The molecular characterisation of *Trichoderma hamatum* effects on plant growth and biocontrol

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Beverley D. Harris

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Abstract

Expanding global populations, unequal food distribution and disease pressure suggest food poverty is increasing. Consequently, much attention is focussed on alternative natural methods in which to increase agricultural yield. Previously, it was observed that *Trichoderma hamatum* strain GD12 and its respective N-acetyl-β-D-Glucosamine mutant Δ*Thnag:hph* promoted plant biomass and fitness that, as a result, may provide a credible natural alternative to synthetic fertilisers. However, on a molecular level, the manner in which this is achieved has not been fully elucidated. In this thesis, I report the biofertiliser effect of GD12 and mutant Δ*Thnag::hph* once applied to autoclaved peat microcosms as sole applications. Furthermore, I demonstrate the biocontrol ability of GD12 when co-inoculated with *Sclerotinia sclerotiorum* or *Rhizoctonia solani* and reveal, that once mycelium co-inoculation has occurred, GD12 increase plant biomass and provide protection; whilst Δ*Thnag::hph* does not. Consequently, I challenged the biocontrol effects of Trichoderma metabolite extract where I validate that both Trichoderma wild type GD12 and mutant Δ*Thnag::hph* are incapable of suppressing pathogen growth. Subsequently, I characterised the up-regulated signatures associated with GD12 and Δ*Thnag::hph* using LC-MS techniques where unique compounds were discovered from each strain of Trichoderma. In conclusion, I provide evidence that N-acetyl-β-D-Glucosamine mutation bring about metabolomic changes that affect the fungal secretome which, in turn, alters plant phenotype, fitness and germination. Furthermore, I have shown that these effects are species specific and depend upon pathogen, plant and fungal properties. However, further investigations are needed to fully elucidate the compound(s) responsible for biocontrol and biofertilisation; especially plant-specific effects that take place as a consequence of fungal activity.
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Additional material:


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### Abbreviations

<table>
<thead>
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<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>CM</td>
<td>Complete Medium</td>
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<tr>
<td>cm</td>
<td>centimetre</td>
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<tr>
<td>d</td>
<td>day</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celcius</td>
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<tr>
<td>dH2O</td>
<td>deionized water</td>
</tr>
<tr>
<td>DIGE</td>
<td>Difference Gel Electrophoresis</td>
</tr>
<tr>
<td>dpi</td>
<td>days post infection</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionisation</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>gL⁻¹</td>
<td>grams per litre</td>
</tr>
<tr>
<td>hex</td>
<td>hexosaminidase</td>
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<tr>
<td>hr</td>
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</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
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<tr>
<td>IPTG</td>
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<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
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<td>Mass Spectrometry</td>
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</table>
ng  nanogram
nm  nanometre
NMR Nuclear Magnetic Resonance
ORF open reading frame
PDA potato dextrose agar
PDB potato dextrose broth
% percentage
% (w:w) percentage weight by weight
RNA ribonucleic acid
rpm revolutions per minute
Chapter 1. General Introduction

1. Introduction to current study

Man has been practising agriculture for thousands of years. Regarding soil fertility, our increased awareness has enabled human populations to improve crop yield and as a consequence flourish. One of the earliest recorded methods of agricultural manipulation occurred in the western Honduras c4500 BCE where the ‘slash and burn’ method of clearing crops indirectly involved the application of burnt organic remains to the soil (Rue, 1987). This practice developed in the Amazonian basin c1000 BCE, whereby burnt bones and bark were applied to the soil in order to increase subsequent crop yields (Guo, 2008).

History has shown modern methods like crop rotation or exogenous nitrogen application improve crop yield. Nonetheless, nutrients are temporarily locked within living systems once consumed and increasing global populations coupled with land used for biofuels and cattle food stock imply demand will soon outweigh supply (Tscharntke et al, 2012). Consequently, food security is threatened if current methods for increasing nutrient availability are not improved. Despite attempts to remedy soil nutrition depletion; the success of man (as a species) is dependent upon soil quality (Dale and Carter, 1955).

Rising demands for intensifying crop yields include optimising pest management control. The excessive use of fertilisers and pesticides is known to bear a negative impact on the environment where the natural nitrogen and phosphorus cycle is affected by an imbalance of these elements. As a consequence, induced leaching and (subsequently) eutrophication is witnessed on a global scale (Smith et al, 1999) with potential to cause more devastation than currently witnessed (Foley et al, 2011; Deegan et al, 2012). Novel strategies must be addressed to
1989). Suggestions of territorial dominance by Glomeromycota approximately 540 Mya is hypothesised to cause increased oxygenation of the atmosphere (Berner et al., 1999) leading to vascular plant development on land (Simon et al., 1993). This resulted in molecular cross talk between fungi and plant that evolved over 400 Mya; developing into an intricate relationship centred on a common functionality (Remy et al., 1994; Parniske, 2008). Importantly, Glomeromycota are associated with up to 90% of arbuscular plants and responsible for plant nutrient uptake essential for plant growth (Smith and Read, 2008; Wang & Qui, 2006).

AMF penetrate roots by initially overcoming the plants’ basal defence via effectors that engage with plant transcription factors allowing entry via hyphopodia and cytoplasmic accumulation at the site of hyphal entry (Genre et al., 2009). Consequently, swollen structures known as arbuscules are formed in the process thus resulting in additional uptake of water, sugar and minerals (Oldroyd et al., 2009; Nehls, 2008; Kleczewski et al., 2010). Furthermore, this was shown to promote molecular cross-talk between plants and fungi, where fungal effectors and plant transcription factors mediate in a similar fashion to bacteria (Kloppholz et al., 2011; Okubo et al., 2012).

### 1.1.1 Beneficial results of symbiosis

Lipochitooligosaccharides (LCOs) are essential for plant colonisation and are a type of cellular receptor molecule similar to bacterial Nod and fungal Myc factors (Simon et al., 1983; Lerouge et al., 1990; Cullimore et al., 2001; Madsen & Stougaard, 2012). Apart from the conserved mechanism in which this occurs, genetic analyses confirm bacterial and fungal LCOs share a common structure; an N-acetyl Glucosamine (NAG) backbone (Dénarié, 1996). Both require the NSP2 gene in order to form endosymbiosis (Maillet et al., 2011) and, as a result of flavonoid secretion, increased
combat crop disease, including alternative methods which are not chemical, but comprise natural moieties. Crop loss attributed to disease coupled with adverse weather conditions mean global food security has become a major concern (FAO, 2006; WRI, 2011). Consequently, much interest surrounds the natural application of PGP and BCA entities in order to increase crop yield in acidic peat (pH 4.2). Therefore, any PGP effects by Trichoderma remain interesting because lettuce prefer a pH range of 6.0–6.7 (Sanders, 2001).

1.1 The evolution of true Fungi; an historical perspective

Since life emerged on Earth 3.8 Gya (G=10⁹) the exact date Fungi diverged from the Tree of Life’s main root has not been fully elucidated (Cavalier-Smith et al, 1999). Hypotheses regarding causes of evolutionary deviance include rapid mutation rates within the lichen genome, resulting in the diversification of mutualistic and pathogenic fungi present today (Lutzoni and Pagel, 1997; Heckmann et al, 2001). Although the protolichen theory is disputed (Lücking et al, 2009), others demonstrate lichens assisted in the diversification of symbiotrophic traits within the Pezizomycotina (Arnold et al, 2009).

Symbiosis is a broadly-used term regarding a mutually beneficial relationship between two species. Consequently, the needs of one organism are met by the presence of another offering a nutritional, defence or ecological advantage (Duhamel et al, 2013). Often associated with territorial ecosystems, mutualism enhances biological diversity and co-evolution events (Aslan et al, 2013). For example, Arbuscular Mycorrhizal Fungi (AMF) are members of the phylum Glomeromycota and as obligate biotrophs, colonise roots to engage directly with the plant on a cellular level. This was discovered from the earliest known true fungal fossil concerning Glomeromycota from the Cambrian era (560 Mya; M=10⁶) (Pirozynski and Dalpé,
transcription rates of *nod* genes are witnessed thus promoting plant symbiosis in the process (Cooper, 2004).

As a result of symbiosis, it is evident bacteria and fungi assist the plant in acquiring a range of nutrients where increased levels of nitrogen, carbon and phosphorus display Plant Growth Promotion (PGP) effects (Nehls, 2008; Kleczewski *et al.*, 2010). Reports show bacteria and fungi co-inoculation bear accumulative PGP effects, thus producing significantly greater plant biomass results than that of a single inoculation with bacteria or fungus alone (Rudresh *et al.*, 2004; Bhattacharjee *et al.*, 2012; Tahira *et al.*, 2012). It is abundantly clear that fungi and plant have evolved to form a mutual relationship based on metabolite commonality. Benign and pathogenic microbes adopt ancient mechanisms which continue to evolve however, not all cross-talk is intra-molecular as with *Trichoderma* spp.

1.1.2 *Trichoderma hamatum* and variability in symbioses

Symbiotic *Trichoderma* spp. is cosmopolitan, where spore counts can exceed 1 x 10^6 per gram of soil (Danielson & Davey, 1973; Sivan & Chet, 1993). Considered as a prerequisite for improved plant viability, *Trichoderma* spp. are known to colonise plants both intra- and extracellular. For example, *T. pseudokoningii, T. harzianum, T. hamatum* and *T. longibrachiatum* displayed endophytic qualities on *Zea mays* by moving to the upper and lower plant internodes (Sobowale *et al.*, 2011). In a similar fashion, one study found that *T. asperellum, H. lixii* (teleomorph of *T. harzianum*), *T. brevicompactum* and *T. virens* formed endosymbiotic relations in banana roots, whereas *T. koningiopsis & T. atroviride* remained on the root surface (Xia *et al.*, 2011). Interestingly, there is little evidence to support the endosymbiotic nature of *T. atroviride* however, it is apparent from the literature that the preferred hosts are North American Red Pine trees (*Pinus resinosa*), shrub genus
indigenous to South America (Espeletia genus) and European Elm trees (Santamaria et al 2012; Miles et al 2012; Diaz et al 2013). In contrast, some Trichoderma spp. are so endophytic that one study failed to disrupt T. virens’ ability to colonise sugar cane plant roots, despite silencing three endochitinase genes associated with endophytism (Romao-Dumaresq et al, 2012).

Examples of intra molecular cross-talk have been reported for plant interactions with endomycorrhizae but not the ectomycorrhizae. In comparison, ectomycorrhiza do not penetrate the plant root and instead surround the root ball with the mantle; a net of hyphae which interact with roots on a close contact level (Felten et al, 2012; Plett et al, 2011). Naturally the mantle extends to the Hartig net which colonise the plant exodermis, which then mediates solute uptake to the plant and increase concentrations of soluble proteins. This promotes lateral root hair formation in the process (Smith & Read, 2008; Rawat & Tewari, 2011; Berta et al, 1995). Ectomycorrhiza participate in a tripartite relationship similar to endomycorrhiza and bacteria where increased plant nitrogen and phosphorus uptake take place (Duponnois & Plenchett, 2003; Diagne et al, 2013).

1.2 Trichoderma spp. and functional variability

Trichoderma is a genus of fungi whose natural habitat involves both woodland territory and the rhizosphere. Considered ubiquitous, Trichoderma spp. possess a repertoire of enzymes that hydrolyse naturally occurring polymers including cellulose, lignin, pectin, chitin and other complex organic compounds; resulting in a plethora of protein up-regulation (Hayes et al, 1992; Donzelli & Harman, 2001). In addition to their ecological diversity, Trichoderma fungi are proficient saprotrophs and often exploited in industry for specific hydrolytic chemistry. This is exemplified by ethanol (biofuel) production via cellulose degradation (Saharay et al, 2010; Balat, 2011),
laccase in paper manufacture (Sadhasivam et al, 2010), hydrophobins in food production (Tchuenbou-Magaia et al, 2009) and the profitable conversion of glucosamine to galactosamine in medicinal organic chemistry (Feng & Ling, 2010).

1.2.1 Trichoderma hamatum and secondary metabolites

*Trichoderma* spp. are renowned for their proteomic and metabolic diversity (Bömke et al, 2008). Recent evidence confirms that Trichoderma and other Ascomycota fungi manipulate post-translational modification processes to produce an array of unusual metabolites generally clustered within the genome (Mukherjee et al, 2006; Tudzynski & Höltter, 1998). Fungi synthesise numerous metabolites that share a common functionality in plants. The phytohormones auxin, Gibberellic Acid (GA), Salicylic Acid (SA) and ABoiscisic Acid (ABA) play significant roles in plant growth and development, maturity and stress tolerance (Contreras-Cornejo et al, 2009; Jin et al, 2011; Inomata et al, 2004). More specifically, it is known Trichoderma promote the up-regulation of these phytohormones within plant tissue (Martinez-Medina et al, 2011; Gutjahr & Paszkowski, 2009).

The most studied auxin is Indole-3-Acetic Acid (IAA), strongly associated with Lateral Root (lateral root) formation and plant development (Hull et al, 2000; Contreras-Cornejo et al, 2009). Some species of fungi produce IAA derivatives and it is known *Trichoderma* spp. secrete and degrade IAA and associative isomers (Gravel et al, 2007; Zhang et al, 2012). Indole-3-Butyric Acid (IBA) is the precursor to IAA, providing a link between plant and microbe chemistry as justified by work showing *Glomus intraradices* inoculated soil increased [plant-associated] IBA synthetase up-regulation (Ludwig-Müller et al, 1997; Fitze et al, 2005).

1.2.2 Plant growth promotion ability of Trichoderma hamatum

Saprotrophs digest decaying material and are widely believed to contribute directly
to carbon and nitrogen recycling via detritus and other organic matter within the soil (Veal & Lynch, 1984; Gadd, 2008; Gorfer et al, 2011). Coupled with Trichoderma spp. mycoparasitic behaviour, saprotrophs contribute to nitrogen sequestration by releasing N-acetyl-β-D-glucosamine (NAG) monomers (Deng et al, 2007; Harman and Bjorkman, 1998; Harman and Shoresh, 2007). Therefore, solubilisation and fixation of essential macronutrients increase plant viability and soil fertility. Trichoderma spp. is also known to increase PGP effects in natural and artificial soil-based microcosms (Ousely et al, 1994; Ryder et al, 2012; Schopmeyer & Fulmer, 1930).

PGP occurs in a number of ways including increasing plant resistance to disease and secreting auxin-like compounds or Volatile Organic Compounds (VOCs) (Shoresh and Harman, 2008; Gravel et al, 2007; Vespermann et al, 2007). Previously, it was hypothesised that Trichoderma induced plant growth complex sugar hydrolysis via hexosaminidase (EC 3.2.1.52, GO:0015929); an exo-chitinase which cleave terminal non-reducing monomers in N-acetyl-β-D-hexosamine (HEX) and NAG polymers abundant within the biosphere (Intra et al, 2008). Recently, it was proven that disruption of the hexosaminidase gene in T. hamatum strain GD12 resulted in enhanced growth promotion of lettuce in acidic peat; albeit contrary to expectations. Further analyses showed that the mutant had a completely altered secondary metabolite profile, leading to the hypothesis that PGP is directly linked to the secretion of water-soluble Secondary Metabolites (SM) (Ryder et al, 2012). Interestingly, this was found to be the case when fungal IAA induced root formation and growth (Hull et al, 2000; Contreras- Cornejo et al, 2009). Additionally, Vinale et al (2008) confirmed 6-n-Pentyl-6H- Pyran-2-one (6PP) emulated an auxin-like compound that promoted plant biomass and improved the phytodefense system (Vinale et al, 2008).
1.2.3 *Trichoderma* spp. as biocontrol agents

*Trichoderma* spp. are prolific secretors which deploy **Cell Wall Degrading Enzymes** (CWDE) to affect cell membrane integrity (Hayes *et al.*, 1992; Lorito *et al.*, 1996; Donzelli & Harman, 2001). Often used in conjunction with CWDE, antimicrobial compounds such as 6PP are deployed in order to overcome the pathogen before hypercoiling and digestion (Chet *et al.*, 1981; Claydon *et al.*, 1987). This reduces phytopathogen numbers that hypothetically increase plant security, potential calorific intake and available colonisation zone (Shoresh and Harman, 2008; Bae *et al.*, 2009). Although both *endo-* and *ecto-* types of mycorrhizae secrete CWDE, ectomycorrhizae generally adopt this as a first line-of-attack to hydrolyse pathogen cell walls.

*Trichoderma* spp. show considerable intra-species allelic variation regarding CWDE, thus contributing various antifungal activities reflecting diverse environmental niches (Sanz *et al.*, 2004; Martinez *et al.*, 2008; Kubicek *et al.*, 2011). For example, some Trichoderma prefer ecto- as opposed to endophyte behaviour (Sobowale *et al.*, 2011; Xia *et al.*, 2011). Other Trichoderma strains secrete various SMs including antibiotics, pyrones and VOCs (Dennis & Webster, 1971a/b; Dong *et al.*, 2011; Stoppacher, 2010). Not only do SMs display biocidal qualities, but some also resemble phytohormones acting as molecular compounds within the plant (Shoresh and Harman, 2008; Gravel *et al.*, 2007; Vespermann *et al.*, 2007). Trichoderma is known to promote ABA, jasmonic acid and salicylic acid up-regulation; stress response phytohormones that regulate systemic defence (Martinez-Medina *et al.*, 2011; Gutjahr & Paszkowski, 2009). Therefore, symbiotic interactions between plant and microorganisms are influenced by a number of factors including soil type, plant species and developmental stage (Lundberg *et al.*, 2012).
1.3 A molecular approach to plant immunity

Eukaryotes have evolved two main defence systems in order to overcome biotic or abiotic stress. Primarily, simple barriers such as skin or the waxy cuticle in the upper epidermis of a leaf is sufficient for topical prevention, else the more complex basal immune system of a cell-mediated response is deployed. In both plant and mammals, the basal immune response involves a cascade of intra and extracellular mechanisms involving molecular receptors, sugar identification molecules, ROS up-regulation and a number of protein kinases (Ausubel, 2005; Rahme et al, 2000; Williams et al, 2011). In a similar fashion to mammals, plants utilise Pattern Recognition Receptors (PRR) such as Toll-like Receptors (Tlateral root) and Nucleotide Oligomerisation Domain (NOD)-like receptors in order to detect foreign bodies (Nürnberger et al, 2004; Dardick & Ronald, 2006; Stuart et al, 2012; Rodríguez et al, 2012); analogous traits hypothesised to be attributed to convergent evolution (Ausubel, 2005).

Depending on the pathogen and the receptor molecule present, plants respond in two major ways: PAMP Triggered Immunity (PTI) or Effector Triggered Immunity (ETI) (Brotman, 2008). Subtle differences occur between PTI and ETI whereby PTI is the initial plant response to Pathogen Associated Molecular Patterns (PAMPs); signature chemicals that reside on the predator’s cell surface and determine self from non-self cells within biological systems. Examples of PAMPs include bacterium Elongation Factor Tu (EF-Tu), LipoPolySaccharide (LPS), flagellin, dsRNA, chitin and swollenin which upon detection, evoke a PTI response (Brotman, 2008). Although PTI is generally sufficient to restore internal homeostasis, a number of pathogens bring about an antagonistic response by secreting effectors to suppress PTI. A number of effectors exist and are species specific where the
most illustrated example includes bacteria and the highly conserved PAMP Type III Secretion System (T3SS). Oomycete effectors include RX lateral root and CRN-type effectors that target E3 ligase activity in a similar fashion to bacterial T3SS (Bos et al., 2010). Transcribed by Avirulence (Avr) genes, successful Avr proteins [effectors] initiate further strategies set out by the plant to overcome the hypersensitive response associated with SAR (review: Zhou & Chai, 2008).

1.3.1 Systemic v induced resistance
The hypersensitive response occurs by Pathogenesis-Related (PR) protein up-regulation via Nucleotide-Bonding Specific Leucine-Rich Receptors (NBS-lateral rootR) and SNF-1-like proteins (Xie et al., 2012). For example, Brassica crops up-regulate the Crr1 gene; an NBS-lateral rootR protein expressed in roots and hypocotyl tissue during Plasmodiophora brassicae Woronin infection however, this does not occur in root hair cells where primary infection occurs (Hatakeyama et al., 2013). Alternatively, it was found that wheat expressed lower levels of Erysiphe graminis–specific NBS-lateral rootRs in root tissue (Gong et al., 2013). Nonetheless, once NBS-lateral rootR genes are up-regulated, ion movement, ROS and callose deposition occurs rapidly to limit pathogen infection, is known as Programmed Cell Death (PCD) (Nandini et al., 2012; Palmieri et al., 2012). Therefore, in a similar fashion to mammalian systems involving NBS-lateral rootR, both PR-related proteins and ultimately Resistant (R) genes are important component of the plants defence system. Combined, both PTI and ETI contribute to the overall defence system as illustrated by the ‘zig zag model’ that involves a gene-for-gene relationship between R and Avr genes; ultimately mediated by salicylic acid (Flor, 1971; Jones & Dangl, 2006).

Co-evolved traits between plant and pathogen show that the way defence
strategies are conveyed within the plant are important. The plant must identify the source of pathogen attack and correspond accordingly. This may involve a more localised response, Induced Systemic Resistance (ISR) or a more systemic response, Systemic Acquired Resistance (SAR). The complex manner in which activation of the plant’s defence system is achieved depends on a number of factors. Chiefly, ISR is independent of salicylic acid and require jasmonic acid and ethylene; phytohormones induced by wounding and biotrophs (Shoresh et al, 2005). Although ISR can induce SAR, ISR is not associated with PR proteins and consequently does not necessitate a hyperoxidative response. On the other hand, SAR is positively regulated by Non-expressor of Pathogenesis Related1 (NPR1), mediating salicylic acid and Mitogen Activated Protein Kinases (MAPKs) up-regulation (review; Grant & Lamb, 2006). Simultaneously, the phytohormones auxin, giberellic acid and ethylene are inhibited in a time-dependent manner (Wang et al, 2012); although not in tomato plants (Alfano et al, 2006).

1.3.2 Sclerotinia sclerotiorum & pre-emergence damping-off disease

Plants are sessile organisms and subsequently, subject to disease by a variety of pathogens including bacteria, viruses, invertebrates, other plants and fungi. Global food security concerns a number of key issues including crop protection from pathogens namely fungi from both Basidiomycota and Ascomycota. Of the most prevalent phytopathogens concerned with crop loss, Fusarium, Magnaporthe, Ustilago, Rhizoctonia, Puccinia and Sclerotinia genera are considered troublesome on a global scale. Pathogenic mode of entry is similar to that of fungi whereby CWDE, toxins, phytohormone-like chemicals and effector proteins are secreted in order to overcome the plants defence system.

Of the phytopathogens addressed within this report, Sclerotinia sclerotiorum is
one of the most devastating necrotrophs; responsible for the destruction of over 400 plant species including arable and wild types. Favoured by moist, cool conditions S. sclerotiorum remain in soil for many years by producing sclerotia; hard melanised fruiting bodies containing a mass of hardened mycelium and nutrients (Coley-Smith et al, 2007). Upon the correct conditions, apothecia emerge from sclerotia and can release up to $7.6 \times 10^5$ ascospores over 20 days in all light damping-off disease, S. sclerotiorum utilises an array of effectors and hydrolytic enzymes in order to successfully overcome the plants defensive system to necrotise plant tissue via oxalic acid and ROS species (Zhu et al, 2013; Kim et al, 2011; Kabbage et al, 2013). Interestingly, it was found that transgenic plants expressing mammalian anti-apoptotic genes displayed reduced S. sclerotiorum disease symptoms (Dickman, 2007). Thus suggesting the exploitation of conserved defence-related genes may bring about a natural remedy to the ever increasing problem.

Crops affected by S. sclerotiorum include the commercially important Brassica, Lactuca and many legume crops resulting in impaired biosecurity (USDA, 2013). In the UK, S. sclerotiorum thrive under cool, moist conditions where potato, carrot and cereals are under threat. Therefore, much interest surrounds S. sclerotiorum control in an environmentally sustainable way not dependent on crop rotation, fungicides or equipment hygiene. However, it is probable discrete environmental pressures induce point mutations that give rise to genomic exclusivity within endogenous S. sclerotiorum populations (Clarkson et al, 2013).

1.3.3 Rhizoctonia solani & post-emergence damping-off disease

Rhizoctonia solani is similar to Sclerotinia sclerotiorum in that it can form sclerotia and cause post-emergence damping-off disease. Considered less pathogenic than
S. sclerotiorum, R. solani cause much crop loss in a variety of plant species including cereals, Solanum and sugarbeet. In a similar fashion to S. sclerotiorum, R. solani emerge from sclerotia where young hyphae penetrate plant tissue via appressoria; swollen structures that penetrate the epidermis of leaves via pegs (Marshall & Rush, 1980; Tariq & Jeffries, 1994). However, in a distinct fashion to S. sclerotiorum, R. solani rarely produce basidiospores and show variation in pathogenicity depending on the plant species. For example, not all R. solani strains induce SA, JA/Eth or auxin pathways in plants (Foley et al, 2013) nor can R. solani tolerate Brassica crops (Angus et al, 1994; Handiseni et al, 2013).

Trichoderma suppress R. solani growth but antagonism depends on plant and fungus species type including methodology. For example, T. harzianum was less effective than T. viride in reducing chick pea infection where mycoparasitism (T. harzianum) and R. solani self destruction (T. viride) occurred via induced R. solani oxidase up-regulation (Srivastava et al, 2012; Huang et al, 2011; Yang et al, 2012). Alternatively, T. reesei was less predatory than T. atroviride and T. virens and the means in which this was achieved differ by either saproptrophic means (T. reesei) or mycotoxin secretion (T. atroviride and T. virens; Atanasova et al, 2013). This is supported by evidence that show expansion of DUF300-type receptors in T. atroviride and PTH-11-like receptors in T. virens when compared to T. reesei; receptors strongly associated with mycoparasitism (Gruber et al, 2013). On the other hand, others found T. reesei and T. longibrachiatum as proficient pathogens in comparison (Chakravarthy et al, 2011).

1.4 Phytohormones and plant health

Abscisic Acid (ABA) was recently discovered in the mammalian immune system where it serves as a regulator of glucose homeostasis and stress tolerance
(Bassaganya-Riera et al, 2010; Bruzzone et al, 2012). Once associated purely with plant abscission, fungi and algae are now known to synthesise ABA but the full role it plays has not been elucidated (Kettner & DörfOing, 2006; Cohen et al, 2009). ABA is a key phytohormone responsible for a number of paramount cellular processes including plant development, water potential and defence; including giberellic acid, jasmonic acid and ethylene antagonism (de Torres-Zabala et al, 2007; Su et al, 2012; Zörb et al, 2013; Duan et al, 2013). Known to be positively-regulated by a number of MAPKs such as SnRK2.6 (for SND-1-relted kinase), ABA is deactivated by phosphorylation (Xie et al, 2012). In contrast, ABA deregulation involves serine-threonine phosphatases, collectively referred to as Protein Phosphatase type 2C (PP2C) here, phosphorylation events antagonise MAPKs and SNF-1 type proteins that finely tune ABA biosynthesis (Meyer et al, 1994; Lynch et al, 2012; Umezawa et al, 2013).

In association with ABA, Salicylic Acid (SA) is a phenol-based phytohormone associated with bringing about SAR. Biosynthesised from phenylalanine or chorismate pathways, SA is converted to the volatile ester methylsalicylate for easy transportation to non-localised plant tissue (Wildermuth et al, 2001; Mauch-Mani & Slusarenko; 1996; Zhang et al, 2012). Previously, SA mutants show a deficiency in photosynthesis rates and impaired SAR whereby the SA Arabidopsis mutant Salicylic acid Induction Deficient 2 (Δsid2) were incapable of producing SA and therefore a potential target for depression by P. syringae pv. DC3000 effectors (Hooft van Huijsduijnen et al, 1986; de Torres-Zabala et al, 2007; Demianski et al, 2012).

1.5 Determination of plant health

Photosynthesis is a fundamental process in which radiation energy is transformed
into chemical energy. The intricate relationship between the shoot and root system of a plant exemplifies this so that photosynthesis (via CO₂ fixation) occurs via the shoots (carbon source) and is responsible for producing oxygen, an electrode potential and the intermediate saccharide triose. Consequently, shoots deliver saccharides to the root system (carbon sink) under a pressure gradient within the phloem. In contrast, the root system is responsible for water and minerals absorption which is then transported to the shoot system via the xylem; delivering vital solvated nutrients to the shoots. More specifically, root absorption provides much needed nitrogen-based ions and photosynthesis provide carbon; both required for plant growth.

Analyses of shoot to root ratios (S:R) is an effective way to deduce a plant’s response to its environment and overall health. Much research has investigated the kinetics of nutrient distribution within plant tissue. Shoot and root development are dependent on each other and, consequently, other factors such as nutrition, drought, phytohormone levels, age, temperature, light and CO₂ levels (Quintero & Bowers, 2012; Davidson, 1969; Thornley, 1972; Tamoi et al, 2011). However, if any abiotic or biotic factor deviates from the norm, shoot and root ratios shift in order to compensate for the change.

1.5.1 Macromolecule availability affects shoot biomass

Mineral soil structure varies and influenced by regional climates, soil management and soil types present (Wobus et al., 2006). Natural weathering processes, gravity and bio-erosion give rise to soil particles that vary in size, texture and physical characteristics thus determining water retention rates; an important factor when considering soil as a life supporting medium.

The soil in South West England is not entirely composed of mineral soil. It contains organic matter formulated by the decomposition and accumulation of
decaying matter over a period of time. In contrast, peat is carbon-rich and mostly organic with any nutrition inaccessible to plants. Consequently it is not used as an exclusive medium in agriculture. This is because peat forms under water-logged conditions and excessive amounts of iron, clay, aluminium and organic compounds accumulate (illuviation; Latin *il* in *luvi*- meaning mud, combined with French *lavere* – meaning to wash; thus to ‘to be washed in’ and increase concentrations). As a consequence, illuviated soil is generally acidic with low nutrient availability and so often used as an alternative fuel source. Generally, reduced PGP effects result from excessive or insufficient soil nutrient levels that impair the ion-cation exchange surface essential for terrestrial chemistry.

1.5.2 Ion-cation exchange properties of soil

Ionic diffusion is an important process occurring in fertile soil and responsible for supporting the diverse array of life witnessed within the biomantle. Accelerated decomposition of oxidised and decaying plant matter by soil microbes contribute to enrich the soil where typically lignin, humin and tannin are used as carbon sources. This eventually degrades over time resulting in increased microbial biomass, nitrogen mobility and air pockets for gaseous and ionic diffusion (Penman, 1940). Sphagnum is a genus of moss commonly found in wetlands such as bogs and mires therefore capable of retaining water. Sphagnum contains high levels of slow decaying phenolic compounds with potentially high carbon content however, the disadvantages of peat concerns a lower pH than fertile soil with nutrients locked within. Alternatively, solvated calcium and magnesium ions induce an acidic environment that increases cation exchange capacity (CEC) where phosphorus, nitrogen and calcium are available for plant assimilation hundreds of years after application (Bruno, 2007; Steiner *et al*, 2007).
1.5.3 Essential nutrients for plant growth

Phosphorus is an essential element used within all living systems. Its ubiquitous nature determines that fundamental life processes cannot occur without it. Phosphorus is a key component in DNA, ATP and metabolic pathway regulation and is the primary ingredient in soil fertilisers and approximately 90% is mined exclusively for agricultural means (Smil, 2000; Rosmarin, 2004). In addition, it is calculated that globally, 3 million tons of phosphorus is annually excreted via urine and faeces (Jönsson et al., 2004). Extraction methods are problematic and are associated with high energy costs, heavy metal pollution and radioactivity from phosphogypsum (Kratz and Schnug, 2006). Furthermore, excessive phosphorus leakage into the surrounding water supplies affects the natural habitat of lakes and other water ways causing eutrophication and toxic algal blooms. As a consequence of reduced natural phosphorus resources, increasing global demand will raise phosphorus prices; thus affecting poorer farming communities.

The negative repercussions of this are astonishing. Since the conception of the ‘Hubbert peak theory’ by Marion Hubbert in 1949 (Hubbert, 1949), his principle has been applied to many areas that concern a finite natural resource and its subsequent availability. Just as Hubbert predicted, American crude oil production would peak between the late 1960’s and early 1970’s (Hubbert, 1956) so others believe that the same principle applies to coal, uranium, helium, gold, fish stocks and phosphorus. Moreover, it is suggested phosphorus rock reserves should be classified as a critical issue in global food security (Koning et al., 2008) and that human waste phosphorus recycling should take place (Dawson and Hilton, 2011; Childers et al., 2011).

Alternatively, nitrogen availability is essential for shoot biomass and
photosynthesis where nitrogen uptake is a linear function of relative growth rate (Shangguan et al, 2004; Ågren & Franklin, 2003). Similarly, water deprivation contributes to increased carbohydrate reservoirs by altering the osmotic potential of the plant cell in order to increase turgor lost by reduced water levels (Hessini et al, 2009; Martinez et al, 2007). On the other hand, it is suggested that macronutrient in-equilibrium produce a variety of changes in plant behaviour. For example, potassium deficiency depressed photosynthesis to such an extent that S:R ratios substantially decreased (Steen, 1984; Andrews et al, 1999); in a similar fashion to low phosphorus levels (Wissuwa, 2005; Hammond & White, 2011; Li et al, 2009).

1.5.4 Phytohormone levels affect plant biomass distribution

Regarding phytohormones, gibberellins and auxins are chiefly responsible for cell elongation and growth. Gibberellic acid (GA) is located within root and shoot tissue, especially in young emerging cells and emerging seeds. Auxins are growth regulators and involved with a number of key processes including shoot and root elongation, cell differentiation, cell repair and various tropisms. The biorhythm of gibberellins and auxins naturally fluctuate as a consequence of different stimuli including light, temperature, sexual maturity and biotic factors. It is known that a fine tune system is in place to ensure auxin concentrations are regulated. An investigation into the effects of phytochromes on shoot development showed that cross talk between auxins and phytochromes/ethylene promoted additional root growth via a delocalised mechanism (Salisbury et al, 2007; Splivallo et al, 2009).

As a result of optimum growth conditions, photosynthesis rates and S:R increase at the expense of root tissue. Conversely, sub-optimum conditions, such as drought, increase shoot ABA levels thus closing stomata and reducing gaseous
efflux in the process. However, the stimulus for ABA up-regulation originates in root tissue as a direct response to dry conditions. Therefore, an intramolecular signalling system controls S:R by abiotic factors that, in turn, increase root mass at the expense of shoot mass (Martin-Vertedor & Dodd, 2011). Resulting in reduced hypocotyl elongation in soybean seedlings (Creelman et al, 1990).

1.6 *Trichoderma hamatum*; hexosaminidase gene disruption

Upon NAG (*N*-acetyl-β-D-glucosaminidase; EC.3.2.1.52) mutagenesis, the resulting mutant Δ*Thnag::hph* (hereafter referred to as Δ*nag*; GenBank: JN107809) displayed altered hyphal chitin deposition, increased vacuolation, decreased sporulation and improved PGP in lettuce plants (Ryder et al, 2012). Consequently, much interest concerns the PGP properties of Δ*nag* once Ryder and co-workers discounted the nitrogen solubalisation hypothesis. To determine a causal link between altered cell morphology and secretion rates, the Trichoderma-specific monoclonal antibody (mAb) MF2 was raised to determine glycoprotein concentrations in mycelium (Thornton et al, 2002). It was discovered that the hyper-secretory potential of Δ*nag* exceed that of GD12 (Figure 1.1). However, what is of particular interest, is the abundance of nitrogen compounds and lack of inorganic elements present within the soil after inoculation with Trichoderma.

Ryder and co-workers found that despite Trichoderma’s ability to solubilise mineral content of soils, GD12 and Δ*nag* did not affect nitrogen soil levels, despite NaNO₃ & NH₄Cl addition to soil. Furthermore, levels of phosphorus, potassium, magnesium, calcium, sodium, manganese, zinc and sulphur were significantly lower in Trichoderma-treated microcosms. This phenomenon is likely to be associated with organic acids secreted by Trichoderma as a bioweapon or chelation effects. We believe PGP biomechanics is more likely to be associated with
a number of factors, each contributing to the overall PGP effect.

A series of investigations were put in place to determine the effects of Trichoderma strains GD12 and Δnag on a range of commercial plant species including lettuce (Lactuca sativa) and tomato (Solanum lycopersicum). More specifically, the PGP effects of Trichoderma could be associated with the up-regulation of specific phytohormones on a subtle molecular level. Interestingly, these effects may take place in a specific time within a plants life cycle or within certain tissues. This chapter will address each of these questions in turn and more specifically, this report will attempt to answer questions concerning the molecular basis on which myco-phyto interactions occur.

Figure 1.1 GD12 and the Δnag mutant hyphae phenotype. GD12 and Δnag were grown on Teflon-coated slides embedded in PDA (Thornton, 2004; 2005) and incubated at 26°C for 24 hr. (A): bright field microscope (Zeiss Axioskop 2) results where the Δnag mutant exhibits hyphal hyper-vacuolation. (B): hyphae immunofluorescence using mAb MF2. Fluorescence of samples was observed by using a Zeiss LSM microscope at 488-nm excitation & 505-to 570-emission wavelengths. Scale bars = 10 μm (Ryder et al, 2012).
Chapter 2. General Materials and Method

2.1 Maintenance of micro-organisms

2.1.1 Trichoderma hamatum

Two *Trichoderma hamatum* strains were used in this study: wild-type ‘GD12’ (NCBI accession number JN107809) (Thornton *et al.*, 2004), and a mutant strain Δ*Thnag::hph* (Ryder *et al.*, 2012). Both were maintained on V8 medium (400 mL H₂O, 100 mL V8, 10 g Agar, 5 g Glucose), Potato Dextrose Agar (PDA) (500 mL dH₂O, 12 g PDA, 10 g Agar) (Sigma-Aldrich) or Potato Dextrose Broth (PDB) (500 mL dH₂O, 12 g PDA). In order to induce sporulation in Δ*Thnag::hph*, caspofungin was incorporated (10% w:v) to the cooled autoclaved PDA through a sterile filter (0.2 μm pore width) (Millipore). Fungal inoculation from original slopes occurred monthly.

2.2 Micro-organism preparation

2.2.1 Trichoderma hamatum spore suspension

*Trichoderma hamatum* spore suspensions were prepared by applying 5 mL sterile H₂O to a petri dish with either 14 days growth of GD12 on PDA or 21 day growth of Δ*Thnag::hph* grown on PDA +Caspofungin (caspofungin; 10% w/v) agar. Once the δH₂O and mycelium had been agitated, the liquid was filtered through sterile miracloth (Calbiochem) and the supernatant was centrifuged at 5000 x g (Beckman JA-17) for 10 minutes at room temperature. The pellet was re-suspended in 1 mL sterile δH₂O, and the concentration of spores was quantified using a haemocytometer.

2.2.2 Trichoderma hamatum mycelium preparation

500 mL of PDB was prepared in 2 L conical flasks and autoclaved at 121°C for 15
minutes. Once cooled, four 5 mm diameter plugs of *T. hamatum* mycelium were
transferred from the leading edge of 3-4 day PDA agar plates to the conical
flasks. The conical flasks were placed on a rotary shaker under a 16 h light/ 8 h dark
regime at 26°C for 5 days before the mycelium was harvested by filtration through
sterile Miracloth and blotted dry with paper towels (Kimberley Clark Corporation) in a
class II microbiological cabinet. It was then placed in a chilled mortar and ground to
a fine powder in liquid nitrogen before storing at -80°C.

### 2.2.3 Trichoderma-bran inoculum preparation

Trichoderma-bran inoculum was prepared in 250 mL conical flasks each containing 10
g bran (Badminton Horse Feeds, UK) and 30 mL M\textsubscript{go}H\textsubscript{2}O. After thoroughly mixing, the
flasks and its contents were autoclaved at 121°C for 20 minutes. Once cooled, four
5-mm diameter plugs of *T. hamatum* mycelium were transferred from the leading
dege of 3-4 day V8 agar plates to the conical flasks and thoroughly stirred to allow
equal distribution of mycelium in the bran. The fungi were allowed to colonise the bran
for a further 5 days under a 16 h light/ 8 h dark regime at 26°C before use.

### 2.2.4 Trichoderma hamatum metabolite extraction

Trichoderma-bran inoculum was prepared as discussed in chapter 2.2.3. Each
flask was then transferred to a Class II microbiological cabinet where 50 mL
M\textsubscript{go}H\textsubscript{2}O was mixed with the fungi-bran mass before spinning on a magnetic stirrer at
700 rpm for one hour at room temperature. The flask contents were then filtered
through sterile Miracloth (Calbiochem, UK) and transferred to sterile Oakridge tubes
(Nalgene). In order to remove bran residues and mycelium, the samples were
centrifuged at 12,500 x g (Beckman JA-17) for 10 minutes at 4°C. The supernatent
was transferred to sterile Oakridge tubes (Nalgene, UK) and this process was
repeated twice more. The final supernatant was then filter-sterilised through Milli-pore Steri-cups (0.2 μm pore size) or for smaller quantities, the Milli-pore (0.2 μm pore size) mini-filter. The samples were then stored at -20°C until further use.

2.3 Plant cultivation

2.3.1 Seed sterilisation

All plant seeds were surface sterilised before use. This involved placing seeds in a sterile eppendorf tube and mixing with ethanol (70%) for 2 minutes followed by 5 rinses/vortex with dH2O. Once the ethanol had been removed, equal aliquots of Sodium Dodecyl Sulphate (SDS; 10% v:v;) and sodium hypochlorite (bleach; 5% w:v) were added to the seeds and mixed for a further 5 minutes. SDS/bleach solution was removed by thorough rinsing with dH2O at least 5 times followed by vortex with dH2O. Seeds were air dried (laminar flow hood) before storing in a dark, cool area.

2.3.2 Lactuca sativa microcosm preparation

One litre of sieved (500-1000 μm) Sphagnum moss peat (Shamrock; Everris, UK) was mixed with 400 mL dH2O and autoclaved at 121°C for 20 min. Once cooled, microcosms (120 x 120 x 12mm) were constructed with 300 g sterilised moss peat only (control) or soil was inoculated with either 8 g fungi-bran inoculum (2.67% w:w) prepared (Chapter 2.2.3.). Each microcosm contained 25 lettuce (Lactuca sativa cultivar Webb’s Wonderful; Unwins Seeds, UK) in a grid system and randomly placed in a growth cabinet (Sanyo) at 24°C with a relative humidity of 90% and a 16 h fluorescent light regime (104m⁻¹/ s⁻¹/ j⁻¹). After 48 hours microcosm lids were removed to inhibit imbibition.
Table 2.1 Microorganism isolates used in this study

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<th>Fungal isolate</th>
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<tr>
<td><em>Trichoderma hamatum</em> GD12</td>
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<td>Laboratory strain (Thornton et al., 2004)</td>
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<tr>
<td>ΔThnag::hph</td>
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<td>Laboratory strain (Thornton et al., 2012)</td>
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<tr>
<td><em>Sclerotinia sclerotiorum</em></td>
<td>&gt; 680 hosts (mostly Brassica spp.)³</td>
<td>CBS strain¹</td>
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<tr>
<td><em>Rhizoctonia solani</em></td>
<td>&gt; 2000 hosts (mostly herbaceous)³</td>
<td>Laboratory strain (Thornton et al., 2012)</td>
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</table>

²Donated by Professor J. West, Plant Biology and Crop Science, Rothamstead Research, West Common, Harpenden, Hertfordshire, AL5 2JQ.

Table 2.2 Plant species used in this study

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<tr>
<td><em>Lactuca sativa</em></td>
<td>Wild type: cv ‘Webbs wonderful’</td>
<td>Unwins Seeds</td>
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<tr>
<td><em>Solanum lycopersicum</em></td>
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Table 2.3 Laboratory suppliers used in this study

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<td>Agar Scientific Ltd</td>
<td>66a Cambridge Rd. Stanstead, Essex, CMC4, 8DA.</td>
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<td>Ermine Business Park, Spitfire Close, Huntingdon, Cambridgeshire, PE29 6XY.</td>
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<td>Little Chalfont, Buckinghamshire, HP7 9NA.</td>
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<td>Anachem House, Charles Street, Luton, Bedfordshire, LU2 0EB.</td>
</tr>
<tr>
<td>BD Biosciences Clontech UK Ltd.</td>
<td>21, In Between Towns Road, Cowley, Oxford, OX4 3LY.</td>
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<td>BDH Ltd.</td>
<td>Broom Road, Poole, Dorset BH12 4NN.</td>
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<tr>
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<td>Calbiochem-Novabiochem Biosoiences UK Ltd.</td>
<td>Boulevard Industrial Park, Padge Road, Beeston, Nottingham, NG9 2JR.</td>
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<tr>
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<td>Difco</td>
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<td>Duchefa</td>
<td>P.O.Box 809, 2003 RV Haarlem, The Netherlands.</td>
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<tr>
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<td>1, The Irwin Centre, Scotland Road, Dry Drayton, Cambridge, CB3 8AR.</td>
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<td>Institute of Microbiology Johanna Wolfgang Goethe-University Frankfurt Marie-Curie-Strasse 9; Building N250 D-60439 Frankfurt, Germany.</td>
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<td>Thermo Life Sciences, Unit 5, Ringway Centre, Edison Road, Basingstoke, RG21 2YH.</td>
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<td>Invitrogen</td>
<td>3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF.</td>
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<td>Millipore (UK) Ltd, 2-4 Fleming Road, Kirkton Campus, Livingston, EH54 7BN.</td>
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<td>Nalgene</td>
<td>Unit 1a, Thorn Business Park, Hereford, HR2 6JT.</td>
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<td>PGC Scientifics</td>
<td>P.O Box 15, Bristol, BS99 5NN.</td>
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<td>Promega UK Ltd.</td>
<td>Delta House, Chilworth Science Park, Southampton SO16 7NS.</td>
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<td>Qiagen</td>
<td>Boundary Court, Gatwick Road, Crawley, West Sussex, RH10 9AX.</td>
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<td>Reichert-Jung (now Leica Microsystems UK)</td>
<td>Davy Avenue, Knowhill, Milton Keynes Bucks. MK5 8LB.</td>
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<td>Roche Diagnostics Ltd.</td>
<td>Bell Lane, Lewes, East Sussex, BN7 1LG.</td>
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<td>Stratagene UK Ltd.</td>
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TAAB
Unwins Seeds
VWR International Ltd.
Waring, Christison Scientific Equipment Ltd.
Web Scientific
Whatman International Ltd.
Zeiss UK

2 Minerva, Calleva Park, Aldermaston Berks. RG7 8NA.
Alconbury Hill, Huntingdon, PE28 4HY.
Merck House, Poole, Dorset, BH15 1TD.
Albany Road, Gateshead, NE8 3AT.
Web Scientific, Crewe, Cheshire, CW2 5PR.
St Leonard’s Road, 20/20 Maidstone, Kent, ME16 0LS.
Carl Zeiss Ltd, 15 - 20 Woodfield Road, Welwyn Garden City, Herts, AL7 1JQ.
Chapter 3. Plant growth promotion effects of *Trichoderma hamatum*

3.1 Introduction

On a macroscopic scale, soil structure varies and is influenced by regional climates, tectonic plate activity, soil management and the ratio of soil types present (Yao et al, 2000; Wobus et al, 2006). Bio-erosion effects contribute to soil development within the earth's mantle, giving rise to soil particles that vary in size, texture and other physical characteristics (Read & Grover, 1977; Viles, 2012). Such characteristics determine water retention rates and the ionic capacity of soil. On a microscopic scale, the rhizosphere is a complex medium directly surrounding the plants' root system where a wealth of microorganisms creates a chemical environment essential for sustaining life. Rhizosphere microbes include α/β proteobacteria, fungi, protozoa, nematodes and algae that can collectively and individually affect a plant's morphology via increased root architecture, shoot biomass and stress responses (Esterbrook & Yoder, 1998; Bais et al, 2001; Bonkowski, 2004; Gillespie et al, 2009; Lynch, 2012).

By considering the number of fungal species within the rhizosphere, a gram of fresh soil will typically sustain Trichoderma, Penicillus, Aspergillus, Fusarium, Rhizoctonia and Mucor genera in spore counts of at least 1 x 10^4/g (Chet & Baker, 1984; Dangar et al, 2010; Palumbo et al, 2010; Zhang et al, 2012). If one considers the saprotrophic role that fungi play within the environment and the population involved, it is evident these organisms must interact with plants through their root systems. For example, nitrogen, carbon and phosphorus contribute to a fertiliser effect (Kleczewski et al, 2010) that is influenced by fungi and bacteria (Bhattacharjee et al,
Nitrogen is a limiting factor for plant growth and it is well known that soil-dwelling bacteria fix atmospheric nitrogen in soil thereby contributing to the nitrogen cycle and soil fertility (Hopkins, 1910; Lechene et al. 2007; Dixon & Khan, 2004; Houlton et al. 2008; Di et al., 2009). Other nitrogen-fixing organisms present in the rhizosphere include protozoa and algae that contribute to the microbial nutrient cycle via dissolved organic matter (Cutler, 1923; Fenchel, 2008). This erosion concept is associated with symbiotic effects fungi have upon plant viability which, in turn, support the hypothesis that plant phenotype is positively affected by such populations (Balogh-Brunstad et al., 2008; Adeleke et al. 2012).

The hydrolysing abilities of *Trichoderma* spp. are a consequence of their Cell Wall Degrading Enzymes (CWDE) that hydrolyse naturally occurring polymers including cellulose, lignin, pectin, chitin and other complex organic pokymeers (Hayes et al., 1992; Donzelli & Harman, 2001). Whether CWDE are deployed for self or non-self means, chitinase is considered important for a number of reasons. Primarily, chitin is a polymer consisiting of N-Acetylβ-D-Glucosamine (NAG) monomers, an essential structural component of anthropods, bacteria and fungi. Not only is chitinase used for exo-skeletal remodelling, but is paramount in mycoparasitism, host-specific invasion and saprotrophism.

Saprotrophism concerns the hydrolysation of dead or decaying organic matter within the soil; hypothesised to contribute to nitrogen sequestration via the release of NAG monomers taken up by the plant to increase plant viability (Deng et al., 2007; Harman and Bjorkman, 1998; Harman and Shoresh, 2007). Consequently, *Trichoderma* spp. are known as effective plant growth promoters in both natural and artificial soil-based microcosms (Ousely et al., 1994; Ryder et al., 2012; Schopmeyer &
Fulmer, 1930). It has been shown that lettuce plants, when sown in potting compost incorporated with 1% (w:w) T. harzianum, are 2-fold larger than controls (Ousely et al, 1993; Ousely et al, 1994). Agar studies with T. atroviride and T. viride displayed similar results (Contreras-Cornejo et al, 2009) with increased PGP effects following autoclaving of spores and metabolite extracts. Consequently, it is hypothesised that Trichoderma mediated PGP occurs via diffusible factors, more specifically, fungal Secondary Metabolites (SM) (Ousely et al, 1993; Ryder et al, 2012).

In addition to CWDE, Trichoderma spp. secrete SM such as Volatile Organic Compounds (VOC), antibiotics, organic acids and phytohormone-like compounds that permeate the rhizosphere; thus having a positive effect on plant growth and defence (Vinale et al, 2008a/2008b; Shoresh and Harman, 2008; Vespermann et al, 2007). For example, fungi have evolved to synthesise a number of SM similar to phytohormones such as auxins and gibberellic acid which are important to plant development and maturation (Contreras-Cornejo et al, 2009; Jin et al, 2011). In addition to PGP effects, Trichoderma spp. are known promote the up-regulation a number of phytohormones which regulate stress response and systemic defence (Martinez-Medina et al, 2011; Gutjahr & Paszkowski, 2009). Defence hormones may promote plant growth indirectly but it is increased levels of auxins in the rhizosphere that is causing scientific interest.

Auxins are a diverse set of cyclic-carboxylic acid phytohormones found within plant tissue where their fundamental effects include cellular division and differentiation. In addition to PGP effects, auxins are responsible for senescence and flower inhibition with additional antimicrobial properties (Somers et al, 2005). Several auxins have been studied including Indole Acetic Acid (IAA), Indole-3-Butyric Acid (IBA), 2-PhenylAcetic Acid (PAA), and 6-Pentyl Pyrone (6PP) which Trichoderma
spp. are known to secrete and degrade (Gravel et al, 2007; Zhang et al, 2012). Hypotheses of how fungal auxins are utilised by plants include plant assimilation and modification of these compounds prior to catabolism (Vinale et al, 2008a).

IBA is the precursor to IAA where biosynthesis is not dependent on the tryptophan pathway (Sitbon et al, 2000). Although both IBA and IAA are alkaloid homologues and enhance root morphology (Hull et al, 2000; Contreras-Cornejo et al, 2009), it was found that soil inoculated with *Glomus intraradices* increased plant-associated IBA synthetase up-regulation that, in turn, induced symbioses (Ludwig-Müller et al, 1997; Fitze et al, 2005). Similarly, PAA and 6PP are found in shoot tissues where cell elongation occurs (Wheeler, 1976; Leuba & LeTourneau, 1990). Although little literature support 6PP’s role in PGP, subtle structural differences in PAA and 6PP (mono-cyclic ring) indicate ambiguous effects.

Much interest surrounds the molecular basis for the PGP effect of *Trichoderma* spp. Overall, this phenomenon is partially attributed to its salvation effects within the soil and its ability to chemically communicate with the plant. To determine any PGP trends, we set out to investigate the effects of *T. hamatum* treatment on plant biomass, phenotype and overall health via carbon and nitrogen partitioning within plant tissues. More specifically, to determine any difference in PGP efficacy, the study of effects of *T. hamatum* wild type GD12 and the mutant △nag (hereafter referred to as △nag) will focus on solid (mycelium biomass) and liquid (metabolite) application.
3.2 Materials and Methods  
3.2.1 Preparation of metabolite extract

*T. hamatum* metabolite extract was prepared (Chapter 2.2.4). For both GD12 and Δ*nag* samples, each extract was fine filtered through a Milli-pore mini-filter (0.2 μm pore size; ‘filtered’) or a sterilised Miracloth (22-25 μm pore size; ‘sieved’). Samples were snap frozen in liquid nitrogen and stored at -80°C until further use.

3.2.2 Preparation of soil extract

Twenty five lettuce seeds (*Lactuca sativa* cv. Webb’s Wonderful) were sown in microcosms prepared as in chapter 2.3.2. After 21 days growth, plants were carefully removed from each microcosm for further analyses and 200 mL *MgH₂O* was applied to each microcosm. Once stirred, the soil and *MgH₂O* was transferred to a sterile 1 L conical flask before stirring at room temperature (1 hr) prior to filtration through sterile Miracloth in a class II microbiological cabinet. In order to remove soil, mycelium or spore residues, the exudate was placed in sterile Oakridge tubes and centrifuged at 12,500 x *g* (Beckman JA-17) for 10 mins at 4°C. This process was repeated twice before samples were snap frozen in liquid nitrogen and stored at -80°C before use.

3.2.3 *Solanum lycopersicum* (cv Ailsa craig) cultivation

One litre of sieved (500-1000 μm) Sphagnum moss peat (Shamrock) was mixed with 400 mL dH₂O and autoclaved at 121°C for 20 min. Once cooled, microcosms (100 x 100 x 100 mm) were constructed with 300 g sterilised moss peat only (Control) or soil was inoculated with 8 g fungi-bran inoculum (2.67% w:w) (Chapter 2.2.3). Each microcosm was sown with one tomato (*Solanum lycopersicum* cv. Ailsa craig) seed and placed in a growth cabinet (Sanyo) at 24°C, relative humidity of 90% and a 16 h fluorescent light regime (105m⁻¹/ s⁻¹/ j⁻¹).
3.3 Results

3.3.1 *Trichoderma hamatum* as plant growth stimulant

A number of experiments was conducted in order to investigate the specific growth effects of *T. hamatum* wild type GD12 and mutant Δnag on lettuce (*Lactuca sativa*; cv Webb’s Wonderful). Trichoderma mycelium was independently applied to replicate microcosms composed of autoclaved peat (2.67% w:w) as discussed in chapter 2.3.2. After specific growth periods, a maximum of 25 data points were retrieved from each of three experimental replicates per treatment for further analyses.

Plant growth rates were determined by extracting three plants from each replicate microcosm on days 14 and 21 with remaining plants excised on day 28. Photographs (Figure 3.1) and data [fresh weights and root morphology] (Figure 3.2) were recorded and analysed using Analysis of Variance (ANOVA; one-way), and Students T-Test. Significant results show Δnag treated microcosms consistently displayed larger plant biomass than GD12 treatment (ANOVA; *P*<0.001). PGP effects are observed within 14 days of treatment where Δnag and GD12-treated plants were 3-fold and 30% larger than the control respectively (ANOVA; *P*<0.05) (Figures 3.1A and 3.2).

Between weeks two and three, GD12 and Δnag-treated plants increased fresh plant weight by 50% and 21% respectively when compared to the control dataset that witnessed a 14% increase (Figure 3.1B). By day 28, GD12-treated plants were 2-fold larger than the control (T-Test; *P*<0.001) while Δnag microcosms were 8-fold larger than the control (T-Test; *P*<0.0001). However, Δnag display accelerated growth rates between weeks three and four (90%) compared to the slower growth rate of GD12. Overall, Δnag induced growth was 2.5, 3 and 4-fold larger than GD12 at days 14, 21 and 28 respectively (ANOVA; *P*<0.05).
3.3.1.1 *Trichoderma hamatum* impact on root morphology

For root morphology (root length and root width), mean values of the Δ*nag* dataset were significantly larger than the control over the duration of the experiment (T-Test; *p*<0.001) (Figures 3.2A & 3.2C). Tap root width was not significantly different between GD12 and Δ*nag*–treated plants on weeks two and three (*p*>0.05). GD12-treated plants’ mean tap root width was not significantly different to the control until week four (T-Test; *p*<0.05) (Figure 3.2C).

![Figure 3.1](image.png)

**Figure 3.1** Plant growth promotion effects of *Trichoderma hamatum* wild type GD12 and Δ*nag* over four weeks. Microcosms containing autoclaved peat were either left untreated (control) or inoculated with GD12 or Δ*nag* bran mix at 2.67% (w:w). Twenty five lettuce seeds were sown in a grid system and placed in a growth room at 26°C. Panel A is week two, B is week three and C is week four. Panel A & B are to scale; panel C scale bar 1:0.7
Figure 3.2 Analyses of lettuce plants treated with *T. hamatum* over four weeks. Fresh root length (A), fresh plant weight (B), fresh root width (C) and mean Lateral Roots (D) of three experimental replicates each containing 25 *Lactuca sativa* seeds sown in a grid system within a 12 x 12 x 2 cm agar plate. Soil remain untreated (control) or inoculated with *T. hamatum* bran mixture (GD12 & Δnag; 2.67% w/w). Five experimental samples were removed from each parameter for analyses in weeks two and three. At week four plants were harvested (maximum of 15 plants/ tray). Each bar represents the mean of five or 15 replicates (±) SE Bars. Data points with same letter denote means not significantly different.
By week two lateral root formation was significantly different where untreated, GD12 or Δnag subsets show values of three, five and 14 lateral roots respectively (ANOVA; p<0.01). By week three, mean values for GD12 and Δnag-treated plants doubled to 10 and 28 lateral roots while the control set failed to significantly increase lateral root formation (Figure 3.2D). Interestingly, a similar trend to growth rates was found with plant weights between weeks three and four. Here, the control and GD12-treated plants grew slowly with mean lateral root values at seven and 12 respectively, whereas Δnag–treated plants displayed a mean value of 66 lateral roots, a 2-fold increase in one week alone (p>0.0001). This correlates increased root architecture with a plants final biomass, and that overall, Δnag exceeds the growth promoting ability of GD12 by 3-fold (root length), 4-fold (plant weight) and 5-fold (lateral root).

![Figure 3.3](image_url)

**Figure 3.3** Shoot to root ratios between lettuce plants treated with *Trichoderma hamatum* GD12 or Δnag. Fungi was incorporated in autoclaved peat at 2.67% (w:w); Control plants were untreated. Three experimental replicates each containing 25 *Lactuca sativa* seeds were sown in a grid measuring (12 x 12 x 2 cm) where n=65. Each bar represents the mean of 25 replicates (±) SE Bars. Data points with same letter denote means not significantly different.
3.3.1.2 *Trichoderma hamatum* and shoot to root ratios

Shoot to root ratios (S:R) were recorded after 21 days growth in order to determine plant viability, where it was found that dry S:R follow a trend within treatments (ANOVA; $p<0.05$, Figure 3.3). The control set had the highest dry S:R compared to $\Delta nag$-treated plants while GD12 displayed intermediary results that were significantly different to the control plants only ($p<0.05$). Table 3.1 provides an overview of the $p$ values associated with each parameter regarding root morphology and plant weight.

**Table 3.1** Comparisons between different $p$ values associated with each fungal treatment. Students T-Tests were conducted between each treatment. Trends shown above reflect that of two experiments conducted separately under the same conditions.

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<td>0.030865</td>
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</table>
3.3.1.3 Establishment rates
Quantification of *T. hamatum* GD12 and Δ*nag* effects upon seed germination rates is of economic importance. In chapter 3.1, a preliminary experiment was conducted to determine the temporal effects of Trichoderma treatment on plant growth. Δ*nag* treatment significantly increase seedling emergence by day two (62.5%) compared to the control which took a further day to match Δ*nag* germination (Figure 3.4). GD12 germination rates were equal to the control on day two (42.18%) but, after day two, GD12 rates remain static at <50% whilst the control and Δ*nag* datasets continue to (albeit not significantly) increase after day five.

![Graph showing establishment rates](image)

**Figure 3.4** Lettuce establishment rates between different Trichoderma treatments over 14 days. Three experimental replicates each containing 25 *Lactuca sativa* seeds were sown in microcosms filled with autoclaved moss peat (control) or a moss peat:Trichoderma mixture (300:8 w/w); *T. hamatum* strains GD12 or Δ*nag*. Mean values were converted to arc sin⁻¹ values for statistical analysis by single-tailed Students t-test/ANOVA. Bars are standard errors.
Therefore, by day 14, GD12 shows significantly lower germination rates than both control and Δnag treated plants at 50, 83.3 and 87.5% respectively (ANOVA; p<0.001).

3.3.2 A dose response to mycelium biomass application

It was hypothesised that the PGP ability of T. hamatum wild type GD12 and Δnag is dose dependant. In order to test this, GD12 and Δnag mycelium inoculation was prepared in bran and incorporated into autoclaved peat at 0.5%, 1%, 1.5%, 2%, 3% and 4% (w:w) as discussed in chapter 2.3.2. Establishment rates were recorded over 10 days and after 21 days (Figure 3.5), plants were harvested for dry weights and S:R determinations using Analysis of Variance (ANOVA; one-way) and Students T-Test analysis.

3.3.2.1 Lettuce establishment rates

Lettuce establishment was significantly reduced in GD12 incorporated soil (~50%). Here, emergence rates decreased as GD12 concentrations increased (Figure 3.6). The lowest emergence rate of the Δnag data set was the 4% inoculum where 66% of seeds germinated by day 10. However, all Δnag-treated plants (except 4% amendment) were similar to the control by day 10 (ANOVA; p>0.05).

Germination rates over a period of 10 days confirm that by day one, more control plants (pale blue) emerged than Δnag (green data points). Both control and Δnag was more efficient than GD12 treatment (grey data points) (ANOVA; p<0.05) (Figure 3.7). Both GD12 and Δnag optimum germination rates involved lower concentrations between 1% and 2%. Here, rapid germination rates occurred between days two and three where Δnag 1.5% (w:w) and 2% (w:w) show 66 & 76% of seeds emerged respectively.
Figure 3.5 *T. hamatum* GD12 and $\Delta$*nag* effects on lettuce after 21 days growth. All microcosms consisted of autoclave peat treated with GD12 or $\Delta$*nag* mycelium extract in varying aliquots or MQH2O (control). Twenty five lettuce seeds were sown in a grid system and placed in a growth room at 26°C and light intensity of 104m$^{-1}$/s$^{-1}$/j$^{-1}$. Left column is GD12-treated plants, right column is $\Delta$*nag* treated plants. Scale bar: 20 mm.
**Figure 3.6** Mean germination (%) of lettuce seeds after 10 days when treated with Trichoderma mycelium at increasing concentrations. Seeds were sown in autoclaved peat amended with GD12 or Δnag or remain untreated. Each bar is the mean arc sin⁻¹ value (±SE). Same letter denote not significantly different between means at 95% significance.
Figure 3.7 Mean daily emergence of lettuce plants over 10 days when treated with increasing concentrations of Trichoderma mycelium. Lettuce seeds were sown in peat inoculated with *T. hamatum* GD12 (A) or Δnag (B) where each data point represents the mean of three replicates. Data was normalised to $\arcsin$ values for statistical analysis by single-tailed Students t-test. (±SE).

![Graph A](image1.png)

![Graph B](image2.png)
Figure 3.8 Effects of increasing Trichoderma inoculum on lettuce morphology after 21 days. Mean dry root (A), dry shoot (B) and Lateral Root (C) values are the mean of three replicates, n=25. (±SE). Same letters denote not significantly different at <95% confidence level.
3.3.2.2 Root and shoot analyses
Dry root (A), dry shoot (B) and lateral roots increased in a dose-dependent fashion (Figure 3.8). Overall, it is evident that $\Delta n$ag inoculation significantly increased shoot and root biomass (ANOVA; $p<1.37E-09$; shoot & $p<8.42E-19$; root). However, dry shoot weight decreased in the GD12+4% (w:w) treatment (T-Test; $p<0.001$).

3.3.3 Plant growth promotion effects of mycelium extracts
In order to compare the PGP effects of metabolite extract and mycelium biomass application to soil, GD12 and $\Delta n$ag were either applied to autoclaved soil at 2.67% (w:w) bran inoculum (see chapter 3.3.2), left untreated, or 200µL aliquots of metabolite extract were applied to L. sativa [cv. Webb’s Wonderful] seedlings on alternate watering days. The metabolite extract consisted of two types, filtered (passage through a Millipore sterile filter, 0.2 µm pore size) or sieved extract (filtered through Miracloth, 22-25 µm). After 21 days growth, 25 plants (maximum) were removed from each microcosm treatment. Growth analysis included dry weights of root and shoots and S:R and germination rates. Visual evidence (Figure 3.9) and data (Figure 3.10) were recorded and analysed using Analysis of Variance (ANOVA; one-way) and Students T-Test.

Here, we report the PGP effects of GD12 and $\Delta n$ag treatment on lettuce plants compared to the control (Figure 3.9). Various treatments include soil incorporated mycelium (left), filtered metabolites (central) and sieved metabolite-treated plants (right). Primarily, fresh root and shoot weights follow similar growth trends, as previously observed, where control plants were smaller than GD12 which, in turn, were smaller than $\Delta n$ag-treated plants. Differences between metabolite or soil inoculum treatment show GD12 metabolite-treatment were much
Figure 3.9 Lettuce plants after 21 days treatment with mycelium or fungal metabolite extract. Control (upper panel), GD12 (mid panel) and Δnag-treated plants (lower panel) include fungal application that was either bran inoculum incorporated into soil at 2.67% (w/w) or 200 μL metabolite extract applied on alternate days. Filtered metabolite extracts were prepared with Millipore filter (0.2 μm pore size) whereas sieved metabolite extracts were prepared with Miracloth (22-25 μm pore size).
Figure 3.10 The effects of different fungal applications on root and shoot morphology. Mean root (A) and shoot (B) dry weights of lettuce plants after 21 days growth; (C) is dry S:R. Treatments include Mill-Q water (control), GD12 and Δnag incorporated in soil at 2.67% (w:w), filtered metabolite extract (0.2 µm size pores), sieved metabolite extract (Mira cloth; 22-25 µm pores). (±SE). Each bar is the mean of three experimental replicates, n=25. Same letter denote no significant difference at 95% confidence level.
larger than GD12 soil treatment to become comparable to the Δ\textit{nag}-inoculum treatment (T-Test; \(p<0.05\)). Similarly, Δ\textit{nag} extract treatment improved plant mass (ANOVA; \(p<0.0001\)). However, Δ\textit{nag} sieved metabolite treatment (>20 \(\mu\)m pore size) was less effective than the Δ\textit{nag} filtered metabolite (<0.2 \(\mu\)m pore size). In contrast, GD12 filtered plants were not significantly different to the GD12 sieved metabolite. Overall, a significant difference between all data sets was observed where Δ\textit{nag} metabolite-treated lettuce were substantially larger than other treatments (ANOVA; \(p<1.0\times10^{-5}\); roots & \(p<1.0\times10^{-8}\); shoots) (Figures 3.10A & 3.10B).

Most fungal treatments exhibited similar dry S:R values (mean 6.2–7.02) except for the control and Δ\textit{nag} sieved metabolite dataset (ANOVA, \(p<0.05\)) (Figure 3.10C). Overall, GD12 filtered and sieved metabolite display similar S:R greater than the control and Δ\textit{nag} sieved treatments (ANOVA; \(p>0.05\)).

3.3.4 Effect of fungal extract on plant growth

Once the PGP effects of Trichoderma GD12 and Δ\textit{nag} exudates had been established, a dose response investigation was undertaken, similar to that conducted for mycelium biomass (Chapter 3.2.2). To achieve this, GD12 and Δ\textit{nag} metabolite extracts were prepared as discussed in chapter 2.2.4. Once the metabolite extract was prepared, aliquots of 50 \(\mu\)L, 100 \(\mu\)L, 200 \(\mu\)L, 300 \(\mu\)L, 400 \(\mu\)L and 600 \(\mu\)L were frozen to reduce treatment bias. A series of six micro-well clear bottom plate microcosms each measuring 127 x 85 x 20 mm (l x w x h) were prepared with autoclaved peat before lettuce seeds were individually sown in each micro-well (34 mm x 20; r x h). Germination rates were recorded over 10 days and after 21 days, plants were harvested for analyses of dry compartment weights and S:R measurements. Plants were photographed and data was analysed using Analysis of Variance (ANOVA; one-way) and Students T-Test (Figures 3.11 - 3.14).
Figure 3.11 The effects of *T. hamatum* metabolite extract on lettuce biomass after 21 days. Individual microcosm dimensions are 34 mm x 20 mm (r x h).
Figure 3.12 The effects of increasing *T. hamatum* metabolite extract dose on lettuce plants after 21 days treatment.
Figure 3.13 Trichoderma metabolite extract effects on lettuce plants upon increasing dose. Mean dry root (A), dry shoot (B) and lateral root (C) after 21 days growth with *T. hamatum* strain GD12 (black bars) or Δnag (grey bars) metabolite extracts applied to plants on alternate days. Control (white bars) was watered with MQH₂O. Each bar is the mean of three experimental replicates, n=25 (±SE). Bars with the same letters denote no significant difference at 95% confidence level.
3.3.4.1 Extract application is dose-dependent

PGP effects were induced by applying varying amounts of either filtered 0.2μm; Millipore filter) metabolite extract or MQH₂O (control) to lettuce plants on alternate days for three weeks (Figures 3.11 and 3.12). The control plants appear to benefit from increasing daily water aliquots, although this is not significant between all of the controls.

Dry weight data confirm GD12 and Δnag-treated lettuce increase dry root weights in a similar fashion where 50, 100 and 200 μL aliquot application steadily increases dry biomass (ANOVA; p<0.05) (Figures 3.13A & B). GD12 treatment fails to significantly increase biomass any further while Δnag-treated lettuce display a significant increase until 300 μL but no further increases at 400 and 600 μL aliquots (T-Test; p>0.05).

Dry shoot weights follow a similar trend except where GD12-treated plants fail to significantly increase dry shoot mass beyond 200 μL aliquots. This PGP effect is linear but reaches a maximum at 400 μl then dry shoot weights begin to decrease at 600 μL (ANOVA; p<0.01). Control plants are not significantly different between treatments, whereas GD12 and Δnag increase in lateral root as inoculation dose increase but this effect does not extend beyond 300 μL (Figure 3.13C). In contrast, Δnag exhibit a rapid decrease in lateral root as extracts are increased to 400 μL.

3.3.4.2 Shoot to root ratios

Control plant dry S:R values were all larger than Trichoderma treated plants with little variation within the untreated parameter (ANOVA; p<0.05; Figure 3.14). Dry S:R display some variation between treatments most notably within the GD12 plants where 100, 200, 400 and 600 μL were significantly reduced compared to that of the 50 μL treated plants (p<0.05). GD12 300 μL metabolite treated plants display the
Figure 3.14 Mean dry S:R after 21 days growth with increasing metabolite extract treatment. Lettuce plants were watered with filtered metabolite extract (0.2 μm size pores) on alternate days and control plants were watered with $\Delta$H$_2$O. Each bar represents the mean of three experimental replicates, n=25 (±SE). Bars with the same letters denote no significant difference at 95% confidence level.

The largest S:R of all treatments. In contrast, only $\Delta$ag 50 μL treatment reveal one significantly larger S:R value within the $\Delta$ag dataset suggesting this dosage does not affect root architecture as effectively as the larger aliquots.

3.3.4.3 Influence of metabolite exudates on germination

After 10 days growth, seedling emergence displays some variability between the different treatments, mostly pronounced in the control (Figure 3.15). GD12 treatments displayed maximum germination rates at 200, 300 and 400 μL but at GD12 100 & 600 μL germination was significantly decreased. In contrast, all $\Delta$ag treatments were not significantly different (p=0.5712) except for comparisons between $\Delta$ag treatment at 50 and 100 μL (p<0.05). This suggests insufficient application of
Distinct trends exist between mean emergence rates of lettuce seeds over 10 days where all control, GD12 or \( \Delta \)nag-treated plants appeared by day three (Figure 3.16). The lowest rate of germination was \( \Delta \)nag treatment at 50 \( \mu \)L aliquots where maximum emergence was not achieved at all (66%) (Figure 3.16C). In contrast, GD12 metabolite treatment significantly improved emergence compared to GD12 bran inoculum and accelerated germination rates compared to \( \Delta \)nag (Figure 3.16B). However, by day 10, GD12 has failed to increase germination whilst \( \Delta \)nag–treated
seeds continue to emerge.

**3.3.5 The effects of spatial boundaries upon plant growth**

It is hypothesised that root architecture is limited by the immediate space surrounding the root ball. Other hypotheses include nutrient availability as a determining factor. Nonetheless, this particular sub-chapter aimed to show that *T. hamatum* inoculum can produce similar PGP as previously witnessed; regardless of spatial limitations. Therefore, in order to deduce the PGP effects of a reduced soil volume and decreased surface area for adventitious roots to develop, two sets of microcosms were prepared. Each contained autoclaved peat only (control) and peat containing a Trichoderma:bran inoculum at 2.67% (w:w) as set out in chapter 2.3.2. Each data set contained a communal microcosm (25 lettuce seeds sown in a grid system) measuring 120 x 120 x 12 mm (l x w x h) (Figure 3.17A) or a smaller individual unit measuring 127 x 85 x 20 mm (l x w x h) each containing six individual wells measuring 34 mm x 20 (r x h) and one seed (Figure 3.17B).

After 21 days growth a maximum of 25 and a minimum of 12 data points were retrieved from each of three experimental replicates [per treatment] for further analyses. Data analysis was performed using Analysis of Variance (ANOVA; one-way) and Students T-Test. Microcosm size has an effect on plant biomass where increased root (Figures 3.17C & 3.17E) and shoot (Figures 3.17D & 3.17F) systems are displayed in communal, larger microcosms (p<0.001). All control, GD12 and Δnag treatments in larger microcosms witness a 56, 78 and 117% increase in fresh root biomass compared to the smaller microcosms respectively. In contrast, fresh shoot increments were not as dramatic where control, GD12 and Δnag each display 37, 42 and 26% improvement in the larger microcosms. Overall, Δnag treatment produced the largest plants where fresh root values validate GD12 and Δnag.
3.16 Germination of *L. sativa* seeds over 10 days when treated with Trichoderma metabolite extract at various concentrations. Mean data values from three replicate microcosms each containing 25 lettuce seeds. Untreated plants (A), GD12-treated plants (B) & ∆nag treated plants (C). Emergence percentages were converted to arc sin⁻¹ values for statistical analysis by single-tailed Students t-test ± SE.
**Figure 3.17** Effects of *T. hamatum*:bran inoculum (2.67% w:w) on lettuce after 21 days. PGP effects in larger, communal microcosms (A) or solitary, smaller microcosms (B). Histograms show fresh root (C) and shoot (D) mass for small microcosms and fresh root (E) and shoot (F) weight for large microcosms. Each bar represents the mean of three experimental replicates, n=25 (±SE). Bars with the same letters denote no significant difference at 95% confidence level.
inoculated plants are 4-fold and as 6-fold larger than the control while fresh shoots are 4- to 4.75-fold larger (Figure 3.18; \(p<0.01\)). Therefore, the \(T. \ hamatum\) mutant \(\Delta \text{nag}\) enhances root architecture more effectively than shoots in both systems; albeit less effectively in smaller systems.

3.3.5.1 Trichoderma PGP effects in recycled soil
To test the longevity of Trichoderma PGP effects in soil, microcosms were prepared as described in chapter 2.3.2 and allowed to grow for three weeks, where fresh root and shoot weights were recorded before new seeds were sown in the used microcosms. The recycled soil was covered and placed under dark conditions at 4°C for 28 days to induce natural vernalization conditions. Following this, lettuce plants were sown in a grid system \((5 \times 5)\) within used microcosms with no additional Trichoderma inoculum or peat. After three weeks growth, plants were excised and weighed in order to compare growth dynamics to the initial bioassay.

The PGP effects of previously used soil inoculated at 2.67% \((w:w)\) with \(T. \ hamatum\) GD12 or \(\Delta \text{nag}\) are not lost in recycled soil for all treatments (Figure 3.18). Fresh root biomass show a 3- and 6-fold increase in the original soil compared to a 3- and 9-fold increase in the recycled soil for GD12 and \(\Delta \text{nag}\) respectively (Figure 3.18A). Shoot weight results were not as impressive where GD12 and \(\Delta \text{nag}\) display a constant 3- and 5-fold increase respectively; regardless if lettuce were grown in used or fresh soil (Figure 3.18B).

S:R values indicate that \(\Delta \text{nag}\) treatment significantly decrease S:R values by 2-fold once seeds are sown in recycled soil \((p>0.01)\) while the control and GD12 plants display some reduction (Figure 3.18C; \(p>0.05\)). Overall, it is evident that Trichoderma persist in the soil and \(\Delta \text{nag}\) continues to decrease S:R post harvest; clearly maintaining PGP properties without reapplication.
Figure 3.18 Histograms depicting PGP effects of recycled soil incorporated with Trichoderma at 2.67% (w:w) on lettuce plants after 21 days. Soil was vernalized for 28 days post harvest, followed by a fresh application of lettuce seeds where untreated (control), GD12 or Δnag are shown (recycled soil). Where mean fresh root (A), shoot (B) and S:R (C) are compared. Each bar is the mean of three experimental replicates, n=25. (±SE). Bars with the same letters denote not significantly different at 95% confidence level.
3.3.6 Trichoderma PGP effects on *Solanum lycopersicum*

In order to deduce the PGP effects of GD12 and $\Delta nag$ on alternative UK crops, *Solanum lycopersicum* (cv. Ailsa Craig) was sown in microcosms each containing autoclaved peat only (control) and peat containing a Trichoderma:bran inoculum at 2.67% (w:w) as discussed in chapter 3.2.3. After 28 days plants were harvested for dry weights where differences in growth were determined using Analysis of Variance (ANOVA; one-way) and Students T-Test.

3.3.6.1 *Solanum lycopersicum* germination rates

Germination and PGP differences were observed after 28 days treatment between untreated plants (upper panel), GD12 (mid panel) and $\Delta nag$-treated plants (lower panel) (Figure 3.19). The effects of GD12 and $\Delta nag$ upon germination clearly prove GD12 mycelium treatment significantly reduce plant emergence while GD12 extract display similar germination rates to the control and $\Delta nag$ extract (Figure 3.21A). In a similar fashion, $\Delta nag$ mycelium application achieved similar emergence rates as untreated and GD12 extract application but at a slower rate. All treatments attained maximum germination by day 4, except GD12 mycelium which failed to achieve 50% emergence (ANOVA; $p<0.001$).

3.3.6.2 *Solanum lycopersicum* morphology

$\Delta nag$ extract significantly improved mean dry root biomass while GD12 extract significantly improved dry shoot mass (T-Test; $p<0.005$) (Figures 3.20B and 3.20C). Interestingly, GD12 mycelium treatment was more detrimental to tomato root growth than GD12 metabolite extract and produce roots 3-fold smaller than the control (T-Test; $p<0.01$). In contrast, GD12 extract increased mean dry shoot weight 2-fold and 60% compared to control and $\Delta nag$-incorporated plants respectively ($p<0.0005$) and increased S:R (ANOVA; $p<0.01$) (Figure 3.20D).
Figure 3.19 The effects of Trichoderma metabolite extract on tomato plants after 28 days. All tomato plants were individually sown in autoclaved peat. Treatment includes untreated peat (control), plants watered with either GD12 or ∆nag metabolite extract or soil inoculated with Trichoderma at 2.67% (w:w) in microcosms measuring 10 x 10 x 10 cm. After 28 days, plants were harvested for fresh and dry weights where differences in growth were determined. Each plant is representative of five replicates. 10 mm standard bar.
Figure 3.20 Growth effects of Trichoderma application on tomato plant emergence and growth. Emergence percentages were converted to arc sin-1 values for statistical analysis by single-tailed Students T-Test (A). Dry root (B), dry shoot (C) and dry S:R (D) are shown where untreated plants ( ; white bar), GD12 mycelium ( ; black bar) and Δnag mycelium ( ; grey bar) are represented. Extracts are depicted as red outline in (A) only. Each bar represents the mean of five replicates each from three experimental repeats (± SE). Same letters are not significantly different at 95% confidence level.
3.4 Discussion

This Chapter has clearly shown that *T. hamatum* GD12 or Δnag treatment significantly increase lettuce biomass whilst persisting in the soil post harvest. Furthermore, this improves if applied as a liquid application; especially following 0.2 micron filtration. Typically, mycelium applications of GD12 and Δnag promoted a mean 3.5-fold and 5-fold increase respectively, which was greater than the 2-fold increase reported previously in soil (Ousely et al, 1993; Ousely et al, 1994) and agar studies treated with *T. atroviride* & *T. viride* (Contreras-Cornejo et al, 2009). Nonetheless, over-expression analyses substantiated that auxin activity was the main PGP benefit associated with Trichoderma. For example, *T. harzianum* IAA mutant T-E5 increased cucumber dry root and shoot mass by 14% and 10% respectively (Zhang et al, 2012). Others have found fresh melon root and shoot weights increased by 1% and & 20%, respectively (Martieze-Medina et al, 2011).

In contrast, metabolite liquid treatments significantly improved lettuce biomass by 4-fold and 7.5-fold in GD12 and Δnag, respectively. However, these PGP effects are dose-dependent and decrease when levels exceed 4% mycelium. Similarly Trichoderma has less effective PGP effects on tomato (*Solanum lycopersicum* cv. Ailsa Craig). Here, GD12 myceliuml application had a similar effect to *T. harzianum* strain T22 application on a variety of tomato cultivars (Tucci et al, 2011). Moreover, *T. hamatum* exhibited less PGP effects in *S. lycopersicum* unless growth medium was enriched with 50% nitrogen (Haque et al, 2012). PGP in tomato was partially restored by GD12 extract application, and dry shoot weights were the largest overall. Similarly, the Δnag extract exhibited PGP properties on root systems, while mycelium treatment failed to promote tomato growth.

Lateral root formation was significantly increased in a dose-dependent manner
following GD12 & Δnag treatment. As previously reported; a plateau in lateral root formation takes place with GD12 mycelium treatment but not with Δnag, regardless of application type. Bran extracts substantially increased lateral root formation as opposed the mycelium application; as confirmed by other studies using T. virens and T. atroviride (Contreras-Cornejo et al, 2009). Although exogenous ABA and nitrogen can modulate S:R (Creelman et al, 1990; Ågren and Franklin, 2003), macronutrient effects are more likely to be responsible (Andrews et al, 1999) or alternative metabolites found within marine-derived strains of Aspergillus yet to be discovered in land dwelling homologues (Anisimov et al, 2012). Others believe fungal secretions of the phytohormone GA promote emergence that in turn is initiated by cyclins (Lee et al, 2002; Masubelele et al, 2005).

So far, it appears that GD12 mycelium treatments can significantly reduce establishment rates of lettuce and tomato seeds in a dose-dependent manner. However, these adverse effects can be abolished once GD12 is applied as a fine filtered extract and becomes more efficient as a PGP agent. This could be a result of concentrating the active property, depending on how the extract was prepared. Nonetheless, this investigation has confirmed the toxic effects of GD12 and excessive mycelium soil inoculations (Ousely et al, 1993). Fascinatingly, both Trichoderma extracts display increased PGP effects once the extract was fine filtered (<0.2 m), suggesting that any antagonistic compounds present are between 0.2 and 20 μm in diameter. Although this was more pronounced in the Δnag extracts, GD12 extracts appear far less antagonistic than the mycelium equivalent. Therefore, differences between mycelium and extract treatment on plant phenotype suggest that physical interactions occur in the soil that reduce PGP effects in tomato and lettuce plants.
PGP levels in the current study exceed those previously reported (Ousely et al, 1994; Contreras-Cornejo et al, 2009; Martinez-Medina et al, 2011; Zhang et al, 2012), and these PGP effects remain post-harvest and persist in the soil. If metabolites play a crucial role, they need to be identified and the intricate molecular dynamics between plant and fungi determined. Therefore, in order to potentiate the role of metabolites associated with wild type GD12 and mutant Δnag treatment, the following Chapter will address biocontrol issues; including phytopathogens responsible for pre-emergence damping-off disease (S. sclerotiorum) and post-emergence damping-off disease (R. solani). This way, it can be determined if NAG mutation has an effect on biocontrol and the ability to parasitise and digest phytopathogens in situ.
Chapter 4.
Biological control by *Trichoderma hamatum*

4.1 Introduction

4.1.1 A commercial perspective to biocontrol

Global food security is a major concern where populations depend on successful crop cultivation to survive (FAO, 2006; WRI, 2011). However, unusual weather patterns, increased disease pressure together with costly fertilisers and biocides may reduce yields (Reddy *et al.*, 2005; Long *et al.*, 2005; Long *et al.*, 2006). If one is to consider crop loss by pathogens such as *Sclerotinia sclerotiorum*, *Rhizoctona solani* and *Pseudomonas syringae* (DC3000): it is reported that $560 million of soybean are devastated by *S. sclerotiorum* in the United States alone (USDA/NASS, 2011); field trials indicate that *R. solani* reduced *Z. mays* L. (maize) yields by 48-42% (Tachibana *et al.*, 1971; Sumner & Minto, 1989); and DC3000 reduced *Phaseolus vulgaris* (common bean) production by as much as 55% (Serfontein, 2007). In view of soybean as a globally important crop, current methods of biocontrol are considered inefficient and new methods need to be developed (Hartman *et al.*, 2011).

Increasing human populations and decreased land mass for production demand intensifying crop yields (Tscharntke *et al.*, 2012). By optimising disease control and increasing yield in an environmentally-friendly way, it may be possible to optimise food demand in a sustainable manner. It is long known that a range of microorganisms including bacteria, fungi and nematodes provide biocontrol against a number of pathogens. The bacterial genus *Rhizobium* and fungal genus *Glomus* are known to inhabit plant tissue and promote a plant’s defence system via intermolecular cross-talk, known as ‘priming’ (Cullimore *et al.*, 2001; Kloeper *et al.*, 2004; Djonovic
et al, 2006). This is an effective strategy deployed to reduce nematode penetration in
tomato plants (Vos et al, 2012) and damping-off by R. solani in chickpea (Hemissi et
al, 2013). Another useful biocide is the fungus Coniothyrium mimitans, an effective
BioControl Agent (BCA) against S. sclerotiorum (Huang, 1977; Melo et al, 2011).
However, this study will focus on the beneficial ascomycete Trichoderma spp., a well
established pathogen suppressor where examples include T. atroviride, T. harzianum
and T. viride. The extent of Trichoderma spp. agricultural success as led to a number
of commercial treatments available including PlantShield®, RootShield® (Bioworks
Inc, New York, USA), and Sentinel® (Agrim Technologies Ltd, New Zealand) (Elad
et al, 1980; Whipps, 1987; Tucci et al, 2011; Ashoub et al, 2009). Furthermore, the
fact that Trichoderma spp. show longevity and spatial distribution within shoot tissue
after soil application and harvest clearly display much potential as a sustainable
biocide (Sobowale et al, 2011). As such, the focus of this study is to determine the
manner in which this occurs and investigate the molecular interactions that take
place between plant and beneficial fungi.

4.1.2 Trichoderma spp. as biocontrol agents

The application of Trichoderma spp. to soil is known to reduce the severity of
plant disease associated with plant pathogens. This is achieved by either invasive or
non-invasive means or a combination of both. For example, Trichoderma spp. secrete
CWDE to affect cell membrane integrity before hypercoiling and digesting soil-dwelling
Additionally, Volatile Organic Compounds (VOCs) such as antifungal and antibiotic
chemicals are used in conjunction with CWDE to suppress pathogen growth to
increase plant health and viability (Bae et al, 2009; Dong et al, 2011).

A more invasive approach involves fungal hyphae engaging with the plant root
in order to penetrate the upper root epidermis. Once in place, chemical signals are exchanged via fungal elicitors that mimic phytohormones which, in turn, induce a more local defence response. Although elicitors are fungal secondary metabolites which promote up-regulation of a number of phytohormones the plant may deploy systemically; they enable the plant to be ‘primed’ in preparation for potential attack (Djonovic et al., 2006; Chagué, 2006; Alfano et al., 2007).

As discussed in detail within Chapter 1.2.2, *Trichoderma* spp. ambiguity in penetrating plant tissue does not prevent priming taking place (review: Ortiz-Castro et al, 2009). In fact, fungal Secondary Metabolite (SM) biosynthesis is known to promote PGP and biocontrol in a number of ways but most importantly, structural similarities between fungi and plant metabolites clearly exist (Richards et al, 2009). Structural similarities may equate to common functionality, although the pathways in which these compounds may participate have yet to be revealed (Niklas et al, 2010). Examples include cyclic compounds deriving from years of evolution; evolutionary traits that exist between the triad of bacteria, fungi and plants. It is no surprise that moieties such as jasmonates, auxins and salicylic acid are up-regulated in plant tissue once *Trichoderma* spp. is inoculated in soil (Martínez-Medina et al, 2011; Gutjahr & Paszkowski, 2009). These appear causal to the activation of a series of chemical modifications that produce novel SMs. Clearly, a more detailed understanding of the chemical process taking place is needed before any agricultural application can be exploited.

**4.1.3 A molecular overview of phytopathogen control**

In the general introduction, activation of plant basal immunity was discussed where ROS, protein kinases and Toll-like receptors such as the Toll-Interleukin 1 Receptor (TIR) domains are deployed to detect invading microbes or initiate the
up-regulation of defence-associated genes (Rodríguez et al., 2012; Nandety et al., 2013; Souza et al., 2023). Depending on the microbe, it is known that plants respond via PAMP Triggered Immunity (PTI) or by an Effector Triggered Immunity (ETI) (Brotman, 2008). Although PTI is sufficient for less severe infections, ETI is triggered by successful pathogen effectors causing an antagonistic response; the SAR-associated Hypersensitive Response (HR) resulting in up-regulation of pathogen-related proteins (Xie et al., 2012). Subsequently, pathogen infection is limited via ROS and callose deposition (Nandini et al., 2012; Palmieri et al., 2012). Together, PTI and ETI contribute to a concerted defence mechanism finely tuned over millions of years.

An induced systemic response is more localised involving JA and ethylene via wounding; whereby SAR is more systemic and necessitates SA (Shoresh et al., 2005). ISR can induce SAR, however induced systemic response is not associated with PR proteins nor a hyperoxidative response. On the other hand, SAR is positively regulated by NPR1 that mediates up-regulation of SA and MAPKs and inhibition of auxin, GA and ethylene (review; Grant & Lamb, 2006; Wang et al., 2012). Nonetheless, not all Trichoderma strains influence SA, JA/Eth or auxin pathways in plants; a pre-requisite for ISR or SAR promotion (Foley et al., 2013).

ABA, SA ethylene are closely regulated by kinases where it was found that impaired MAPK function resulted in decreased pathogen resistance against DC3000 (Hettenhausen et al., 2012). On the other hand, MAPK play an essential role in defence against pathogenic fungi where suppression of plant MAPK pathways enables successful entry (Zhao et al., 2007). Although fungi are known to metabolise or biosynthesise ABA (Cohen et al., 2009), SA (Staunton & Weissman, 2001) and ethylene (Zhu et al., 2012), the full role ABA in modulating responses to fungi has
not been fully elucidated.

ABA is a key phytohormone responsible for development, water potential and defence that antagonises GA, JA and ethylene (Su et al, 2012; Zörb et al, 2013; Duan et al, 2013). By considering the negative regulation of JA and ethylene by ABA, and the up-regulation of JA/Eth-associated genes by T. asperelloides (Brotman et al, 2012), it is suggested that ABA impairment may increase the effects of JA and ethylene within plant tissue. Others suggest co-inoculation of rhizobia and fungi may bring about increased resistance to phytopathogens via stomata closure (Khatabi et al, 2012). Nevertheless, phytohormone roles and regulation is complex, especially if one considers that over expression of ethylene-associated genes increased defence responses via HR initiation and PCD, or that Arabidopsis auxin mutants displayed a compromised SAR Liu et al, 2008; Truman et al, 2010; ward et al, 2010). Others have proven that Trichoderma spp. increase plant health via priming or the up-regulation of IAA, JA and SA (Shoresh et al, 2005; Brotman et al, 2012; Segarra et al, 2009). Therefore, in order to deduce the full extent to which SAR occurs, two phytopathogens will be examined within this chapter to determine the full extent of T. hamatum’s effects on plant health and viability. This includes the Ascomycete Sclerotinia sclerotiorum and the Basidiomycete Rhizoctonia solani.

4.1.4 Phytopathogen synopsis
Sclerotinia sclerotiorum is a devastating disease responsible for pre-emergence damping-off and necrosis of over 400 plant species including arable and wild types (Broad Institute of Harvard and MIT; http://www.broadinstitute.org/). S. sclerotiorum persists in the soil by forming sclerotia; hard melanised fruiting bodies containing mycelium where apothecia emerge releasing thousands of spores (Coley-Smith et al, 2007; Clarkson et al, 2003). Its prevalence and success is mostly attributed to
sclerotia thus providing an interesting target for the prevention of *S. sclerotiorum* persistence. By terminating the reproductive cycle at the storage stage, it may be possible to eradicate the persistent manner in which they remain in the soil (Smith *et al*, 2012).

In a similar fashion, *Rhizoctonia solani* causes post-emergence damping-off disease and forms sclerotia in order to survive periods of adverse [plant] weather conditions. Crops affected include cereals, Solanum and sugarbeet; but not Brassica crops due to an inherent intolerance (Angus *et al*, 1994; Handiseni *et al*, 2013). Necrotrophs such as *R. solani* and *S. sclerotiorum* secrete the CtaG/cox11domain effector or integrin-like effectors and hydrolytic enzymes in order to infect and necrotise plant tissue via oxalic acid or ROS species (Zhu *et al*, 2013; Kim *et al*, 2011; Kabbage *et al*, 2013; Zheng *et al*, 2013). Because of this, much crop loss include commercially important crops (USDA, 2013).

Just as pathogens display variability in pathogenicity, Trichoderma vary in biocontrol ability, where pathogen antagonism depends on the species. For example, some *Trichoderma* spp. act as efficient mycoparasites (*T. hamatum*) or up-regulate oxidase activity (*T. viride*) (Srivastava *et al*, 2012; Huang *et al*, 2011; Yang *et al*, 2012). In contrast, other Trichoderma species prefer to secrete mycotoxins (*T. atroviride*, *T. viride*) or CWDE (*T. reesei*) (Atanasova *et al*, 2013; Gruber *et al*, 2013). Highly conserved mechanisms are present in all three kingdoms and of the few reports available, it is evident an historical tripartite relationship between plants, fungi and bacteria is in place (Frias *et al*, 2012). Although much is known about the roles SA, ABA, JA and ethylene play in plant resistance to disease, little is known about the effects Trichoderma has upon plant viability when grown under disease pressure. Therefore, it is highly desirable to have a bioagent with PGP and BCA characteristics.
This Chapter sets out to explore the biocontrol ability of GD12 and $\Delta Thnag$:hph (hereafter referred to as $\Delta$ nag) upon $S$. sclerotiorum and $R$. solani. Furthermore, I present a series of experiments that investigate the physical interactions between beneficial and harmful pathogenic fungi at various concentrations of each fungus. This way, it can be determined whether inoculum application rates effects plant survival rates or if this occurs in a dose-dependent manner. Therefore, the results presented in this study may contribute to a fuller understanding of the concerted chemical relationship that finely tunes hormonal mediated responses of the plant.
4.2 Materials and Methods

4.2.1 Maintenance of micro-organisms

4.2.1.1 Sclerotinia sclerotiorum

Five strains of the plant pathogen Sclerotinia sclerotiorum were used in this study; SS1 (NCBI accession number FJ984493) and four UK strains BFS, GFR1, GFR11 & M448 (donated by West, J., Rothamstead Research, 2011). All were maintained on V8 media (400 mL dH2O, 100 mL V8, 10 g Agar, 5 g Glucose) or Potato Dextrose Agar (PDA) (500 mL dH2O, 12 g PDA, 10 g Agar) (Sigma) under a 16 h fluorescent light/ 8 h dark regime at 26°C. Any sclerotia were removed with sterilised tweezers and placed in a sterile universal tube and stored at 4°C until further use.

4.2.1.2 Rhizoctonia solani

Plant pathogen Rhizoctonia solani (NCBI accession number Z54277) was grown on Potato Dextrose Agar (PDA) (500 mL dH20, 12 g PDA, 10 g Agar) (Sigma) or V8 media (400 mL dH20, 100 mL V8, 10 g Agar, 5 g Glucose) under a 16 h fluorescent light/ 8 h dark regime at 26°C.

4.2.2 Micro-organism preparation

4.2.2.1 Sclerotinia sclerotiorum sclerotia preparation

After 2 weeks growth each sclerotium was removed from the agar plate with sterilised tweezers and placed in a sterilised universal tube until further use. Before use, sclerotia were remove from universals and placed in 70% ethanol for 2 min, removed from the ethanol and thoroughly rinsed three times with dH2O before slicing the sclerotia in two pieces with a fresh blade. Each portion was placed face down (fleshy, pale colour mycelium) onto freshly prepared media for three days.
4.2.2.2 Phytopathogen-poppy seed inoculum preparation
Small conical flasks containing 10 g black poppy seeds and 12.5 mL dH2O were well mixed and autoclaved at 121°C for 20 min. Once cooled, 10 x 2 mm diameter plugs of *S. sclerotiorum* or *R. solani* mycelium were transferred from the leading edge of five day PDA plates to the conical flasks and stirred to allow equal distribution of mycelium in the poppy seeds. The fungi were allowed to colonise the poppy seeds for a further seven days under a 16 h light/ 8 h dark regime at 26°C before use.

4.2.2.3 *Lactuca sativa* pathogen co-inoculation
Soil (prepared as in Chapter 2.3.2) samples include untreated microcosms (positive control) either treated with *S. sclerotiorum* or *R. solani*-poppy seed inoculum at 2.67% only (w:w; negative control), Trichoderma-peat inoculum only (2.67% w:w; PGP only) or microcosms were inoculated with both pathogen and Trichoderma at 2.67% (w/w; BCA). In addition to mycelium incorporated within the soil, Trichoderma metabolite-treated plants were grown in soil inoculated with each phytopathogen. Seeds were either pre-treated with 400 µL metabolite exudate or MOH2O. Each microcosm was sown with 25 lettuce (*Lactuca sativa*) cultivar Webb’s Wonderful in a grid system and randomly placed in a growth cabinet (Sanyo) at 24°C with a relative humidity of 90% and a 16 h fluorescent light regime. Light intensity: 108 m⁻¹/ s⁻¹/ j⁻¹. Each microcosm remained covered for 48 hr to induce simultaneous germination and to secure establishment rates.
4.3 Results

4.3.1 *Trichoderma hamatum* and phytopathogen suppression

Once the PGP effects of GD12 and Δ*Thnag::hph* were deduced, tests to quantify their effectiveness as a BCA were conducted against two specific pathogenic fungi: the polyphagous plant pathogen *S. sclerotiorum* (GenBank accession FJ984493), and the anastomosis group 1 post-emergence lettuce pathogen *R. solani* (CBS323.84). After 21 days growth, plants were harvested for dry compartment weights and shoot to root ratios (S:R). These samples were subjected to analyses using Analysis of Variance (ANOVA; one-way), Students T-Test (One-Way) analysis.

4.3.1.1 Emergence rates and plant establishment

Overall, it is evident that co-inoculation of GD12 with *S. sclerotiorum* (GD12+Ss) or *R. solani* (GD12+Rs) significantly improve germination rates of lettuce (Figures 4.1A and 4.1B). Here, the inhibitory effects of *S. sclerotiorum* and *R. solani* upon lettuce seed germination is evident when inoculated in peat at 2.67% (w:w). Comparisons between untreated (+ve Control), *S. sclerotiorum*-only (-ve control) and *R. solani*-only (-ve control) clearly show *S. sclerotiorum* pathogenicity is greater than *R. solani* where 60%, 3% and 56% germination is witnessed respectively (T-Test; p<0.01). However, *S. sclerotiorum* pathogenic ability is reversed upon GD12 co-inoculation where we see GD12+Ss seed emergence increase to 77% which is greater than GD12-only with 63% emergence (T-Test; p<0.05). In contrast, Δ*nag* failed to increase germination rates upon infection where Δ*nag*-only (+ve control) and Δ*nag+Ss* display 70% and 43% emergence respectively (T-Test, p<0.01).

*R. solani* infection is less severe than *S. sclerotiorum* where the control (untreated) plants show intermediate germination rates to *R. solani*-only (56%) and 20% affording a 77% emergence rate in GD12+Rs compared to 62% in GD12-only.
Figure 4.1 Biological control of pre-emergence of *S. sclerotiorum* and post-emergence of *R. solani* by GD12 and \( \Delta \text{nag} \). *S. sclerotiorum* (upper left) and *R. solani* (lower left) suppression by GD12 (centre) and \( \Delta \text{nag} \) (right) (A). Mean emergence when grown in untreated (Control), single (pathogen or Trichoderma strains only) or mixed (pathogen+Trichoderma strain) microcosms (B). Emergence percentages were converted to \( \text{arc sin}^{-1} \) values and are from three replicate microcosms each containing 25 lettuce seeds (± SE). Same letters are not significantly different at <95% confidence level (ANOVA; One Way and T-Test).
\( \Delta n a g \)-only (70\%) (T-Test; \( p>0.05 \)). Alternatively, GD12 improved emergence rates by and 64\% in \( \Delta n a g+R s \) (T-Test, \( p<0.01 \)).

### 4.3.1.2 Plant compartmentalisation

Ratios between dry root and shoot weights determine plant compartmentalisation that, in turn, is determined by the nutritional effects of the growth medium. Here, any PGP effects witnessed by single or double inoculation will be compared to lettuce grown under disease-free conditions. As previously witnessed in Chapter three, the PGP-only data set followed similar trends however, GD12+Rs treatment significantly increased dry root mass by 2.5-, 2-fold and over 4-fold compared to the control (+ve), GD12-only and \( R. \ solani \)-only plants (Figure 4.2). In comparison, GD12+Ss completely restored germination from \( S. \ sclerotiorum \)-only, producing plants with weights similar to \( \Delta n a g \)-only. Furthermore, GD12+Ss increase dry root biomass by 2-fold and 50\% compared to the control (+ve) and GD12 respectively (T-Test; \( p<0.01 \)). However, \( \Delta n a g \) produce intermediate BCA results and here, \( \Delta n a g+R s \) display dry root mass 2.5- and 50\% larger than \( R. \ solani \)-only and control (+ve) respectively (T-Test, \( p<0.05 \)) while partially restoring some germination in \( \Delta n a g+S s \) with similar root mass to the controls (+ve) (T-Test, \( p>0.05 \)). Therefore, once under disease pressure, GD12 and \( \Delta n a g \) display dry root mass that is significantly greater than the negative controls. However, this becomes insignificant once \( \Delta n a g+S s \) is compared to the positive control. Dry shoot mass values followed similar trends to dry root biomass where GD12 enhanced PGP shoot effects once co-inoculated with \( R. \ solani \) or \( S. \ sclerotiorum \) (Figure 4.2B). Here, a 3-fold increase occurs in comparison to the control plants (\( p<0.001 \); Figure 4.2B). These differences are reduced when compared to GD12-only and the control (+ve) where a 33\% (T-Test; \( p<0.05 \)) and 8\% increase occurs (T-Test; \( p>0.05 \)). Alternatively, \( \Delta n a g \)-disease plants were significantly smaller
Figure 4.2 The effects of GD12 and ∆nag on disease pressure in lettuce plants after 21 days. No treatment (white) or GD12 (black) and ∆nag (grey) were inoculated or co-inoculated with S. sclerotiorum 1 (stripe) or R. Solani (spot). Mean dry root (A), dry shoot (B) and dry S:R (E) bars are mean values from three replicate microcosms each containing 25 lettuce seeds (± SE). Same letters denote not significantly different at <95% confidence level (ANOVA; One Way and T-Test).
than Δnag-only but larger than the (+ve) control by 50% (T-Test; \( p<0.05 \)). Dry S:R significantly increase upon co-inoculation of Trichoderma but decline once co-inoculated with phytopathogens (Figure 4.2C). However, these values remain significantly larger than the positive control plants (T-Test; \( p<0.05 \)).

4.3.2 Trichoderma metabolite; biocontrol effectiveness

*T. hamatum* GD12 and Δnag significantly reduce phytopathogen effects on plant growth. It was shown in Chapter 3.3.3, that Millipore filtered metabolite extract (<0.2 μm) improved plant biomass up to 8-fold. In order to quantify the biocontrol effects of metabolite extract, microcosms were prepared, as in Chapter 2.3.2, with autoclaved peat as a positive control. Twenty five lettuce seeds were sown in individual microcosms where the negative control consisted of *S. sclerotiorum* or *R. solani* incorporated at 2.67% (w:w) and the positive control contained no pathogen. Plant treatment consisted of 200 μL (<0.2 μm filter) of GD12 or Δnag metabolite extract on alternate days however, of these seeds one half was pre-watered with 400 μL metabolite extract upon sowing while the other half were not. After 48 hours, microcosm lids were removed and here plants were watered on alternate days with metabolite extract (200 μL) or \( \text{MoH}_2\text{O} \) only (control groups). After 21 days growth, plants were excised, weighed and analysed using Analysis of Variance (ANOVA; one-way) and Students T-Test; one way.

4.3.2.1 Germination rates

Overall, seed pre-treatment with metabolite extract (Met+) does not significantly benefit germination compared to seeds that were not (Met-; red outline) \( (p>0.05) \) (Figure 4.3). Moreover, germination rates of Trichoderma-only treated plants were depressed upon metabolite pre-treatment compared to the control. Over 10 days, the positive control set achieved the greatest germination (74% post arc-sin\(^{-1}\)
normalisation; Figure 4.3, ANOVA; \( p<0.001 \) which was 13\%, 9\% and 20\% greater than GD12 Met+, \( \Delta \text{nag Met+/-} \) and Trichoderma+\( Rs \) data sets respectively (T-Test; \( p<0.05 \)). Because metabolite treatment failed to suppress \( S. \ sclerotiorum \), no plants germinated or survived and are therefore omitted for clarity (Figure 4.3).

![Histogram displaying plant establishment at day 10. Control plants (white), GD12 (black), \( \Delta \text{nag} \) (grey), \( R. \ solani \) (spot) and no metabolite pre-treatment (red outline) are shown. Each bar represents the mean of three replicate microcosms where \( n=25 \). (±SE). Same letter denote not significantly different at <95\% confidence level (ANOVA; One Way and Student’s T-Test).](image)

**Figure 4.3** Histogram displaying plant establishment at day 10. Control plants (white), GD12 (black), \( \Delta \text{nag} \) (grey), \( R. \ solani \) (spot) and no metabolite pre-treatment (red outline) are shown. Each bar represents the mean of three replicate microcosms where \( n=25 \). (±SE). Same letter denote not significantly different at <95\% confidence level (ANOVA; One Way and Student’s T-Test).

### 4.3.2.2 Growth in the presence of soil pathogens

\( T. \ hamatum \) GD12 or \( \Delta \text{nag} \) liquid application has no biocontrol effect against \( S. \ sclerotiorum \) (far left) but has potential effects against \( R. \ solani \) (centre) (Figure 4.4). Seed pre-treatment makes no difference to shoot mass therefore in order to determine the true biocontrol and PGP effects of GD12 and \( \Delta \text{nag} \), dry shoot and root weights were recorded and compared.
GD12 metabolite seed pre-treatment significantly improved root biomass in both GD12-only (30%) and GD12+Rs (90%) (Figure 4.5A). In contrast, Δnag pre-treatment made no difference in dry root mass regardless if diseased (T-Test; p>0.05). All Δnag plants were larger than the untreated (+ve control) plants by at least 2-fold (ANOVA; p<0.05) while GD12 pre-treatments afforded roots 4–fold larger than the positive control (ANOVA; p<0.01).

Dry shoot weights display similar trends to the dry root data where seed pre-treatment in GD12 and Δnag significantly improve shoot biomass compared to their non- treated counterparts by 40% to 75% more (ANOVA, p<0.001). The non pre-treated datasets GD12+Rs and Δnag+Rs were similar in value to the untreated plants and the Rs–only (T-Test, p>0.05). Overall, mean dry shoot weight was significantly larger when grown under disease pressure. However, this only occurred upon seed pre-treatment and metabolite treatment is not as effective as mycelium:bran inoculum in reducing soil dwelling pathogens (Figure 4.5B).

Mean dry S:R values are significantly reduced upon Trichoderma inoculation and Trichoderma:pathogen co-inoculation when compared to the control plants (ANOVA; p <0.01) (Figure 4.5C). Moreover, S:R continue to decrease when seeds are pre-treated with 400 μL of filtered Δnag extract thus suggesting enhanced root structure (T-test; p<0.01).

4.3.3 Pathogen effects of four UK Sclerotinia sclerotiorum strains

During the course of this work the strain Ss1 lost its pathogenicity. Four UK strains of Sclerotinia sclerotiorum were donated by Jon West (Rothamstead Research, UK) to use. Initial studies were conducted to determine the most appropriate strain followed by a series of bioassays to determine pathogenicity. Lactuca sativa (cv Webb’s Wonderful) were co- inoculated with T. hamatum and S.
Figure 4.4. The biocontrol properties of Trichoderma GD12 or Anag metalloprotease against Phytophthora S. scelerotiorum and R. solani after 21 days. Left panel is the R. solani treatment, mid panel is the R. solani and the right panel represents Trichoderma metalloprotease treatment. Upper row: control data sets, mid row: GD12 treated; lower row: Anag treated plants. Metabolite pre-treatment (400 µL) is shown as (+) or (-). Microcosm width is 120 mm.
Figure 4.5 The effects of GD12 and $\Delta$nag metabolite treatment on lettuce biomass when deployed as a seed pre-treatment. Untreated plants (white) or GD12 (black) and $\Delta$nag (grey) seeds/plants were pre-treated (Met+; no outline) or not (Met--; red outline). Co-inoculation include $R$. solani (spot) where each bar is the mean of three data points each from three experimental replicates measuring mean dry root (A), dry shoot (B) and dry S:R (C) ($\pm$SE). Same letters denote not significantly different at $<95\%$ confidence level (ANOVA; One Way and Student’s T-Test). $S$. sclerotiorum-treated lettuce failed to survive thus omitted for clarity.
sclerotiorum in autoclaved peat at 2.67% (w:w) in order to test T. hamatum’s biocontrol ability against these new S. sclerotiorum strains. After 21 days growth, plants were excised, weighed and analysed using Analysis of Variance (ANOVA; one-way) and Students T-Test analysis.

4.3.3.1 Survival rates of Lactuca sativa
The necrotrophic effects of S. sclerotiorum upon lettuce seed viability, is evident after 21 days (Figure 4.6). Here it is shown that no plants survived within the negative control set and of the few seeds that germinated, rapid necrosis occurred within 48 hrs of challenge (Figure 4.6; left column). The most aggressive S. sclerotiorum strain appeared to be GFR1 that produced an average of 0.67 seedlings that, by day 21, was reduced to no viable plants. M448 inoculated lettuce plants fared slightly better than their GFR1 inoculated counterparts where the control group had an average of one seedling emerge (control), with 12 in the GD12 group and no survivors in the Δnag inoculated data set. This was followed by the GFR11 sub group that witnessed a mean one seedling (control), 17 seedlings (GD12) and nine seedlings (Δnag) emerge. Of the emerged seedlings, none survived in the control and Δnag set however, GD12 inoculated plants were the only data set bearing viable plants. Therefore, Figure 4.6 highlights the strong biocontrol ability of GD12 compared to Δnag that produced more surviving plants. Evidently, GD12’s show some biocontrol against the necrotroph S. sclerotiorum whereas Δnag display a complete lack of protection against all S. sclerotiorum strains except BFS.

4.3.3.2 A comparative study of root and shoot biomass
Overall, co-inoculation of GD12 and S. sclerotiorum significantly increase dry root and shoot weights in L. sativa. UK strain GFR11 show more effective PGP properties on root structure as opposed to UK strain M448 that improved shoot
Figure 4.6 Four UK strains of *Sclerotinia sclerotiorum* co-inoculated in sterilised peat with equi-amounts of *T. hamatum* (2.67% w/w). Upper row is the positive control, 2nd row is BFS, 3rd row is GFR1, 4th row is GFR11 and 5th row is strain M448. Far right column is co-inoculated with ∆nag, mid column is GD12 and far left column has no *T. hamatum*. Tray width: 12 cm where each tray represents the mean of three experimental replicates, n=25.
Figure 4.7 Trichoderma as a biocontrol agent on four UK strains of S. sclerotiorum. Untreated (white) plants are compared against plants treated with GD12 (black), $\Delta$nag (grey) or S. sclerotiorum (pattern). Both Trichoderma & Sclerotinia were incorporated at 2.67% (w/w). Each bar is the mean value of three experimental replicates n=25 (±SE). Same letters are not significantly different at <95% confidence level.
mass when upon co-inoculation. Here, GD12+GFR11 dry root mass is 3.5- and 18-fold larger compared to the GD12-only and untreated plants respectively (ANOVA; \(p<0.01\)) (Figure 4.7A). Alternatively, GD12+GFR1 and GD12+M448 treatment increase dry root mass by 2-fold with respect to GD12-only (ANOVA; \(p<0.05\)). In contrast, GD12+BFS failed to increase dry root and shoot mass as effectively. Root mass was not significantly different to GD12-only plants and 6-fold larger than the untreated plants, respectively while dry shoot mass was 17% and 8-fold larger than GD12-only and untreated plants (ANOVA, \(p<0.05\); Figure 4.7B). However, this report clearly confirm that GD12+M448 treatment significantly increase shoot mass by 20- and 2.5-fold to the untreated and GD12-only plants (ANOVA; \(p<0.001\)). This is more effective than GD12+GFR1 and GD12+GFR11 that induced a 50% increase to untreated lettuce.

4.3.3.3 Seedling establishment rates under disease pressure

Of the four UK \(S. sclerotiorum\) strains used, all reduced seedling establishment by 100% when inoculated without GD12 (Figure 4.7C). Upon GD12 co-inoculation, seedling emergence and establishment significantly improved (ANOVA; \(p<0.001\)). \(\Delta\text{nag}\) improves germination with \(S. sclerotiorum\) strains BFS and M448 only, where values were intermediate to the negative controls and GD12. All the GD12+Ss strains were similar in value with relation to each other and less effective as GD12-only, except GD12+M448 that was not significantly different to GD12-only treatment.

4.3.4 Graduated inoculation of \(S. sclerotiorum\) strain BFS

To ascertain whether inoculum concentration of \(S. sclerotiorum\) was a limiting factor, individual microcosms were prepared with Trichoderma GD12 or \(\Delta\text{nag}\) inoculated at 2.67% (w:w) and \(S. sclerotiorum\) strain BFS inoculated in soil at varying concentrations of 1, 1.5, 2 and 3% (w:w). The positive control was
Figure 4.8 The effects of *S. sclerotiorum* strain BFS on plant viability after 21 days. Varying concentrations of BFS was co-inoculated with *T. hamatum* (2.67% w/w). Upper row is the positive control, 2nd row is BFS at 1%, 3rd row is 1.5%, 4th row is 2% and 5th row is 3% (w:w) inoculation. Far right column is co-inoculated with $\Delta$*nag*, mid column is GD12 and far left column has no *T. hamatum*. Tray width: 12 cm; each tray represents the mean of three experimental replicates, n=25.
autoclaved peat only and the negative control was *S. sclerotiorum* only. After 21 days
growth, plants were excised and dry weight was determined using statistical
analyses; T-Test and ANOVA (one-way).

4.3.4.1 Biocontrol boundaries of Trichoderma against Sclerotinia
As *S. sclerotiorum* strain BFS concentrations increase, very little difference is
witnessed amongst the GD12 data set whereas ∆nag treatment fails to provide any
biocontrol against BFS at any concentration (Figure 4.8). The far left column in Figure
4.8 exhibits *S. sclerotiorum*-only at varying concentrations, where *S. sclerotiorum*
mycelium growth increase with inoculum density. With GD12 treatment, no *S.
sclerotiorum* mycelium is present on the soil surface regardless of concentration. An
abundance of green mycelium suggests that as *S. sclerotiorum* concentrations
increase, GD12 growth improves resulting in larger lettuce plants. By contrast, ∆nag
appears to be incapable of suppressing *S. sclerotiorum* pathogenicity on lettuce
seeds with no seeds emerging or surviving.

4.3.4.2 Effects of biocontrol on establishment rates
Germination rates of lettuce (*Lactuca sativa* cv. Webb’s Wonderful) seeds were
recorded over 10 days where untreated and ∆nag plants were the most efficient within
the data set (ANOVA; *p*<4.0E-23) (Figure 4.9). PGP followed previous trends where
control emergence rates were greater than ∆nag that, in turn, was greater than
GD12. Furthermore, the plants receiving PGP-only treatment was 2-fold larger than
the GD12+BFS treatment (ANOVA; *p*<1E-09). Little difference exist between
GD12+BFS treatments, and although increased *S. sclerotiorum* inoculation had no
effect on GD12’s inability to suppress, seed establishment rates were intermediate the
PGP and *S. sclerotiorum*-only treatments displaying a mean 35% emergence. ∆nag
failed to display BCA remaining similar to the negative control sets.
4.3.4.3 Analysis of root and shoot phenotype

Under no disease pressure, mean dry root mass followed the same PGP trend whereby △nag dry root mass is greater than GD12 which, in turn, is larger than that of the control plants. However, it is evident that upon *S. sclerotiorum* inoculation, PGP effects of GD12 are enhanced in a dose-dependent manner. GD12+BFS plants were larger than the PGP treatment by a mean 2-fold (with the exception of GD12+BFS 3%). Within the GD12+Ss treatment, increased *S. sclerotiorum* inoculation maximised plant root biomass at BFS 2% decreasing by 77% at 3% BFS concentration (Figure 4.10A; ANOVA, *p*<0.01).

GD12+BFS treated plants were significantly different in dry shoot weights where Trichoderma-only plants were similar in value (Figure 4.10B). The biocontrol effects follow a graduated PGP response peaking at GD12+BFS2% only to decrease at 3%. GD12+BFS 2% mean dry shoot weight is 67% and 39% larger than the GD12+BFS 1.5% and GD12+BFS 3% (ANOVA; *p*<0.05).

S:R values indicate mass allocation within plant tissue where higher S:R indicate increased shoot biomass has occurred at the expense of root biomass.
Figure 4.10 Plant phenotype when grown in co-inoculated peat at increasing concentrations of *S. sclerotiorum* strain BFS. Each bar is the mean value of dry root (A), dry shoot (B) and dry S:R (C) values each with three replicates; n=25 (±SE). Same letters are not significantly different at 95% confidence level.
GD12+BFS treatments increase dry S:R values in a dose dependent manner (Figure 4.10C) while the PGP dry S:R values validate that GD12-only were larger than untreated and $\Delta_{nag}$ by 15% and 45% respectively (ANOVA; $p<0.05$).

4.3.5 Extended inoculation of *S. sclerotiorum* UK strain BFS

Previously, dry root/shoot mass and S:R improved with increased Ss+GD12 inoculation. As a continuation of Chapter 4.3.5 above, where BFS soil inoculation maximised at 3%, this section addresses excessive *S. sclerotiorum* inoculation where rates up to 10% are investigated. Trichoderma strains were inoculated in soil at 2.67% (w:w) with *S. sclerotiorum* inoculation at 2, 5, 7.5 and 10% (w:w). After 21 days growth, plants were excised and dried prior statistical analyses.

4.3.5.1 Trichoderma as an effective biocontrol agent

It is evident that GD12 mycelium growth substantially increase as BFS concentrations increase; despite Trichoderma concentrations remaining at 2.67% (w:w) (Figure 4.11). Therefore, in order to determine Trichoderma’s effect on BFS growth, soil samples were removed before washing with DIW. Upon filtration and centrifugation, 1 cm$^3$ triplicate samples were removed before microscopic analysis. Microscope studies of the negative control data set (BFS only) show that by day 21, BFS spore counts increase with inoculation rates however, the introduction of GD12 prevented the production of BFS spores (ANOVA; $p<0.09E-21$, Figure 4.12). Conversely, each $\Delta_{nag}$ treatment displays mean Sclerotinia spore values similar to the *S. sclerotiorum* control where increasing BFS infiltration rates increase Sclerotinia spore counts that $\Delta_{nag}$ cannot counteract effectively (T-Test; $p>0.05$).

4.3.5.2 Co-inoculation effects on plant morphology

Once the biocontrol effect of GD12 was ascertained, any further PGP effects resulting from increasing amounts of *S. sclerotiorum* inoculum were determined.
Figure 4.1.1 Temporal effects of Trichoderma strains GD12 and \( \Delta \text{na} \) upon B. \( \Delta \text{na} \) growth after three, five, seven, and 21 days. Upper row: Positive controls (no S. sclerotiorum) and lower rows show microcosms with increasing S. sclerotiorum dose. Within the four panels depicting each day, left column is control, mid column is GD12, and the far right column is \( \Delta \text{na} \). Trichoderma inoculum remained at 2.67% (w/w). Each microcosm represents the mean of three replicates.
Figure 4.12 Histogram of mean *S. sclerotiorum* soil spore count. Each bar is the mean of three replicates each from three independent experiments. (±SE). Same letters denote not significantly different at a 95% confidence level.

Control  GD12  Δnag  GD12+BFS at 2%  GD12+BFS at 5%  GD12+BFS at 7.5%  GD12+BFS at 10%

Figure 4.12A Overview of lettuce plant morphology after 21 days treatment with or without disease pressure. *T. hamatum* GD12 or Δnag at 2.67% (w:w), *S. sclerotiorum* UK strain BFS at 2, 5, 7.5 or 10% inoculation rate and untreated (control). Scale bar: 1mm.
Figure 4.13 Plant morphology when grown in peat co-inoculated with increasing concentrations of *S. sclerotiorum* strain BFS (2-10%). Peat-only (control), PGP only (GD12 or Δnag @ 2.67% w:w) and BCA treatment display mean dry root (A), dry shoot (B) and dry S:R (C) values. Each bar is the mean of three experimental replicates; n=25 (±SE). Same denotes not significantly different at 95% confidence level.
Plant biomass significantly increased compared to the PGP-only and control treatment (ANOVA; p<0.001). Dry root weights increased in a dose-dependent manner all of which were greater than the GD12-only treatment by 1.5 to 3-fold (Figure 4.12A). In contrast, the GD12+BFS treatments show dry shoot weights are 3-fold larger than GD12-only, but not significantly different between each other (Figure 4.13B). Dry S:R values in the PGP-only treatment are significantly reduced as opposed to increased S:R in all GD12+BFS, except GD12+BFS 2% that was insignificant to GD12-only (Figure 4.13C); Δnag-treated plants held the lowest S:R.

4.4 Discussion

N-Acetyl Glucosaminidase (NAG) is an essential gene involved with a number of key processes including cell wall regulation, spore production, carbohydrate metabolism and hydrolysis; including CWDE and chitin hydrolysis (Gruber & Seidi-Seiboth, 2012; Donzelli & Harman, 2001; Kellner & Vandenbol, 2010). Much is known about chitinase and its role in biocontrol efficacy, however, little evidence exists regarding Trichoderma and the effects of NAG mutation upon biocontrol; especially when deployed as a metabolite treatment. Of the few reports available, most are concerned with the identification and quantification of the effects of specific fungal secondary metabolites in vitro and not the metabolite extract in situ (Clayden et al, 1987; Vinale et al, 2006; El-Hasan et al, 2009). This can become problematic if one considers the effects of species variation upon secondary metabolite production and, ultimately, BCA (Alvares et al, 2012; Ojaghian, 2011; Amin et al, 2010).

This study set out to determine the BCA effects of both T. hamatum wild type GD12 and the N-acetyl-β-D-glucosamine mutant ΔThnag::hph. So far, we have effectively displayed differences between biocontrol and biofertiliser effects of T. hamatum which depend on application type and genotype. As a liquid metabolite
application, GD12 and $\Delta n$ag are highly effective PGP agents. However, neither was effective as BCA’s. In contrast, *T. hamatum* soil treatment proved to be less efficient than the metabolite treatment as a PGP but was highly effective as a BCA. Thus suggesting NAG is causally related to PGP and/or BCA activity.

Disruption of the Trichoderma NAG gene results in reduced biocontrol abilities. Germination rates of soil inoculated $\Delta n$ag treated lettuce were intermediate between *R. solani*-only and GD12+Rs plants. The impaired *R. solani* suppression is corroborated by other reports using *T. viride* and *T. atroviride* NAG mutants (Romão-Dumaresq *et al.* 2012; Omann *et al.*, 2012). Regarding plant productivity, $\Delta n$ag treatment improves root and shoot mass when plants are co-inoculated with *R. solani*. However, the effects were not as significant as GD12 nor were they as significant as $\Delta n$ag-only plants. Therefore, $\Delta n$ag may provide some protection against *R. solani* but this occurs at the expense of plant survival rates and limited biocontrol.

By contrast, GD12 suppressed *R. solani* growth and significantly reduced spore numbers (Ryder *et al.*, 2012; Brunner *et al.*, 2005). Coupled with improved plant emergence, production and PGP, GD12 interacted with *R. solani* and showed greater PGP than GD12-only, i.e. without disease pressure. GD12+Rs induced large, healthy plants with a root biomass significantly larger than $\Delta n$ag-only. Of the few reports involving *R. solani* suppression, similar germination rates with some PGP effects were witnessed when *T. viride* and arbuscular mycorrhiza fungal isolates were deployed giving rise to a mean 2-fold biomass increase and 76% disease reduction (Srivastava *et al.*, 2012; Lewandowski *et al.*, 2013). Moreover, other reports confirm *T. harzianum* reduce Fusarium-disease incidence in maize by 79% with a 94% emergence, again, with some associated PGP effects (Harleen & Chandler,
2011). On the other hand, shoot mass comparisons between disease free data sets and GD12+Rs were no different. GD12 and Δnag metabolite application fail to improve plant emergence rates however, all plants displayed significantly reduced root weights compared to the disease-free data sets.

GD12 and Δnag metabolite treatment failed to increase seed viability. As a mycelium application to soil, it is shown Δnag had little or no biocontrol against *S. sclerotiorum* infection. Of the five pathovars tested, one strain of *S. sclerotiorum* was suppressed by Δnag enough for viable plants to be harvested: strain *Ss1*. This interaction resulted in intermediate germination rates and root/shoot weights with S:R similar to the positive control. In contrast, *S. sclerotiorum* strains BFS, M448, GFR1 and GFR11 appeared to be more pathogenic than *Ss1* in the presence of Δnag where only 6% of BFS+Δnag and 6% of M448+Δnag plants emerged by day ten, only to be necrotised by day 21.

By contrast, GD12-treated soils display reduced *S. sclerotiorum* sporulation rates, apothecia and sclerotia frequency. While Δnag treatment showed a 60% decrease in sclerotia, GD12 completely abolished sclerotia formation. This is greater than the 37% and 30% decrease involving wild type *T. harzianum* and a commercial application of *T. harzianum*. Others found similar suppression by *T. viridecens* thus supporting interspecies differences exist in biocidal effects (Wang *et al.* 2012; Zeng *et al.*, 2012). Of these reports, no PGP effects were witnessed and no *T. hamatum* was incorporated within these studies.

Others found up to 90% of sclerotia are potentially suppressed by Trichoderma thus reducing *S. sclerotiorum* populations (Dos Santos & Dhingra, 1982; Kim & Knudsen, 2008). How this is achieved is uncertain, but it is hypothesised that physical interactions must take place to ensure hydrolysing enzymes overcome
pathogen cell wall integrity (Chet et al, 1981; Whipps, 1987). Others confer that decreasing soil acidity needed for sclerotia formation or suppression of Shk1 gene; a histidine kinase responsible for sclerotigenesis and fungicide resistance suppress pathogen populations (Chen et al, 2004; Duan et al, 2013). Importantly, novel fungal SMs continue to emerge including sclerosin, an antifungal lipopeptide excreted by Pseudomonas and P. tolaasii or xantholysins secreted by Pseudomonas (Berry et al, 2012; Li et al, 2013).

It will be interesting to explore the role of secondary metabolites in GD12’s suppression of S. sclerotiorum strains. Similar seed germination rates are evident in all GD12+Ss treatments by day seven when GD12 mycelium are applied to the soil at a constant 2.67% (w:w). GD12 provide adequate biocontrol at increasing concentrations of S. sclerotiorum growth. A weak association with Δnag and S. sclerotiorum growth rates was found resulting in reduced S. sclerotiorum mycelium levels over seven days. Although no plants survived the bioassay, some reduction in S. sclerotiorum sporulation and sclerota formation by Δnag was evident.

As a fully functional wild type, GD12 display impressive BCA results against S. sclerotiorum, R. solani and Magnaporthe oryzae when applied as a bran-inoculum application (Elad et al, 1980; Ryder et al, 2012). This property is evident in a range of Trichoderma spp. (Amin et al, 2010). However, evidence of both PGP and BCA effects are rare especially when R. solani and S. sclerotiorum are concerned (Ojaghian et al, 2011). Because the Trichoderma metabolite extract failed to provide protection against R. solani and S. sclerotiorum or reduce spore numbers, this lead us to hypothesise that in order for efficient BCA to occur, physical interactions must exist between Trichoderma and pathogen; a phenomenon strongly associated with parasitism followed by hydrolysing enzymes and secondary
metabolites (Chet et al., 1981; Elad et al., 1982; Schirmböck et al., 1994; Matroudi et al., 2009).

This study provides evidence for increased GD12 mycelium growth as a consequence of increased S. sclerotiorum populations. GD12 proliferation coupled with reduced S. sclerotiorum populations improves dry root biomass up to 19-fold compared to that of untreated plants whereas the GD12-only plants were 7-fold larger than the control. One report shows T. hamatum increased plant viability against S. cepovorum where emergence increased from 10% to 55% with a 6-fold PGP increase (Leta & Selvaraj, 2013). Others deployed selective mutation within the gene coding for the TransMembrane 17 (TM17) region of MRP1 (Multidrug Resistant Protein) (Zhang et al., 2002) in T. harzianum (w+); results in similar BCA and PGP against M. phaseolina in sunflower. However, within this report TM17 improved plant viability and dry root biomass by 100% and 11-fold respectively whereas the wild type elicited a 50% survival and a 6.5-fold increase in biomass (Nagamani et al., 2011). Some PGP effects of T. harzianum under disease pressure are less impressive than those presented here. A 40% PGP increase occurred in Mungbean accompanied by a 87% decrease of stem rot in V. radiata (Dubey et al., 2009). It is hypothesised that increased predation improve nutrient availability (Altomare et al., 1999; Shoresh and Harman, 2008; Bae et al., 2009). However, of the evidence supporting BCA effects at higher pathogen inoculum rates, few reports confirm the PGP effects of co-inoculation (Metcalf et al., 2004; Villalta et al., 2004).

Of the reports concerning BCA and PGP coupled with increasing phytopathogens dose, reports of T. harzianum soil application afforded a 25% and 40% plant biomass increase that decreased with additional S. sclerotiorum concentrations (Elad et al., 1980; Singh, 1991). Others show co-inoculation with
Trichoderma and *S. sclerotiorum* reduced pathogenic fungi numbers, but failed to improve plant biomass when compared to untreated plants (Menendez & Godeas, 1998; Rabeendran *et al*, 2006; Daoubi *et al*, 2009; Amin *et al*, 2010; Ojaghian, 2011). This implies the potential double effect of BCA and PGP is unattainable in a natural strain of *Trichoderma* spp.

We have clearly reported that PGP occurs in a *S. sclerotiorum* dose dependent manner not previously witnessed. Therefore, by increasing *S. sclerotiorum* inoculum concentrations GD12 mycelium growth rates increase and, as a result, PGP effects are enhanced further. Furthermore, upon co-inoculation of GD12 and *S. sclerotiorum*, this current work failed to reveal the toxic effects associated with high concentrations of *Trichoderma* spp. (Thimann, 1937; Vinale *et al*, 2008; Daoubi *et al*, 2009). Therefore, efficient BCA is a combined effect of molecular, genomic and environmental fitness; thus suggesting specific mutations compromise Trichoderma’s phenotype to produce an inefficient BCA. This implies a molecular trade-off exists between plant, benign fungi and pathogens that ultimately affects the phenotype and commercial availability of the plant. Coupled with biofertiliser capability both, when utilised, could be exploited to suppress disease and increase yield simultaneously in an environmentally and economical way.

Questions surrounding the method in which biocontrol and biofertiliser affects are achieved continue to arise, though it may involve hydrolysed pathogen material contributing to increased GD12 and plant growth via nutritional means. Alternatively, the Trichoderma secretome may contain resolution to the problem associated with transcriptional activity. It is hypothesised that intricate molecular interactions are involved and mutagenesis of specific binding proteins could produce less virulent phytopathogenic strains (Mentlak *et al*, 2012; Penn & Daniel, 2013; Lecompte *et al*,
2013). Of course, this can work in reverse where genetic modification can produce fungal strains that bear more protection against pathogens. Furthermore, identification and extraction of particular metabolites could prove worthwhile especially in a mutant fungus with hypersecretory potential. Therefore, in order to determine reasons for the different effects of GD12 and $\Delta Thnag::hph$ on plant viability, further analyses of each fungal metabolite extract was assayed and discussed further in the following chapter.
Chapter 5. The putative identification of low molecular mass metabolites of *Trichoderma hamatum*

5.1 Introduction

5.1.1 Secondary metabolite synthesis

Primary Metabolites (PM) are a diverse group of compounds involved with essential cellular processes whereas Secondary Metabolites (SMs) are more specialised and often phylogenetically distinct (Luckner, 1984). SMs are exploited for their medical bioactivity often displaying antimitotic, fungicidal, antibacterial or antimicrobial effects (Smith *et al*, 2012; Pan *et al*, 2012; Santos *et al*, 2012). However, the true purpose of SM biosynthesis is generally for defensive or invasive means especially with sessile organisms like plants that deploy toxic SM to deter pathogens (Jin *et al*, 2011).

In fungi, SM biosynthesis is regulated by conserved coding regions often located in clusters at specific regions within the genome. PolyKetides (PKs) and Non-Ribosomal Peptides (NRPs) are synthesised in a similar fashion to fatty acids that deploy malonyl-CoA or acetyl CoA as starter units. PKs and NRPs are constructed by a series of coding domains within a section of DNA, and subject to many modifications producing a range of bioactive compounds. Structural differences between each cluster-type protein are determined by the series of coding regions comprising the module. PK and NRP [synthesis] modules overlap and often consist of similar units explaining why PKS/NRPS hybrid clusters are found. Typically, PKS clusters contain modules that each hold a number of coding regions (domains) each responsible for a specific enzyme (Figure 5.1).

Initially, the ACP starter domain codes for an Acyl Carrier Protein (ACP) responsible for transporting the initial building block via a phosphopantetheine loading arm. Depending on the SM, various domains follow in which other residues are added.
Figure 5.1 Type 1 PKS cluster associated with erythromycin biosynthesis. Each new starter unit is shown in red with modules numbered above. Reading from left to right, the loading domain (LD) initiates synthesis where acyl transferase (AT), acyl carrier protein (ACP), β–keto synthase (KS), β–Keto reductase (KR), dehydratase (DH) and enoyl reductase (ER) coding regions are held within modules.

Elongation and modification continues where domains encoding for Acyl Transferase (AT), DeHydratase (DH), Enoyl Reductase (ER), β–Keto Synthase (KS), Methyl Transferase (MT) and β–Keto Reductase (KR) activities are found terminating with ThioEsterase (TE). Depending on the fungal species, PKS or NRPS clusters display much variation in composition and position within the genome. Within Trichoderma spp., 28, 16, and 10 NRPS cluster copies are found in T. virens, T. atroviride and T. reesei respectively while NRPS/PKS hybrid sequences are repeated four, two and one times respectively within the genome (Kubicek et al, 2011).

5.1.2 Secondary metabolite regulation
SM gene expression is stimulated by external factors and is strictly regulated by DNA
methylation, acetylation and phosphorylation events (Maymon et al, 2009). Hypercoiled DNA (heterochromatin) is inaccessible to modification and often hypoacetylated therefore, before transcription takes place, histone acetylation usually occurs in order to reduce chromatin density (Noma et al, 2001). Chromatin mediated control of SM biosynthesis in filamentous fungi is mediated by the hepA (heterochromatin protein1), hdA (histone deacetylase) and laeA (putative methyltransferase) genes. In Aspergillus, mutagenesis of hdA and laeA resulted in reduced gliotoxin and other general SM production and loss of pathogenicity (Lee et al, 2009; Bok et al, 2005; Reye et al. 2010). Others show laeA disruption reduced all cellulase transcription in T. reesei (Seiboth et al, 2013). Although transgenic T. reesei laeA mutants were not restored by the A. nidulans orthologue, this suggests reduced conservation occurs between both fungi (Karimi-Aghcheh et al, 2013). Nonetheless, transcriptional activity is species dependent and although the full function of LaeA is not yet defined, it is hypothesised that LaeA is the first protein to be identified as self methylating (Patananan et al, 2013).

5.1.3 Metabolite collective diversity and function

Suggestions of horizontal gene transfer events leading to structural and functional similarities between SM are considered to bring about self-serving environmental advantages (Khaldi et al, 2008; Richards et al, 2009; Niklas et al, 2010). Furthermore, these similarities are exploited by plants and microorganisms to confer a selective genetic advantage that is distinct from evolutionary pressure; suggesting SM clustering survival is partly dependent on horizontal transfer (Walton, 2000).

A number of fungal and plant SMs share common functionality such as auxins, gibberellins and abscisic acid; significant phytohormones in plant development, maturity and stress tolerance (Contreras-Cornejo et al., 2009; Jin et
al., 2011; Inomata et al., 2004). Some fungi initiate symbioses and induce lateral root (lateral root) formation by secreting lipochitooligosaccharides (LCOs) (Martinez-Medina et al., 2011; Gutjahr & Paszkowski, 2009). Interestingly, lateral root formation is generally associated with plant auxins while symbiosis initiation occurs via NOD or MYC factors (Maillet et al., 2011; Nehls, 2008). Suggestions of molecular cross talk were confirmed by work that showed T. virens and Citrus sinensis share a sucrose/symporter system with 50% sequence similarity therefore enabling symbioses (Vargas et al., 2010). Alternatively, the pathogen B. cinerea assimilate and convert plant hexose to mannitol, trehalose and glucogen. This way, fungal penetration ability increases via conidiation and hyphae differentiation whilst suppressing the plant’s hypersensitive response simultaneously (Dulermo et al., 2009; Calmes et al, 2013; Smirnov & Cumbes, 1989). Therefore, it is hypothesised that suppression of hexose conversion would increase hexose levels that, in turn, promote plant assimilation of carbon and increase shoot mass. Other reports support this and maintain that any loss in mannitol activity would decrease multi-stress tolerance and virulence potential (Wang et al, 2012). Alternatively, over expression of mannitol dehydrogenase in plants increased resistance to B. Cinerea (Williamson et al, 2013).

With this in mind, a balance of two factors contributes to plant SM production. Together, photosynthetic ability and environmental resources determine defence strategies where a trade off exist between growth and biocontrol. For example, conditions that restrict growth but not photosynthesis favour SM production as determined by C:N ratios (Müller et al, 2013). Therefore, if carbon levels are high, carbon-based SM such as kaempferol increases (Royer et al, 2013). This finely tuned mechanism is influenced by soil dwelling microbes such as bacteria and fungi
that solvate inaccessible nutrients or secrete SM that are subsequently assimilated by plants and employed for either biofertiliser, biocontrol or a combination of both effects.

5.1.4 Plant growth promotion metabolites

Although carbohydrate metabolism is a vital common denominator in molecular cross-talk, it is well documented Trichoderma spp. secrete volatile compounds allowing antibiotics, water soluble organic acids and other auxin-like compounds to permeate the rhizosphere (Vinale et al., 2008). Auxins are cyclic-carboxylic acids including \textit{Indole-3-Acetic Acid} (IAA), \textit{2-PhenylAcetic Acid} (PAA), \textit{Indole-3-Butyric Acid} (IBA) and \textit{6-Pentyl Pyrone} (6PP) that are strongly associated with root formation and plant development (Gravel et al., 2007) (Figure 5.2B). Auxin regulation in plant and fungi reveal \textit{A. thaliana} auxotrophs adopt the tryptophan-independent pathway with indole-3-glycerol phosphate as a precursor (Normanly et al., 1993, Zhang et al., 2008). Alternatively, others have shown that \textit{Nicotiana tabacum} L. protoplasts adopt the tryptophan-based pathway while the necrotroph \textit{U. maydis} preferred the non-tryptophan pathway (Figure 5.2A) (Sitbon et al., 2000; Rao et al, 2010). Regardless of this, both pathways involve IAA chemical modification including conjugation and hydrolysis (Davies et al., 2010; Zhang et al., 2008).

However, plant-specific chemistry can affect fungal SM efficacy as exemplified by exogenous IAA that restored the root hair deficient \textit{A. thaliana rhd6} mutant as opposed to reduced root growth in lignonberry (Banner, 1996; Contreras-Cornejo et al., 2009). Some fungi mimic plant auxins especially IAA-like compounds under a tightly regulated pathway via synthesis and hydrolysis of IAA-conjugates (LeClere et al., 2002). Consequently, fungal secreted IAA is sequestered and possibly modified
Figure 5.2 The IAA biosynthetic pathway and associated metabolites. The Shikimate pathway precludes the tryptophan-based pathway (A) producing auxins involved with plant growth promotion (B). Indole acetic acid (IAA), indole-3- butyric acid (IBA), 2-phenylacetic acid (PAA) and 6-pentyl pyrone (6PP) display a common functionality to include a conjugated system that supports a carbonyl functional group (red box). IAA and IBA are homologues, with each containing a fused ring system whereby, PAA nor 6PP are a fused ring system. PAA and 6PP are auxins with additional biocontrol activity; this may be attributed to the carbonyl group as part of the single (non-fused) ring system. These auxins are associated with fungal secretion.
once assimilated by plants (Vinale et al., 2008). This suggests isomerism is a key factor in bioactivity (Daoubi et al., 2009; Dong et al., 2011) or that fungal metabolites are toxic when concentrations exceed the micro molar range (Thimann, 1937). Therefore before auxins are extracted, purified and used in application, the efficacy of structural isomers must be considered. SM structure is highly varied and a number of structural and functional isomers may exist each displaying varying degrees of bioactivity. Three structural isomers of a compound with a molecular mass and formula of 320.1624 and C_{18}H_{24}O_{5} exemplify this (Figure 5.3). The spatial orientation of the hydroxyl functional group (circle 1) differs between α-Zearalenol (A) and β-Zearalenol (B) in that (A) afford an equatorial position and (B) is axial. In contrast, isomer C has a ketone functional group (circle 1)

![Chemical Structures](image)

**Figure 5.3** Comparative illustration of chemical functional groups associated with functional and structural isomerism. Metabolite A and B are structural isomers to each other and metabolite C is a functional isomer to A and B.
with reduced saturation (circle 2); each with different chemical characteristics.

5.1.5 Biocontrol metabolites

Trichoderma spp. secretes CWDE to affect cell membrane integrity before hypercoiling and digestion (Chet et al, 1981; Claydon et al, 1987; Donzelli & Harman, 2001). Additionally, Volatile Organic Compounds (VOCs), antifungal and antibiotic chemicals are used in conjunction with CWDE to suppress pathogen growth to increase plant security and health (Amin et al, 2010; Bae et al, 2009; Dong et al, 2011). In a similar fashion, phytopathogens secrete effectors and hydrolytic enzymes in order to colonise plant tissue (Zhu et al, 2013). However, others hypothesise more intricate molecular interactions are responsible therefore mutagenesis of specific binding proteins could produce less responsible therefore mutagenesis of specific binding proteins could produce less virulent phytopathogenic strains (Mentlak et al, 2012; Penn & Daniel, 2013; Lecompte et al, 2013).

Just as pathogens vary in pathogenicity, Trichoderma vary in biocontrol ability via mycoparasitism (T. hamatum), oxidase up-regulation (T. viride), mycotoxins (T. atroviride, T. viride) or CWDE (T. reesei) (Atanasova et al, 2013; Gruber et al, 2013; Huang et al, 2011; Srivastava et al, 2012; Yang et al, 2012). Reports of Trichoderma spp. successfully suppressing S. sclerotiorum growth exist and of 11 Trichoderma spp. tested, T. viridescens reduced S. sclerotiorum mycelium growth and sclerotia formation by 95% and 90% respectively (Amin et al, 2010). In contrast, T. viride displayed 31% and 66% reduction in S. sclerotiorum mycelium and sclerotia formation, respectively (Amin et al, 2010). Little is known about the metabolites responsible for fungicidal activity and these have been suggested to be associated with G-protein regulation or simple excreted compounds like oxalic acid (Reithner et al, 2005; Willimas et al, 2011). However, a causal link exists between
sugar metabolism and G-protein activity as confirmed by gpr1-silenced transformants that displayed reduced G-protein expression rates coupled with impaired nag1 and ech42 transcription. Consequently, gpr1 display ineffective mycoparasite overgrowth despite exogenous cAMP (Omann et al, 2012). This has been confirmed by others who found that upon confrontation with F. solani, T. harzianum increased expression of NAG, tubulin and the G-protein β subunit (Steindorff et al, 2012).

Chitin adopts PAMP qualities thus eliciting defense responses in plants. As a monomeric constituent of chitin, NAG residues constitute the active site of NOD and MYC factors that up-regulate hydrolytic enzymes needed for parasitism and endosymbiosis (Maillet et al, 2011). Furthermore, NAG is effective against plant tumours and reverse the inhibitory effects of hexokinase suppression by D-allose; thus exerting positive regulatory effects on GA synthesis in a similar fashion to mannoheptulose (Richardson et al, 2013; Fukumoto et al, 2011). Furthermore, this BCA property has been coupled with PGP where chitosan treatment increased lignin type compounds and therefore strengthening stem structure in the process (Hadwinger et al, 1984).

Much interest concerns the chemical interactions between plant and microorganisms during symbiosis, infection and defence. Pyrosequencing techniques prove mutualistic results vary and depend on host specificity depending on soil type, location and genus (Lundberg et al, 2012). Furthermore, mutualism effects contribute to plant hormone and secondary metabolite up-regulation, in a species-dependent manner. This report has shown that upon NAG disruption, impaired biocontrol occurs thus suggesting a molecular trade off exists with reduced commercial availability. NAG mutagenesis effects upon host- specific protection produce diverse results. For example, reduced nag1 and ech42 transcription in T. atroviride show
increased levels of low molecular weight antifungal metabolites coupled with decrease levels of chitinase, 6PP and sesquiterpene structures; compounds strongly associated with biocontrol (Reithner et al, 2005; Rubio et al, 2008). Strain variability of Trichoderma spp. and pathogen display subtle differences in antagonism, mycoparasitic and secretory ability (Alvares et al, 2012; Ojaghian, 2011; Amin et al, 2010).

So far, we report Δnag as a highly effective PGP while GD12 is an efficient BCA against S. sclerotiorum and R. solani. Following this, it was found that all metabolite extracts failed to prevent S. sclerotiorum infection in lettuce plants and that physical interactions between both fungi is needed. Consequently, because little is known about the effect NAG mutation has on fungal SM productivity, this chapter sets out to characterise low molecular mass signatures and to determine if NAG mutation alters T. hamatum secretome and if so, to what degree.
5.2 Materials and Methods

5.2.1 HPLC-Mass Spectrometry

Freshly filtered metabolite extract was prepared as discussed in chapter 2.3 where replicate 15 mg samples of Trichoderma-bran extract were inactivated by freeze-drying then stored at -80°C for 48 hrs. Metabolite profiling was performed using a QToF 6520 mass spectrometer (Agilent Technologies, Palo Alto, USA) coupled to a 1200 series Rapid Resolution HPLC system. 5 µl of sample extract was loaded onto a Polaris 3 C18-A 1.8 µm, 2.0 x 250 mm reverse phase analytical column (Agilent Technologies, Palo Alto, USA). For detection using positive ion mode, mobile phase A comprised 0.1% formic acid in water and mobile phase B was 95% acetonitrile with 0.1% formic acid in water. For negative ion mode, mobile phase A comprised 5mM ammonium formate in water and mobile phase B was 95% acetonitrile.

The following gradient was used: 0 min–0% B; 10 min–0% B; 30 min–100% B; 40 min–100% B; 40.5 min–0% B; 10 min post time. The flow rate was 0.2 ml min⁻¹ and the column temperature was held at 35 °C for the duration. The source conditions for electrospray ionisation were: gas temperature was 325 °C with a drying gas flow rate of 9 l min⁻¹ and a nebuliser pressure of 35 psig. The capillary voltage was 3.5 kV in both positive and negative ion mode. The fragmentor voltage was 115 V and skimmer 70 V. Scanning was performed using the auto MS/MS function with a survey scan of 5 scans sec⁻¹, and MS/MS scan rate of 4 scans sec⁻¹. A sloped collision energy of 3.5 V/100 Da with an offset of 5 V was applied for untargeted collision induced dissociation. Once samples were aligned using Kernel Feature Alignment (Perera et al, 2011; unpublished) the raw data files were processed using the ExSpec Analytical Pipeline to give untargeted profiling data.
5.3 Results

5.3.1 Metabolite overview

Putative metabolite detection involved comparisons between bran, GD12 and $\Delta n$ag bran extracts as prepared in Chapter 2.2.4. After five days, any low molecular mass metabolite was captured and prepared for LC mass spectroscopy (Chapter 5.2.2). Data was processed as described (Perera et al, 2012). In total 20,499 features (signatures) were detected with <1.0E-05 confidence. Of these, 9103 were up-regulated by 2-fold or more relative to basal secretion levels and contained distinct feature profiles where 7970 (87.5%) remain common amongst all treatments and 728 were distinct with bran, GD12 and $\Delta n$ag each showing 209, 317 and 202 unique signatures respectively (Figure 5.4A).

Following this, identification of all features involved the METLIN databank (Custodio, 2005). This includes many spectra profiles ranging between 100 and 1200 daltons where 65,000 metabolites with $m/z$ (mass-to- charge ratio) values, retention times and high accuracy mass values at less than 1ppm are stored. Highly accurate measurements deploy LIPID MAPS (http://www.lipidmaps.org/), Kyoto Encyclopedia of Genes and genomes (KEGG; http://www.genome.jp/kegg/), KNApSAck (http://kanaya.naist.jp/KNApSACK/), National Institute of Standards and Technology (NIST; www.nist.gov/), MassBank (www.massbank.jp/), Human Metabolome Project (HMP; http://www.hmdb.ca/) and ARM (www.metabolome.jp) as additional libraries. Any signatures that failed to correlate with METLIN were verified using Chemspider (Pence & Williams, 2010).

Upon closer inspection, bran and $\Delta n$ag extracts contain more hydrophilic features than hydrophobic (Figure 5.4B & C) while GD12 maintained similar levels of each. Further analyses of Figures 5.4B & C include feature distribution up-
**A: complete data set**

![Venn diagram showing the distribution of metabolites between the control (bran), GD12/bran, and ∆nag/bran groups.](image)

**B: hydrophobic**

**C: hydrophillic**

**Figure 5.4** Feature distribution between the control, GD12/bran and ∆nag/bran metabolite extract. Control (bran; blue), GD12/bran (yellow) and ∆nag/bran (green) display (A) 9103 putative metabolites consist of (B) 3657 (40.17%) hydrophobic and (C) 5446 (59.83%) hydrophillic signatures.
Table 5.1 Up-regulation of unique putative signatures found within each *Trichoderma hamatum* secretome. ‘All’ represents any feature up-regulated by 2-fold or more, ‘4-fold+’ are features up-regulated by 4- to 7-fold. Where 1°: primary; 2°: secondary and +/-: simultaneously up and down-regulated.

<table>
<thead>
<tr>
<th></th>
<th>GD12 Hydrophilic All</th>
<th>4-fold+</th>
<th>Hydrophobic All</th>
<th>4-fold+</th>
<th>Δnag Hydrophilic All</th>
<th>4-fold+</th>
<th>Hydrophobic All</th>
<th>4-fold+</th>
</tr>
</thead>
<tbody>
<tr>
<td>1° metabolism</td>
<td>45</td>
<td>24</td>
<td>36</td>
<td>32</td>
<td>22</td>
<td>10</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>2° metabolism</td>
<td>73</td>
<td>45</td>
<td>67</td>
<td>38</td>
<td>64</td>
<td>31</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>Amino acid</td>
<td>14</td>
<td>6</td>
<td>14</td>
<td>4</td>
<td>23</td>
<td>11</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Organic acid</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Plant-like</td>
<td>15</td>
<td>9</td>
<td>24</td>
<td>16</td>
<td>11</td>
<td>5</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>Unknown+/-</td>
<td>9</td>
<td>6</td>
<td>13</td>
<td>4</td>
<td>15</td>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Expressed +/-</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>161</td>
<td>94</td>
<td>156</td>
<td>94</td>
<td>141</td>
<td>69</td>
<td>61</td>
<td>48</td>
</tr>
</tbody>
</table>

regulated 2-fold or more (‘all’) and those significantly expressed 4-fold or more (‘4-fold+’) (Table 5.1). Here, hydrophobic and hydrophillic parameters are categorised further into PM, SM, acids, plant-associated features and those unidentifiable or replicated within each secretome. Interestingly, Δnag secrete fewer PM, SM and phyto-like compounds than GD12 but more hydrophillic amino acids and organic acids. Furthermore, all SM-like expression exceeded that of PM where GD12 and Δnag display a 1.75 and 2.5-fold increase in SMs. To gain further insight of fundamental metabolism, each *T. hamatum* secretome was investigated further to include signatures associated with sugar metabolism in order to determine any NAG mutational effects.
Figure 5.5 Glucose metabolism and compounds up-regulated 4-fold or more in GD12 and ∆nag. Table (A) and illustration (B) compare GD12 (red) and ∆nag (green) where the mean of five replicates ($p<1.0E-06$). RT: retention time, Mw: molecular weight, TCA: tricarboxylic acid, AA: amino acid, FA: fatty acid, PPP: pentose phosphate pathway, Ala/Asp/Gln/Glu: AA residues.
5.3.1.1 Putative signatures related to sugar metabolism
GD12 discriminate signatures are mostly associated with gluconeogenesis and the mid to later stages of the *TriCarboxylic Acid* cycle (TCA) while $\Delta nag$ display potential compounds associated with FA, AA and trehalose catabolism (Figure 5.5A). GD12 display increased levels of fumaric acid, succinates, $\alpha$-ketoglutarate and oxaloacetate ($p<0.05$; T-Test) however, $\Delta nag$ show no trace of erythrose or seduheptulose; key compounds in gluconeogenesis. In fact, of the TCA- associated compounds, the $\Delta nag$ extract display increased levels of features associated with earlier stages of sugar metabolism (Figure 5.5B).

5.3.1.2 Putative primary metabolite identification
Essential PM up-regulated by 4-fold or more were categorised further where in this instance DNA, amino acid, sugar or FA compounds were compared against a number of databanks producing potential candidates with significant similarity ($p<1.0E-05$). Overall, $\Delta nag$ and GD12 display 10 and seven hits respectively with unique results. GD12 is predicted to secrete cUMP, xanthone, guanosine and propioloc acid involved in purine metabolism, RNA and $\beta$-alanine synthesis (Table 5.2A). Alternatively, $\Delta nag$ is predicted to secrete moieties such as phosphate, nitric acid, uracil, thymine, histidine, xanthine-like metabolites and Shikimate-associated proteins (Table 5.2B). Uniquely, GD12 afforded PQQH$_2$, a reduced pyrroloquinoline quinone co-factor involved with electron transfer in PSII while $\Delta nag$ secreted the dopamine antagonist thiethylperazine.

5.3.2 Putative secondary metabolite identification and function
Of the 9103 potential metabolites quantified within this report, only a possible 77 potentially discriminatory bioactive SM could be identified within 1ppm confidence limits. GD12 and $\Delta nag$ each secreted 48 and 29 unique low molecular weight
signatures 4-fold or more respectively. Once a potential identity was ascertained, peer-reviewed reports were used to help verify structure or activity. In a bioactive sense, all metabolites were assigned either biocontrol or biofertiliser; combined factors

**Table 5.2** Putative chemical features up-regulated by GD12 and Δnag detected by MS at a significant value of 1.0E-06 or less. Mean value of five biological replicates from each experimental treatment (p<1.0E-06). Where RT: retention time; Mw: monoisotopic weight, n: number of signatures detected; PPP: Pentose Phospate Pathway and FA: fatty acid.

<table>
<thead>
<tr>
<th>RT</th>
<th>Mw</th>
<th>n</th>
<th>Putative Identity</th>
<th>Molecular formula</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5577</td>
<td>70.047</td>
<td>1</td>
<td>Propionic acid</td>
<td>C3H2O2</td>
<td>β-Alanine metabolism</td>
</tr>
<tr>
<td>1.0328</td>
<td>166.05</td>
<td>1</td>
<td>D/L-xylolate</td>
<td>C5H10O6</td>
<td>PPP, sugar metabolism</td>
</tr>
<tr>
<td>1.0590</td>
<td>196.05</td>
<td>1</td>
<td>Xanthone-type</td>
<td>C13H8O2</td>
<td>Purine metabolism</td>
</tr>
<tr>
<td>2.5283</td>
<td>308.02</td>
<td>1</td>
<td>cUMP</td>
<td>C9H11N2O8P</td>
<td>Cyclic nucleotide</td>
</tr>
<tr>
<td>9.1511</td>
<td>283.09</td>
<td>1</td>
<td>Guanosine</td>
<td>C10H13N5O5</td>
<td>Nucleoside (RNA slicing)</td>
</tr>
<tr>
<td>6.0556</td>
<td>327.03</td>
<td>1</td>
<td>PQQH2</td>
<td>C14H8N2O8</td>
<td>Essential co-factor</td>
</tr>
<tr>
<td>2.3100</td>
<td>58.980</td>
<td>1</td>
<td>Propamine</td>
<td>C3H9N</td>
<td>Simple amine</td>
</tr>
<tr>
<td>2.2012</td>
<td>62.990</td>
<td>1</td>
<td>Nitric acid</td>
<td>HNO3</td>
<td>Basic metabolism</td>
</tr>
<tr>
<td>10.787</td>
<td>97.980</td>
<td>1</td>
<td>Phosphate</td>
<td>H3O4P</td>
<td>Essential chemical</td>
</tr>
<tr>
<td>3.3836</td>
<td>112.03</td>
<td>1</td>
<td>Uracil</td>
<td>C4H4N2O2</td>
<td>Pyrimidine; RNA &amp; protein</td>
</tr>
<tr>
<td>4.2392</td>
<td>126.04</td>
<td>1</td>
<td>Thymine</td>
<td>C5H6N2O2</td>
<td>Nucleotide base</td>
</tr>
<tr>
<td>1.0254</td>
<td>136.04</td>
<td>2</td>
<td>Hypoxanthine</td>
<td>C5H4N4O</td>
<td>Purine derivative Isomer</td>
</tr>
<tr>
<td>2.7410</td>
<td>188.04</td>
<td>2</td>
<td>Allopurinol</td>
<td>C5H5N2O2</td>
<td>Purine derivative</td>
</tr>
<tr>
<td>13.150</td>
<td>165.07</td>
<td>1</td>
<td>L-Histidine</td>
<td>C6H9N3O2</td>
<td>Essential amino acid</td>
</tr>
<tr>
<td>1.0083</td>
<td>168.05</td>
<td>2</td>
<td>Methylxanthine</td>
<td>C6H8N4O2</td>
<td>Purine derivative</td>
</tr>
<tr>
<td>2.7180</td>
<td>202.03</td>
<td>1</td>
<td>Demethylmethoxsalen</td>
<td>C11H6O4</td>
<td>Coumarin; shikimate pathway</td>
</tr>
<tr>
<td>9.971</td>
<td>399.18</td>
<td>1</td>
<td>Thiethylperazine</td>
<td>C22H26N3S2</td>
<td>Dopamine antagonist</td>
</tr>
</tbody>
</table>
that may contribute to overall plant biofitness once plants are grown in *T. hamatum* amended soil. Therefore, to deduce any potential trends, each feature was further categorised into biocontrol (antibiotic, antifungal, antimicrobe, antimitotic, pesticide) or biofertiliser specific SMs to include plant-like compounds.

**Table 5.3** Potential features up-regulated 4-fold or more by GD12 and Δ*nag* after five days growth in autoclaved bran. Each data point represents the mean of five replicates from three experimental repeats where *p*<1.0E-06.

<table>
<thead>
<tr>
<th>SM type</th>
<th>GD12</th>
<th>Δ<em>nag</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Defence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aflatoxin</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Antibiotic</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Fungicide</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>Antimicrobe</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Antimitotic</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Pesticide</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>General</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

5.3.2.1 **Putative signatures associated with defence-like qualities**
Overall, the GD12 secretome display 2-fold more biocontrol signatures than Δ*nag* and of these, GD12s signatures chiefly relate to antibiotic and pesticide-like compounds while Δ*nag* is more general in nature (Table 5.3). For example, of the seven antibioactive-like compounds GD12 is predicted to excrete one medical related (matteuorienate B); two broad spectrum (thiocarbohydrazide and burseran); one *Legionella* spp. specific (atracic acid), one that negates biofertilizer effects (koninginin) and two which remain unquantified (hydroxymethylphosphonate and hydroxyvanillyl; Table 5.4). In contrast Δ*nag* exhibited three with antibiotic effects; one that inhibit gram negative bacteria such as *N. gonorrhoeae* (angucyclin,
deoxypodorhizone) and gentiopicrin; effective against a number of pathogenic bacteria excluding *P. Fluorescens* and *L. Monocytogenes* (Table 5.5). Furthermore, four pesticide-associated metabolites were discovered in GD12 (chlorophenoxyacetate, baclofen, mitragynine and prochlorperazine-N-oxide) while two were identified in Δnag (fluenetil and nigakilactone M).

In bran inoculum, GD12 secretes 4-fold more fungicidal compounds than Δnag with twelve and three expressed respectively. Of the twelve compounds detected in GD12, five are inconclusively verified (hispidulin, trichodenone A, probenecid, colneleic acid and phenytoin-N-glucuronide), five are effective against phytopathogens (ascladiol, epoxydon, (+)-cyclophellitol, 5-Methyl-1,2-oxazol-3-yl β-D-glucopyranoside and prinomide), one is implicated in plant/ human infection (p-2-chlorobenzhydrol) and another in human mycoses (pyocyanin). Fungicides secreted by Δnag were dimethylphenanthrene, imperatorin and verbascoside; effective against white-rot, Candida infection and the plant pathogen *Penicillium digitatum* respectively.

Few antimicrobe moieties were secreted. GD12 secretes the more general antimicrobial compounds sulochrin, fluoren-9-one and terrain, while Δnag secreted compounds effective against fungi (hydroxyanthraquinone) and bacteria (isophylloflavan). On the other hand, no antimitotic compounds were identified in the Δnag secretome while GD12 displayed five: deacetylvinodoline and colchicines (antimitotic); aspidin (pro-apoptosis); wikstromol (dopamine receptor antagonism) and one via an unknown mechanism: artonin l. Interestingly, Δnag produced three features, two natural plant pigments with pro-apoptotic properties (α-peroxytetrahydrobiopterin and cyanidin 3-rutinoside-5-glucoside) and the more general compound gramine; a toxic alkaloid generally deployed as a PSM.
Table 5.4 Putative Δnag biocontrol SM associated features up-regulated by 4-fold or more after five days growth in autoclaved bran. Each mean value is of five biological replicates from each experimental treatment ($p<1.0E-06$).

<table>
<thead>
<tr>
<th>RT</th>
<th>Mw</th>
<th>n</th>
<th>Putative identity</th>
<th>n-fold</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0628</td>
<td>206.11</td>
<td>2</td>
<td>Dimethylphenanthrene</td>
<td>5</td>
<td>Hashemi &amp; Latibari, 2011</td>
</tr>
<tr>
<td>2.6677</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.294</td>
<td>224.06</td>
<td>1</td>
<td>Hydroxyanthraquinone</td>
<td>4</td>
<td>Liu et al, 2007</td>
</tr>
<tr>
<td>1.6634</td>
<td>258.11</td>
<td>1</td>
<td>Fluenetil</td>
<td>4</td>
<td>No record</td>
</tr>
<tr>
<td>1.0626</td>
<td>270.09</td>
<td>1</td>
<td>Imperatorin</td>
<td>7</td>
<td>Mishra et al, 2010</td>
</tr>
<tr>
<td>9.8667</td>
<td></td>
<td></td>
<td>Medicarpin/ESR1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0368</td>
<td>273.11</td>
<td>2</td>
<td>α-peroxytetrahydrobiopoterin</td>
<td>4</td>
<td>Samolski et al, 2009</td>
</tr>
<tr>
<td>2.1589</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19.668</td>
<td>400.15</td>
<td>1</td>
<td>Deoxypodhorizone</td>
<td>6</td>
<td>Pettit et al, 2004</td>
</tr>
<tr>
<td>2.3728</td>
<td>174.12</td>
<td>1</td>
<td>Gramine</td>
<td>6</td>
<td>Adams et al, 2012</td>
</tr>
<tr>
<td>8.2458</td>
<td>356.11</td>
<td>2</td>
<td>Gentioticpin</td>
<td>6</td>
<td>Liu et al, 2007</td>
</tr>
<tr>
<td>8.4612</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>17.403</td>
<td>792.22</td>
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<td>Cyanidin 3-rutinoside-5-glucoside</td>
<td>4</td>
<td>Fredes &amp; Montenegro, 2013</td>
</tr>
<tr>
<td>2.4348</td>
<td>660.17</td>
<td>2</td>
<td>Iso/phyelloflavan</td>
<td>4</td>
<td>Kolodziej et al, 2001</td>
</tr>
<tr>
<td>5.8274</td>
<td>468.14</td>
<td>1</td>
<td>Angucyclin</td>
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<td>Novakova et al, 2010</td>
</tr>
<tr>
<td>1.2266</td>
<td>624.21</td>
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<td>Acteoside or Verbasidose</td>
<td>4</td>
<td>Zhao et al, 2011</td>
</tr>
<tr>
<td>1.3383</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cyourou et al, 2013</td>
</tr>
</tbody>
</table>

5.3.2.2 Putative signatures associated with plant growth promotion

To determine potential contributions to biofertiliser effects, features up-regulated 4-fold or more were investigated and analysed to determine if any trends in expression patterns exist and if so, the level and manner in which they may contribute to PGP. Here, GD12 (Table 5.6) and Δnag (Table 5.7) excrete a similar number of potential PGP SM, especially those associated with DNA and acid metabolites whilst GD12 up-regulated more phytohormone-like features than Δnag. What is of interest are the difference in DNA associated metabolites secreted. GD12 display increased levels
Table 5.5 Putative GD12 biocontrol secondary metabolite features detected after five days growth in autoclaved bran. *Available as a commercial application. Each mean value is of five biological replicates from each experiment (p<1.0E-06).

<table>
<thead>
<tr>
<th>RT</th>
<th>Mw</th>
<th>n</th>
<th>Putative identity</th>
<th>n-fold</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0378</td>
<td>106.03</td>
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<td>Thiocarboxyhydrizide</td>
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<td>Singh et al, 2011.</td>
</tr>
<tr>
<td>2.0480</td>
<td>111.99</td>
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<td>Naidu et al, 2012.</td>
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<td>1.2162</td>
<td>124.05</td>
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<td>Trichodenone A</td>
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<td>Santos et al, 2012.</td>
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<td>1.4534</td>
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<tr>
<td>2.8409</td>
<td>154.06</td>
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<td>Terrein (phobic) Methoxyltoluene</td>
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<td>Ascladiol (hydrophobic)</td>
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<td>2.3020</td>
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<td>2.8654</td>
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</tr>
<tr>
<td>7.0968</td>
<td>156.04</td>
<td>2</td>
<td>Epoxoydon (hydrophillic)</td>
<td></td>
<td>Krohn &amp; Schulz, 2013</td>
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<tr>
<td>7.3352</td>
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<td></td>
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<td>1.0606</td>
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<td>2</td>
<td>(+)-Cyclophellitol</td>
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<td></td>
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<td>180.06</td>
<td>1</td>
<td>Fluoren-9-one</td>
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<td>Venkatesan et al, 2012</td>
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<td>Chlorophenoxyacetate</td>
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<td>Fulthorpe et al, 1994</td>
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<td>2.7185</td>
<td>196.07</td>
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<td>Atracic acid</td>
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<td>Roel &amp; Baiahmad, 2011</td>
</tr>
<tr>
<td>3.2056</td>
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<td></td>
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<td>2.7170</td>
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<td>Sudhakar et al, 2013</td>
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<td>6.9700</td>
<td>213.06</td>
<td>2</td>
<td>Baclofen/Carbanolate</td>
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</tr>
<tr>
<td>9.0924</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1.0333</td>
<td>218.05</td>
<td>1</td>
<td>p-2-Chlorobenzhydrol</td>
<td>6</td>
<td>El Hage et al, 2011</td>
</tr>
<tr>
<td>11.084</td>
<td>261.09</td>
<td>1</td>
<td>β-D-Glucopyranoside, 5-methyl-3-isoxazolyl</td>
<td>4</td>
<td>Metzen et al, 2012* hymexazol</td>
</tr>
<tr>
<td>8.3918</td>
<td>267.10</td>
<td>1</td>
<td>Primidone* or CGS 12094</td>
<td>5</td>
<td>Parrish et al, 1997</td>
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<tr>
<td>2.3295</td>
<td>284.20</td>
<td>1</td>
<td>Koninginin</td>
<td>5</td>
<td>Mori et al, 2002</td>
</tr>
<tr>
<td>1.1145</td>
<td>285.11</td>
<td>1</td>
<td>Probenicid</td>
<td>4</td>
<td>Dacey &amp; Sande, 1974</td>
</tr>
<tr>
<td>2.0420</td>
<td>294.22</td>
<td>1</td>
<td>Colineic acid</td>
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<td>Schuck et al, 2012</td>
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<tr>
<td>2.4600</td>
<td>328.06</td>
<td>1</td>
<td>Atloxacin G1 or M1</td>
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<td>8.7657</td>
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<td>1.2112</td>
<td>389.13</td>
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<td>9.7948</td>
<td>398.23</td>
<td>2</td>
<td>Mitragynine/phoncodine</td>
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<td>Wungsintaweekul et al, 2012</td>
</tr>
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<td></td>
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<tr>
<td>1.3444</td>
<td>399.17</td>
<td>1</td>
<td>Colchicine</td>
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<td>Sivakumar, 2013</td>
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</table>
of MeValonic Acid (MVA) as opposed to the Methyl Erythrose Phosphate (MEP) monomers in Δnag. However, this is not to state fundamental metabolism is affected by the mutation of a single gene. It is suggested that NAG mutation may circumvent other pathways to ensure basic metabolism occurs; pathway associations that have not been investigated. Moreover, GD12 display compounds concerned with DNA acetylation and methylation events (D-mylo-inositol tetraphosphate and azacytidine) where in contrast, Δnag secreted the nucleotide thymidine.

GD12 contained four putative phytohormones including two betaines (Trimethylglycine and hyaphorine), indole 3-acetamide and gibberellin A2; whereby Δnag secreted indoleamine. However, phytohormones and their derivatives display overlapping roles as exemplified by the three SA-like acids (methyisalicylate, pimelic acid, S-nitroso-L-glutathione) secreted by GD12. Similarly Δnag secreted three acids that afforded one each of ABA (lunularic acid), IAA-like (3-bromopropylphthalimide) and SA (valeroyl salicylate). Other GD12 hits involved carbon (gluconolactone) and phosphorus (phytic acid) degradation. Interestingly, GD12 displayed increased levels of calcium pantothenate (vitamin B5; dimer) and NAG monomers while Δnag secreted the B5 monomer (pantetheine), 6-hydroxy hexanoic acid, four miscellaneous SMs (fruticosonine, methyl 1,3,4,9- tetrahydro-2H-β-carboline-2-yacetate, physovenine and syringaldehyde); no NAG monomers.
Table 5.6 Putative GD12 plant-associated signatures up-regulated more than 4-fold after five days growth in autoclaved bran. Each mean value is of five biological replicates from each experimental treatment ($p<1.0E-06$).

<table>
<thead>
<tr>
<th>RT</th>
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<th>Formula</th>
<th>Putative identity</th>
<th>n-fold</th>
<th>Reference</th>
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<td></td>
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<tr>
<td>1.9958</td>
<td>148.07</td>
<td>C8H12O4</td>
<td>Mevalonic Acid</td>
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<td>Graebe, 1972</td>
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<tr>
<td>6.7121</td>
<td>244.08</td>
<td>C8H12N4O5</td>
<td>Azacytidine or Ribavirin</td>
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<td>Williams, 2008</td>
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<tr>
<td>17.090</td>
<td>499.93</td>
<td>C6H16O18P4</td>
<td>D-my-o-Instol tetraphosphate</td>
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<td>Watson et al, 2012</td>
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<tr>
<td>Acids</td>
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<td></td>
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</tr>
<tr>
<td>2.4289</td>
<td>152.05</td>
<td>C8H8O3</td>
<td>Methyl salicylic acid</td>
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<td>Park et al, 2007</td>
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<td>1.4296</td>
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<td>Pimelic acid</td>
<td>5</td>
<td>du Vigneaud et al, 1942</td>
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<tr>
<td>8.5330</td>
<td>178.05</td>
<td>C6H11O6</td>
<td>L-Glutatolate</td>
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<td>Blakely et al, 1957</td>
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<td>8.6791</td>
<td>297.12</td>
<td>C14H19NO6</td>
<td>Phénylaminoglycucronide</td>
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<td>Grogan, 2009</td>
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<td>5.3442</td>
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<td>S-Nitroso-L-glutathione</td>
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<td>Phytohormones</td>
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<td>1.3295</td>
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<td>Betaine</td>
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<td>174.08</td>
<td>C10H10N2O</td>
<td>Indole 3-acetamide</td>
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<td>Vandeputte et al, 2005</td>
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<td>248.14</td>
<td>C14H18N2O2</td>
<td>Hypaphorine A</td>
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<td>Ditengou, 2003</td>
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<td>5.5617</td>
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<td>8.7393</td>
<td>221.09</td>
<td>C8H15NO6</td>
<td>Glucosamine/NAG</td>
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<td>Hadwinger et al, 1984</td>
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<td>1.3057</td>
<td>478.17</td>
<td>C18H32CaN2O10</td>
<td>Calcium pantothenate</td>
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<td>Askari-Khorasgani et al, 2013</td>
</tr>
</tbody>
</table>

5.3.3 Fatty Acid putative signatures

GD12 up-regulated 2-fold more FA than and $\Delta$nag and of these GD12 showed two saturated FA (3-hydroxy-sebacic acid and pentadecanoic acid) while $\Delta$nag display no unsaturated FA (Table 5.8). Of the six unsaturated FA secreted by GD12 one was an oleic acid derivative (7-oxo-11 (E) Z-tetradecenoic acid), two were choline based
Table 5.7 Putative Δnag plant-associated signatures up-regulated more than 4-fold after five days growth in autoclaved bran. Each mean value is of five biological replicates from each experimental treatment ($p<1.0E-06$).

<table>
<thead>
<tr>
<th>RT</th>
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<th>Formula</th>
<th>Putative identity</th>
<th>n-fold</th>
<th>Reference</th>
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<tr>
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<td>DNA</td>
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</tr>
<tr>
<td>7.7971</td>
<td>216.04</td>
<td>C5H13O7P</td>
<td>Methyl-D-erythritol-4-phosphate</td>
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<td>Zhao et al, 2013</td>
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<tr>
<td>1.0966</td>
<td>242.09</td>
<td>C10H14N2O5</td>
<td>Thymidine</td>
<td>5</td>
<td>Wang &amp; Kool, 1995</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Acids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.3746</td>
<td>118.06</td>
<td>C5H10O3</td>
<td>(2R,3R) Butyric acid</td>
<td>7</td>
<td>Nieminen et al, 2008</td>
</tr>
<tr>
<td>9.0425</td>
<td>129.04</td>
<td>C5H7NO3</td>
<td>Pyroglutamic acid</td>
<td>7</td>
<td>Adney et al, 2003</td>
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<tr>
<td>2.3998</td>
<td>132.08</td>
<td>C6H12O3</td>
<td>6-hydroxy hexanoic acid</td>
<td>6</td>
<td>Cheng et al, 2000</td>
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<tr>
<td>1.0164</td>
<td>258.09</td>
<td>C15H14O4</td>
<td>Lunularic acid or hydroxyphenylsalicylate</td>
<td>4</td>
<td>Yoshikawa et al, 2002</td>
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<td>4.0781</td>
<td>268.11</td>
<td>C11H10BrNO2</td>
<td>3-Bromopropylphthalimide</td>
<td>5</td>
<td>Bisewska et al, 2012</td>
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<td>6.0666</td>
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<td>22.866</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Phytohormone</td>
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<td></td>
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<td>2.4835</td>
<td>132.07</td>
<td>C6H8N2</td>
<td>Indoleamine</td>
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<td>Zhang et al, 2013</td>
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<td>1.8136</td>
<td>182.06</td>
<td>C9H10O4</td>
<td>Syringadhyde</td>
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<td>2.4083</td>
<td>244.12</td>
<td>C14H18N2O2</td>
<td>Methyl 1,3,4,9-tetrahydro-2H-b-carbolin-2-yacetate</td>
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<td>Soledad et al, 1993</td>
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<td>8.7776</td>
<td>262.13</td>
<td>C14H18N2O3</td>
<td>Physovenine</td>
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<td>Dale et al, 1970</td>
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<td>2.0972</td>
<td>312.22</td>
<td>C20H28N2O</td>
<td>Fruticosonine</td>
<td>5</td>
<td>Chaichit et al, 1979</td>
</tr>
</tbody>
</table>

(2-acetyl-sn-glycero-3-phosphocholine, 1-acetyl-2-acetyl-sn-glycero-3-phosphocholine) and three were associated with the arachidonic acid synthesis pathway (13-hydroperoxy-9,11-octadecadienoic acid, 9-hydroperoxy-12,13-dihydroxy-10-octadecenoic acid and 2,3-thromboxane B1). In contrast, Δnag FA hits were not modified and consisted of oleic acic, linoleic acid, pentenoic acid and glycerophospholipid.
**Table 5.8** Putative chemical signatures up-regulated by GD12 and Δnag after cultivation in bran for five days. Each mean value is of five biological replicates from each experimental treatment ($p<1.0E-06$).

<table>
<thead>
<tr>
<th>RT</th>
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<td></td>
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<tr>
<td>2.7558</td>
<td>218.12</td>
<td>3-hydroxy-sebamic acid</td>
<td>C10H18O5</td>
<td>Kamiyo <em>et al.</em>, 1997</td>
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<td>2.4167</td>
<td>240.17</td>
<td>7-oxo-11 (E) Z-tetradecenoic acid</td>
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<td>18.975</td>
<td>258.22</td>
<td>Pentadecanoic acid</td>
<td>C15H30O3</td>
<td>Wewer <em>et al.</em>, 2013</td>
</tr>
<tr>
<td>1.0710</td>
<td>299.11</td>
<td>2-acetyl-sn-glycero-3-phosphocholine</td>
<td>C10H22NO7P</td>
<td>Liu <em>et al.</em>, 2013</td>
</tr>
<tr>
<td>2.4967</td>
<td>341.04</td>
<td>1-acetyl-2-acetyl-sn-glycero-3-phosphocholine</td>
<td>C12H24NO8P</td>
<td>Liu <em>et al.</em>, 2013</td>
</tr>
<tr>
<td>13.838</td>
<td>312.23</td>
<td>13-hydroperoxy-9,11-octadecadienoic</td>
<td>C18H32O4</td>
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<td>8.8120</td>
<td>346.24</td>
<td>9-hydroperoxy-12,13-dihydroxy-10-octadecenoic acid</td>
<td>C18H34O6</td>
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<td>Kupfahl <em>et al.</em>, 2012</td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>9.9438</td>
<td>114.03</td>
<td>Pentenoic acid deriv</td>
<td>C6H10O2</td>
<td>Poole &amp; Whitaker, 1997</td>
</tr>
<tr>
<td>22.309</td>
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<td>Linoleic acid</td>
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<td>22.602</td>
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<tr>
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<td>C18H34O2</td>
<td>Manimuthu <em>et al.</em>, 2013</td>
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<td>2.2507</td>
<td>656.44</td>
<td>Glycerophospholipid</td>
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5.4 Discussion

I have reported features with potential PGP and BCA effects were uniquely recovered from GD12 and $\Delta n$ag. Furthermore, it is evident that, once an hygromycin cassette had been inserted within the N-acetyl-\(\beta\)-D-glucosamine gene sequence, the T. hamatum secretome produce a unique range of low molecular mass compounds with bioactivity (Table 5.9). A number of auxin-like compounds were recovered from GD12 and $\Delta n$ag, with different functionalities. For example, $\Delta n$ag show indoleamine and 3-bromopropylphalamide modification coupled with \(\alpha\)-peroxytetrahydrobiopterin biotransformation to 5- hydroxytryptophon potentially resulting in increased IAA levels (Gravel, 2007; Bisewska et al, 2012; Wahyuni et al, 2013; Zhang et al, 2013). In contrast, GD12 deploys the more volatile MSA that, upon methylation, reduces SA levels and increases SA-conjugates reporting to lead to increased root mass and reduced PR-related gene expression (Trupiano et al, 2013). This suggests the more alkylated valerlyl SA predicted in $\Delta n$ag samples, has more potent PGP effects than MSA with reduced BCA. However, exogenous SA at nano and femto levels give positive effects on root growth (Bayat et al, 2012; Samolski et al, 2009) and is often exploited by phytopathogens to improve pathogenicity and colonisation ability (Vandeputte et al, 2005). Consequently IAA antagonism can take place where structural similarities enable inhibition, resulting in reduced root hair development or negating etiolated wheat coleoptile growth (Ditengou et al, 2000; Cutler et al, 1991). Two negative auxin regulators were discovered in GD12 therefore, the combination of both inhibitory and promotional compounds in GD12 may account for the reduced PGP effect compared to $\Delta n$ag as illustrated in Chapter 3 and Chapter 4.

Here, GD12 synthesised the methylated GA2 as opposed to the alkene
group in GA3 and GA1. Both are similar in efficacy but not as potent as GA2 (Sondheimer & Galson, 1966). Again, subtle structural similarities remain however, as in MSA and GA2, methylation occurs. Increased methylation increases hydrophobicity which appears to improve PGP effect; here, Δnag deploy propyl groups (Figure 5.6). Other compounds emerging as PGP compounds include nitrates and phosphates where we hypothesise NAG modification brings about alternative methods of NO synthesis. GD12 deploys the NO synthase generated GSNO while Δnag display increased levels of propamine; an amine donor that generate NO upon oxidation.

**Table 5.9.** Overview of PGP signatures up-regulated by 4-fold or more in GD12 or Δnag. PGP: plant growth promotion, NO: nitric oxide, N14: nitrogen, P15: phosphorus and (-): negative effects. *Both BCA and PGP qualities.

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<td></td>
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<td>Pantetheine (monomer)</td>
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<td>α-peroxytetrahydrobipterin</td>
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<td>Pyroglutamic acid</td>
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<td>Giberellic Acid A2</td>
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<td>3-bromopropylphthalamide</td>
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<td>S-Nitrosoglutathione</td>
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<td>P15 source</td>
<td>Phosphorous</td>
<td></td>
<td>P15 source</td>
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<td>Pimelic acid</td>
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<tr>
<td>Koninginin</td>
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<td>PGP (-)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>N-acetyl Glucosamine</td>
<td>4</td>
<td>PGP*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Considering that NO is associated with primary metabolism, plant cell wall strengthening and adventitious root formation, the efficacy of GSNO and propamine has not been quantified (Arasimowicz-Jelonek et al., 2013; Molilina-Favero et al., 2007). However, it is hypothesised that induced lateral root formation occur via interactions with root brassinosteroids, NO and IBA (Kolbert et al., 2008; Tossi et al., 2013; Schlicht et al., 2013). Additionally, phosphate solvation increase phosphorus levels within the rhizosphere where it has been shown T. harzianum deploy acid phosphatase to liberate phosphorus (Leitão et al., 2010). Little information supporting phytase expression in Trichoderma exist except for the isolation and characterisation of Trhxt1, a putative glucose transporter gene that was expressed under anoxic conditions or when glucose levels are high (Ramos et al., 2006). However, up-regulation of phytic acid and inositol tetraphosphate suggests a fully functional NAG gene may contribute to phosphorus hydrolysis in grains (Mosblech et al., 2010; Kryštofová et al., 1998).

Regarding BCA, we report NAG mutation affects the biosynthesis of potential bioactive SM associated with biocontrol. Immediately, we see GD12 affords 3-fold more potential biocontrol signatures than Δnag. Of these, five are associated with jasmonic acid regulation (Pan et al., 2012; Badri et al., 2008; Fu et al., 2006; Mosblech et al., 2009) while Δnag up-regulated the SA-like compound lunularic acid (Figure 5.7). The JA-associated metabolites secreted by GD12 display alternative functions involving lignin-based compounds (wikstromol; Tandon & Rastogi, 1976) with mutagenic (hispidulin; Lin et al., 2010) and antimicrobe effects (deacetylvindoline; Sivakumar, 2013). Fungal inoculation significantly increases plant vindoline production with expression occurring in A. fumigatus but not in Trichoderma (Tiwari et al., 2013; Anisimov et al., 2012).
Figure 5.6 Comparison of reference PGP compounds to metabolites secreted by Trichoderma. Reference (A), GD12 (B) and Δnag (B) include negative growth regulators (-), IAA: indole-3-acetic acid, IBA: indolebutyric acid, SA: salicylic acid and GA: giberellic acid.
Table 5.10 Overview of biocontrol signatures up-regulated in GD12 or ∆nag by 4-fold or more. Where SAR: systemic acquired resistance, JA: jasmonic acid and SA: salicylic acid.

<table>
<thead>
<tr>
<th>Putative identity</th>
<th>n-fold</th>
<th>Function</th>
<th>Putative identity</th>
<th>n-fold</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>GD12</td>
<td></td>
<td></td>
<td>∆nag</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyocyanin</td>
<td>5</td>
<td>SAR</td>
<td>Dimethylphenanthrene</td>
<td>5</td>
<td>Fungicide</td>
</tr>
<tr>
<td>Hispidulin</td>
<td>4</td>
<td>JA-like</td>
<td>Verbascoside</td>
<td>4</td>
<td>Antimicrobe</td>
</tr>
<tr>
<td>Colmeleic acid</td>
<td>6</td>
<td>JA-like</td>
<td>Imperatorin</td>
<td>7</td>
<td>Antimicrobe</td>
</tr>
<tr>
<td>Deacetylvinodoline</td>
<td>6</td>
<td>JA-like</td>
<td>Lunularic acid</td>
<td>4</td>
<td>SA-like</td>
</tr>
<tr>
<td>Wikstromol</td>
<td>5</td>
<td>JA-like</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhamnoside</td>
<td>5</td>
<td>Sugar</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aflatoxin</td>
<td>4</td>
<td>Toxin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trichodenone A</td>
<td>5</td>
<td>Cytotoxin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-2-chlorobenzhydrol</td>
<td>6</td>
<td>Antimicrobe</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenyltoin</td>
<td>5</td>
<td>Carcinogen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulochrin</td>
<td>5</td>
<td>Antimicrobe</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hymexazol</td>
<td>4</td>
<td>Fungicide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prinomide</td>
<td>5</td>
<td>Fungicide</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Interestingly, this chapter has shown that GD12 up-regulate features with various predicted functions; potentially contributing to the BCA effect observed in Chapter four. However, the large number of antifungal compounds retrieved from the GD12 bran extract had no effect as a metabolite treatment.

Therefore, the biocidal effect of GD12 against S. sclerotiorum must be attributed to physical interactions that may, potentially, involve structural or functional isomers of the compounds retrieved so far. Fungicidal compounds up-
Figure 5.7 Comparative illustration of jasmonic acid (JA) associated metabolites found within the GD12 secretome. This includes the SA-like metabolite found within Δnag (Lunularic acid). JA is a reference and (n)=n-fold increase.

regulated by GD12 bear overlapping functions with the anti-microbial metabolites (hispidulin and colneleic acid). Overall, Δnag secreted two (dimethylphenanthrene & imperatorin) effective against white-rot fungi and S. sclerotiorum and similar in structure to GD12’s pyocyanin (Hosseini Hashemi & Latibari, 2011). However, GD12 display two features predicted to be fungicides used commercially; hymexazol and prinomide - effective against post/pre emergence damping-off disease. Interestingly, GD12 secrete the sugar conjugate (β-D-glucopyranoside, 5- methyl-3-isoxazolyl) generally associated with cellular toxin removal. Isoxazoles inhibit High Osmolarity Glycerol (HOG) activity via two- component histidine kinase and benefits crops.
against *S. sclerotiorum* infection by inhibiting kinase *shk1*; essential for sclerotia development and antifungal resistance (Duan *et al.*, 2013). This may explain GD12’s proficiency in *S. sclerotiorum* inhibition and sclerotia formation however, other explanations must be considered. This includes GD12 ability to remove toxins naturally found within bran extract via glycosylation or that GD12 is capable of hymexazol and prinomide biosynthesis. If this is the case, this is the first report of prinomide or hymexazol biosynthesis in Trichoderma. To discount this possibility,
one can point to additional research that confirm prinomide and hymexazol are not used as a fungicide in oat but associated with sugarbeet, tomato and rice protection (European Food Safety Authority; 2010). This strongly suggests these commercial fungicides are unlikely to be present within the original oat bran used in the original inoculum material and are, in fact, hypothesised to be biosynthesised by GD12.

As a conclusion to the biocidal effects of GD12, it is worthy to note increased aflatoxin G1 levels were observed. Generally, aflatoxin is a potent mycotoxin with a number of isomers with varying degrees of toxicity; G, being slightly less toxic than the most potent B1. Indigenous to Aspergillus spp., aflatoxin is found in cereals and live food stock causing much concern. Biodegradation to less toxic compounds involve ascladiol, a breakdown product of patulin naturally biodegraded by fungi and present within the GD12 secretome (review: McCormick, 2013). Furthermore, comparative analysis of oat disease indicates Aspergillus does not infect oat fields and instead, is consumed by phytopathogenic strains that do produce aflatoxin G1 such as Fusarium, Rhizoctonia, Pythium and Ustilago to name a few.

This chapter has found signature up-regulation is enhanced by NAG functionality. Coupled with increased levels of azacitidine, an epigenetic modifier that inhibit DNA methylation in pathogenic fungi and aflotoxin biosynthesis in A. flavus (Jupe et al, 1986; Lin et al, 2013; Asai et al, 2011), GD12 also secreted the vitamin B5 dimer calcium pantothenate in contrast to ∆nag that secreted increased levels of the pantothenate monomer pantetheine. This suggests ∆nag increase intracellular levels of acetyl coenzyme A via pantothenate hydrolysis to yield pantetheine monomers; coenzyme A precursors with increased bioactivity (Keefe et al, 1995). Alternatively, the abundance of pantetheine within the ∆nag secretome may suggest failure in the
incorporation of (phospho)pantetheine essential for ACP activity in polyketide and FA synthesis. Coupled with the fact GD12 contains a gene that encodes the protein phosphopantetheine (Studholme et al, 2013). It could be inferred that low levels of pantetheine in GD12 imply SM synthesis reduces such levels and instead increases SM production by polyketide activity as opposed to Δnag that failed to up-regulate as many SMs as GD12.

Essentially, NAG impairment contributes to less effective BCA that, in turn, reflect the myriad of roles NAG play within fungal and plant systems. MYC and NOD factors are essential for cellular immune responses with a generic structure Composing of three to five NAG monomers in the active site (Cullimore et al, 2001). NAG is effective against plant tumours in a dose dependant manner (Richardson et al, 2013) and that it is known NAG elicit the HR response in plants that consequently compromise plant growth thus explaining reduced growth rates in GD12-treated lettuce as opposed to Δnag–treated plants. However, others confer chitosan treatment leads to increased lignin type compounds in plants ultimately resulting in stem strengthening (Hadwinger et al, 1984). However, NOD factors have been associated with PGP properties in pea (Siczek et al, 2013).

In this chapter we have proven GD12 and Δnag display different secondary metabolite profiles when cultured independently under no disease pressure. Furthermore, GD12 appears to up-regulate the MVA as opposed to MEP pathways and that a high volume of fungicide compounds were recovered. In order to determine differences in genomic content and transcription rates, the following chapter addresses the GD12 and Δnag genome via bioinformatic approaches. SM regulation and pleiotropic effects on phenotype may highlight any discrepancies; especially after hygromycin cassette disruption during NAG insertion mutagenesis.
Chapter 6 Bioinformatic identification and analysis of putative secondary metabolite-associated genes in the GD12 genome

6.1 Introduction

6.1.1 Coding sequence alignment from a bioinformatic perspective

DNA provides the blueprint of an organism and encodes the genetic potential to adapt to different environmental challenges. Secondary metabolites (SMs) are specialised proteins responsible for species-specific functions not essential for survival. For comparing secreted SM against genome content, protein sequences provide information available at the time of expression and are easily comparable. Amino acid sequences contain both physiochemical information about the protein itself and provide inferences on how related proteins have evolved to differ from homologues from related organisms. For example, tBLASTn analysis is used to translate all six possible reading frames into their cognate amino acids to facilitate identification of proteins. Post-transcriptional events such as splicing, methylation, acylation or protein folding (chaperonin activity) mean the final protein composition will differ in content to the original DNA sequence.

Current comparative proteomic methods depend on sophisticated alignment tools to deduce potential structural and evolutionary relationships between species (Zhang et al, 2000; Altschul et al., 1997). Predicted Open Reading Frames (ORFs) are compared against known/validated sequences to provide insight into potential function before experimental quantification can determine the true identity. De novo analysis of entire genomes results in numerous contigs representing sections of DNA containing ORFs. In this instance, the T. hamatum GD12 genome was fully sequenced in-house using Illumina HiSeq paired end sequencing followed by
de novo assembly using the Velvet version 1.1.04 software to produce a number of putative contigs with unverified mapping data, ie ‘nodes’ (Zerbino & Birney, 2008). This GD12 genome has been submitted to NCBI under the taxonomic identifier 1247866 and GenBank accession number AY247559.

6.1.2 Trichoderma atroviride database construction
Aspergillus and Trichoderma spp. are filamentous ascomycetes contained within the Pezizomycotina sub division. Recent bioinformatic analysis confirm two major divergence events have produced sister classes; Eurotiomycetes (genus Aspergillus) and Sordariomycetes (genus Trichoderma) where consequently, both species are phylogenetically similar based upon morphology, physiology and molecular data (Figure 6.1) (Hao et al, 2009). On a molecular level, Trichoderma and Aspergillus share transcription functional homology as witnessed upon targetted gene disruption assays. Here, increased cellulase and β- glucosidase expression in A. nidulans were introduced to the T. reesei genome producing a functional homologue (Denton & Kelly, 2011; Nakazawa et al, 2012). Consequently, Trichoderma and Aspergillus are often used as comparative gene sequence databanks for enzyme and gene characterisation (Kubicek et al, 1996; El-Bondkly, 2006; Schuster et al, 2011). However, GD12 affords a unique genome with more similarities at the DNA level to T. atroviride than T. reesei where it was found 52% of the GD12 genome sequence aligned with T. atroviride and only 6% aligned against the T. reesei genome (Studholme et al, 2013). Because of this, T. atroviride is preferred to T. reesei for inferring function of proteins, thus explaining their continued use as comparative protein homologues (Voight & Wostemeyer, 2000; Drouin et al., 1995).
Figure 6.1. Two phylogenetic trees each depicting actin gene homology. (1A) compares coding sequences from a wide range of taxa (Drouin et al, 1995) and 1B) concern fungi only (Voigt & Wostemeyer, 2000).
6.1.3 Secondary metabolite quantification

Fungal SM coding sequences are clustered at specific regions within the genome. PolyKetides (PKs), Non- Ribosomal Peptides (NRPs) and fatty acids are synthesised in a similar fashion and are contained within a series of domains within DNA (Martinez et al, 2008, Reyes-Dominguez, 2010). Post-translational modifications produce bioactive compounds with subtle structural differences (Maymon et al, 2009). Chapter five discussed the variability between PKS and NRPS cluster frequency of different fungal genera and moreso within Trichoderma (Kubicek et al, 2011). However, filamentous fungi control SM biosynthesis by chromatin regulation that is mediated by hepA, hdaA and laeA genes; genes associated with pathogenicity (Reye et al. 2010). Nonetheless, protein differentiation and activity can be determined by chaperonin activity, which is important for correct function, otherwise inactive or increased toxicity occurs (Mayer, 2010).

Chaperones are specialised protein complexes that fold polypeptides into the correct three dimensional structures. Chaperones contain the subunits GroEL, GroES or other Heat Shock Proteins (HSP) that reduce denaturation under adverse conditions by increasing thermal stability. DNA loss-of-function analysis reveals that reduced HSP ability affected iron superoxidedismutase activity thus reducing chloroplast superoxide dismutase (Kuo et al, 2013).

IAA is a tryptophan-based indolamine synthesised by the enzyme Tryptophan Synthase [Bifunctional] (TRPB). Indoleamines are essential in fungal SM production and present in all microorganisms but not within the Animalia genome. IAA-like compounds are deployed by bacteria for virulence or endophytic reasons and upon mutagenesis, these interactions were attenuated (Chu et al, 2011; Taté et al, 1999). Chapter five shows GD12 and Δnag promote root formation as a result of the
putative IAA derivatives detected. Therefore, as a continuum of SM biosynthesis, the GD12 genome will be mined for these coding sequences to determine if strong evolutionary traits exist between *A. nidulans*, *T. atroviride* and *T. hamatum*.

As a concluding chapter to this work, potential coding regions contained within the GD12 genome were studied, focussing on genes encoding SM production. This report will incorporate the recently sequenced GD12 genome where a 40-fold coverage show most coding sequences recovered display a 15-fold to 30-fold coverage (Studholme *et al*, 2013).
6.2 Material and Methods

6.2.1 Fungal protein sequence dataset construction

In order to identify *Trichoderma atroviride* coding regions for comparative analyses against *T. hamatum*, a simple dataset containing specific *A. nidulans* genes was constructed as a mining tool (Galagan *et al.*, 2005). In order to achieve this, the ‘Aspergillus Comparative Database’ was accessed via the home page of BROAD Institute (http://www.broadinstitute.org/). Here, genes identified within the *A. nidulans* fully annotated genome were used in order to determine corresponding protein sequences in *T. atroviride* (US Dept. of Energy’s Joint Genome Institute (JGI) website; http://genome.jgi-psf.org/ (Nierman *et al.*, 2005).

A BLAST alignment on the *T. atroviride* v2.0 databank(s) followed using *A. nidulans* protein sequences as templates to include masked, unmasked, All Gene Models and Gene Catalog databanks for tBLASTn analyses. Additionally, images of linear plots and hit scaffolds were recorded and all nucleic sequences were later cross referenced against their protein sequence counterparts for correlation. Once protein sequences of *T. atroviride* were downloaded they were subsequently used as a reference in a BLAST search against the GD12 and Δnag genome.

6.2.2 *Trichoderma hamatum* database mining

In order to determine the location of specific genes within the Trichoderma genome, a local windows-based alignment tool (file name: blast-2.2.26-x64-win64.exe; was downloaded from the NCBI website (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/release/LATEST/) (Zhang *et al.*, 2000; Altschul *et al.*, 1997). Once the *T. hamatum* reference sequences were formatted, each *T. atroviride* sequence file was searched against *T. hamatum* (Figure 6.2). Statistical parameters were set at $p<1.0E-05$ with a maximum of 250 alignment hits.
Figure 6.2 Screenshot of tBLASTn alignment program performed on host computer. Contents of the BLAST folder are shown in upper screen (red circle) whilst the lower screen are programming codes used for tBLASTn alignment using the ‘formatdb’ and ‘blastall’ command prompts against the GD12 genome as a reference sequence. This procedure was repeated for the ∆nag reference genome sequence (DNA) using T. atroviride protein sequences as mining tools.
6.3 Results
6.3.1 Genome overview of *Trichoderma hamatum*

Complete genome sequence alignment of GD12 and Δnag was carried out to determine SM clusters frequency and position. Once *A. nidulans* sequences were obtained from the BROAD institute (Galagan *et al.*, 2005), they were used as a template for mining *T. atroviride* sequences (Kubicek *et al.*, 2011). Once in place, alignment analyses bewteen *T. atroviride* and *T. hamatum* sequences followed. Here, with a 40-fold coverage, the GD12 database show 11643 potential Open Reading Frame (ORF) sequences consisting of 38.176⁶ bases. Overall, the GD12 genome is larger than those of *T. reesei* and *T. atroviride* and similar in size to *T. virens*. Of the Trichoderma strains displayed, GD12 has the largest number of predicted genes with undetermined mean gene length and exons per gene (Table 6.1).

**Table 6.1** Genomic content of reference genomes used within this chapter. *denote incomplete annotated genome content at time of press.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genome (Mb)</th>
<th>n Contigs</th>
<th>n Genes</th>
<th>Mean aa length</th>
<th>Exons/gene</th>
<th>% GC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. nidulans</em></td>
<td>30.07</td>
<td>248</td>
<td>10701</td>
<td>485</td>
<td>3.07</td>
<td>50.32</td>
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<tr>
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<td>29</td>
<td>11863</td>
<td>455</td>
<td>2.86</td>
<td>49.78</td>
</tr>
<tr>
<td><em>T. reesei</em> QM6a</td>
<td>33.45</td>
<td>89</td>
<td>9129</td>
<td>469</td>
<td>3.1</td>
<td>52.81</td>
</tr>
<tr>
<td><em>T. virens</em></td>
<td>38.77</td>
<td>93</td>
<td>11643</td>
<td>471</td>
<td>2.93</td>
<td>49.44</td>
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<tr>
<td>GD12</td>
<td>38.18</td>
<td>73*</td>
<td>12391</td>
<td>-</td>
<td>-</td>
<td>48.6</td>
</tr>
</tbody>
</table>
6.3.2 Genomic distribution of polyketide synthesis genes

*A. nidulans, T. atroviride* and *T. hamatum* display similar number of gene copies for SM production, more specifically polyketide biosynthesis. Coding sequences for Acyl Carrier Protein (ACP), Acyl Transferase (AT), DeHydratase (DH), β-keto Reductase (KR), Enoyl Reductase (ER), Thioesterase (TE)/ (SH), Methyltransferase (MT), PKS and NRPS were compared. Here, all three genomes (*T. atroviride, A nidulans* and GD12) contain one ACP and DH gene sequences except ∆nag that did not produce an alignment (Table 6.2). Protein sequences repeated throughout the genome include KR, MT, PKS and NRPS that were found in higher frequencies in *A. nidulans T. atroviride* and GD12 but less in ∆nag because of insufficient coverage.

**Table 6.2** Gene sequences for proteins associated with secondary metabolite biosynthesis. *A. nidulans* and *T. atroviride* were deployed as reference genomes for data extraction within the *T. hamatum* GD12 and ∆nag genomes.

<table>
<thead>
<tr>
<th>Protein</th>
<th>n residues</th>
<th>Interpro</th>
<th>GD12</th>
<th>∆nag</th>
<th>A. nidulans</th>
<th>T. atroviride</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACP</td>
<td>139</td>
<td>IPR009081</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>AT</td>
<td>421</td>
<td>IPR014043</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>DH</td>
<td>557</td>
<td>IPR013328</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>KR</td>
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<td>13</td>
<td>41</td>
<td>37</td>
</tr>
<tr>
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<td>415</td>
<td>IPR014358</td>
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<td>0</td>
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<td>9</td>
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<td>972</td>
<td>IPR013624</td>
<td>21</td>
<td>21</td>
<td>9</td>
<td>11</td>
</tr>
</tbody>
</table>
Figure 6.3 Alignment profiles displaying ACP and AT homology. ACP (A) and AT (B) upper alignment displays the protein sequence gene location within the *T. atroviride* genome. Lower profile is alignment of GD12 against *T. atroviride* by tBLASTn (unmasked); alignment legend is displayed to the upper right.
Using tBLASTn alignment, a high degree of conservation between T. Atroviride and A. nidulans transcript of ACP (IPR009081) and DH exist (IPR013328) (p=0). Although all four genomes contained one AT (IPR014043) gene, the alignment profile was not as robust as ACP and DH (p=2.84E-12) (Figures 6.3 and 6.4). Overall, GD12 contained multiple copies of ACP, AT and PKS-like DH within individual nodes (p<1.0E-016) while T. atroviride and A. nidulans did not (Figure S6.1).

BLAST alignment profiles with ER (IPR014358) show T. atroviride and A. nidulans with one (duplicated) copy on 1 node while GD12 contained three, each on individual nodes (Table 6.2). The ER protein alignment between T. atroviride and A.

Figure 6.4 Alignment profiles demonstrating the DH sequence. Upper alignment is the protein sequence gene location within the T. atroviride. Lower alignment show GD12 against T. atroviride by tBLASTn (unmasked); alignment legend is displayed to the upper right.
Figure 6.5 Alignment profiles displaying the ER gene sequence. (A) is the alignment between *T. atroviride* (upper contig) and *A. nidulans* (lower contig). Below each alignment is the gene location within the *T. atroviride* genome that was aligned against GD12 by tBLASTn (unmasked); alignment legend displayed upper right.
nidulans remain highly similar ($p=4.99\times10^{-12}$; Figure 6.5A). Upon closer inspection, T. atroviride ER sequence contain introns while GD12 remain continuous ($p=0$; Figure 6.5B).

The TE (IPR001031) sequence was more conserved between T. atroviride and A. nidulans than ER ($p=4.42\times10^{-16}$; Figure 6.6A). The $\Delta$nag genome failed to align as a consequence of incomplete sequencing while GD12 reveal three copies of TE, each on individual nodes (contigs) with +1 frameshift and 96% identity ($p<1E^{-131}$; Figure 6.6B) (Supplementary Figure S6.1).

Alignment profiles confirm T. atroviride and A. nidulans each contain three SH (IPR002155) coding sequences while GD12 and $\Delta$nag display seven and one SH alignment respectively (node 1821). All SH were contained within separate nodes with high similarity ($p<1E^{-95}$). GD12 display seven SH ORFs over four nodes where two nodes contained multiple copies ($p<1E^{-132}$). In contrast, T. atroviride and A. nidulans display duplicated SH sequences within the same nodes.

MT (IPR013217) sequence alignment show 16 and 22 predicted MT sequences times within the T. atroviride and A. nidulans genome respectively. GD12 and $\Delta$nag each contain 24 and five distinct nodes each containing a number of MT sequences. T. atroviride and A. nidulans alignments reveal PKS-MT copies were contained within separate loci and distributed throughout the genome with close alignment ($p<3E^{-95}$; Figure 6.7A). Of these, three aligned with high homology ($p=0$). GD12 contain 41 MT sequences distributed over 24 nodes in which nine were perfectly aligned ($p=0$; Figure 6.7B), most were highly similar ($p<1E^{-60}$) and five display little homology ($p<1E^{-60}$).

T. atroviride and A. nidulans contained 12 and 16 individual loci containing 41 and 37 KR (IPR001395) respectively. Of these KR sequences, most show
Figure 6.6 Alignment profiles highlighting TE gene similarity. (A) is the alignment between *T. atroviride* (upper contig) and *A. nidulans* (lower contig). Below each alignment is the gene location within the *T. atroviride* genome that was aligned against GD12 by tBLASTn (unmasked); alignment legend displayed upper right.
Figure 6.7 Alignment of the MT sequence. (A) display the *T. atroviride* (upper contig) and *A. nidulans* (lower contig) sequences. (B) MT location within the *T. atroviride* genome and the alignment of *T. atroviride* against GD12. tBLASTn (unmasked) alignment; legend displayed upper right.
moderate homology ($p<1\text{E}-030$). In contrast, GD12 and $\Delta n$ag each display 25 and 13 KR sequences spanning over five and four nodes respectively; with reduced similarity ($p<6\text{E}-009$). However, GD12 encodes one KR sequence on node 3715 with excellent alignment to the $T. \text{atroviride}$ sequence ($p<1\text{E}-178$).

6.3.3 Polyketide Synthase multi complex enzyme

PKS (IPR013968) alignment analysis reveal $A. \text{nidulans}$ and $T. \text{atroviride}$ each contain 29 and 19 PKS clusters compared to GD12 that display 33 and 9 PKS clusters respectively. Of these, $A. \text{nidulans}$ and $T. \text{atroviride}$ contain two alignments identical at the amino acid level while others show high alignment ($p<1\text{E}-126$). $T. \text{atroviride}$ PKS cluster distribution show nine different contigs (Figure 6.8B) while GD12 and $A. \text{nidulans}$ display 24 and 13 nodes respectively. $A. \text{nidulans}$ and $T. \text{atroviride}$ contain 3 PKS hits aligned in one contig (Figure 6.8A). Although the $T. \text{atroviride}$ are distinct compared to $A. \text{nidulans}$ and GD12, $T. \text{atroviride}$ show even distribution within the genome. GD12 contain 10 nodes, each with more than two PKS clusters (Figure S6.2).

6.3.4 Non-Ribosomal Peptide Synthase identification

$A. \text{nidulans}$ and $T. \text{atroviride}$ reveal nine and seven NRPS (IPR013624) clusters respectively where only one sequence reveal perfect alignment on contig 23 ($T. \text{atroviride}$) ($p=0$; Figure 6.9A). All other alignments showed less conservation between the species with incomplete NRPS sequences and lower probability values ranging between $p<6\text{E}-07$ and $p<4\text{E}-24$. Similarly, GD12 displays approximately 37 NRPS arranged over 21 nodes; including repeats and incomplete alignments ($p<5\text{E}-06$ to $p<2\text{E}-41$; Table S6.1). GD12 has one perfect alignment at the amino acid level (node 4386; $p=0$). Furthermore, three and six PKS/NRPS hybrid clusters were found in $A. \text{clavatus}$ and GD12 respectively.
Figure 6.8 PKS protein sequence alignment. (A) is between *T. atroviride* (upper contig) and *A. Nidulans*; (B) is the gene location within *T. atroviride* genome and the alignment between GD12 and *T. atroviride* by tBLASTn (unmasked) alignment; legend displayed in upper right.
Figure 6.9 Alignment of NRPS protein cluster sequence. (A) is between T. atroviride (upper contig) and A. nidulans (lower contig). (B) Position of each NRPS cluster within the T. atroviride genome and alignment between GD12 and T. atroviride using tBLASTn (unmasked) alignment; legend is displayed to the upper right.
Figure 6.10 LaeA protein sequence alignment. (A) is between *T. atroviride* (upper contig) and *A. nidulans* (lower contig). (B) LaeA sequence position within *T. atroviride* only. (B) Alignment profile between *T. atroviride* and GD12 using tBLASTn (unmasked) alignment; legend is displayed to the upper right.
6.3.5 Combinatory factors of protein expression and clustering

Other proteins associated with SM productivity were determined via BLAST search including LaeA, GroES, bifunctional tryptophan synthase (TRPB) or antibiotic biosynthesis components (dTDP-glucose 4,6-dehydratase, Vancomycin; bleomycin and MDRP). Initial alignments between A. nidulans and T. atroviride show two and 12 ORFs for the LaeA gene respectively (Figure 6.10). T. atroviride display two contigs with duplicated and incomplete copies of LaeA while GD12 contained four copies on four nodes with varying sequence similarity in the protein sequence (node 7251, \( p=0; 6493, \ p=2E-81; 558, \ p=2E-41; 6219, \ p=6E-11 \)).

The BLAST search for GroES (IPR011032) protein coding sequences found one hit in A. nidulans \( (p<5E-40) \) and six in T. atroviride of which three are highly significant \( (p<5E-40) \) and three are less so \( (p<1E-07; \) Figure 6.11A). GD12 contained two hits, both on different nodes with different alignment scores. The GD12 chaperonin sequence in node1893 \( (p=1E-45) \) display more aligned amino acids than the sequence found on node 7288 \( (p=2E-32; \) Figure 6.11B). Both T. hamatum GroES sequences contained 14% gaps (Figure S6.1).

BLAST analysis confirms that the T. atroviride genome contains two copies of bifunctional tryptophan synthase (TRPB) within two different contigs (IPR008244). Of these, contig 24 show 100% sequence similarity while contig 21 was incomplete (Figure 6.12A). Similarly, A. nidulans displayed two hits associated with tryptophan biosynthesis; one TRPB and one tryptophan synthase with reduced alignments score on the tryptophan synthase. The T. hamatum hits show GD12 contain two copies of TRPB on node 5330 \( (p=0; \) Figure 6.12B) with 97-99% positive identities and a -1 to -3 frameshift in some locations.

A number of alignments corresponding to antibiotic synthesis were determined.
Figure 6.11 Alignment of the GroES protein sequence. (A) is the alignment between *T. atroviride* (upper contig) and *A. nidulans* (lower contig) while (B) display position within *T. atroviride* genome and alignment against GD12 using tBLASTn (unmasked) alignment; legend shown in upper right.
Figure 6.12 TRPB protein sequence alignment. (A) is between *T. atroviride* (upper contig) and *A. nidulans* (lower contig); (B) TRPB location in *T. atroviride* and alignment between GD12 and *T. atroviride* using tBLASTn (unmasked) alignment; legend is upper right.
Figure 6.13 dTDP-glucose 4,6-dehydratase protein sequence alignment. (A) is between T. atroviride (upper contig) and A. nidulans (lower contig). (B) Protein location within the T. atroviride genome and alignment of T. atroviride against GD12 using tBLASTn (unmasked) alignment; legend is upper right.
Protein coding sequence for vancomycin group of antibiotics display 2 coding sequences for dTDP-glucose 4,6-dehydratase (IPR005888) were recovered from *T. atroviride* (Figure 6.13B) and one in *Aspergillus* spp. and *N. fischeri*. Other fungi such as *A. oryzae* contain one identified copy and two putative copies compared to *A. nidulans* that failed to show any alignments; hence the alignment with *A. oryzae*.

In GD12 and Δ*nag* three and one BLAST hits were retrieved respectively. GD12 hits were found in three nodes with node 4563 having the highest e-value with 84% positive identifications (*p*=0). Nodes 5623 and 3246 were less similar and contain 13% gaps (*p*<7E-10). Hygromycin biosynthesis was investigated where no alignment hits were recovered from *T. atroviride* or GD12 regardless if using *Streptomyces hygroscopicus*, *E. coli* or *G. zeae* compound coding sequences. On the other hand, the protein sequence encoding for bleomycin biosynthesis (IPR004360) show one accurate alignment in both *T. atroviride* and *A. nidulans* (*p*=0) while GD12 contains one hit on node 5142 (*p*<9E-050) (data not shown).

**Multi-Drug Resistance Protein** (MDRP) alignment between *A. nidulans* and *T. atroviride* shows high similarity (*p*<3E-089) (Figure 6.14). *A. nidulans* and *T. atroviride* each display four and 16 MDRP coding regions respectively. *A. nidulans* reveal three identical hits whilst the other hits are not so confident (*p*<3E-50). In contrast, GD12 displays 10 nodes each containing a coding sequence varying in probability values with 50% to 63% positives and a mean 3% gap (*p*<3E-06 to *p*<2E-69).

### 6.3.6 Potential secondary metabolite gene clustering in GD12

A combination of genetic coding regions associated with SM biosynthesis contributes to SM clustering. By comparing and aligning regions of high coding densities, it is possible to potentially identify approximate genomic locations in relation to each other; although this needs further quantification.
Figure 6.14 Alignment analyses of the MDRP protein sequence. (A) is alignment between *T. atroviride* (upper contig) and *A. nidulans* (lower contig). (B) is the coding sequence location within the *T. atroviride* genome that was aligned against GD12 using tBLASTn (unmasked) alignment (partially shown); legend shown in upper right.
Protein coding regions within the GD12 genome appear clustered while PKS, MT and NRPS codes are distributed evenly throughout the genome. PKS clusters are adjacent to an MT sequence in at least 16 clusters out of a possible 33 PKS sequences. In contrast, MT only shared the same node with NRPS four times and this was in conjunction with a PKS sequence (see supplementary figures).

NRPS clusters were frequent between nodes 435 and 2284. More specifically, regions of high density NRPS clusters appear between nodes 1312 to 1713 (23 NRPS) and nodes 1929 to 2284 with 11 NRPS clusters. However, specific clusters exist within the GD12 genome and four potential PKS/NRPS clusters appear on node 3835, 5087, 5766 and 7104. Furthermore, three putative PKS/NRPS clusters were located at nodes 4143/4140, 4768/4798 and 6007/6017 (≤30 nodes apart) (Supplementary figure S6.1). These were assigned putative because the GD12 genome has not been fully quantified with chromosome numbers and defined contigs. Until this has taken place, it cannot be verified if these are, in fact, PKS/NRPS hybrid clusters or individual modules arranged concurrently. In contrast, LaeA sequences are located at extreme ends of the genome read (nodes 558 and 7251) with two copies on node 6219 and 6493.

6.4 Discussion

SM gene clusters are found in all filamentous fungi and usually expressed under specific conditions. Actinorhodin was the first complete PKS–type SM identified in S. coelicolor and since then, many more PKS and NRPS-like SMs have been identified, located and characterised (Malpartida & Hopwood, 1984; Manning, 2013; Collemare et al, 2008; Manning et al; 2013). Comparative genomic analyses between various Trichoderma strains show diversity exists. This is to say differences in genome size and content reflect the role each play within their respective niches. Genomic and
proteomic studies confirm *T. atroviride*, *T. virens* and *T. reesei* share between 78-96% conserved gene order. However, upon further inspection, *T. hamatum* is more closely related to *T. atroviride* than most Trichoderma strains (Kubicek *et al.*, 2011). Therefore, phylogenetic similarities between *A. nidulans*, *T. atroviride* and *T. hamatum* predict where novel sequences not experimentally verified are located.

Since the publication of GD12s genome sequence, investigations can identify novel coding sequences possibly contributing to GD12s biocontrol and plant growth promoting ability. This study has found GD12, *A. nidulans* and *T. atroviride* contain dissimilar NRPS and PKS/NRPS hybrid cluster numbers but with similar PKS-associated domains. This may suggest a series of events have led to higher conservation within coding regions of PKS or just simply that less research has focussed on these NRPS-like clusters. Nonetheless, this analysis suggests that GD12 encodes more NRPS clusters than *A. nidulans* and *T. atroviride*. These were found concentrated between nodes 1312 and 2284 followed by a series of duplication events at nodes 6007 to 7104. Until the GD12 genome is fully annotated, it is not certain if GD12 NRPS sequences cluster in subteleomeric regions in a similar fashion to *A. nidulans* (Schroeck *et al.*, 2009). However, future investigations could ascertain if GD12s unique proteome affords unique genomic coding regions such as the NRPS-derived antibiotics surfactin and fengycin (Studholme *et al.*, 2013).

Alternatively, PKS/NRPS hybrid clusters are less frequent in fungi and are emerging as complex coding regions with no particular sequence similarity. Recent phylogenetic analyses discovered nine PKS/NRPS clusters in *M. grisea* while *Chaetomium globosum* encodes six (Collemare *et al.*, 2008); a similar number found within the GD12 genome. Recently, a supercluster was discovered in *T. reesei* that revealed two NRPS/PKS hybrid clusters followed by a TL, NRPS, P450, DH and a
MFS domain; only found once previously in Ascomycota (Mukherjee et al, 2012).

*T. atroviride* contain single copies of specific PKS housekeeping genes (ACP, AT, MT, ER) contained within small intragenic regions while TE and DH remained as a continuous code albeit with varying probability values. Uniquely, GD12 showed multiple copies of ER and TE within the same node while the ER sequence was repeated three times between nodes 5050 and 6644; thus suggesting their organisation is conserved within the same scaffold despite no formal quantification of the GD12 chromosomal structure. It is suggested repetitive elements are associated with novel gene development and the rate in which this occurs is not uniform, but determined by speciation events (Manning et al, 2013). Here, transduplication events were predicted for NRPS, MT and other PKS-associated genes found in GD12 implying increased SM production and divergence; a property not all filamentous fungi display, especially strains of Trichoderma that are more adept to a cellulolytic lifestyle (Martinez et al, 2008).

In contrast, all the multi drug resistance, methylation, protein folding and SM production (MDRP, MT, GroES and LaeA) sequences were randomly distributed throughout the scaffold in GD12 and *A. nidulans*, while remaining clustered with fewer introns in *T. atroviride*. Approximately 50% of LaeA genes in *Aspergillus* spp. are subtelomeric including an LaeA-regulated supercluster recently described in *A. fumigatus* (Anderson et al, 2011). The function of this supercluster remains unclear. Here we report a putative super cluster in GD12 (nodes 3835-3857 and 4336-4386) warranting further investigation. However, it must be noted that the GD12 genome has not been fully verified with definitive scaffolds or chromosomal location and that coding regions are referred as ‘nodes’. Therefore, assigning the task of defining telomere locations or definitive SM ORF positions with relation to these
telomeres remain inconclusive. Regardless, this Chapter has set out to determine if specific trends exist within the GD12 genome when compared against others such as *A. nidulans* and *T. atroviride*. In contrast to the 9-fold coverage genome shotgun sequencing of *T. reesei* (syn. *Hypocreopsis jechorina*) (Martinez et al, 2008), here our analysis include a 40-fold coverage of GD12 with most coding sequences identified as showing 15-fold to 30-fold coverage (Studholme et al, 2013).

In conclusion, this chapter has confirmed previous reports of PKS, NRPS and PKS/NRPS cluster frequency found within other filamentous fungi (Kubicek et al, 2011; Mukherjee et al, 2012). Furthermore, this chapter suggests that variability in exon duplication and intron frequency occurs within these genomes and that GD12 show more similarity to *A. nidulans* than *T. atroviride* for the components involved in the synthesis of the secondary metabolites investigated here. Our initial investigations may highlight specific trends that exist between these fungi and that their individual lifestyles reflect their genome. The fact remains, further analysis of the genome coupled to a detailed transcriptomic study is needed in order to fully determine the proteinous compounds responsible for plant growth promotion and biocontrol.
Chapter 7 General Discussion

7.1 General Discussion

*Trichoderma* spp. are acknowledged for their PGP and biocontrol bioactivity (Chet *et al*, 1981; Shoresh and Harman, 2008; Yang *et al*, 2012). Often commercially exploited in agriculture, medicine and industry it was assumed enzyme activity was responsible for PGP and BCA via lysis activity (Veal & Lynch, 1984; Hayes *et al*, 1992; Intra *et al*, 2008; Sadhasivam *et al*, 2010; Feng & Ling, 2010). Therefore, to test the nutrient release theory, a unique *T. hamatum* strain (GD12) was recovered from Whiddon Down in Devon (UK) and underwent mutagenesis to create the hexosaminidase deficient mutant *Thnag::hph (Δnag)* (Ryder *et al*, 2012). Upon nutrient composition analysis, no significant difference was found in soil mineral levels. Suggesting nutritional chitinolysis is not directly responsible for PGP. However, Δnag displayed greater PGP effects than GD12 which led to the hypothesis the hypersecretory phenotype and diffusible proteins are responsible.

Few reports discuss the fungal secretome and its effect on plant health and phytopathogen control. Furthermore, there is little known about the BCA and PGP mechanism, nor has this been fully explored on a proteomic and genomic level. As a consequence, the intention of this study was to determine the molecular basis of *T. hamatum* upon plant growth and biocontrol. For this purpose, I conducted a number of bioassays to determine the PGP and BCA of GD12 and Δnag against the fungal phytopathogens responsible for pre- and post-emergence damping-off disease *Sclerotinia sclerotiorum* and *Rhizoctonia solani* respectively. I also set out to determine if the PGP and BCA effects of GD12 were dose-dependent and if these effects persist in soil.
In Chapters six and three, I show GD12 has a unique genome that upon alteration, shows greater PGP qualities in a dose dependent manner (Ryder et al, 2012; Studholme et al, 2013). As a solid application, Δnag displayed no toxic effects at higher doses which is in contrast to GD12 (Thimann, 1937; Vinale et al, 2008; Daoubi et al, 2009). However, this report clearly prove that fine filtration (<0.2 μm pore size) of bran-inoculum extract reduced GD12 mycelium toxicity in soil for both lettuce and tomato plants (Tucci et al, 2011). This suggests potential toxins are larger and easily removed from a crude mixture.

Root and shoot morphology are determined by nitrogen or carbon availability respectively and in this report I reveal S:R differences occur as a result of application type (Andrews et al, 1999; Quintero & Bowers, 2012; Shangguan et al, 2004). Here, the re-used soil bioassay and bran extracts significantly increased root biomass and, consequently, reduced S:R in the process. Interestingly, GD12 and Δnag bran inoculum oppose this trend and increased S:R in a dose dependent manner thereby increasing shoot mass at the expense of root. Root architecture is influenced by auxins that significantly increase lateral root formation and root biomass (Contreras-Cornejo et al, 2009; Zhang et al, 2012). In addition, low soil phosphorus levels are known to increase root mass at the relocation from shoots thus reducing S:R (Hammond & White, 2011; Liu et al, 2009). Considering T. viridae increases phosphate solubilisation (Rawat & Tewari 2011), and the T. harzianum genome encode for the phosphatase gene (Leitão et al, 2010), it is possible GD12 increase phosphorus availability thereby reducing root mass as a consequence. Incidentally, Chapter 5 confirmed that GD12 afforded D-myo-Inistol tetraphosphate and phytic acid while Δnag secreted the inorganic ion phosphate, suggesting plant phosphorus uptake is more rapid with Δnag. Other compounds synonymous with PGP
are auxins and GA. Here, GD12 afforded GA₂ and auxin-like compounds expressed at lower frequencies with very similar structures to phytohormones. In contrast, I have shown GD12 is a less effective PGP while secreting increase levels of two putative growth inhibitors (>5-fold; hyaphorine A and koninginin A) (Cutler et al, 1999; Mori et al, 2002). In relation to Δnag, I reveal nitrogen based compounds with similar skeletal structure to indoleamine are secreted; a potent tryptophan-based compound structurally related to IAA (Gravel, 2007; Bisewska et al, 2012; Wahyuni et al, 2013; Zhang et al, 2013). Furthermore, alignment analysis confirms the enzyme TRPB is highly conserved between all three fungi. Nonetheless, SM clusters are strongly associated with filamentous fungi; more so in the biocontrol associated Trichoderma strains (Martinez et al, 2008). Little is known about the pleioptropic effects of HEX1 mutation upon a strain of T. hamatum and less is known of any biocontrol ability.

Chapter four set out to determine the BCA of GD12 and Δnag where it was discovered NAG impairment significantly reduced BCA against two well established phytopathogens. As a mycelium application, GD12 inhibits S. sclerotiorum and R. solani mycelium and sclerotia formation in a similar fashion to T. viridescens (Amin et al, 2010). Alternatively, Δnag mycelium application resulted in some reduction of sclerotia and spore numbers in S. sclerotiorum; supporting the hypothesis that increased NAG expression is crucial in biocontrol (Steindorff et al, 2012). In contrast, GD12 and Δnag metabolite extracts fail to provide any biocontrol; supporting the theory that physical interactions must take place for biocontrol to take place (Whipps, 1987; Kim & Knudsen, 2008).

I have shown S. sclerotiorum biocontrol is impaired upon HEX1 mutation and no simultaneous PGP effects are witnessed (Romão-Dumaresq et al. 2012; Metcalf et al, 2004; Villalta et al, 2004). In contrast, I present data that suggests Δnag is an
efficient BCA against *R. solani*. Few reports show simultaneous PGP and BCA and of those, a <40% gain (Elad *et al.*, 1980; Singh, 1991; Dubey *et al.*, 2009). This is species-specific as justified by a 6-fold gain witnessed when the *T. harzianum* MDRP mutant was co-inoculated with *Macrophomina phaseolina* (Nagamani *et al.*, 2011; Leta & Selvaraj, 2013). Ultimately, our results confirm that GD12 displays impressive PGP and BCA when co-inoculated in soil as a mycelium application with *S. sclerotiorum* and *R. solani*. Uniquely, I confirm that GD12 mycelium application provides adequate protection against increasing *S. sclerotiorum* concentrations maximising at 10% inoculation (w:w). Others fail to report such findings and instead, show reduced biocontrol effects as pathogen inoculum increase (Elad *et al.*, 1980; Singh, 1991); thus illustrating the powerful BCA of GD12. I report that GD12 proliferation occurs as a direct result of increasing *S. sclerotiorum* inoculum, thus potentiating a positive feed-back loop with BCA and PGP effects. Moreover, I have shown that upon disease-pressure, GD12 reduces S:R greater than GD12+Ss.

In a similar fashion to *Pseudomonas*, GD12 contains coding sequences for some antibiotics and other resistance-type genes with high conservation. This includes a NRPS coding region for the antibiotic surfactin or fengycin (Studholme *et al.*, 2013). However, the putative biocontrol signatures contained in the bran extract were secreted with no disease pressure; thus potentiating false positives. However, transcriptional comparisons between wild type *T. atroviride* and mutant (M7) reveal defence SM were produced when grown on standard PDA under no disease pressure (Mukherjee *et al.*, 2006). Furthermore, a number of signatures were up-regulated in GD12 and Δ*nag* as a result of anaerobic respiration; confirming the mechanics of a closed biosystem. This suggests biocontrol SM are potentially expressed as a result of increased carbon dioxide and other waste levels.
Physical interactions between *A. nidulans* and the phytopathogen *S. hygroscopicus* up-regulate unique SMs such as orsellinic acid and the lichen SM lecanoric acid in *A. nidulans*; only discovered once before in Ascomycotes (Schroeckh *et al*, 2009). Here, a number of putative signatures were discovered and are of particular interest. Contained within the GD12 secretome were two commercial antifungal compounds prinomide and hymexazol. Investigations to their authorised use show these products are not used against oat bran infections. Therefore, to determine if this is the first reported case of their biosynthesis in Trichoderma, further investigations are needed. Overall, this report suggests that GD12 secrete JA derivatives available for plant assimilation. However, *A. thaliana* display reduced Eth/JA and ABA levels when grown in GD12 amended soil (Studholme *et al*, 2013). The reasons for this are not clear but may involve less effective isomers of JA that are not captured by plants.

Upon NAG mutation, it is clear PGP ability increases while BCA decreased. Additionally, GD12 and Δnag each display unique SM signatures when grown in bran-inoculum. It was hypothesised small soluble secreted proteins were responsible and that SM biosynthesis may play a role in this phenomenon. Consequently, this report confirms the GD12 genome affords similar amounts of PKS coding sequences while displaying more NRPS and PKS/NRPS clusters than *A. nidulans* or *T. atroiride*. This may explain GD12s BCA against *S. sclerotiorum* including sclerotia abolishment. Therefore, to determine how GD12 reduced sclerotia, attempts were made to obtain the coding sequence for the antifungal lipopeptide sclerosin; an effective agent against *S. sclerotiorum* (Berry *et al*, 2012; Li *et al*, 2013). Upon investigation, no sclerosin coding sequence was available and therefore uncharacterised; thus a useful screening tool once the sequence is available.
7.2 Future investigations

Additional future experimental work will include extraction of the bran metabolite filtrate residue to collect any particles >0.2 μm. This way, captured toxins could be assessed in plate assays against a range of pathogens to determine BCA. Similarly, the residue could be quantified using MS or NMR to identify potential signatures.

In order to determine the bioactivity of compounds identified in Chapter 5, further tests will include soil-based bioassays involving crop plants and commercially available compounds.

Radio-active labelled IAA compounds could determine plant assimilation pathways via N$_{15}$ or C$_{13}$ incorporation. This way, root and shoot compartmentalisation could be determined.

The mode of BCA action could be analysed using agar plate confrontational assays between GD12, $\Delta n$ag and Ss. The interaction zone can be excised for RNA determination and proteomic activity.

Disruption analyses involving single copy gene sequences found within the PKS cluster system include ACP, AT, DH or the unusual PKS/NRPS cluster contained in the GD12 genome. Mutant assays against S. sclerotiorum and other pathogens may highlight the effects of SM mutation against GD12s BCA.

To test the hypothesis NAG plays a crucial role in fungal defence systems, genetic manipulation of MYC factors may determine if similar effects of NAG mutation takes place. Moreover, the secretome of such a mutant may exhibit a similar phenotype or PGP effects.
Previous attempts to deduce the identity of bioactive compounds in peat-based microcosms when under disease pressure remain inconclusive. Methods to increase the concentration of metabolites associated \textit{in situ} need improvement before effective MS analysis can take place.
### Table S6.1

Protein coding regions from *T. hamatum* GD12 genome associated with polyketide biosynthesis as determined by BLAST sequencing.

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Saprotrophic competitiveness and biocontrol fitness of a genetically modified strain of the plant-growth-promoting fungus *Trichoderma hamatum* GD12

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Summary

Trichoderma species are ubiquitous soil fungi that hold enormous potential for the development of credible alternatives to agrochemicals and synthetic fertilizers in sustainable crop production. In this paper, we show that substantial improvements in plant productivity can be met by genetic modification of a plant-growth-promoting and biocontrol strain of Trichoderma hamatum, but that these improvements are obtained in the absence of disease pressure only. Using a quantitative monoclonal antibody-based ELISA, we show that a N-acetyl-β-D-glucosaminidase-deficient mutant of T. hamatum, generated by insertional mutagenesis of the corresponding gene, has impaired saprotrophic competitiveness during antagonistic interactions with Rhizoctonia solani in soil. Furthermore, its fitness as a biocontrol agent of the pre-emergence damping-off pathogen Sclerotinia sclerotiorum is significantly reduced, and its ability to promote plant growth is constrained by the presence of both pathogens. This work shows that while gains in T. hamatum-mediated plant-growth-promotion can be met through genetic manipulation of a single beneficial trait, such a modification has negative impacts on other aspects of its biology and ecology that contribute to its success as a saprotrophic competitor and antagonist of soil-borne pathogens. These findings have important implications for the development of GM Trichoderma strains for use in plant agriculture.
INTRODUCTION

*Trichoderma* species are ubiquitous soil saprotrophs that have attracted sustained scientific interest as biological control agents of plant disease. In addition to their biocontrol properties, certain strains have been shown to enhance crop productivity by stimulating plant growth (Contreras-Cornejo et al., 2009; Harman et al., 2004; Ortíz-Castro et al., 2009; Vinale et al., 2008, 2009). While genetic modification of *Trichoderma* strains by constitutive over-expression of chitinase, β-glucanase and proteinase genes has allowed the development of strains with improved biocontrol capabilities (Flores et al., 1997; Baek et al., 1999; Limón et al., 1999; Viterbo et al., 2001; Djonovic et al., 2007), less attention has been paid to enhancing the P-G-P activities of these fungi via genetic modification, and the impact that any such modification might have on their saprotrophic competence and fitness as biocontrol agents.

In a previous study, we showed that a naturally occurring strain of *Trichoderma hamatum* was able to promote plant growth in low pH, nutrient-poor peat soils (Thornton, 2008). These soils contain a significant pool of nitrogen sequestered in chitin of insect and fungal origin (Kerley and Read, 1998), and fungal N-acetyl-β-D-glucosaminidase has been shown to be a key chitinolytic enzyme releasing sequestered nitrogen for assimilation by plants in peat ecosystems (Leake & Read, 1990; Kerley & Read, 1995, 1998; Read & Perez-Moreno, 2003; Lindahl & Taylor, 2004). We hypothesized that a similar mechanism might be driving the *T. hamatum* P-G-P phenomenon, since *T. hamatum* is a well-characterized producer of extracellular chitinases including N-acetyl-β-D-glucosaminidase, an enzyme also shown to be integral to the biocontrol of root-infecting pathogens (Chet & Baker, 1981; Chet et al., 1981; Lorito, 1998; Brunner et al., 2003; Harman et al., 2004). To investigate the role of the enzyme in *T. hamatum* P-G-P, we
disrupted \textit{N}-acetyl-\(\beta\)-D-glucosaminidase production in the fungus by insertional mutagenesis of the corresponding gene and found, contrary to our expectations, that enzyme inactivity dramatically increased the growth-promotional activity of the fungus in sterilized soil systems.

The ability to manipulate the P-G-P properties of \textit{Trichoderma} biocontrol strains holds enormous potential for the development of sustainable alternatives to agrochemicals for plant disease control and for the development of crop growth stimulants. The dramatic increase in \textit{T. hamatum} P-G-P activity as a result of the genetic modification, and the potential for use of the mutant strain as a genetically engineered microorganism with enhanced plant growth stimulant properties, led us to determine whether the mutation conferred an ecological advantage to the fungus during antagonistic interactions with the saprotrophic competitor \textit{Rhizoctonia solani} in soil. Furthermore, we set out to investigate whether its ability to control pre- and post-emergence diseases of lettuce seedlings caused by \textit{Sclerotinia sclerotiorum} and \textit{R. solani} respectively had been modified. Using laboratory-based microcosms, we show that while loss of \textit{N}-acetyl-\(\beta\)-D-glucosaminidase activity imparts a dramatic improvement in P-G-P activity in the absence of plant disease pressure, the mutation has a negative impact on its ability to compete saprotrophically with \textit{R. solani} in soil. Furthermore, the ability of the mutant to promote seedling emergence and growth is constrained by the presence of soil-borne pathogens.

This work demonstrates that trade-offs exist in the genetic engineering of \textit{Trichoderma} strains that exhibit the dual beneficial traits of P-G-P and biocontrol. Genetic manipulation of a single attribute has consequences for other aspects of the organism’s biology and ecology.
Competitive saprotrophic ability and biocontrol fitness of GM *Trichoderma hamatum* GD12

**METHODS**

**Fungal strains, growth conditions, and DNA analysis.** All strains of *T. hamatum* used in this study are derived from strain GD12 (GenBank accession AY247559)(Thornton *et al.*, 2004; Thornton, 2005; Thornton, 2008), and were maintained on potato dextrose agar (PDA) or V8 agar as described previously. The anastomosis group 1 post-emergence lettuce pathogen *Rhizoctonia solani* (CBS323.84) and polyphagous plant pathogen *Sclerotinia sclerotiorum* (GenBank accession FJ984493) were grown on PDA. Gel electrophoresis, restriction enzyme digests, gel blots, and sequencing were performed according to standard procedures (Sambrook *et al.*, 1989).

**PCR of N-acetyl-β-D-glucosaminidase genes and production of enzyme-deficient mutant.** The degenerate primers NagA (GTC CTG (AC) (AG)₅ G(GC)₅ (CT)₃ GA(AG) AC₅ TT(CT) (AT) (GC)₅ CA) and NagB (TTG AG (CT) TC(AG) TC₅ CC(AGCT) CC₅ GT(AG) TG(AG) AA(AG) TA) were designed based on multiple sequence alignments of known *Trichoderma* Nag proteins, and were used to amplify a 575 bp fragment from GD12 genomic DNA. The 575 bp PCR amplicon was used to probe restriction enzyme digests of genomic DNA to determine *N*-acetyl-β-D-glucosaminidase gene copy number and was subsequently cloned into pGEM-T. Positive clones were identified by restriction enzyme digests with NcoI and NotI. To amplify a larger fragment of the *T. hamatum* NAG gene for insertional mutagenesis, primers were designed using the sequenced 575 bp fragment and the *T. harzianum* EXC2Y nucleotide sequence retrieved from the NCBI databases ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). The primer set 30.2 (CGTCATTATTCATTAATAGTTGCC) and M13.52 (TCCTGTGTGAAATTGTTATCCGCTG...
CCAGCATCCAA), and primer set 5.1 (TAGGCACATACTCCTCCCTCTCTC) and M13.32
(GTCGTGACTGGAAAAACCTTGGCGGACGCCATACTC), were used to amplify 1.0 kb and
919 bp of the target gene respectively. Insertional mutagenesis was performed using a fusion-
based PCR method. The HPH gene from Neurospora crassa under the N. crassa TRPC
promoter, conferring resistance to hygromycin, was cloned into pBluescript (Stratagene) as a 1.4
kb EcoRI-Xba fragment. To amplify the split HPH templates, the primer set M13F
(CGCCAGGGTTTCCCAGTCACGA) and HY (GGATGCCTCCGCTCGAAGTA), and
primer set M13R (AGCGGATAACAATTCACACAGGA) and YG (CGTTGCAAGACCTGC
CTGAA), were used. A third round PCR was performed using the nested primers 5.2
(TTGACCAGACGGTCCAGGTAACCT) and 30.1 (GCACATCAACCTGAGATGTGGTGT)
to join the constructs together. T. hamatum GD12 protoplasts were transformed with 2 µg DNA
of the third round PCR product. ∆Thnag::hph mutants were selected for resistance to 300 µg ml⁻¹
hygromycin. The T. hamatum GD12 NAG gene and protein sequence were submitted to
GenBank and an accession number obtained (JN107809).

Complementation of the N-acetyl-β-D-glucosaminidase-deficient mutant. A 4.29 kb
amplicon consisting of the 1.86 kb Thnag ORF, 1.93 kb of promoter region and 0.5 kb of 3’ un-
translated region, was amplified from genomic DNA. The complete gene sequence was obtained
from in-house sequencing of the T. hamatum GD12 genome using an Illumina GA2 sequencer.
Primer sequences for amplification were NagC: AGGGATGAGAGCCTTGATGTATT
and NagD: TTTCCTATAAGAGCGATCTG. PCR was performed in an Applied Biosystems
GeneAmp® PCR system 2400 cycler using Herculase® polymerase in Herculase x10 buffer
(Stratagene). An initial Hot Start and denaturation step was carried out at 94 °C for 5 min
followed by PCR cycling parameters of 94 °C for 30 sec, 50 °C for 30 sec, 72 °C for 5 min (10 cycles), 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 5 min, and 72 °C for 10 min (20 cycles). The resulting 4.29 kb PCR fragment was gel purified using a Wizard® (Promega) kit and cloned into the 3 kb pGEM-T vector. Positive clones were confirmed by restriction digest with ApaI and SpeI. The 4.29 kb Thnag fragment was subsequently liberated from pGEM-T using ApaI and SpeI, and ligated into the pCB1532 vector containing the ILVI gene encoding resistance to sulfonyleurea (Sweigard et al., 1997). The resulting plasmid was used to transform the N-acetyl-β-D-glucosaminidase mutant ΔThnag::hph. Putative ΔThnag::hph:NAG re-transformants were selected for resistance to 1 mg ml⁻¹ sulfonyleurea.

**Determination of chitinase activities.** For enzyme activity assays, fungal strains were grown for 4 d at 26 °C in replicate flasks containing sterilized wheat bran and 1.0% (w/v) chitin (Sigma C7170). Extracts were prepared by mixing the contents of flasks with 50 ml dH₂O for 1 h at 23 °C, followed by centrifugation at 16000 g to remove bran and fungal biomass. N-acetyl-β-D-glucosaminidase activity in polyacrylamide gels was determined according to Tronsmo & Harman (1993) using the substrate 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide, the preferred fluorescent substrate of this enzyme (Duo-Chuan, 2006). For colorimetric estimations of enzyme activities, a commercial chitinase assay kit (Sigma CS0980) was used.

**Spore production and sensitivities to antibiotics.** Mycelium from GD12 and ΔThnag::hph were inoculated onto PDA, on to PDA containing 200 µg ml⁻¹ calcofluor white, or PDA containing 200 µg ml⁻¹ caspofungin. Colony diameters were measured over a 4 d growth period at 26 °C, except for caspofungin plates which were measured over 14 d. Spore production was
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quantified after 21 d using a haemocytometer following suspension of spores in 20 ml dH₂O and filtration through Miracloth (Thornton, 2008).

Plant-growth-promotion and soil nutrient analysis. One litre of sieved (500-1000 µm) Sphagnum moss peat (Shamrock) was mixed with 400 ml distilled water (dH₂O) and sterilized by autoclaving at 121 °C for 15 min. Inoculum of *T. hamatum* was prepared by inoculating sterile autoclaved wheat bran mix (10 g wheat bran and 30 ml dH₂O) with five 5-mm diameter plugs of mycelium taken from the growing edge of PDA plate cultures of the fungus. After incubation at 26 °C for 5 d under a 16 h fluorescent light regime to allow complete colonization of the bran by mycelium, microcosms (120 x 120 x 12 mm) were constructed with 8 g bran inoculum and 300 g sterilized peat (1:37.5 w/w), and were sown with 25 seeds of lettuce (*Lactuca sativa* cultivar Webb’s Wonderful). Microcosms were placed in a fully randomized design in a growth cabinet (Sanyo) at 24 °C with a relative humidity of 95% and a 16 h fluorescent light regime. After 14 d growth, plants were harvested and dry weights of shoots and roots obtained. Differences in dry weights were analyzed by one-way analysis of variance (ANOVA) and post-hoc Tukey tests were used to determine significance. For soil nutrient analysis, replicate microcosms consisting of the peat and bran inoculum mix were assayed by NRM Laboratories (Berkshire, UK) after 14 d incubation under the condition described. Control microcosms consisted of sterilized peat with uncolonized autoclaved bran only. Student’s t-tests were used to determine statistical significance.

Secretion assay and microscopy. Secretion was determined in shake culture experiments by using an enzyme-linked-immunosorbent assay (ELISA) with a *Trichoderma*-specific monoclonal
antibody (mAb) MF2 that binds to an extracellular, constitutively expressed, glycoprotein antigen secreted from the hyphal tip (Thornton et al., 2002; Thornton, 2004). Potato dextrose broth contained in 250 ml flasks was sterilized by autoclaving and the flasks inoculated with five plugs (3 mm diameter) of mycelium taken from the growing edge of PDA plate cultures of the fungi. The flasks were incubated as shake cultures at 26 °C and, at 3-day intervals, culture fluids were collected by straining contents through Miracloth. Fluids were then centrifuged for 5 min at 16000 g and 50 µl samples transferred to microtiter wells for assay by ELISA. There were three replicates for each treatment. Absorbance values in ELISA were converted to units of protein equivalents by using standard curves of chromatographically purified antigen, prepared from doubling dilutions of a phosphate buffered saline solution of the antigen in microtiter wells (Thornton et al., 2002). Dry weights were obtained by drying the collected mycelium to constant weight at 80 °C. For immunofluorescence microscopy, fungi were grown on Teflon-coated glass slides embedded in PDA (Thornton et al., 2002; Thornton, 2004), were fixed and processed using mAb MF2 and FITC-conjugated secondary antibody (Thornton et al., 2002; Thornton, 2004), or were immersed in calcofluor white solution (50 µg ml⁻¹) without fixation (Elorza et al., 1983). Fluorescence of samples was observed by using either a Zeiss Axioskop 2 fluorescence microscope using 495-nm excitation and 500- to 550-nm emission wavelengths or a Zeiss LSM confocal microscope using 488-nm excitation and 505 to 570-nm emission wavelengths.

**Quantification of competitive saprotrophic abilities.** The competitive saprotrophic abilities of *Trichoderma* strains GD12 and 6.1 were quantified during antagonistic interactions with *R. solani* in peat microcosms by using the immunological approach described by Thornton (2004). One litre of sieved (500-1000 µm) sphagnum moss peat (Shamrock) was mixed with 400 ml
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Dilute H₂O and 0.5% (w/v) wheat bran in 2 L flasks and sterilized by autoclaving at 121 °C for 15 min. For preparation of pathogen inoculum, 10 g of white poppy seeds and 5 ml D₂O were autoclaved at 121 °C for 15 min. The seeds were inoculated with five 5-mm plugs of mycelium taken from the leading edge of PDA culture plates, incubated for 15 d at 26 °C, and the colonized seeds air-dried at 23 °C under sterile conditions. *Trichoderma* strains were incorporated as spore inoculum (Thornton, 2004). For the generation of spores, strains GD12 and 6.1 were grown on PDA containing 200 μg ml⁻¹ caspofungin and spore suspensions prepared from 3-wk-old plates as described. Poppy seed inoculum of *R. solani* was added to the sterilized peat at 0.1% (w/v), while *Trichoderma* strains were added as spore suspensions (1 ml) containing 10⁴ conidia ml⁻¹ D₂O. The spores from both *Trichoderma* strains, and the *R. solani* poppy seed inoculum, showed >95% germinability when grown on PDA. The contents of the flasks were mixed thoroughly and the mixtures used to construct microcosms for antigen extraction and assay by *R. solani*-specific ELISA over a 21 d incubation period, using the procedures described previously (Thornton & Gilligan, 1999; Thornton, 2004).

**Biocontrol assays.** The fitness of *T. hamatum* strains GD12 and 6.1 as biocontrol agents was determined by their abilities to control pre- and post-emergence diseases of lettuce caused by the root-infecting pathogens *S. sclerotiorum* and *R. solani* respectively. Lettuce microcosms (120 x 120 x 12 mm) were constructed as described with peat amended with *Trichoderma* strains only (8g of bran inoculum and 300 g peat), pathogen only (8g of poppy seed inoculum and 300 g peat), or both. Plants were grown under the conditions described and percentage emergence and dry weights of plants determined after 21 d. Differences in dry weights of shoot and root...
Competitive saprotrophic ability and biocontrol fitness of GM *Trichoderma hamatum* GD12 materials were analyzed by single-tailed t-test. Differences in percentage emergence were determined by single-tailed t-test after transformation of data using the arc sin$^{-1}$ function.
RESULTS

Confirmation of \(N\)-acetyl-\(\beta\)-D-glucosaminidase disruption and complementation

A targeted gene disruption of the \textit{Trichoderma hamatum NAG} gene (GenBank accession JN107809) was carried out by insertion of a 1.4 kb gene cassette conferring hygromycin resistance into the open reading frame. Southern blot analysis showed that the hygromycin-resistant transformant 6.1 (hereafter referred to as \(\Delta\text{Thng}::\text{hph}\)) contained the correct size insertion (Supplementary Fig. 1). Southern blot analysis also confirmed complementation of mutant \(\Delta\text{Thng}::\text{hph}\) (C17, hereafter referred to as \(\Delta\text{Thng}::\text{hph}:\text{NAG}\))(Supplementary Fig. 1).

To investigate the effect of the mutation on enzyme activity, \(N\)-acetyl-\(\beta\)-D-glucosaminidase activities of GD12, \(\Delta\text{Thng}::\text{hph}\) and the complemented strain were determined colorimetrically, and by using an in-gel assay for \(N\)-acetyl-\(\beta\)-D-glucosaminidase activity. In-gel activity assays (Fig. 1a) and colorimetric assays showed disruption of \(N\)-acetyl-\(\beta\)-D-glucosaminidase activity in the \(\Delta\text{Thng}::\text{hph}\) mutant and confirmed our initial findings that only a single copy of the \(N\)-acetyl-\(\beta\)-D-glucosaminidase gene exists in \textit{T. hamatum} (Supplementary Fig. 2). Tests with \(\Delta\text{Thng}::\text{hph}:\text{NAG}\) showed that the complementation had restored enzyme activity (Fig. 1b). Colourimetric determinations of enzyme activities showed a decrease in chitinase activities consistent with the results of Brunner \textit{et al.} (2003), who showed that loss of \(N\)-acetyl-\(\beta\)-D-glucosaminidase activity in \textit{T. atroviride} affected the formation of other chitinases. Complementation of \(\Delta\text{Thng}::\text{hph}\) restored chitinase activities (Table 1).
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*N-acetyl-β-D-glucosaminidase disruption enhances plant-growth-promotion, but is not associated with nutrient release*

The enzyme-deficient mutant $\Delta$Thnag::hph was tested for its ability to enhance growth promotion of lettuce (*Lactuca sativa*). We originally hypothesized that disruption of *N*-acetyl-β-D-glucosaminidase, a key chitinolytic enzyme implicated in the de-polymerisation of soil chitin and release of nitrogen for plant growth (Leake & Read, 1990; Kerley & Read, 1995; Kerley & Read, 1998; Read & Perez-Moreno, 2003; Lindahl & Taylor, 2004), might decrease or even eliminate plant-growth-promotion by *T. hamatum*. Contrary to our expectations, we found that disruption of the *NAG* gene dramatically enhanced the growth of lettuce seedlings as shown in Fig. 1c. Treatment with GD12 resulted in a 4-fold increase in leaf and shoot dry weights ($P=0.05$) and a 5-fold increase in root dry weights ($P<0.001$) when compared to the control. The increase in plant growth was more dramatic with the *N*-acetyl-β-D-glucosaminidase-deficient mutant. Treatment with $\Delta$Thnag::hph resulted in a 13-fold increase in shoot and leaf dry weights ($P<0.001$) and an 11-fold increase in root dry weights ($P<0.001$)(Fig. 1d). The complemented strain essentially behaved like the wild type strain in these experiments (Fig. 1d), thus establishing that the effect was due to enzyme loss-of-function.

The plant-growth-promotion effects witnessed with certain *Trichoderma* strains have been linked to the solubilization of phosphates and micronutrients (Altomare *et al.*, 1999), but the evidence presented here shows that nutrient release as a consequence of saprotrophic activity of the fungus could not account for the observed plant-growth-promotion with GD12 or with the $\Delta$Thnag::hph mutant. Indeed, there were no significant decreases in the amounts of available nutrients as a consequence of saprotrophic colonization of peat by the two strains of fungi when compared to
the uncolonized controls. While concentrations of ammonia-N, nitrate-N and total soluble N were higher in \( \Delta \text{Thag::hph} \) microcosms compared to GD12, the differences were not significant (Student’s t-test, Table 2). Nevertheless, to test whether the increase in N availability observed in the \( \Delta \text{Thag::hph} \) microcosms might contribute to the increase in plant growth promotion found with the mutant, we added ammonium and nitrate in the form of soluble \( \text{NH}_4\text{Cl} \) and \( \text{NaNO}_3 \) to identical levels found in \( \Delta \text{Thag::hph} \)-colonised microcosms. However, no increase in plant growth resulting from these treatments was found (results not shown). We were also able to discount the possibility that growth promotion occurs in response to the control of minor root pathogens (Windham et al., 1986), because our studies were conducted with sterilized peat in the absence of disease pressure. We therefore conclude that plant-growth-promotion by the fungus does not occur through nutrient release.

**The \( N \)-acetyl-\( \beta \)-D-glucosaminidase-deficient mutant has altered chitin deposition at the hyphal tip**

Growth tests of the \( \Delta \text{Thag::hph} \) mutant revealed no significant reduction in hyphal growth *in vitro* compared to GD12 (Figs. 2a,b). However, spore production was absent in the mutant both in axenic culture and in soil (Figs. 1e & 2a). The spore concentration of axenic cultures of the wild-type strain was \( 2.7 \times 10^7 \) spores ml\(^{-1}\). Chitin is a structural component of the cell wall of *Trichoderma* species and \( N \)-acetyl-\( \beta \)-D-glucosaminidase is one of a number of chitinase enzymes that are believed to play a role in cell wall turn-over and remodeling during hyphal development (Reyes et al., 1989 a,b; Rast et al., 1991; Sahai & Manocha, 1993; Horsch et al., 1997; White et al., 2002). To test whether \( N \)-acetyl-\( \beta \)-D-glucosaminidase functions in cell wall biogenesis of this fungus, mycelium of GD12 and \( \Delta \text{Thag::hph} \) were inoculated onto standard medium
containing calcofluor white, and sensitivity assessed over a 4 day period. Calcofluor white preferentially binds to polysaccharides containing 1,4-linked D-glucopyranosyl units and alters the assembly of chitin microfibrils in fungi (Elorza et al., 1983). Sensitivity to this compound is therefore closely related to the chitin content of cell walls. After 4 days growth, there was no significant difference in growth of the mutant compared to the wild-type strain (Figs. 2a,b). However, when calcofluor white was used in microscopy staining tests, intense fluorescence was observed at the tips of mutant hyphae, whereas no such pattern was observed in GD12 (Fig. 3a). This is consistent with a defect involving incorrect deposition of chitin polymers at the hyphal tip of the ∆Thnag::hph mutant.

The significant (P<0.05, Student’s t-test) decrease in growth of ∆Thnag::hph during exposure due to caspofungin (Figs. 2a and 2b), an echinocandin antifungal drug that inhibits β-1,3-glucan synthases (Lesage et al., 2004; Walker et al., 2008; Fuchs and Mylonakis, 2009), was not unexpected since chitin in the fungal cell wall is covalently linked via a peptide linkage to β-glucan. Chitin and β-glucan are extruded at the hyphal apex and, following modification, result in the formation of chitin microfibrils cross-linked to a glucan matrix (Wessels, 1993). Consequently, the combination of incorrect chitin deposition at the hyphal tip due to N-acetyl-β-D-glucosaminidase deficiency and inhibition of β-1,3-glucan synthesis by caspofungin would be predicted to further significantly inhibit cell wall biosynthesis and growth of the mutant compared to the wild type strain. Despite this, after 3 weeks growth, sporulation had been partially restored in the ∆Thnag::hph mutant (Fig. 2c), so that spore concentrations for GD12 and ∆Thnag::hph were 1x10^7 ml^-1 (±0.2x10^7 ml^-1) and 6.4x10^4 ml^-1 (±1x10^4 ml^-1) respectively. Furthermore, the ∆Thnag::hph spores were viable. Single spore isolates germinated on PDA to
produce non-sporulating colonies (Fig. 2d). This showed that sporulation in the N-acetyl-β-D-
glucosaminidase mutant can be induced by exposure to caspofungin, but that the ability to
produce spores is lost following release from the drug. Little is known about β-1,3-glucan
synthases and sporulation in filamentous fungi, but in the human pathogen Aspergillus
fumigatus, exposure to the drug results in up-regulation of chitin biosynthetic genes and
stimulation of chitin synthesis (Fortwendel et al., 2010). It is reasonable to speculate that a
similar process occurs in ∆Thnag::hph during caspofungin exposure, leading to improved cell
wall integrity which supports transient and partial restoration of sporulation.

The N-acetyl-β-D-glucosaminidase-deficient mutant shows increased secretion of a
Trichoderma-specific extracellular antigen

Secretion by filamentous fungi is a process that occurs at the hyphal apex (Woosten et al., 1991;
Wessels, 1993; Gordon et al., 2000; Thornton, 2004). The altered deposition of chitin at the
hyphal tip of the mutant led us to investigate whether ∆Thnag::hph showed an altered pattern of
secretion compared to GD12. To study secretion, we examined production of an extracellular,
constitutively expressed, glycoprotein antigen by immunofluorescence and by quantitative
enzyme-linked-immunosorbent assay (ELISA)(Thornton et al., 2002). A Trichoderma-specific
monoclonal antibody (mAb) MF2 raised against the antigen (Thornton et al., 2002) was used to
determine glycoprotein contents in the culture filtrates. Because the antibody binds to antigen
produced during active growth of the fungus (Thornton, 2004) we were able to quantify, using a
standard curve of purified antigen (Fig. 3c), the protein concentration per unit biomass of the
fungus. Using this procedure, MF2 antigen production was found to be up to 35-fold higher in
∆Thnag::hph compared to the wild-type strain (Fig. 3d) showing hyper-secretion of the antigen
by the mutant. Consistent with these data, immunofluorescence microscopy of the hyphal tips of wild-type and mutant strains with mAb MF2 showed the antigen was bound to the cell wall of GD12 but there was additional elevated production of the antigen in a halo surrounding the swollen tip of \( \Delta \text{Thnag::hph} \) hyphae (Fig. 3b).

**The N-acetyl-\( \beta \)-D-glucosaminidase-deficient mutant has reduced competitive saprotrophic ability and impaired biocontrol fitness**

The competitive saprotrophic abilities of GD12 and \( \Delta \text{Thnag::hph} \) were determined by quantitative ELISA, during antagonistic interactions with the pathogen *R. solani* in soil-based microcosms. Because the mutant \( \Delta \text{Thnag::hph} \) was shown *in vitro* to hyper-secrete the MF2 antigen used in a previous study to quantify *Trichoderma* saprotrophic growth dynamics in soil (Thornton, 2004), we used a *Rhizoctonia*-specific ELISA (Thornton & Gilligan, 1999; Thornton, 2004) to quantify the effects of *T. hamatum* on the pathogen’s saprotrophic growth. Using this method, we were able to determine the competitive saprotrophic abilities of the two *Trichoderma* strains (Fig. 4a). The population dynamics of *R. solani* were determined in microcosms containing the pathogen only or in mixed species microcosms inoculated with the pathogen and GD12 or \( \Delta \text{Thnag::hph} \). In the absence of *Trichoderma*, active biomass of *R. solani* increased between days 2 and 3 followed by a decline between days 3 and 4 (○). From day 4 onwards, there was a rapid increase in active biomass production up to day 10, with a steady decline thereafter up to the last day of sampling (day 21). In the presence of *T. hamatum* GD12 (◇), a similar trend in *R. solani* biomass production was shown up to day 4, but from days 4 to 21 no further active biomass of the pathogen was produced. In contrast, the mutant \( \Delta \text{Thnag::hph} \) showed impaired interference competition, allowing saprotrophic growth of the pathogen.
throughout the 21 day sampling period (Δ). Specificity of the *R. solani* mAb EH2 was shown using extracts from microcosm containing GD12 only (□) or ∆*Thnag::hph* only (●), where no antigen was detected throughout the 21 day sampling period.

Biocontrol fitness of GD12 and ∆*Thnag::hph* was determined in lettuce microcosms inoculated with the lettuce pathogens *S. sclerotiorum* and *R. solani* (Fig. 4b). In the absence of disease pressure, GD12 increased significantly the mean dry weights of shoot and root materials compared to the control (lettuce plants with no treatment)(Figs. 4d,e). Additional further increases in mean dry weights of plant materials were shown in ∆*Thnag::hph* treated microcosms in the absence of disease pressure (Figs. 4d,e). The pre-emergence damping-off pathogen *S. sclerotiorum* prevented emergence of all lettuce plants in the absence of *Trichoderma* (Figs. 4b-e). *T. hamatum* GD12 controlled pre-emergence damping-off disease caused by *S. sclerotiorum* (Figs. 4c-e) and significantly increased seedling emergence and mean dry weights compared to the control. Furthermore, seedling emergence and mean root dry weight were significantly increased in the mixed species microcosms compared to the microcosms with GD12 treatment only (Figs. 4c,e). While treatment of *S. sclerotiorum* microcosms with the mutant ∆*Thnag::hph* significantly increased establishment of lettuce seedlings compared to *S. sclerotiorum* only (Figs. 4b,c), percentage emergence was significantly reduced compared to the control. Furthermore, mean dry weights of shoot and root materials in the *S. sclerotiorum* + ∆*Thnag::hph* microcosms were significantly reduced compared to microcosms containing the pathogen and GD12 (Fig. 4c-e). The post-emergence lettuce pathogen *R. solani* had no significant effect on seedling emergence and mean root dry weight compared to control microcosms (Figs. 4c,e), but significantly reduced mean shoot dry weight (Fig. 4d). Mixed
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species microcosms containing *R. solani* and GD12 showed significant increases in emergence
and mean dry weights compared to the control and to the *R. solani* and GD12 only microcosms
(Figs. 4c-e). While similar trends were shown in microcosms containing the pathogen and
\(\Delta Thnag::hph\), the increases in mean shoot and root dry weights compared to *R. solani* only were
significantly less than those of the *R. solani* + GD12 microcosms and, in the case of shoot dry
weight, microcosms containing \(\Delta Thnag::hph\) only (Figs. 4c-e).
DISCUSSION

With increasing demands for sustainable alternatives to agrochemicals and synthetic fertilizers in food production, there is renewed interest in exploiting the beneficial properties of soil microorganisms. One such beneficial microorganism is *Trichoderma*, a common soil fungus that has been shown to be a credible alternative to pesticides in the control of plant disease (Harman et al., 2004; Verma et al., 2007). In addition to disease control, certain strains also exhibit plant-growth-promotion activities (Bae et al., 2009; Contreras-Cornejo et al., 2009; Harman et al., 2004; Ortiz-Castro et al., 2009; Verma et al., 2007; Vinale et al., 2008, 2009). These dual attributes make *Trichoderma* species attractive both as biocontrol agents and as plant growth stimulants.

Currently, strict governmental regulations prevent the deployment of genetically modified microorganisms for use in human food production (Weaver et al., 2005). One reason for this is the risk of escape of these organisms from the site of application and the competitive advantage any mutation might have on the biological and ecological fitness of the organism. In this study, we aimed to determine whether a genetic modification that enhances the plant growth stimulant properties of *Trichoderma hamatum* imparts additional advantages in terms of biocontrol fitness and competitive saprotrophic ability. While a previous study has examined genetic stability and ecological persistence of *T. virens* genetically modified with a hygromycin resistance gene and a gene encoding an organophosphohydrolase (Weaver et al., 2005), this is the first time, to our knowledge, that a study has been undertaken to determine the saprotrophic competitiveness and biocontrol fitness of a GM strain of *T. hamatum* with biocontrol and P-G-P activities.
Mutagenesis of the gene \((NAG)\) encoding \(N\text{-acetyl-}\beta\text{-D-glucosaminidase, resulted in a non-sporulating mutant of GD12 with altered secretion, as shown by increased production of an extracellular \textit{Trichoderma} glycoprotein antigen. The cell walls of ascomycete fungi contain a mixture of fibrillar components and amorphous or matrix materials (Horsch et al., 1997). The main fibrillar component is chitin, a straight-chain \(\beta(1-4)\)-linked polymer of \(N\text{-acetylglucosamine. Filamentous fungi grow by extension at the flexible apex of the hypha, a process that requires re-modeling of the fibrillar chitin component of the cell wall to allow extension at the growing tip. This re-modeling requires coordinated production of constitutive chitinase enzymes such as \(N\text{-acetyl-}\beta\text{-D-glucosaminidase and endochitinase, in addition to biosynthetic chitin synthases (Horsch et al., 1997; Rast et al., 1991) and \(\beta\text{-glucan synthases (Wessels, 1993). Consequently, a deficiency in \(N\text{-acetyl-}\beta\text{-D-glucosaminidase in GD12 appears to result in abnormal re-modeling of the hyphal tip and would account for the intense staining with calcofluor white found in the swollen hyphal apices of the \(\Delta Thnag::hph\) mutant, indicative of aberrant chitin deposition.\n
Unlike the work of Brunner et al. (2003), which showed that disruption of \(N\text{-acetyl-}\beta\text{-D-glucosaminidase activity in \textit{T. atroviride did not effect sporulation of the fungus, the \(\Delta Thnag::hph\) mutant of \textit{T. hamatum lacked conidiation. Similar reductions in sporulation efficiency were found in \textit{Aspergillus} mutants altered in chitin deposition (Borgia et al., 1996; Horiuchi et al., 1999; Müller et al., 2002). Alterations in chitin deposition could also account for the increased secretion of the MF2 antigen in \(\Delta Thnag::hph\), since secretion in fungi is a process that occurs at the flexible growing tip (Woosten et al., 1991; Wessels, 1993; Gordon et al., 2000; Thornton, 2004) and is governed by the structure of the cell wall (Kruszewska et al., 1999;
Competitive saprotrophic ability and biocontrol fitness of GM *Trichoderma hamatum* GD12 Perlinska-Lenart *et al.*, 2006). This is consistent with the increased secretion of glycoproteins observed in the related fungus *Trichoderma reesei* as a consequence of mutations in chitin distribution in the hyphal cell wall (Perlinska-Lenart *et al.*, 2006).

Despite the abnormal morphology of ∆Thnag::hph we found, contrary to our expectations, that the mutation increased the ability of the fungus to promote plant growth. The discovery that the mutation had resulted in a biocontrol strain with improved P-G-P activity, led us to investigate whether the mutation also conferred increased fitness as a soil saprotroph and as an antagonist of plant pathogens. We found that exposing ∆Thnag::hph to the drug caspofungin allowed us to switch-on spore production in an ordinarily non-sporulating mutant. This allowed us to generate inoculum for incorporation into soil microcosms and to undertake comparative studies of the competitive saprotrophic abilities (CSA) of the two *Trichoderma* strains.

Saprotrophic competitiveness was investigated in soil microcosms during antagonistic interactions with the pathogen *Rhizoctonia solani*, an aggressive colonizer of nutrient reserves in soil (Garrett, 1970). Because the mutant strain was found to hyper-secrete the MF2 antigen, we were unable to use the MF2 quantitative ELISA developed in a previous study (Thornton, 2004) to track the population dynamics of the *Trichoderma* strains. Instead, we used a *Rhizoctonia*-specific mAb EH2 (Thornton *et al.*, 1993) to quantify the population dynamics of the pathogen in mixed species microcosms (Thornton & Gilligan, 1999; Thornton, 2004). Using this procedure, we were able to show that the mutant ∆Thnag::hph has reduced CSA compared to GD12, and allows saprotrophic growth of the pathogen in soil.
Saprotrophic competitiveness is an important aspect of biological control, since it enables the biocontrol agent to compete with pathogens for nutrient resources in soil. The plurivorous necrotrophic fungus *Sclerotinia sclerotiorum* is an important pathogen of a diverse range of hosts including lettuce (Malvarez *et al*., 2007). *T. hamatum* GD12 was effective not only in preventing colonization of lettuce seeds by the pathogen, but also in further stimulating seedling emergence in mixed species microcosms. In contrast, the mutant ∆Thnag::hph displayed a significant reduction in its ability to stimulate emergence in the presence of the pathogen. However, loss in emergence was compensated by an increase in the weight of established plants. This showed that in the presence of an aggressive pre-emergence damping-off pathogen, loss of biocontrol fitness was balanced by the improved P-G-P activity of the mutant. A similar trend was apparent with *R. solani*. Significant increases in the emergence and dry weights of plants were found in mixed species microcosms containing GD12 and the pathogen compared to microcosms containing GD12 only. However, the ability of ∆Thnag::hph to promote plant growth was significantly impaired by the pathogen.

This work demonstrates that substantial improvements can be gained in plant productivity by genetically manipulating the growth stimulant properties of a biocontrol and plant-growth-promoting strain of *Trichoderma*. However, in the case of *T. hamatum* ∆Thnag::hph, the genetic modification leading to improved P-G-P activity in the absence of disease pressure, does not bestow ecological or biological advantages to the antagonist. On the contrary, the mutation decreased the saprotrophic competitiveness and biocontrol fitness of the fungus, and its ability to promote plant growth was constrained by the presence of soil-borne pathogens.
529 ACKNOWLEDGEMENTS
530 The work was funded by the UK Biotechnology and Biological Sciences Research Council, to whom we are grateful.
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Competitive saprotrophic ability and biocontrol fitness of GM *Trichoderma hamatum* GD12

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Competitive saprotrophic ability and biocontrol fitness of GM Trichoderma hamatum GD12


**Table 1.** Chitinase activities of *T. hamatum* GD12 and the mutant strains ∆Thnag::hph and ∆Thnag::hph:NAG.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Chitobiosidase</th>
<th>Endochitinase</th>
<th>N-acetyl-β-D-glucosaminidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>GD12</td>
<td>0.70 ± 0.04</td>
<td>1.40 ± 0.30</td>
<td>10.80 ± 0.90</td>
</tr>
<tr>
<td>∆Thnag::hph</td>
<td>0.04 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>∆Thnag::hph:NAG</td>
<td>0.68 ± 0.12</td>
<td>1.08 ± 0.41</td>
<td>9.03 ± 0.40</td>
</tr>
</tbody>
</table>

*Figures are the mean of 3 replicates ± SE*
Table 2. Peat nutrient analysis following saprotrophic colonization by *T. hamatum* GD12 and the *N*-acetyl-β-D-glucosaminidase-deficient mutant ∆*Thnag::hph*

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Units</th>
<th>Control</th>
<th>GD12</th>
<th>∆<em>Thnag::hph</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Conductivity</td>
<td>uS cm⁻¹</td>
<td>169±7</td>
<td>69±1</td>
<td>72±1</td>
</tr>
<tr>
<td>pH</td>
<td>-</td>
<td>4.40±0.01</td>
<td>4.86±0.03</td>
<td>4.90±0.03</td>
</tr>
<tr>
<td>Dry matter*</td>
<td>%</td>
<td>25.1±0.0</td>
<td>23.3±0.3</td>
<td>23.1±0.3</td>
</tr>
<tr>
<td>Density</td>
<td>kg m⁻³</td>
<td>557±7</td>
<td>565±15</td>
<td>559±24</td>
</tr>
<tr>
<td>Dry density</td>
<td>kg m⁻³</td>
<td>99±1</td>
<td>87±2</td>
<td>87±3</td>
</tr>
<tr>
<td>Chloride</td>
<td>mg l⁻¹</td>
<td>29±2</td>
<td>21±1</td>
<td>22±2</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>mg l⁻¹</td>
<td>240±14</td>
<td>79±2</td>
<td>82±2</td>
</tr>
<tr>
<td>Potassium</td>
<td>mg l⁻¹</td>
<td>231±10</td>
<td>36±1</td>
<td>39±1</td>
</tr>
<tr>
<td>Magnesium</td>
<td>mg l⁻¹</td>
<td>21±1</td>
<td>0.70±0.09</td>
<td>0.76±0.09</td>
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<tr>
<td>Calcium</td>
<td>mg l⁻¹</td>
<td>6.4±0.1</td>
<td>1.4±0.3</td>
<td>1.4±0.2</td>
</tr>
<tr>
<td>Sodium</td>
<td>mg l⁻¹</td>
<td>30±3</td>
<td>21±1</td>
<td>15±1</td>
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<tr>
<td>Ammonia-N†</td>
<td>mg l⁻¹</td>
<td>22±1</td>
<td>33±3</td>
<td>37±4</td>
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<tr>
<td>Nitrate-N†</td>
<td>mg l⁻¹</td>
<td>1.43±0.03</td>
<td>1.03±0.33</td>
<td>2.53±0.21</td>
</tr>
<tr>
<td>Total soluble N†</td>
<td>mg l⁻¹</td>
<td>24±3</td>
<td>34±3</td>
<td>39±4</td>
</tr>
<tr>
<td>Sulphate</td>
<td>mg l⁻¹</td>
<td>33±1</td>
<td>22±1</td>
<td>21±2</td>
</tr>
<tr>
<td>Boron</td>
<td>mg l⁻¹</td>
<td>0.137±0.007</td>
<td>0.117±0.009</td>
<td>0.120±0.015</td>
</tr>
<tr>
<td>Copper</td>
<td>mg l⁻¹</td>
<td>&lt;0.006</td>
<td>&lt;0.006</td>
<td>&lt;0.006</td>
</tr>
<tr>
<td>Manganese</td>
<td>mg l⁻¹</td>
<td>0.167±0.009</td>
<td>&lt;0.006</td>
<td>&lt;0.006</td>
</tr>
<tr>
<td>Zinc</td>
<td>mg l⁻¹</td>
<td>0.067±0.007</td>
<td>&lt;0.006</td>
<td>&lt;0.006</td>
</tr>
<tr>
<td>Iron</td>
<td>mg l⁻¹</td>
<td>0.130±0.02</td>
<td>0.100±0.006</td>
<td>0.113±0.023</td>
</tr>
</tbody>
</table>

*Percentages converted to degrees using arc sin⁻¹ transformation
†Differences between means of GD12 and ∆*Thnag::hph* not significant at 95% confidence level (Student’s t-test)
FIGURE LEGENDS

**Fig. 1.** (a) In-gel activity assay showing loss of N-acetyl-β-D-glucosaminidase activity in the Δ*Th*na*g::hph* mutant 6.1. (b) In-gel activity assay showing restoration of N-acetyl-β-D-glucosaminidase activity in the Δ*Th*na*g::hph*:NAG complemented strain C17. No other fluorescent products were visible on the gels. (c) Lettuce plants grown for 3-wk in peat microcosms containing the *T. hamatum* strain GD12 and the N-acetyl-β-D-glucosaminidase-deficient mutant Δ*Th*na*g::hph*. Scale bar, 18mm. (d) Histogram showing dry weights of lettuce leaf (black bars) and root (white bars) material after 3-wk growth in peat microcosms with GD12, Δ*Th*na*g::hph* and the complemented strain C17. Each bar is the mean of replicate values ± SE. Bars with different letters are significantly different at 95% confidence level. (e) Sporulation of GD12 and lack of sporulation of Δ*Th*na*g::hph* in peat following incorporation of bran inoculum.

**Fig. 2.** Growth of *T. hamatum* GD12 and Δ*Th*na*g::hph* on PDA amended with calcofluor white and caspofungin (a) Photomicrographs showing growth of GD12 and Δ*Th*na*g::hph* after 4 d (PDA only, PDA + calcofluor white) or after 14 d (PDA + caspofungin)(Scale bar, 15 mm). (b) Colony diameters of GD12 (open squares) and Δ*Th*na*g::hph* (open circles) measured over 4 d (PDA only, PDA + calcofluor white) or over 14 d (PDA + caspofungin). Each point is the mean of three replicate values ± SE. (c) Sporulation of *T. hamatum* GD12 and Δ*Th*na*g::hph* after 3 weeks growth on PDA amended with caspofungin, and (d) subsequent colony morphologies of single spore isolates after sub-culture to PDA only.
Fig. 3. Phenotypic analysis of the ΔThnag::hph mutant. (a) Microscopic analysis of hyphae of GD12 and ΔThnag::hph following exposure to calcofluor white and observation under UV light, showing intense fluorescence at hyphal tips of the N-acetyl-β-D-glucosaminidase-deficient mutant. Scale bar, 12 µm. (b) Immunofluorescence of GD12 and ΔThnag::hph hyphae with mAb MF2, showing hyper-secretion of the glycoprotein antigen around the swollen tip of the mutant hypha. (Scale bar, 10 µm). (d) Quantification of extracellular glycoprotein antigen concentrations in GD12 (open circles) and ΔThnag::hph (closed circles) shake culture filtrates. Antigen concentrations in (d) were determined by converting absorbance values from ELISA with the Trichoderma-specific monoclonal antibody MF2 to equivalents of glycoprotein concentration by using a calibration curve of purified Trichoderma antigen (c). Each point is the mean of three replicate values ± SE.

Fig. 4. Competitive saprotrophic abilities and biocontrol efficacies of T. hamatum GD12 and ΔThnag::hph. (a) Population dynamics of R. solani in the presence of GD12 (◇), ΔThnag::hph (△) and in the absence of Trichoderma (○). The specificity of the R. solani-specific monoclonal antibody EH2 was shown using extracts from microcosms containing T. hamatum GD12 (□) or ΔThnag::hph (●) only. Absorbance values were converted to biomass equivalents (expressed as [mg LM (2g of mix)⁻¹]) using a standard calibration curve of R. solani lyophilized mycelium. The mean ± SE (based on three replicate values) was then calculated for each set of samples from the populations on each day of sampling. (b) Biological control of pre-emergence (S. sclerotiorum) and post-emergence (R. solani) disease of lettuce by T. hamatum GD12 and ΔThnag::hph. Note control control of disease by the wild-type strain GD12, but sporadic control of disease by the mutant ΔThnag::hph. (c) Histograms showing mean emergence (as a
percentage) and mean shoot and root dry weights of lettuce plants grown in single (pathogens only or *Trichoderma* strains only) or mixed species (pathogens and *Trichoderma* strains) peat-based microcosms. Control microcosms contained lettuce only. Data are the mean values ± standard errors from 3 replicate microcosms each containing 25 lettuce seeds. Emergence percentages were converted to arc sin\(^{-1}\) values for statistical analysis by single-tailed t-test. Bars with different letters are significantly different at 95% confidence level.
SUPPLEMENTARY FIGURE LEGENDS

Fig. 1. Southern blot analysis of the $N$-acetyl-$\beta$-D-glucosaminidase-deficient mutant $\Delta Thnag::hph$ (6.1) and its complementation. (a) Genomic DNA was isolated from the hygromycin-resistant and enzyme-defective strain 6.1 and from sulphonyl urea/hygromycin-resistant $\Delta Thnag::hph:NAG$ complemented strains using the CTAB method. (b) DNA was subjected to BglII digestion, fractionated by agarose gel electrophoresis, blotted to Hybond-N and hybridised with the 575 bp fragment of the $ThNAG$ gene. Insertional mutation in strain 6.1 is indicated by a 1.4 kb size difference compared to the wild-type GD12. The presence of two hybridising bands at 3.3 kb and 1.4 kb in transformants C6 and C17 shows complementation of the $\Delta Thnag::hph$ mutant 6.1.

Fig. 2. Southern blot analysis showing $NAG$ gene copy number. (a) Genomic DNA extracted from $T. hamatum$ GD12 was digested with restriction enzymes (lane 1 BamHI; lane 2, EcoRI; lane 3 Hind III; lane 4, PstI; lane 5 BamHI, EcoRI; lane 6 BamHI, Hind III; lane 7 EcoRI, PstI; lane 8 ApaI; lane 9 ApaI, BamHI; lane 10 ApaI, EcoRI). (b) Southern blot probed with a 575 bp fragment of the $NAG$ gene. The presence of single hybridizing restriction fragments in lanes 1-10 confirms that $NAG$ is a single copy gene in $T. hamatum$.  

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Figure 1 Ryder et al.
Figure 2 Ryder et al.
Figure 3 Ryder et al.
Figure 4 Ryder et al.
Investigating the beneficial traits of Trichoderma hamatum GD12 for sustainable agriculture - insights from genomics.

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Abstract

*Trichoderma hamatum* strain GD12 is unique in that it can promote plant growth, activate biocontrol against pre- and post-emergence soil pathogens and can induce systemic resistance to foliar pathogens. In this paper we characterise GD12 using a combination of *de novo* genome sequence data and transcriptomics. We compare GD12 with other published *Trichoderma* genomes and show that *T. hamatum* GD12 contains unique genomic regions with the potential to encode novel bioactive metabolites that may contribute to GD12’s agrochemically important traits.

Introduction

With global populations estimated to reach 9 billion by 2050, current plant breeding approaches alone will not support the increased demand for food. There is an urgent need to investigate alternative, sustainable approaches to enhance agricultural production. Additional pressures on food production such as existing and emerging pathogens (Anderson et al, 2004; Fisher et al, 2012), soil erosion (Montgomery, 2007), reduced water and nutrient availability (Powlson et al, 2011; Sauer et al, 2010), climate change (Schmidhuber & Tubiello, 2007) and competition for available land from other sectors such as house building and biofuels (Harvey & Pilgrim, 2011), will add further pressure on agricultural systems to maximize crop productivity. Moreover, hazard-based criteria for assessing pesticides could lead to a range of agrochemicals being withdrawn from European markets, leading to the potential loss of the only effective fungicide groups against major crop diseases.

Faced with these developments, alternative sustainable agricultural strategies are being investigated, with a strong focus on exploiting beneficial organisms. Members of the fungal genus *Trichoderma* have the potential for reducing existing dependence on the use of environmentally damaging and unsustainable chemicals required for disease control, and fertilizers (Fantke et al, 2012), by providing an opportunity to sustainably improve crop productivity while reducing the likelihood of development of fungicide resistant pathogens.

*Trichoderma* is a member of the Ascomycota, the largest group of fungi. Asexual reproduction occurs through the production and germination of asexual conidia (Steyaert et al, 2010) and in some species of *Trichoderma*, sexual teleomorphic stages (*Hypocrea* spp.) have been identified (Seidl et al, 2009), although *Trichoderma* is now the accepted holomorph nomenclature (International Botanical Congress 2011). *Trichoderma* has been exploited in many industries including paper,
textile, biofuel and agriculture due to its prolific secretion of degrading enzymes and biocontrol activities (Chaverri et al, 2003; Giraldo et al, 2007; Kuhad et al, 2011; Miettinen-Oinonen & Suominen, 2002; Pere et al, 2001).

Biocontrol encompasses a variety of mechanisms working singularly or synergistically during the interaction between a biological control agent, plant pathogen and plant to achieve effective disease control (Howell, 2003). These mechanisms can be either indirect - competition for nutrients and space, antibiosis and stimulation of plant-defense mechanisms, direct mycoparasitism, or a combination thereof. Mycoparasitism involves direct antagonism of soil-borne pathogens by a combination of enzymatic lysis through secretion of chitinases, glucanases, proteases, antibiotic production, and competition for space and substrates (Harman, 2006; Lorito et al, 2010). Since the 1930’s, *Trichoderma*’s mycoparasitic biocontrol activities have been extensively used in agriculture. Research has focussed predominately on *Trichoderma virens*, *T. atroviride*, *T. asperelloides*, *T. asperellum* and *T. harzianum* (Benitez et al, 2004; Howell, 2003). However, mycoparasitism is widespread. More than 1,100 *Trichoderma* strains from 75 molecularly defined species displayed mycoparasitic potential against the pathogens *Alternaria alternata*, *Botrytis cinerea* and *Sclerotinia sclerotiorum* (reported in Druzhinina et al. 2011). Yet, despite its agronomic importance, our current knowledge about the mechanistic basis for mycoparasitism is rudimentary.

Certain *Trichoderma* strains have been shown to stimulate plant growth through the production of plant-growth-promoting (PGP) compounds (Chang et al, 1986; Contreras-Cornejo et al, 2009; Ousley et al, 1994; Vinale et al, 2009) although both biological control and PGP traits are rarely found together. Often, PGP is unpredictable and is influenced by environmental factors (Maplestone et al, 1991; Ousley et al, 1993). The mechanisms for PGP are thought to variously arise from direct effects on plants, decreased activity of microflora, and inactivated toxic compounds in the root zone (Harman et al, 2004). *Trichoderma* species can also ameliorate a wide range of abiotic stresses such as salinity, temperature and drought; improve photosynthetic efficiency; enhance nutrient uptake; significantly increase nitrogen use efficiency in crops – all attributes that can contribute to enhanced PGP characteristics often evident upon inoculation (Bae et al, 2009; Djonovic et al, 2006; Harman et al, 2004; Shoresh et al, 2010). Strains stimulate PGP through the production of, yet to be defined, PG compounds (Contreras-Cornejo et al, 2009; Ryder et al, 2012; Vinale et al, 2009), most likely through a combination of one or more of the remarkably diverse array of secondary metabolites and proteins such as pyrones, peptaibols, and terpenes (Lorito et al, 2010) that *Trichoderma* produces.
In addition to mycoparasitism and PGP, some \textit{Trichoderma} strains can induce broad spectrum systemic resistance (ISR) in foliar leaves (Shoresh et al, 2010). Collective deployment of these positive agronomic traits of growth promotion, enhanced tolerance to abiotic stress and broad spectrum systemic immunity are striking and unique. Generally it is accepted that, in agricultural systems, the activation of defense responses generates a “trade-off” in terms of reduced growth or enhanced susceptibility to other stresses (Heidel et al, 2004; van Hulten et al, 2006). Remarkably however, \textit{Trichoderma} inoculation can ameliorate these traditionally perceived “costs” suggesting it can locally suppress MAMP (Microbe Associated Molecular Pattern) triggered immunity (MTI) and systemically activate or prime induced plant immunity. Suppression of MTI has been recently demonstrated for the plant growth promoting rhizobacterium (PGPR) \textit{Pseudomonas fluorescens} strain WCS417r, which grows endophytically or on root surfaces (Millet et al, 2010).

Modifications of hormonal balance by host or microbe are key drivers in determining the outcome of plant-pathogen interactions, including suppression of MTI (Grant & Jones, 2009). ISR induced by \textit{P. fluorescens} WCS417r is mediated through jasmonic acid/ethylene (JA/ET) signalling (Ton et al, 2002). The role for hormone signalling in \textit{Trichoderma} ISR is often contradictory with various \textit{Trichoderma} strains activating ISR through different signaling modules. \textit{T. asperellum} T34 mediated ISR appears to parallel WCS417r JA/ET based “priming” events, resulting in enhanced resistance to obligate biotrophs, hemi-biotrophs and necrotrophs (Segarra et al, 2009). By contrast, \textit{T. harzianum} T22 inoculated maize had constitutive expression of some PR proteins in the absence of a pathogen. In melon, \textit{T. harzianum} can control \textit{Fusarium} wilt through induction of basal resistance and the attenuation of \textit{Fusarium oxysporum} induced hormonal disruption of abscisic acid (ABA), salicylic acid (SA) and ET signalling (Martinez-Medina et al, 2010; Martinez-Medina et al, 2011). Recently, ISR, induced by \textit{T. hamatum} T382 against \textit{Botrytis cinerea} in \textit{A. thaliana} was reported to involve both an initial priming and a post infection response (Mathys et al, 2012).

Thus current knowledge suggests that induction of ISR depends upon the specific strain. Host genotype also contributes as genetic variability between tomato lines determines the outcome of PGP and biocontrol interactions with \textit{T. atroviride} and \textit{T. harzianum} (Tucci et al, 2011). Remarkably, rhizosphere interactions can also be influenced by foliar signals. The bacterial phytoxin and jasmonate mimic, coronatine (COR), or the bacterial MAMP, flagellin, induce a shoot-to-root signal that acts to recruit the gram positive PGPR \textit{Bacillus subtilis} FB17 and requires a functional cognate receptor for jasmonate or flagellin (Lakshmanan et al, 2012). Cell culture filtrates of FB17 suppress flagellin induced MTI, suggesting that a diffusible bacterial component enables rhizobacterial colonization and establishment of host–mutualistic associations. Moreover,
root binding of FB17 restricts the stomata-mediated entry of virulent *Pseudomonas* as part of an ISR response (Kumar et al, 2012).

*Trichoderma hamatum* is a naturally occurring rhizosphere dwelling member of the genus which has attracted academic and industrial interest due to its ability to increase plant biomass and its potential as a biological control agent (Chet et al, 1981; Elad, 2000; Harman, 2006). A previously reported strain of *Trichoderma hamatum* (GD12) isolated from soil in Devon, UK, promotes plant growth in low pH, nutrient poor peat and displays biological control protection against pre- and post-emergence diseases of lettuce seedlings caused respectively by *Sclerotinia sclerotiorum* and *Rhizoctonia solani*, under the same conditions (Ryder et al, 2012; Thornton, 2005; Thornton, 2008). While plant growth promotion and biocontrol by *Trichoderma* have been well documented, both traits rarely occur together (Contreras-Cornejo et al, 2009; Vinale et al, 2009). Thus, the genome sequence of GD12 would provide a valuable insight into the genetic potential underlying these important agronomic traits.

Whole genome sequences are becoming increasingly available, with the industrial strain *T. reesei*, and biological control strains *T. atroviride, T. harzianum,* and *T. virens, T. longibrachiatum, T. citrinoviride, T. asperellum* now accessible in public repositories (www. http://genome.jgi.doe.gov/pages/home.jsf?core=genome&query=Trichoderma&searchType=Keyword). Genome comparisons between the mycoparasitic *T. atroviride* and *T. virens* species with the saprophytic *T. reesei* identified components predicted to contribute to a parasitic lifestyle and a genome reduction in *T. reesei* (Kubicek et al, 2011).

Here we present further characterisation of the dual PGP and biocontrol strain *T. hamatum* GD12. We undertook whole genome sequencing of this strain and compared it to sequenced *Trichoderma* strains (*T. atroviride* and *T. virens*) to predict important components involved in PGP and biological control fitness of *T. hamatum* GD12. This work demonstrates differences between strains which allowed us to identify genomic regions/clusters unique to GD12 that can be further studied to gain a more comprehensive understanding of genetic basis for PGP and biological control. Finally, we capture a global snapshot of gene expression in *Arabidopsis thaliana* plants grown in GD12 amended compost, which suggests GD12 modifies hormonal balance and activates transposons during its interaction with *A. thaliana*. This study provides a foundation for further dissection of GD12’s ability to promote beneficial attributes.
Materials and Methods.

Plant growth promotion and biological control assays.
One litre of sieved sphagnum moss peat (Shamrock, Scotts Professional, UK) was mixed with 400 ml dH$_2$O and sterilized by autoclaving. Twenty-five lettuce seeds (*Lactuca sativa* cultivar Webb’s Wonderful) were sown into 120 mm x 120 mm x 12 mm square plastic culture dishes (Greiner, Bio-One, UK) containing 300 g sterile peat. For plant growth promotion and biological control assays, microcosms were supplemented with: 8 g *T. hamatum* bran inoculum, *Sclerotinia* poppy seed inoculum or both (Ryder et al, 2012). Bran inoculum was prepared by inoculating a sterile bran mixture (250 ml conical flask containing 10 g wheat bran (Badminton Horse Feeds, UK and 30 ml sterile dH$_2$O) with five 4 mm plugs of agar from the leading edge of a 3-day-old *T. hamatum* culture, grown on Potato Dextrose Agar (Sigma-Aldrich). The inoculum was incubated for 5 days at 26°C under a 16 h light regime. *S. sclerotiorum* poppy seed inoculum was prepared by inoculating a sterile poppy seed mixture (250 ml conical flask containing 10 g black poppy seeds with 10 ml sterile dH$_2$O) with ten 1 mm plugs of agar from the leading edge of a 3-day-old *S. sclerotiorum* culture grown on PDA. The inoculum was incubated for 10 days at 26°C under a 16h light regime. Microcosms were maintained at 24°C under a 16h light 8h dark cycle at 90% humidity. Following the removal of lids after 48h, microcosms were watered daily with sterile dH$_2$O. After 21 days, plants were harvested, washed and oven dried (75°C) to a constant weight. Shoot and root dry weights were determined and the data analysed by using ANOVA and Tukey Post hoc tests.

Rewatering assays.
100 ml dH$_2$O was added to triplicate 5 day old *Trichoderma hamatum* bran inoculum flasks (prepared as above) for each strain to be tested. Samples were mixed for 1 h and filtered through miracloth (Caliobiochem) into 2x 50 ml aliquots. Samples were centrifuged at 10, 000 g for 10 min. and vacuum filtered through 5 µm filter paper (Whatman) and autoclaved for 15 min. at 121 °C. Seedlings were watered with filtrate every alternate days for 21 days.

Biocontrol assay. *Magnaporthe oryzae* leaf infection assays were carried out using dwarf Indica rice (*oryza sativa*) cultivar CO-39, which is susceptible to rice blast. Eight seedlings of CO-39 were planted in 15 pots (7 cm) and grown for 14 days (2-3 leaf stage) in soil containing *Trichoderma*-bran inoculum prior to *M. oryzae* strain Guy-11 infection. Disease symptoms were scored after 5 days according to Valent et al. (1991).
**Bioinformatics methods**

We used Velvet version 1.1.04 (Zerbino & Birney, 2008) for de novo assembly of genome sequence. For ab initio gene prediction we used FgenesH ([http://linux1.softberry.com/berry.phtml?topic=fgenesh](http://linux1.softberry.com/berry.phtml?topic=fgenesh)). SignalP 3.0 (Bendtsen et al, 2004) and Phobius (Kall et al, 2004) were used for prediction of signal peptides and transmembrane domains. Alignments were visualised using the Artemis Comparison Tool (Carver et al, 2005). To generate Venn diagrams we used the Venn Diagram Generator ([http://bioinformatics.psb.ugent.be/webtools/Venn/](http://bioinformatics.psb.ugent.be/webtools/Venn/)). PfamScan (Punta et al, 2012) was used to search for conserved domains in protein sequences.

**Results.**

*Trichoderma hamatum* promotes growth of lettuce (Ryder et al, 2012) and *Arabidopsis thaliana* (Fig. 1A) in acidic, nutrient-poor, organic peat soils (Thornton, 2005). In previous work, we hypothesised that the PGP properties of GD12 might occur through the enzymatic release of nitrogen from chitin. Remarkably however, rather than reduced or loss of PGP, disruption of the GD12 N-acetyl-b-D-glucosaminidase gene (∆Thnag1::hph strain) dramatically enhanced the growth of lettuce seedlings, indicating that increased production of stimulatory compound(s) was due to N-acetyl-b-D-glucosaminidase deficiency (Ryder et al, 2012). This PGP activity was found to be present in water-soluble extracts derived from both bran grown GD12 and ∆Thnag1::hph. PGP activity was heat stable, withstanding autoclaving at 121°C for 15 min. Notably ∆Thnag1::hph bran extracts promoted enhanced lettuce growth (Fig. 1B) consistent with the predicted hyper-secretion capability of ∆Thnag1::hph.

In contrast to enhanced PGP, loss of N-acetyl-b-D-glucosaminidase activity drastically impaired GD12’s competitive saprotrophic ability and biocontrol fitness (Ryder et al, 2012). Figure 2A shows that mycoparasitism of four *Sclerotinia sclerotiorum* strains was abolished in the ∆Thnag1::hph mutant. By contrast, *Trichoderma hamatum* GD12 not only showed strong biocontrol of *S. sclerotiorum*, but strikingly PGP was dramatically enhanced as well compared to GD12 amendment alone (Fig. 2B). We interpret these data to suggest that cryptic metabolomic pathways, ordinarily silent in GD12 in axenic culture, are induced during antagonistic interactions in soil.

**Genome informed metabolic predictions.**

Production of plant-growth-promoting (PGP) compounds and mycoparasitism by certain strains of *Trichoderma* are well documented (Contreras-Cornejo et al, 2009; Vinale et al, 2009) although both
traits are rarely found together. Some *Trichoderma* strains additionally possess the ability to activate ISR to a broad range of pathogens. To investigate the robustness of *T. hamatum* GD12 biocontrol properties we examined the ability of GD12 and the ∆Thnag1::hph mutant to confer resistance to the rice blast pathogen *Magnaporthae oryzae*. GD12 and, to a greater extent, ∆Thnag1::hph both reduced lesion formation compared to non-inoculated rice plants. Thus, although ∆Thnag1::hph has lost the ability to mycoparasitise *S. sclerotiorum* it has the capacity to elicit a strong induced systemic resistance response (Fig. 3).

**Draft genome sequence of *T. hamatum* GD12**

We hypothesised that the genome sequence of *T. hamatum* GD12 might provide valuable insight into the genetic potential underlying the unique PGP, mycoparasitism and ISR inducing properties of this saprotrophic fungus. A GD12 genome sequence could facilitate secondary metabolite pathway predictions, mapping of mRNA-seq data to genomic clusters and capture unique genes and gene families not coded by other *Trichoderma* genomes.

We therefore assembled a draft genome sequence of GD12 from 12 million pairs of Illumina GA2 paired-end 73-bp reads using Velvet 1.1.04. This yielded 2,770 scaffolds with a N50 length of 41.6 Kb. The total length of the assembly was 38.2 Mb. The whole genome shotgun data have been deposited at DDBJ/EMBL/GenBank under the accession ANCB00000000. Using FgenesH (trained on *Neurospora crassa*) we predicted 12,391 protein-coding genes in GD12.

**Comparison with other *Trichoderma* genomes**

The genome sequence of GD12 shares little similarity with previously sequenced *Trichoderma* genomes at the nucleotide sequence level. The three sequenced *Trichoderma* genomes analysed in detail, *T. atroviride* (~36.4 Mb), *T. virens* (~38.8 Mb) and *T. reesei* (~34 Mb) show remarkably conserved gene order (78 - 96%), with > 50% of annotated genes having orthologous in the related ascomycetes *Neurospora crassa* and *Gibberella zeae*. (Kubicek et al, 2011). Strikingly, only 52% of the GD12 genome sequence aligned against that of *T. atroviride* and only 6% aligned against the more distantly related *T. reesei* (using the dnadiff tool from the Mummer package). To ensure this limited sequence homology was not due to sample contamination, a geographically distinct *T. hamatum* isolate, strain 11, was sequenced. Strain 11 showed 98% sequence identity to GD12.

At the level of amino acid sequence, 62.4% of the GD12 predicted proteins (*i.e.* 7,773 proteins; Fig. 4A) had a close homologue in at least one of *T. atroviride, T. harzianum, T. reesei* or *T. virens* species compared (here, we define a close homologue as sharing at least 80% sequence identity
over at least 90% of the length of the query sequence). Of the GD12 predicted proteins, only 5,531 (59%) are highly conserved in *T. atroviride* (at least 80% amino acid sequence identity over at least 90% of the sequence length; Fig. 4A). Thus GD12 contains novel genomic regions with the potential to encode novel, agrochemically important gene products leading to unique bioactive metabolites that may contribute to GD12’s PGP and biocontrol activities.

These *T. hamatum*-specific genomic regions likely hold the key to the unique biological interactions observed in this species. For example, we identified a 47-kbp *T. hamatum*-specific region described in Table 1 and illustrated in Figure 5, which appears to encode several enzymes (Genes 3, 5, 6, 7) and transporters (Genes 2, 4) that might contribute to novel secondary metabolism pathways. Predicted gene 3 encodes a protein containing an attachment site for phosphopantetheine, a prosthetic group that acts as a ‘swinging arm’ for the attachment of activated fatty acid and amino-acid groups. It also contains a domain characteristic of AMP-binding enzymes. Gene 5 encodes a protein with an ATP-grasp domain, characteristic of enzymes that possess ATP-dependent carboxylate-amine ligase activity. Gene 6 encodes a putative aminotransferase while Gene 7 is predicted to encode a polyketide synthase, which provide important sources of naturally occurring small molecules such as antibiotics and other industrially important polyketides.

While the vast majority of the unique GD12 genes were of unknown function, there were some interesting candidate genes that might contribute to both PGP and GD12’s biocontrol activities. These included enzymes for cyclobenzene, benzoate (2-keto-4-pentenoate hydratase) and nicotinate (nicotinamidase) degradation; potential synthesis of the plant phytohormone zeatin (adenylate isopentenyltransferase) and the insect hormone ecdysone (ecdysone oxidase). Most notable are the nonribosomally synthesized cyclic lipopeptide antibiotics such as surfactin and three of the five *Bacillus subtilis* fengycin synthetases that nonribosomally synthesise fengycin a lipopeptidic antibiotic (Wu et al, 2007). Thus a rich reservoir of metabolic potential exists in the unique genomic regions of *T. hamatum*.

**The secretome**

Given the dual plant growth promotion and biocontrol properties of *T. hamatum* constituents of the “secretome” represent candidates in the molecular dialogue between soil pathogens and the plant rhizosphere. Of the 12,391 hypothetical GD12 genes, 1,014 (8.2%) were predicted to be encode secreted proteins based upon SignalP 3.0 (Bendtsen et al, 2004) and absence of a typical transmembrane domain as determined by Phobius (Kall et al, 2004)(Supplementary File 1). Of
these, 370 were unique to *T. hamatum*, more than entire the “core” secretome shared by the 5 *Trichoderma* species (Fig. 4B). Only 469 (55.5%) of *T. hamatum* secretome proteins are conserved in *T. atroviride* suggesting some divergence in the nature of the secreted bioactive proteins. The secretome of *T. atroviride* is, surprisingly, enriched for 26 proteins containing the fungal-specific Zn(2)Cys(6) transcription factor domain (Pfam:PF04082; http://genomebiology.com/2011/12/4/R40). The predicted secretome of GD12 is also similarly enriched, with 11 proteins containing this transcription-factor domain. One striking feature of the GD12 secretome is the enrichment for putative AMP-binding enzymes; 14 of the GD12 secreted proteins contain an AMP-binding domain (Pfam: PF00501; Supplementary File 2).

**Small secreted (cysteine-rich) proteins (SSCRPs)**

We identified potential SSCRPs in the predicted secretomes of *T. hamatum* and *T. atroviride* as proteins whose length was 300 amino acids or fewer and which contained at least four cysteine residues, as defined in Kubicek et al. 2011. There were 153 proteins in *T. hamatum* satisfying these criteria (Supplementary File 3), of which 83 had no close homologue in *T. harzianum, T. reesei, T. virens* or *T. atroviride* (i.e. no BLASTP hit with at least 80% sequence identity over at least 90% of the protein’s length). For comparison, in *T. atroviride* there were 170 proteins satisfying these criteria, of which 106 had no close homologue in *T. harzianum, T. reesei, T. virens* or *T. hamatum*. Thus there is a complement of ~60-70 SSCRPs that may constitute a “core” effector complement. The *T. hamatum* SSCRP’s contained a diverse range of Pfam domains (Supplemental Files 4 & 5) suggesting a complex array of biological activities associated with these SSCRPs.

**LysM motifs.**

The LysM motif binds different peptidoglycans in bacteria and chitin-like compounds in eukaryotes (Buist et al, 2008; de Jonge & Thomma, 2009). Recent studies have shown that fungal LysM motifs can bind and suppress chitin oligomers that would be recognized by plant pattern recognition receptors, preventing the activation of an innate immune response (de Jonge et al, 2010). Seven hypothetical GD12 proteins contain a LysM domain (Pfam: PF01476) although none of these are predicted to be secreted. This is similar to the numbers reported previously for *T. reesei* (6), *T. virens* (7) and *T. atroviride* (9).

**The impact of *T. hamatum* GD12 on the *Arabidopsis thaliana* transcriptome.**

We undertook a preliminary microarray study to broadly survey the molecular signatures underlying establishment of PGR/ISR in GD12 vs. mock inoculated whole *Arabidopsis thaliana*
Col-0 (roots and shoots). Total RNA was extracted 6 days after inoculation of 2-week old plants, coincident with the first visible differences in growth (see Fig. 1 for indicative growth differences) and hybridised to Affymetrix ATH1 arrays (see NASCARRAYS-162; http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl for outputs and metadata). Although this experiment lacks the replication to derive statistically supported conclusions, it provides a global insight into the whole plant transcriptional re-programming induced by *T. hamatum* GD12. In total, 241 genes were suppressed (Supplementary File 6), and 123 genes induced greater than two fold (Supplementary File 7) in response to *T. hamatum* GD12 soil amendment.

Some interesting patterns emerge from these data. Genes encoding components involved in ethylene (ET), abscisic acid (ABA) and most notably, jasmonic acid (JA) signalling pathways were suppressed by GD12 (see Supplementary File 6 for details). The JA biosynthesis components LOX3 (At1g17420), AOC3 (At3g25780), OPR3 (At2g06050) and OPC8 CoA ligase (At1g20510) were all suppressed, as were four negative regulators of JA signaling, *JAZ 1,5,7,8* (At1g19180; At1g17380; At2g34600; At1g30135) which are usually co-induced with JA biosynthetic genes (Santner & Estelle, 2007). The key JA response genes PDF1.2A (At5g44420) and PDF1.2B (At2g26020) were also suppressed as were many other genes annotated as JA inducible. The latter included key components of the flavonoid pathway, induced by JA including *TYROSINE AMINOTRANSFERASE 3* (Atg24850), *DIHYDROFLAVONOL 4-REDUCTASE* (At4g35420) and *UDP-GLUCOSE: FLAVONOID 3-O-GLUCOSYLTRANSFERASE* (At5g54060) which were all at least two fold lower in plants in which GD12 was added compared to the control. These particular expression patterns are interesting as flavonoids are postulated to be possible long distance signaling molecules (Buer et al, 2007; Buer et al, 2008).

By contrast to suppressed genes, up-regulated genes were mostly uncharacterized (Supplementary File 7). Annotated transcripts were notably over-represented by genes involved in growth and development, for example *AGAMOUS-LIKE 23* (At1g65360), 36 (At5g26645), 42 (At5G62165), 82 (At5g58890), *HYPONASTIC LEAVES 1* (At1g09700), *JAGGED* (At1g68480), *SHI-RELATED SEQUENCE 4* (At2g18120), *DEFECTIVELY ORGANIZED TRIBUTARIES 4* (At4g18750), *BRASSINOSTEROID-INSENSITIVE 4* (At4g39400), *LSD1-LIKE2* (At3g13682), *MYB-RELATED PROTEIN 1* (At5G18240). Additionally, various ion transporters/channel proteins including *ATOCT2* (At1G79360), *ATGLR2.8* (At2g29110), *ATCHX1* (At1g16380), *CNCGC15* (At2g28260), *GORK1* (At5g37500), *RAPID ALKALINIZATION FACTOR 12* (At2g19040) and *DEHYDRIN XERO 1* (At3g50980), were induced, implicating modification of cellular ion homeostasis during PGP/ISR.
Interestingly, among the top 20 GD12 induced genes were 5 transcripts involved in transposition (At2g16000, At1g40230, At1g41860, At5g28870 and At2g31080). While specific expression patterns would need to be individually validated, these data provide a global insight into how GD12 modifies the Arabidopsis transcriptome and suggests that suppression of hormone responses, in particular JA signalling, represents a key mechanism in establishment of PGP/ISR.

Discussion and conclusions.

Fossil evidence predicts that the mycoparasitic lifestyle evolved more than 400 million years ago (Taylor & Berbee, 2006). Like T. virens and T. atroviride, the T. hamatum genome encodes a vast arsenal of cell wall degrading enzymes such as chitinases, glycoside hydrolases, β-1,3-glucanases and N-acetyl-b-D-glucosaminidases that are presumably deployed to degrade the carbohydrate defenses of its biocontrol targets. It also encodes a wealth of proteases, polyketide syntheses and nonpeptide syntheses consistent with its mycoparasitic lifestyle. However, approximately half the T. hamatum proteome and its constituent secretome is unique to GD12 and vice versa.

Recent comparative genomic experiments have revealed that the T. reesei genome contraction has occurred, with consequent loss of mycoparasitic ability. At 38.8 Mbp, the T. virens genome is nearly 5 Mbp larger than T. reesei and 2.7 Mbp larger than T. atroviride. Unique to T. virens and T. atroviride are secondary metabolite gene clusters localized on non-syntenic islands that are likely to contribute to mycoparasitism. Notable in the larger T. virens genome, are a repertoire of non-ribosomal peptide synthetases (NRPS) expanded to 28, twice that present in other fungi (Kubicek et al, 2011; Martinez et al, 2008).

T. hamatum is phylogenetically most closely related to T. atroviride (Kubicek et al, 2011) yet, with the incomplete GD12 assembly at ~ 38.1 Mbp, nearly as large as the T. virens genome. Despite the close phylogenetic relationship, there were striking differences between T. atroviride and T. hamatum homology, with approximately 40% of the GD12 proteome being unique. These differences most likely reflect the strong evolutionarily genomic potential and additional beneficial traits of plant growth promotion and induced systemic resistance encoded by GD12, as well as components required for niche differentiation. A simple analysis of GD12 unique regions identified a range of components implicated in secondary metabolism including evidence for production of nonribosomally synthesized lipopeptide antibiotics such as surfactin and the antibiotic fengycin (Wu et al, 2007).
T. hamatum encoded over 4658 unique proteins and shared a core proteome of 3620 predicted proteins with the four other Trichoderma’s (T. atroviride, T. virens, T. reesei, T. harzianum). An additional 2096 proteins were unique to GD12 and T. atroviride reflecting the closer evolutionarily relationship between these two species. Some of these genes may specify enzymes responsible for the breakdown of polymeric organic molecules into a form that can be absorbed, or in the secretion of fungal synthesised compounds that have roles in antibiosis or are signals molecules facilitating communication with mutualistic partners. How the unique component of the GD12 genome has been acquired and is deployed remains to be determined. Figure 5 highlights a 47-kbp T. hamatum-specific region that encodes several biosynthetic enzymes and transporters with potential to contribute to novel secondary chemistries, including two NRPS components. Moreover, like T. atroviride (Baker et al, 2012), the T. hamatum genome has a number gene clusters encoding polyketide synthases. PKSs play important roles in synthesis of secondary metabolites such as in the plant pathogen Ustilago maydis (Kamper et al, 2006) and a hybrid NRPS/PKS has recently been implicated in ISR in maize (Mukherjee et al, 2012).

We predicted 370 unique proteins in the secretome of T. hamatum and a core of 327 proteins shared across T. atroviride, T. virens, T. reesei and T. harzianum. GD12 and T. atroviride shared 164 unique putative secreted proteins, nearly 20 times as many as any of the other species (Fig. 4). The distinct genomic potential is also reflected in the deployment of small secreted cysteine rich peptides. Approximately 50% of the SSCRPs were shared between the two species.

A striking feature of the GD12 secretome was the enrichment for putative AMP-binding enzymes; 14 of the GD12 secreted proteins contain the AMP-binding domain (Pfam: PF00501; Supplementary File 2). Interestingly, many of these proteins are capable of acyl:adenyl ligase activities that can positively or negatively modulate bioactivity through the ligation of residues such as amino acids. Cochliobolus carbonum race 1 HC-toxin synthetase produces the cyclic tetrapeptide called HC-toxin (Scott-Craig et al, 1992; Walton, 2006). Plant acyl:adenyl ligases include enzymes generating bioactive amide hormone conjugates such as JA-Ile and JA-Trp from JA and IAA-Trp from indole acetic acid (Staswick, 2009; Staswick & Tiryaki, 2004). Virulent phytopathogens such as Pseudomonas syringae synthesise IAA-lysine synthetase which can inactivate plant IAA to IAA-lysine (Romano et al, 1991)
Microarray analysis of the early stages of PGP induced by GD12 in Col-0 plants supported the central role for hormonal modulation by *Trichoderma* spp. in plant growth promotion. Data support suppression of hormonal signaling by *T. hamatum*, most notably JA and ABA. In contrast, *T. asperellum* T34 activates JA/ET based priming events that underpin ISR and broad-spectrum enhanced resistance (Segarra et al, 2009). More robust experiments, including capturing the transcriptome of GD12 are needed to validate these findings.

Overall, it is an exciting and opportune time to exploit the remarkable genetic and chemical potential for beneficials for sustainable agriculture. Co-evolution with hosts has endowed *Trichoderma* spp. with a range of agronomically important traits. The genome of *T. hamatum* GD12 encodes the genetic potential to promote growth and induce ISR in a range of plants. The arsenal of genes in GD12 also enables it to effectively mycoparasitise *S. sclerotiorum*, a successful and persistent pathogen of agronomic crops. Strikingly, mycoparasitism of *S. sclerotiorum* results in additional PGP. Importantly, culture filtrates that promote plant growth (Fig. 1B) are incapable of suppressing the pathogenic effects of *Sclerotinia* suggesting that, additional enhanced plant growth stimulation occurs during interactions with soil pathogens. We hypothesise that antagonism between GD12 and root pathogens in the plant rhizosphere leads to transcriptional activation of cryptic secondary metabolite pathways that are phenotypically silent in axenic culture. This is in agreement with recent reports of activation of silent gene clusters in *Aspergillus nidulans* following co-cultivation of the fungus with other microorganisms which has led to the identification of novel secondary metabolites (Schroeckh et al, 2009). Thus the genome sequence of GD12, and comparisons with other *Trichoderma* genomes will facilitate genetic dissection of these traits. Genome informed predictions will help to identify and experimentally validate novel secondary metabolism implicated in adaptation to specific ecological niches and promotion of beneficial traits.

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**Figure Legends**

**Figure 1:** Plant growth promotion by *Trichoderma hamatum* GD12. (A). Amendment of peat compost with *T. hamatum* GD12 promotes growth of *Arabidopsis thaliana* accession Col-0. Photographed at 3 weeks, (B). Soluble, autoclaved bran extracts from GD12 or the *N*-acetyl-β-D-glucosaminidase knockout mutant (Δ*Thnag1::hph*) promote growth of lettuce (*Lactuca sativa*...
cultivar Webb’s Wonderful) in sterile peat. Lettuce were supplemented with the indicated amount of bran extract every second day. Photographs were taken 21 days after sowing.

**Figure 2:** Active biocontrol of the pre-emergence pathogen, *Sclerotinia sclerotiorum* by *T. hamatum*, GD12 results in additional plant growth promotion. (A). *T. hamatum*, GD12 but not the N-acetyl-β-D-glucosaminidase mutant Δ*Thnag1::hph* is able to suppress *S. sclerotiorum* and allow germination of lettuce seedlings. Photograph taken 7 days post sowing. (B). Mycoparasitism of *Sclerotinia sclerotiorum* by *T. hamatum*, GD12 results in enhanced plant growth promotion, compared to amendment with GD12 alone.

**Figure 3.** *T. hamatum* biocontrol and plant disease suppression. (A). Leaf segments of rice (cultivar CO-39) showing rice blast symptoms. (B). Suppression of rice blast disease by *T. hamatum*. Growth of rice cultivar CO-39 in soil amended with *T. hamatum* GD12 (white bars) and the N-acetyl-β-D-glucosaminidase deficient mutant Δ*Thnag1::hph* (grey bars) reduced the size of the lesions caused by the rice blast *Magnaporthe oryzae*. This was most pronounced in soil amended with Δ*Thnag1::hph* where disease was restricted to type 1 (lesion 0.5 mm in length) and type 2 (lesion ~ 1 mm in length). Plants grown in the absence of *T. hamatum* (black bars) developed lesion types 1 and 2 and also type 3 (lesions ~ 2 mm in length) and type 4 (lesions ~ 3-4 mm in length) lesions (Valent et al, 1991).

**Figure 4.** Venn diagrams showing the conservation of the *Trichoderma hamatum* GD12 predicted proteome and secretome in previously sequenced *Trichoderma* species. We used BLASTP to search for similar sequences to each of the 12,391 predicted GD12 proteins. We performed BLASTP searches against the previously published predicted proteomes of *T. atroviride*, *T. virens*, *T. reesei* and *T. harzianum* as well as against the GD12 predicted proteome. A protein was counted as conserved in a species if there was a BLASTP hit with least 80% amino acid sequence identity covering at least 90% of the query sequence. We present the results for the full 12,391 predicted proteins and also for the subset of these comprising 1,014 predicted secreted proteins. (A). *T. hamatum* GD12 shares a core proteome of 3620 predicted proteins, with *T. hamatum* with *T. atroviride*, *T. harzianum*, *T. reesei* and *T. virens* and has 4658 unique proteins. The GD12 proteome is most homologous to that of *T. atroviride*. (B) The 1,014 proteins predicted to encode secreted proteins based upon secretion signals (SignalP) and lack of a typical transmembrane domain (Phobius) were compared to similarly derived secretomes from *T.
atroviride, T. harzianum, T. reesei and T. virens. GD12 shares a core secretome of 327 proteins and has 370 predicted unique secretomes.

Figure 5. A genomic region unique to *Trichoderma hamatum* GD12 with the capacity to encode novel secondary metabolites. (A) This 47-kbp region (GenBank: KB232787) has no detectable nucleotide sequence similarity to previously sequenced *Trichoderma* genomes except for the two short regions indicated by rectangles, which share 85% and 78% nucleotide sequence identity with *T. atroviride* scaffold 19. (B) Arrows indicating predicted protein-coding genes, which are described in Table 1.
Supplementary Files.

Supplementary File 1:
GD12.secretome.no-TMs.faa.pfamscan.html. Frequencies of Pfam domains in predicted secreted proteins encoded in the GD12 genome.

Supplementary File 2:
Supp2_AMP-binding_secreted.faa.txt. AMP-binding domain proteins are over-represented in the secretome.

Supplementary File 3:
Supp3_GD12.secretome.no-TMs.SSCRPs.faa.txt
Sequences of candidate small secreted cysteine-rich proteins (SSCRPs) encoded in the GD12 genome. Amino acid sequences are given in FastA format.

Supplementary File 4:
Supp4_GD12.secretome.no-TMs.SSCRPs.faa.pfamscan.txt. Pfam domains in each predicted small secreted cysteine-rich proteins (SSCRPs) encoded in the GD12 genome.

Supplementary File 5:
Supp5_GD12.secretome.noTMs.SSCRPs.faa.pfamscan.html. Frequencies of Pfam domains in predicted small secreted cysteine-rich proteins (SSCRPs) encoded in the GD12 genome.

Supplementary File 6:
Arabidopsis thaliana genes suppressed 2 fold, six days after T. hamatum addition to peat compost.

Supplementary File 7:
Arabidopsis thaliana genes induced 2 fold, six days after T. hamatum addition to peat compost.

References


Thornton CR (2005) Use of monoclonal antibodies to quantify the dynamics of alpha-galactosidase and endo-1,4-beta-glucanase production by Trichoderma hamatum during saprotrophic growth and sporulation in peat. *Environmental microbiology* 7: 737-749


**Figure 3.**

**A**  
*M. oryzae* disease index

**B**  
*T. hamatum* disease severity score

- **M. oryzae** disease index:
  - 0: No visible lesions
  - 1: Mild lesions
  - 2: Moderate lesions
  - 3: Severe lesions
  - 4: Extreme lesions

- **T. hamatum** disease severity score:
  - Lesion score 1-4:
    - ΔThnag::hph
    - GD12
    - Control
  - Number of lesions per 5 cm²

Bar graph showing the number of lesions at different lesion scores.