Chemical composition, antibacterial and antifungal activities of *Trichoderma* sp. growing in Tunisia

Fethi Bel Haj KHETHR[1], Samia AMMAR[2], Dhouha SAÏDANA[1], Majda DAAMI[3], Jihane CHRIAA[4], Kaouthar LIOUANE[5], Mohamed Ali MAHJOUB[2], Ahmed Noureddine HELAL[1], Zine MIGHRI[2]*

[1]Laboratory of Genome, Diagnostic and Valorisation, Faculty of Pharmacy, 5000 Monastir; [2]Laboratory of Natural Substances Chemistry and Organic Synthesis, Faculty of Sciences, 5000 Monastir; [3]Laboratory of Phytopathology, The Regional Pole of Agricultural Research-development, Chott-Mariem, Sousse; [4]Laboratory of Environment Microbiology, Faculty of Pharmacy, 5000 Monastir; [5]Laboratory of Genetic, Biodiversity and Bio-Resources Valorisation, Biotechnology Institute of Monastir, 5000 Monastir, Tunisia

Abstract - *Trichoderma* species are common soil-inhabiting fungi that have been developed as effective biocontrol agents against various phytopathogenic microorganisms. The chemical composition of butanolic extract prepared from cultivated *Trichoderma* sp. was analysed using GC-FID and GC-MS. Six components were identified. Limonene, the terpenoid compound, was found to be the major component in the tested extract (92.6%). Antibacterial and antifungal activities were also investigated against five pathogenic Gram positive and Gram negative bacterial strains and five pathogenic fungi. In tested concentrations, the prepared extract showed positive antibacterial values, but no antifungal activity was detected.

Key words: *Trichoderma* sp., chemical composition, butanolic extract, antibacterial activity, antifungal activity.

INTRODUCTION

In recent years plant pathologists and commercial companies have shown considerable interest in the application of biological control agent. *Trichoderma* sp., an anamorphic fungal genus, contains fungi frequently found on decaying wood and in soil (Samuels, 1996; Klein and Eveleigh, 1998). This genus has received particular attention as agent for the biological control plant fungi pathogens and appears to be likely candidates for successful exploitation in the future (Cardoza *et al*., 2006). The mechanisms suggested being involved in biocontrol by these fungi are formation of infection structures (e.g. coiling), production of hydrolytic enzymes, secretion of antifungal metabolites, and induction of defence responses in plants, that work synergistically to achieve disease control (Howell, 2003; Harman *et al*., 2004).

Trichoderma species are known to produce over 40 different metabolites that may contribute to their mycoparasitic and antibiotic action (Sivasithamparam and Ghisalberti, 1998). After

recognising the presence of a potential host fungus, *Trichoderma* inhibits or kills the plant pathogen by parasitising its hyphae, thereby employing hydrolytic enzymes to degrade the host's cell wall (Chet *et al*., 1998; Kubicek *et al*., 2001).

In the present work, we are interested to the natural compounds extracted from *Trichoderma* sp. Chemical composition was then investigated using Gas Chromatography-Flame Ionisation Detector (GC-FID) and Gas Chromatography-Masse Spectrometry (GC-MS) methods. Antibacterial and antifungal activities were tested to search new eventual active principles responsible of such activities.

MATERIALS AND METHODS

***Trichoderma* sp. strains.** *Trichoderma* sp. is native fungus, collected from the forest area of Ain Draham (Tunisia).

A voucher specimen was deposit in the Laboratory of Natural Substances Chemistry and Organic Synthesis, Faculty of Sciences, Monastir, Tunisia.

* Corresponding author. Phone: 216 73500276; Fax: 216 73500278; E-mail: zine_mighri2002@yahoo.fr

Culture conditions and preparation of the butanolic extract. *Trichoderma* sp. strain was grown on potato dextrose agar (PDA; Merck, Germany) at 28 °C until sporulation. Indeed, the PDA is the most effective culture media in the isolation this fungus (Vargas Gil *et al.*, 2006). *Fusarium oxysporum* F.33.03, *Fusarium sambucinum* F.48.03, *Aspergillus niger*, *Alternaria* sp. and *Penicillium* sp. were used as plant pathogenic host fungi and were obtained from the collection of the Phytopatology Laboratory, Regional Pole of Research-Development Agriculture, Chott Mariem, Sousse, Tunisia. All the strains were inoculated on agar plates (90 mm in diameter, 20 ml medium/Petri dish) containing malt extract (12.7 g/l), glucose (10 g/l) and agar (20 g/l) (MEA).

The antimicrobial activity was evaluated against five selected Gram positive and Gram negative species: *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* NCIMB 8853, *Escherichia coli* ATCC 5218 and *Micrococcus luteus* NCIMB 8166. Mueller Hinton Agar (MHA; g/litre): beef infusion solids 4.0, starch 1.5, casein hydrolysate 17.5, agar 15.0) was used for the bacteria medium growth.

Trichoderma sp. was grown in PDA as described previously (Vargas Gil *et al.*, 2006). The liquid culture of the fungus (3.5 l) was filtered under vacuum. Two media have been obtained: the intracellular and the extracellular media. In our study we interested only to the extracellular medium which was extracted by butanol to obtain the butanolic extract (1.5 g). The resultant extract was concentrated under reduced pressure, taken to dryness under vacuum and stored at 4 °C until tested.

Analyses of the butanolic extract compounds.
Gas chromatograph. HP 5890-series II equipped with: Flame ionisation detectors (FID) and HP-5, 30 m * 0.25 mm ID, 0.25 μm film thickness fused capillary column. The carrier gas was nitrogen (1.2 ml/min). The oven temperature program was 1 min isothermal at 50 °C, then 50-280 °C at a rate of 5 °C/min and held isothermal for 1 min. The injection report temperature was 250 °C, detector 280 °C. Volume injected: 0.1 μl of 1% solution (diluted in hexane).

Mass spectrometer. HP5972 recording at 70 eV; scan time 1.5 s; mass Range 50-550 amu. Software adopted to handle mass spectra and chromatograms was a HP Chem-Station.

Identification of the compounds. The components of the butanolic extract were identified by comparing of their mass spectra with those of a computer library (Wiley 275 library). Further confirmation was done by referring to retention indices data generated from a series of alkanes (C_9-C_{28}) (Shibamoto, 1987; Adams, 1995).

Antimicrobial activity.
Disc diffusion method. The qualitative antimicrobial assay of the butanolic fraction of *Trichoderma* sp. was carried out by the disc diffusion method (Bauer *et al.*, 1966; Chabbert, 1972; Marmonier, 1987). This assay was performed using culture growth at 37 °C during 18 h and adjusted to approximately 10^5 colony forming unit per millilitre (CFU/ml). The used culture mediums were MHA for the bacteria and PDA for the fungi. Five hundred microlitres of the inoculum were spread over plates containing MHA or PDA, and a Whatman paper disc (5 mm) impregnated with 0.2, 0.5 or 1 mg/disc of the butanolic fraction was placed on the surface of the media. The plates were left 30 min at room temperature to allow the diffusion of the ingredient. The bacteria were incubated at 37 °C for 24 h and the fungi at 37 °C for 48 h. After incubation period, the inhibition zone obtained around the disc was measured. Two control discs (Standard antibiotics, Bio-Rad Laboratories, Inc.) were used in the test: ampicillin used to control the sensitivity of the tested bacteria and carbendazine to control the tested fungi. A negative control, involving the presence of microorganisms without test material (blank) was also used. The experiments were run in triplicate, and the developing inhibition zones were compared with those of references discs.

Dilution method. The minimal inhibitory concentration (MIC) and minimal bacterial concentration (MBC) of the tested extract were determined using the Mueller Hinton broth (MHB) dilution method (Vanden Berghe and Vlietinck, 1991). All tests were performed in MHB supplemented with 5% Tween 80 (Hicheri *et al.*, 2003). Bacterial strains were cultured overnight in MHB at 37 °C. Tubes of MHB containing various concentrations of butanolic extract, mentioned in Table 1, were inoculated with 10 μl of 10^5 CFU/ml of microorganism suspensions. They were incubated in a shaker (120 rpm) at 37 °C for 24 h (May *et al.*, 2000). Control tubes without tested samples were assayed simultaneously. All samples were tested in triplicates. The MIC was defined as the lowest concentration preventing visible growth (May *et al.*, 2000; Delaquis *et al.*, 2002; Burt, 2004).

Referring to the result of the MIC essay, the tubes showing complete absence of growth were identified and 10 μl of each tube were transferred to Trypto-Caseine-Soja agar (TCSA) plates (Ronda and Rybak, 2001) and incubated at previously mentioned times and temperatures. The complete absence of growth was considered as the MBC (Canillac and Mourey, 2001).

TABLE 1 - Chemical composition of the *Trichoderma sp*. butanolic extract

Pic number	Compound	RI apolar	Percentage
1	Limonene	1104	92.6
2	3,7-dimethyl octa-1,6-dien-3-ol = ß-linalool	1147	2.45
3	1,7,7-trimethyl Bicyclo[2.2.1]heptan-2-one.	1183	1.78
4	Dodecane	1221	1.12
5	Tetradecane	1376	0.89
6	12-Methyl-tridecanoate of methyl	1462	1.03
Terpens			92.6
Hydrocarbons			2.01
Alcohols			2.45
Ketones			1.78
Esters			1.03
Total			99.87

The components and their percentages are listed in order of their elution on apolar column (BP-1).

RESULTS AND DISCUSSION

Chemical composition of the butanolic extract

The butanolic extract of *Trichoderma* sp. contained essentially a terpenoid compound identified as limonene (92.6%) (Fig. 1, 2). The other constituents represented weak proportions and belonged to alcohols, ketones, hydrocarbons and fatty acid esters families (Table 1). The presence of the terpenoid compound, limonene, was in agreement with previous studies. Cardoza *et al.* (2005) and Sivasithamparam and Ghisalberti (1998) reported that filamentous fungi, including *Trichoderma*, produce large series of terpenes, with many applications.
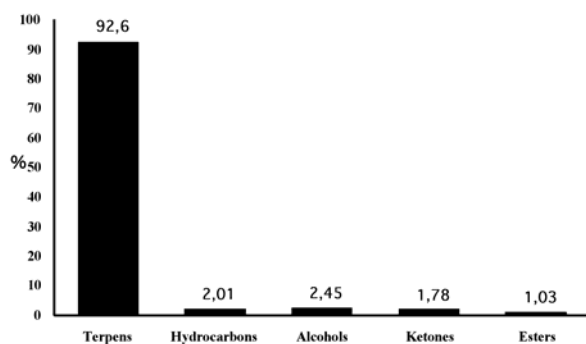

FIG. 1 - Repartition of compound classes in the *Trichoderma* sp. butanolic extract.
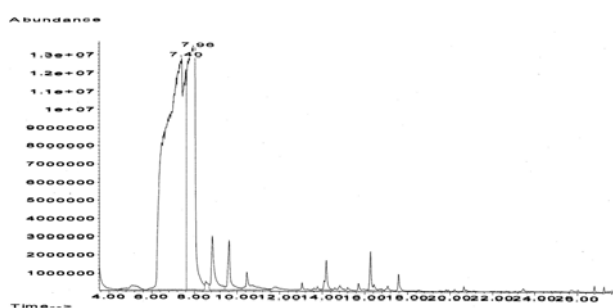

FIG. 2 - Chromatogram of the *Trichoderma* sp. butanolic extract.

Antibacterial activity

The antibacterial activity of the butanolic extract was tested *in vitro* by using disc diffusion and liquid dilution methods with the microorganisms as seen in Table 2. The results of the bioassays showed that the butanolic extract exhibited a significant antibacterial activity against all Gram positive cocci and against the Gram negative rods *E. coli*. Although butanolic extract of *Trichoderma* sp. was not effective against *P. aeruginosa* at the tested concentrations, the fungal strain *Trichoderma viride* grown on four alternative plant polysaccharides as C sources, had induced enzymes able to degrade the bacterial biofilm matrix of *Pseudomonas fluorescens* (Orgaz *et al.*, 2006)

Visible growth of *S. aureus*, *S. epidermidis* and *M. luteus* was prevented at a concentration of 500 µg/ml while for *E. coli* it was at a concentration of 1 mg/ml. This result was in agreement with many studies realised on plant species such as those reported by Kokoska *et al.* (2002), Bougatsos *et al.* (2003) and Yayli *et al.* (2005).

The higher resistance among Gram negative bacteria could be due to the differences in the cell membrane of these bacterial groups. Indeed, the external membrane of Gram negative bacteria renders their surfaces highly hydrophilic (Smith-Palmer *et al.*, 1998) whereas the lipophilic ends of the lipoteichoic acids of the cell membrane of Gram positive bacteria may facilitate penetration by hydrophobic compounds (Ultee *et al.*, 1999; Cox *et al.*, 2000). The butanolic extract of *Trichoderma* sp. appeared effective against *E. coli*. Previous study shows that trichoviridin was the first antibiotic compounds isolated from a strain of *Trichoderma koningii*, it was active against *E. coli* and *Trichophyton usteroides* (Yamano *et al.,* 1970).

As can be seen in Table 2, the tested extract exhibited an antibacterial activity against *S. epidermidis* at a concentration of 1 mg/ml (MBC).

Butanolic extract composition was represent-

TABLE 2 - Antimicrobial activity of *Trichoderma sp.* butanolic extract

Microorganism	Ampicillin inhibition zone (mm) at disc concentration				MIC* (µg/ml)	MBC** (µg/ml)
	10 mg	0.2 mg	0.5 mg	1 mg		
Gram positive bacteria						
Staphylococcus aureus ATCC 29213	17	7	7	7.5	500	> 800
Staphylococcus epidermidis NCIMB 8853	18	7	7	7	500	1000
Micrococcus luteus NCIMB 8166	17	5.5	5.5	6	500	> 800
Gram negative bacteria						
Escherichia coli ATCC 35218	10	6	6	6	n.d.	1000
Pseudomonas aeruginosa ATCC 27853	9	(-)	(-)	(-)	n.d.	n.d.

* MIC: minimum inhibitory concentration, ** MBC: minimum bactericidal concentration.
ATCC: American Type Culture Collection, NCIMB: National Collections of Industrial Marine and Food Bacteria.
(-): no activity detected, n.d.: not determined.

ed for 92.6% by limonene, a terpenoid compound which could be responsible for the observed antibacterial activity against the tested bacteria. It is known that *Trichoderma* species produce several terpenoid compounds with strong antimicrobial activities that have been related to the antagonism and biocontrol capacities of *Trichoderma* strains (Corley *et al.*, 1994; Sivasithamparam and Ghisalberti, 1998). Furthermore, *,*-linalool, which is an aliphatic alcohol, seemed to ameliorate the antibacterial activity. According to Delaquis *et al.* (2002), fractions rich in long chain alcohols were active against bacteria. The antimicrobial properties of alcohols were known to increase with molecular weight (Morton, 1983, Pauli, 2001). The compounds present in the greatest proportions are not necessarily responsible for the higher share of the total activity. Thus, the involvement of the less abundant constituents should be considered. And then, the activity could be attributed to the presence of minor compounds or at least to a synergistic effect between all components. In fact, the synergistic effects of the diversity of major and minor constituents present in the extract should be taken into consideration to account for their biological activity (Burt, 2004).

Results of both methods employed for the antibacterial activity are comparable (diffusion method, micro-dilution method). However, MIC and MBC values were lower than the positive control (Table 2). We can suggest that the inhibition zone does not reflect the real antibacterial effectiveness of the sample, since it is affected by the solubility of the extract, the diffusion range in the agar, the evaporation, etc. This point was in agreement with Kim *et al.* (1995) and Cimanga *et al.* (2002) suggestions.

Antifungal activity
At the tested concentrations, the butanolic extract, showed no antifungal activity against the four pathogenic fungi. Previous study reported that terpenoid compounds were known by their antifungal properties (Corley *et al.*, 1994; Vicente *et al.*, 2001; Nielsen *et al.*, 2005); whereas, *Trichoderma* strains were considered as important fungicides for the biological control of plant diseases (Vizcaíno *et al.*, 2005). Some *Trichoderma* species are known to possess good antagonistic abilities against plant pathogenic fungi, e.g., *Fusarium*, *Pythium*, *Rhizoctonia* and *Sclerotinia* species (Lewis and Papavizas, 1987, Sivan and Chet, 1993). *Trichoderma harzianum* inhibited growth of *Aspergillus niger* Van Tieghem, *Pestalotia rhododendrii* Cuba and several saprophytic bacteria (Hutchinson and Cowan, 1972). Mycoparasitic *Trichoderma* strains are able to recognise the host hyphae, to coil around them, develop haustoria, penetrate the cell wall of the host with cell-wall degrading enzymes like chitinases, glucanases and proteases, and utilise the contents of the host hyphae as nutrient source (Elad *et al.*, 1982, Calvet *et al.*, 1989).

The absence of the antifungal activity in our research could be due to the low concentration of extract used. Furthermore, antifungal susceptibility is influenced by the type of medium, pH, inoculum size, temperature and the time of incubation (Ghannoum *et al.*, 1996). Hence, we can suggest that higher concentrations of the butanolic extract could engender a more potent effect against all microorganisms.

CONCLUSION

This preliminary screening is an evaluation of the potential antimicrobial activity of the fungus *Trichoderma* sp. The results obtained indicate that further assays are useful. Our next approach will be focused on isolating and testing pure active compounds. These active metabolites should provide models for the synthesis of better bactericides and fungicides from *Trichoderma* sp.

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