Journal of Plant Protection Research ISSN 1427-4345

ORIGINAL ARTICLE

The influence of cobalt ions on growth and enzymatic activity of entomopathogenic fungi used in biological plant protection

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Vol. 60, No. 1: 58–67, 2020

DOI: 10.24425/jppr.2020.132207

Received: May 16, 2019
Accepted: September 27, 2019

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Abstract

This study focused on the effect of heavy metal cobalt ions (at concentrations of 1–1000 ppm) on the development and enzymatic activity of four entomopathogenic fungi: *Beauveria bassiana*, *Beauveria brongniartii*, *Isaria fumosorosea* and *Metarhizium robertsii*, commonly used in biological plant protection. It was found that each of the tested species of fungi reacted individually to contact with the Co^{2+} ions at their various concentrations. Depending on the variants of the experiment carried out, there were changes in the development of the mycelia (mainly growth inhibition) and their morphological features (color and structure) in comparison to the control samples. Co^{2+} ions had a fungistatic effect on all fungal strains, whereas a fungicidal effect was noted at concentrations of 750 ppm and 1000 ppm against *M. robertsii* and *I. fumosorosea*, respectively. In addition, there was a discrepancy in enzymatic activity between the tested fungal species developing in the medium with varying concentrations of metal salt. The inhibitory effect of Co^{2+} ions on lipase production was observed in *I. fumosorosea*. Protease production was stimulated in *B. bassiana* at all Co^{2+} concentrations, whereas in *M. robertsii* this effect was noted at 1 ppm. The changing dynamics of extracellular fungal hydrolases, due to the action of Co^{2+} ions, may translate into the role of these microorganisms in the processes of insect pathogenesis. This work suggests that severe pollution of the environment by cobalt could be a restrictive factor for the development and pathogenicity of entomopathogenic fungi and must be taken into account for their successful application in biological plant protection.

Keywords: cobalt, entomopathogenic fungi, enzymatic activity, heavy metals

Introduction

Entomopathogenic fungi (EPF) are one of several highly specialized groups of microorganisms developing on arthropods, including insects that are pests of crop plants (Fiedler and Sosnowska 2017; Singh *et al.* 2017). Their ability to infect pests makes this group of microorganisms particularly important in biological plant protection (Pečiulytė and Dirgінčiutė-Volodkienė 2012). The biochemical activity of entomopathogens, determining their effectiveness in biological pest control, depends on the development of conditions for these fungi in the environment. Natural ecosystems and agroecosystems are subject to constant changes resulting from human activity, which often lead to the pollution of the environment due to growing industrial waste production (Baldrian 2003; Tkaczuk 2005; Baghban *et al.* 2014). Numerous anthropogenic products and materials including steel, batteries, glass, pigments and polyesters (produced with the use of Co) may be some of the factors leading to environmental pollution, especially in industrial areas (Tripathi and

Srivastava 2007; Hartikainen *et al.* 2013). Man's contribution to cobalt (Co) concentrations in the terrestrial environment has risen mainly from mining, smelting and industrial activities (Collins and Kinsela 2010). Man also adds cobalt to the soil, primarily through three mechanisms. The major mechanism is the use of cobalt salts, e.g. cobalt sulphate, as a feed additive to keep cattle and crops healthy in areas where there is insufficient natural bioavailable cobalt. Smaller amounts of cobalt also enter the soil from the airborne transport of particulate emissions and application of sewage sludge onto fields. Especially in agriculture, the utilization of pesticides, chemical fertilizers and preservatives contributes not only to fluctuations in the chemical composition of the ecosphere, but also to the disturbance of interactions between soil organisms. Consequently, life forms which are continuously exposed to potentially toxic factors (including heavy metal ions) have evolved mechanisms of metal homeostasis and metal resistance to cope with varying amounts of these elements in their environments. This requires a cellular ability to recognize the metal species, as well as relative concentrations (Tripathi and Srivastava 2007). The introduction of heavy metal compounds into the environment generally induces morphological and physiological changes in microbial communities (Ezzouhri *et al.* 2009; Hasanzadeh *et al.* 2012; Tkaczuk *et al.* 2019). It is clear, that the interference of heavy metals with physiological, enzymatic and reproductive processes of organisms has ecological consequences. Limitations in growth or reproduction in the presence of metals lead to changes in population structures. The effects of heavy metals on enzymatic activity influences energy flux around the ecosystem (Baldrian 2003). 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 the fungal species, metal concentration and physicochemical factors (Tkaczuk 2005; Tkaczuk *et al.* 2019).

Cobalt is an essential metal for microorganisms, required as a trace element at nanomolar concentrations for a range of metabolic activities and structural organization, being an essential component of vitamin B_{12} and an integral requirement for enzymes like transcarboxylase and amidino aspartase (Tripathi and Srivastava 2007; Rasha 2017). However, at micro- and millimolar concentrations, Co^{2+} ions may be toxic to cells, inhibiting cellular respiration and the citric acid cycle (Nies 1992; Tripathi and Srivastava 2007; Rasha 2017). Toxicity of cobalt has been reported in plants (Palit *et al.* 1994), bacteria (Ranquet *et al.* 2007) as well as fungi (Hartikainen *et al.* 2013) but the exact mechanism of this toxicity is still poorly understood (Rasha 2017). Cobalt in soils throughout the world results from a combination of natural and human activities. Cobalt soil concentrations depend on a number of factors including local geology, atmospheric deposition of cobalt-containing dust, land use and associated amendments, mineral particle distribution, soil age, and climactic and transport factors affecting localized concentrations. The average cobalt concentration in European soils is between 1–20 mg · kg^{-1} dry weight, although this can become much higher in areas which are geologically rich in cobalt (Wendling *et al.* 2009). Paveley (1988) found natural levels of cobalt at over 2,500 mg · kg^{-1} dry weight in soil. His study noted that the area had a healthy ecosystem that had adapted to these naturally high cobalt concentrations. Most of the cobalt in the soil is not biologically available, i.e., cobalt forms stable carbonate and hydroxide minerals that cannot be absorbed by animal or plant life (Perez-Espinosa *et al.* 2004). Consequently, a very large amount of cobalt would have to be introduced into a volume of soil before local wildlife would be adversely affected. However, free cobalt ions present in the environment may influence the physiology of microorganisms. There is a lack of knowledge about the impact of cobalt on the growth and biochemical activity of EPF. Thus, the aim of this study was to assess the impact of Co on the growth and enzymatic activity of four EPF: *Beauveria bassiana*, *Beauveria brongniartii*, *Isaria fumosorosea* and *Metarhizium robertsii*.

Materials and Methods

Fungal strains

Beauveria bassiana (Bals.-Criv.) Vuill. (UPH34), *Beauveria brongniartii* (Sacc.) Petch (UPH42), *Isaria fumosorosea* (Wize) (UPH62) and *Metarhizium robertsii* J.F. Bisch., S.A. Rehner & Humber (WA27856) fungal strains were obtained from the Fungal Collection at the Department of Plant Protection and Breeding, Siedlce University of Natural Sciences and Humanities (Siedlce, Poland). The strains were isolated near Siedlce (Masovian district, Poland) from the soil of arable fields by means of a *Galleria* bait method (Zimmermann 1986). Before the experiment, all isolates were grown on a Sabouraud medium (Biocorp, Poland) and kept at 4°C.

Media and inocula preparations

The influence of metal on fungi was tested on solid potato dextrose agar (PDA) medium (Biocorp, Poland) supplemented with cobalt (II) chloride (anhydrous, Sigma Aldrich). $CoCl_2$ salt was added separately to

a PDA medium after autoclaving (at approx. 50°C) to reach concentrations of: 1; 10; 50; 100; 250; 500; 750; 1000 ppm of Co^{2+} ions and then placed on a magnetic stirrer. When cooled, it was poured into 90 mm Petri dishes. PDA medium, which was devoid of metal salt, served as a control medium. Inocula were prepared from 10-day old fungal colonies grown on pure PDA medium by collecting aerial hyphae of particular fungal strains and preparing a suspension of fungal spores in sterile physiological saline (concentration of fungal spores approximately 1.0×10^9/ml determined by the use of a Thoma counting chamber). Twenty microliters drops of each fungal suspension were transferred by an automatic pipette to the center of the test plates (five repetitions for each Co^{2+} concentration and control) and incubated at 25°C for 18 days.

The growth response study and determination of the minimum inhibitory concentration

The development of fungi was expressed by the use of tolerance index (TI) according to Fazli *et al.* (2015). To compare the tolerance index of the fungal strains, the radius of colony extension on PDA medium supplemented with $CoCl_2$ at different concentrations was measured against the control medium (PDA devoid of cobalt). The radial growth was evaluated from four measurements (in millimeters) that passed through the center of an inoculated portion. When TI values are lower than 1 there is growth inhibition, 1 means that there is no influence, whereas values higher than 1 indicate that there is growth stimulation. Minimum inhibitory concentration (MIC) was defined as the minimum inhibitory concentration of heavy metal in medium that inhibited the visible growth of tested fungal strains. If no apparent growth of fungi was observed after the incubation period, the particular metal concentration was considered the highest metal concentration tolerated by the tested EPF.

The determination of fungi enzymatic activity

The API-ZYM test (bioMérieux, Lyon, France) was used to semi-quantitatively determine the activity of 19 hydrolytic enzymes of the fungi: alkaline phosphatase (2), esterase (C4) (3), esterase lipase (C8) (4), lipase (C14) (5), leucine arylamidase (6), valine arylamidase (7), cystine arylamidase (8), trypsin (9), chymotrypsin (10), acid phosphatase (11), naphthol-AS-BI-phospohydrolase (12), α-galactosidse (13), β-galactosidase (14), β-glucuronidase (15), α-glucosidase (16), β-glucosidase (17), N-acetyl-β-glucosoaminidase (18), α-mannosidase (19) and α-fucosidase (20), following the manufacturer's instructions. The API-ZYM strips were inoculated in two repetitions with matured (14 day) fungal cultures grown on PDA without and with cobalt at concentrations of 1, 100 and 500 ppm (transferred into sterile physiological saline solution), and then incubated at 30°C for 4 h. Hydrolytic activity was determined in nanomoles of hydrolyzed substrate, in a 5-grade color scale from 0 to 5 provided by the manufacturer indicating the reactions: 0 – negative reaction (no enzyme production), 1 – 5 nM, 2 – 10 nM, 3 – 20 nM, 4 – 30 nM and 5 – 40 nM and more.

Results

The influence of cobalt on growth of entomopathogenic fungi (EPF)

The results of the present study demonstrated that different species of EPF showed various levels of tolerance to cobalt (manifested in varied TI and MIC values) depending on its concentrations. All fungal strains exhibited growth at lower concentrations of metal (up to 50 ppm) but it became reduced in the presence of higher concentrations due to the increase in length of the lag phase as compared to the control sample (Figs. 1–4). This is evidence of fungistatic activity of Co^{2+} ions on entomopathogenic fungi. Fungicidal activity of Co^{2+} ions was observed at concentrations of 750 ppm and 1000 ppm against *M. robertsii* and *I. fumosorosea*, respectively. While all concentrations had an inhibiting influence on *I. fumosorosea* and *M. robertsii* growth (TI values lower than 1), it is worth noting that growth of *B. bassiana* and *B. bongniartii* was even stimulated by low (1 and 10 ppm) concentrations of Co^{2+}. The MIC values were different for all analyzed strains. *Beauveria bassiana* and *B. brongniartii* were able to grow in all Co concentrations, no *I. fumosorosea* growth was observed in a concentration of 750 ppm, while the MIC value for *M. robertsii* was 500 ppm. On exposure to cobalt, morphological changes were observed in all fungal strains which may indirectly indicate a different degree of tolerance. It was noted that at concentrations of 50 and 100 ppm there was no formation of aerial hyphae of *B. bassiana*. Similarly, in 250 ppm and in higher concentrations, aerial hyphae vanished in *B. brongniartii* and *M. robertsii*.

The influence of cobalt on the enzymatic activity of entomopathogenic fungi (EPF)

The results of determining the enzymatic activity of EPF by the use of the API-ZYM test are presented in Table 1. The *B. bassiana* growth in the presence of Co^{2+} at a concentration of 1 ppm resulted in increased

production (compared to the control) of the following enzymes: leucine arylamidase (6), naphtol-AS-BI--phosphohydrolase (12), β-glucosidase (17), N-acetyl--β-glucosaminidase (18) and α-mannosidase (19). The higher concentration of Co^{2+} ions (100 ppm) was responsible for the reduction of esterase (C4) (3), lipase (C14) (5), naphtol-AS-BI-phosphohydrolase (12), β-galactosidase (14) activities and a simultaneous increase in lecucine arylamidase (6), acid phosphatase (11), β-glucosidase (17) and α-mannosidase (19) activity. No esterase (C4) (3) activity was detected at a concentration of 100 ppm. The highest Co^{2+} concentration (500 ppm) increased the activity of esterase (C4) (3), lipase (C14) (5) and N-acetyl-β-glucosaminidase (18),

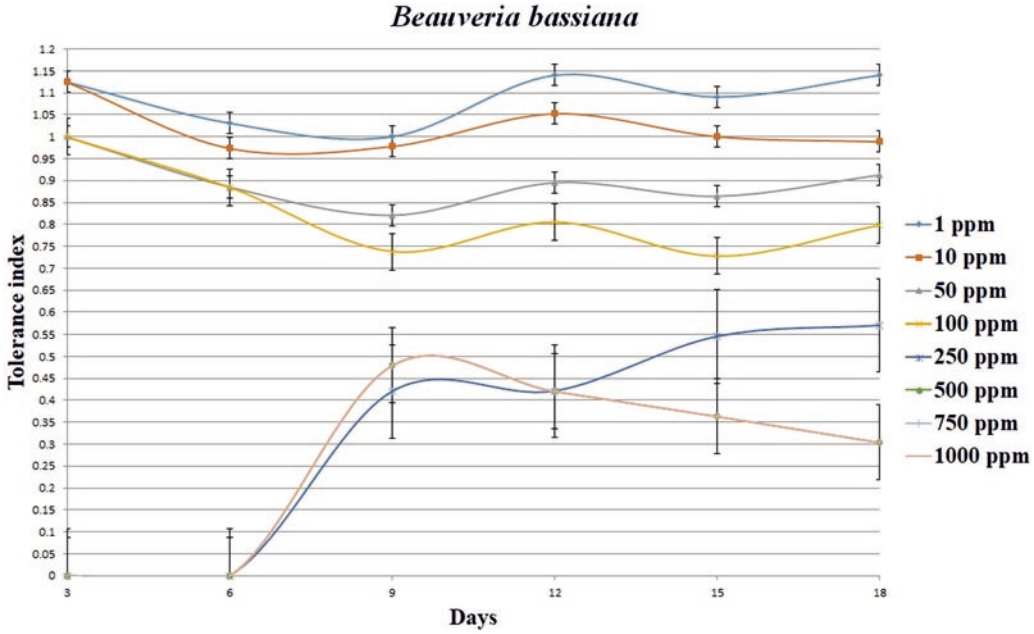

Fig. 1. Effect of various Co^{2+} concentrations on the tolerance index of *Beauveria bassiana* during an 18-day incubation period, bars present means and whiskers SD (mean ± SD)

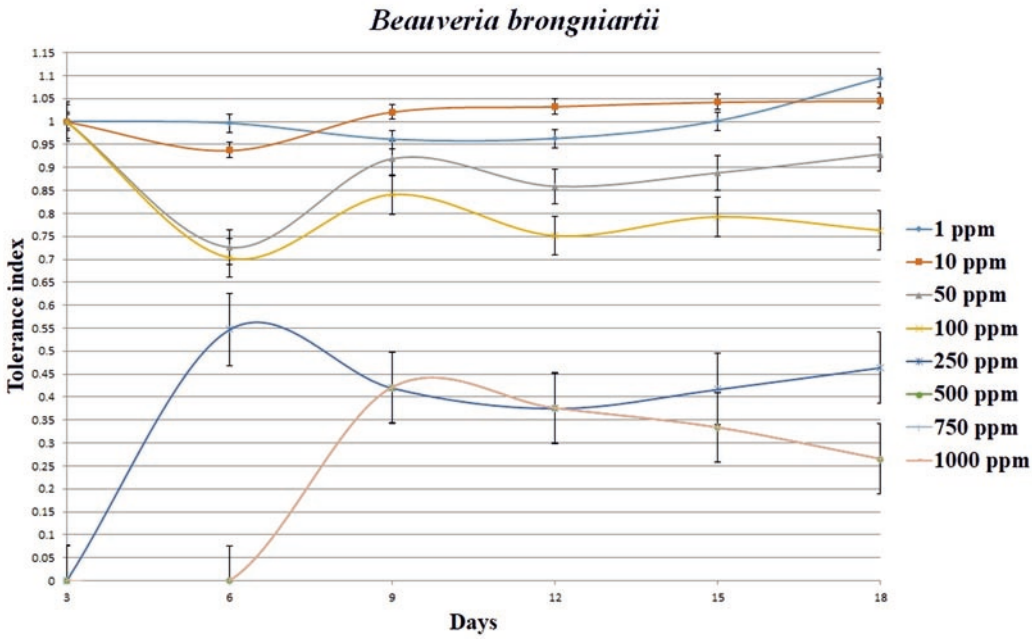

Fig. 2. Effect of various Co^{2+} concentrations on the tolerance index of *Beauveria brongniartii* during an 18-day incubation period, bars present means and whiskers SD (mean ± SD)

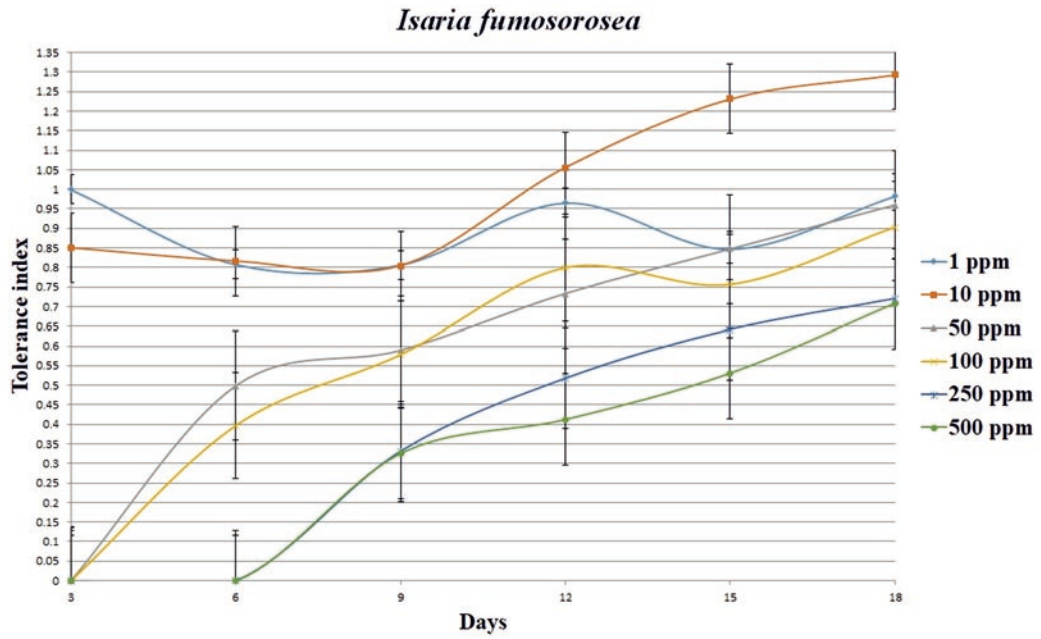

Fig. 3. Effect of various Co^{2+} concentrations on the tolerance index of *Isaria fumosorosea* during an 18-day incubation period, bars present means and whiskers SD (mean ± SD)

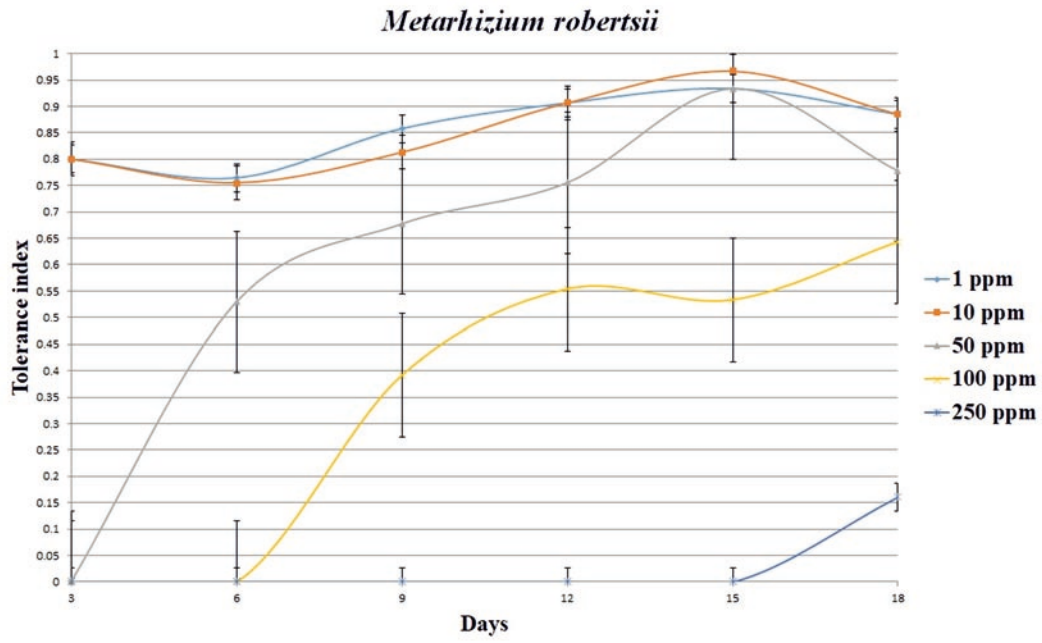

Fig. 4. Effect of various Co^{2+} concentrations on the tolerance index of *Metarhizium robertsii* during an 18-day incubation period, bars present means and whiskers SD (mean ± SD)

but weakened the initially active leucine arylamidase (6), β-glucosidase (17) and N-acetyl-β-glucosaminidase (18). The change in *B. brongniartii* development conditions due to the presence of Co^{2+} ions in the medium contributed to enzymatic disorders. The concentration of this metal, amounting to 1 ppm, significantly increased the activity of lipase esterase (C8) (4) and acid phosphatase (11), which at the highest concentration of Co^{2+} (500 ppm) was produced at the level of ≥40 nmol. At Co^{2+} concentrations of 1, 100 and 500 ppm, stimulation of N-acetyl-β-glucosaminidase (18) production was observed. In addition, α-mannosidase (19) activity from 10 nmol to 20–30 nmol increased in the culture medium at 100 ppm. In the

Table 1. The enzymatyic activity of entomopathogenic fungi determined by the use of API-ZYM test

Enzyme		*Beauveria bassiana*				*Beauveria brongniartii*				*Isaria fumosorosea*				*Metarhizium robertsii*			
		C	Co_1	Co_2	Co_3	C	Co_1	Co_2	Co_3	C	Co_1	Co_2	Co_3	C	Co_1	Co_2	Co_3
2	Alkaline phosphatase	2	2	2	1	3	3	3	1	1	1	1	1	1	4	3	1
3	Esterase (C4)	1	1	1	1	3	3	3	3	1	1	1	1	1	2	2	3
4	Esterase lipase (C8)	2	2	1	2	1	3	1	1	1	1	1	1	1	3	2	1
5	Lipase (C14)	2	2	1	2	1	1	1	1	1	1	1	1	1	2	1	1
6	Leucine arylamidase	2	3	2	1	3	2	3	2	1	1	1	1	1	3	3	2
7	Valine arylamidase	1	2	1	1	2	2	2	1	1	1	1	1	1	2	3	1
8	Cystine arylamidase	1	1	1	1	2	1	2	1	1	1	1	1	1	2	1	1
9	Trypsin	1	1	1	1	1	2	1	1	1	1	1	1	1	2	2	1
10	Chymotrypsin	1	1	1	1	1	2	1	1	1	1	1	1	1	2	2	1
11	Acid phosphatase	1	2	5	3	3	4	4	5	2	1	2	2	1	4	5	4
12	Naphthol-AS-BI-phospohydrolase	2	2	1	3	4	4	4	5	3	2	3	3	1	4	5	4
13	α-Galactosidase	1	2	1	1	1	1	3	1	1	1	1	1	1	2	3	1
14	β-Galactosidase	2	2	1	2	3	3	5	1	2	1	2	1	1	2	4	1
15	β-Glucuronidase	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1
16	α-Glucosidase	1	1	1	1	1	2	2	1	1	1	1	1	1	1	3	1
17	β-Glucosidase	1	1	3	3	1	3	4	3	2	1	1	1	3	3	4	3
18	N-Acetyl-β-glucosoaminidase	2	4	1	1	2	4	5	3	1	1	1	4	4	5	4	3
19	α-Mannosidase	1	1	1	1	2	2	4	1	1	1	1	1	1	3	2	1
20	α-Fucosidase	0	0	1	0	1	2	2	1	1	1	2	1	1	3	3	1

C – control; cobalt concentrations: Co_1 – 1 ppm; Co_2 – 100 ppm; Co_3 – 500 ppm

control sample, however, the presence of this hydrolase was found at a lower level (10 nmol). Co^{2+} also influenced the enzymatic activity of *I. fumosorosea*. It was found that in relation to the control sample (without cobalt), the highest productivity was detected from naphtol-AS-BI-phosphohydrolase (12), which, under the conditions of undisturbed development by cobalt, showed a level of 20 nmol. The activity of other enzymes was usually the same and within the limits of 5–10 nmol. At the lowest concentration (1 ppm) of Co^{2+}, acid phosphatase (11), β-galactosidase (14) and β-glucosidase enzymatic activity was limited in comparison to the control. However, with increased concentrations of Co^{2+} ions, the activity of naphtol-AS-BI-phosphohydrolase (12) remained at the same level. The activity of N-acetyl-β-glucosaminidase (18) increased from 5 nmol to as much as 30 nmol only at the highest tested concentration of Co^{2+} (500 ppm). *Metarhizium robertsii* in the control test showed high activity of two enzymes: β-glucosidase (17) and N-acetyl-β-glucosaminidase (18) at a level of 20 nmol and 30 nmol, respectively. The activity of the remaining hydrolases was at the same level, with the exception of β-galactosidase (14) and β-glucosidase (17). The disturbance of the enzymatic balance of *M. robertsii* occurred in the case of Co^{2+} ions already at their lowest and medium concentrations (1 and 100 ppm). In this combination, the production of almost all tested enzymes (except β-glucuronidase and α-glucosidase at 1 ppm, and lipase C14 (5), cystine arylamidase at 100 ppm) increased as compared to the control, which along with the increase in concentration to 500 ppm was weakened or remained unchanged at the same level (except esterase (C4), in which production was stimulated).

Discussion

Based on the results it is clear that cobalt ions may influence the EPF physiology. Similar observations were made by other researchers who studied the influence of heavy metals on fungal growth (Colpaert *et al.* 2000; Tkaczuk 2005; Anahid *et al.* 2011; Fazli *et al.* 2015). The reduced tolerance index reflects the inhibitory growth function of heavy metals. The variation in the metal tolerance may be due to the presence of one or more strategies of tolerance or resistance mechanisms exhibited by fungi (Iram *et al.* 2009;

Fazli *et al.* 2015). Some fungal strains show resistance to high, while others are sensitive even to low concentrations of cobalt. It must also be taken into account that the contamination at polluted sites is usually not caused by a single metal and that the selection is probably caused either by the most toxic element or by different metals acting synergistically (Gabriel *et al.* 1994; Iram *et al.* 2009). Several experiments have shown that *Fusarium* isolates are widely distributed in heavy metal contaminated environments and are resistant to many toxic metals, including Co (Zafar *et al.* 2007; An *et al.* 2015). Sanglimsuwan *et al.* (1993) studied 21 strains of 16 species of wood-rotting fungi for their resistance to metals (including Co). The resistance differed from species to species with *Pleurotus ostreatus* (Jacq.) P. Kumm. and *P. cystidious* O.K. Miller being the most resistant. In the study of Palmans *et al.* (1995) some white-rot fungi species [such as *Trametes versicolor* (L.) Lloyd] were resistant to heavy metals including Co, but some were sensitive. Anahid *et al.* (2011) studied the tolerance of *Aspergillus niger* Tiegh, *A. foetidus* Thom & Raper and *Penicillium simplicissimum* Thom to heavy metals (including Co). They showed that *Aspergillus* species presented poor tolerance (up to 500 ppm and 1500 ppm for *A. foetidus* and *A. niger*, respectively) to Co and *P. simplicissiumum* had moderate tolerance to Co in medium (up to 2500 ppm). Hasanzadeh *et al.* (2012) observed that Co could stop the growth of nematophagus fungi at concentrations ranging from 500 to 2000 ppm. Aishwarya *et al.* (2017) isolated endophitic fungus *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. which was tolerant to high heavy metal concentrations and was able to grow in the presence of Co at a concentration 600 ppm. There are reports about high resistance of *Neurospora* against Co (Joshi 2014; Desai *et al.* 2016). Pal *et al.* (2006) isolated fungi belonging to *Aspergillus*, *Mortierella*, *Paecilomyces*, *Penicillium*, *Pythium*, *Rhizopus* and *Trichoderma* genera from serpentine soil that were tolerant to Co^{2+} concentrations higher than 6 mM. In comparison to those results, entomopathogenic fungi seem to be poorly and moderately resistant to cobalt. In contrast, Hartikeinen *et al.* (2013) observed that some of the basidiomycetous and ascomycetous fungi were sensitive to Co even below the levels of contaminant limits in the environment.

Disorders of conidiophore production are often a reaction of fungi to stress conditions (Frank *et al.* 1993). Similar disorders were observed in the present study. Those changes may suggest low tolerance of the tested strains to Co^{2+} ions. A possible explanation of these different morphological changes among the tested strains may be due to the vast detoxification/tolerance mechanisms that each fungus applies (Fazli *et al.* 2015). In general, two mechanisms have been proposed for the heavy metal tolerance of fungi: 1. Extracellular (chelation and cell wall binding) sequestration, or 2. Intracellular physical sequestration of metal by binding to proteins or other ligands to prevent it from damaging the metal sensitive cellular targets (Anahid *et al.* 2011; Rasha 2017). Bankar *et al.* (2018) analyzed morphological changes of *Yarrowia lipolitica* (Wick., Kurtzman & Herman) Van der Walt & Arx under cobalt stress and observed that the normal ellipsoidal shape of yeast cells was changed into elongated shapes with bipolar scars, and formed pseudohyphae in response, which is probably the result of heavy metal-induced oxidative stress in fungal cells. The mechanism of cobalt distribution in entomopathogenic fungi is unknown. Frank *et al.* (1993) analyzed the distribution of cobalt in *Trichoderma viride* Pers. conidia and mycelia using the isotope ^{60}Co. The distribution of Co in fungal structures varied depending on the concentration. At 0.5 mM there was more Co in conidia than in mycelia, while, conversely at 1 mM the Co level was higher in mycelia, while at 5 mM no conidia formation was observed. As has been shown on the *Saccharomycs cerevisiae* Meyen ex E.C. Hansen model, amino acid histidine is one of the essential elements, and strains with a lack of histidine biosynthesis are more sensitive to cobalt in the environment (Pearce and Sherman 1999). Intracellular histidine may bind divalent metals such as Co^{2+}. On the other hand, proline is also reported as a crucial amino acid involved in neutralization of heavy metals (Sarathchandran *et al.* 2014).

Several researchers have reported the formation of colorful mycelia or pigment production as a response to heavy metals in media (Fazli *et al.* 2015). It has been previously suggested that production of pigments and organic acids (such as oxalic and citric acids) in fungal cells or their secretion into the environment is accompanied with a precipitation of metal ions (Sarathchandran *et al.* 2014; Fazli *et al.* 2015). Such reactions have also been observed in this study. A claret-colored halo was observed around *B. brongniartii* colonies in Co^{2+} concentrations of 1 and 10 ppm. Moreover, it was noted that a 50 ppm Co^{2+} concentration stimulated the formation of a white halo around *B. bassiana* colonies. It seems that this reaction is not specific, because the secretion of a white extracellular precipitate around *Aspergillus nidulans* (Eidam) G. Winter exposed to cobalt was also observed by Tripathi and Srivastava (2007).

Only a little is known about the impact of Co^{2+} ions on the enzymatic activity of fungi. The results of the present study provide new information on the possibilities of their impact on fungal physiology and demonstrate that they influenced the enzymatic activity of entomopathogenic fungi depending on species and metal concentration in medium. In the context of the lack of data on the fungi cobalt tolerance mechanism,

these studies have practical significance because they constitute a key to understanding the consequences of modifying the biochemical activity of these microorganisms in the aspect of the effectiveness of their use in biological plant pest control. Proteases, lipases, esterases, lipoxygenases and chitinases play important roles in the pathogenicity of the EPF (Wang *et al*. 2002; Pedrini *et al*. 2013; Sánchez-Pérez *et al*. 2014; Firouzbakht *et al*. 2015). Therefore, any disruption of their synthesis may change the course of pathogenesis of insects caused by these microorganisms. The epicuticle, the outer layer of the insecticide cuticle, acts as the first barrier against pathogen attack. It is composed of a heterogeneous mixture of lipids, long-chain alkenes, esters and fatty acids. Lipases hydrolyze ester bonds in lipoproteins. Fats and waxes are contained in the outer insect cover. Cobalt seems to have a limited influence on entomopathogenic fungi lipases. The inhibitory effect of Co^{2+} ions was noted only at a concentration of 100 ppm in *I. fumosorosea*. Another role of lipases is to increase the degree of adhesion of fungus spores to the epicuticle (Beys da Silva *et al*. 2010). Lipases can also affect changes in the permeability of biological membranes (as a result of the hydrolysis of lipid compounds contained in them). Proteases are one of the most important factors in the pathogenicity of entomopathogenic fungi. After the dissolution of the epicuticle by lipases and esterases, EPF secrete large amounts of proteases, capable of degrading protein and peptide substances. Dissolved proteins are degraded by peptidases (arylamidases) and amino acid exopeptidases that serve as nutrients for fungi which consequently may accelerate the development of mycoses in insects (Wang *et al*. 2002; Sánchez-Pérez *et al*. 2014). All tested Co^{2+} concentrations stimulated the synthesis of proteases in *B. bassiana*, and a similar reaction was observed at a concentration of 1 ppm in *M. robertsii*. N-Acetyl-β-glucosaminidase (NAG) is a high molecular-weight hydrolytic lysosomal enzyme. It breaks chemical bonds of glycosides and amino sugars that form structural components in many tissues. It is necessary for the degradation and disposal of various parts of the cell, including the cell membrane (Pusztahelyi and Pócsi 2014). The lowest (1 ppm) Co^{2+} concentration stimulated the production of NAG in *B. brongniartii* and *M. robertsii*, whereas the highest (500 ppm) stimulated NAG activity in *I. fumosorosea*. Activity of alkaline and acid phosphatases was also observed in the studied strains of fungi. These enzymes are involved in the catalysis of the dephosphorylation of various phosphate esters. The differences of enzymatic activities as a result of Co^{2+} present in medium might suggest that in a natural environment, the pollution caused by this metal may influence the pathogenicity of entomopathogenic fungi. Co is a transition element and is a pivotal component of several enzymes and co-enzymes. According to Anahid *et al*. (2011) cobalt, even at low concentrations, could exert toxic effects, such as blockage of the functional groups of fungal enzymes. The toxic activities of cobalt and its compounds depend on the physicochemical properties of these complexes, including their electronic structure, ion parameters (charge-size relations) and coordination (Palit *et al*. 1994). Several studies reported the influence of cobalt on the enzymatic activity of fungi belonging not only to the entomopathogenic group. Hartikainen *et al*. (2013) observed a color reaction of some acomytectous and basidiomycetous fungi with ABTS grown in the presence of Co, indicating that it may have a stimulative effect on the production of extracellular oxidative enzymes involved in biopolymers depolymerization processes essential in the cycling of nutrients in well balanced ecosystems. Machida and Nakanishi (1984) observed that the pyranose oxidase activity in *Trametes versicolor* was inhibited in the presence of Co^{2+}. Falih (1997) observed that cellulase of *Phanerochaete chrysosporium* P. Karst. was inhibited in the presence of Co.

In conclusion, the novel finding in the present study is that the growth of tested entomopathogenic fungi was sensitive to Co^{2+} which modified their development and biochemical activity. This metal could be a restrictive factor for their development in the environment and it could influence the fungal communities in contaminated soils. These changes can imply the successful use of entomopathogenic fungi in the biological control of insects and in the cycling of carbon in the ecosystem.

Acknowledgements

We gratefully acknowledge Marta Koniuszek for her assistance in conducting this study.

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