Characterization of secondary metabolites from *Trichoderma* species isolated from Libyan soils and their biodegradation ability of methyl tertiary butyl ether

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Abstract:

The current study was conducted at University of Napoli, Italy to characterize and identify secondary metabolites produced by three locally isolated *Trichoderma* spp., *T. longibrachiatum* UAMH7955 (Lib1), *T. harzianum* (Lib2) and *T. longibrachiatum* UAMH7956 (Lib3) from Libyan soils at the Biotechnology Research Centre, Tripoli, Libya and their potential in bioremediation of 0.1, 0.2, 0.4, 0.4, 1.0, 1.0% (v/v) Methyl *tert*-butyl ether (MTBE). Results showed two major components extracted from Lib1 corresponding to lipo-carbohydrate and Peptaibol. Both Lib1 and Lib2 ioslates showed good tolerance to toxic pollutant up to concentration of 0.4% compared to control treatment. While , Lib3 was affected negatively by the presence of MTBE at the lowest concentrations treatment.

Key words: *Trichoderma, secondary metabolites, bioremediation, MTBE*

المستخلص:

أجريت هذه الدراسة بجامعة نابولي، إيطاليا وذلك لوصف وتعريف منتجات الأيض الثانوية الناتجة عن ثلاثة عزلات محلية من فطر *Trichoderma*، *T. longibrachiatum* UAMH7955 (Lib1)، *T. harzianum* (Lib2)، *T. longibrachiatum* UAMH7956 (Lib3) تم عزلها من الترب الليبية بمركز التقنيات الحيوية، طرابلس، ليبيا وتقييم قدرتهم على المعالجة الحيوية للملوث السام Methyl *tert*-butyl ether (MTBE) عند مستوى 0.1، 0.2، 0.4، 0.8، 1.0، 1.5% (v/v). بينت النتائج وجود مركبين رئيسيين تم استخلاصهما من عزلتي Lib2 هما lipo-carbohydrate و Peptaibol. أظهرت العزلتان Lib1 و Lib2 قدرة على تحمل الملوث السام لحد تركيز 0.4% مقارنة بمعاملة الشاهد. بينما كان تأثير MTBE سلبي على نمو العزلة Lib3 وذلك عند المعاملة بأقل تركيز منه.

كلمات مفتاحية: *Trichoderma، منتجات الأيض الثانوية، المعالجة الحيوية، MTBE*

Introduction:

Trichoderma species have long been recognized as biological control agents (BCAs) for the control of plant diseases and for their ability to increase plant growth and development. They are widely used in agriculture, and some of the most useful strains demonstrate a property known as "rhizosphere competence" (Harman, 2000). Much of the known biology and many of the uses of these fungi have been documented (Harman and Kubicek, 1998; Harman *et al*., 2004a; Kubicek and Harman, 1998). It is found nearly in all temperate and tropical soils.

Trichoderma produces a variety of lytic enzymes characterized by high diversity of structural and kinetic properties, thus increasing the probability of this fungus to counteract the inhibitory mechanisms used by neighboring microorganisms (Ham *et al*., 1997). Further, *Trichoderma* hydrolytic enzymes have been demonstrated to be synergistic, showing an augmented antifungal activity when combined with themselves, other microbial enzymes, PR proteins of plants and some xenobiotic compounds (Lorito *et al*., 1994a; 1994b; 1996; 1998; Fogliano *et al*., 2002; Schirmböck *et al*., 1994; Woo *et al*., 2002). *Trichoderma* strains seem to be an inexhaustible source of antibiotics, from the acetaldehydes gliotoxin and viridin (Dennis and Webster, 1971), to alpha-pyrones (Keszler *et al*., 2000), terpenes, polyketides, isocyanide derivatives, piperacines, and complex families of peptaibols (Sivasithamparam and Ghisalberti, 1998). All these compounds produce synergistic effects in combination with CWDEs, with strong inhibitory activity to many fungal plant pathogens (Lorito *et al*., 1996; Schirmböck *et al*., 1994).

Industrialization combined with increased urbanization and changing agricultural practices have caused a rise in the level of contaminants found in the environment, resulting in a negative impact on human health. Methods used for cleanup of polluted sites by the removal of hazardous compounds is a serious problem, which requires a multi-faceted approach for obtaining suitable solutions. Physical and chemical treatments have been the most commonly used methods for remediation of soil pollutants to date, however, their high costs have increased the search for alternative methods based on biological systems, such as bioremediation (involving microbes) and phytoremediation (involving both microbes and plants) techniques for detoxification of xenobiotic compounds (Eapen et al., 2007). In Libya, large portion of economy is supported by the petroleum industry. During the refining process many pollutants may be released in the environment, air and groundwater sources. Methyl *tert*-butyl ether (MTBE) is a compound frequently added to gasoline in order to increase octane number. Unfortunately, it frequently contaminates groundwater when gasoline containing MTBE is spilled or leaked in storage and is difficult to clean up due to its high solubility in water (Levchuk *et. al*, 2014; Roslev *et. al*., 2014)

Numerous *Trichoderma* strains are resistant to or capable of degrading hydrocarbons, chlorophenolic compounds, polysaccharides and the xenobiotic pesticides used in agriculture (Harman and Kubicek, 1998; Harman *et al*., 2004b). The capacity of these organisms to sequester, metabolize, release and exchange substances may represent a potential application for bioremediation or phytobioremediation in the cleanup of

contaminated sites. In this strategy, the fungus could accumulate toxicants or breakdown the compounds, as well as stimulate the growth and development of the plant which in turn augments its capacity to accumulate and metabolize the noxious substances, then these plants could be eventually removed from the site (Harman *et al.*, 2004b).

Although numerous commercial products containing *Trichoderma* are available for use in greenhouse and field, the effectiveness and reliability of these products under diverse environmental conditions, i.e. temperature, can limit growth and development. In Libya, interest has been oriented to the potential use of biocontrol in agriculture. The antagonistic activities of local Libyan *Trichoderma* isolates has been reported against many fungal plant pathogens (*Fusarium* sp., *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Botrytis cinerea*), (Duzan *et. al*., 2007, Eshlaibek *et. al*., 2021, Abadi *et. al*., 2017, Elguail, *et al*, 2021). However, there is a general lack of information about type of secondary metabolites produced by the Libyan isolates Further, little is known about type of compounds and their ability to interact with commercial products and possible applications. Thus, the aim of this study is to characterize the secondary metabolites produced by three Libyan isolates *T. longibrachiatum* UAMH 7955 (Lib1); *T. harzianum* (Lib2); *T. longibrachiatum* UAMH 7956 (Lib3) previously isolated and showed their efficacy to antagonize the plant pathogens; *Rhizpctonia* sp., *Fusrium* sp. and *Alternaria* sp. (Duzan *et. al*., 2007); *Sclerotinia sclerotiorum* (Eshlaibek, 2021); *Botrytis cinerea* (Abadi *et. al*., 2017) and the potential biotechnological use of these isolates in bioremediation, detoxification of MTBE.

Materials and Methods:

Isolation and growth conditions of *Trichoderma.*

Trichoderma isolates were collected from nine agricultural areas in the northwestern part of Libya, including Al-Khums, Al-Gharahboli, Tajoura, Al-Nofleen, Tareek Al-Matar, Ghasser Ben-Ghasheer, El-Azizia and Yefren, and were identified based on ITS sequence analysis (Duzan *et., al*., 2007) in order to determine the fungal population density and obtain a representative set of isolates. Soil samples were placed in polyethylene bags, and stored at 5° C until plated. The fungal isolations were performed by using a serial dilution technique (Tuite, 1969).

Potato dextrose agar (PDA; SIGMA, St. Louis, MO, USA) medium was prepared according to the manufacturer's instructions, and augmented with Lactic acid and Rose Bengal to suppress bacterial growth, then poured into 90 mm Petri plates. One hundred grams of soil samples were added to 100 ml distilled water and homogenized for 1 min.; then a dilution series was prepared (0, 10, 10^2, 10^3, 10^4) in sterile water. One hundred microliters of each dilution was inoculated to the surface of plates containing PDA, spread evenly with a sterile spreader and incubated in the dark for 5-7 days at 25° C. Emerging fungal colonies were isolated, stained with methylene blue, identified by observations under a microscope. Colonies of *Trichoderma* were selected, transferred to new PDA plates, then pure cultures were obtained, and maintained on PDA slants at 25°C. Conidia from 4 day old cultures were collected in water and any mycelial debris was separated by determined using with a haemocytometer and adjusted when

necessary. Spore suspensions were stored at -20° C in 20% v/v glycerol solution until used. *T. atroviride* strain P1 (ATCC 74058) and *T. harzianum* strain T22 (ATCC 20847), commonly used as biocontrol agents (Harman, 2000; Tronsmo, 1989), were included as controls.

Agar plugs of the *Trichoderma* cultures were inoculated to the center of plates containing PDA, Salt Medium (SM) and incubated at 25° and 30° C in the dark. The growth of the fungal colony was measured daily throughout the incubation period. The composition of SM in one liter of water was as follows: KH_2PO_4 680 mg L^{-1}, K_2HPO_4 870 mg L^{-1}, KCl 200 mg L^{-1}, NH_4NO_3 1 g L^{-1}, $CaCl_2$ 200 mg L^{-1}, $MgSO_4.7H_2O$ 200 mg L^{-1}, $FeSO_4$ 2 mg L^{-1}, $MnSO_4$ 2 mg L^{-1}, $ZnSO_4$ 2 mg L^{-1}, Sucrose 10 g L^{-1}, agar 10 g L^{-1} (all purchased from SIGMA).

Isolation and characterization of secondary metabolites.

Secondary metabolites were isolated from *Trichoderma* culture filtrates as described by Vinale et al. (2006). Briefly, two 7-mm diameter plugs of each Libyan *Trichoderma* isolate, obtained from actively growing margins of PDA cultures, were inoculated into 5 L conical flasks containing 1 L of sterile one-fifth (1/5 X) strength Potato Dextrose Broth (PDB). The stationary cultures were incubated for 31 days at 25° C. The cultures were filtered under vacuum through filter paper (Whatman No. 4), and the filtrates stored at 2° C for 24 h. The filtered culture broth (2 L) of each isolate was extracted exhaustively with ethyl acetate (EtOAc). The combined organic fraction was dried (Na_2SO_4) and evaporated under reduced pressure at 35 ^oC. The recovered red-brown residue was subjected to flash column chromatography (Si gel; 50 g), eluting with a gradient of EtOAc:petroleum ether (8:2 to 10:0). Column chromatography was carried out using silica gel 60 GF_{254} and GF_{60} 35-70 mesh (merck, Darmstadt, Germany). Analytical and preparative thin-layer chromatographies (TLC) were performed on silica gel (Kieselgel 60, GF254, 0.25 and 0.5 mm, respectively, Merck); compounds were detected with UV radiation (254 or 366 nm) and/or by spraying the plates with CeSO4 (10% w/v in water) or H_2SO_4 (5% v/v in ethanol) and heating at 110° C for 10 min. Fractions showing similar TLC profiles were combined and further purified by using RP-18 column (H2O: Methanol gradient form 100 to 0 of H2O). All purified compounds were analyzed by 1H, 13C NMR and LC/MS. 1H and 13C NMR spectra were recorded with a Bruker AM 500 spectrometer operating at 500 (1H) and 125 (13C) MHz using residual and deuterated solvent peaks as reference standard. Low and high resolution mass spectra were obtained by using a VG Autospec mass spectrometer (EI mode).

Detoxification and compatibility with toxic pollutants.

Liquid cultures of the three *Trichoderma* isolates (Lib1, Lib2, Lib3) were screened for their ability to growth in presence of Methyl tert-butyl ether (MTBE), a common contaminant of ground water when gasoline with MTBE is spilled or leaked at gas stations.

Fungal inoculum (prepared from plate cultures as described above) was inoculated in

flasks containing sterile medium (SM) amended with different concentrations of MTBE (SIGMA). The cultures were incubated at 25° C, in orbital agitation of 150 rpm for 6 d. The mycelial biomass was collected by filtration, dried at 120° C for 2 h (or until dry) and then weighed. Moreover, the ability of the isolates to degrade the toxic compound was quantified by determining the residue of MTBE present in the culture filtrate after removing the fungal mycelium. Separation and quantification of MTBE in the liquid culture was performed by using Gas Chromatography - Flame Ionization Detector (GC-FID) on an Agilent 7890A gas chromatographer (Agilent Technologies) with an HP-5 column. The sample injection port was maintained at 300° C, and all samples were eluted through the column with a flow rate of 1.2 ml/min. The concentration of the MTBE was determined by comparison to a standard curve with concentrations ranging from 0.1 to 10% (v/v). All samples were analyzed at least in duplicates.

Results:

Metabolic profile of Libyan isolates.

Although our data confirmed that the Libyan Trichoderma strains do not produce 6-n-pentyl-6H-pyran-2-one (TLC analysis), the most characterized and important of the Trichoderma antibiotics (Ghisalberti *et al*., 1990), other compounds with antibiotics activity were detected. Unfortunately the organic fractions obtained from culture filtrates of Lib2 and Lib3 isolates didn't allow to properly identify the secondary metabolites produced. On the other hand, when the methanolic fraction extracted from Lib1 culture filtrate was analyzed, the mixture showed two major components, corresponding to lipo-carbohydrate and Peptaibol. This fraction was further separated by preparative RP flash chromatography. Fraction n. 4 gave a major component that was further analysed by using NMR spectroscopy. The isolated compound showed 1H (Fig. 1A) and 13C (Fig. 2). spectra similar to those reported in literature (Fig. 1–B) (Auvin-Guette *et al*., 1992). Moreover, the COSY bidimensional NMR spectrum of fraction n. 4 (Fig. 3-A) suggested that the isolated compound could be assigned to the lipopeptaibols class of natural compounds, and in particular resulted closely related to the Trichogin A IV, previously isolated from T. longibrachiatum (Peggion *et al*., 2003; Fig. 3-B).

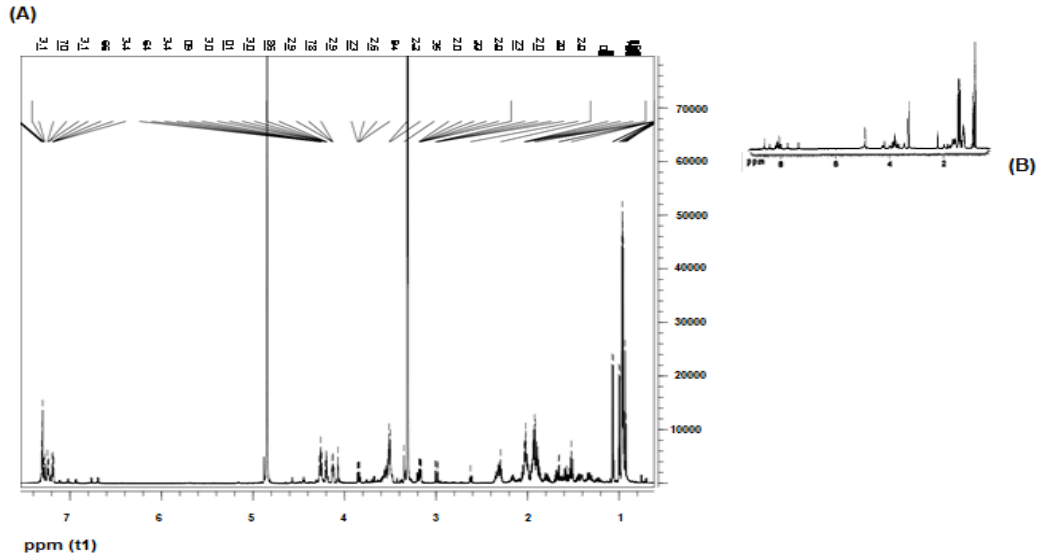

Fig. 1: ^{1}HNMR spectra of fraction °4 isolated from Lib1 culture filtrate and recorded in CD_3OD (A) and Trichogin AIV recorded jn *d6*-DMSO (B) (Auvin-Guette *et. al*., 1992). Instrument: Bruker600 MHz.

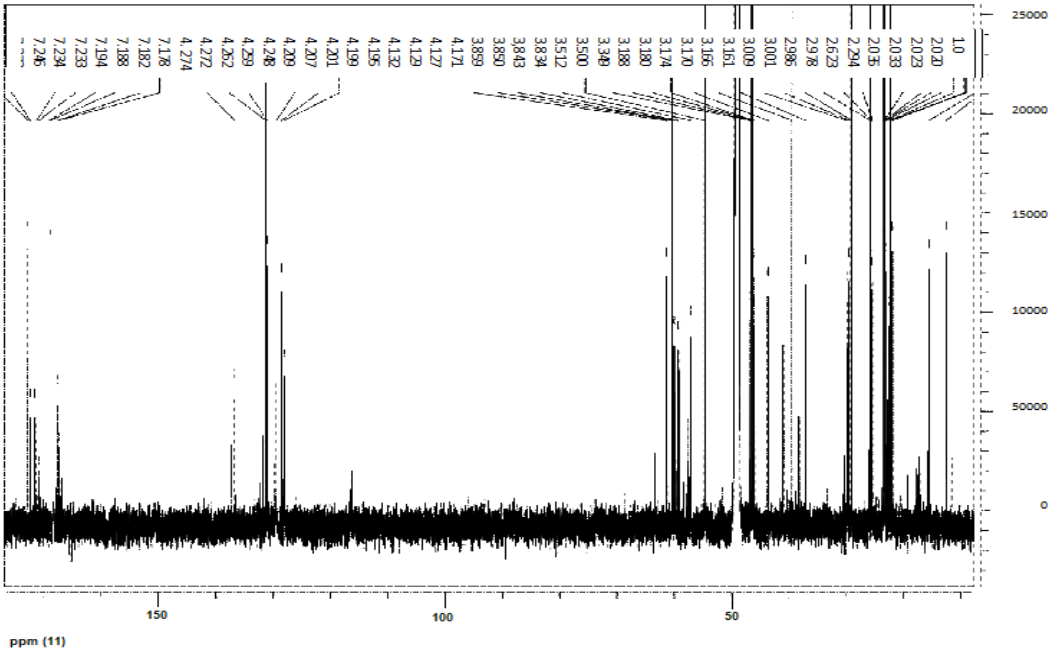

Fig. 2: ^{13}C NMR spectrum of fraction n° 4 isolated from Lib1 culture filtrate and recorded in CD_3OD. Instrument: Burker 600 MHz.

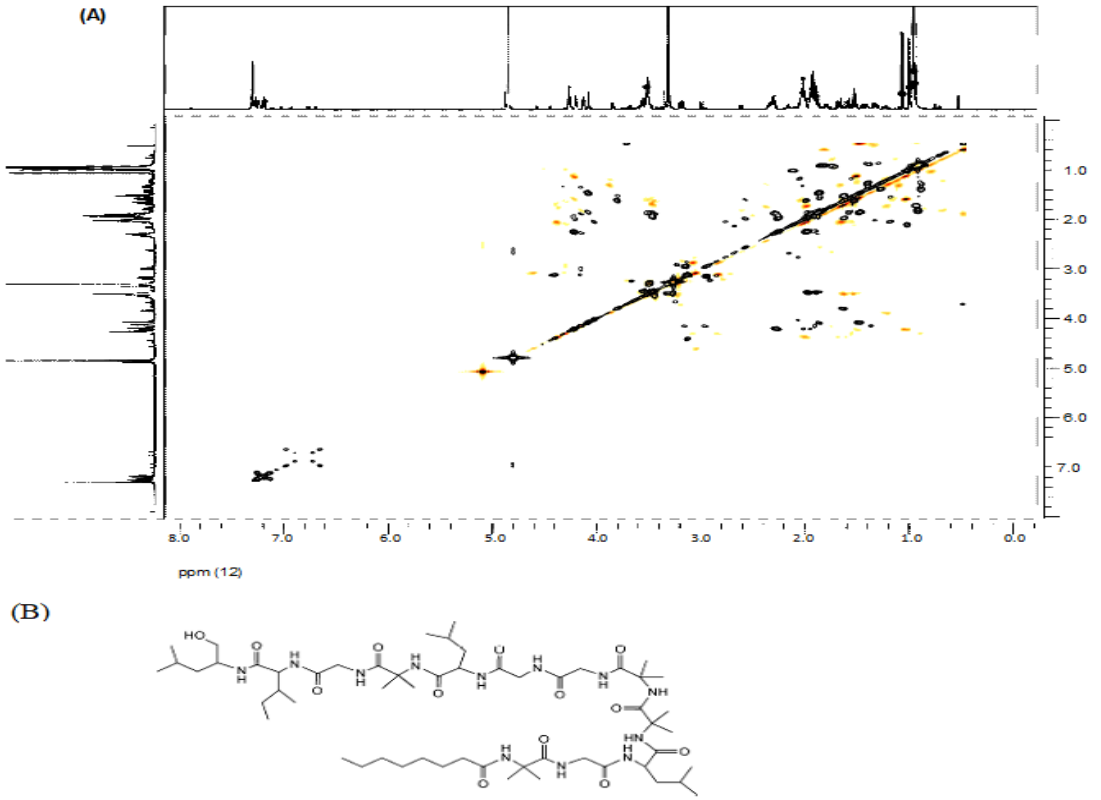

Fig. 3: COSY bidimensional NMR spectrum of fraction n° 4 isolated from Lib1 culture filtrate (A) and structure of Trichogin A IV isolated from *T. longibrachiatum* by Peggion *et al*., 2003(B).

Detoxification abilities of Libyan isolates.

The *Trichoderma* strains were also tested for their ability to grow in contaminated substrates, in order to evaluate their possible biotechnological application as "bioremediating microbes". *In vitro* assays were performed to analyze their growth in liquid medium amended with different concentrations of the toxic pollutant Methyl *tert*-butyl ether (MTBE) ranging from 0.1 to 1.5 % (v/v). Both Lib1 and Lib2 isolates showed good tolerance to the pollutant up to a concentration of 0.4%, compared to the untreated control. Conversely, the biomass of isolate Lib3 was negatively affected by the presence of MTBE even at lower concentrations (Fig. 4).

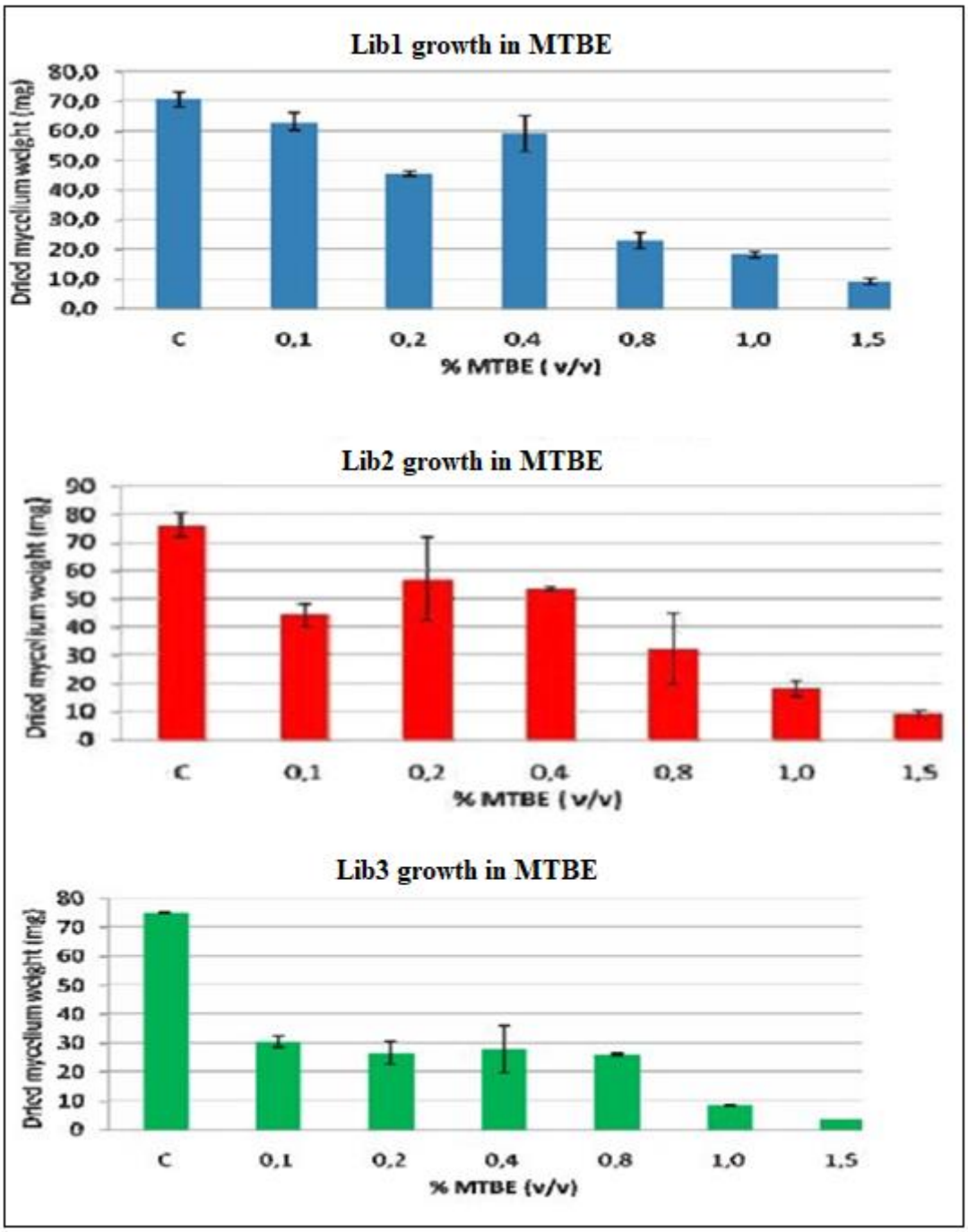

Fig. 4: *In vitro* growth of three Libyan isolates (top Lib1, middle Lib2, bottom Lib3) in the presence of different concentrations of MTBE (0.1 to 1.5 % v/v). C = control without MTBE. Fungal mycelium was harvested by filtration, dried and weighed.

Analysis by gas chromatography of the fungal culture filtrates grown in the presence of MTBE showed a decrease in quantity of MTBE with all three of Trichoderma isolates. Although all isolates demonstrated similar trends in their chromatographic profiles, the Lib2 isolate showed the highest degradation of the contaminant particularly at 4 days after inoculation, as compared to the other two isolates, and only results from this representative are shown (Fig. 5).

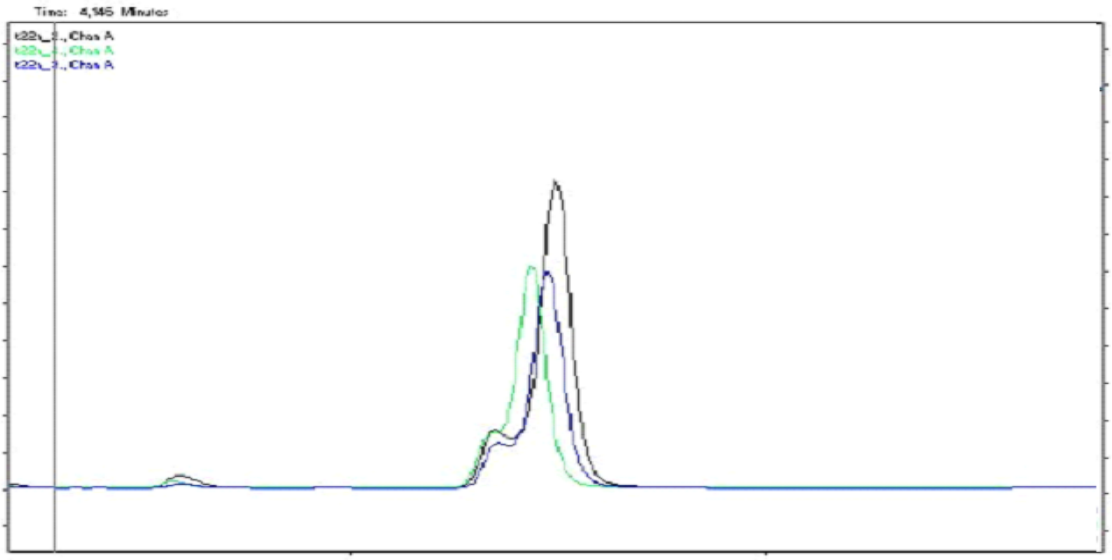

Figure 5. GC-FID analysis of Lib2 isolate culture filtrate grown in presence of 0.2% MTBE after removal of fungal mycelium. Black line: 2d after inoculum; Green Line: 4d after inoculum; Blue line: 6d after inoculum.

Discussion:

The compounds produced by the BCA in the fungal culture filtrates contained various secondary metabolites, like peptaibols, which may also act as elicitors of plant defence mechanisms against pathogens. In fact, the application of peptaibols were found to activate a defence response in tobacco plants (Benítez *et al.*, 2004; Viterbo *et al.*, 2007). Similarly, the peptaibol isolated and identified from the Lib1 culture could represent a molecular factor possibly involved in the induction of defence mechanisms in Trichoderma-treated plants (Abadi *et. al*., 2017). Many secondary metabolites produced by Trichoderma have antibiotic activity and have been demonstrated to play a role in biological control against various phytopathogens, however, their effect on the plant in the BCA-plant interaction are not known. On the other hand, Vinale *et al*. (2008) reported that some Trichoderma compounds, such as 6-pentyl-α-pyrone (6PP) acted as effectors on plant growth, possibly by acting in an auxin-like manner or by stimulating the hormone production in the plant, thus enhancing growth of the root system and plant size. Further, when some fungal BCA secondary metabolites were applied to tomato or

canola plants, they stimulated ISR to subsequent treatments with the foliar pathogens *B. cinerea* or *Leptosphaeria maculans*, respectively, and activated the production of several PR proteins associated with plant defense.

A large portion of the Libyan economy is supported by the petroleum industry. During the refining process many pollutants may be released in the environment, air and groundwater sources. Methyl *tert*-butyl ether (MTBE) is a compound frequently added to gasoline in order to increase octane number. Unfortunately, it frequently contaminates groundwater when gasoline containing MTBE is spilled or leaked in storage and is difficult to clean up due to its high solubility in water. In order to test if the fungal isolates could tolerate toxic compounds, and to determine their ability to degrade and survive such substance, investigations were conducted in presence of MTBE. Contaminated liquid media were inoculated with Trichoderma strains and the toxic content, and fungal growth was monitored. Preliminary results demonstrated that the growth of the local isolates Lib1 and Lib2 didn't differ from controls until a concentration of 0.4% MTBE, however, Lib3 showed a reduced biomass weight also at lower doses. Lib1 grew the best in the presence of this toxic compound.

The ability of Trichoderma isolates to degrade MTBE in liquid culture was also confirmed by GC-FID demonstrating a significant reduction in the level of this pollutant even 4 days after inoculation. In particular, Lib2 performed the best among the Libyan isolates. These results represents potential biotechnological applications for the isolated microbes in decontamination of polluted areas, as used alone or in combination with plants (phytoremediation). Various microorganisms are being studied to evaluate their ability to remediate various chemicals often present at contaminated industrial sites. Also, scientists are currently looking into genetically engineering certain microorganisms to increase their ability to metabolize specific chemicals, such as hydrocarbons, in contaminated sites. More research is required in order to completely understand the complex microbial processes for potential bioremediation, especially the bioremediation of metals. On the other hand selectivity of some microorganisms are better at degrading one kind of chemical than another is required.

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