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

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*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 an *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. The work has important implications for fungal morphogenesis, demonstrating a clear link between hyphal architecture and secretory potential. Furthermore, it highlights the need for a holistic approach to the development of genetically modified *Trichoderma* strains for use as crop stimulants and biocontrol agents 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; Ortiz-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; Djonović et al., 2007), less attention has been paid to enhancing the plant-growth-promoting (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 & 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 N-acetyl-β-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 *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 *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 micro-organism 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 *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 *Sclerotinia sclerotiorum* and *R. solani*, respectively, had been modified. Using laboratory-based microcosms, we show that while loss of N-acetyl-β-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 *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 *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.

**METHODS**

**Fungal strains, growth conditions and DNA analysis.** All strains of *T. hamatum* used in this study are derived from strain GD12 (GenBank accession no. AY247559) (Thornton et al., 2004; Thornton, 2005, 2008) and were maintained on potato dextrose agar (PDA) or V8 agar as described previously. Strain GD12 is free-living and does not form an association with plant roots. The anamorph group 1 post-emergence lettuce pathogen *R. solani* (CBS323.84) and polyphagous plant pathogen *S. sclerotiorum* (GenBank accession no. 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 [5’-GTCCTTG(AC)(AG)3G(GC)5(CT)TGAAG(A)CG3TTT(CT)(AT)(GC)5C-3’] and NagB [5’-TTGAG(CT)TGCAG(AG)CC(AG)(GT)(AT)G(GA)AA(G)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 amplification was 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 *Neol* 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 *Trichoderma harzianum* EXC2Y nucleotide sequence retrieved from the NCBI databases (www.ncbi.nlm.nih.gov). The primer set 30.2 (5’-CGCTATTACATATATAGTGGC-3’) and M13.52 (5’-TCTCCTTGAATAATTTACGCTGGCAGATC-3’) and primer set 5.1 (5’-TAGGGACATACCTCTCCCTCTC-3’) and M13.32 (5’-GTGGTAGCTGGAAAAACCTCGGGGAGGCCATAC-3’) 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* TRP promoter, conferring resistance to hygromycin, was cloned into pBluescript (Stratagene) as a 1.4 kb EcoRI-XhoI fragment. To amplify the split HPH templates, the primer set M13F (5’-CGCAGGTTTCCCCAGTCACAGC-3’) and HY (5’-GGATGCTCCGCTGAAGATA-3’) and primer set M13R (5’-AGCGGATAAACATTTCTACACAGGA-3’) and YG (5’-CCTTGCAAGACCTGCTGGA-3’) were used. A third round PCR was performed using the nested primers 5.2 (5’-TGAGCACAGCGGTCAGGCTGTAACCCGCTTAATGGTGAACCGGCAATCTGCCTC-3’) and 30.1 (5’-GCATCAACCTGTAGATGGTGCTG-3’) to join the constructs together. *T. hamatum* GD12 protoplasts were transformed with 2 μg DNA of the third round PCR product. ΔThnag::hph mutants were selected for resistance to 300 μg hygromycin ml⁻¹. The *T. hamatum* GD12 Nag gene and protein sequence were submitted to GenBank under accession no. JN107809.

**Complementation of the N-acetyl-β-D-glucosaminidase-deficient mutant.** A 4.29 kb ampiclon consisting of the 1.86 kb Thnag ORF, 1.93 kb of the promoter region and 0.5 kb of the 3’ untranslated 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 (5’-AGGGATGAGAGCTTGATGATT-3’) and NagD (5’-GGATGCTCCGCTGAAGATA-3’). PCR was performed in an Applied Biosystems GeneAmp PCR system 2400 cycler using Herculease polymerase in Herculase ×10 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 s, 50 °C for 30 s, 72 °C for 5 min (10 cycles), 94 °C for 30 s, 55 °C for 30 s, 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 Apal and Spel. The 4.29 kb Thnag fragment was subsequently liberated from pGEM-T using Apal and Spel, and ligated into the pCB1532 vector containing the ILVI gene encoding resistance to sulfonylurea (Sweigard et al., 1997). The resulting plasmid was used to transform the N-acetyl-β-D-glucosaminidase mutant ΔThnag::hph. Putative ΔThnag::hph:Nag retransformants were selected for resistance to 1 mg sulfonylurea ml⁻¹.

**Determination of hyphal chitin contents and chitinase activities.** For enzyme activity assays, fungal strains were grown for 4 days at 26 °C in multiple 250 ml flasks containing sterile autoclaved wheat bran mix [10 g wheat bran and 30 ml distilled water (dH₂O)] and 1.0% (w/v) chitin from shrimp shells (Sigma C7170). Flasks were inoculated with five 5 mm diameter plugs of mycelium taken from the growing edge of 4-day-old PDA plate cultures. Under these conditions, the bran-chitin mixture was fully colonized by both fungi by day 4. Extracts were prepared by mixing the contents of flasks with 50 ml dH₂O for 1 h at

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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 the method of Tronson & Harman (1993) using the substrate 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide, the fluorescent substrate of this enzyme (Duo-Chuan, 2006). For colorimetric estimations of enzyme activities, a commercial chitinase assay kit (Sigma C89980) was used. Chitin contents of hyphae were determined by colorimetric estimations of N-acetylglucosamine released by alkaline digestion. Three replicate 250 ml flasks containing 100 ml autoclaved potato dextrose broth (Fluka P6685) were inoculated with five 5 mm diameter plugs of mycelium taken from the growing edge of 4-day-old PDA plate cultures. The flasks were incubated at 26°C with shaking for 3 days and 100 mg samples of fresh hyphal biomass assayed for chitin content according to the nitrous acid – 3-methyl-2-benzothiazolone hydrazone hydrochloride – ferric chloride assay (Kaminsky & Heath, 1982; Thornton et al., 1991). Chitin concentrations were expressed as µg glucosamine (g fresh mycelium)-1. A Student’s t-test was used to determine statistical significance.

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

**P-G-P and soil nutrient analysis.** One litre of sieved (500–1000 µm) Sphagnum moss peat (Shamrock) was mixed with 400 ml dH2O and sterilized by autoclaving at 121°C for 15 min. *T. hamatum* inoculum 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 4-day-old PDA plate cultures of the fungus. After incubation at 26°C for 5 days 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 Wonder). 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 21 days growth, plants were harvested and dry weights of shoots and roots were obtained. Differences in dry weights were analysed by one-way analysis of variance (ANOVA) and post-hoc Tukey tests were used to determine significance.

Water extracts from bran inoculum were also used to determine whether P-G-P could be achieved in the absence of the fungus. Five-day-old bran inoculum (10 g) was mixed thoroughly for 1 h with 50 ml dH2O and extracts were centrifuged at 12000 g for 5 min. Clarified extracts were filtered through Miracloth (Calbiochem) and then filter-sterilized by passing through a 0.2 µm membrane (Millipore). Microcosms were constructed with 300 g sterilized peat and 30 ml sterile extract, and were sown with 25 lettuce seeds. Thereafter, conditions were as described above. After 7 days growth, dry weights of shoots and roots were obtained.

For soil nutrient analysis, replicate microcosms consisting of the peat and bran inoculum mix were assayed by NRM Laboratories after 21 days incubation under the conditions 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 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 in 250 ml flasks was sterilized by autoclaving and the flasks were 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 were transferred to microtitre wells for assay by ELISA. There were three replicates for each treatment. Absorbance values from ELISA were converted to units of protein equivalents by using standard curves of chromatographically purified antigen, prepared from doubling dilutions of a PBS solution of the antigen in microtitre wells (Thornton et al., 2002). Dry weights were obtained by drying the collected mycelium to a 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 505–570 nm emission wavelengths or a Zeiss LSM confocal microscope using 488 nm excitation and 505–570 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). Sieved (500–1000 µm) sphagnum moss peat (1 l; Shamrock) was mixed with 400 ml dH2O and 0.5% (w/v) wheat bran in 21 flasks and sterilized by autoclaving at 121°C for 15 min. For preparation of pathogen inoculum, 10 g white poppy seeds and 3 ml dH2O 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 days at 26°C, and the colonized seeds were 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 caspofungin ml-1 and spore suspensions were prepared from 3-week-old plates. 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 106 conidia ml-1 in dH2O. 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 were used to construct microcosms for antigen extraction and assay by *R. solani*-specific ELISA over a 21 day 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 (8 g bran inoculum and 300 g peat), pathogen only (8 g poppy seed inoculum and 300 g peat) or both. Plants were grown under the conditions described and percentage emergence and dry weights of plants were determined after 21 days. Differences in dry weights of shoot and root materials were analysed by single-tailed t-test. Differences in percentage emergence were determined by single-tailed t-test after transformation of data using the arcsin function.
RESULTS

Confirmation of N-acetyl-β-D-glucosaminidase disruption and complementation

A targeted gene disruption of the \textit{T. hamatum} NAG gene (GenBank accession no. 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 \textit{ΔThnag::hph}) contained the correct sized insertion (Supplementary Fig. S1, available with the online version of this paper). Southern blot analysis also confirmed complementation of mutant \textit{ΔThnag::hph} (C17, hereafter referred to as \textit{ΔThnag::hph:NAG}) (Supplementary Fig. S1). To investigate the effect of the mutation on enzyme activity, \textit{N-acetyl-β-D-glucosaminidase} activities of GD12, \textit{ΔThnag::hph} and the complemented strain were determined colorimetrically, and by using an in-gel assay for \textit{N-acetyl-β-D-glucosaminidase} activity. In-gel activity assays (Fig. 1a) and colorimetric assays showed disruption of \textit{N-acetyl-β-D-glucosaminidase} activity in the \textit{ΔThnag::hph} mutant and confirmed our initial findings that only a single copy of the \textit{N-acetyl-β-D-glucosaminidase} gene exists in \textit{T. hamatum} (Supplementary Fig. S2). Tests with \textit{ΔThnag::hph:NAG} showed that the complementation had restored enzyme activity using both the in-gel activity assay (Fig. 1b) and colorimetric assay (Table 1). The chitin content of mutant hyphae was significantly reduced (\textit{P}<0.05, Student’s \textit{t}-test) compared with GD12. The mean hyphal chitin concentration of \textit{ΔThnag::hph} was 6.9±0.9 μg glucosamine (g mycelium)$^{-1}$ compared with 17.9±2.6 μg glucosamine (g mycelium)$^{-1}$ for GD12.

\textit{N-acetyl-β-D-glucosaminidase} disruption enhances P-G-P but is not associated with nutrient release

The enzyme-deficient mutant \textit{ΔThnag::hph} was tested for its ability to enhance growth promotion of lettuce (\textit{L. sativa}). We originally hypothesized that disruption of \textit{N-acetyl-β-D-glucosaminidase}, a key chitinolytic enzyme implicated in the depolymerization of soil chitin and release of nitrogen for plant growth (Leake & Read, 1990; Kerley & Read, 1995, 1998; Read & Perez-Moreno, 2003; Lindahl & Taylor, 2004), might decrease P-G-P by \textit{T. hamatum}. In contrast with our expectations, we found that disruption of the \textit{NAG} gene dramatically enhanced the growth of lettuce seedlings as shown in Fig. 1c. Treatment with GD12 resulted in a fourfold increase in leaf and shoot dry weights (\textit{P}=0.05) and a fivefold increase in root dry weights (\textit{P}<0.001) compared with the control. The increase in plant growth was more dramatic with the \textit{N-acetyl-β-D-glucosaminidase}-deficient mutant. Treatment with \textit{ΔThnag::hph} resulted in a 13-fold increase in shoot and leaf dry weights (\textit{P}<0.001) and an 11-fold increase in root dry weights (\textit{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 P-G-P effects seen with certain \textit{Trichoderma} strains have been linked to the solubilization of phosphates and micronutrients (Altomare \textit{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 P-G-P with GD12 or with the \textit{ΔThnag::hph} mutant. Indeed, there were no significant increases in the amounts of available nutrients as a consequence of saprotrophic colonization of peat by the two strains of fungi when compared with the uncolonized controls. While concentrations of ammonia-nitrogen, nitrate-nitrogen and total soluble nitrogen were higher in \textit{ΔThnag::hph} microcosms compared with GD12, the differences were not significant (Student’s \textit{t}-test, Table 2). Nevertheless, to test whether the increase in nitrogen availability observed in the \textit{ΔThnag::hph} microcosms might contribute to the increase in P-G-P found with the mutant, we added ammonium and nitrate in the form of soluble NH$_4$Cl and NaNO$_3$ to identical levels found in \textit{ΔThnag::hph}-colonized microcosms. However, no increase in plant growth resulting from these treatments was found (results not shown). A number of elements (P, K, Mg, Ca, Na and sulphate) showed a reduction in availability in the \textit{Trichoderma}-treated microcosms. The reasons for this are unclear, but may be due to chelation by the fungi (Altomare \textit{et al.}, 1999). We were able to discount the possibility that growth promotion occurs in response to the control of minor root pathogens, because our studies were conducted with sterilized peat in the absence of disease pressure. This is consistent with other studies showing growth promotion by \textit{Trichoderma} species in the absence of detectable disease (Chang \textit{et al.}, 1986) and in sterile soil (Windham \textit{et al.}, 1986). We therefore conclude that P-G-P by the fungus does not occur through nutrient release or control of minor root pathogens.

Increases in the growth of lettuce plants in response to water extracts from bran inoculum of GD12 and \textit{ΔThnag::hph} showed that growth promotion was due, at least in part, to the production of a water-soluble growth enhancing compound(s) (Supplementary Fig. S3). Growth promotion was more pronounced with extracts from the \textit{N-acetyl-β-D-glucosaminidase}-deficient mutant than with extracts from GD12, indicating increased production of the stimulatory compound(s) by \textit{ΔThnag::hph}.

The \textit{N-acetyl-β-D-glucosaminidase}-deficient mutant has altered chitin deposition at the hyphal tip

Growth tests of the \textit{ΔThnag::hph} mutant revealed no significant reduction in hyphal growth \textit{in vitro} compared with GD12 (Fig. 2a, b). However, spore production was completely absent in the mutant in both axenic culture and soil (Figs 1e and 2a). The spore concentration of axenic cultures of the wild-type strain was 2.7×10$^7$ spores ml$^{-1}$. 

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Chitin is a structural component of the cell wall of *Trichoderma* species and *N*-acetyl-β-D-glucosaminidase is one of a number of chitinase enzymes that are believed to play a role in cell wall turnover and remodelling during hyphal development (Reyes et al., 1989a, b; Rast et al., 1991; Sahai & Manocha, 1993; Horsch et al., 1997; White et al., 2002). To test whether *N*-acetyl-β-D-glucosaminidase functions in cell wall biogenesis of this fungus, GD12 and

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**Fig. 1.** (a) In-gel activity assay showing loss of *N*-acetyl-β-D-glucosaminidase activity in the ΔThnag::hph mutant 6.1. (b) Zymograms showing restoration of *N*-acetyl-β-D-glucosaminidase activity in the ΔThnag::hph:NAG complemented strain C17. No other fluorescent products were visible on the gels. (c) Lettuce plants grown for 3 weeks in peat microcosms containing the *T. hamatum* strain GD12 and the *N*-acetyl-β-D-glucosaminidase-deficient mutant ΔThnag::hph. Bar, 18 mm. (d) Histogram showing dry weights of lettuce leaf (black bars) and root (white bars) material after 3 weeks growth in peat microcosms with GD12, ΔThnag::hph and the complemented strain C17. Data shown are the mean ± SEM of three replicate trays, each containing 25 plants. Bars with different letters are significantly different at 95% confidence level. (e) Sporulation of GD12 (production of spore masses, indicated by arrow) and lack of sporulation (absence of spore masses) of ΔThnag::hph in peat following incorporation of bran inoculum.
Glucosaminidase-deficient mutant partially 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 (Fig. 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 ΔThnag::hph mutant.

The significant (P<0.05, Student’s t-test) decrease in growth of ΔThnag::hph during exposure to caspofungin (Fig. 2a, b), an echinocandin antifungal drug that inhibits β-1,3-glucan synthases (Lesage et al., 2004; Walker et al., 2008; Fuchs & 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, 1994). 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 synthase by caspofungin would be predicted to further significantly inhibit cell wall biosynthesis and growth of the mutant compared with the wild-type strain. Despite this, after 3 weeks growth, sporulation had been partially restored in the ΔThnag::hph mutant (Fig. 2c), so that spore concentrations for GD12 and ΔThnag::hph were 1×10^7 ml^-1 (±0.2×10^7 ml^-1) and 6.4×10^4 ml^-1 (±1×10^4 ml^-1), respectively. Furthermore, the ΔThnag::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 upregulation of chitin biosynthetic genes and stimulation of chitin synthesis (Fortwendel et al., 2010). It is reasonable to speculate that a similar process occurs in ΔThnag::hph during caspofungin exposure, leading to improved cell wall integrity which supports transient and partial restoration of sporulation.

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

Data are the mean ± SEM of triplicate values.

<table>
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<tr>
<th>Strain</th>
<th>Enzyme activity (units ml^-1)</th>
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<tr>
<td></td>
<td>Chitobiosidase</td>
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<tr>
<td>GD12</td>
<td>0.70 ± 0.04</td>
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<tr>
<td>ΔThnag::hph</td>
<td>0.04 ± 0.01</td>
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<tr>
<td>ΔThnag::hph:NAG</td>
<td>0.68 ± 0.12</td>
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</table>

ΔThnag::hph mycelia were inoculated onto standard medium containing calcofluor white, and sensitivity was 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 (Fig. 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 ΔThnag::hph mutant.

The significant (P<0.05, Student’s t-test) decrease in growth of ΔThnag::hph during exposure to caspofungin (Fig. 2a, b), an echinocandin antifungal drug that inhibits β-1,3-glucan synthases (Lesage et al., 2004; Walker et al., 2008; Fuchs & 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, 1994). 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 synthase by caspofungin would be predicted to further significantly inhibit cell wall biosynthesis and growth of the mutant compared with the wild-type strain. Despite this, after 3 weeks growth, sporulation had been partially restored in the ΔThnag::hph mutant (Fig. 2c), so that spore concentrations for GD12 and ΔThnag::hph were 1×10^7 ml^-1 (±0.2×10^7 ml^-1) and 6.4×10^4 ml^-1 (±1×10^4 ml^-1), respectively. Furthermore, the ΔThnag::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 upregulation of chitin biosynthetic genes and stimulation of chitin synthesis (Fortwendel et al., 2010). It is reasonable to speculate that a similar process occurs in ΔThnag::hph during caspofungin exposure, leading to improved cell wall integrity which supports transient and partial restoration of sporulation.

Table 2. Peat nutrient analysis following saprotrophic colonization by T. hamatum GD12 and the N-acetyl-β-D-glucosaminidase-deficient mutant ΔThnag::hph

The units of measurement are mg l^-1 for all analytes except conductivity (μS cm^-1), dry matter (g converted to degrees using arc sin^-1 transformation), and density and dry density (kg m^-3). No units for pH.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Control</th>
<th>Strain GD12</th>
<th>ΔThnag::hph</th>
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</thead>
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<tr>
<td>Conductivity</td>
<td>169 ± 7</td>
<td>69 ± 1</td>
<td>72 ± 1</td>
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<tr>
<td>pH</td>
<td>4.40 ± 0.01</td>
<td>4.86 ± 0.03</td>
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<td>23.1 ± 0.3</td>
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<tr>
<td>Density</td>
<td>557 ± 7</td>
<td>565 ± 15</td>
<td>559 ± 24</td>
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<tr>
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<td>99 ± 1</td>
<td>87 ± 2</td>
<td>87 ± 3</td>
</tr>
<tr>
<td>Chloride</td>
<td>29 ± 2</td>
<td>21 ± 1</td>
<td>22 ± 2</td>
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<tr>
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</tr>
<tr>
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<td>0.76 ± 0.09</td>
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<tr>
<td>Sodium</td>
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<td>21 ± 1</td>
<td>15 ± 1</td>
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<tr>
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<td>33 ± 3</td>
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<td>Nitrate-nitrogen*</td>
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<td>1.03 ± 0.33</td>
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<td>Total soluble</td>
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<tr>
<td>Sulphate</td>
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<td>21 ± 2</td>
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<td>Boron</td>
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<td>0.117 ± 0.009</td>
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<td>Copper</td>
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<td>Manganese</td>
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<tr>
<td>Iron</td>
<td>0.130 ± 0.02</td>
<td>0.100 ± 0.006</td>
<td>0.113 ± 0.023</td>
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</tbody>
</table>

* Differences between means of GD12 and ΔThnag::hph not significant at 95% confidence level (Student’s t-test).
Fig. 2. Growth of *T. hamatum* GD12 and $\Delta T_{\text{Thnag}}::\text{hph}$ on PDA amended with calcofluor white and caspofungin. (a) Photographs showing growth of GD12 and $\Delta T_{\text{Thnag}}::\text{hph}$ after 4 days (PDA only, PDA+calcofluor white) or after 14 days (PDA+caspofungin). Bar, 15 mm. (b) Colony diameters of GD12 (□) and $\Delta T_{\text{Thnag}}::\text{hph}$ (○) measured over 4 days (PDA only, PDA+calcofluor white) or over 14 days (PDA+caspofungin). Each point is the mean±SEM of three replicate values. (c) Sporulation of *T. hamatum* GD12 and $\Delta T_{\text{Thnag}}::\text{hph}$ after 3 weeks growth on PDA amended with caspofungin, and (d) subsequent colony morphologies of single spore isolates after subculture to PDA only. Note lack of green spore masses in $\Delta T_{\text{Thnag}}::\text{hph}$ cultures grown in the absence of caspofungin.
The \textit{N}-acetyl-\textit{\beta}-\textit{D}-glucosaminidase-deficient mutant shows increased secretion of a \textit{Trichoderma}-specific extracellular antigen

Secretion by filamentous fungi is a process that occurs at the hyphal apex (Wösten \textit{et al.}, 1991; Wessels, 1994; Gordon \textit{et al.}, 2000; Thornton, 2004). The altered deposition of chitin at the hyphal tip of the mutant led us to investigate whether \textit{ΔThnag::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 ELISA (Thornton \textit{et al.}, 2002). A \textit{Trichoderma}-specific mAb MF2 raised against the antigen (Thornton \textit{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

\textbf{Fig. 3.} Phenotypic analysis of the \textit{ΔThnag::hph} mutant. (a) Microscopic analysis of hyphae of GD12 and \textit{ΔThnag::hph} following exposure to calcofluor white and observation under UV light, showing intense fluorescence at hyphal tips of the \textit{N}-acetyl-\textit{\beta}-\textit{D}-glucosaminidase-deficient mutant. Bar, 12 μm. (b) Immunofluorescence of GD12 and \textit{ΔThnag::hph} hyphae with mAb MF2, showing hyper-secretion of the glycoprotein antigen around the swollen tip of the mutant hypha. Bar, 10 μm. (d) Quantification of extracellular glycoprotein antigen concentrations in GD12 (○) and \textit{ΔThnag::hph} (●) shaking culture filtrates. Antigen concentrations in (d) were determined by converting absorbance values from ELISA with the \textit{Trichoderma}-specific mAb MF2 to equivalents of glycoprotein concentration by using a calibration curve of purified \textit{Trichoderma} antigen (c). Each point is the mean ± SEM of three values.
antigen production was found to be up to 35-fold higher in ΔThnag::hph compared with 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 ΔThnag::hph hyphae (Fig. 3b).

The N-acetyl-β-D-glucosaminidase-deficient mutant has reduced competitive saprotrophic ability and impaired biocontrol fitness

The competitive saprotrophic abilities of GD12 and ΔThnag::hph were determined by quantitative ELISA during antagonistic interactions with the pathogen R. solani in soil-based microcosms. Because the mutant ΔThnag::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 ΔThnag::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 ΔThnag::hph showed impaired interference competition, allowing saprotrophic growth of the pathogen throughout the 21 day sampling period (△). Specificity of the R. solani mAb EH2 was shown using extracts from microcosm containing GD12 only (□) or ΔThnag::hph only (●), where no antigen was detected throughout the 21 day sampling period.

Biocontrol fitness of GD12 and ΔThnag::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 the mean dry weights of shoot and root materials significantly compared with the control (lettuce plants with no treatment) (Fig. 4d, e). Additional further increases in mean dry weights of plant materials were shown in ΔThnag::hph-treated microcosms in the absence of disease pressure (Fig. 4d, e). The pre-emergence damping-off pathogen S. sclerotiorum prevented emergence of all lettuce plants in the absence of Trichoderma (Fig. 4b–e). T. hamatum GD12 controlled pre-emergence damping-off disease caused by S. sclerotiorum (Fig. 4c–e) and significantly increased seedling emergence and mean dry weights compared with the control. Furthermore, seedling emergence and mean root dry weight were significantly increased in the mixed species microcosms with the microcosms with GD12 treatment only (Fig. 4c, e). While treatment of S. sclerotiorum microcosms with the mutant ΔThnag::hph significantly increased establishment of lettuce seedlings compared with S. sclerotiorum only (Fig. 4b, c), percentage emergence was significantly reduced compared with the control. Furthermore, mean dry weights of shoot and root materials in the S. sclerotiorum + ΔThnag::hph microcosms were significantly reduced compared with 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 with control microcosms (Fig. 4c, e), but the mean shoot dry weight was significantly reduced (Fig. 4d). Mixed species microcosms containing R. solani and GD12 showed significant increases in emergence and mean dry weights compared with the control and the R. solani- and GD12-only microcosms (Fig. 4c–e). While similar trends were shown in microcosms containing the pathogen and ΔThnag::hph, the increases in mean shoot and root dry weights compared with R. solani only were significantly less than those of the R. solani+GD12 microcosms and, in the case of shoot dry weight, microcosms containing ΔThnag::hph only (Fig. 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 micro-organisms. One such beneficial micro-organism 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 P-G-P activities (Bae et al., 2004; Verma et al., 2009). In combination with major beneficial activities, such as increased plant growth and reduced pre-emergence damping-off pathogen R. solani infection and enhanced disease control of R. solani, the increases in mean shoot and root dry weights compared with R. solani only were significantly less than those of the R. solani+GD12 microcosms and, in the case of shoot dry weight, microcosms containing ΔThnag::hph only (Fig. 4c–e). Trichoderma is attractive as both biocontrol agents and plant growth stimulants.

Currently, strict governmental regulations prevent the deployment of genetically modified (GM) micro-organisms 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 T. hamatum imparts additional advantages in terms of biocontrol fitness and competitive saprotrophic ability (CSA). While a previous study has examined genetic stability and ecological persistence of Trichoderma 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*.

Mutagenesis of the gene (NAG) encoding N-acetyl-β-D-glucosaminidase, resulted in a non-sporeulating variant of GD12 with altered secretion, as shown by increased production of an extracellular *Trichoderma* glycoprotein antigen. The cell walls of ascomycete fungi contain a mixture of fibrillar components and amorphous or matrix

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**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 (○) and ΔThnag::hph (△) and in the absence of *Trichoderma* (●). The specificity of the *R. solani*-specific mAb 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 lyophilized mycelium (2 g peat-bran mix)] using a standard calibration curve of *R. solani* lyophilized mycelium. The mean ± SEM (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 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 (pathogens and *Trichoderma* strains) species peat-based microcosms. Control microcosms contained lettuce only. Data are the mean ± SEM from triplicate 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.
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 ΔThnag::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 of the two *Trichoderma* strains.

Saprotrophic competitiveness was investigated in soil microcosms during antagonistic interactions with the pathogen *R. 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). This mAb binds to an extracellular antigen secreted during hyphal growth of the pathogen (Thornton et al., 1993). Estimations of active biomass of the pathogen in microcosm samples required comparison with calibration curves. In the absence of a source of purified EH2 antigen, extracts from lyophilized mycelium (LM) were used as a quantifiable and repeatable source of antigen for the construction of a standard curve of *R. solani* biomass (Thornton & Gilligan, 1999). This curve was used to convert the absorbance values of microcosm extracts in ELISA to LM biomass equivalents [expressed as mg LM (2 g peat-bran mix)⁻¹], thereby allowing comparative estimates to be made of the abilities of GD12 and ΔThnag::hph to limit the growth of *R. solani*. In doing so, we were able to investigate the CSAs of GD12 and the mutant strain in relation to *R. solani* proliferation in mixed species microcosms. Using this procedure, we showed that the mutant ΔThnag::hph has reduced CSA compared with GD12, thereby permitting saprotrophic growth of the pathogen in soil. The interference competition of GD12 (as defined by Wicklow, 1992) is unlikely to be mediated through direct physical contact with the live host, since this strain of the fungus does not display coiling of *R. solani* hyphae *in vitro*, a property that has been demonstrated with other antagonistic *T. hamatum* strains (Chet et al., 1981). It is feasible that loss of overall chitinase activities due to N-acetyl-β-d-glucosaminidase deficiency was a contributing factor for the mutant’s inability to compete with *R. solani* for nutrient reserves (bran) in mixed species microcosms, since *Trichoderma* chitinolytic enzymes have been shown to have substantial inhibitory effects on the hyphal elongation of chitin-containing fungi such as *R. solani* (Lorito, 1998).

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 *S. sclerotiorum* is an important pathogen of a diverse range of hosts including lettuce (Malvárez et al., 2007). *T. hamatum* GD12 was effective in not only preventing colonization of lettuce seeds by the pathogen but also further stimulating seedling emergence in mixed species microcosms. In contrast, the mutant ΔThnag::hph displayed a significant reduction in its ability to stimulate emergence in the presence of the pathogen. However, a loss of emergence was compensated for 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 with microcosms containing GD12 only. However, the ability of ΔThnag::hph to promote plant growth was significantly impaired by the pathogen.

This work demonstrates that substantial improvements in plant productivity can be gained by genetically manipulating the growth stimulant properties of a biocontrol and P-G-P strain of *Trichoderma*. However, in the case of *T. hamatum* ΔThnag::hph, the genetic modification leading to improved P-G-P activity in the absence of disease pressure, does not confer 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.

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