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Prenylated *Trans*-Cinnamic Esters and Ethers against Clinical *Fusarium* spp.: Repositioning of Natural Compounds in Antimicrobial Discovery

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Abstract: Onychomycosis is a common nail infection mainly caused by species belonging to the *F. oxysporum*, *F. solani*, and *F. fujikuroi* species complexes. The aim of this study was to evaluate the in vitro susceptibility of six representative strains of clinically relevant *Fusarium* spp. toward a set of natural-occurring hydroxycinnamic acids and their derivatives with the purpose to develop naturally occurring products in order to cope with emerging resistance phenomena. By introducing a prenylated chain at one of the hydroxy groups of *trans*-cinnamic acids **1**–**3**, ten prenylated derivatives (coded **4**–**13**) were preliminarily investigated in solid Fusarium minimal medium (FMM). Minimal inhibitory concentration (MIC) and lethal dose 50 (LD_{50}) values were then determined in liquid FMM for the most active selected antifungal *p*-coumaric acid 3,3′-dimethyl allyl ester **13**, in comparison with the conventional fungicides terbinafine (TRB) and amphotericin B (AmB), through the quantification of the fungal growth. Significant growth inhibition was observed for prenylated derivatives **4**–**13**, evidencing ester **13** as the most active. This compound presented MIC and LD_{50} values (62–250 μM and 7.8–125 μM, respectively) comparable to those determined for TRB and AmB in the majority of the tested pathogenic strains. The position and size of the prenylated chain and the presence of a free phenol OH group appear crucial for the antifungal activity. This work represents the first report on the activity of prenylated cinnamic esters and ethers against clinical *Fusarium* spp. and opens new avenues in the development of alternative antifungal compounds based on a drug repositioning strategy.

Keywords: onychomycosis; mycoses; *Fusarium* spp.; drug development; antifungal activity; phenolic inhibitors; hydroxycinnamic acid derivates; *p*-coumaric acid 3,3′-dimethyl allyl ester

Citation: Oufensou, S.; Casalini, S.; Balmas, V.; Carta, P.; Chtioui, W.; Dettori, M.A.; Fabbri, D.; Migheli, Q.; Delogu, G. Prenylated *Trans*-Cinnamic Esters and Ethers against Clinical *Fusarium* spp.: Repositioning of Natural Compounds in Antimicrobial Discovery. *Molecules* **2021**, *26*, 658. https://doi.org/10.3390/molecules26030658

Academic Editor: Giulio Rastelli
Received: 28 December 2020
Accepted: 24 January 2021
Published: 27 January 2021

1. Introduction

Phenolics are among the most desirable food components because of their excellent antioxidant activity and nutraceutical properties. They also find wide-ranging application in medicine and agriculture in virtue of their antimicrobial, anti-inflammatory, and antitumoral activities [1,2]. Among the phenolic compounds, cinnamic acids are a group of aromatic carboxylic acids formed through the biochemical route of shikimate pathway, leading to the synthesis of lignin, the polymeric material that provides mechanical support to the plant cell wall [3,4]. *p*-Coumaric acid **1**, caffeic acid **2**, and ferulic acid **3** are the most common cinnamic acids, consisting of a *trans*-α, β-unsaturated carboxylic chain bonded to a phenol, catechol, and guaiacyl unit, respectively. They possess three distinctive

structural motifs that may possibly contribute to the free radical scavenging capability of these compounds (Figure 1).

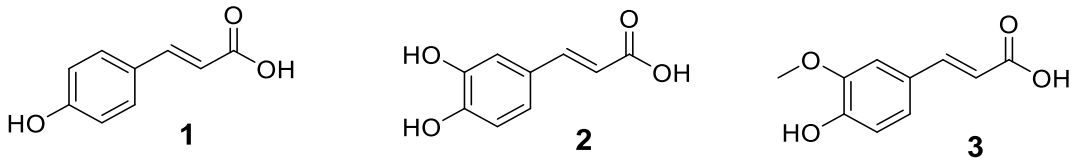


Figure 1. Chemical structures of *p*-coumaric acid **1**, caffeic acid **2**, and ferulic acid **3**.

The presence of an electron donating group on the benzene rings provides the additional property of terminating free radical chain reaction. The carboxylic acid group with a conjugated C-C double bond provides additional quenching sites for free radicals. These features of cinnamic acids are reflected in many biological processes. Cinnamic acids, the main constituents of plant defense, prevent the effects of reactive oxygen species (ROS) formed during fungal infection [5]. The carboxylic acid group of cinnamic acid can act as an anchor by which the compound binds to the lipid bilayer, providing some protection against lipid peroxidation, a process spread out across mammal and human cells [6].

Cinnamic acids **1**–**3** are widely distributed in fruits (e.g., apple, pear, grape, orange, tomato, and berries), vegetables (e.g., bean, potato, and onion), and cereals (e.g., maize, oat, and wheat bran) [7]. They occupy a key place as intermediates in the synthesis of pharmaceuticals, dyes, flavorings, cosmetics, thermoplastics, and materials [8,9]. Because of their high-promoting health capacities and commercial value and given the availability of cinnamic acids **1**–**3** in different plants, extracting processes from biomass are also well studied even though the main accessibility of these compounds comes from synthetic or microbial processes [10,11].

Drug repositioning implies the identification of novel biological targets for natural-occurring compounds that may find application in fields where safety and efficiency are still lacking. One approach relies on slight structural modification of the natural compound, aiming to enhance its biological activity toward a specific target. When the structural modification of the compound concerns the introduction of a natural unit to the molecule framework, the final compound acquires a natural-like feature. Often, such slight structural modifications are devoted to improving bioavailability and selectivity of the parent compound [12–16].

Onychomycosis is a chronic fungal nail infection mainly caused by *Fusarium* spp., particularly those belonging to three specie complexes: *F. oxysporum* (FOSC), *F. solani* (FSSC), and *F. fujikuroi* species complex (FFSC) [17–21]. *F. oxysporum* Schlechtend. emend. Snyder & Hansen; *F. solani* (Mart.) Sacc.; *F. petroliphilum* (Q.T. Chen & X.H. Fu) Geiser, O'Donnell, D.P.G. Short, & N. Zhang; *F. keratoplasticum* Geiser, O'Donnell, D.P.G. Short, & Ning Zhang; *F. falciforme* (Carrión); and *F. verticillioides* (Saccardo) Nirenberg are the most representative species responsible for onychomycosis; moreover, they also infect skin and hair and are considered as emerging pathogens from superficial mycoses as dermatomycoses. *Fusarium* spp. are increasingly reported among the world population. Besides dermatomycoses or onycomycoses, they are responsible for disseminated infections, particularly in patients undergoing cancer therapy or those affected by immunological deficiency [22–24].

Systemic antifungals are the most effective treatment, with meta-analyses showing mycotic cure rates of 76% for terbinafine, 63% for itraconazole with pulse dosing, 59% for itraconazole with continuous dosing, and 48% for fluconazole [25,26]. The use of these agents is discouraged in patients suffering from liver, renal, or heart disease, and in those receiving medications with which there may be significant drug–drug interactions [27]. Terbinafine (TRB) belongs to the allylamine class of synthetic antimycotic agents, and inhibits the squalene epoxidase, a key enzyme involved in the early phase of the ergosterol biosynthetic pathway [28]. Amphotericin B (AmB) is a broad-spectrum antifungal agent belonging to the polyene class; its mechanism of action targets membrane function by

forming channels in the fungal cell membrane, hence allowing ions and organic compounds of the cytoplasm to escape [29].

Susceptibility to fungicides among different *Fusarium* spp. may vary greatly, and clinical *Fusaria* showing multiple resistance to most applied antifungal drugs are increasingly being reported [30–32]. Numerous factors have been cited to explain the lack of response to therapy, such as nonadherence to treatment, incorrect diagnosis, or advanced disease [25]. Additionally, antimycotic prophylaxis in high-risk patients may enhance selective pressure, which favors multidrug-resistant fungi, including *Fusaria* [33]. This urges a massive investment in the development of novel agents to treat emerging and resistant fungi [34].

Considering the antimicrobial activity of cinnamic acids **1**–**3** [35], their low toxicity, and large market availability, the aim of this work was to investigate these compounds against a selection of representative strains belonging to the *F. oxysporum* (FOSC), *F. solani* (FSSC), and *F. fujikuroi* (FFSC) species complexes; namely, *F. oxysporum*, *F. solani F. keratoplasticum*, and *F. verticillioides*.

Aiming to improve the bioavailability and to enhance antimicrobial activity, we transformed cinnamic acids **1**–**3** in esters and ethers using short, medium, and long prenylated chains as alcoholic unit. The efficiency of prenylated phenols in crossing bacterial and fungal membranes [36], as well as their role in exerting antimicrobial activity [37], are generally acknowledged. *O*-prenylated phenols are secondary metabolites of plants. Even though they have been considered for years as biosynthetic intermediates of the most widespread *C*-prenylated derivatives, recently, *O*-prenylated chains are assuming a key role in the bioactivity of molecules into which they are embedded [38,39]. We thus predicted that the preparation of prenylated ethers and prenylated esters of cinnamic acids **1**–**3** (Figure 2) could offer an alternative to the conventional antifungal drugs used against clinical *Fusaria*, enhancing the antimycotic effect of the parent acid.

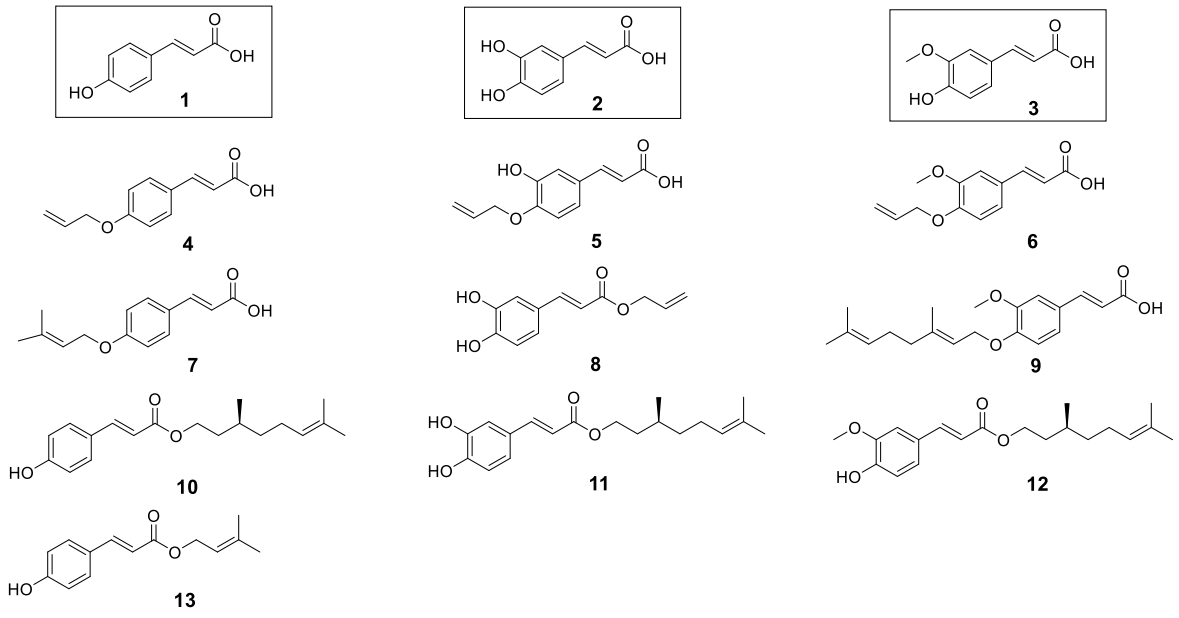


Figure 2. Chemical structure of the tested compounds numbered from **1** to **13**.

An in vitro assay of clinical isolates of *Fusarium* spp. grown on solid Fusarium minimal medium (FMM) amended with compounds **1**–**13** was carried out and a structure–activity relationship was described. Minimal inhibitory concentration (MIC) and lethal dose 50 (LD_{50}) values were then determined in liquid FMM for the most active selected antifungal compound, in comparison with the conventional fungicides terbinafine (TRB) and amphotericin B (AmB). The presence of a *O*-prenylated chain in natural occurring cinnamic acids

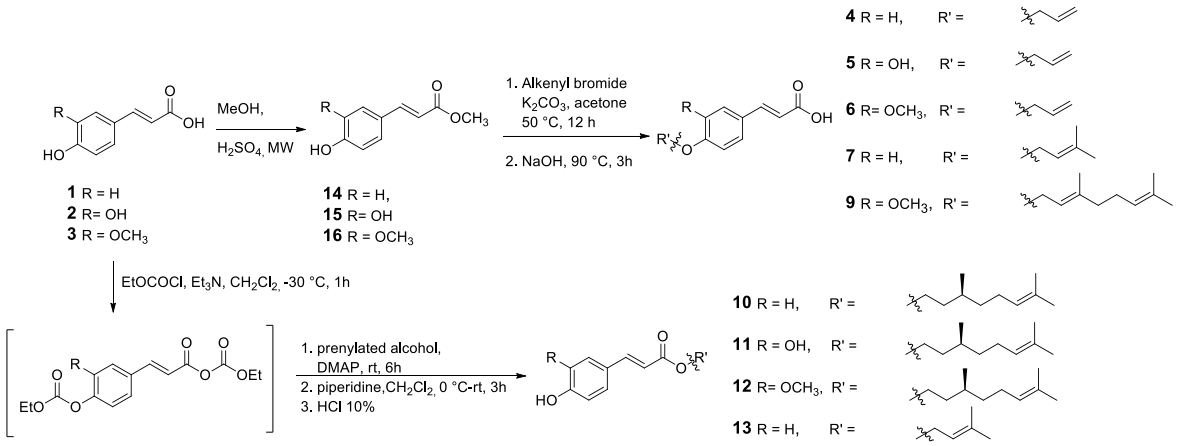
may share a different mode of action compared to conventional antifungal drugs and offers a successful example of drug repositioning.

2. Results

2.1. Chemistry

Prenylated esters **4**–**7** and **9** and prenylated ethers **10**–**13** were prepared, starting from the corresponding cinnamic acid **1**–**3** and, according to the reaction, allyl, 3,3′-dimethyl allyl (prenyl), geranyl, and citronellyl bromide or alcohol. Because of the contemporary presence of two hydroxyl groups in the starting hydroxy cinnamic acid, different synthetic approaches were applied when ether or ester was the final product.

In order to functionalize selectively the phenolic-OH group with a prenylated chain, esterification of the carboxylic group was mandatory (Scheme 1).



Scheme 1. Synthesis of hydroxyl cinnamic ethers **4**–**7** and **9** and esters **10**–**13** and **14**–**16**.

Ethers **4**–**7** and **9** were obtained by the Williamson reaction, starting from the corresponding methyl ester and the appropriate alkenyl bromide under basic conditions and further ester hydrolysis. Yields varied in the range of 53 and 85%. Methyl esterification of acids **1**–**3** was carried out under acid conditions under microwave treatment.

For compound 5, the selectivity of etherification reaction at the *p*-phenolic-OH was confirmed by Nuclear Overhauser Effect Spectroscopy-NMR (NOESY) experiment and by comparison of NMR spectra with the corresponding methyl ester reported in the literature (see Materials and Methods for references).

Esters **10**–**13** were prepared with yields ranging between 37 and 47%, starting from the corresponding parent acid (i.e., 4-hydroxy cinnamic acid **1**, caffeic acid **2**, and ferulic acid **3**) via activation of the carboxylic group with ethyl chloroformate and triethylamine and further addition of the appropriate prenylated alcohol (Scheme 1) and hydrolysis of the phenyl ethyl carbonate.

Compound **8** was obtained via Fischer esterification of acid **2** using allyl alcohol as solvent under acidic conditions as reported in the literature. Compound **13** was also obtained in 87% yield by enzymatic transesterification of the corresponding ethyl ester, in turn achieved by the microwave method, as well as 3,3′-dimethyl allyl alcohol, in mild conditions.

The purity of all new compounds was judged to be >98% by ^{1}H-NMR spectral determination.

The remarkable different lipophilicity estimated, as the value of the logarithm of the partition coefficient of compounds **1**–**13** for *n*-octanol/water (LogP), allowed us to evaluate the possible influence of this property in the antifungal activity of each compound (Table 1).

Table 1. Values of the logarithm of LogP of compounds **1**–**13** for *n*-octanol/water, estimated by ChemBioDraw Ultra 13.

Compound	LogP	Compounds	LogP	Compounds	LogP
1	1.15	**6**	2.37	**11**	4.51
2	1.42	**7**	3.04	**12**	4.78
3	1.54	**8**	2.11	**13**	3.04
4	2.50	**9**	4.44		
5	2.11	**10**	4.90		

As expected, in all compounds studied, the lipophilicity increases as the number of carbon atoms in the prenylated chain increases. Among the three natural occurring acids, the lipophilicity changes in the following order: ferulic acid **3** > caffeic acid **2** > *p*-coumaric acid **1**. The trend is also retained both in the series of geranyl esters **10** > **12** > **11** and in the series of allyl ethers **4** > **6** > **5**. No difference in LogP value resulted in caffeic acid derivatives when the *p*-phenolic-OH or the carboxylic functionalities were protected by an allyl group (i.e., compounds **5** and **8**) or by a 3,3′-dimethyl allyl chain (i.e., compounds **7** and **13**).

2.2. Antifungal Activity of the Parent Compounds **1**–**3** *and Derivatives*

The three naturally occurring hydroxycinnamic acids **1**–**3** and their ethers and esters derivatives **4**–**13** (Figure 2) were used in a preliminary in vitro screening to compare their antifungal activity against six *Fusarium* spp. clinical isolates associated to onychomycosis in a hospital in Milan (Italy) (Table 2).

Table 2. List of *Fusarium* spp. isolates tested in this study.

Species/Species Complex/Sequence Type (ST)	NRRL n.[a]	PVS-Fu n.[b]	Diagnosis	Isolate Source	Date
F. oxysporum/FOSC/ST33	46603	89	Onychomycosis	Toe	2004
F. oxysporum/FOSC/ST33	46606	91	Onychomycosis	Toe	2005
F. keratoplasticum/FSSC/ST2bb	46443	93	Dermatomycoses	Foot	2004
F. solani/FSSC/ST5aa	44903	96	Onychomycosis	Toe	2006
F. verticillioides/FFSC	46599	87	Onychomycosis	Toe	2007
F. verticillioides/FFSC	46442	115	Onychomycosis	Toe	2005

[a] NRRL n. Collection number of Agricultural Research Service (ARS); [b] PVS-Fu n. Collection number of Dipartimento di Agraria, Sezione Patologia Vegetale ed Entomologia, Sassari, Italy.

In the first screening, carried out on solid FMM, exposure to cinnamic acids **1–3** did not determine any significant reduction of mycelium fungal growth compared with the untreated control, with the exception of caffeic acid **2**, which induced a slight inhibition on *F. oxysporum* 89 colony diameter (Figure S1).

In the case of FOSC and FFSC, we noted a significant inhibition of vegetative growth upon exposure to all the compounds derived from cinnamic acids **1**–**3** compared with the untreated control, except for compound **5**, which did not induce any significant reduction of colony diameter in the two FOSC isolates. FOSC isolates were particularly sensitive (>53% and >56% of inhibition for *F. oxysporum* 89 and *F. oxysporum* 91, respectively) to compounds **4**, **7**, **8**, **9**, and **13**, whereas in the case of FFSC, the most effective compounds were **5**, **8**, **9**, **10**, **11**, and **13**. In both species complexes, compound **13** was by far the most effective inhibitor of fungal growth, leading to complete inhibition of the two *F. verticillioides* isolates (Figures S1–S3).

The two representative isolates of the FSSC displayed a different level of sensitivity to compounds **4**–**13**: compounds **4**, **7**, **10**, **11**, and **13** determined >25% inhibition of radial growth on *F. keratoplasticum* 93, whose vegetative growth on solid FMM was completely inhibited by compound **13**, whereas *F. solani* 96 was only partially inhibited by compounds **10**, **11** (36–30% inhibition, respectively), and **13** (63% inhibition; Figure S2).

These preliminary data demonstrate unequivocally that compound **13** has the highest antifungal activity towards all *Fusarium* strains investigated (Figures S1–S3). Citronellyl *p*-coumaric ester **10** was the second most effective inhibitor towards FSSC and FFSC strains, while FOSC representative isolates proved more sensitive to compound **8**. Compound **9** was more effective on FOSC and FFSC representatives compared with FSSC ones. The inhibitory activity of compound **9** towards FOSC isolates was comparable to that displayed by the ethers **4** and **7**, presenting a shorter prenylated chain. Overall, the ester functionality in the cinnamic acid derivatives appears more efficient in conferring inhibitory properties to the tested compounds compared with the ether one.

2.3. Determination of the Minimal Inhibitory Concentration (MIC) and Lethal Dose 50 (LD_{50}) for p-Coumaric acid 3,3′-Dimethyl Allyl Ester **13**

In a further screening performed on FMM liquid medium, the minimal inhibitory concentration (MIC) and the lethal dose 50 (LD_{50}) of compound **13** and of two conventional fungicides were determined (Table 3). *p*-Coumaric acid 3,3′-dimethyl allyl ester **13** confirmed its remarkable good antifungal activity already shown in solid FMM compared with TRB and AmB, with an MIC range comprised between 125 and 250 μM in FOSC (Figures S4 and S5), 62 and 125 μM in FSSC (Figures S6 and S7), and 125 and 500 μM in FFSC (Figures S8 and S9) representative isolates. With respect to the LD_{50}, the most effective compound was TRB (LD_{50} ranging from 2.0 to 64 μM), followed by AmB (LD_{50} ranging from 1.0 to 67.5 μM) and ester **13** (LD_{50} ranging from <7.8 to 125 μM), respectively, for almost all strains. In the case of *F. verticillioides* 115, which was less sensitive to AmB (LD_{50} 8.40–16.8 μM) than to TRB (LD_{50} 2.0–4.0 μM) and **13**, the latter was able to reduce 50% of the fungal growth at a concentration <7.8 μM (Table 3, Figure S9).

Table 3. In vitro susceptibility of strains belonging to the three *Fusarium* species complexes, isolated from onychomycosis and dermatomycosis against the selected compound *p*-coumaric acid 3,3′-dimethyl allyl ester **13** and the two conventional drugs TRB [b] and AmB [c].

Species/Species Complex/Sequence Type (ST)	[a] PVS-Fu n.	Ester 13		[b] TRB		[c] AmB	
		[d] MIC (μM)	[e] LD_{50} (μM)	MIC (μM)	LD_{50} (μM)	MIC (μM)	LD_{50} (μM)
F. oxysporum/FOSC/(ST33)	89	>125–250	31–62	>256	8–16	>135	16.8–33.7
F. oxysporum/FOSC/(ST33)	91	>125–250	62–125	>256	16–64	>135	33.7–67.5
F. keratoplasticum/FSSC/(ST2bb)	93	62	<7.8	128–256	2.0–4.0	33.7–67.5	>2.1–4.2
F. solani/FSSC/(ST5aa)	96	62–125	<7.8	64–128	2.0–4.0	4.2–2.1	1.0
F. verticillioides/FFSC	87	500	62–125	>256	2.0–4.0	>135	2.1–4.2
F. verticillioides/FFSC	115	125–250	<7.8	>256	2.0–4.0	>135	8.4–16.8

[a] PVS-Fu n. Collection number of Dipartimento di Agraria, Sezione Patologia Vegetale ed Entomologia, Sassari, Italy; [b] TRB: terbinafine; [c] AmB: amphotericin B; [d] minimal inhibitory concentration (MIC) range determined visually according to mOD absorbance values (nm) detected after 48 h of incubation at 25 °C; [e] lethal dose 50 (LD_{50}) range determined visually according to mOD absorbance values (nm) detected after 48 h of incubation at 25 °C.

The effects of compounds **13**, TRB, and AmB applied at the same concentration as the MIC determined in liquid FMM on the morphology of representative isolates of *F. solani*, *F. keratoplasticum*, *F. oxysporum*, and *F. verticillioides* were examined by optical microscopy after 72 h (Table 4). While in the untreated controls, a regular morphology of the mycelium with abundant presence of microconidia as well as normal spore germination were observed, in the presence of 250 μM of compound **13**, degradation of the hyphae and vacuolisation of the cytoplasm were evident, along with an alteration of the rarely formed conidia, showing a compromised germination. The release of cell constituents was also noticeable, which can explain the progressive increase in the absorbance signal over time in liquid FMM (Figures S4–S9).

After 72 h of exposure to 256 μM TRB, a marked reduction on mycelium formation was observed, and fungal hyphae and spores showed a distorted morphology with tortuous growth (Table 4). This concentration of TRB could not totally inhibit the mycelium growth or the spore production but caused an evident swelling of conidia. The effects of TRB were