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Letter to Editor

The Effect of Selected Heavy Metal Ions on the Growth and Conidial Germination of the Aphid Pathogenic Fungus *Pandora neoaphidis* (Remaudiére et Hennebert) Humber

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Abstract

The effect of heavy metal ions (Cd, Cr^{3+}, Cu^{2}, Ni, Pb^{2+} and Zn) on the growth and germination of conidia of the aphid pathogenic fungus *Pandora neoaphidis* was studied. The metal ions were added into the culture medium in three concentrations: A – concentration corresponding to the mean content of that metal in Polish soils, B – concentration 10-times higher and C – 100-times higher than the mean ones. The investigated heavy metal ions, except for Ni, added to the media at the concentrations corresponding to the mean content of these metals in Polish soils, did not affect the growth of aphid-pathogenic fungus *Pandora neoaphidis.* Ni, Cu, Zn and Cr, added to the media at a concentration 100-times higher than in Polish soils, prevented the growth of the pathogen. All the tested metal ions, except for Cu, added to the media at the mean soil concentration, did not affect conidial germination of the fungus. The conidia of *P. neoaphidis* were unable to germinate in the presence of the Cr, Cu, Pb and Zn ions in the medium at a concentration that was 100-times higher than the mean one. Cu and Zn caused a significant reduction of conidial germination even at a concentration that was 10-times higher than the mean content of these metals in Polish soils. This work suggest that strong pollution of soil by some heavy metals could be a restrictive factor of development and pathogenicty of entomophthoralean fungi in the environment.

Keywords: *Pandora neoaphidis*, heavy metals, mycelial growth, germination of conidia

Introduction

Fungi from the order Entomophthorales play an important role in the regulation of the aphid population comparable to that done by parasitoids and predators. *Pandora neoaphidis* (Zygomycetes, Entomophthorales) is one of the most widely distributed fungal pathogens of aphids, and it is an important natural factor reducing pest aphid numbers in many crops [1-4]. *P. neoaphidis* has been recorded from more than 70 species of aphids and its potential for biological control has been investigated in several studies [5-8]. As with the other members of the Entomophthorales, the primary conidia of *P. neoaphidis* are actively ejected from the mycelium sporulating on infected cadavers under humid conditions. If the conidia land on a non-host surface, e. g. leaf or soil, they are able to resporulate, forming several series of infective secondary conidia [4, 6].

The development and infectivity of entomopathogenic fungi may be limited by many abiotic factors, i. e. pesticide use [9-13], mineral fertilization [14], and also by heavy metal pollution [15-20]. The influence of heavy

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metal ions on the development of *P. neoaphidis* or other entomophthoralean fungi has not been studied previously. Several laboratory experiments have demonstrated that ions of heavy metals may prevent the growth or restrict biomass increment of some entomopathogenic Hyphomycetales, like *Beauveria bassiana*, *Paecilomyces fumosoroseus* [21-23], *P. farinosus* or *Lecanicilliun lecanii* (=*Verticillium lecanii*) [18]. Surprisingly, Nordgren et al. [16] in their study of the microfungal flora in soils polluted by heavy metals, have noted that some of the fungi found in increased frequencies near a smelter were entomogenous species. They were later found to be tolerant to elevated concentrations of Cu [24]. Ropek and Frączek [25] stated that municipal landfill sites may negatively affect the surrounding environment through soil contamination with leachates containing heavy metals. This negatively influences soil organisms including, among other factors, entomopathogenic fungi and nematodes.

There are many crops in Poland which grow in regions with high industry emission levels and heavy metal contaminated soils, especially in the Upper Silesia region [26]. It is well known that increasing pollution of the environment (soil and air) by heavy metals increases the number of sucking insect-pests, e. g. aphids, spider mites or grasshoppers [27, 28]. Jaworska and Gospodarek [28] have reported that aphids on plants grown on soil polluted by heavy metals formatted more numerous colonies and infested a greater number of plants than those from plants grown on soil with natural metal content. Little is known about the influence of heavy metal pollution on the natural enemies and pathogens of aphids.

Soil provides the matrix for the maintenance of a natural reservoir of many entomopathogenic fungi and can be inoculated with entomopathogenic fungi either by an infected insect entering the soil and subsequently dying or by the deposition of spores on the soil surface [29]. Many species of entomogenous fungi from the order Entomophthorales overwinter in the soil in the stage of resting spores. *Pandora neoaphidis* resting spores have not been recorded in the field, and the mechanism of its overwintering is largely unknown. The fungus may persist during winter as hyphal bodies in cadavers [30] or it may reproduce at a slow rate in overwintering aphids [31, 32]. Nielsen et al. [33] have suggested that *P. neoaphidis* may overwinter in soil as thick-walled "loriconidia," which are similar to resting spores.

The aim of these studies was to determine the effects of 6 metal ions (Cd, Cr^{3+}, Cu^{2}, Ni, Pb^{2+} and Zn) on the growth and conidial germination of aphid pathogenic fungus *P. neoaphidis.*

Material and Methods

Effects on Growth

The effect of 6 aforementioned heavy metal ions on the growth and germination of the *P. neoaphidis* conidia was studied. The isolate of *P. neoaphidis* used in this experiment was originated from pea aphid (*Acyrtosiphon pisum* Harris), belonging to the collection of the Plant and Invertebrate Division, Rothamsted Research, UK.

The metal ions in the form of nitrates were added to the liquid Sabouraud medium enriched with egg yolk and milk powder in three concentrations: A – concentration corresponding to the mean content of that metal in Polish soils [26], B – concentration 10-times higher and C – 100-times higher than mean ones. The metal ions in the A (mean) concentration were added into culture medium in the following quantities: Cd – 0.22 mg/l, Cr – 12 mg/l, Cu – 6.5 mg/l, Ni – 6.5 mg/l, Pb – 13.8 mg/l and Zn – 33 mg/l. The Saboraud medium contains the buffer system ($H_2PO_4^-$ and HPO_4^{2-}), which stabilized its acidity on the constant level of 6.4 pH. After solidification medium was inoculated by fungus and the plates were kept at 20°C and the colony diameter was measured 5, 10, 15 and 20 days after inoculation. Each metal combination and the control with no metals were replicated four times. The analysis of variance and Duncan t-test were used to assess the differences between means at P=0.05.

Effects on Conidial Germination

In order to determine the effects of metal ions on conidia germination, microscope slides were covered with a thin layer of water agar containing the concentrations of the metal ions described previously and placed in Petri dishes filled with wet filter paper. The acidity of medium was buffered to achieve similar and constant pH levels as used in a previous experiment. Sporulating mummies of *A. pisum* infected by *P. neoaphidis* were attached individually to the underside of the Petri dish lid and then positioned directly above the microscope slide onto which primary conidia were deposited. The mummies were left in this position for 10 minutes. For the purpose of avoiding production of secondary conidia, the slides were inverted instantly after the end of spore deposition. The germination of primary conidia was assessed microscopically 6 and 12 hours after the collection of the conidia. The number of germinated spores was calculated by counting 100 spores per replication. The experiment was replicated four times for each combination. The results were expressed as a percentage of the control value.

Results and Discussion

Effects on Growth

Among all the heavy metals used in the experiment, Ni showed the greatest inhibitory effect on the growth of *P. neoaphidis*. The fungus was unable to grow on the medium containing Ni at 10-times and 100-times higher concentrations than the mean dose (Table 1). The fungus displayed low sensitivity to all the metal ions present in the

medium at the A concentration, except for Ni. The low toxic effect of Cu, Cd, Ni, Pb and Zn ions, added to the medium in the above-mentioned concentrations, on the growth of entomopathogenic fungi like: *B. bassiana*, *B. brongniartii* and *P. fumosoroseus*, was reported by Tkaczuk [23] and Tkaczuk et al. [22, 34]. *P. neoaphidis* was unable to grow on the media containing Cu, Zn and Cr at the highest (C) concentration. Cu and Zn strongly inhibited fungal growth even at a 10-times higher level than mean content of individual metals in the soil. Strong toxic effects of Cu, Cr and Zn on the growth of *P. fumosoroseus* was reported by Jaworska et al. [21]. Arnebrant et al. [24] found that another entomogenous fungus, *Verticillium lecanii*, did not grow on media contaminated with more than 400 mg Cu/l. Cu exhibited the most potent effect on the growth of *V. lecanii* among the other tested metal ions, including: Cd, Ni, Pb or Zn [18]. Cu and Zn, present in the medium at 100-times higher concentration than the mean content of these metals in Polish soils, strongly inhibited mycelial growth of *B. bassiana*, *M. anisopliae*, *M. flavoviride* and *Hirsutella* sp. [34], but did not affect their growth at 10-times higher concentration. This means that *P. neoaphidis* seems to be more sensitive to the Cu and Zn ions than entomogenous Hyphomycetes.

Cd and Pb added to the medium at the highest level considerably reduced the size of fungal colonies (Tab. 1), but the same metal ions added to the medium at the concentration 10-times higher than the mean ones, did not limit fungal growth significantly. Entomopathogenic fungi *P. fumosoroseus* [23] and *B. brongniartii* [22] showed very low sensitivity to the toxic effect of these metals in all the above-mentioned concentrations. Chromium, which completely inhibited the growth of *P. neoaphidis* at the highest (C) concentration, did not have a negative effect on the growth of the pathogen at the A and B concentrations.

Effects on Conidial Germination

All the metal ions used at (A) concentration, corresponding to the mean content of those metals in Polish

Table 1. Effects of different concentrations of heavy metal ions on radial growth (mean growth in mm) of *Pandora neoaphidis* in vitro.

Metal ions	Concentration*	The size of the colonies (in mm) of fungus after 5-20 observation days			
		5	10	15	20
Cd	A	5.5 (±0.4) b	13.8 (±0.6) b	34.0 (±1.2) ab	57.4 (±1.5) a
	B	8.3 (±0.3) a	17.0 (±1.1) ab	38.9 (±1.2) a	52.7 (±1.5) ab
	C	6.0 (±0.3) b	8.2 (±0.4) cd	17.6 (±0.6) cd	40.0 (±2.3) c
Cr^{3+}	A	7.3 (±0.4) a	19.4 (±1.1) a	41.5 (±1.7) a	54.8 (1.6) a
	B	5.6 (±0.2) b	13.4 (±0.5) bc	34.4 (±1.8) b	54.1 (±0.7) a
	C	0 d	0 d	0 d	0 d
Cu	A	7.0 (±0.5) a	18.3 (±0.8) ab	38.4 (±1.5) a	54.4 (±0.9) a
	B	3.4 (±0.2) c	5.8 (±0.2) cd	11.3 (±0.6) cd	15.0 (±0.7) cd
	C	0 d	0 d	0 d	0 d
Ni	A	6.0 (±0.4) b	12.8 (±0.5) c	19.8 (±0.7) c	30.9 (±1.7) c
	B	0 d	0 d	0 d	0 d
	C	0 d	0 d	0 d	0 d
Pb	A	7.0 (±0.4) a	17.2 (±0.8) ab	39.0 (±2.5) a	55.0 (±1.3) a
	B	6.6 (±0.5) a	16.3 (±0.8) ab	39.2 (±1.2) a	53.6 (±0.8) a
	C	5.2 (±0.4) b	10.6 (±0.5) c	19.8 (±0.7) c	30.8 (±1.0) c
Zn	A	7.8 (±0.2) a	14.7 (±0.8)b	38.8 (±1.2) a	53.2 (±0.8) a
	B	5.9 (±0.4) b	12.4 (±0.9) c	20.0 (±0.9) c	32.1 (±1.0) c
	C	0 d	0 d	0 d	0 d
Control		7.4 (±0.4) a	19.8 (±0.8)a	40.0 (±1.2) a	57.6 (±0.8) a

*A – concentration corresponding to the mean content of that metal in Polish soils, B – concentration 10-times higher, C – concentration 100-times higher than the mean ones. Means in column followed by different letters are significantly different (at P= 0.05)

soils (with the exception of Cu), did not affect significantly the process of conidia germination (Figs. 1 and 2). The spores of *P. neoaphidis* were unable to germinate in the presence of Cu, Zn, Cr and Pb at the highest (C) concentration of these metals in the medium. Cu also prevented the germination of conidia at the B dose, and even at the lowest level of copper in the medium, only a few percent of spores were able to germinate.

The influence of heavy metal ions on the conidia germination of *P. neoaphidis* have not previously been investigated. However, fungicide Miedzian 50 WP, based on copper in the shape of copper sulphate, strongly inhibited the germination of the fungus conidia in vitro [35]. The ions of Al, Cd, Cr, Cu, Li, Ni, Pb and Zn added to the medium in the form of nitrates at 100-times higher concentration than the mean content of given metals in Polish soils, completely inhibiting the spore germination of entomopathogenic fungus *P. fumosoroseus.* The same metal ions strongly limited spore germination of *P. fumosoroseus,* even at 10-times the concentration of the mean ones [23]. On the other hand, the conidia of insect pathogenic species *P. farinosus* germinated well on media containing Cu at a concentration of 400 mg l^{-1} [15]. Zn ions caused a significant reduction in the germination of *P. neoaphidis* at 10-times greater concentration than the mean soil levels (Figs. 1 and 2). Chromium ions present in the water agar at the C dose permitted spore germination. The percentage of germinated *P. neoaphidis* conidia on the media containing Pb ions at B concentration was also significantly reduced in relation to control. Among all the heavy metal ions tested, Cd and Ni exhibited a weak inhibitory effect on the germination of the conidia. Cadmium, added to the medium in all the tested levels, did not negatively affect germination process of the pathogen's spores. The Ni ions were not toxic to *P. neoaphidis* conidia at mean concentration (A), and even at the concentration 10-times higher (B).

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 physico-chemical factors [36-38].

Metals in soil are present as free metal ions, soluble metal complexes, exchangeable metal ions, organically bound metals, percipitated or insoluble compounds such as oxides, carbonates and hydroxides [39]. The mobility of metals in soil is dependent of their speciation, which is controlled by hydrochemical variables (pH, redox potential, presence of complexing inorganic and organic anions, ionic strength) as well as by their interactions with solid surface. The toxicity of metals in soil depends on their bioavailability, which, according to Berthelin et al. [40], is a function not only of their total concentration but also of physico-chemical (e. g. ph, Eh, organic matter, clay content) and biological (e. g. biosorption, boaccumulation and solubilization) factors. It is known that fungi are able to accumulate significant amounts of metals [37, 41]. The cell walls of fungi are composed of polysaccharides, proteins and lipids [42] which contain functional groups with potential metal complexing capacities. Some entomopathogenic fungi are considered strongly tolerant or resistant to soil contents of heavy metals [24]. Insect pathogenic fungi: *B. bassiana*, *M. anisopliae* and *P. farinosus* proved to be resistant to high concentrations of copper ions in the environment [16] Laboratory observations by Popowska et al. [20], have shown that entomopathogenic fungi can accumulate high metal contents in their fruiting bodies, and that metal accumulation varied between species. The isolates of *M. anisopliae* isolated from soils of high contents of heavy metals could develop some adaptive mechanisms, like increased accumulation of metals in mycelium, to survive in contaminated habitats [20]. However, studies under laboratory conditions [19, 21, 22, 43] have shown that some heavy metal ions, especially at high concentrations, can considerably limit growth, sporulation and patogenicity of entomogenous fungi.

A majority of soils in Poland do not reveal strong pollution with heavy metals. But in some regions of the country, e. g. Upper Silesia, elevated concentrations of some heavy metals are observed [26].

Fungus *Pandora neoaphidis* seems to be insensitive to low concentrations of heavy metal ions tested in this experiment, but at the highest concentrations some met-

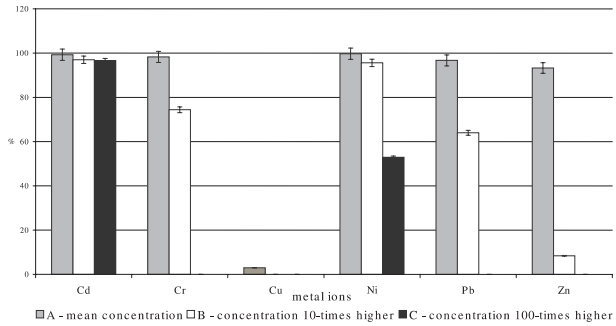

Fig.1. Effects of different concentrations of heavy metal ions on conidial germination of *Pandora neoaphidis* (% in relation to the control) in vitro after 6 hours. Error bars denote the standard deviation.

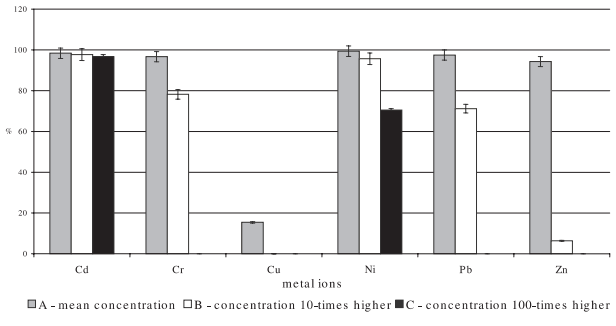

Fig. 2. Effects of different concentrations of heavy metal ions on conidial germination of *Pandora neoaphidis* (% in relation to the control) in vitro after 12 hours. Error bars denote the standard deviation.

als could prevent or inhibit the growth and conidial germination of this fungus. It means that the strong pollution of soil, by some heavy metals could be a restrictive factor for development of this pathogen in the environment and its successful use in biological control against insects.

Conclusions

1. The investigated heavy metal ions, except for Ni, added to the media at the concentrations corresponding to the mean content of these metals in Polish soils, did not affect the growth of the aphid-pathogenic fungus *Pandora neoaphidis.* The ions of Ni, Cu, Zn and Cr, added to the media at a 100-times higher concentration, prevented the growth of the pathogen.
2. All the tested metal ions, except for Cu, added to the media at the concentrations corresponding to the mean content of these metals in Polish soils, did not affect a conidial germination of the fungus. The conidia of *Pandora neoaphidis* were unable to germinate in the presence of the Cr, Cu, Pb and Zn ions in the medium at the concentration that was 100-times higher than the mean one. Cu and Zn caused a significant reduction of conidial germination even at the concentration that was 10-times higher than mean content of these metals in Polish soils.
3. Strong pollution of soil by some heavy metals could be a restrictive factor of development and pathogenicty of entomophthoralean fungi in the environment.

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