ANALYSIS OF CROSS-TALK BETWEEN *TRICHODERMA ATROVIRIDE* AND *PSEUDOMONAS FLUORESCENS*

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SUMMARY

Application of mixtures of biocontrol agents has been shown to more effectively control the level of several plant pathogens. However, very little is known about possible interactions between co-inoculated biocontrol strains at the molecular level. In this study, we have analyzed the effects of two biocontrol agents, *Trichoderma atroviride* and *Pseudomonas fluorescens*, in the control of take-all, a disease caused by *Gaeumannomyces graminis*. The results showed that *T. atroviride* combined with one of several strains of *P. fluorescens* isolated from wheat fields in north-west Iran [including stains Z7, B119 (DAPG-producing), P4 (Phenazine-producing), P6, and P21] significantly reduced the severity of take-all in wheat, albeit with different efficiencies. While combination of *T. atroviride* with bacterial isolates Z7 or B119 improved take-all suppression in comparison to treatment with the individual biocontrol agents, combination of the P4, the P6 or the P21 isolate with *T. atroviride* had a negative effect on biocontrol activity compared to the individual agents. Since chitinases and DAPG are known to be important biocontrol substances produced by *T. atroviride* and *P. fluorescens*, respectively, we have studied changes in their levels of expression under co-inoculation conditions. The results showed that combined inoculation of *T. atroviride* and *P. fluorescens* represses expression levels of the *Ech42* endochitinase gene both at the transcriptional and translational levels. In contrast, in the presence of the bacterial strains, expression of *Nag1*, another chitinase gene of *T. atroviride*, was either slightly enhanced or remained unchanged. Interestingly, when co-inoculated with *T. atroviride*, *PhlD*-positive strains of *P. fluorescens* produced significantly higher levels of DAPG antibiotic. Our results provide further evidence to support the hypothesis that mixing antagonists can have either positive or negative effects on the expression of their key biocontrol genes.

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INTRODUCTION

Take-all, caused by the soil-borne ascomycete *Gaeumannomyces graminis* (Sacc.) Arx et Olivier var. *tritici* Walker (*Ggt*) is an important crown and root rot disease of wheat and barley (Duffy *et al*., 1997). Because of the economic importance of the disease and the lack of efficient alternative control methods, biological control of wheat take-all has been investigated intensively (Duffy *et al*., 1996). The most prominent antagonistic microorganisms that can suppress *Ggt* are fluorescent Pseudomonads (de Souza *et al*., 2002; Duffy and Weller, 1995; Ownley *et al*., 1992; Raaijmakers *et al*., 1997; Weller *et al*., 1988) and *Trichoderma* spp. (Dewan and Sivasithamparam, 1988; Duffy *et al*., 1996, 1967). Although many experiments have demonstrated the potential benefits of biocontrol, inconsistent performance in different places and seasons has led to delay in their commercialization to manage take-all (Duffy *et al*., 1996). One possible way to overcome this problem is to use combinations of the biocontrol agents to provide an environment more similar to the natural microbiota present in the soil (Duffy and Weller, 1995; Genowati, 2001; Raupach and Kloepper, 1998; Rini and Sulochana, 2006). Although several studies have recommended combining biocontrol agents to more effectively control plant pathogens (Duffy and Weller, 1995; Genowati, 2001; Pierson and Weller, 1994; Raupach and Kloepper, 1998; Rini and Sulochana, 2006), very little is known about possible interactions between co-inoculated biocontrol strains at the molecular level. For example, providing biocontrol agents as a mixture could have positive or negative effects on the expression of key biocontrol genes in one or both partners of the mixture.

Trichoderma atroviride and *Pseudomonas fluorescens* have been extensively studied and applied as biocontrol agents (Lutz *et al*., 2004). Since chitin is a major component of the cell walls of many fungi, the potential antifungal role of chitinolytic enzymes secreted by *Trichoderma* spp. have received particular attention (Viswanathan

et al., 2006). It has been shown that *T. atroviride* secretes several cell wall-degrading enzymes, including the ECH42 endochitinase encoded by *Ech42* and N-acetyl-β-D-glucosaminidase encoded by *Nag1* (Mach *et al*., 1999; Woo *et al*., 1999). On the other hand, the polyketide metabolite 2,4-diacetylphloroglucinol (DAPG) is one of the most effective antimicrobial metabolites produced by several strains of fluorescent Pseudomonads, and various studies have demonstrated a role for DAPG in the suppression of a variety of soil-borne diseases (Haas and Keel, 2003; Maurhofer *et al*., 2004).

The objective of this study was to determine the compatibility of *T. atroviride* with several fluorescent *Pseudomonas* spp. against take-all. In this study, we have first examined the effects of co-inoculated *P. fluorescens* strains and *T. atroviride* on the control of *Ggt* under growth chamber conditions. Then, we have analyzed the effect of these combinations on the expression levels of *Ech42* and *Nag1* genes in *T. atroviride* as well as on DAPG production in *P. fluorescens*.

MATERIALS AND METHODS

Fungal and bacterial isolates. *T. atroviride* strain P1 (kindly gifted by Prof. M. Lorito, Naples, Italy) was cultured on potato-dextrose agar (PDA; Merck, Germany) at 24°C. *Ggt* was isolated from wheat rhizosphere and maintained on 1/4 PDA at 4°C. *P. fluorescens* strains were isolated from wheat rhizosphere samples collected from different wheat fields in north-west Iran according to the method described by Raijmakers *et al*. (1997).

PCR Assay for PhzC/PhzD and PhlD. To evaluate the production of phenazine and DAPG antibiotics by *P. fluorescens* strains, PCR analysis was performed using two pairs of specific primers (Raaijmakers *et al*., 1997) to detect *PhzC/PhzD* and *PhlD* genes, that are respectively involved in the phenazine and DAPG biosynthetic pathways. Since *P. fluorescens* CHA0 has been studied extensively, it was used as a standard for comparisons of the biological control activity of other *Pseudomonas* strains.

Biocontrol assay. Growth chamber assays were conducted as described previously (Crozier, 1999; Duffy and Weller, 1995; Ownley *et al*., 1992). Inocula of *Ggt* and *T. atroviride* were prepared as described by Genowati (2001) and Crozier (1999), and Duffy *et al.* (1996, 1997), respectively. Inocula were then removed from the flask, air-dried, and stored at 4°C in darkness until use. For bacterization, seeds of winter wheat (cv. Falat) were surface-sterilized with 1% NaOCl for 10 min. Seeds were then coated with bacteria as described (Pierson and Weller, 1994). Bacterial populations in treatments generally ranged from 10^7 to 10^8 CFU per seed as determined by dilution-plate methods (Duffy *et al*., 1996; Mazzola *et al*., 1995). Both *T. atroviride* and *Ggt* inocula were added to the soil at a rate of 2% wt/wt (based on soil fresh weight). PVC tubes (22 × 5 cm in size) were closed at the bottom with a cotton ball and filled with 40 g of sterile perlite and 200 g of infested soil. The tubes were then incubated for 24 h at 20 to 25°C prior to planting. Four treated seeds were placed on the soil surface in each tube and covered with a 5-cm^3 layer of sterile perlite followed by watering with 10 ml of sterile tap water. To facilitate seed germination, tubes were covered with clear plastic and incubated at room temperature (20-25°C) for 48 h. Tubes were then transferred to a growth chamber (photoperiod cycle: 12 h light, 18°C and 12 h dark, 14°C; 50% relative humidity). After emergence, plants were watered twice weekly with 10 ml of one-third strength Hoagland's solution (macro-elements only) (Duffy and Weller, 1995). After 4 weeks, plants were harvested, roots were excised, washed with tap water, and placed in 70% ethanol. Take-all severity was evaluated by rating disease symptoms on a scale of 0 to 8 as described (Duffy and Weller, 1995; Duffy *et al*., 1996; Mazzola *et al*., 1995; Ownley *et al*., 1992) where 0 = healthy plant with no visible symptoms; 1 = less than 10% black roots; 2 = 10 to 25% black roots; 3 = 25 to 50% black roots; 4 = 50 to 100% black roots; 5 = all roots with lesions and lesions at base of stem; 6 = lesions extending up the stem; 7 = plants chlorotic and severely stunted; and 8 = plant dead or nearly so. Roots were weighed after being blotted with paper towels and air-dried for 10-12 min. Shoots were also dried at 70°C overnight, and weighed after cooling to room temperature (Crozier, 1999). To test for possible antagonistic activity of *T. atroviride* and *P. fluorescens*, an experiment was arranged with a completely randomized design with 15 seedlings (replicas) for each treatment (five tubes with three seedlings per tube). The statistical comparison was performed by one-way ANOVA using SPSS software, and means were compared using the Duncan test ($P<0.05$).

Semi-quantitative RT-PCR. Semi-quantitative RT-PCR was used to evaluate the effects of *P. fluorescens* strains on the expression of *Ech42* and *Nag1,* two important biocontrol genes of *T. atroviride*. Briefly, the bacterial strains were cultured on LB medium (27°C, 160 rpm, overnight) and diluted to OD_{600}= 0.125 (equivalent to 10^8 CFU/ml). Four bacterial spots (20 µl each) were placed equidistantly on the circumference of a circle of 3 cm from the center of a plate containing 1.5% malt extract agar. The bacteria were then inoculated with an inverted plug of a fresh culture of *T. atroviride* placed in the center of the plate. After 66 h incubation at 24°C in darkness, the mycelial surface area of the fungus was measured using a planimeter. Mycelia were then scraped from the plates and used for RNA extraction with a Tri-Reagent kit (Sigma-Aldrich, USA)

according to the manufacturer's instructions. After treatment with DNase I (Roche, Germany), the concentration and quality of RNA samples were measured by spectrophotometer and agarose gel electrophoresis. cDNA was then synthesized using similar amounts of RNA (4 µg) from each sample using a Superscript III RT kit according to the manufacturer's instructions. For *Ech42*, a 1,263 bp fragment was amplified using the specific primers Ech42-F (5'-ATGTTGGGTTTCCTCGGA-3') and Ech42-R (5'-TCATTCGGGATGGTTGTCA-3'). A *Nag1*-specific primer pair (Nag1-F: 5'-CTGGTTCCAACTTCAACAGCAAGG-3', and Nag1-R: 5'-ATTTATGCGAACAAGTGCAAGCCG-3') was also used to amplify a 1,569 bp fragment from the synthesized cDNA library. The PCR program included an initial denaturation step at 94°C for 4 min followed by 30 cycles of 94°C for 20 sec, 50°C for 20 sec, and 72°C for 45 sec, and a final 10-min extension at 72°C. The housekeeping gene, Actin, was used as an internal control to normalize quantification of expression (primer pair Actin-F: 5'-AGAAGTTGCTGCCCTCGTTATCG-3', and Actin-R: 5'-TCAGCCAGGATCTTCATCAG-3'). The experiment was conducted with three technical replicas. To further confirm the results obtained by RT-PCR, the experiment was repeated for two bacterial isolates (CHA0, P6) using another specific primer pair for each gene, i.e Ech42-F2 (5'-AACCCCAACTCTTCACCATACAACACC-3') and Ech42-R2 (5'-CGGCCTTGGGAAGAACCTTG-3') for *Ech42* gene, and Nag1-F2 (5'-CTGGTTCCAACTTCAACAGCAAGG-3') and Nag1-R2 (5'-TCAATGACCTTGTAGCCAGACTCG-3') for *Nag1* gene (data not shown). Quantification of bands was performed using Image J software on pictures captured from agarose gels after electrophoresis of RT-PCR products.

Glucose oxidase activity assay. To further investigate expression of *Ech42* and *Nag1* genes, *T. atroviride* mutants containing the glucose oxidase gene fused to the regulatory elements of these genes (kindly provided by Prof. M. Lorito) were studied as described by Lutz *et al.* (2004).

High-performance liquid chromatography assay. A high-performance liquid chromatography (HPLC) assay was performed to evaluate the effects of *T. atroviride* on 2,4-diacetylphloroglucinol (DAPG) accumulation in two DAPG-producing *P. fluorescens* strains, CHA0 and B119. *T. atroviride* was cultured in 500 ml flasks containing 250 ml of Czapek-Dox medium followed by incubation in the dark (24°C, 180 rpm). After 7 days, fungal biomass was collected by centrifugation at 2,200 g for 15 min. The supernatant was then filtrated through a 0.2-µm filter to remove fungal cells, and stored at 2°C for no longer than 2 days (Lutz *et al.*, 2004). The overnight cultures of bacterial strains grown in 10 ml of LB broth (27°C, 160 rpm) were diluted to OD_{600}=0.001. Aliquots of 60 µl were used for inoculation of 48 ml of KB broth supplemented with 12 ml of fungal filtrates. In control treatments, bacteria were grown in KB broth supplemented with Czapek-Dox medium. In all experiments, after 22, 46, and 72 h incubation at 27°C at 160 rpm, samples of 15 ml were used to measure DAPG production by HPLC, according to Duffy and Defago (1999). The HPLC instrument was using a system supplied by Shimadzu-SPD-6VA consisting of a Shimadzu LC-6A Liquid chromatograph pump and a Shimadzu-SPD-6VA UV-Vis spectrophotometric detector. The mobile phase was a mixture of 100 mM KH_2PO_4 (pH=5.8) and acetonitrile (60:40, v/v). Separation was carried out on a C-18 column [Perfectsil Target ODS-3 (5 µM), 250×4.6 mm] with a C-18 guard column [Perfectsil TargetODS-3 (5 µM), 10×4 mm]. The flow rate was 1 ml/min, and maximum UV absorbance and approximate retention times for detection were 283 nm and 10.6 min, respectively. A solution of 100 µM pure DAPG (kindly provided by Dr. M.P. Lutz, Zurich, Switzerland) was used as standard for quantifications.

RESULTS

Twenty six strains of *P. fluorescens* were isolated from wheat fields in the north-west Iran. Based on *in vitro* tests (data not shown), five strains including B119, Z7 (with high antagonistic activity), P4 (with medium antagonistic activity) and P21 and P6 (with low antagonistic activity) were selected for further analysis. These bacterial strains were shown to contain *PhzC/PhzD* (genes involved in phenazine antibiotic biosynthesis) and *PhlD* (a gene involved in DAPG antibiotic biosynthetic pathway) by PCR assay. PCR analysis using specific primers for *PhlD* gene indicated that B119 and Z7 contain this gene, thus were expected to produce DAPG. In contrast, the *PhlD* gene could not be detected in strains P21, P6 and P4. The P4 isolate was the only isolate in which the specific fragment for *PhzC/PhzD* genes could be detected (data not shown) and was therefore expected to produce the antibiotic phenazine.

Growth chamber assays was used to verify the ability of the biocontrol agents to suppress take-all. Virtually all of the treatments significantly reduced the severity of take-all on wheat and resulted in increased shoot and root weight in comparison to the infected control, except for strain P4 (Table 1). As shown in Table 1, in comparison with the infected control, *T. atroviride* significantly reduced the disease severity rate from 6.30 to 4.40, whereas the root and shoot weight were increased from 55.80 and 18 mg to 124.40 and 32 mg, respectively. Also, individual bacterial treatments with CHA0, B119, and Z7 suppressed take-all significantly (Table 1). The *phlD*-positive isolates were also the most effective

Table 1. Alteration of wheat take-all severity by application of different combinations of *Trichoderma atroviride* with different fluorescent Pseudomonad strains (CHA0, B119, Z7, P21, P6, P4) in growth chamber assays.

Treatment	Root Necrosis Rate (0-8)	Shoot weight (mg)	Root weight (mg)
Control	0.00^{a}	66.00^{a}	309.80^{a}
Ggt	6.30^{i}	18.00^{gh}	55.80^{h}
T+Ggt	4.40^{f}	32.00^{def}	124.40^{de}
CHA0+Ggt	3.00^{d}	29.40^{def}	153.00^{d}
B119+Ggt	3.10^{de}	33.60^{de}	170.40^{c}
Z7+Ggt	3.50^{de}	29.00^{def}	130.80^{d}
P21+Ggt	4.50^{f}	24.20^{fg}	130.80^{de}
P6+Ggt	4.90^{f}	27.00^{ef}	100.00^{fg}
P4+Ggt	3.70^{e}	14.00^{h}	49.00^{h}
CHA0+Ggt+T	2.20^{c}	36.60^{cd}	153.70^{c}
B119+Ggt+T	1.40^{b}	56.40^{b}	210.80^{b}
Z7+Ggt+T	1.40^{b}	41.80^{c}	167.40^{c}
P21+Ggt+T	5.60^{gh}	25.80^{f}	81.00^{g}
P6+Ggt+T	5.00^{fg}	27.00^{ef}	100.00^{fg}
P4+Ggt +T	5.80^{hi}	12.00^{h}	24.80^{i}

Data were analyzed using SPSS statistical software, and means were compared using the Duncan Test. Values indicated by different letters in the superscript, show significant difference at $p<0.05$, in comparison to the corresponding column values. T: *Trichoderma atroviride*.

in inhibiting mycelial growth of *Ggt* in *in vitro* assays (data not shown). Combination of *T. atroviride* with the aforesaid three bacterial isolates improved take-all suppression in comparison to each individual treatment (Table 1). In contrast, when *T. atroviride* was combined with P6, P21, and P4 isolates, the biocontrol activity of individual agents either was reduced or remained unchanged (Table 1).

As mentioned, the experiments using combination of *T. atroviride* and different strains of *P. fluorescens* resulted in diverse changes in their biocontrol activity in comparison to the individual application of these agents. Thus, it was reasonable to hypothesize that certain mixtures of biocontrol agents could provoke antagonistic effects on each other at the molecular level. Therefore, molecular and biochemical experiments were conducted to analyze possible effects of a biocontrol agent on some of the key factors involved in the biocontrol activity of the other agent. To this end, changes in the expression levels of two important chitinolytic genes from *T. atroviride*, *Ech42* and *Nag1*, were measured in co-inoculation with a strain representing each of the three distinct groups of antagonistic activity (Z7: high, P4: medium, P6: low) from *P. fluorescens*, using semi-quantitative RT-PCR. Also, DAPG (an important antibiotic involved in the biocontrol activity of several *P. fluorescens* strains) production was measured by HPLC to study the possible effect of *T. atroviride* on the level of DAPG secretion by *P. fluorescens* strains.

RT-PCR analysis showed that *T. atroviride Ech42* transcript levels were decreased by co-inoculation with *P. fluorescens* strains, albeit to different amounts (Fig. 1, 2). In contrast RT-PCR revealed that *Nag1* expression at the transcriptional level was increased in the presence of all four bacterial strains.

To gain a better understanding of the regulation of expression of *Ech42* and *Nag1*, experiments were carried out with *T. atroviride* mutants containing the glucose oxi-

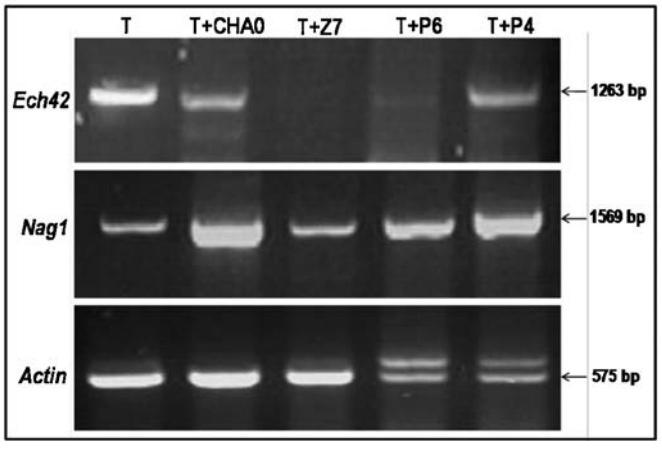

Fig. 1. RT-PCR analysis of the expression of *Ech42* and *Nag1* genes from *T. atroviride* when co-inoculated with *P. fluorescens* strains, including CHA0, Z7, P6, and P4. T = *T. atroviride*. Actin was used as an internal control to normalize gene expression.

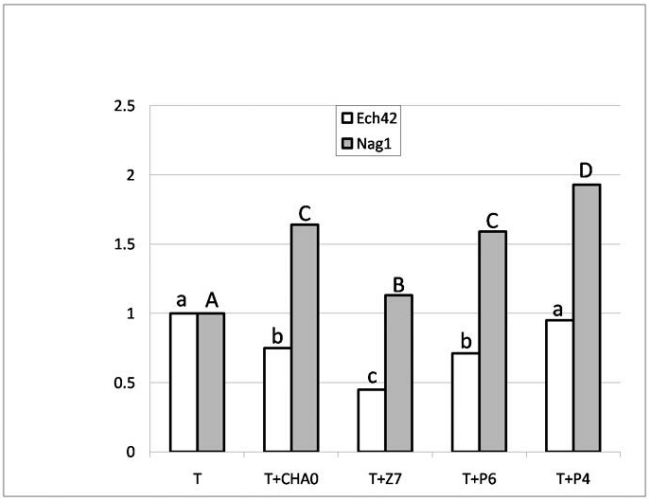

Fig. 2. Comparison of *Ech42* and *Nag1* transcript levels following co-inoculation of *T. atroviride* with different strains of *P. fluorescens*. The expression levels are shown as a ratio of the expression level in *T. atroviride* alone. Significant differences at $p<0.05$ according to the Duncan's Multiple Test are indicated by different small (for *Ech42*) and capital (for *Nag1*) letters.

dase gene fused to the regulatory elements of these genes. For the *Ech42* gene, changes in the glucose oxidase activity correlated well with the results obtained from RT-PCR experiments (Fig. 2, 3A), i.e. glucose oxidase activity of the *Ech42* fusion mutant was generally lower in the presence of the bacterial strains. These results provide evidence that expression of the *Ech42* gene in *T. atroviride* is mainly regulated at the transcriptional level during co-inoculation. In contrast, the *Nag1* fusion mutant showed either unchanged or only slightly elevated levels of glucose oxidase activity when *T. atroviride* was inoculated in combination with the bacterial strains (Fig. 3B). However, transcript levels of this gene were significantly increased when co-inoculated with the *P. fluorescens* strains (Fig. 2). These results indicate that *Nag1* gene expression is tightly regulated at the post-transcriptional level.

The effects of *T. atroviride* on DAPG antibiotic production by two *PhlD*-positive bacterial strains, CHA0 and B119, were investigated by HPLC analysis. The result show that DAPG production increased significantly after 22 h co-inoculation with *T. atroviride*. Increased levels of DAPG were also observed after 48 and 72 h incubation (Fig. 4). In both strains, the highest level of DAPG production was observed after 72 h incubation. It should be noted that addition of fungal filtrate to the medium of the bacterial strains did not affect bacterial population size (data not shown).

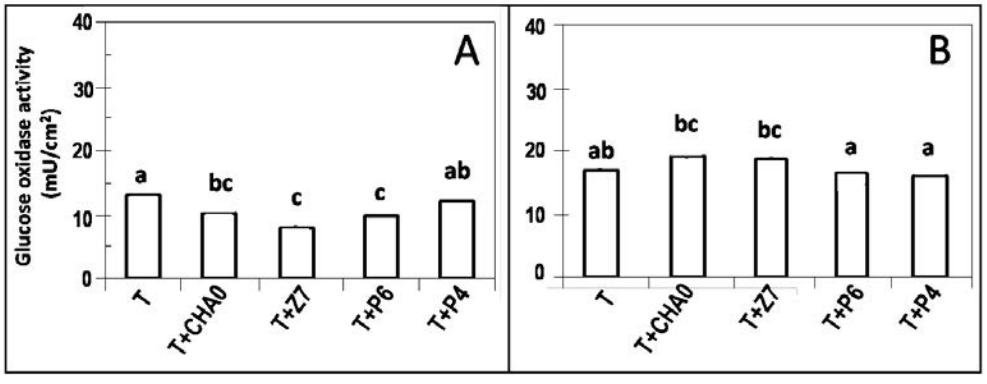

Fig. 3. Glucose oxidase activity of *T. atroviride* mutants expressing an *Ech42-Gox* (A) or a *Nag1-Gox* (B) fusion in combination with various *P. fluorescens* strains. Bars indicated by different letters, show significant difference at $p<0.05$ according to the Duncan's Multiple Test.

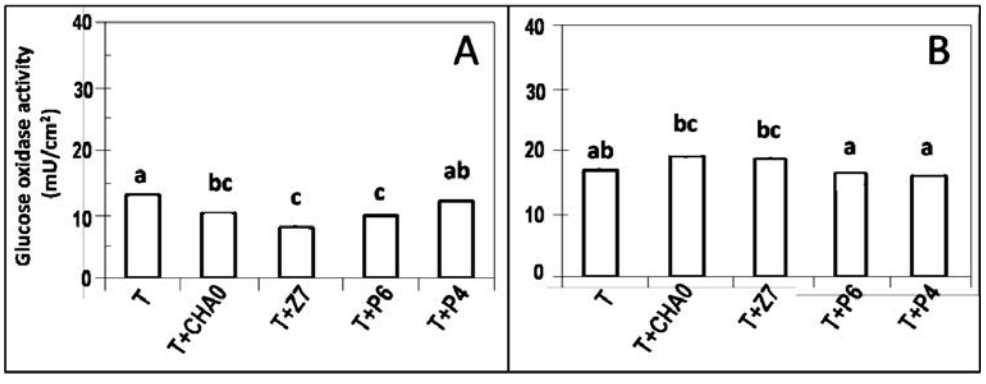

Fig. 4. DAPG antibiotic production in two *PhlD*-positive strains of *P. fluorescens*, CHA0 and B119, after 22, 48 and 72 h incubation with or without *T. atroviride* culture filtrate (T). Data are expressed as µg DAPG per ml of culture.

To confirm the inhibitory effect of DAPG on *Ech42* transcription, the effect of direct addition of DAPG (0.1 mM) to the growth medium of *T. atroviride* on *Ech42* transcript levels was analyzed by RT-PCR. The results showed that DAPG significantly decreases *Ech42* expression at the transcriptional level (Fig. 5).

Analysis of fungal mycelial growth after co-inoculation with different strains of *P. fluorescens*, was in agreement with the above results. Thus, the mycelial surface area was more widely reduced (albeit to different extents) by the DAPG-positive strains (CHA0 and Z7) than by DAPG-negative strains (Fig. 6). In addition, of the two DAPG-negative strains, the Phenazine-positive P4 strain reduced fungal growth more than Phenazine-negative P6 strain (Fig. 6).

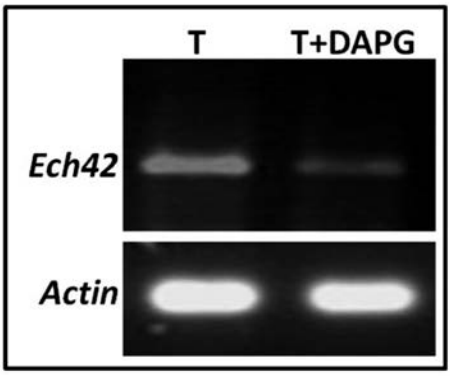

Fig. 5. RT-PCR analysis to study the expression of the *T. atroviride Ech42* gene in the absence (T) or presence (T+DAPG) of DAPG (0.1 mM) in the culture medium. Actin was used as an internal control.

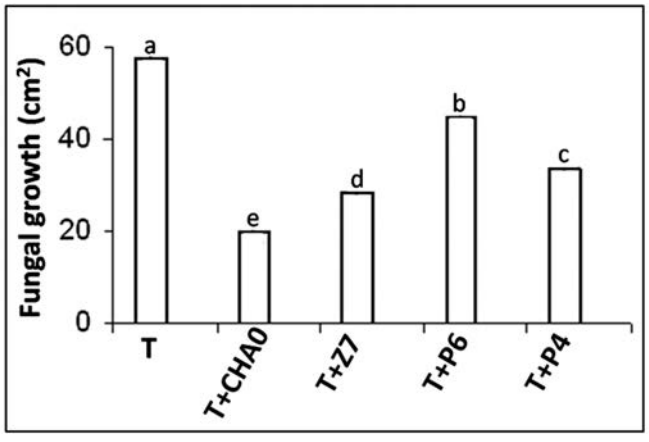

Fig. 6. Growth of *T. atroviride* P1 (T) in the presence of *P. fluorescens* strains CHA0, Z7 (DAPG-positive), P6 (DAPG-negative) and P4 (phenazine-positive). Each value is the mean of three experiments with six replicas per treatment. Bars with different letters are significantly different according to Duncan's Multiple Test ($p<0.05$).

DISCUSSION

Several studies have shown that combining biocontrol agents is often a more effective method to control a variety of plant pathogens, including wheat take-all, than inoculation of a single agent (Duffy and Weller, 1995; Duffy *et al.*, 1996; Genowati, 2001; Pierson and Weller, 1994). However, there are exceptions to this rule as decreased biocontrol activity has been observed in several strain mixtures (Dandurand and Knudsen, 1993; de Boer *et al.*, 1999). In this study, we have studied the effects of combination of *T. atroviride* P1 with several strains of *P. fluorescens* (B119, Z7, P21, P6 and P4), isolated from wheat fields in the north-west Iran. Our results showed that combination of *T. atroviride* with the DAPG-producing bacterial strains B119 and Z7 have the potential for greater biocontrol activity against take-all than observed with individual application of these biocontrol agents. In contrast, combination of P21, P6 and P4 strains with *T. atroviride* either reduced or did not change the biocontrol activity of the individual treatments.

Several factors can be responsible for variations in biocontrol activity of certain strain mixtures. One important factor may be related to differential regulation by the other strain in the mixture of the biosynthetic genes responsible for the production of secondary metabolites involved in disease suppression. *Ech42* and *Nag1* are two important *T. atroviride* genes responsible for the production of chitinolytic enzymes involved in pathogen growth repression (Mach *et al.*, 1999). Using RT-PCR technique, we have demonstrated that *P. fluorescens* strains significantly decrease *Ech42* expression in *T. atroviride* at the transcriptional level (Fig. 1, 2). These results correlated well with the results obtained from glucose oxidase assay (Fig. 3). This finding not only provides evidence that expression of *Ech42* gene is controlled at the transcriptional level, but that *P. fluorescens* strains secret one or more compounds which can transcriptionally down-regulate the *T. atroviride Ech42* gene. One or more antibiotics produced by certain of the *P. fluorescens* strains might be involved in transcriptional suppression of *Ech42*. Indeed, addition of DAPG antibiotic into growth medium of *T. atroviride* significantly decreased transcription of *Ech42* as demonstrated by RT-PCR analysis (Fig. 5). Nevertheless, DAPG is probably not the only compound affecting expression of *Ech42*, because significant reduction in transcription levels of this gene was also observed in combinations of *T. atroviride* with DAPG-negative strains of *P. fluorescens* (Fig. 1, 2). In contrast to the situation with the *Ech42* gene, the results for expression of the *Nag1* gene using the glucose oxidase assay did not correlate well with data obtained from RT-PCR analysis. Thus, whereas RT-PCR revealed a strong increase in transcript levels of *Nag1* in the presence of *P. fluorescens* strains (Fig. 2),

little if any significant increase was observed in the glucose oxidase assay (Fig. 3). Therefore, it appears that the expression level of *Nag1* is largely controlled at post-transcriptional levels. Addition of DAPG to the growth medium of *T. atroviride* had negative effects on *Nag1* transcription as analyzed by RT-PCR assay. However, the expression level of *Nag1* showed a slight increase in glucose oxidase assay (data not shown). The results thus indicate that increased transcript level can even have a negative effect on the expression level of *Nag1*, and that there are other unknown compounds produced by bacterial strains that can positively regulate transcription of *Nag1*. Differentially alteration of *Ech42* and *Nag1* expression by *P. fluorescens* strains is not surprising, because distinct regulatory mechanisms have been reported for these genes (Mach *et al*., 1999).

In previous studies, increased expression of *Phl* genes (involved in DAPG biosynthetic pathway) from *P. fluorescens* in the presence of *T. atroviride* has been reported using a *PhlA'-'lacZ* fusion (Lutz *et al*., 2004). In this study, we have verified these results by direct HPLC measurement of DAPG antibiotic content. Our observations show changes in DAPG level at three time periods (22, 48, 72 h) after co-inoculation with *T. atroviride* (Fig. 4). Interestingly, addition of DAPG to the growth medium of *T. atroviride* reduced fungal mycelia growth (data not shown). In agreement with these results, the mycelial surface area of fungus was more extensively reduced by co-inoculation with DAPG-positive strains of *P. fluorescens* in comparison to DAPG-negative strains (Fig. 6). It has also been reported that DAPG causes various types of disorganization in hyphal tips in *P. ultimum* var. *sporangiiferum* (de Souza *et al.*, 2003).

Comparison of the results obtained from RT-PCR and glucose oxidase assays indicated that the bacterial antimicrobial agent DAPG plays an important role in repression of *Ech42*, but not of *Nag1* chitinases. This conclusion is in partial agreement with earlier results reported by Lutz *et al*. (2004), although our experiments go further to provide the first comparisons between transcriptional and translational regulation of *Ech42* and *Nag1* expression in the presence of various *P. fluorescens* strains. Our results also show that metabolites produced by bacterial antagonists influence expression of *Ech42* and *Nag1* in different ways. While *Ech42* expression was decreased by all antibiotic-producing, or non-antibiotic-producing bacterial strains, *Nag1* expression was slightly enhanced by DAPG-producing strains and remained unchanged by other strains. Based on these results, DAPG production and its over-expression in the presence of *T. atroviride* may be the primary reason for increased biocontrol activity in this mixture of agents, because the mixture of *T. atroviride* with DAPG-producing bacteria led to elevated DAPG level and therefore, enhanced biocontrol activity. When, on the other hand, non-DAPG producing strains are mixed with *T. atroviride*, down-regulation of biocontrol genes such as *Ech42* chitinase by these strains could be a possible reason for the reduced biocontrol activity of mixture. This reduction may be compensated for by higher DAPG production in DAPG-producing strains. We conclude that there is strong cross-talk between *P. fluorescens* and *T. atroviride* affecting regulation of various metabolic pathways involved in the biocontrol process.

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