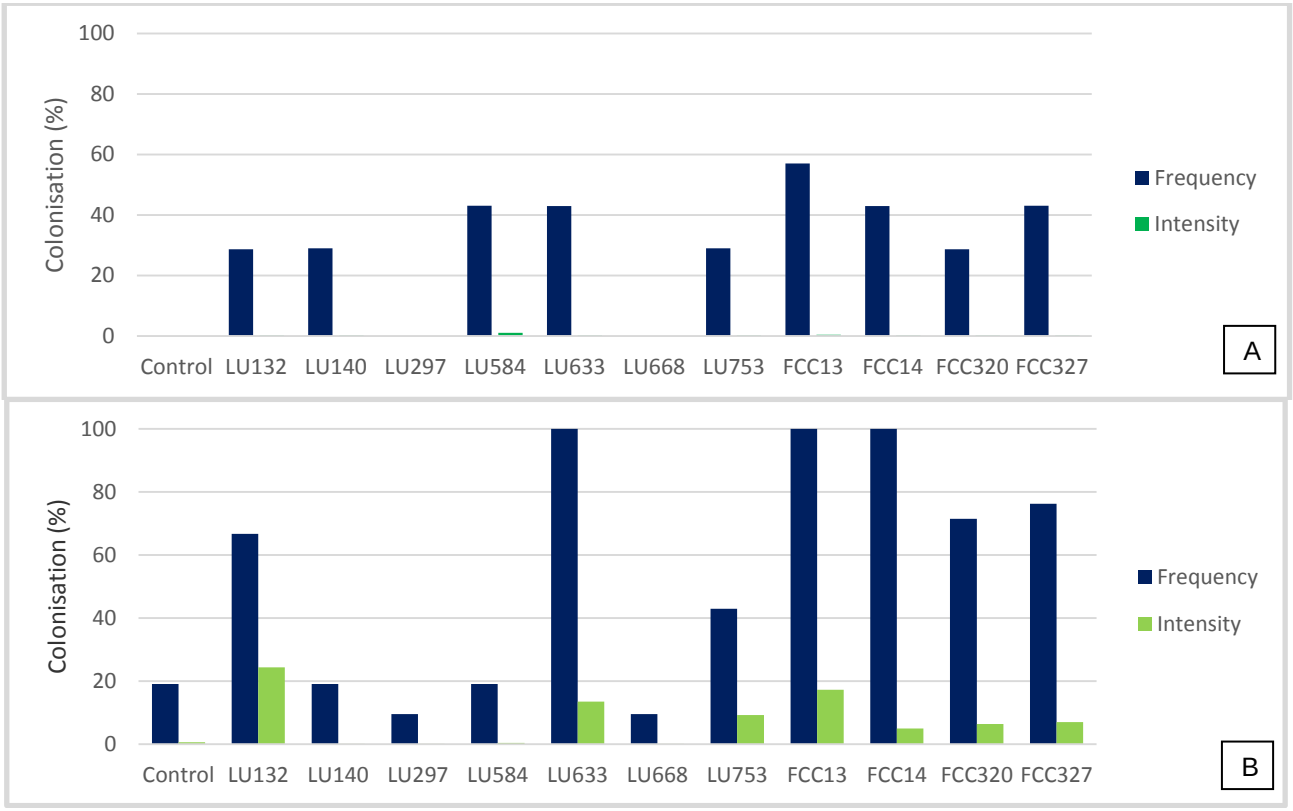


Figure 12: Colonisation frequency and intensity of *Trichoderma i*solates in *P. radiata* roots A) 3 days before emergence and B) 1 day after emergence based on fluorescent labelling data.

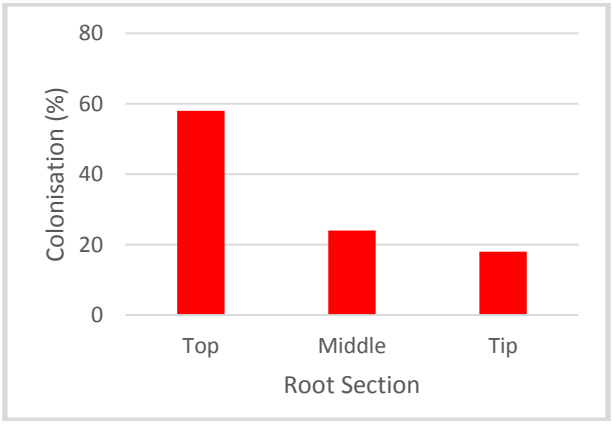


Figure 13: Colonisation (%) of different sections of the roots 1 day after emergence in *P. radiata* seedlings, based on fluorescent labelling data. Mean of all *Trichoderma* treatments.

During early root growth of *P. radiata*, most *Trichoderma* colonisation occurred In the root section closest to the potting mix surface ('top'; Figure 13) when averaged over all *Trichoderma* treatments. Fewer (18%) infections were observed at the tip of the root.

4.1.3 Comparison of Colonisation Methods and Timing of Colonisation

Both direct plating and microscopy showed that the *Trichoderma* strains colonised roots 3 days before emergence (Figures 3a and 12a) Both methods also indicated a similar level of root colonisation at each sampling time (Figure 14).

Isolates LU633, FCC327, FCC13 and FCC14 were shown to be strong colonisers during early seedling growth (Figure 14a and b).

P. radiata was colonised within 10 days of planting and *Trichoderma* inoculation (this being about 3 days before emergence when roots were between 10 and 20mm long).

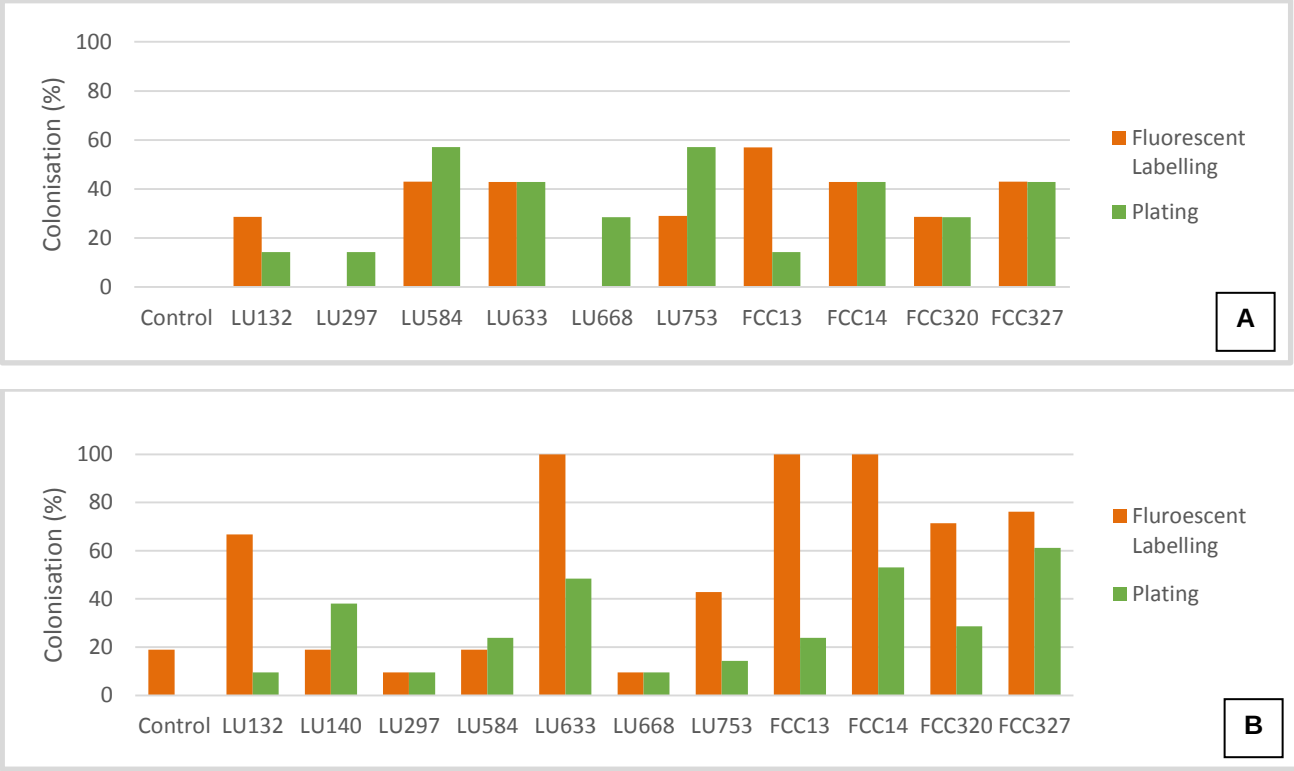


Figure 14: *Colonisation of* P. radiata *roots A) 3 days before emergence and B) 1 day after emergence, measured by fluorescence labelling and plating with MRB.*

CONCLUSION

The exact time when colonisation initially occurs could be further defined by sampling *P. radiata* seedlings before, and immediately after, emergence. Further experimentation should focus on isolates determined to strongly colonise roots(eg FCC13, FCC14, FCC320, FCC327, LU140, LU633 and LU668)

To improve the visualisation of the fungal material in further work the labelling method could potentially be refined in several ways including:

i) the addition of propidium iodide to the WGA-AF488 solution to better define plant cell walls and the position of the fungal hyphae.

ii) destaining of WGA-AF488 from root tissue with water for one week to improve the contrast between fungal structures and root cell walls in *P. radiata.*

iii) changing the concentration of WGA-AF488 to improve the contrast between fungal structures and root cell walls.

iv) changing the soaking time in KOH to improve the clearing of cell contents.

REFERENCES

Ayliffe, M., Periyannan, S., Feechan, A., Dry, I., Schumann, U., Wang, M., Pryor, A. and Lagudah, E. 2013. A simple method for comparing fungal biomass in infected plant tissue. Molecular Plant-Microbe Interactions. 26: 658-667.

Deshmukh, S., Hückelhoven, R., Schäfer, P., Imani, J., Sharma, M., Weiss, M., Waller, F. and Kogel, K., 2006. The root endophytic fungus Piriformospora indica requires host cell death for proliferation during mutualistic symbiosis with barley. Proceedings of the National Academy of Science USA 103: 18450–18457

Emde, B., Heinen, A., Gödecke, A. and Bottermann, K. 2014. Wheat Germ Agglutinin Staining as a Suitable Method for Detection and Quantification of Fibrosis in Cardiac Tissue after Myocardial Infarction. European Journal of Histochemistry 58: 315-319.

Martín-Rodrigues, N., Sanchez-Zabala, J., Salcedo, I., Duñabeitia, M.K., 2015. New insights into Radiata pine seedling root infection by *Fusarium circinatum*. Plant Pathology 64: 1336-1348.

Trouvelot, A., Kough, J. and Gianinazzi-Pearson, V. 1986. Mesure du taux de mycorhization VA d'un systeme radiculaire. Recherche de methods d'estimation ayant une signification fonctionnelle. In: Gianinazzi-Pearson V. and Gianinazzi, S., Eds., Physiological and Genetical Aspects of Mycorrhizae, Inra, Paris, 217-221.

Vierheilig, H., Schweiger, P. and Brundrett, M., 2005. An overview of methods for the detection and observation of arbuscular mycorrhizal fungi in roots. Physiologia Plantarum 125: 393-404

APPENDICES

Appendix A: Experimental Dates

Event	
	P. radiata
Planting and Inoculation Date	23 Aug 2016
Emergence Date	05 Sept
Sample 1 Root Plating WGA-AF488 Sections Taken	02 Sept (3 days before emergence) ✓ ✓ Tip
Sample 2 Root Plating WGA-AF488 Sections Taken	06 Sept (1 day after emergence) ✓ ✓ Top / Middle / Tip
Sample 3 Root Plating WGA-AF488 Sections Taken	12 Sept (7 days after emergence) ✓ X Top / Middle / Tip

✓ = measured
X = not measured

Appendix B: Malt Yeast Extract Agar (MYE) Recipe

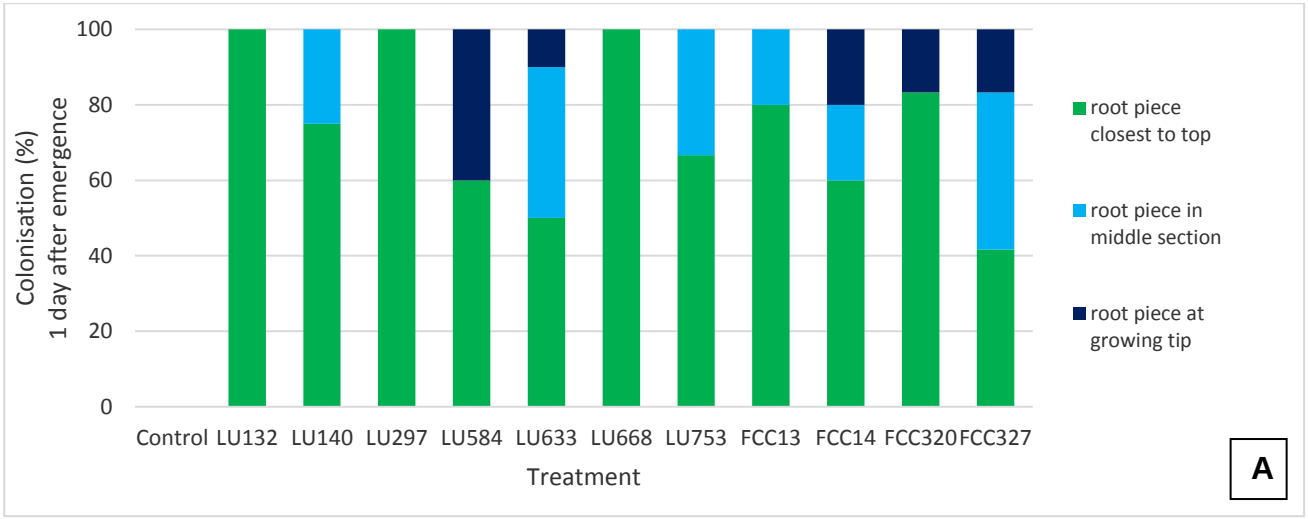
Malt extract	10 g
Yeast extract	1 g
Agar	20 g

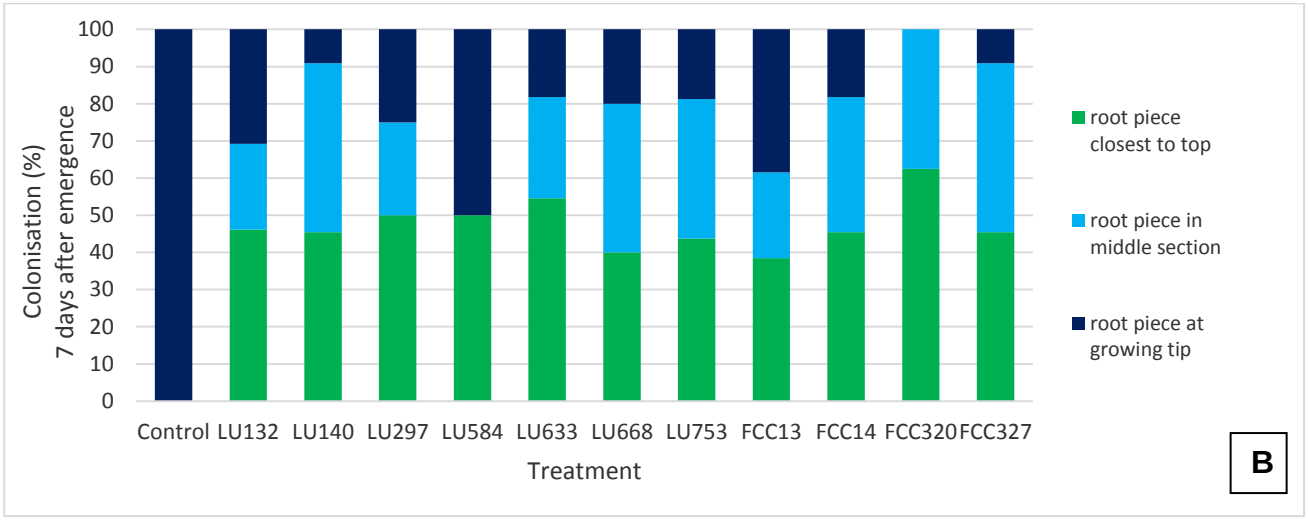
Make up to 1L with distilled water.

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: *Colonisation (%) of different sections of* P. radiata *roots by* Trichoderma *isolates 1 day (A) and 7 days (B) after emergence.*