Do plant endophytic and free-living *Chaetomium* species differ?

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Abstract

The carbon nutrition of leaf endophytes and free-living members of the genus *Chaetomium* were compared to better understand the interaction between endophytes and their host, and the potential for endophytes to have complex life cycles. Broadly similar potential utilisation of several carbon sources was observed among endophytes and free-living isolates of *Chaetomium*. All fungi developed measurable biomass on glucose, cellobiose, carboxymethyl cellulose and xylan, and crystalline cellulose was slowly cleared from agar. Pectin was poorly utilised by these isolates. Sequence analysis of the fungi indicates that diverse taxa of *Chaetomium* may colonise wheat leaves, and that fungi with similar sequences are found in other habitats. Endophytic isolates of *Chaetomium* may complete part of their life cycle in other habitats.

Key words: Carbon nutrition, polysaccharide, plant fungus interaction, cellulose, pectin, xylan.

Introduction

Fungal endophytes are ubiquitous in leaf and stem tissues of plant species (Petrini 1991; Marshall *et al*. 1999; Schulz *et al*. 1999). Specific information on the interaction between endophytes and their plant host has been derived from studies of *Neotyphodium* species and their systemic association with Pooid grasses (Saikkonen *et al*. 1998; Faeth & Fagan 2002). Transmission of *Neotyphodium* species is vertical, from host to seed, and colonisation of the plant may increase plant resistance to herbivory, pathogens and a range of abiotic stresses (Breen 1993; Crous *et al*. 1995; Marshall *et al*. 1999). While endophytic *Neotyphodium* species obtain spatial, nutritional and protective benefits from the host, the interaction may range from mutualistic through neutral to antagonistic and varies between each endophyte and host combination (Saikkonen *et al*. 1998; Faeth & Fagan, 2002).

Endophytes with horizontal transmission between hosts are widespread. For instance, endophytic *Chaetomium* species have been isolated from healthy leaves of wheat (*Triticum* sp.: Crous *et al*. 1995; Dingle & McGee 2003; Istifadah & McGee 2006) where it grows between cells of the leaf (Istifadah 2005). Morphological identification indicates a close relationship between endophytic and free-living *Chaetomium* (Istifadah 2005).

Unlike *Neotyphodium* species, horizontally transmitted endophytes have a portion of their lifecycle outside the host (Petrini 1991; Dingle & McGee 2003; Kriel *et al*. 2000; Wilson 1995; Saikkonen *et al*. 1998). The nutritional biology of endophytic *Chaetomium* species is unclear. Endophytic species of *Chaetomium* presumably obtain carbon from photosynthate when colonising plants, but they may also derive carbon from plant cell-wall constituents such as cellulose, hemicellulose and pectin, access that might harm the host. Fungal access to carbon in plant polysaccharide is dependant on the expression of an array of cellulases, hemicellulases, pectinases and accessory enzymes (de Vries & Visser 2001). Some free-living species of *Chaetomium* produce these enzyme activities (Fähnrich & Irrgang 1982; Markham & Bazin 1991; Czakaj & Czuba 2003; Latif *et al*. 2006) though their activity in endophytic isolates of *Chaetomium* remains unknown.

The aims of the current study were thus to compare groups of plant endophytic and free-living *Chaetomium* isolates for their potential to degrade plant cell-wall polysaccharides and to investigate whether the two groups of *Chaetomium* were genetically distinct.

Materials and methods

Fungal Isolates

The 14 isolates of *Chaetomium* used in this study were isolated from a variety of sources (Table 1). Briefly, six endophytic fungi, CA, CB2, CB3, CB5, CW2 (from Camden, NSW) and NC1 (from Narrabri, NSW), were isolated from surface-sterilised leaves of field grown wheat (Istifadah & McGee 2006). The non-endophytic isolates were obtained from a number of sources: two isolates (NSW2 and NSW3) were obtained from soil collected at Narrabri, NAD2 and NAD3 were obtained from different batches of surface sterilised seeds of cranberries (*Vaccinium macrocarpon*) while the remainder (NA12, NA16, NA25 and NA26) were isolated from scats of the common brushtailed possum (*Trichosurus vulpecular*) collected from different locations around the University of Sydney. Endophytic fungi were previously morphologically identified by Dr Michael Priest (Istifadah & McGee 2006) while other fungi were morphologically identified (Watanabe 2002; Domsch *et al*. 1993; Bell 2005). Prior to all experiments, all isolates were maintained on potato dextrose agar (PDA, Sigma) in the dark at 20°C with sub-culturing every 3–4 wk.

Solubilisation of crystalline cellulose

Four replicates of all isolates of *Chaetomium* were grown in 9 cm diam. Petri dishes containing 25 mL of carbon free Czapek media (Levinskaite 2004) supplemented with 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0%, 4.5% and 5.0% microcrystalline cellulose powder (Sigma) at 20ºC for 14 d. For each concentration four Petri dishes were left uninoculated as negative controls. An isolate of *Trichoderma viride*, a known cellulolytic fungus, was included as a positive control. The presence and extent of cellulose solubilsation around and under growing mycelium was assessed and compared to the controls daily.

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Table 1 Closest matches determined from BLASTN searches between 5.8S, ITS1 and ITS2 regions of *Chaetomium* isolates of field samples and sequences from the GenBank nucleotide database.

Accession *Isolate*	Source	Morphological identity	GenBank closest match[1]	ID | OL[2]	Tentative identity
EU035794 *CA*[3a]	Wheat leaves	*C. dolichotrichum*	AJ279450[4] *Chaetomium funicola*	100 | 540	*Chaetomium* sp.
EU035795 *CB2*	Wheat leaves	*C. olivaceum*	AJ279468[4] *Chaetomium* sp. 6/97-38	99 | 534	Chaetomium *sp.*
EU035796 *CB3*[3c]	Wheat leaves	*C. globosum*	AY625061[4] *Chaetomium globosum* strain UAMH 7142	100 | 536	*Chaetomium globosum*
EU035797 *CB5*[3b]	Wheat leaves	*C. cochliodes*	AY560520[4] *Chaetomium* sp. 73-19-O-Mexico	99 | 535	*Chaetomium* sp.
EU035798 *CW2*[3a]	Wheat leaves	*C. dolichotrichum*	AJ279450[4] *Chaetomium funicola*	100 | 540	*Chaetomium* sp.
EU035799 *NA12*[3b]	Possum scats	*C. globosum*	AY560520[4] *Chaetomium* sp. 73-19-O-Mexico	99 | 535	*Chaetomium* sp.
EU035800 *NA16*[3b]	Possum scats	*C. globosum*	AY560520[4] *Chaetomium* sp. 73-19-O-Mexico	99 | 535	*Chaetomium* sp.
EU035801 *NA25*	Possum scats	C. globosum	AY560520[4] *Chaetomium* sp. 73-19-O-Mexico	99 | 535	*Chaetomium* sp.
EU035802 *NA26*	Possum scats	*Chaetomium* sp.	AJ458185 *Chaetomium nigricolor*	94 | 496	*Chaetomium* sp.
EU035803 *NAD2*[3b]	Cranberry seeds	*C. globosum*	AY560520[4] *Chaetomium* sp. 73-19-O-Mexico	99 | 535	*Chaetomium* sp.
EU035804 *NAD3*[3b]	Cranberry seeds	*C. cochliodes*	AY560520[4] *Chaetomium* sp. 73-19-O-Mexico	99 | 535	*Chaetomium* sp.
EU035805 *NC1*[3c]	Wheat leaves	*C. globosum*	AY625061[4] *Chaetomium globosum* strain UAMH 7142	100 | 536	*Chaetomium globosum*
EU035806 *NSW2*	Soil	*C. globosum*	AY429056 *Chaetomium globosum* isolate Cg9	99 | 534	*Chaetomium globosum*
EU035807 NSW3	Soil	*C. globosum*	AY429056 *Chaetomium globosum* isolate Cg9	99 | 535	*Chaetomium globosum*

[1] The entire sequence consisting of 18S partial sequence; ITS1, 5.8S, ITS2 complete sequence; 28S partial sequence used in BLASTN searches

[2] ID | OL: Percentage identity | overlap region

[3] Identical sequences: a) CA, CW2; b) CB5, NA12, NA16, NAD2, NAD3; c) CB3, NC1

[4] AJ279450 and AJ279468 (Wirsel *et al*. 2001); AY560520 (Perez-Vera *et al*. 2005); AY625061 (Melkin *et al.* 2004)

Use of soluble carbohydrates

Prior to this experiment all 14 *Chaetomium* isolates were cultured on a carbon-free, low nitrogen, modified Melin Norkrans (MMN) agar (Marx & Bryan 1975; Midgley *et al*. 2004) for 7 d in order to deplete internal carbon stores. To determine utilisation of the various carbon substrates, carbon-free low CN MMN liquid medium (Midgley *et al*. 2006) was supplemented with either D-glucose, D-cellobiose, sodium carboxymethyl cellulose (CMC, Sigma), pectin (Sigma) or birchwood xylan (Roth) to a final concentration of 4 g carbon L^{-1}. A carbon-free treatment was included to determine the amount of growth from carbon remaining in the inocula. For all treatments, one plug of inoculum (5 mm diam) was excised from the leading edge of colonies growing on carbon-free MMN agar and inoculated into Petri dishes containing 25 mL liquid medium. All treatments were replicated four times and cultures were incubated in the dark at 20ºC. After incubation for 9 d mycelia were manually removed from the dishes, blotted briefly on absorbent paper, dried overnight at 80ºC and weighed.

Statistical analyses

The biomass on each source of carbon by each isolate was analysed using SPSS version 14.0. As some data were non-parametrically distributed and transformation did not normalize the data, all data were analysed using the Kruskal-Wallis test ($P<0.05$) and, where significant differences were observed, Mann-Whitney U-tests were used to identify significantly different pairs ($P<0.05$).

ITS rDNA sequencing

Because the nutrition of most isolates appeared similar, the identity of isolates was checked using molecular sequences. Each isolate was grown for 7 d in liquid malt extract agar containing 2% glucose, 2% malt extract and 1% peptone (Sigma). DNA was extracted (Cassago *et al*. 2002). The resulting DNA was purified using the Wizard® DNA Clean Up System (Promega) according to the manufacturers instructions.

The internal transcribed spacer region (ITS) was amplified from genomic DNA using either the ITS1 and ITS4 primers (Gardes and Bruns 1993) or using the ITS1F and ITS4A primer pair (Larena *et al*. 1999). The PCR was conducted in an Eppendorf Mastercycler Gradient Thermocycler using the following program: 4 min at 94ºC, followed by 35 or 30 cycles of 94ºC for 1 min, 52ºC or 50ºC for 1 min, 72ºC for 2 min or 1 min, with a final extension of 72ºC for 10 min or 8 min for the ITS1/ITS4 primer pair and ITS1F/ITS4A primer pair respectively. Amplicons were purified with the QIAquick® PCR Purification kit (QIAGen) and were nucleotide sequenced at Sydney University Prince Alfred Macromolecular Analysis

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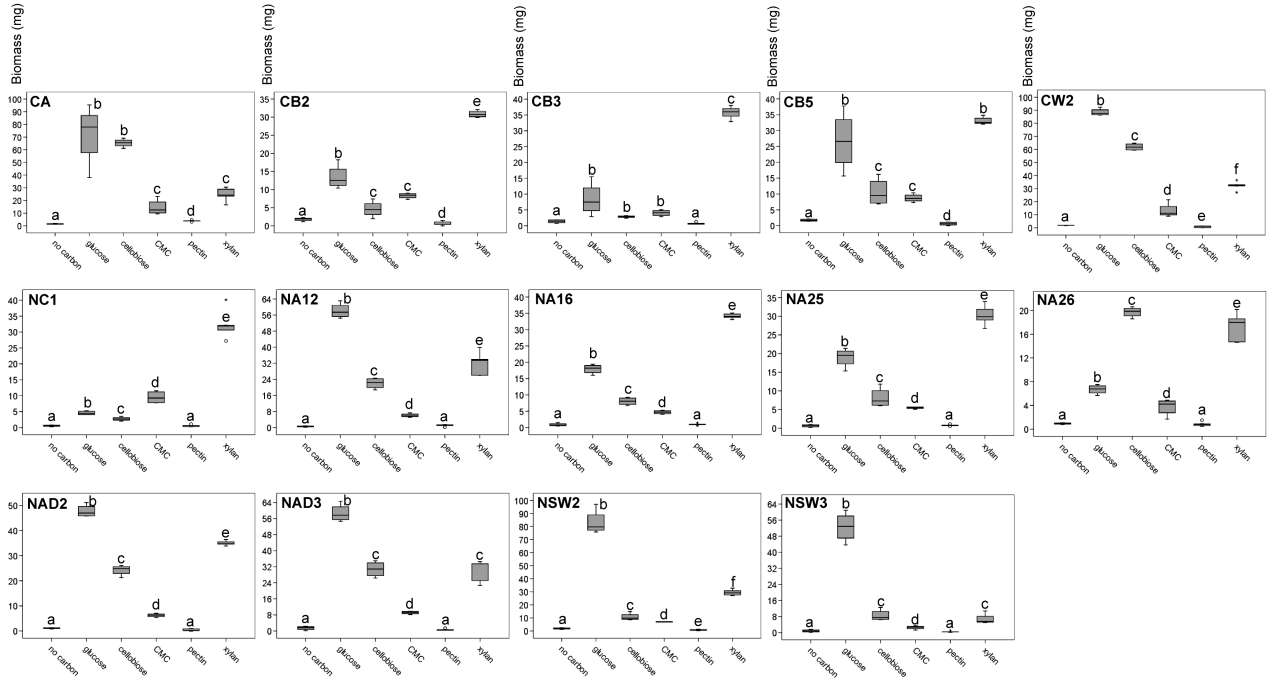

Fig. 1 Boxplots of biomass yields (mg) of *Chaetomium* isolates in liquid MMN media either supplemented with glucose, cellobiose, CMC, pectin, xylan or without supplementation (no carbon). Boxes represent interquartile range (IQR), dark centre bar represents the median biomass. Whiskers indicate the maximum and minimum yields. Open circles indicate outliers 1.5xIQR, asterisks indicate outliers 3xIQR. Lowercase letters on boxes indicate significant differences ($P < 0.05$) between carbon sources as determined by the Kruskal-Wallis test and post-hoc Mann-Whitney U-tests. Scales differ between box plots.

Centre, Sydney or Macrogen (Seoul, South Korea).

Similarity searches were performed against the non-redundant database maintained by the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) using the BLASTN algorithm (Altschul *et al*. 1990) on the entire sequence containing the ITS1, 5.8S rRNA and ITS2 regions. The sequence for each isolate was submitted to the GenBank nucleotide database under the accession numbers EU035794 to EU035807. Sequences were aligned using the CLUSTALW program (Thompson *et al*. 1994). Phylogenetic relationships were deduced with the neighbor-joining method, internal nodes were computed using 1000 bootstrap replicates and consensus trees were constructed using PAUP version 4.0b (http://paup.csit.fsu.edu/).

Results

Solubilisation of crystalline cellulose

Regardless of the cellulose concentration, faint clearing zones were evident from the third day for all isolates except NA26. For NA26 faint clearing zones appeared after 14 d. Clearance zones were more obvious at the lower concentrations, while more perithecia were evident at higher concentrations of cellulose. No clearing was apparent on control plates. In comparison, clearing of cellulose under *Trichoderma viride* was rapid and extended to the perimeter of the plate.

Use of soluble carbohydrates

With the exception of pectin, all fungi produced measurable biomass on all carbon sources tested. Only CA produced measurable biomass on pectin. For all other fungi, biomass produced on pectin was not significantly different to, or significantly less than, the no carbon control. Seven isolates, CA, CW2, NA12, NAD2, NAD3, NSW2 and NSW3, produced most biomass on glucose ($P<0.05$). The five isolates, CB2, CB3, NC1, NA16 and NA25, produced most biomass on xylan ($P<0.05$). CB5 produced most biomass on glucose and xylan. NA26 produced most biomass on cellobiose. Pectin aside, biomass produced on CMC was lowest for CW2, NA12, NA16, NA25, NA26, NAD2, NAD3, NSW2 and NSW3 (Fig. 1).

Molecular analysis

ITS-PCR produced a single amplicon (*ca.* 490 bp) for each isolate. Comparison of ITS sequences with sequences available in the GenBank nucleotide database indicated that the DNA sequences from CB2, CB3, CB5 (all from Camden, NSW), NC1, NSW2 and NSW3 (all three from Narrabri, NSW) had 99–100% sequence identity with *C. globosum* (Table 1). Two endophytic isolates, CA (Camden, NSW) and CW2 (Cowra, NSW) are 100% identical across their ITS sequence and had ITS sequences that were identical to *C. funicola*. The ITS sequence from NA26 had 94% sequence identity and closest match with *C. nigricolor*, while the closest BLAST match for CB5, NAD2, NAD3, NA12, NA16 and NA25 was to an undescribed species of *Chaetomium* isolated from *Eucalyptus grandis* leaves in Mexico (Perez-Vera *et al*. 2005). ITS sequences from CA and CW2 shared greatest identity with a root endophytic *Chaetomium* species from *Phragmites australis* (Wirsel *et al*. 2001) (Table 1). No previous reference sequences for *C. dolichotrichum, C. olivaceum* and *C. cochliodes* were found in the GenBank nucleotide database.

The overall branching of the phylogram (Fig. 2), based on the complete ITS1, 5.8S rDNA and ITS2 regions, reflected the morphological groupings of the isolates. For example two endophytic fungi, CA and CW2, identified by DNA sequence as *C. funicola* and morphologically identified as *C. dolichotrichum* are on

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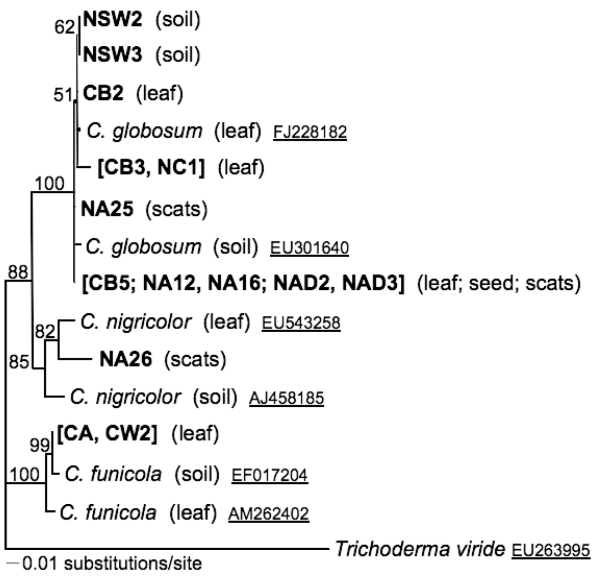

Fig. 2 Phylogram depicting the neighbour-joining consensus tree of *Chaetomium* isolates from Table 1 and reference DNA sequences. Bootstrap values are indicated on the branch nodes. Isolates in square brackets have 100% identity. GenBank accession IDs are underlined. Accession EU263995 (Hanada *et al.* 2006).

the same node. NA26, an unidentified species of *Chaetomium*, appears on a separate node and is distant from the other isolates. CB2, CB3 and CB5 were all isolated from leaves, were morphologically distinct and are on separate nodes. Endophytic CB3 and NC1 are on the same node and were both identified as *C. globosum*. The isolates from soil (NSW2 and NSW3) identified as *C. globosum* group together on the same node. *C. funicola* has a distinct lineage from *C. globosum* and *C. nigricolor*.

Discussion

Broad trends in nutrition are apparent in this study. Data from a limited number of isolates from various substrates indicate that *Chaetomium* isolates, regardless of their origin, have broadly similar patterns of biomass production on plant cell-wall related polysaccharides. With the exception of pectin, all fungi examined produced measurable biomass on all carbon sources tested. For the majority of isolates biomass production was greatest on glucose. This was unsurprising as glucose was the simplest carbon source examined and is readily used by many fungi (Jennings 1995).

Five isolates, CB2, CB3, NC1, NA16 and NA25, produced the greatest biomass on the hemicellulose, xylan. After cellulose, xylan is the second most abundant cell-wall component in nature (Taiz & Honigman 1976). Complete degradation of branched xylan requires an endo-acting β-1,4 xylanase, β-xylosidase and accessory enzymes (Couglan & Hazlewood 1993; Tenkanen 1998). Xylanolytic activity has been previously observed in two *Chaetomium* species (Baraznenok *et al*. 1999; Czakaj & Czuba 2003; Latif *et al*. 2006). Production of biomass and the generally large yields on xylan by all isolates indicates the presence of xylanolytic activity in these isolates. The reason for greater biomass on xylan compared to glucose in these isolates is unclear. It may be that the pH of the media (pH 5.0) was biased towards xylan transport and utilization rather than glucose.

Degradation of cellulose in fungi is facilitated by the synergistic action of endo-acting β-1,4-glucanohydrolase, cellobiohydrolase and β-1,4 glucosidase activities (Tuohy *et al*. 2002). These enzyme activities have been previously demonstrated for *C. cellulolyticum* and *C. globosum* (Watanabe 1968a,b; Fähnrich & Irrgang 1982) and growth by all isolates in the present study on crystalline cellulose, CMC and cellobiose indicates that the fungi also produce a complete complex of cellulolytic enzymes. In comparison to *T. viride*, growth and clearing of cellulose by the isolates of *Chaetomium* used in the present study was slow. Slow clearing may reflect intrinsic differences in rates of growth or differences in rates of expression of cellulolytic enzymes between the two genera.

The lack of biomass of most isolates on pectin as a sole source of carbon was intriguing. These endophytes spread through leaf tissue between cells (Istifadah 2005) indicating penetration of the middle lamella. All four *C. globosum* isolates in the present study failed to use pectin for growth. Pectinase activity in *C. globosum* was reported by Czakaj & Czuba (2003). The acidic growth conditions may have reduced pectin utilization (Griffin 1994) and its absence in these isolates requires further investigation.

Plant cell wall degrading enzymes may be produced by endophytic *Chaetomium* during initiation of colonisation of the host. Whether this expression continues inside plant tissues has not been established. Many fungi repress their cellulolytic and hemicellulolytic systems in the presence of low molecular weight carbon sources such as those found in photosynthate (Merivuori *et al*. 1985). Down regulation may occur once the endophyte has access to photosynthate within the plant tissues. In this study the potential for endophytes to utilise various cell wall substrates is indicated.

The phylogenetic analysis supports the morphological identification of CA and CW2 as being distinct from *C. globosum*. CA and CW2 were morphologically identified as *C. dolichotrichum*, and the identical ITS sequence identity between the two isolates strongly supports conspecificity.

Although only the DNA sequence from the ITS region was examined, the phylogenetic analysis indicates that endophytic *Chaetomium* are genetically similar to *Chaetomium* from scats, seeds and soil. Indeed, *Chaetomium* species isolated from various substrates in South East Asia and Europe shared similar identity with isolates from leaf, scats and soil isolates collected from central and south eastern NSW. Genetic similarity strongly supports the view that some species of *Chaetomium* potentially inhabit many environments, including living plant tissue.

The data presented in the current study indicate endophytic and free-living isolates of *Chaetomium* have physiologically similar carbon nutrition. Furthermore, ITS sequence data indicate that endophytic *Chaetomium* are genetically similar to free-living forms. Taken together, the data indicate several interpretations. One possibility is that an individual fungus may cycle between multiple habitats. That is, soil, scats and seeds

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may be colonised from spores released from endophytic genotypes. Indeed, the possibility is indicated if isolates from scats originated in the leaf material eaten by the possums. Alternatively, different genotypes of the same *Chaetomium* species may have different life strategies with some genotypes specialising in endophytic associations with plants. If the former hypothesis is supported by further work, then some *Chaetomium* species may be better described as opportunistic endophytes. We have found no evidence to indicate differing life strategies among the isolates.

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