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ORIGINAL PAPER

CHEMICAL CHANGES IN SPORES OF THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ROBERTSII* AFTER EXPOSURE TO HEAVY METALS, STUDIED THROUGH THE USE OF FTIR SPECTROSCOPY*

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Abstract

Heavy metals, currently present in the environment beyond permissible limits, are extremely hazardous elements which have been recognized as having influence on fungal physiology. Exposure to heavy metals results in changes in physiological processes, but also in the chemical composition and content of macromolecules (nucleic acids, proteins, lipids and polysaccharides, etc.) of fungal cells. Fourier-transform infrared spectroscopy (FTIR) is a powerful technique for the study of those changes because of the simplicity of sample preparation and avoidance of any chemicals (lower cost and lesser environmental impact), reliability and short measurement times, compared to other available methods. The present study was designed to characterize the chemical changes in *Metarhizium robertsii* spores as a result of exposure to cadmium, cobalt, lead and tin. The spores were analysed by the use of FTIR spectroscopy after cultivation of the fungus in presence of metal ions at concentrations: 0.998 mg dm^{-3}, 9.982 mg dm^{-3}, 49.910 mg dm^{-3}, 99.821 mg dm^{-3} (corresponding to 1, 10, 50 and 100 ppm, respectively). The results revealed that heavy metals influenced the chemical composition (proteins, lipids, polysaccharides, nucleic acids) of *M. robertsii* spores, and depending on their concentration in the medium affected all chemical groups present in fungal cells. Hydroxyl, carbonyl, methyl, ester, amino and phosphoryl functionalities played a significant role in the response of the fungus to the presence of metals in the medium. These results indicate that FTIR is a useful technique to determine the chemical changes in fungi exposed to heavy metals.

Key words: entomopathogenic fungi, heavy metals, cadmium, cobalt, lead, tin, FTIR, *Metarhizium*.

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INTRODUCTION

Metals are an integral part of all ecosystems and occur in both elemental and ore forms throughout nature. Life has evolved in an environment rich in various metals and all cells have incorporated certain metal ions into their essential cellular functions (Tripathi, Srivastava 2007). Industrialization and urbanization in this and preceding centuries have generated a tremendous amount of soil, water and air pollution (Tkaczuk 2005, Oves et al. 2013, Alyemeni, Almohisen 2014, Rahman, Singh 2016). Heavy metals, such as Cu, Cr (III), Zn, Mn, Co, and Mo have biological importance at low concentrations, but in high concentrations and with long-term exposure cause detrimental effects on several biomolecules (Alyemeni, Almohisen 2014). On the other hand, Hg, Cd, Cr(VI), As, and Pb are very toxic, even at very low concentrations. Heavy metals are extremely hazardous and currently occur in the environment beyond permissible limits. They accumulate in biological systems and concentrate in the food chain at each trophic level (Rahman, Singh 2016). Consequently, life forms are continuously being exposed to potentially toxic conditions and therefore most organisms have evolved mechanisms of metal homeostasis and metal resistance to cope with varying amounts of these metals in their environments. This requires a cellular ability to recognize the metal species as well as relative concentrations (Tripathi, Srivastava 2007). The introduction of heavy metal compounds into the environment generally induces morphological and physiological changes in microbial communities (Ezzouhri et al. 2009, Wyszkowska et al. 2013). A wide spectrum of potentially toxic interactions between metals and fungi in almost every aspect of their metabolism, growth, germination and differentiation may change, depending on a fungal species, metal concentration and physico-chemical factors (Tkaczuk 2005).

Fungi represent one of the essential components of the biotic systems where they are pivotal players in nutrient turnover. Entomopathogenic fungi are a separate group, and play a key role in the regulation of insect populations, particularly soil-dwelling insect pests (Pečiulytė, Dirginčiutė-Volodkienė 2012). The insect-pathogenic fungus *Metarhizium robertsii* J.F. Bisch., S.A. Rehner&Humber is a common inhibitant of soils worldwide (Bidochka et al. 2001), and has been studied and used as an insect pathogen for biocontrol (Hunter et al. 2001, Lomer et al. 2001) and is also an endophyte that stimulates plant root development (Sasan, Bidochka 2012). The development and infectivity of entomopathogenic fungi may be limited by many abiotic factors, i.e. pesticide use, mineral fertilization and also heavy metal pollution (Tkaczuk 2005). Some heavy metals are essential for fungal metabolism, whereas others have no known biological role. Both essential and nonessential heavy metals are toxic to fungi when present in excess amounts. There is evidence that soil pollution with heavy metals can inhibit or kill these fungi (Pečiulytė, Dirginčiutė-Volodkienė 2012).

Several laboratory experiments have demonstrated that heavy metal ions may prevent the growth or restrict the biomass increment of some entomopathogenic fungi (Jaworska et al. 1996, Tkaczuk et al. 1999, Ropek, Para 2002, Tkaczuk 2003, Popowska-Nowak et al. 2004, Tkaczuk 2005).

Fourier transform infrared (FTIR) spectroscopy has been shown to be a powerful technique in the study of biological macromolecules and of complex biological systems, such as tissues and cells (Orsini et al. 2000, Essendoubi et al. 2005, Nie et al. 2007, Erukhimovitch et al. 2010, Salman et al. 2010, Lecellier et al. 2014). The detection and identification of microorganisms by using FTIR spectroscopic techniques is a promising and invaluable tool due to its sensitivity, speed, low cost and simplicity. FTIR spectroscopy not only provides competitive and rapid identification methods, but also allows the study of microorganisms in their intact state. It also appears to be a very promising tool for the study of microbial metabolism, antibiotic susceptibility and other interactions with drugs (Orsini et al. 2000). There is great potential for FTIR spectroscopy in tandem with appropriate mathematical tools for simple and rapid discrimination and identification of various fungi genera (Salman et al. 2010). Moreover, the chemical composition of the samples can also be visualized simultaneously. Hence, FTIR spectroscopy can aid in the understanding of complex chemical processes during fungal development. FTIR may be a useful tool for the visualization of chemical changes in fungal cells due its contact with heavy metals, as was previously used in the study of marine brown alga (D'Souza et al. 2008), bacteria (Kazy et al. 2006, Oves et al. 2013, Afzal et al. 2017) and fungi (Simonescu, Ferdeş 2012, Damodaran et al. 2013, Kariuki et al. 2017).

The aim of the present study was to evaluate the chemical changes in spores of *M. robertsii* after exposure to heavy metals through the use of FTIR spectroscopy.

MATERIALS AND METHODS

Fungal strain

The *Metarhizium robertsii* WA27856 fungal strain was obtained from the Fungal Collection at the Department of Plant Protection and Breeding, Siedlce University of Natural Sciences and Humanities (Siedlce, Poland), and its taxonomic status had been previously confirmed by means of morphological and molecular studies as well (Różalska et al. 2013). The strain was isolated near Siedlce (Masovian district, Poland) from the soil under an arable field, by means of a *Galleria* bait method (Zimmerman 1986). Before the experiment, an isolate of *M. robertsii* had been grown on a Sabouraud medium and kept at 4°C.

Media preparations

The influence of a metal on fungal spores was tested on solid PDA media (Merck, Germany) supplemented with metal salts. $CdSO_4$; $CoCl_2$; $Pb(NO_3)_2$ and $SnCl_2 \times 2H_2O$ (Sigma Aldrich) were added separately to a PDA medium after autoclaving to reach concentrations: 0.998 mg dm^{-3}, 9.982 mg dm^{-3}, 49.910 mg dm^{-3}, 99.821 mg dm^{-3} (corresponding to 1, 10, 50 and 100 ppm, respectively) and then placed on a magnetic stirrer. When cooled, it was poured into 90 mm Petri dishes. The PDA medium, which was devoid of any metal salt addition, served to maintain non-exposed *M. robertsii* colonies. Inocula (0.5 cm^2 plugs) were prepared from 10-day old colonies grown on a pure PDA medium. A single plug was transferred to the centre of the test plates and these were incubated at 25°C in darkness for 14 days.

Spores collection and preparation

The spores were collected from mature (14 days) *M. robertsii* colonies by the use of sterile physiological saline solution. The spores were washed down from the surface of the colonies into sterile tubes, centrifuged (1000 rpm, 5 min), and repeatedly washed by sterile physiological saline and then centrifuged three times. Subsequently, the saline was removed and the spores were dried at room temperature for 24 h.

FTIR measurements

Fourier transform infrared spectra of the *M. robertsii* spore samples were measured using a FTIR spectroscopy (Perkin Elmer Spectrophotometer, Spectrum 100, Waltham, MA, USA), operated at a resolution of 4 cm^{-1} over 64 scans in order to minimize noise and improve band separation. The samples were then spread on crystal as a thin layer. The spectra were recorded at a wavelength of 650-4000 cm^{-1}. All spectra were baseline corrected and vector normalized using SPECTRUM software.

RESULTS AND DISCUSSION

FTIR spectroscopy is based on the vibrational excitation of molecular bonds through the absorption of infrared light energy. The sum vibrational spectra for a cell's macromolecules content (nucleic acids, proteins, lipids and polysaccharides, etc.) can be thought of as a spectral "fingerprint" for a particular organism (Subburaj et al. 2013). The simplicity of the sample preparation and avoidance of any chemicals (lower cost and lesser environmental impact), reliability and short measurement times (approximately 1 min), compared to other available methods means that the FTIR technique is suitable for the large scale screening of fungal samples and routine analysis (Salman et al. 2010). Combining the advantages of FTIR spectroscopy

with other routinely used techniques, it offers an opportunity to improve the efficiency of fungal classification and identification. In this study, the FTIR technique was used to determine the chemical compositional changes of the fungal spores as a result of exposure of *M. robertsii* to various concentrations of heavy metals.

The FTIR spectra of *M. robertsii* spores exposed to different concentrations of cadmium are shown in Figure 1. At 3279 cm^{-1}, the absorbance values decreased in all concentrations of Cd, being the lowest at the 99.821 mg dm^{-3} concentration. Only the 99.821 mg dm^{-3} concentration caused noticeable growth of absorbance at bands 2923.97 cm^{-1} and 2854.22 cm^{-1}, respectively, while the concentrations 0.998 mg dm^{-3}, 9.982 mg dm^{-3} and 49.910 mg dm^{-3} resulted in a small growth at 1744.01 cm^{-1}, which were attributed to lipids, while this significantly increased at 99.821 mg dm^{-3}. At 1634.84 cm^{-1} (amide I), there was remote decreasing of absorbance when the concentration of Cd was 0.998 mg dm^{-3} and it was higher at 99.821 mg dm^{-3}, whereas the 49.910 mg dm^{-3} concentration showed an increase in absorbance, and the 9.982 mg dm^{-3} concentration matched the control sample. In contrast, all concentrations of Cd caused a decrease in absorbance in the amide II region. At 1454.77 cm^{-1} and 1380.43 cm^{-1}, these bands were attributed to the asymmetric bending of methyl groups in proteins and stretching C-N vibrations of cytosine-guanine, respectively, but there was a general decline when the concentration of Cd was increased. At 1233.13 cm^{-1} imputed to PO^2,

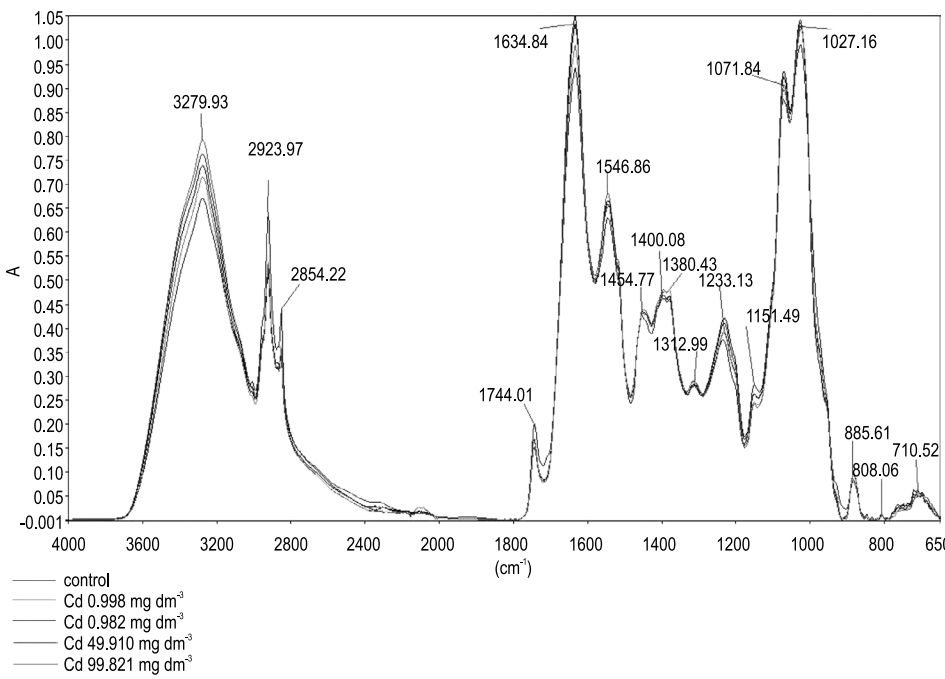

Fig. 1. The FTIR spectra of *M. robertsii* spores after exposure to various cadmium concentrations

there was a decrease in absorbance at 0.998 mg dm^{-3} and 99.821 mg dm^{-3}, while 9.982 mg dm^{-3} and 49.910 mg dm^{-3} induced a growth. In bands at 1151.49 cm^{-1} and 1027.16 cm^{-1} derived from glycogen and chitin C-O-C and C-C bonds, there was increasing absorbance up to concentration 49.910 mg dm^{-3}, whereas 99.821 mg dm^{-3} caused a growth at 1151.49 cm^{-1} and, in contrast, a decline at 1027.16 cm^{-1}. At 1071.84 cm^{-1}, which was attributed to skeletal DNA vibrations, a decline in absorbance at 0.998 mg dm^{-3} and 99.821 mg dm^{-3} was noted, whereas at 9.982 mg dm^{-3} and 49.910 mg dm^{-3} the absorbance value increased. Cadmium is known as one of the most toxic trace elements in soil and water, even in low concentrations. Cadmium is released into the ecosystem by mine tailing, effluents from the textile, leather, tannery, electroplating and galvanizing industries, as well as cadmium batteries (Wyszkowska et al. 2013). It is non-biodegradable and tends to accumulate in living organisms, causing significant threats to both the environment and public health. Compared to other heavy metals, Cd has a relatively higher mobility in soil and therefore is quickly absorbed (An et al. 2015). It has to be noted that cadmium exerts its toxic effects directly on the plasma membrane, where it interferes with solute transport and other membrane phenomena. Howlett and Avery (1997) observed that cadmium may cause *Saccharomyces cerevisiae* membrane permeabilization (associated with K efflux), as well as changes in the membrane's composition. These data are in accordance with results of this study, where changes in lipids and PO^{2-} (which is part of phospholipids in the cell membrane) peak intensity were observed. Cadmium may bind to aromatic amino acid residues in enzyme molecules, and can also cause oxidative damage to proteins by the induction of oxidative stress associated with the production of reactive oxygen species, such as hydroxyl or superoxide radicals (Baldrian 2003).

The FTIR spectra of *M. robertsii* spores exposed to different concentrations of cobalt are shown in Figure 2. At 3279.11 cm^{-1}, there was decrease in absorbance values at the concentrations 9.982 mg dm^{-3} and 49.910 mg dm^{-3} of Co, while at 99.821 mg dm^{-3} there was an increase. At band 2924.86 cm^{-1}, all concentrations of Co caused a decrease in absorbance values, and similarly at 2854.69 cm^{-1}, but at 49.910 mg dm^{-3} an increase was noted. At 1744.70 cm^{-1}, attributed to lipids, the concentrations 0.998 mg dm^{-3}; 9.982 mg dm^{-3} and 49.910 mg dm^{-3} resulted in a slight decrease in absorbance and at 99.821 mg dm^{-3} this change was explicit. At 1634.84 cm^{-1} (amide I) and at 1546.97 cm^{-1} (amide II), there was a remote increase in absorbance when the concentration of Co was 0.998 mg dm^{-3} and 9.982 mg dm^{-3}, and it was higher when it was 49.910 mg dm^{-3} and 99.821 mg dm^{-3}. At 1450.90 cm^{-1}, this was attributed to asymmetric bending of the methyl groups in protein samples from 9.982 mg dm^{-3} and 99.821 mg dm^{-3}, and this was the same as the control sample, whereas the concentrations of 0.998 mg dm^{-3} and 49.910 mg dm^{-3} caused an increase in absorbance values. At 1393.46 cm^{-1}, there was an increasing tendency when the concentration of Co increased, attributed to stretching C-N vibrations of cyto-

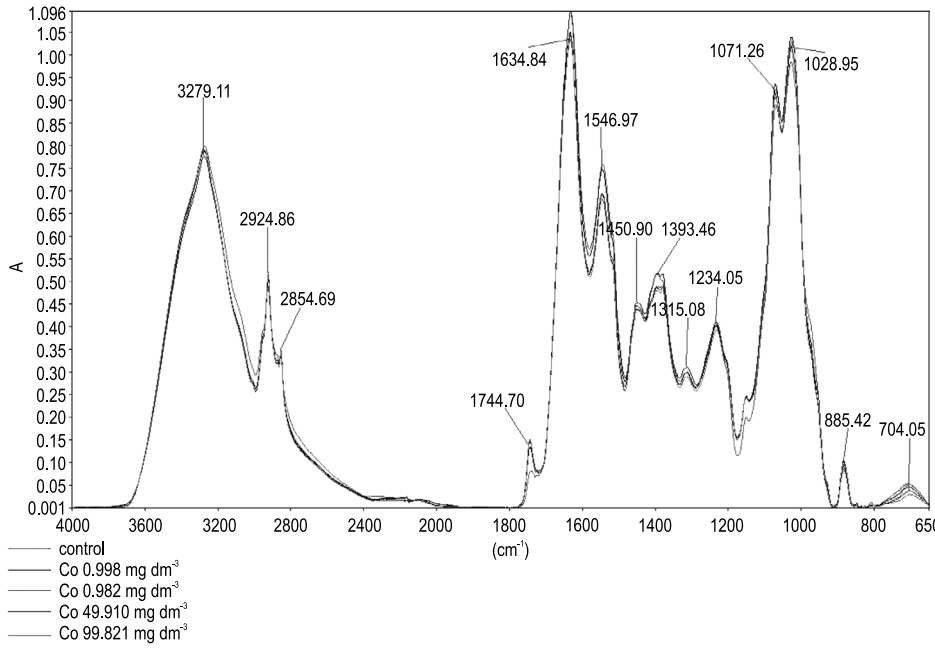

Fig. 2. The FTIR spectra of *M. robertsii* spores after exposure to various cobalt concentrations

sine-guanine pairs. At 1234.05 cm^{-1}, all concentrations of Co caused a slight decrease in absorbance values. At 1071.84 cm^{-1}, a decline of absorbance at 99.821 mg dm^{-3} was noted, whereas at the other concentrations absorbance values increased. At band 1151.49 cm^{-1} Co, the concentrations 0.998 mg dm^{-3}, 9.982 mg dm^{-3} and 49.910 mg dm^{-3} caused a slight increase in absorbance, while 99.821 mg dm^{-3} caused a clear decline. At band 1027.16 cm^{-1}, a noticeable decrease was observed in samples exposed to 0.998 mg dm^{-3}; 49.910 mg dm^{-3} and 99.821 mg dm^{-3}, while 9.982 mg dm^{-3} caused a slight increase in absorbance. Co is used extensively for industrial purposes, such as steel production, electroplating, and for pigment production. Just as other heavy metals, Co is an essential metal required in trace amounts by organisms for a range of metabolic activities and structural organization. It is an essential component of vitamin B_{12} and an integral requirement for enzymes, such as transcarboxylase and amidino aspartase. However, at higher concentrations, Co is toxic to cells, as it inhibits cellular respiration and citric acid cycle enzymes (Tripathi, Srivastava 2007). Our results are supported by the results of Afzal et al. (2017), who observed changes in peak in the 3500-3200 cm^{-1} region spectrum of *Klebsiella variicola* exposed to Co, considered the binding of Co with amino and a hydroxyl group. Similarly, a change in peak in the 1500-1750 cm^{-1} region of the spectrum of Co was observed, which indicated the binding of Co with a carboxyl group (Afzal et al. 2017).

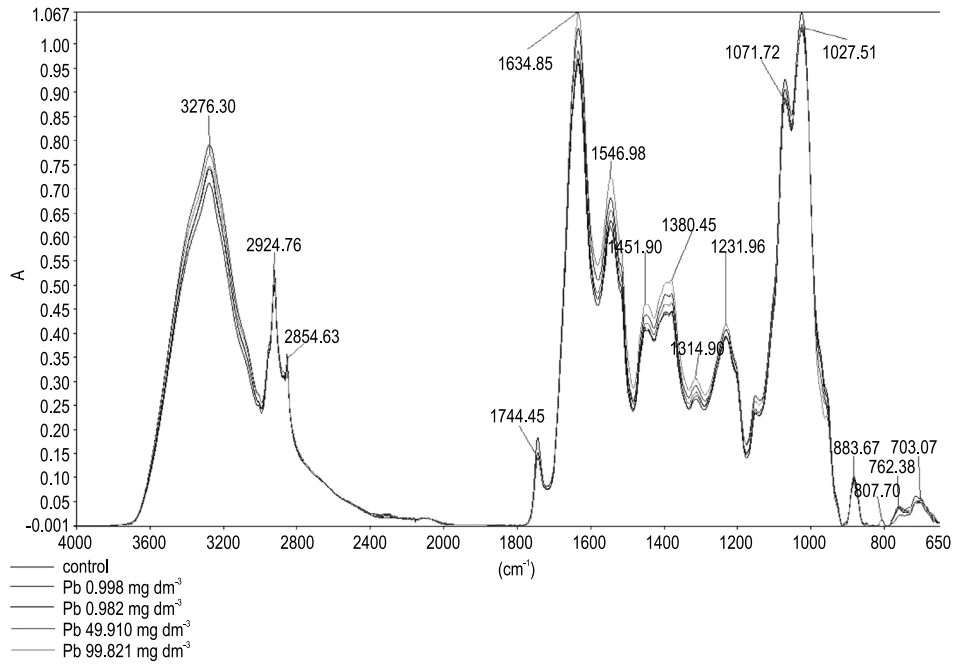

Fig. 3. The FTIR spectra of *M. robertsii* spores after exposure to various lead concentrations

The FTIR spectra of *M. robertsii* spores exposed to different concentrations of lead are shown in Figure 3. At 3276.30 cm^{-1}, at all concentrations of Pb, there was decrease in absorbance values, which were the highest at the 99.821 mg dm^{-3} concentration. At band 2924.76 cm^{-1}, the control sample and 99.821 mg dm^{-3} sample were similar, but 9.982 mg dm^{-3} caused a slight decrease in absorbance, whereas 0.998 mg dm^{-3} and 49.910 mg dm^{-3} resulted in a decline. At the 2854.63 cm^{-1} band, only 9.982 mg dm^{-3} caused the peak intensity growth, while all other concentrations resulted in a decrease. At 1744.70 cm^{-1}, attributed to lipids, the concentrations 0.998 mg dm^{-3}; 49.910 mg dm^{-3} and 99.821 mg dm^{-3} resulted in slight decreases in absorbance, while 9.982 mg dm^{-3} caused an increase. At 1634.8 cm^{-1} (amide I), at 1546.98 cm^{-1} (amide II) and at 1451.90 cm^{-1} (the asymmetric bending of methyl groups from proteins), there was a tendency for lower absorbance at the concentrations 0.998 mg dm^{-3}; 9.982 mg dm^{-3} and 49.910 mg dm^{-3}, but within the aforementioned wavelengths, the highest Pb concentration caused an explicit growth of absorbance, suggesting that high concentrations of Pb stimulate high protein production. At 1231.96 cm^{-1} (PO^{2-}), there was also a noticeable growth of peak intensity caused by 9.982 mg dm^{-3} and 99.821 mg dm^{-3}. At 1071.84 cm^{-1}, the decline in absorbance at 49.910 mg dm^{-3} and 99.821 mg dm^{-3} was noted and it was attributed to skeletal DNA vibrations, whereas at 0.998 mg dm^{-3} and 9.982 mg dm^{-3} the absorbance value increased. Exposure to heavy metals induces the production of various meta-

bolites, such as citric acid, homogeneous proteins, heterogeneous proteins and peroxidases, by fungi. SARATHCHANDRAN et al. (2014) observed high synthesis of proline in the presence of Pb^{2+} in *Aspergillus niger*, which indicates that Pb^{2+} acts as a chief inducer for proline synthesis, a protective mechanism of fungus to detoxify heavy metals by acting as a scavenger of hydroxyl radicals formed due to metal toxicity inside the cell. Likewise, as noted in our results, changes in amide I and amide II peaks were observed, suggesting that exposure to lead could influence the amino acid composition of proteins. GOLA et al. (2017) observed a shifting and masking of the amines, alkanes, aliphatic amines, aromatic organic and alkyl halides groups in the FTIR spectrum of *Beauveria bassiana* exposed to Pb. Shifts in the spectra of FTIR in the presence of Pb^{2+} showed the involvement of functional groups, representing protein (N-H) stretching, lipid-protein stretching, carbohydrate stretching, ester fatty acids stretching, protein amide (C=O) stretching, protein-lipid bending of methyl and carbohydrate of polysaccharides (GOLA et al. 2017).

The FTIR spectra of *M. robertsii* spores exposed to different concentrations of tin are shown in Figure 4. At 3279.93 cm^{-1}, at all the concentrations of Sn, there was a decrease in absorbance values, which fell the lowest at the 99.821 mg dm^{-3} concentration. At 2924.85 cm^{-1} and 2854.57 cm^{-1} bands, only the concentration of 0.998 mg dm^{-3} caused a noticeable growth of absorbance, while at 1744.58 cm^{-1}, attributed to lipids, the concentrations of 0.998 mg dm^{-3} and 9.982 mg dm^{-3} resulted in a growth and 49.910 mg dm^{-3}

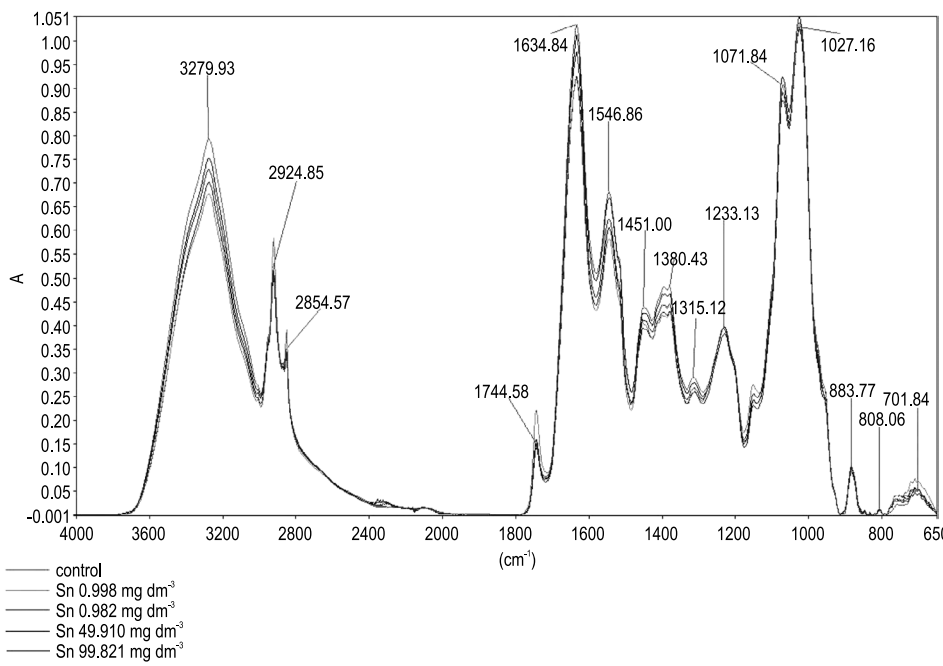

Fig. 4. The FTIR spectra of *M. robertsii* spores after exposure to various tin concentrations

caused an increase. At 1634.84 cm^{-1} (amide I), there was a remote decrease in absorbance when the concentrations of Sn were 49.910 mg dm^{-3} and 9.982 mg dm^{-3}, but absorbances were higher at 99.821 mg dm^{-3}. In contrast, all concentrations of Sn caused a decrease in absorbance in the amide II region. At 1451 cm^{-1} and 1380.43 cm^{-1}, which were attributed to the asymmetric bending of methyl groups in proteins and stretching C-N vibrations of cytosine-guanine, respectively, there was a tendency of a decline when the concentration of Sn was higher. At 1233.13 cm^{-1} imputed to PO^{2-}, there was only a decrease in absorbance at 99.821 mg dm^{-3}. In the band at 1151.49 cm^{-1} derived from glycogen and chitin C-O-C and C-C bonds, the concentration 49.910 mg dm^{-3} was similar to the control sample, there was an increase in absorbance at 0.998 mg dm^{-3} and 9.982 mg dm^{-3}, whereas 99.821 mg dm^{-3} caused a noticeable decline, though at 1027.16 cm^{-1} there was a slight decline at 49.910 mg dm^{-3}, but the other concentrations caused growth. At 1071.84 cm^{-1}, attributed to skeletal DNA vibrations, the decline in absorbance at 0.998 mg dm^{-3}, 49.910 mg dm^{-3} and 99.821 mg dm^{-3} was noted, whereas at 9.982 mg dm^{-3} the absorbance value increased. Most studies on the toxic effects of tin have concentrated on organotin compounds. Toxicity studies of inorganic tin compounds are rather limited, presumably due to the widespread belief that inorganic tin species would hydrolyze to form insoluble, nontoxic SnO_2 or hydroxides. However, Hallas et al. (1982) have shown that a small amount of tin (as $SnCl_4 \cdot 5H_2O$) may reduce microbial populations. Also, observations by Łopusiewicz et al. (2019) proved the influence of Sn^{2+} ions on the growth and enzymatic activity of entomopathogenic fungi.

Our comparison of FTIR data of both control and those *M. robertsii* spore samples that had been exposed to heavy metals provided direct evidence to show that hydroxyl, carbonyl, amino and phosphoryl functionalities played a significant role in the fungal response to metals. Our results are supported by Rahman et al. (2015), who observed changes in the spectra of *Trichoderma* exposed to heavy metals (including cadmium). Kariuki et al. (2017) observed that hydroxyl, carbonyl and carboxyl functional groups were responsible for binding copper and lead ions. According to Simonescu and Ferdeș (2012), interactions between heavy metal ions and fungal biomass involve hydroxyl, amino, and carbonyl groups as shifting peaks characteristic of these groups are registered in the FTIR spectra. In our study, the most remarkable difference was at 3000-3800 cm^{-1} representing hydroxyl (-OH) stretching, as well as the bands representing carbonyl (C=O) stretching (derived mainly from proteins). Noticeable differences have been also observed in bands attributed to lipids, nucleic acids and polysaccharides. Due to the complexity of the absorption of various cellular polysaccharides, specific determinations are rather difficult. The cell surface of microorganisms is negatively charged owing to the presence of various anionic structures, such as glucan and chitin. This gives microorganisms the ability to bind metal ions. Within the intracellular mechanism, metal transport proteins may be involved in metal tole-

rance, either by extruding toxic metal ions from the cytosol of a cell or by allowing metal sequestration into the vacuolar compartment (Anahid et al. 2011). The results of the present study are in line with the results of Park et al. (2005), who performed a chromium biosorbtion study on marine algae and noted that a peak in the 3500-3200 cm^{-1} region was due to the stretching of the N–H bond of amino groups indicating a bound hydroxyl group. Kazy et al. (2006), in a study on lanthanum biosorbtion, noted that absorption peaks at 2900-3000 cm^{-1} were attributed to the asymmetric stretching of C–H bond of the $–CH_2$ groups combined with that of $–CH_3$ groups. These authors maintained that the main functional groups that were responsible for bioadsorption process were the hydroxyl, amino, carbonyl, carboxyl, sulfonate, amide, imidazole, phosphonate and phosphodiester groups.

CONCLUSIONS

1. The results of the FTIR spectroscopy provide good methodology and offer insight into the determination of chemical changes in fungal spores as a result of exposure to heavy metals.

2. It is possible to identify the broad chemical composition of biological samples using FTIR, which offers a non-invasive extraction-less method, and may help in understanding the complex chemical processes during their growth in the presence of heavy metals.

3. Cadmium, cobalt, lead and tin caused different chemical changes in *M. robertsii* spores depending on their concentration in a medium, and influenced all of the chemical groups present in fungal cells.

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