Forests **2012**, *3*, 83-94; doi:10.3390/f3010083

OPEN ACCESS

forests

ISSN 1999–4907

www.mdpi.com/journal/forests

Article

Transformation of *Cyclaneusma minus* with Green Fluorescent Protein (GFP) to Enable Screening of Fungi for Biocontrol Activity

Rebecca McDougal [1,†,*], Alison Stewart [2] and Rosie Bradshaw [1]

[1] Institute of Molecular BioSciences, College of Sciences, Massey University, Private Bag 11–222, Palmerston North, 4442, New Zealand; E-Mail: r.e.bradshaw@massey.ac.nz

[2] Bio-Protection Research Centre, P.O. Box 84, Lincoln University, Christchurch 7647, New Zealand; E-Mail: alison.stewart@lincoln.ac.nz

[†] Current Address: Scion, New Zealand Forest Research Institute Ltd., Private Bag 3020, Rotorua, 3046, New Zealand.

[*] Author to whom correspondence should be addressed; E-Mail: rebecca.mcdougal@scionresearch.com; Tel.: +647-343-5534; Fax: +647-348-0952.

Received: 23 November 2011; in revised form: 10 January 2012 / Accepted: 19 January 2012 / Published: 1 February 2012

Abstract: Cyclaneusma needle-cast has a major impact on the New Zealand forest industry. The causal agent, *Cyclaneusma minus*, causes most severe damage to 11–20 year-old trees and currently there are no economically viable procedures for control of the disease in New Zealand. Here we present a method for genetic transformation of *C. minus* using protoplasts generated by incubation with Glucanex™ enzyme. *C. minus* was transformed with a gene encoding green fluorescent protein (GFP) and expression was stable after successive sub-culturing of the strain in the absence of selective pressure. Expression of the *gfp* gene allowed us to utilize an *in vitro* GFP-based screening method to identify strains of *Trichoderma* with potential for biocontrol of this disease. The strain that showed the most promise as a potential biocontrol candidate exhibited a low level of inhibition by uncharacterized metabolite(s) that *C. minus* secretes into the medium, and consistently caused a loss of GFP expression from the GFP-labeled *C. minus* strain. The interaction between *C. minus* and the biocontrol strain, in the interaction zone where GFP expression was lost, was determined to be fungicidal. The utility of such biocontrol strains is discussed. This study represents the first genetic manipulation of *C. minus* and will pave the way for further studies of the life cycle and infection biology of this organism.

Keywords: Cyclaneusma; needle-cast; protoplast; transformation; antagonism; biocontrol; GFP

1. Introduction

Cyclaneusma minus (Butin) DiCosmo, Peredo and Minter [1] is an ascomycetous fungus associated with disease in *Pinus* spp. Like many fungi, *Cyclaneusma* has undergone name changes during its research history and this has been documented elsewhere [2,3]. In addition, taxonomic classification of *Cyclaneusma* and *Naemacyclus* has been ambiguous [4]. Recent phylogenetic analysis has suggested that *Cyclaneusma* and *Naemacyclus* be treated as *Leotiomycetes incertae sedis* [5].

C. minus causes needle-cast in *Pinus* species worldwide, such as in Christmas tree plantations (*P. sylvestris* L.) [6,7]. Its association with disease is complicated by the fact that it can be found in asymptomatic tissue, and hence is often regarded as an endophyte or latent pathogen [8–10]. In Europe, *Cyclaneusma minus* is frequently reported as an endophyte. It has been recently isolated in Estonia although it is not acting as a serious pathogen [11]. In South Africa *C. minus* occurs mainly as a saprophyte on dead needles and is not associated with disease symptoms [12]. *C. minus* infects many *Pinus* species including *P. radiata* D. Don which is the predominant plantation species in New Zealand. Cyclaneusma needle-cast (CNC) currently causes the greatest economic loss compared to any other disease in the *P. radiata* plantation industry in New Zealand, costing $38 million each year [13].

CNC is most severe in *P. radiata* plantations with trees aged 11 to 20 years old, in New Zealand, although infection with *C. minus* is observed in trees from age three years [2]. Early observations showed a predominance of severe infections in isolated trees in a stand [14], as opposed to a part of a stand or a whole stand being infected. But more recently the disease has presented as mild infection in part of the stand or the whole stand, with individually infected trees observed less often [15]. This change in disease pattern has been attributed to the elimination of highly susceptible tree genotypes from planting stock in New Zealand [13]. Until recently the species of *Cyclaneusma* present in New Zealand were reported be *C. niveum* and *C. minus* [3]. *C. minus* has also been shown to consist of the "verum" and "simile" varieties [16], although recent research has suggested that these two varieties are actually separate species [17]. There is an urgent need for more detailed studies of the genetics, epidemiology and infection biology of this pathogen. Development of a genetic transformation system for *C. minus* would provide a useful tool to facilitate these studies. For example the use of reporter genes such as the green fluorescent protein (*gfp*) gene has become a popular method for monitoring fungal interactions [18,19]. GFP-labeling has been used to observe interactions of fungi with their plant hosts or with other fungi [20,21]. Therefore, GFP-labeling could provide a useful tool for studies of *C. minus* infection biology.

Several options for control of CNC have been investigated. In New Zealand, planting of tree genotypes less susceptible to disease during the 1990s has resulted in reduced levels of infection compared to those planted in the 1970s [22]. Genetic resistance has also been observed in *P. sylvestris* [7]. Silvicultural trials showed that stocking density and pruning had no effect on disease, although delayed thinning did provide control of CNC [2]. Fungicide trials performed in the 1980s

achieved reductions in infection levels, but not at a level where the cost of spraying could be justified [23], hence fungicide treatment is not a viable option for control. It has been shown by modeling that CNC disease severity is sensitive to environmental conditions. This information will provide a baseline to predict the impact of CNC under future climate scenarios [24].

Research into biocontrol for diseases of pine has gained momentum in recent times, including studies of biocontrol for nursery diseases [25,26], wood stain fungi [27,28] and wood decay [29]. *Trichoderma* species have been investigated for control of *Phythophthora cinnamomi* Rands in *P. radiata* roots [25] and for enhancing induced resistance to infection by *Diplodia pinea* (Desm.) Kickx [30]. Biocontrol has also been successfully used for control of *Armillaria*-induced *P. radiata* seedling mortality with a formulation of *Trichoderma* spores (ArborGuard™) [31–33]. A recent study has also investigated the potential for *Trichoderma* and *Clonostachys* species to control *Fusarium circinatum* Nirenberg and O'Donnell, the causal agent of pitch canker [34].

The use of biocontrol for *Dothistroma septosporum* (Dorog.) Morelet, another fungal pathogen of *P. radiata*, has been previously studied [35]. Screening of various fungal and bacterial strains using a novel method with green fluorescent protein (GFP)-labeled *D. septosporum* revealed several biocontrol strains with potential for use in control of dothistroma needle blight (DNB). This screening method uses GFP as an indicator of metabolic activity in the pathogen and also utilizes the hygromycin resistance selection marker (also encoded by the plasmid containing the *gfp* gene) to determine if the interaction is fungicidal or fungistatic. This method also takes into account the effect of inhibitory metabolites, produced by the pathogen, on the biocontrol strains [35]. The ability of biocontrol strains to provide control for CNC, as well as DNB, would present a unique opportunity for dual disease control for the forestry industry in New Zealand, especially considering no other means of control currently exist for CNC.

The aims of this work were, firstly, to develop a transformation system for *C. minus*, using the green fluorescent protein (*gfp*) gene and, secondly, to use the *C. minus gfp* transformant to determine if strains of *Trichoderma* spp. shown to be antagonistic to another foliar pine pathogen, *D. septosporum,* are also antagonistic to *C. minus.*

2. Materials and Methods

2.1. Source and Maintenance of Fungal Cultures

Cyclaneusma minus strain NZFS3617 was isolated from a symptomatic *Pinus radiata* tree in Kaingaroa Forest, New Zealand (Margaret Dick, Scion, Rotorua, New Zealand). All *Trichoderma* strains tested as potential biocontrol candidate strains were obtained from the Bio-Protection Research Centre, Lincoln University, New Zealand. *Dothistroma septosporum* strain FJT20 [35] was utilized as a positive control in some experiments as it has been previously transformed with the *gfp* gene. *E. coli* strain XL1-Blue [36] was used for plasmid propagation.

Dothistroma cultures were routinely maintained on dothistroma medium (DM) [37]. *C. minus* and *Trichoderma* strains were maintained on potato dextrose agar (PDA; Merck, Dramstadt, Germany). *E. coli* strain XL-1 was maintained on Luria-Bertani (LB) medium [38]. Pine minimal medium with glucose (PMMG) was used as an alternative medium to PDA for growth experiments and competition

assays [35]. All fungal culture incubations were performed at 22 °C, and *E. coli* incubations were performed at 37 °C.

2.2. Plasmid and Propagation

Plasmid pCT74, containing the *gfp* gene, has been described previously [18]. The plasmid was propagated by growth in *E. coli* XL-1 and extracted using an alkaline lysis method [38]. Plasmid DNA was restriction digested with SalI to confirm the correct size and quantified using a Nanodrop-1000 spectrophotometer (Thermo-Fischer Scientific, Waltham, MA, USA).

2.3. Protoplast Preparation and Transformation of C. minus

Protoplast generation and transformation were performed using a method based on that of Bradshaw *et al.* [39]. Ground mycelium of *C. minus* was inoculated onto PDA medium overlaid with sterile cellophane, and incubated at 22 °C for 6 days. Cellophanes with *C. minus* growth were removed from the agar and incubated with a sterile Glucanex™ (Novozymes Corp., Bagsværd, Denmark) solution (20 $mg \cdot mL^{-1}$ in OM buffer; 1.4 M $MgSO_4$, 10 mM Na_2HPO_4, pH adjusted to 5.8 with 100 mM NaH_2PO_4) at 30 °C with shaking at 110 rpm, for approximately 18 h. Protoplasts were placed into Corex centrifuge tubes in 5 mL aliquots and overlaid with 2 mL of ST buffer (0.6 M sorbitol, 100 mm Tris-HCl pH 8.0). Following centrifugation for 5 min at 20 °C and 1085× *g*, protoplasts were collected from the interface and washed twice with 5 mL of STC buffer (1 M sorbitol, 50 mm $CaCl_2$, 50 mm Tris-HCl pH 8.0) with further centrifugation for 5 min at 20 °C and 1085× *g*. The protoplasts were resuspended in STC buffer at a concentration of 10^7–10^8 per mL. Twenty µL of polyethylene glycol (PEG) 6000 solution (40% w/v PEG, 1 M sorbitol, 50 mM $CaCl_2$, 50 mM Tris-HCl, pH 8.0) was added to 80 µL aliquot of protoplasts. Approximately 2.5 µg of pCT74 DNA was added to the protoplasts, mixed gently and incubated on ice for 30 min. Additional PEG solution (900 µL) was added and protoplasts were mixed gently and incubated at 20 °C for 20 min. Aliquots (100 µL) were added to sterile, molten, non-selective medium (potato dextrose broth [PDB] containing 0.8 M sucrose and 0.8% [w/v] agar) and poured over base agar (PDB containing 0.8 M sucrose and 1.5% [w/v] agar). Plates were incubated at 22 °C overnight. Selective overlay medium containing hygromycin B (100 µg/mL) was prepared and poured over the plates. Incubation was continued at 22 °C.

2.4. DNA Extraction

The wild-type strain and a putative transformant of *C. minus* were grown in PDB for eight days with shaking at 200 rpm. *D. septosporum* strain FJT20 was grown in DM broth for seven days with shaking at 200 rpm. Mycelia were harvested by centrifugation at 4000 rpm for 10 min (Eppendorf centrifuge 5810) and stored at −20 °C prior to freeze-drying. DNA was extracted using a previously described method [40]. DNA was further purified using a Perfect Prep Gel Cleanup column (Eppendorf, Hamburg, Germany) starting from step five of the manufacturer's instructions. DNA was eluted in 60 µL of sterile milliQ water and quantified using a Nanodrop-1000 spectrophotometer (Thermo-Fischer Scientific, Waltham, MA, USA).

2.5. PCR Amplification of the gfp *Gene from the Transformant*

To confirm the presence of the *gfp* gene in putative transformants, DNA was amplified using primers rtGFPfwd1 and rtGFPrev1 [35]. DNA from wild-type *C. minus* and *D. septosporum* FJT20 were used as negative and positive controls respectively, as well as a no-template control to check for contamination. PCR reactions were performed using the FIREPol® DNA Polymerase kit (Solis BioDyne, Tartu, Estonia), according to the manufacturer's instructions. Each 25 μL PCR reaction typically contained 10× buffer BD (2.5 μL), 1.5 mM $MgCl_2$, 0.4 μM of each primer, 1.25 U of FIREPol® DNA Polymerase, 1 μL of DNA (approx 50 ng) and PCR-grade water (up to 25 μL total volume). The cycling conditions consisted of an initial denaturation step of 95 °C for 4 min, then 30 cycles of 94 °C (1 min), 55 °C (1 min) and 72 °C (1 min), and a final extension step of 72 °C (10 min). Gel electrophoresis was performed with 1% (wt/vol) agarose in TBE and visualized by UV transillumination after staining with ethidium bromide.

2.6. Southern Hybridization

For hybridization, digested genomic DNA from wild-type and putative transformant strains, as well as *D. septsporum* FJT20 was transferred to Amersham Hybond-N^+ nylon membranes (GE Healthcare Ltd., Buckinghamshire, UK) by capillary transfer [38]. A *gfp* gene probe was labelled using a DIG high-prime DNA labelling system (Roche Applied Science, Penzberg, Germany). Hybridizations were carried out at 42 °C in Roche standard hybridization buffer containing formamide [50% deionized formamide, 5× SSC, 0.1% (w/v) *N*-lauroylsarcosine, 0.02% (w/v) SDS, and 2% (w/v) blocking reagent].

2.7. Comparison of Growth Rates

Growth of the *C. minus* wild-type and the confirmed transformant were compared on PDA and PMMG (pH 4.0 and 7.0) to determine if transformation of *C. minus* with the *gfp* gene had any detrimental effects on growth. A 6 mm mycelial plug was inoculated onto each medium for both strains. Plates were prepared in triplicate and incubated at 22 °C. Growth measurements were performed at time intervals over 20 days. Data were analyzed in Microsoft Excel; a TTEST was used to determine if there was significant difference in growth rates between the strains.

2.8. Screening Potential Biocontrol Strains

Competition assays were performed with *gfp*-labeled *C. minus* (FJT95) as described by McDougal *et al.* [35], on both PDA and PMMG medium, except that the *gfp*-labeled *C. minus* strain was incubated for nine days prior to co-inoculation with biocontrol strains. For assessment of fungicidal or fungistatic effects, plugs were cut from colonies of FJT95 on the biocontrol challenge plates that had completely lost GFP fluorescence. These were then sub-cultured onto PDA medium, containing hygromycin B at pre-determined minimum inhibitory concentrations (MIC) ranging from 100–180 $\mu g \cdot mL^{-1}$.

3. Results

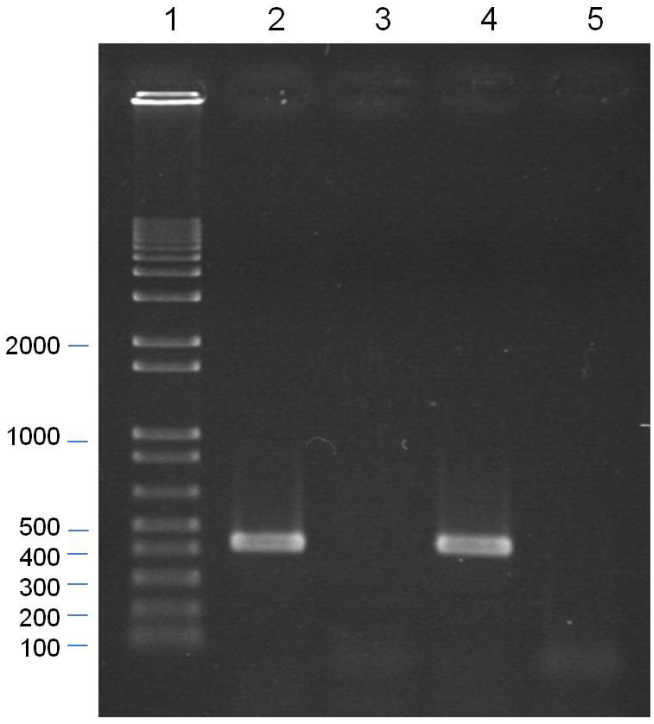
3.1. C. minus *Protoplast and Transformation Yield, and GFP Expression*

Approximately 6×10^8 protoplasts were obtained from mycelium grown on four cellophane discs. A higher yield of protoplasts was obtained after 24 h incubation with Glucanex™ compared to 8 h incubation (data not shown). Growth of mycelia and regeneration of protoplasts of untransformed *C. minus* (no DNA control) were completely inhibited by 100 µg/mL hygromycin B. Transformation efficiency was very low with only four putative transformant colonies observed on selective medium. GFP was present in one colony (out of four) when observed under UV light, indicating that the *gfp* gene was expressed in one transformant. GFP expression was absent in the *C. minus* wild-type strain. The GFP expression was determined to be stable in the transformant by successive sub-culturing, on non-selective medium, without loss of GFP. The *C. minus gfp* transformant was designated FJT95.

3.2. Molecular Characterization of FJT95

DNA yields were high (average 0.7 µg DNA mg^{-1} dry weight of mycelium) from *C. minus* FJT95, *C. minus* wild-type (untransformed) and *D. septosporum* FJT20. PCR was performed to determine the presence of the *gfp* gene using rtGFPfwd1 and rtGFPrev1 primers with DNA from *C. minus* FJT95. PCR products of the expected size (approximately 450 bp) were obtained with DNA from *C. minus* FJT95 and the positive control strain *D. septosporum* FJT20, but no products were obtained with DNA from *C. minus* wild-type or in the no-template control (Figure 1).

Figure 1. Detection of *gfp* gene by PCR in *C. minus* transformant strain FJT95. Lane 1, 1 kb^+ ladder; Lane 2, *C. minus* FJT95; Lane 3, *C. minus* wild-type; Lane 4, *D. septosporum* strain FJT20; Lane 5, no-template control.



The presence of the *gfp* gene in strain FJT95 was verified by Southern hybridization. The *gfp* probe hybridized to DNA fragments of approximately 7.5 kb in the *C. minus* FJT95 transformant and a DNA fragments of approximately 8.0 kb in *D. septosporum* FJT20 (positive control), but no hybridization was observed to DNA from *C. minus* wild-type (data not shown).

3.3. Growth of FJT95 Compared to C. minus *Wild Type*

To determine whether transformation of *C. minus* with pCT74 had an effect on the growth of FJT95, strains were grown on PDA and PMMG medium and growth rates were determined over time. No significant difference ($p > 0.05$) was observed in the growth rates of the FJT95 transformant compared to *C. minus* wild-type on PDA or PMMG medium: FJT95 exhibited a growth rate of 1.4 ± 0.1 mm·day^{-1} on PDA and 1.6 ± 0.0 mm·day^{-1} on PMMG medium, whereas the *C. minus* wild-type strain had a growth rate of 1.5 ± 0.1 mm·day^{-1} on PDA and 1.5 ± 0.2 mm·day^{-1} on PMMG.

3.4. Screening Fungal Strains for Antagonistic Activity towards C. minus *FJT95*

Strains of *Trichoderma* were screened against *C. minus gfp* transformant FJT95 in an *in vitro* screening assay that utilizes GFP expression by the pathogen as an indicator of metabolic activity. The strains tested included some of those previously shown to exhibit antagonistic activity toward *D. septosporum* such as FBC2, FBC4 and FBC6 [35]. It has been observed that *C. minus* produces a yellow-colored metabolite (uncharacterized) that is secreted into the medium during growth. The toxicity of this metabolite to other fungi is not known. Therefore, as with *D. septosporum*, growth measurements of the biocontrol strains were recorded to determine if their growth was inhibited in the presence of *C. minus* FJT95. The levels of inhibition ranged from 6.3% to 44.2%, with an average of 17.8%. The strain showing the lowest level of inhibition was FBC4 (Table 1).

Loss of GFP expression by the *C. minus* FJT95 strain when grown in dual culture was used as an indication of antagonistic activity by potential biocontrol strains as previously described with *D. septosporum* [35]. FJT95 did not show any loss of GFP fluorescence after growth with FBC11 on PDA medium (Figure 2a,b), suggesting no antagonistic effect of FBC11. Loss of GFP from strain FJT95 was observed when grown with FBC4 on both PDA (Figure 2c,d) and PMMG media (data not shown), suggesting antagonistic activity of FBC4 towards *C. minus*. While other strains also showed some ability to cause a loss of GFP fluorescence, their antagonist effects appeared to be inconsistent and influenced by the type of media used (summarized in Table 1).

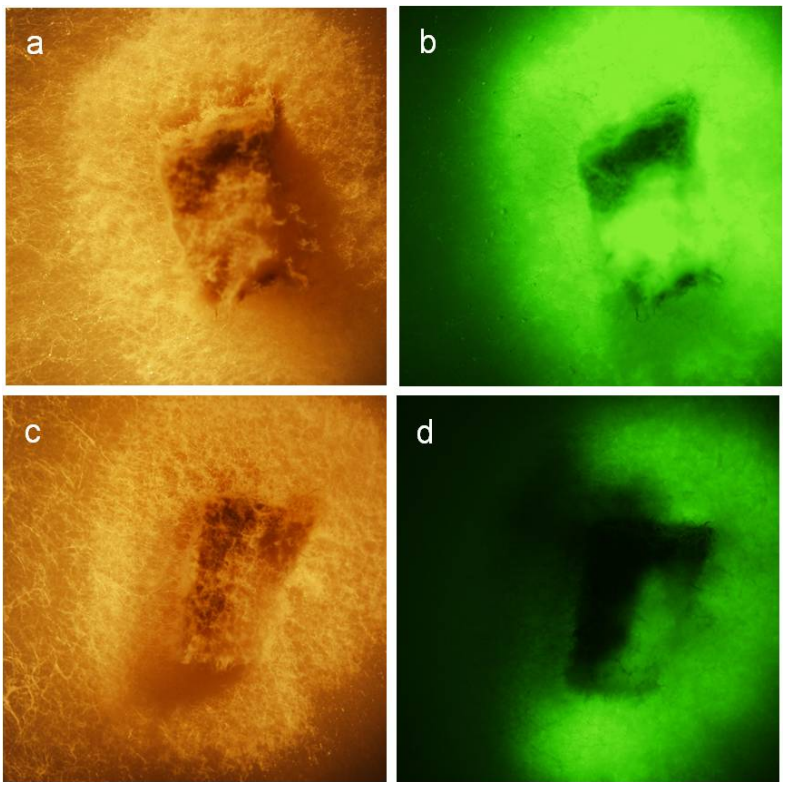
To determine if FJT95 was viable in regions where GFP fluorescence was lost, a small region of the interaction zone containing both the FJT95 and FBC4 mycelia were sub-cultured onto selective media in which only strain FJT95 would grow, as previously described with *D. septosporum* [35]. No growth was observed from the FJT95 plugs taken from colonies overgrown by FBC4 strains, whereas the FJT95 positive control grew on the selective plates, and the *C. minus* (wild-type) negative control did not grow (data not shown). The results indicated that FBC4 had a fungicidal effect on strain FJT95.

Table 1. Inhibition of FBC strains by *C. minus* FJT95 and post-competition green fluorescent protein (GFP) fluorescence of FJT95.

Trichoderma species and strain		% Inhibition of *Trichoderma* by *C. minus* [1]		Loss of GFP fluorescence in *C. minus* [2]	
		PDA [3]	PMMG [4]	PDA	PMMG
T. atroviride	FBC2	11.9 ± 7.7	**12.3 ± 8.4**	+	+/−
T. atroviride	FBC4	**13.9 ± 3.9**	6.3 ± 5.0	-	-
T. hamatum	FBC6	**33.4 ± 10.4**	**44.2 ± 3.5**	-	+/−
T. atroviride	FBC9	**10.5 ± 7.0**	**13.5 ± 4.0**	-	+/−
T. atroviride	FBC10	13.0 ± 0.0	15.5 ± 4.2	+	-
T. atroviride	FBC11	15.8 ± 5.6	**26.8 ± 2.2**	++	-
T. atroviride	FBC12	**14.7 ± 4.3**	**16.8 ± 4.9**	+	-

[1] % Inhibition of growth of FBC strains by *C. minus* (produces uncharacterized metabolites), calculated from radial growth measurements toward and away from *C. minus* (data not shown). Values are the mean of four replicate assays ± standard deviation. Text in bold indicates no significant difference ($p \leq 0.05$) between growth towards and away from *C. minus*. [2] ++: no loss of GFP fluorescence (indicates no antagonistic activity); +: partial loss GFP fluorescence; -: complete loss of GFP fluorescence (indicates antagonistic activity); +/−: equal number of + and – assays observed. Typical results from four replicate assays. [3] PDA: Potato dextrose agar (see Section 2.1). [4] PMMG: Pine minimal medium with glucose (see Section 2.1).

Figure 2. GFP fluorescence of *C. minus* FJT95 after *in vitro* interaction with *Trichoderma atroviride* strains FBC4 and FBC11. (**a**) FJT95 with FBC11 under visible light; (**b**) FJT95 with FBC11 under UV light; (**c**) FJT95 with FBC4 under visible light; (**d**) FJT95 with FBC4 under UV light; note loss of fluorescence on the left. FBC4 and FBC11 grew towards FJT95 from left hand side of the photographs.



4. Discussion

The ability to perform genetic manipulation with plant pathogenic fungi paves the way for studies of complex host-pathogen or pathogen-biocontrol agent interactions. In this study we have shown that *C. minus* is amenable to transformation, although the transformation efficiency was poor despite obtaining good numbers of protoplasts. Further studies are required to optimize the transformation efficiency. Nevertheless, transformation was achieved and the *C. minus* GFP-transformant, strain FJT95, did not exhibit any differences to the wild-type strain with respect to growth rate on the two media tested. The GFP-labeled *C. minus* strain FJT95 was used for further studies to investigate the biocontrol potential of *Trichoderma* strains.

The use of a previously published method for screening potential biocontrol agents of *D. septosporum* [35] was applied to *C. minus* strain FJT95 in this study. This method was previously shown to be effective for *D. septosporum*, a slow-growing fungus that secretes metabolites into the growth medium. These are also characteristics of *C. minus* and the method proved very effective with this organism. The colony morphologies of *D. septosporum* and *C. minus* are quite different, the former having raised, dense colonies and the latter more flat and filamentous. Despite this the GFP based-method still provided clear results indicating that the *Trichoderma* strain that showed greatest antagonistic activity against *C. minus* was also antagonistic toward *D. septosporum* [35], and suggests that this method could be used to test the biocontrol potential of other fungi.

The results of the screening method, utilizing GFP expression and data from colony measurements, were used to determine which *Trichoderma* strains showed potential for biocontrol of *C. minus*. For simplicity, we present data for six strains of *T. atroviride* and one strain of *T. hamatum*, although the method was tested using a total of 20 strains including *Trichoderma* spp (belonging to four species) as well as one strain each of *Clonostachys rosea* and one strain of *Ulocladium* sp. Of all the strains tested *T. atroviride* strain FBC4 had the greatest antagonistic activity against *C. minus in vitro*, as evidenced by consistent loss of GFP from strain FJT95 on both media. Strain FBC4 also grew well in the presence of *C. minus* as shown by low growth inhibition levels, whilst other strains such as *T. hamatum* FBC6 were inhibited, leading us to speculate that *C. minus* produces antifungal metabolites. Interestingly, *T. atroviride* strain FBC4 also showed a high level of antagonism toward *D. septosporum*, whereas FBC2 was less effective at reducing GFP expression from *C. minus* compared to *D. septosporum* [35]. In addition, loss of GFP expression also correlated with fungicidal activity of the biocontrol strains, as determined by sub-culturing on medium containing hygromycin. Lack of growth on hygromycin by FBC4-challenged *C. minus* FJT95 (that contained a hygromycin resistance selectable marker gene as well as the *gfp* gene), indicated that it had lost viability.

Nursery and forest trials are required to determine if the biocontrol candidates identified in this *in vitro* study have potential for control of CNC. Because *C. minus* generally only causes disease on *P. radiata* from an age of approximately three years [2], it would be difficult to perform *in planta* biocontrol trials with seedlings. However, it may be possible to perform nursery trials with small plants grown from cuttings of trees that are older than three years.

GFP-labeling of plant pathogens opens the door to many techniques for studying their pathogenicity. For example further characterization of the infection process, or elucidating the mechanism involved in its endophytic or latent pathogen lifestyle will be facilitated by use of the GFP-labeled *C. minus* strain.

To the best of our knowledge, this is the first time genetic transformation of *C. minus* has been achieved, thus providing a GFP-labeled strain and ability to use this strain to further validate a recently published method for biocontrol screening. *In planta* testing of potential biocontrol strains identified using this type of approach will provide further screening to elucidate the most promising strains for biocontrol.

Acknowledgments

RM was supported by funding from The Agricultural and Marketing Development and Research Trust (AGMARDT). Margaret Dick (Scion, New Zealand) is thanked for providing *C. minus* NZFS3617. Robert Hill (Bio-Protection Research Centre, New Zealand) is thanked for advice on screening strains for biocontrol. Rebecca Ganley and John Bain are thanked for comments on the manuscript. This work was carried out in compliance with the current laws governing genetic experimentation in New Zealand.

Conflict of Interest

The authors declare no conflict of interest.

References

1. DiCosmo, F.; Peredo, H.; Minter, D.W. *Cyclaneusma* gen. nov., *Naemacyclus* and *Lasiostictis*, a nomenclatural problem resolved. *Eur. J. For. Path.* **1983**, *13*, 206–212.
2. Bulman, L.S.; Gadgil, P.D. *Cyclaneusma needle-cast in New Zealand, Forest Research Bulletin No. 222*; Forest Research: Rotorua, New Zealand, 2001.
3. Gadgil, P.D. Cyclaneusma (Naemacyclus) needle-cast of *Pinus radiata* in New Zealand. 1. Biology of *Cyclaneusma minus*. *New Zeal. J. For. Sci.* **1984**, *14*, 179–196.
4. Gernandt, D.S.; Platt, J.L.; Stone, J.K.; Spatafora, J.W.; Holst-Jensen, A.; Hamelin, R.C.; Kohn, L.M. Phylogenetics of Helotiales and Rhytismatales based on partial small subunit nuclear ribosomal DNA sequences. *Mycologia* **2001**, *93*, 915–933.
5. Lantz, H.; Johnston, P.R.; Park, D.; Minter, D.W. Molecular phylogeny reveals a core clade of Rhytismatales. *Mycologia* **2011**, *103*, 57–74.
6. Kistler, B.R.; Merrill, W. Etiology, symptomology, epidemiology and control of Naemacyclus needle-cast of Scots pine. *Phytopathology* **1977**, *68*, 267–271.
7. Merrill, W.; Wenner, N.G. *Cyclaneusma* needle-cast and needle retention in Scots Pine. *Plant Dis.* **1996**, *80*, 294–298.
8. Helander, M.L.; Sieber, T.N.; Petrini, O.; Neuvonen, S. Endophytic fungi in Scots Pine needles—Spatial variation and consequences of simulated acid-rain. *Can. J. Bot.* **1994**, *72*, 1108–1113.
9. Kowalski, T. Fungi in living symptomless needles of *Pinus sylvestris* with respect to some observed disease processes. *J. Phytopathol.* **1993**, *139*, 129–145.
10. Sieber, T.N.; Rys, J.; Holdenrieder, O. Mycobiota in symptomless needles of *Pinus mugo* ssp. *uncinata*. *Mycol. Res.* **1999**, *103*, 306–310.
11. Drenkhan, R.; Hanso, M. Recent invasion of foliage fungi of pines (*Pinus* spp.) to the Northern Baltics. *For. Stud.* **2009**, *51*, 49–64.

12. Crous, P.W.; Wingfield, M.J.; Swart, W.J. Shoot and needle diseases of *Pinus* spp. in South Africa. *South African For. J.* **1990**, 60–66.
13. Bulman, L. Quantifying pest impact and spread: Cyclaneusma needle-cast. In *Forest Biosecurity and Protection Annual Science Report*; Scion: Rotorua, New Zealand, 2009. Available online: http://www.scionresearch.com/general/science-publications/science-publications/technical-reports/forest-protection/forest-biosecurity (accessed on 20 November 2011).
14. Gadgil, P.D. Symptoms and pathogenicity. In *Cyclaneusma needle-cast in New Zealand, Forest Research Bulletin No. 222*; Bulman, L.S., Gadgil, P.D., Eds.; Forest Research: Rotorua, New Zealand, 2001; pp. 2–5.
15. Bulman, L. Scion (New Zealand Forest Research Institute Ltd), Rotorua, New Zealand. Personal Communication, 2011.
16. Dick, M.A.; Somerville, J.G.; Gadgil, P.D. Variability in the fungal population. In *Cyclaneusma needle-cast in New Zealand, Forest Research Bulletin No. 222*; Bulman, L.S., Gadgil, P.D., Eds.; Forest Research: Rotorua, New Zealand, 2001; pp. 12–19.
17. Glen, M.; Prihatini, I.; Smith, T.; Wardlaw, T.; Mohammed, C. Spring Needlecast in Tasmania—Fungal Communities and Environmental Factors. Presented at ACPP APPS 2011: New Frontiers in Plant Pathology for Asia and Oceania, Darwin, Australia, 26–29 April 2011. Available online: http://www.appsnet.org/Publications/Proceedings/APPS%202011%20Handbook.pdf (accessed on 20 November 2011).
18. Lorang, J.M.; Tuori, R.P.; Martinez, J.P.; Sawyer, T.L.; Redman, R.S.; Rollins, J.A.; Wolpert, T.J.; Johnson, K.B.; Rodriguez, R.J.; Dickman, M.B.; Ciuffetti, L.M. Green fluorescent protein is lighting up fungal biology. *App. Environ. Microbiol.* **2001**, *67*, 1987–1994.
19. Larrainzar, E.; O'Gara, F.; Morrissey, J.P. Applications of autofluorescent proteins for *in situ* studies in microbial ecology. *Annu. Rev. Phytopathol.* **2005**, *59*, 257–277.
20. Lu, Z.X.; Tombolini, R.; Woo, S.; Zeilinger, S.; Lorito, M.; Jansson, J.K. *In vivo* study of *Trichoderma*-pathogen-plant interactions, using constitutive and inducible green fluorescent protein reporter systems. *Appl. Environ. Microbiol.* **2004**, *70*, 3073–3081.
21. McLean, T.; Fourie, P.H.; McLeod, A. Reporter gene transformation of the trunk disease pathogen *Phaeomoniella chlamydospora* and biological control agent *Trichoderma harzianum*. *Australas. Plant. Path.* **2009**, *38*, 153–167.
22. Bulman, L.S. Economic impact of Cyclaneusma Needlecast in New Zealand. In *Client Report No. 16815*; Scion: Rotorua, New Zealand, 2009.
23. Hood, I.A.; Bulman, L.S. Chemical control. In *Cyclaneusma Needle-Cast in New Zealand, Forest Research Bulletin No. 222*; Bulman, L.S., Gadgil, P.D., Eds.; Forest Research: Rotorua, New Zealand, 2001; pp. 50–54.
24. Watt, M.; Rolando, C.; Palmer, D.; Bulman, L.S. Predicting the severity of *Cyclaneusma minus* on *Pinus radiata* in New Zealand. *Forest Health News* **2011**, *217*. Available online: http://www.scionresearch.com/general/science-publications/science-publications/science-newsletters/forest-health-newsletter (accessed on 20 November 2011).
25. Reglinski, T.; Dick, M. Biocontrol of forest nusery pathogens. *New Zeal. J. For. Sci.* **2005**, *50*, 19–26.

26. Valiente, C.; Diaz, K.; Gacitúa, S.; Martinez, M.; Sanfuentes, E. Control of charcoal root rot in *Pinus radiata* nurseries with antagonistic bacteria. *World J. Microb. Biot.* **2008**, *24*, 557–568.
27. Held, B.W.; Thwaites, J.M.; Farrell, R.L.; Blanchette, R.A. Albino strains of *Ophiostoma* species for biological control of sapstaining fungi. *Holzforschung* **2003**, *57*, 237–242.
28. Vanneste, J.L.; Hill, R.A.; Kay, S.J.; Farrell, R.L.; Holland, P.T. Biological control of sapstain fungi with natural products and biological control agents: A review of the work carried out in New Zealand. *Mycol. Res.* **2002**, *106*, 228–232.
29. Schoeman, M.W.; Webber, J.F.; Dickinson, D.J. The development of ideas in biological control applied to forest products. *Int. Biodeter. Biodegr.* **1999**, *43*, 109–123.
30. Reglinski, T.; Rodenburg, N.; Taylor, J.T.; Northcott, G.L.; Chee, A.A.; Spiers, T.M.; Hill, R.A. *Trichoderma atroviride* promotes growth and enhances systemic resistance to *Diplodia pinea* in radiata pine (*Pinus radiata*) seedlings. *For. Pathol.* **2012**, *42*, 75–78.
31. Hill, R.A.; Paderes, D.E.; Wigley, P.J.; Broadwell, A.H. Growth of *Pinus radiata* seedlings in the nursery with novel microbial formulations. *N. Z. Plant Protect.* **2007**, *60*, 305.
32. Hill, R.A.; Stewart, A.; Hohmann, P.; Braithwaite, M.; Clouston, A.; Minchin, R. Enhancing growth and health of *Pinus radiata* in New Zealand and *Acacia mangium* in Malaysia with selected *Trichoderma* isolates. In *Proceedings of the 11th International workshop on Trichoderma and Gliocladium*, Haifa, Israel, 13 October 2010.
33. Paderes, D.E.; Hill, R.A.; Wang, W.-Y.; Stewart, A. Development of a bio-protection system for *Pinus radiata* with *Trichoderma* (ArborGuard™). In *Proceedings of the 4th Australasian Soilborne Diseases Symposium*, Queenstown, New Zealand, 3–6 September 2006.
34. Moraga-Suazo, P.; Opazo, A.; Zaldúa, S.; González, G.; Sanfuentes, E. Evaluation of *Trichoderma* spp. and *Clonostachys* spp. strains to control *Fusarium circinatum* in *Pinus radiata* seedlings. *Chil. J. Agri. Res.* **2011**, *71*, 412–417.
35. McDougal, R.; Yang, S.; Schwelm, A.; Stewart, A.; Bradshaw, R. A novel GFP-based approach for screening biocontrol microorganisms *in vitro* against *Dothistroma septosporum*. *J. Microbiol. Meth.* **2011**, *87*, 32–37.
36. Bullock, W.O.; Fernandez, J.M.; Short, J.M. XL1-Blue—A high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. *Biotechniques*. **1987**, *5*, 376–378.
37. Bradshaw, R.E.; Ganley, R.J.; Jones, W.T.; Dyer, P.S. High levels of dothistromin toxin produced by the forest pathogen *Dothistroma pini*. *Mycol. Res.* **2000**, *104*, 325–332.
38. Sambrook, J.; Fritsch, E.F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd ed.; Cold Spring Harbour Laboratory Press: New York, NY, USA, 1989.
39. Bradshaw, R.E.; Bidlake, A.; Forester, N.; Scott, D.B. Transformation of the fungal forest pathogen *Dothistroma pini* to hygromycin resistance. *Mycol. Res.* **1997**, *101*, 1247–1250.
40. Doyle, J.J.; Doyle, J.L. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem. Bull.* **1987**, *19*, 11–15.