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Signaling between bacterial and fungal biocontrol agents in a strain mixture

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Abstract

The use of bacterial and fungal strain mixtures is a promising way to improve efficacy of biocontrol treatments. Certain *Pseudomonas* and *Trichoderma* strains belong to the most common studied biocontrol agents. One key factor for the biocontrol efficacy of several *P. fluorescens* strains is the synthesis of 2,4-diacetylphloroglucinol (DAPG). Production of chitinases, such as the ECH42 endochitinase and the NAG1 *N*-acetyl-β-D-glucosaminidase, is a primary mechanism of action for *T. atroviride*. We examined the molecular interactions between the DAPG-producing *P. fluorescens* strains CHA0 and Q2-87 and chitinase-producing *T. atroviride* P1. Interactions were monitored using the reporter gene constructs, *phlA'-'lacZ* translational fusion in *P. fluorescens* CHA0 and *ech42-goxA* or *nag1-goxA* fusions in *T. atroviride* P1. We found that DAPG enhanced *nag1*, but not *ech42* expression, whereas an unidentified substance from *P. fluorescens* CHA0 repressed expression of both *Trichoderma* chitinases. Addition of *T. atroviride* P1 culture filtrates to growing cultures of *P. fluorescens* enhanced *phlA* expression transiently during growth. These results indicate that negative and positive effects on expression of key biocontrol genes may occur while mixing antagonists.

Keywords: *Pseudomonas*; *Trichoderma*; Biocontrol strain combination; Gene expression; 2,4-Diacetylphloroglucinol; Chitinase

1. Introduction

Introduction of two or more antagonists in a strain mixture has been proposed as an approach to improve the level and reliability of biocontrol treatments across an expanded broader range of environments [1]. Strain mixtures have indeed proven in some cases to be more effective than single strain treatments against a variety of plant diseases [2–7]. There are, however, other reports of biocontrol strain combinations that had no effect on efficacy or even reduced the level of disease suppressive activity of individual antagonists [8–12]. An important prerequisite for designing effective strain mixtures is choosing strains that complement rather than interfere with the antagonistic activity of each antagonist. Surprisingly, little is currently known at the molecular level about interactions between co-inoculated biocontrol strains despite the level of interest in strain mixtures.

The objective of this study was to determine in vitro the impact of two of the most studied biocontrol organisms, *Pseudomonas fluorescens* and *Trichoderma atroviride*, on the expression of each other's primary biocontrol genes. Well-characterized model strains of each organism were used. These organisms have distinct mechanisms in biocontrol and distinct regulation of key biocontrol genes. *P. fluorescens* CHA0 and Q2-87 produce various secondary metabolites with antimicrobial activity, including hydrogen cyanide and metal-chelating

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siderophores. Strain CHA0 also produces salicylic acid and the polyketide antimicrobial compound, pyoluteorin. For both strains, one of the most important is the polyketide antimicrobial compound 2,4-diacetylphloroglucinol (DAPG) [13]. DAPG is widely distributed in antagonistic *P. fluorescens* strains that occur in natural disease suppressive soils [14]. It has a wide range activity against bacteria, fungi, and helminths [15,16]. The DAPG synthesis is influenced by edaphic parameters (e.g., pH, mineral concentrations and clay quality) [17,18], host genotype, host age, pathogen infection [19], genetic instability of inoculants [20], and is inhibited by certain pathogen toxins [21]. The DAPG biosynthetic genes, *phlACBDE*, are organized as an operon [13]. In this study, strain CHA0, harboring a translational fusion of the promoter proximal DAPG biosynthetic gene, *phlA*, to the reporter gene *lacZ* on plasmid pME6259 was used to monitor expression of the DAPG biosynthetic gene. *Trichoderma* biocontrol activity is mostly due to mycoparasitism facilitated by the production of a suite of cell wall-degrading enzymes (CWDE) [22]. *T. atroviride* strain P1 (formerly classified as *T. harzianum*) [23], which we have used in our work, has previously been shown to have broad-spectrum biocontrol activity [24]. The CWDEs of strain P1 act synergistically to advance mycoparasitism of phytopathogenic fungi. Of these, the most important are the ECH42 endochitinase encoded by *ech42* and an *N*-acetyl-β-D-glucosaminidase encoded by *nag1* [25,26]. Expression of the *nag1* chitinase gene is repressed by a pathogen toxin [27]. To our knowledge, no other biotic factors influencing chitinase gene expression were identified. Much of the insight of regulation of these genes has been achieved using sensitive reporter fusions of the chitinase genes with the *Aspergillus niger* glucose-oxidase encoding gene, *goxA* [25,27]. These are the constructs we have used in our experiments.

In this study, we demonstrate that metabolites from a bacterial and a fungal antagonist alter, positively and negatively, the expression of each other's most critical biocontrol genes.

2. Materials and methods

2.1. Microorganisms and culture conditions

The *P. fluorescens* strains and their derivatives used in this study are described in Table 1. Plasmid pME6259 is a derivative of pME6010 and contains a *phlA'-'lacZ* translational fusion and a tetracycline resistance gene [29]. Plasmid pME6010 has approximately six copies per chromosome and is maintained stably in *P. fluorescens* in the absence of antibiotic selection [34]. Preliminary work has shown that pME6259 retention in strain CHA0 is 98.8 ± 0.9% after 20 generations. Expression of the *phlA'-'lacZ* translational fusion reflects DAPG biosynthesis [19,29]. Bacteria were routinely cultivated at 27 °C on King's B agar (KB) [35] or LB medium (Difco, Detroit, MI) with the addition of tetracycline (125 μg/ml) as required, unless otherwise mentioned.

T. atroviride strain P1 and derivatives carrying *goxA*-reporter gene fusions with the *ech42* (derivative *ech42-gox*) or the *nag1* (derivative *nag1-gox*) chitinase biosynthetic gene, have previously been described [25]. These strains display a glucose oxidase activity pattern that is consistent under various conditions with expression of the native *ech42* and *nag1* genes, as assayed by Northern analysis [25]. The strains were stored as spore suspensions in 20% glycerol (vol/vol) at −20 °C. Four days before the start of experiments, *T. atroviride* strains were grown on PDA media [4.8 g potato dextrose broth (Difco), 12 g agar (Oxoid, Hampshire, UK), pH 6.5] at 24 °C in darkness.

2.2. Measurement of gene expression

To measure glucose oxidase activity, 10 ml of a phosphate buffer (KH_2PO_4: 1.2 g/l, K_2HPO_4: 2.6 g/l, pH 7.1) were added to each plate. Plates were shaken for 30 min at 80 rpm, the liquid containing mycelia fragments was transferred to 15 ml Falcon™ centrifuge tubes (Greiner Labortechnik, Kremsmünster, Austria), and centrifuged for 5 min at 1800*g*. The supernatant was

Table 1
Pseudomonas fluorescens and *Trichoderma atroviride* strains used

Strain	Relevant characteristic	Reference
Pseudomonas fluorescens		
CHA0	Wild-type, DAPG$^+$, Plt$^+$, Swiss natural disease suppressive soil	[28]
CHA631	Δ*phlA*/DAPG$^-$, Plt$^+$	[29]
CHA660	Tn*5*-insertion, Km^r, DAPG$^+$, Plt$^-$	[30]
CHA89	*gacA*::Ω-km, Km^r, DAPG$^-$, Plt$^-$	[31]
Q2-87	Wild-type, DAPG$^+$, Plt$^-$, Washington state, natural disease suppressive soil	[1,32]
Q2-87::Tn*5*-1	Q2-87 *phlD*::Tn*5*-1, DAPG$^-$, Km^r	[32]
Trichoderma atroviride		
P1	Wild-type, isolated from wood chips	[33]
P1 *ech42-gox*	Endochitinase Ech42$^+$, *ech42-gox* fusion Hyg^r	[25]
P1 *nag1-gox*	*N*-Acetyl-β-D-glucosaminidase Nag1$^+$, *nag1-gox* fusion, Hyg^r	[25]

DAPG, 2,4-diacetylphloroglucinol; Plt, pyoluteorin; Km^r, kanamycin resistant; Hyg^r, hygromycin resistant.

used for quantification of glucose oxidase activity according to Mach et al. [25].

β-Galactosidase assays were conducted according to the method of Miller [36]. Activities were expressed as units per 10^8 cfu. Unit values were calculated according to Miller [36] following the formula:

$$1000 \times OD_{420}/(\text{time [min]} \times \text{sample volume [ml]}) = \text{units of } \beta\text{-galactosidase.}$$

2.3. Influence of P. fluorescens and derivatives lacking production of specific metabolites on expression of key biocontrol genes of T. atroviride P1

Unless otherwise mentioned, overnight cultures of the *P. fluorescens* wild-type strain CHA0 grown in 10 ml of LB broth (27 °C, 160 rpm) were diluted to an optical density at 600 nm (OD_{600}) of 0.125, which is equivalent to 10^8 cfu/ml. The bacteria were inoculated with four spots of 20 μl in equidistance on the circumference of a circle of 3.2 cm from the center of a 1.5% malt extract agar (MA) plate. One plug of an actively growing culture of *T. atroviride* P1 reporter strain *ech42-gox* or *nag1-gox* was placed inverted in the centre of the plate and incubated at 24 °C in darkness. After 66 h, the mycelial surface area of *T. atroviride* P1 was measured using a planimeter and glucose oxidase activity was measured as described above. Data from four experiments with six replicate plates were pooled after determining there was no significant trial × treatment interaction ($P = 0.439$, and 0.700 for *ech42*-, and *nag1*-expression) using Systat version 9.0 (Systat Inc., Evanston, IL). Means of four experiments with six replicates each were separated using Fisher's protected ($P \leqslant 0.05$) least significant difference (LSD) test.

To evaluate a possible influence of secondary metabolites of *P. fluorescens* CHA0 on chitinase gene expression in *T. atroviride* P1, the reporter strains *nag1-gox* and *ech42-gox* were grown in the presence of the following derivatives of *P. fluorescens* CHAO: CHA631 (DAPG-minus), CHA660 (pyoluteorin-minus) and CHA89 (*gacA*-minus).

In a second set of experiments, the role of DAPG in the interaction of the two antagonists was determined. *T. atroviride* P1 reporter strain *nag1-gox* was grown in presence or absence of *P. fluorescens* wild-type strains CHA0 and Q2-87 and their non-DAPG-producing derivatives CHA631 and Q2-87::Tn*5*-1 as described above. All treatments were tested on plates prepared with as well as without the addition of synthetic DAPG (0.1 mM).

2.4. Influence of secondary metabolites of P. fluorescens CHA0 on ech42 and nag1 expression in T. atroviride P1

MA plates were amended with synthetic DAPG, pyoluteorin or salicylic acid to a final concentration of 0.1 mM. Salicylic acid was also tested at 0.5 mM. All substances were dissolved in methanol, and the control treatment received the same amount methanol (final concentration: 5%). One plug of an actively growing culture of *T. atroviride* P1 reporter strain *ech42-gox* or *nag1-gox* was placed inverted in the middle of a plate and incubated for 66 h at 24 °C in darkness. The mycelial surface area was measured using a planimeter and glucose oxidase activity was measured as described above. Data from repeated trials were pooled after determining no significant treatment × trial interaction ($P = 0.156$, and 0.106 for *ech42*-, and *nag1*-expression), and analyzed as described above.

2.5. Influence of T. atroviride P1 filtrates on bacterial gene expression in vitro

Czapek-Dox medium (Oxoid, Hampshire, UK) (250 ml in 500 ml baffled flasks) was inoculated with four 7-mm diameter plugs taken from an actively growing culture of *T. atroviride* wild-type strain P1. Cultures were incubated for 7 days at 24 °C on a rotary shaker (180 rpm) in darkness. Fungal biomass was collected by centrifugation at 2200*g* for 15 min. To remove cells, the supernatant was filtered through a 0.8 μm-membrane (Nalgene, Rochester, NY) and stored at 2 °C for no longer than 1 week. Mid- to end-exponential growth-phase LB cultures of *P. fluorescens* CHA0 carrying the *phlA'-'lacZ* reporter construct were diluted to an OD_{600} of 0.001 and 30-μl aliquots were used for inoculation in 24 ml of KB broth amended with 6 ml of fungal filtrates. Bacteria grown in KB amended with plain Czapek-Dox medium served as a control. Cultures were incubated with rotary shaking at 160 rpm at 27 °C. In all experiments, samples of 1 ml were taken throughout the exponential and early stationary growth phase and β-galactosidase activities were determined as described above. Bacterial growth was estimated at OD_{600}, after having established the correlation with cfu by dilution plating on KB agar. The experiment included four replicate flasks and was repeated four times with similar results in all trials.

2.6. Influence of volatile substances from T. atroviride P1 on expression of a phlA'-'lacZ reporter gene fusion in P. fluorescens CHA0

The experiment was conducted on dual compartment dishes to avoid physical contact between the microorganisms but to allow air transfer. One compartment contained KB agar and was inoculated with a 100 μl drop of a diluted ($OD_{600} = 0.1$) overnight culture of the *P. fluorescens* reporter strain CHA0/pME6259 grown in 10 ml of LB broth (27 °C, 160 rpm). The other compartment contained MA and was inoculated with one 7-mm-diameter plug of an actively growing culture of *T. atroviride*

wild-type strain P1. The fungal biocontrol agent was inoculated 48 h prior to the bacterial agent. After 17, 20 and 23 h bacterial incubation at 27 °C in darkness, bacteria were scraped off the plate with 5 ml of saline solution (0.9%). The β-galactosidase activities of bacterial suspensions were determined as described above. Bacterial growth was estimated at OD_{600} and by dilution plating on KB agar supplemented with tetracycline.

3. Results

3.1. P. fluorescens *strains altered growth and chitinase gene expression in* T. atroviride *P1*

When *T. atroviride* P1 was grown alone, *ech42* expression which is indicative of ECH42 biosynthesis per mycelial surface was 28.6 mU/cm^2 (Fig. 1A). Expression of the *ech42-gox* fusion was significantly repressed, between 38.5% and 45%, by co-inoculation with *P. fluorescens* CHA0 or one of its derivatives (Fig. 1A). In contrast, *nag1* expression in *T. atroviride* P1 which is indicative of NAG1 biosynthesis was not altered in the presence of *P. fluorescens* CHA0 or its derivative CHA660, both DAPG-producers and ranged between 21 to 22 mU/cm^2 (Fig. 1C). The DAPG-non-producing derivatives CHA631 and CHA89 repressed expression of the *nag-gox* fusion by 36.5% (Figs. 1C and 2). In contrast to strain CHA0, the wild-type strain Q2-87 enhanced significantly expression of *nag1* by 97%. The DAPG non-producing derivative Q2-87::Tn*5*-1 had no influence on *nag1* expression (Fig. 2).

After 66 h on MA plates, *T. atroviride* P1 reporter strain *nag1-gox* grew to cover a surface area of 54.5 cm^2. Fungal growth was reduced by co-inoculation with the wild-type *P. fluorescens* strains CHA0 (by 50%) (Fig. 1D) and Q2-87 (by 40%) (data not shown). Growth reduction with their derivatives was 51% (CHA660), 19% (CHA631), 15% (CHA89) (Fig. 1D), and 11% (Q2-87::Tn*5*-1) (data not shown). Growth of *T. atroviride* P1 reporter strain *ech42-gox* was similar to that of the *nag1-gox* reporter strain (Fig. 1B).

3.2. *Induction of* nag1 *expression with synthetic DAPG*

Amendment of the media with 0.1 mM synthetic DAPG enhanced *nag1* expression in *T. atroviride* (Fig. 2). In presence of strain CHA0 and CHA631 the *nag1* expression was lower than in presence of Q2-87 and Q2-87::Tn*5*-1, suggesting the existence of an inhibitory factor in CHA0, which is absent in strain Q2-87. On DAPG amended media, *nag1* expression was not lower in the presence of the DAPG-negative mutants than in the presence of their correspondingly wild-type strains on pure media indicating that the DAPG amendment was able to compensate the loss of DAPG production by the mutants.

3.3. *Secondary metabolites of* P. fluorescens *enhanced expression of a* nag1-gox *and* ech42-gox *reporter gene fusion in* T. atroviride *P1*

Expression of *ech42* in *T. atroviride* P1 was slightly induced by DAPG (0.1 mM), pyoluteorin (0.1 mM), and

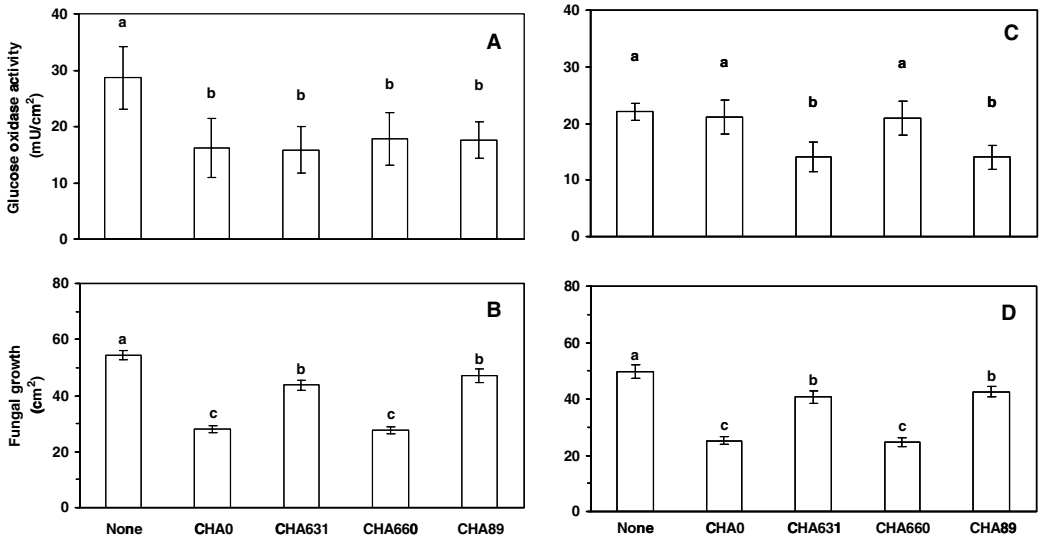

Fig. 1. Expression of the *ech42-gox* (A) and *nag1-gox* (C) fusion and growth of *T. atroviride* P1 derivatives *ech42-gox* (B) and *nag1-gox* (D) in presence of *P. fluorescens* wild-type strain CHA0 or its derivatives CHA631 (DAPG-minus), CHA660 (pyoluteorin-minus), and CHA89 (*gacA*-mutant). Bacteria were inoculated as four 20 μl spots in 3.2 cm distance of *Trichoderma* and plates were incubated for 66 h at 24 °C in darkness. Each value is the mean of four experiments with six replicates per treatment. Bars with the same letter are not significantly different according to Fisher's protected LSD test ($P \leqslant 0.05$).

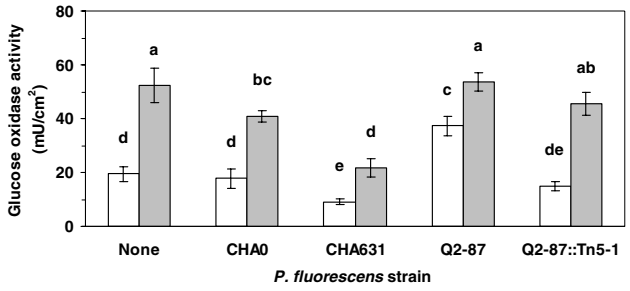

Fig. 2. Expression of the *nag1-gox* fusion in *T. atroviride* P1 grown on MA plates (empty bars) or MA plates containing DAPG (0.1 mM) (grey bars) in presence of *P. fluorescens* wild-type strains CHA0 and Q2-87 and their non-DAPG-producing derivatives CHA631 and Q2-87::Tn*5*-1. Bacteria were inoculated as four 20 μl spots in 3.2 cm distance of *Trichoderma* and plates were incubated for 66 h at 24 °C in darkness. Each value is the mean of three experiments with six replicate plates. Bars with different letters are significantly different according to Fisher's protected LSD test ($P \leqslant 0.05$).

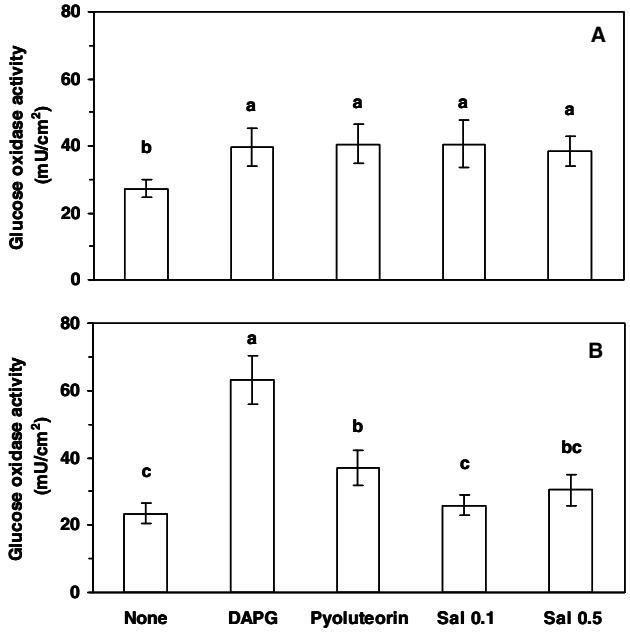

Fig. 3. Expression of the *ech42-gox* (A) and *nag1-gox* (B) fusions in *T. atroviride* P1 grown on MA agar plates amended with DAPG (0.1 mM), pyoluteorin (0.1 mM), and salicylic acid (Sal at 0.1 and 0.5 mM) for 66 h at 24 °C in darkness. The control treatment (None) received same amount of methanol solvent. Each value is the mean of four experiments with six replicate plates. Bars with different letters are significantly different according to Fisher's protected LSD test ($P \leqslant 0.05$).

salicylic acid (0.1 and 0.5 mM) (Fig. 3A), with expression levels ranging between 27.2 and 40.6 mU/cm^2. In contrast, *nag1* expression was strongly induced by DAPG (169%) and pyoluteorin (57%). 0.1 mM salicylic acid did not significantly alter *nag1* expression, but 0.5 mM salicylic acid enhanced *nag1* expression by 30% (Fig. 3B). In the absence of *P. fluorescens* CHA0 secondary metabolites, *nag1* expression was 23.5 mU/cm^2 fungal surface area. When growth effects of the bacterial metabolites on *gox* reporter gene expression are accounted for [37], we found that for *ech42-gox* all metabolites (DAPG, pyoluteorin and salicylic acid) were actual inducers (Fig. 4A). In contrast, for *nag1-gox* only DAPG had a growth independent inducer activity (Fig. 4B).

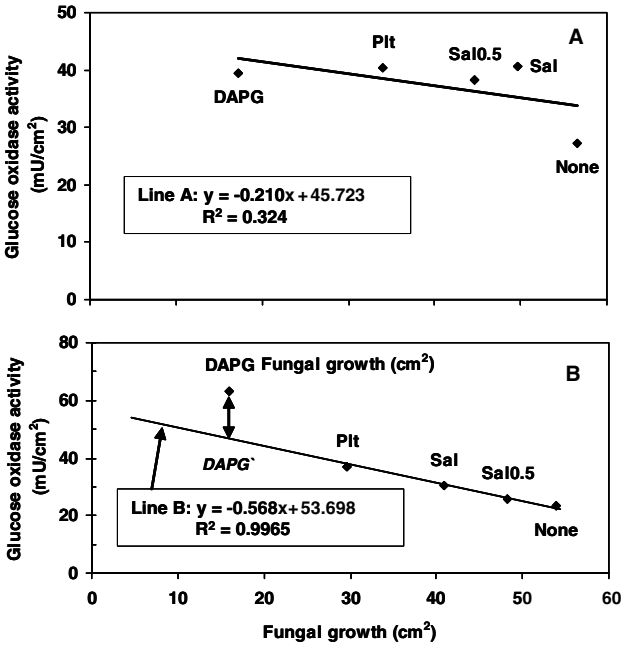

Fig. 4. Correlation between fungal growth and expression of the *ech42-gox* (A) and *nag1-gox* (B) fusions in *T. atroviride* P1 in the presence of DAPG (0.1 mM), pyoluteorin (Plt) (0.1 mM), and salicylic acid (Sal at 0.1 and 0.5 mM). The control treatment (None) received the same amount of methanol solvent. Lines A and B represent the expected values supposing growth and expression would be linked. *DAPG'* is the expected value for the DAPG treatment in the case that *nag1*-expression is only growth dependent. The arrow indicates the growth-independent part of the DAPG mediated induction of *nag1*-expression. Each value is the mean of four experiments with six replicate plates. Equation and R^2-values were calculated by using Systat version 9.0.

3.4. T. atroviride *P1 culture filtrates enhanced the expression of DAPG biosynthetic genes in* P. fluorescens *CHA0*

Fungal metabolites in the filtrates increased expression of the translational *phlA'-'lacZ* reporter indicative of DAPG biosynthesis transiently during bacterial growth (Fig. 5A). Normal levels of β-galactosidase activity were 30 U/10^8 cfu (after 14 h) and 404 U/10^8 cfu (after 20 h), with a subsequent decline to 320 U/10^8 cfu after 23 h growth. When of *T. atroviride* culture filtrates were added to the bacterial growth media at a ratio of 5:1 *phlA* expression was increased by 76% after 14 h of growth. This enhancing effect declined steadily until 23 h of growth when no significant impact of culture filtrates

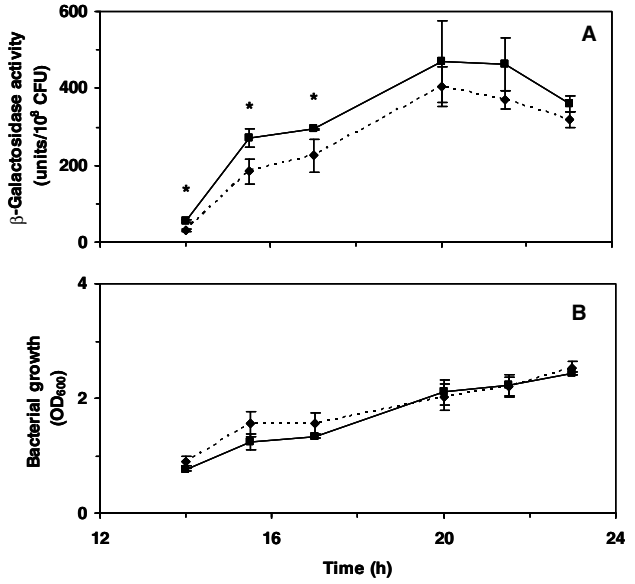

Fig. 5. Expression of *phlA'-'lacZ* (A) and growth (B) of *P. fluorescens* CHA0/pME6259 in media amended with *T. atroviride* wild-type strain P1 culture filtrates. Bacteria were grown in 24 ml KB media amended with 6 ml Czapek-Dox media (···) or in 24 ml King's B medium amended with 6 ml culture filtrates of *T. atroviride* P1 (—) at 27 °C. Values are means of one representative experiment with four replicate flasks. Asterisks (*) indicate means that are significantly different according to Fisher's LSD protected test ($P \leqslant 0.05$).

was observed anymore (Fig. 5A). Cell-free culture filtrates of *T. atroviride* wild-type strain P1 had no significant effect on bacterial growth when added to liquid media at a ratio of 5:1 (Fig. 5B).

3.5. T. atroviride *P1 volatile substances enhanced expression of DAPG biosynthetic genes in* P. fluorescens *CHA0*

The influence of volatiles emitted by *T. atroviride* P1 on the expression of a *phlA'-'lacZ* fusion in *P. fluorescens* strain CHA0 was monitored by growing both organisms in a two-compartment petri dish that physically separated the two organisms but allowed gas exchange. In the absence of *T. atroviride* P1 *phlA*-expression in CHA0 was highest 17 h after inoculation (193 U/10^8 cfu) and decreased to 80 and 121 U/10^8 cfu after 20 h and 24 h, respectively (Fig. 6). Volatiles from *T. atroviride* P1 induced *phlA* expression. Induction was highest after 20 h, where expression of the *phlA'-'lacZ* fusion was 60% higher in the presence of fungal volatiles than in their absence (Fig. 6).

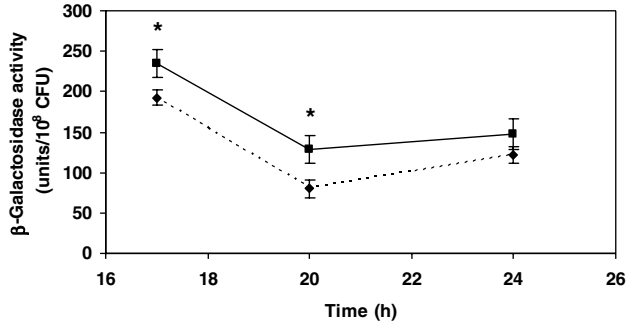

Fig. 6. Expression of *phlA'-'lacZ* in *P. fluorescens* CHA0/pME6259 grown on a two-compartment petri dish co-inoculated (—) or not (···) with *T. atroviride* P1. Means of two experiments with four replicate plates are presented. Asterisks (*) indicate means that are significantly different according to Fisher's LSD protected test ($P \leqslant 0.05$).

4. Discussion

Combining biocontrol agents in strain mixtures is one approach to improve the level and reliability of performance [1]. We found that when a bacterial and a fungal biocontrol agent were combined in a strain mixture, there were positive effects on the expression of key biocontrol genes in both antagonists. This is the first report of a beneficial interaction between fungal and bacterial antagonists at the molecular level, and it offers an explanation for improved biocontrol activity with *Trichoderma* and *Pseudomonas* mixtures [38].

Comparison of wild-type strains and mutants indicated that the bacterial antimicrobial compound DAPG was the primary mechanism behind the stimulation of the chitinase gene. The DAPG level we used is similar to what has been reported to be produced by the bacteria in the rhizosphere of different crops [19,39]. Also expression of both chitinases was repressed by an unknown modulator produced by *P. fluorescens* CHA0; a phenomenon which was absent with *P. fluorescens* Q2-87. The strains used in this study belong to different ARDRA groups (ARDRA = analysis of restriction pattern of amplified DNA coding for 16s rRNA) showed by comparison of *phlD* restriction patterns [40] and have therefore genetically different backgrounds. It would be interesting, if members of the same ARDRA group generally alter chitinase expression similarly. Further studies to identify the inhibitory components of CHA0 would enable selection of strains that only improve interactions, or finding ways to selectively shut-off the negative signal. Not surprisingly, *ech42* and *nag1* were altered differentially by *Pseudomonas* and its secondary metabolites. These genes have distinct regulatory mechanisms [25] and it has been shown that the *Fusarium*-mycotoxin deoxynivalenol repress only *nag1*-expression [27]. Stress factors are known as inducers for *ech42*-, but not for *nag1*-expression. This indicates that the chitinase gene induction observed was not in consequence of physiological stress.

Environmental factors influence the production of antimicrobial compounds, including DAPG, by fluorescent pseudomonads [13,20]. Here, we report a new biotic modulator for DAPG biosynthetic gene expres-

sion. Some biotic factors, which interfere with *phlA*-expression, interact with the *phlF* gene encoding a repressor protein of DAPG biosynthesis [13]. *T. atroviride* P1 might produce an inducer that binds to PhlF and thereby prevents the interaction of the repressor protein with the *phl* promoter leading to the enhanced *phlA* expression observed. Volatile organic compounds (VOC) modulate interactions between a wide range of soil bacteria and fungi. These effects include both stimulation and inhibition of growth and enzyme production [41]. In our study, *T. atroviride* volatiles had a significant stimulatory effect on *phlA* gene expression. Further research is needed to identify and characterize the volatile fraction responsible for this effect.

Our results show for the first time that the compatibility of antagonists applied as strain mixtures is not only dependant on compatible utilization of nutrients, minerals or space but can also be influenced by molecular signaling between the individual antagonists. In addition to the benefit of enhancing the biocontrol mechanisms of individual strains in the mixture, there is potential for synergistic activity. Fogliano et al. [42] reported that *Pseudomonas* lipodepsipeptides primed pathogen cells such that CWDE penetration and activity was increased. It has been reported recently that DAPG causes different stages of disorganization in hyphal tips in *P. ultimum* var. *sporangiiferum*, including alteration of the plasma membrane, vacuolization and cell content disintegration [43].

It has been shown that some mycotoxins produced by fungal pathogens, like fusaric acid and deoxynivalenol are able to down-regulate expression of genes involved in biocontrol activity of antagonists [27,29,44]. These effects have been interpreted as a potential pathogen self-defense mechanism [21]. Interestingly, in this study, metabolites produced by antagonists positively influence expression of key biocontrol factors.

Despite the positive influence of the bacterial antagonist on biocontrol gene expression in the co-inoculated fungal antagonist, we observed a slight but significant reduction in *T. atroviride* growth when co-inoculated with wild-type strains of *P. fluorescens*. The bacterial antimicrobial compound DAPG was the likely culprit since we observed less inhibition with the *P. fluorescens* DAPG non-producing mutants. Even though DAPG has been known to have a broad-spectrum antimicrobial activity [15], ours is the first demonstration of non-target inhibition of plant-beneficial fungus, and it provides a reasonable explanation for early observations of ineffective biocontrol with *Trichoderma* in North American and Australian soils that have large populations of indigenous pseudomonads [8,45,46]. In contrast, the fungal antagonist in our strain mixture had no adverse effect on growth of *P. fluorescens*. The cosmopolitan occurrence of DAPG-producing pseudomonads [14] and *Trichoderma* [24] strains in agricultural soils, particularly strains with disease suppressive activity, increases the importance of looking at the molecular interactions we describe when designing biocontrol inoculants.

In order to overcome the drawback of non-target growth inhibition, it may be feasible to select *Trichoderma* strains that have enhanced tolerance to DAPG. Natural diversity within populations of pathogenic fungi has been reported for sensitivity to *Pseudomonas* and *Trichoderma* metabolites [21]. Recently, active efflux with ABC transporters has been reported to increase tolerance in several fungi to bacterial antibiotics [47]. Nevertheless, whereas compatibility in terms of growth is certainly desirable it is by no means exclusive in terms of improved efficacy of strains mixtures. Pierson and Weller [1] reported that some of the most effective mixtures of *Pseudomonas* strains were those containing mutually growth inhibitory cohorts. Similarly, Duffy et al. [4] reported that *Pseudomonas* and *Trichoderma* strain mixtures were effective even when the bacterial cohort inhibited growth of the fungus, probably because the antagonists were spatially separated in the rhizosphere. Induction of expression of genes involved in biocontrol activity (e.g., DAPG effects on *nag1* chitinase gene) might compensate for slight growth inhibitory effects.

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References

[1] Pierson, E.A. and Weller, D.M. (1994) Use of mixtures of fluorescent pseudomonads to suppress take-all and improve the growth of wheat. Phytopathology 84, 940–947.

[2] Park, C.-S., Paulitz, T.C. and Baker, R. (1988) Biocontrol of Fusarium wilt of cucumber resulting from interactions between *Pseudomonas putida* and nonpathogenic isolates of *Fusarium oxysporum*. Phytopathology 78, 190–194.

[3] Paulitz, T.C., Ahmad, J.S. and Baker, R. (1990) Integration of *Pythium nunn* and *Trichoderma harzianum* isolate T-95 for the biological control of Pythium damping-off of cucumber. Plant Soil 121, 243–250.

[4] Duffy, B.K., Simon, A. and Weller, D.M. (1996) Combination of *Trichoderma koningii* with fluorescent pseudomonads for control of take-all on wheat. Phytopathology 86, 188–194.

[5] Leeman, M., den Ouden, F.M., van Pelt, J.A., Cornielsen, C., Matamala-Garros, A., Bakker, P.A.H.M. and Schippers, B. (1996) Suppression of fusarium wilt of radish by co-inoculation of fluorescent *Pseudomonas* spp. and root-colonizing fungi. Eur. J. Plant Pathol. 102, 21–31.

[6] Raupach, G.S. and Kloepper, J.W. (1998) Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathology 88, 1158–1164.

[7] Guetzky, R., Shtienberg, D., Elad, Y. and Dinoor, A. (2001) Combining biocontrol agents to reduce the variability of biological control. Phytopathology 91, 621–627.

[8] Hubbard, J.P., Harman, G.E. and Hadar, Y. (1983) Effect of soilborne *Pseudomonas* spp. on the biological control agent, *Trichoderma hamatum*, on pea seeds. Phytopathology 73, 655–659.

[9] de la Cruz, A.R., Poplawsky, A.R. and Wiese, M.V. (1992) Biological suppression of potato ring rot by fluorescent pseudomonads. Appl. Environ. Microbiol. 58, 1986–1991.

[10] Dandurand, L.M. and Knudsen, G.R. (1993) Influence of *Pseudomonas fluorescens* on hyphal growth and biocontrol activity of *Trichoderma harzianum* in the spermosphere of pea. Phytopathology 83, 265–270.

[11] de Boer, M., van der Sluis, I., van Loon, L.C. and Baker, P.A.H.M. (1999) Combining fluorescent *Pseudomonas* spp. strains to enhance suppression of Fusarium wilt of radish. Eur. J. Plant Pathol. 105, 201–210.

[12] Molina, L., Constantinescu, F., Michel, L., Reimmann, C., Duffy, B. and Défago, G. (2003) Degradation of pathogen quorum-sensing molecules by soil bacteria: a preventive and curative biological control mechanism. FEMS Microbiol. Ecol. 45, 71–81.

[13] Haas, D. and Keel, C. (2003) Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant diseases. Annu. Rev. Phytopathol. 41, 117–153.

[14] Weller, D.M., Raaijmakers, J.M., Gardener, B.B.M. and Thomashow, L.S. (2002) Microbial populations responsible for specific soil suppressiveness to plant pathogens. Annu. Rev. Phytopathol. 40, 309–348.

[15] Keel, C., Schnider, U., Maurhofer, M., Voisard, C., Laville, J., Burger, U., Wirthner, P., Haas, D. and Défago, G. (1992) Suppression of root diseases by *Pseudomonas fluorescens* CHA0: importance of the bacterial secondary metabolite 2,4-diacetylphloroglucinol. Mol. Plant-Microbe Interact. 5, 4–13.

[16] Cronin, D., Moënne-Loccoz, Y., Fenton, A., Dunne, C., Dowling, D.N. and O'Gara, F. (1997) Role of 2,4-diacetylphloroglucinol in the interactions of the biocontrol pseudomonad strain F113 with the potato cyst nematode *Globodera rostochiensis*. Appl. Environ. Microbiol. 63, 1357–1361.

[17] Duffy, B. and Défago, G. (2000) Controlling instability in *gacS-gacA* regulatory genes during inoculant production of *Pseudomonas fluorescens* biocontrol strains. Appl. Environ. Microbiol. 66, 3142–3150.

[18] Ownley, B.H., Duffy, B.K. and Weller, D.M. (2003) Identification and manipulation of soil properties to improve the biological control performance of phenazine-producing *Pseudomonas fluorescens*. Appl. Environ. Microbiol. 69, 3333–3343.

[19] Notz, R., Maurhofer, M., Schnider-Keel, U., Duffy, B., Haas, D. and Défago, G. (2001) Biotic factors affecting expression of the 2,4-diacetylphloroglucinol biosynthesis gene *phlA* in *Pseudomonas fluorescens* biocontrol strain CHA0 in the rhizosphere. Phytopathology 91, 873–881.

[20] Duffy, B. and Défago, G. (1999) Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. Appl. Environ. Microbiol. 65, 2429–2438.

[21] Duffy, B., Schouten, A. and Raaijmakers, J.M. (2003) Pathogen self-defense: mechanisms to counteract microbial antagonism. Annu. Rev. Phytopathol. 41, 501–538.

[22] Lorito, M. (1998) Chitinolytic enzymes and their genes. In: *Trichoderma* and *Gliocladium* (Harman, G.E. and Kubicek, C.P., Eds.) (Ed.),. In: Enzymes, Biological Control and Commercial Application, vol. 2, pp. 73–99. Taylor & Francis, London.

[23] Kullnig, C.M., Krubica, T., Lorito, M., Mach, R.L., Rey, M., Benitez, T. and Kubicek, C.P. (2001) Confusion abounds over identities of *Trichoderma* biocontrol isolates. Mycol. Res. 105, 769–772.

[24] Harman, G.E. and Björkman, T. (1998) Potential and existing uses of *Trichoderma* and *Gliocladium* for plant disease control and plant growth enhancement. In: *Trichoderma* and *Gliocladium* (Harman, G.E. and Kubicek, C.P., Eds.) (Ed.),. In: Enzymes, Biological Control and Commercial Application, vol. 2, pp. 229–265. Taylor & Francis, London.

[25] Mach, R.L., Peterbauer, C.K., Payer, K., Jaksits, S., Woo, S.L., Zeilinger, S., Kullnig, C.M., Lorito, M. and Kubicek, C.P. (1999) Expression of two major chitinase genes of *Trichoderma atroviride* (*T. harzianum*) P1 is triggered by different regulatory signals. Appl. Environ. Microbiol. 65, 1858–1863.

[26] Woo, S.L., Donzelli, B., Scala, F., Mach, R., Harman, G.E., Kubicek, C.P., Del Sorbo, G. and Lorito, M. (1999) Disruption of the *ech42* (endochitinase-encoding) gene affects biocontrol activity in *Trichoderma harzianum* P1. Mol. Plant-Microbe Interact. 12, 419–429.

[27] Lutz, M.P., Feichtinger, G., Défago, G. and Duffy, B. (2003) Mycotoxigenic *Fusarium* and deoxynivalenol production repress chitinase gene expression in the biocontrol agent *Trichoderma atroviride* P1. Appl. Environ. Microbiol. 69, 3077–3084.

[28] Stutz, E.W., Défago, G. and Kern, H. (1986) Naturally occurring fluorescent pseudomonads involved in suppression of black root rot of tobacco. Phytopathology 76, 181–185.

[29] Schnider-Keel, U., Seematter, A., Maurhofer, M., Blumer, C., Duffy, B., Gigot-Bonnefoy, C., Reimmann, C., Notz, R., Défago, G., Haas, D. and Keel, C. (2000) Autoinduction of 2,4-diacetylphloroglucinol biosynthesis in the biocontrol agent *Pseudomonas fluorescens* CHA0 and repression by the bacterial metabolites salicylate and pyoluteorin. J. Bacteriol. 182, 1215–1225.

[30] Maurhofer, M., Keel, C., Haas, D. and Défago, G. (1994) Pyoluteorin production by *Pseudomonas fluorescens* strain CHA0 is involved in the suppression of *Pythium* damping-off of cress, but not ofcucumber. Eur. J. Plant Pathol. 100, 221–232.

[31] Laville, J., Blumer, C., Von Schroetter, C., Gaia, V., Défago, G., Keel, C. and Haas, D. (1998) Characterization of the *hcnABC* gene cluster encoding hydrogen cyanide synthase and anaerobic regulation by ANR in the strictly aerobic biocontrol agent *Pseudomonas fluorescens* CHA0. J. Bacteriol. 180, 3187–3196.

[32] Vincent, M.N., Harris, L.A., Brackin, J.M., Kovacevich, P.A., Mukerij, P., Weller, D.M. and Pierson, E.A. (1991) Genetic analysis of antifungal activity of a soilborne *Pseudomonas aureofaciens* strain. Appl. Environ. Microbiol. 57, 2928–2934.

[33] Tronsmo, A. (1989) *Trichoderma harzianum* used for biological control of storage rot on carrots. Nor. J. Agric. Sci. 3, 157–161.

[34] Heeb, S., Itoh, Y., Nishijyo, T., Schnider, U., Keel, C., Wade, J., Walsh, U., O'Gara, F. and Haas, D. (2000) Small stable shuttle vectors based on the minimal pVS1 replicon for use in gram-negative, plant-associated bacteria. Mol. Plant-Microbe Interact. 13, 232–237.

[35] King, E.O., Ward, M.K. and Raney, D.E. (1954) Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44, 301–307.

[36] Miller, J.H. (1992) A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria. Cold Spring Harbor Laboratory Press, Plainview, NY.

[37] Leveau, J.H.J. and Lindow, S.E. (2001) Predictive and interpretive simulation of green fluorescent protein expression in reporter bacteria. J. Bacteriol. 183, 6752–6762.

[38] Duffy, B.K., Ownley, B.H. and Weller, D.M. (1997) Soil chemical and physical properties associated with suppression of take-all of wheat by *Trichoderma koningii*. Phytopathology 87, 1118–1124.
[39] Bonsall, R.F., Weller, D.M. and Thomashow, L.S. (1997) Quantification of 2,4-diacetyphloroglucinol produced by fluorescent *Pseudomonas* spp in vitro and in the rhizosphere of wheat. Appl. Environ. Microbiol. 63, 951–955.
[40] Ramette, A., Moënne-Loccoz, Y. and Défago, G. (2001) Polymorphism of the polyketide synthase gene *phlD* in biocontrol fluorescent pseudomonads producing 2,4-diacetylphloroglucinol and comparison of *phlD* with plant polyketide synthases. Mol. Plant Microbe Interact. 14, 639–652.
[41] Wheatley, R.E. (2002) The consequences of volatile organic compound mediated bacterial and fungal interactions. Anton. Leeuw. Int. J. Gen. Mol. Microbiol. 81, 357–364.
[42] Fogliano, V., Ballio, A., Gallo, M., Woo, S., Scala, F. and Lorito, M. (2002) *Pseudomonas* lipodepsipeptides and fungal cell wall-degrading enzymes act synergistically in biological control. Mol. Plant-Microbe Interact. 15, 323–333.
[43] de Souza, J.T., Arnould, C., Deulvot, C., Lemanceau, P., Gianinazzi-Pearson, V. and Raaijmakers, J.M. (2003) Effect of 2,4-diacetylphloroglucinol on *Pythium*: cellular responses and variation in sensitivity among propagules and species. Phytopathology 93, 966–975.
[44] Notz, R., Maurhofer, M., Dubach, H., Haas, D. and Défago, G. (2002) Fusaric acid-producing strains of *Fusarium oxysporum* alter 2,4-diacetylphloroglucinol biosynthetic gene expression in *Pseudomonas fluorescens* CHA0 in vitro and in the rhizosphere of wheat. Appl. Environ. Microbiol. 68, 2229–2235.
[45] Simon, A. and Sivasithamparam, K. (1989) Pathogen-suppression: a case study in biological suppression of *Gaeumannomyces graminis* var. *tritici* in soil. Soil Biol. Biochem. 21, 331–337.
[46] Kredics, L., Antal, Z., Manczinger, L., Szekeres, A., Kevei, F. and Nagy, E. (2003) Influence of environmental parameters on *Trichoderma* strains with biocontrol potential. Food Technol. Biotechnol. 41, 37–42.
[47] Schoonbeek, H.-J., Raaijmakers, J.M. and de Waard, M.A. (2002) Fungal ABC transporters and microbial interactions in natural environments. Mol. Plant-Microbe Interact. 15, 1165–1172.