

ORIGINAL ARTICLE

Factors affecting the production of *Trichoderma harzianum* secondary metabolites during the interaction with different plant pathogens

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Keywords

antibiotics, biopesticides, fungal pathogens, elicitors, LC/MS, *Trichoderma*, secondary metabolites.

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2008/1928: received 10 November 2008, revised 15 January 2009 and accepted 23 January 2009

doi:10.1111/j.1472-765X.2009.02599.x

Abstract

Aims: Strains of *Trichoderma* spp. produce numerous bioactive secondary metabolites. The *in vitro* production and antibiotic activities of the major compounds synthesized by *Trichoderma harzianum* strains T22 and T39 against *Leptosphaeria maculans*, *Phytophthora cinnamomi* and *Botrytis cinerea* were evaluated. Moreover, the eliciting effect of viable or nonviable biomasses of *Rhizoctonia solani*, *Pythium ultimum* or *B. cinerea* on the *in vitro* production of these metabolites was also investigated.

Methods and Results: T22azaphilone, 1-hydroxy-3-methyl-anthraquinone, 1,8-dihydroxy-3-methyl-anthraquinone, T39butenolide, harzianolide, harzianopyridone were purified, characterized and used as standards. In antifungal assays, T22azaphilone and harzianopyridone inhibited the growth of the pathogens tested even at low doses (1–10 µg per plug), while high concentrations of T39butenolide and harzianolide were needed (>100 µg per plug) for inhibition. The *in vitro* accumulation of these metabolites was quantified by LC/MS. T22azaphilone production was not enhanced by the presence of the tested pathogens, despite its antibiotic activity. On the other hand, the anthraquinones, which showed no pathogen inhibition, were stimulated by the presence of *P. ultimum*. The production of T39butenolide was significantly enhanced by co-cultivation with *R. solani* or *B. cinerea*. Similarly, viable and nonviable biomasses of *R. solani* or *B. cinerea* increased the accumulation of harzianopyridone. Finally, harzianolide was not detected in any of the interactions examined.

Conclusions: The secondary metabolites analysed in this study showed different levels of antibiotic activity. Their production *in vitro* varied in relation to: (i) the specific compound; (ii) the phytopathogen used for the elicitation; (iii) the viability of the elicitor; and (iv) the balance between elicited biosynthesis and biotransformation rates.

Significance and Impact of the Study: The use of cultures of phytopathogens to enhance yields of *Trichoderma* metabolites could improve the production and application of novel biopesticides and biofertilizers based on the active compounds instead of the living microbe. This could have a significant beneficial impact on the management of diseases in crop plants.

Introduction

Fungal strains of the genus *Trichoderma* are important biocontrol agents successfully applied as biopesticides worldwide, and are well-known producers of secondary metabolites with antibiotic activity (Sivasithamparam and Ghisalberti 1998; Reino et al. 2008; Vinale et al. 2008a). *Trichoderma* spp. are known to be involved in complex interactions with host plants and resident microbial communities, which include an often beneficial combination of antibiosis and other mechanisms, such as mycoparasitism, competition for nutrients/space, promotion of plant growth and induction of systemic resistance (Harman and Kubicek 1998; Benítez et al. 2004; Harman et al. 2004; Vinale et al. 2008a). The involvement of secondary metabolites in the ability of *Trichoderma* spp. to activate plant defence mechanisms and regulate plant growth has been recently studied in depth (Vinale et al. 2008b).

Microbial secondary metabolites are produced during microbial development and sporulation, and are among the most important natural products used to inhibit microbial growth (Sekiguchi and Gaucher 1977; Mapleston et al. 1992; Stone and Williams 1992; Vinale et al. 2008a). The spectrum of secondary metabolites secreted by *Trichoderma* is species- and strain-dependent and includes volatile and nonvolatile antifungal substances (Sivasithamparam and Ghisalberti 1998). In a recent paper, we reported the isolation and characterization of the major secondary metabolites produced by *Trichoderma harzianum* T22 and T39, two commercial strains successfully used as biopesticides and biofertilizers in greenhouse and open field production (Vinale et al. 2006).

In the present work, we tested the antibiotic activities of the major secondary metabolites synthesized by *T. harzianum* strain T22 and T39 against the fungal pathogens *Leptosphaeria maculans*, *Phytophthora cinnamomi* and *Botrytis cinerea*. We also determined their *in vitro* production during the interaction with various fungal pathogens by using LC/MS analysis, and evaluated the usefulness of viable and nonviable pathogen biomasses to elicit secondary metabolites production in *T. harzianum*.

Materials and methods

Fungal strains and culture conditions

The fungal pathogens *R. solani*, *P. ultimum*, *B. cinerea* and *P. cinnamomi* as well as the antagonistic strains T22 and T39 of *T. harzianum* were maintained on potato dextrose agar (PDA) slants at room temperature and subcultured bimonthly during the study. Similarly, *L. maculans* isolate UWA P11 was grown on V8 agar plates and

cultured as above. The pathogens were isolated from field crops in Italy.

Production and characterization of secondary metabolites

The filtered culture broths of *T. harzianum* strains T22 and T39 were prepared as previously described (Vinale et al. 2006). Major secondary metabolites were extracted and characterized according to the protocol described by Vinale et al. (2006), quantified by LC/MS and then used as pure standards.

Plate antifungal assays

The antifungal activity of the purified *Trichoderma* compounds was tested *in vitro* against the fungal pathogens *L. maculans*, *P. cinnamomi* and *B. cinerea*, as described by Vinale et al. (2006). Briefly, agar plugs cut from actively growing margins of each pathogen colony were placed at the centre of one-fifth strength PDA plates. Purified compounds (10 µl) dissolved in filter sterilized ethyl acetate (EtOAc) at concentrations ranging from 0.01 to 200 µg per plug was applied on top of each plug and the solvent was left to completely evaporate under a laminar flow. The controls included the application of 10 µl of the solvent alone. The pathogen growth was measured daily and reported as colony diameter (mm). Each treatment consisted of three replicates and each experiment was repeated twice.

Co-cultures of *Trichoderma* and fungal pathogens

Four 7 mm diameter plugs of each *Trichoderma* and pathogen (*R. solani*, *P. ultimum*, *B. cinerea*) strain, obtained from actively growing margins of PDA cultures, were co-inoculated into 500-ml conical flasks containing 100 ml sterile one-fifth strength potato dextrose broth (PDB). The stationary cultures were incubated for 30 days at 25°C. Broth cultures of *T. harzianum* without fungal pathogens served as controls.

Trichoderma cultures in the presence of nonviable pathogen biomasses

Trichoderma harzianum inoculum was prepared as reported above. The fungal pathogens *R. solani* and *P. ultimum* were grown in PDB at 25°C stirring at 120 rev min⁻¹. After 7 days, the mycelia were separately harvested, sterilized (120°C for 30 min) and freeze-dried. The sterilized mycelia were then ground in a mortar to obtain a fine powder and added separately to the broth at a concentration of 10 g l⁻¹. To prepare the cell walls of

B. cinerea, the fungal mycelium was scraped from PDA dishes and homogenized in 10 volumes of 0.1% (w/v) SDS. Purification of cell walls was carried out as reported by Messner and Kubicek (1990). The purified cell walls were ground to a fine powder under liquid nitrogen and added to the broth at a concentration of 10 g l⁻¹. Culture broths of *T. harzianum* without amendment with fungal pathogens were used as controls.

Production of secondary metabolites in the presence of viable or nonviable pathogen biomasses

The production of major secondary metabolites by *T. harzianum* in liquid cultures amended with viable (living mycelium) or nonviable (sterilized mycelium or cell walls) biomass of different fungal pathogens (*R. solani*, *P. ultimum*, *B. cinerea*) was evaluated. The stationary cultures were incubated for 30 days at 25°C and then filtered under vacuum. The filtered broths were extracted exhaustively with EtOAc. The combined organic fractions were dried with Na₂SO₄ and evaporated under reduced pressure at 35°C. The recovered residues were re-suspended in methanol and subjected to LC/MS analysis for the quantification of metabolites.

Chromatographic separation was performed using an HPLC apparatus equipped with two Micropumps Series 200 (Perkin Elmer, Shelton, CT, USA), and a Gemini 5 µm C18 110 A column (150 × 42 mm) (Phenomenex, Torrance, CA, USA). The eluents were **A** = water +0.1% formic acid, and **B** = methanol. The gradient program for T22azaphilone, 1-hydroxy-3-methyl-anthraquinone and 1,8-dihydroxy-3-methyl-anthraquinone was as follows: 40–100% B (8 min), 100% B (2 min) at a constant flow of 0.2 ml min⁻¹. The gradient program for T39butenolide, harzianolide and harzianopyridone was as follows: 50–100% B (10 min), 100% B (3 min) at a constant flow of 0.2 ml min⁻¹. Injection volume was 20 µl.

Mass spectrometry (MS) analyses were performed on an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Toronto, Canada) equipped with a heated nebulizer source working in the positive or negative ion mode. Only for T22azaphilone a TurboIonspray source was used. Acquisitions were performed using a multiple reaction monitoring (MRM) approach. Specific transitions (precursor and product ions) for each metabolite are also monitored as reported in Table 1.

Results

The major secondary metabolites (1–6; Fig. 1) obtained from culture filtrates of T22 and T39 *Trichoderma* strains were purified and characterized as previously reported (Vinale et al. 2006). In plate antifungal assays, 1–10 µg of

Table 1 LC/MS transitions monitored for each metabolite by multiple reaction monitoring

Metabolite	Precursor ion [M + H] ⁺ m/z	Product ions [M + H] ⁺ m/z
Positive ion mode		
T22azaphilone	345	259, 241
1-Hydroxy-3-methyl-anthraquinone	239	211
1,8-Dihydroxy-3-methyl-anthraquinone	255	209
T39butenolide	221	203, 157, 133
Harzianolide	222	204, 158, 134
Negative ion mode		
Metabolite	Precursor ion [M – H] ⁻ m/z	Product ions [M – H] ⁻ m/z
Harzianopyridone	280	250, 265, 183

T22azaphilone (**1**; Fig. 1) completely inhibited the growth of the fungal pathogens *P. cinnamomi*, *B. cinerea* and *L. maculans* (Fig. 2). This is in agreement with the inhibitory effect of 1–100 µg of T22azaphilone on *Gaeumannomyces graminis* var. *tritici*, *R. solani* and *P. ultimum* (Vinale et al. 2006). Anthraquinones (**2**, **3**; Fig. 1), instead, demonstrated no antifungal activity against *P. cinnamomi*, *B. cinerea* and *L. maculans* (data not shown), as previously reported for *R. solani* and *P. ultimum* (Vinale et al. 2006).

Relatively to the secondary metabolites isolated from *T. harzianum* T39, the most effective was the harzianopyridone, which exhibited in the same plate assay 100% growth inhibition of *L. maculans* and *P. cinnamomi* at 1 and 100 µg application rate, respectively (Fig. 2). T39butenolide (**4**; Fig. 1) was able to completely stop the growth of *L. maculans* and *P. cinnamomi* (Fig. 2), as well as that of *G. graminis* var. *tritici* and *R. solani* at 100 µg (Vinale et al. 2006). Finally, harzianolide (**5**; Fig. 1) showed less

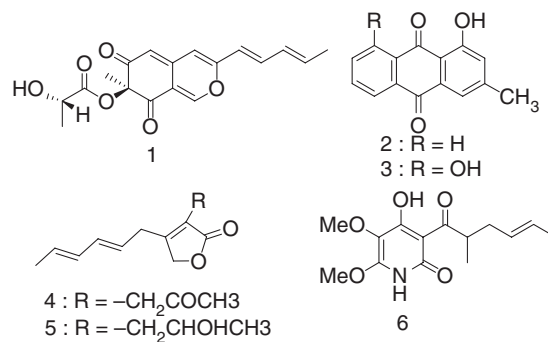


Figure 1 *Trichoderma* secondary metabolites. 1: T22azaphilone; 2: 1-hydroxy-3-methyl-anthraquinone; 3: 1,8-dihydroxy-3-methyl-anthraquinone; 4: T39butenolide; 5: harzianolide; 6: harzianopyridone.

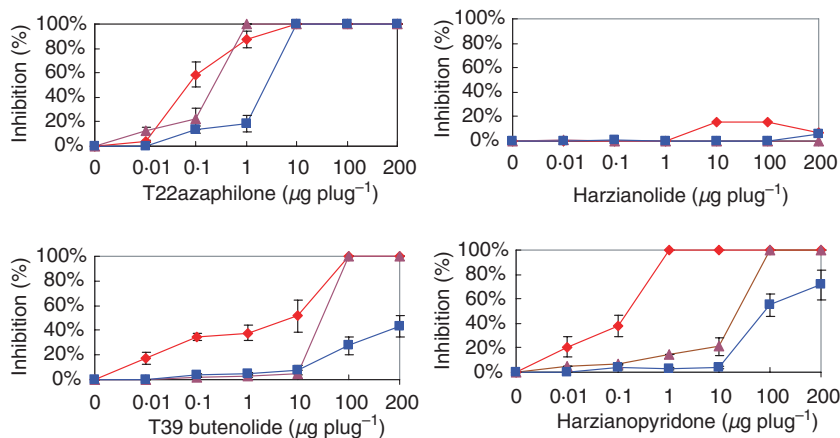


Figure 2 Antibiotic activity of the purified *Trichoderma* secondary metabolites: (—♦—) *Leptosphaeria maculans*; (—▲—) *Phytophthora cinnamomi*; (—■—) *Botrytis cinerea*. Concentrations ranging from 0.01 to 200 μg per plug.

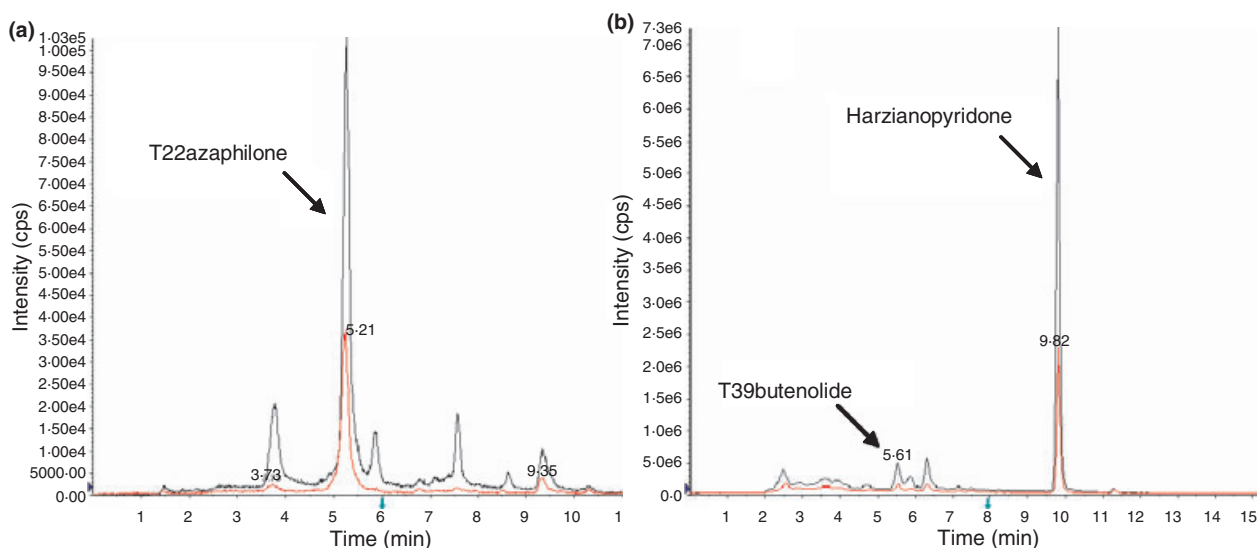


Figure 3 (a) LC/MS of extracts obtained from control (T22 alone – black) and co-culture of *Trichoderma harzianum* T22 and *Botrytis cinerea* (red); (b) LC/MS of extracts obtained from control (T39 alone – red) and co-cultures of *T. harzianum* T39 and *Rhizoctonia solani* (black).

than 25% growth inhibition at the maximum application rate of 200 μg (Fig. 2).

The isolated secondary metabolites were used as pure standards in LC/MS-based quantification of the amount of these compounds accumulated in the medium during *in vitro* interaction of *Trichoderma* with fungal pathogens (examples shown in Fig. 3). For these experiments *R. solani*, *P. ultimum* and *B. cinerea* were used because of their economical importance in agriculture and the very different response to the tested metabolites, as determined also in an earlier work (Vinale et al. 2006). The production of major secondary metabolites by *T. harzianum* in liquid cultures was differently affected by the presence of viable or nonviable biomasses of the three phytopathogens tested. Among the compounds produced in liquid co-cultures by *T. harzianum* strain T22, no significant

differences were found in the accumulation of T22azaphilone between control and *R. solani*- or *B. cinerea*-treated samples (Table 2). Instead, the presence of nonviable biomasses of the pathogens, as well as the co-culture with the living mycelium of *P. ultimum*, significantly reduced or completely inhibited T22azaphilone production compared to the untreated control. Interestingly, the secretion of anthraquinones (2, 3; Fig. 1) was elicited by the interaction with both viable and nonviable *P. ultimum* biomasses, but not by the other treatments.

Co-cultures of *T. harzianum* strain T39 and *R. solani* or *B. cinerea* enhanced the production of T39butenolide, but there was no difference compared to controls containing nonviable pathogen biomasses (Table 2). Similarly, *R. solani* and *B. cinerea* stimulated the accumulation of harzianopyridone during the interaction of

Table 2 Production of secondary metabolites (μg) by *Trichoderma harzianum* strains T22 and T39 during the interaction with fungal pathogen

Treatment	Produced compounds (μg)				
	<i>Trichoderma harzianum</i> strain T22			<i>Trichoderma harzianum</i> strain T39	
	T22azaphilone	1-Hydroxy-3-methyl-anthraquinone	1,8-Dihydroxy-3-methyl-anthraquinone	T39 butenolide	Harzianopyridone
Control	372 \pm 60	260 \pm 40	114 \pm 30	508 \pm 50	52 \pm 20
Co-culture					
<i>Rhizoctonia solani</i>	326 \pm 50	114 \pm 30	93 \pm 30	1700 \pm 150	212 \pm 30
<i>Pythium ultimum</i>	6 \pm 1	542 \pm 30	260 \pm 5	450 \pm 50	4 \pm 1
<i>Botrytis cinerea</i>	302 \pm 40	157 \pm 40	88 \pm 20	850 \pm 60	158 \pm 30
Nonviable biomass					
<i>R. solani</i>	8 \pm 2	15 \pm 2	8 \pm 1	401 \pm 70	85 \pm 5
<i>P. ultimum</i>	nd	1060 \pm 200	357 \pm 10	475 \pm 30	3 \pm 1
<i>B. cinerea</i>	nd	134 \pm 10	39 \pm 20	357 \pm 25	169 \pm 30

nd: not detected. Co-culture: *T. harzianum* + living mycelium of *R. solani*, *P. ultimum* or *B. cinerea*. Nonviable biomass: *Trichoderma* + lyophilized *R. solani* or *P. ultimum* mycelia (10 g l^{-1}), or *Trichoderma* + *B. cinerea* cell walls (10 g l^{-1}). Control: *Trichoderma* alone. Quantification performed by LC/MS analysis. Values are means of three replicates (\pm SD).

T. harzianum with both living and dead fungal pathogens. Finally, harzianolide was not induced nor detected in any of our experiments (data not shown).

Discussion

Production of secondary metabolites in fungi is a complex process associated with morphological development (Calvo *et al.* 2002). Secondary metabolites are of tremendous importance in biotechnological applications, but in some cases seem not to have clear function for the producing microbe. In a previous work, we analysed the major secondary metabolites produced by two commercial strains (T22 and T39) of *T. harzianum* (Vinale *et al.* 2006). Six compounds (1–6) obtained from fungal culture filtrates were isolated and characterized. These metabolites showed different levels of antibiotic activity against the fungal pathogens *G. graminis* var. *tritici*, *R. solani* and *P. ultimum* (Vinale *et al.* 2006). In this paper, their antifungal activity against other important phytopathogens such as *L. maculans* (causal agent of the blackleg disease on crucifers), *P. cinnamomi* (causal agent of the forest dieback disease), *B. cinerea* (causal agent of various grey mould diseases) is reported. Both T22azaphilone and harzianopyridone produced by *T. harzianum* strain T22 showed a broad spectrum of activity even if applied at low concentrations (approx. 1–10 μg per plug in a standard plate assay), while T39butenolide and harzianolide secreted by strain T39 significantly inhibited pathogens

growth only when used at higher concentrations (approx. > 100 μg per plug; Fig. 2). These data confirmed and contributed to our knowledge of the spectrum of antifungal activity of these secondary metabolites (Vinale *et al.* 2006).

We also quantified by LC/MS the production of T22 and T39 secondary metabolites in liquid cultures amended with viable or nonviable pathogen biomasses. Interestingly, in *T. harzianum* strain T22 the interaction with fungal pathogens seemed not to be correlated with the accumulation of antibiotic compounds. In fact, the secretion of T22azaphilone, a compound that demonstrated strong antifungal activity *in vitro*, was not particularly induced in co-culture with *R. solani* or *B. cinerea*, and appeared to be even repressed when nonviable pathogen biomass or *P. ultimum* living mycelia were applied (Table 2). Conversely, increased amounts of anthraquinones were found in *P. ultimum*-treated samples, even though these compounds did not demonstrate significant antifungal properties. Similarly, Donnelly and Sherida (1986) isolated anthraquinones in elicited co-culture of *T. polysporum* and *Heterobasidion annosum*, but no marked inhibition of pathogen growth was observed. In fact, anthraquinones are pigments and have been considered among the most abundant fungal natural products giving colour to spores, appressoria, sclerotia, sexual bodies and other developmental structures (Yu and Keller 2005). In addition because of their chemical structure, anthraquinones could act as ROS scavengers in *Trichoderma*

and their accumulation could be stimulated by using appropriate elicitors (i.e. fungal pathogen biomasses) to increase resistance of beneficial strains against biotic or abiotic stresses (Yu and Keller 2005).

T39butenolide and harzianopyridone production was elicited in co-cultures with *B. cinerea* and *R. solani*. In contrast, harzianolide, a reduced form of T39butenolide, was not detected in the interactions between *T. harzianum* strain T39 and pathogen biomasses, as previously observed in plate confrontation assays with *R. solani* (Vinale et al. 2006). Serrano-Carreón et al. (2004) found that the production of 6-pentyl- α -pyrone (6PP) was stimulated by the cultivation with nonviable pathogen mycelium, but not in the co-culture condition. Therefore, they suggested that 6PP production in *T. harzianum* is elicited by *R. solani* as an antagonistic response determined by the physiological status of the host.

It has been demonstrated that virulent phytopathogenic fungi are able to bio-transform metabolites which would otherwise be toxic (Zikmundova et al. 2002). This strategy helps pathogens to overcome host defences (i.e. antibiotics produced by plants) or to withstand adverse environmental conditions, such as those where toxic compounds are produced by antagonistic agents (Bowyer et al. 1995; Friebe et al. 1998). Different fungal pathogens can transform the *Trichoderma* 6PP into isomeric mixtures of monohydroxylated derivatives or, in some cases, into carboxylic acids by further oxidation (Cooney et al. 1997; Poole and Whitaker 1997; Cooney and Lauren 1999). Therefore, the quantity of secondary metabolites accumulated by *Trichoderma* in co-cultures with fungal pathogens depends on the balance between the eliciting effect and the rate of biotransformation exerted by the host pathogen.

Our data indicate that the amount of secondary metabolites produced in *T. harzianum* may depend on the state of the host as well as on the following important factors: (i) the strain considered; (ii) the eliciting microbe or condition; and (iii) the balance between biosynthesis and biotransformation of the metabolite. The understanding and manipulation of the above issues will improve the production process of natural compounds secreted by antagonistic fungi or other microbes. The direct application of the relative eliciting molecules may represent a novel approach to develop biopesticides and biofertilizers based on bioactive compounds.

Acknowledgments

This work was supported by the following projects: FIRB 2002 prot. RBNE01K2E7; MIUR-PON numbers: DD12935, DD5266, 5911, 10418; EU TRICHOEST QLK3-2002-02032; EU 2E-BCAs; Endure Noex 2007-2010;

Università degli Studi di Napoli “Federico II” Programma di Scambi Internazionali per la Breve Mobilità. The authors thank Felice Scala and Michelina Ruocco for their contribution to the manuscript.

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