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Occurrence of entomopathogenic fungi in cultivated soils in Slovakia in the intensive agricultural system production

Występowanie grzybów entomopatogenicznych w glebach uprawnych Słowacji o intensywnym systemie produkcji rolniczej

Abstract. The aim of the study was to identify the types of entomopathogenic fungi (EPF) and to determine the intensity of their occurrence in intensively cultivated agricultural soils in the Danubian Lowland, Slovakia. Soil samples were collected on two dates (spring, autumn) from seven sites where the following crops were grown: soybean, barley, alfalfa, sugar beet, maize, maize with the addition of a biostimulator and from wasteland. The entomopathogenic fungi from individual soil samples were isolated using the isolation method on a selective medium. Entomopathogenic fungi were identified microscopically based on the morphology of their microstructures and the morphology of colonies, using standard keys. The number of EPF was presented in CFU g^{-1} of dry matter of soil. Both in spring and autumn, EPF belonging to three genera were identified: *Beauveria*, *Metarhizium* and *Cordyceps*. Analyzing the average density of infectious units of the identified genera of EPF in both study dates on arable land, it was found that fungi of the genus *Metarhizium* spp. formed more infectious units in the spring study date, whereas fungi of the genera *Beauveria* spp. and *Cordyceps* spp. formed more infectious units in the autumn study date, and these differences were statistically significant.

Keywords: entomopathogenic fungi, intensive agricultural system production, cultivated soils in Slovakia

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INTRODUCTION

Agriculture productivity today faces many challenges in meeting the requirements of a growing people population. Progressing climate change and pressure from pests are two of the main factors influencing the limited growth of agricultural production [Thirumeni et al. 2024]. The development of agricultural mechanization and automation, breeding more productive and resistant plant varieties have influenced the increase in production, however, it is necessary to search for and implement alternative strategies to increase the quantity and quality of crops obtained while reducing chemical inputs in agriculture [Welch and Graham 2004, Mateo-Sagasta et al. 2017, FAO et al. 2018, Gonzalez-Guzman et al. 2021].

Entomopathogenic fungi (EPF) are microorganisms that control insect host density in nature. Soil is considered the natural habitat of EPF because it is where they deposit their infectious spores [Tkaczuk 2008, Medo and Cagàń 2011, Sharma et al. 2018]. Besides their long-known ability to control insect populations [Vega et al. 2009, Jordan et al. 2021, Khun et al. 2021], EPF possess other traits which have only recently been exposed. One such trait is their ability to colonize plants [Vega et al. 2008, Bamisile et al. 2018], which has been partly ascribed to their strong competition in the rhizosphere [Hu and Leger 2002] and to their interactions being facilitated by certain molecular mechanisms [Wang and Leger 2007, Fang and Leger 2010, Liao et al. 2013]. The EPF are currently being tested as environmentally friendly, providing an alternative to chemical insecticides in the control of insect pests [Tkaczuk 2008, Dara 2017, Fenibo et al. 2021, Kim et al. 2023]. Soil, which is the main EPF reservoir, has been deemed the most suitable habitat for application of these biocontrol microbes [Lomer et al. 2001]. Additionally, the physical and chemical properties of soil, such as pH, carbon, nitrogen, and soil elements [Hawkes et al. 2011, Wang et al. 2021], also have impacts on soil fungi distributions and diversity, while the concentration of soil nutrients influences fungal diversity most [Yang et al. 2022]. These microorganisms are easily produced, multiplied, applied, and dispersed in the field, and can be used, in combination or individually, with low impact on non-target organisms [Domingues et al. 2020]. It is known that pesticides used on a large scale in intensive agricultural production may have a different effect on the growth, sporulation and pathogenicity of EPF. Therefore, it is necessary to search for and test new solutions in plant protection that will reduce the negative impact of chemicals on the environment, people and microorganisms inhabiting the soil environment [Majchrowicz and Poprawski 1993, Tkaczuk 2001, Rashid et al. 2010, Hernandez et al. 2012, Tkaczuk et. al. 2012, 2015, Pelizza et al. 2015, Fiedler and Sosnowska 2017, Holka and Kowalska 2023].

The aim of the study was to identify the types of EPF and to determine the intensity of their occurrence in intensively cultivated agricultural soils in the Danubian Lowland, Slovakia.

MATERIAL AND METHODS

The study material consisted of samples collected from the humus horizon of arable soils in 2024 (spring – June, autumn – September) at the University Agricultural Enterprise SPU, Oponice, Slovakia, in the Nitra Region, Topolcany District in the Danubian

Lowland (geographic coordinates: 48°28'05"N 18°08'39"E). Soil samples were collected from 7 sites using a soil stick to a depth of about 15–20 cm. In each site, 4 zones (repetitions) were designated, spaced about 10–15 m apart. About 6 samples were collected from each repetitions, and then a mixed sample was prepared. After changing the sampling location, the soil stick was surface sterilized with 70% ethyl alcohol each time. Soil samples were placed in plastic zip-lock bags and stored at approximately 4°C until laboratory experiments began. The tested soils were classified as chernozem, quality class I – the best arable soil. Crop characteristics, crop area and pesticide protection used are presented in table 1.

Table 1. Crop area, forecrop and use of chemical protection

Cultivation	Area (ha)	Forecrop	Dose of applied plant protection products
Glycine willd L. (soybean)	27.16	*Triticum aestivum* L. (common wheat)	Soleto 500 SC (2.67 dm^3 ha^{-1}), Pulsar 40 (1 dm^3 ha^{-1}), Thifen 75 WDG (12 g ha^{-1}), Amistar Gold Max (1 dm^3 ha^{-1})
Hordeum L. (barley)	23.57	*Zea mays* L. (maize)	Cythrin Max EC (50 cm^3 ha^{-1}), Slape trio (700 cm^3 ha^{-1}), Starane forte (0.60 dm^3 ha^{-1}), Stabilan 750 SL (1 dm^3 ha^{-1}), Baghira 040 EC (0.80 dm^3 ha^{-1}), Supersect Max EC (50 cm^3 ha^{-1})
Medicago satvia L. (alfalfa)	27.94	*Sorghum bicolor* L. (sorghum bicolor)	Stomp Aqua 455 CS (2.2 dm^3 ha^{-1})
Beta vulgaris L. (sugar beet)	34.63	*Hordeum* L. (barley)	Asahi SL (0.25 dm^3 ha^{-1}), Beetup Flo (150 cm^3 ha^{-1}), Conviso One (0.50 dm^3 ha^{-1}), Mero Stefes (1.47 dm^3 ha^{-1}), Bettix Combi 500 SC (2 dm^3 ha^{-1}), Supersect Max EC (50 cm^3 ha^{-1}), Bagani 125 ME (0.80 dm^3 ha^{-1}), Karate Zeon 5 CS (0.15 dm^3 ha^{-1}), Belanty (1.47 dm^3 ha^{-1})
Zea mays L. (maize)	16.43	*Glycine willd* L. (soybean)	Spandis 54 WG (0.49 kg ha^{-1}), Certo (0.20 kg ha^{-1}), Vesticor SC (100 cm^3 ha^{-1})
Zea mays L. (maize) + biostimulator	6.35	*Glycine willd* L. (soybean)	Spandis 54 WG (0.49 kg ha^{-1}), Certo (0.20 kg ha^{-1}), Vesticor SC (100 cm^3 ha^{-1}) + Neosol (150 kg ha^{-1})
Agricultural wasteland	–	–	–

Before starting the cultivation, soil tests were performed to determine: pH_{KCl} – potentiometrically, and available forms of phosphorus, potassium and magnesium, which were determined in extracts using the ICP-AES method (tab. 2).

The entomopathogenic fungi from individual soil samples were isolated using the selective medium method developed by Strasser et al. [1996]. In order to determine the generic composition and quantitative assessment of infectious units of EPF in the tested soils, tests were carried out using a selective medium with the following composition: 20 g glucose, 10 g peptone, 18 g agar, which were dissolved in 1 dm^3 of distilled water and then sterilized in an autoclave at 121°C for 20 minutes.

Selective medium were added to the prepared substrate after cooling to 50°C: 0.6 g of streptomycin, 0.05 g of tetracycline and 0.1 g of dodine. The selective medium was poured into Petri dishes. From the collected soil sample, 2 g of soil was weighed and then covered with 18 cm^3 of distilled water with the addition of a surface tension reducing agent (Triton X-100).

Table 2. Results of agrochemical soil testing

Cultivation	pH_{KCl}	P (mg kg^{-1})	K (mg kg^{-1})	Mg (mg kg^{-1})
Glycine willd L.	7.4	81.0	270.0	381.0
Hordeum L.	6.3	76.0	211.0	228.0
Medicago satvia L.	6.5	70.0	191.0	371.0
Beta vulgaris L.	7.3	58.0	187.0	312.0
Zea mays L.	7.1	94.0	263.0	371.0
Zea mays L. + biostimulator	7.3	92.0	260.0	368.0
Agricultural wasteland	6.3	–	–	–

Using an automatic pipette, 0.1 cm^3 of soil solution was collected and transferred to the surface of the selective substrate. Inoculations were performed in four replicates for each soil sample. The dishes with the applied soil solution were transferred to an incubator at 21°C without access to light and after 10 days the number of colony-forming units (CFU) of EPF developing on the culture medium was determined. The number of EPF was presented in CFU g^{-1} of dry matter of soil. Entomopathogenic fungi were identified microscopically based on the morphology of their microstructures (by determining the size and shape of conidia, conidiogenic cells) and the morphology of colonies, using standard keys [Rehner and Buckley 2005, Rehner et al. 2011, Humber 2012, Inglis et al. 2012]. Considering that only morphological methods were used to identify the fungi, they were assigned to the rank of genus because, as shown by the latest phylogenetic studies based on DNA sequencing [Bischoff et al. 2006, 2009, Kepler et al. 2017], there are many species of fungi within the genera *Beauveria*, *Metarhizium* and *Cordyceps*, the distinction of which is almost impossible without the use of molecular methods.

The obtained results were statistically processed using the Statistica 13.3 TIBCO Software Inc. One-way analysis of variance (ANOVA) and Tukey's post-hoc test were performed. The calculated means were combined into homogeneous groups at the significance level of $\alpha < 0.05$. Simple correlation coefficients were calculated for the studied soil parameters and the identified types of EPF. The standard deviation was calculated.

RESULTS AND DISCUSSION

The conducted studies showed that the types and intensity of EPF occurrence were different depending on the species of the cultivated plant and the time of soil material collection for testing. Both in spring and autumn, EPF belonging to three genera were identified: *Beauveria*, *Metarhizium* and *Cordyceps* (tabs 3 and 4). The entomopathogenic fungi of the genera *Beauveria*, *Metarhizium* and *Cordyceps* are the dominant fungi found in soil environments in Europe, as confirmed by studies conducted by Samson et al. [1988], Keller and Zimmermann [1989], Landa et al. [2002], Prenerová et al. [2009], Medo and Cagáň [2011], Augustyniuk-Kram and Kram [2012], Majchrowska-Safaryan and Tkaczuk [2021], Kovač et al. [2021], Majchrowska-Safaryan et al. [2023].

Table 3. Identified fungal genera and the density of their colony – forming units (CFU × 10^3 g^{-1}) in the tested cultivated soils (spring date)

Cultivation	Genera of entomopathogenic fungi		
	Beauveria spp.	*Metarhizium* spp.	*Cordyceps* spp.
Agricultural wasteland	0.83 ±0.23 ^{ab}	3.0 ±0.40 ^{a}	–
Glycine willd L.	1.0 ±0.40 ^{ab}	1.8 ±0.47 ^{b}	–
Hordeum L.	0.5 ±0.0 ^{b}	1.7 ±0.62 ^{b}	–
Medicago satvia L.	1.7 ±0.24 ^{a}	1.8 ±0.24 ^{b}	0.3 ±0.24 ^{a}
Beta vulgaris L.	0.5 ±0.0 ^{b}	0.5 ±0.41 ^{d}	0.3 ±0.24 ^{a}
Zea mays L.	1.0 ±0.41 ^{ab}	0.7 ±0.24 ^{cd}	0.2 ±0.24 ^{a}
Zea mays L. + biostimulator	0.7 ±0.24 ^{b}	1.3 ±0.62 ^{bcd}	0.2 ±0.24 ^{a}
F value	191.95	289.31	217.35
P value	0.0	0.0	0.0

± standard deviation; abcd – means within columns with the same lowercase letters are not significant at $\alpha < 0.05$

Table 4. Identified fungal genera and the density of their colony – forming units (CFU × 10^3 g^{-1}) in the tested cultivated soils (autumn date)

Cultivation	Genera of entomopathogenic fungi		
	Beauveria spp.	*Metarhizium* spp.	*Cordyceps* spp.
Agricultural wasteland	1.5 ±0.0 ^{b}	2.2 ±1.02 ^{a}	–
Glycine willd L.	1.3 ±0.23 ^{b}	1.3 ±0.23 ^{ab}	–
Hordeum L.	0.7 ±0.24 ^{c}	1.2 ±0.62 ^{ab}	–
Medicago satvia L	3.3 ±0.62 ^{a}	1.3 ±0.94 ^{ab}	0.7 ±0.24 ^{ab}
Beta vulgaris L.	0.7 ±0.24 ^{c}	0.2 ±0.24 ^{b}	1.0 ±0.0 ^{a}
Zea mays L.	1.5 ±0.0 ^{b}	0.3 ±0.47 ^{b}	0.5 ±0.0 ^{c}
Zea mays L. + biostimulator	1.2 ±0.62 ^{b}	1.2 ±0.62 ^{ab}	0.3 ±0.23 ^{c}
F value	437.83	76.45	223.88
P value	0.0	0.0	0.0

± standard deviation; abcd – means within columns with the same lowercase letters are not significant at $\alpha < 0.05$

Fungi of the genus *Beauveria* spp. formed the greatest number of colony forming units (CFU) in soils where the cultivated plant was *Medicago stiva* L., while in autumn the number of CFU was high, 49% higher than in spring. On both dates of the study, the smallest number of CFU were found in arable soil where *Hordeum* L. and *Beta vulgaris* L. were grown, these differences amounted to 29%. Konopická et al. [2022] examined the generic composition of EPF in arable soils of the Czech Republic and found that the genus *Beauveria* occurred only in two locations (Meziříčí, Mlýn Podhora) and the number of CFU units was small. The amount of CFU fungi per 1 cm^3 mL of soil ranged from 1.04×10^2 to 5.93×10^2 per 1 cm^3. The effect of sampling site on CFU of the genus *Beauveria* was not significant (F = 1.3379; df = 7,36; P = 0.2614). In contrast, Medo and Cagáń [2011] found that the influence of field crop species was a significant factor influencing the number of isolated EPF infectious units from the soils in Slovakia (v2 = 28.604; df = 10; P = 0.001) only in the case of *B. bassiana*, with a higher detection rate in samples from alfalfa and clover fields than other crops. Medo et al. [2016] conducting studies on the phylogenetic structure of 109 soil-borne entomopathogenic *Beauveria* isolates acquired using the *Galleria mellonella* bait method from different habitat types in Slovakia, found three *Beauveria* species: *B. bassiana*, *B. pseudobassiana* and *B. brongniartii*, represented by 51.4%, 43.1% and 5.5% of acquired isolates, respectively. Correlation analysis with the habitat type and individual habitat characteristics showed strong preferences of the most prevalent haplotypes for agricultural (*B. bassiana* A1) and forest habitats (*B. pseudobassiana*) which has possible implications for conservative biocontrol strategies.

Entomopathogenic fungi of the genus *Metarhizium* spp. produced colony forming units in all tested soil samples, regardless of the date of the study. In both the spring and autumn periods of the study, the highest number of CFU of this genus was found in soils of uncultivated agricultural land. More in the spring period by over 25% compared to the autumn period. Tkaczuk et al. [2014] examined the occurrence of EPF in Polish soils cultivated in conventional and organic systems and found that *M. anisopliae* was the most frequently isolated species where intensive chemical protection was used. The lowest number of CFU was produced by fungi of the genus *Metarhizium* spp. in soils of arable land where *Beta vulgaris* L. was cultivated, respectively: spring period 0.5 CFU $\times 10^3$ g^{-1} and autumn period 0.2 CFU $\times 10^3$ g^{-1}.

Medo and Cagáń [2011] studied the occurrence of EPF in the soils of Slovakia and found that *M. anisopliae* was isolated mainly in field and meadow samples, and that production intensification did not show any negative impact. It was most often isolated from lowland soils with a neutral or alkaline pH. Uzman et al. [2019] investigated the occurrence of EPF in soils from conventional and organic vineyards in the Rhinehessen region in Germany. In both types of vineyards, EPF taxa of the genera *Metarhizium* spp., *Beauveria* spp. were found. The presence of *Metarhizium* in all habitats, confirms reports of a strong association of species of this fungus with soils in cultivated habitats, particularly field crops [Quesada-Moraga et al. 2007, Domingues et al. 2022]. Domingues et al. [2022] found a significant occurrence of infectious entities of EPF of the genus *Metarhizium* in arable soils where soybean was cultivated.

In the case of the conducted studies, CFU of EPF of the genus *Cordyceps* spp. were not found in the soils collected from the field which was an agricultural wasteland or in the objects where the cultivated plants were *Glycine willd* L. and *Hordeum* L. in both study dates. The highest number of CFU of this genus was formed in the soil where *Beta vulgaris* L. was cultivated (1.0 CFU $\times 10^3$ g^{-1}) collected in the autumn term of the study. Also in the spring term, CFU of *Cordyceps* spp. were found in this object, but their number was significantly lower. Medo and Cagáń [2011] found the presence of *Cordyceps* (= *Isaria*

spp.) fungi in Slovak soils using the selective medium method at the level of 8%, with the highest number in soils collected from mid-field trees and meadows.

This relationship was also confirmed in the studies carried out in Poland by Majchrowska-Safaryan et al. [2023], who found 11.5 CFU × 10^3 g^{-1} in the soil of field trees and 2.2 CFU CFU × 10^3 g^{-1} in meadow. An analysis of simple correlation coefficients was carried out for the identified genera of EPF and selected parameters of the tested soils, which did not reveal any statistically significant correlations at any time during the study (tabs 5 and 6).

Table 5. Correlations between selected properties of the studied soils and the number of colony-forming units (CFU × 10^3 g^{-1}) of the identified genera of EPF (spring date)

Correlation	*Beauveria* spp.	*Metarhizium* spp.	*Cordyceps* spp.	pH_{KCl}	P	K	Mg
Beauveria spp.	1.00	0.22	0.32	0.17	0.078	0.04	0.30
Metarhizium spp.	–	1.00	–0.65	–0.64	–0.69	–0.70	–0.72
Cordyceps spp.	–	–	1.00	0.31	0.31	0.26	0.54
pH_{KCl}	–	–	–	1.00	0.53	0.64	0.66
P	–	–	–	–	1.00	0.98*	0.91*
K	–	–	–	–	–	1.00	0.93*
Mg	–	–	–	–	–	–	1.00

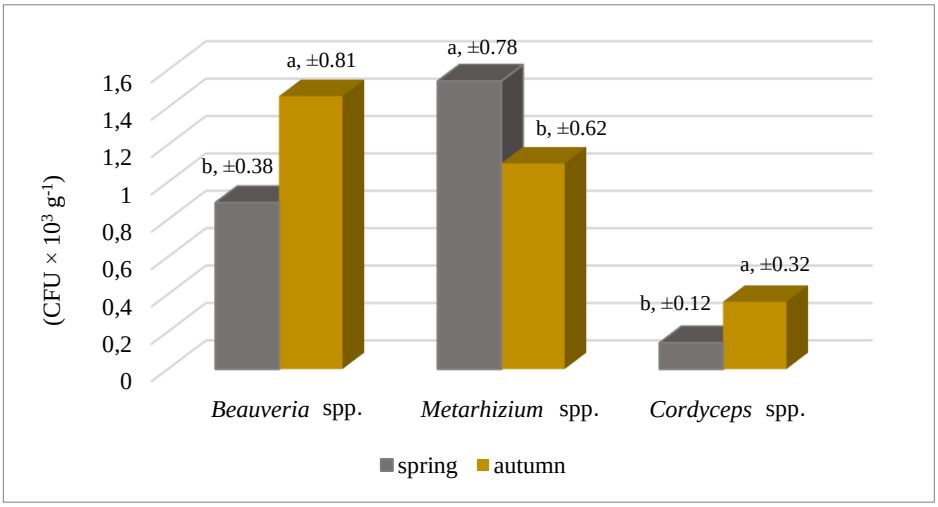
* the marked correlation coefficients are significant with $\alpha < 0.05$

Table 6. Correlations between selected properties of the studied soils and the number of colony-forming units (CFU × 10^3 g^{-1}) of the identified genera of EPF (autumn date)

Correlation	*Beauve-ria* spp.	*Metarhi-zium* spp.	*Cordyceps* spp.	pH_{KCl}	P	K	Mg
Beauveria spp.	1.00	0.26	0.23	–0.30	–0.03	–0.09	0.18
Metarhizium spp.	–	1.00	–0.70	–0.57	–0.61	–0.63	–0.65
Cordyceps spp.	–	–	1.00	0.31	0.17	0.16	0.43
pH_{KCl}	–	–	–	1.00	0.53	0.64	0.66
P	–	–	–	–	1.00	0.98*	0.91*
K	–	–	–	–	–	1.00	0.93*
Mg	–	–	–	–	–	–	1.00

* the marked correlation coefficients are significant with $\alpha < 0.05$

Analyzing the average density of CFU of the identified genera of EPF in both study dates on arable land, it was found that fungi of the genus *Metarhizium* spp. formed more infectious units in the spring study date, whereas fungi of the genera *Beauveria* spp. and *Cordyceps* spp. formed more CFU in the autumn study date, and these differences were statistically significant (fig. 1). This relationship is confirmed by earlier studies by Majchrowska-Safaryan et al. [2023] on the occurrence of EPF in soils of habitats with diverse use in eastern Poland. Gorczyca et al. [2018] also studied the occurrence of EPF in agricultural and natural soils of south-eastern Poland and confirmed that in the autumn period of the study the dominant species was *B. bassiana*.



a, b – significance at the $\alpha < 0.05$; ± standard deviation

Fig 1. Mean density of EPF colony forming units (CFU) at both test dates on arable soil

Many studies indicate that environmental factors related to the habitat are the main selective forces shaping the structure of facultative EPF [Vänninen 1996, Bidochka et al. 2002, Meyling and Eilenberg 2007, Quesada-Moraga et al. 2007, Tkaczuk 2008, Meyling et al. 2009, Medo and Cagán 2011, Tkaczuk et al. 2016, Ciceoi et al. 2021, Glare et al. 2021, Majchrowska-Safaryan et al. 2023].

CONCLUSION

1. The conducted studies showed that the genera and intensity of EPF occurrence were different depending on the species of the cultivated plant and the time of soil material collection for testing in soils used. In the intensive cultivation system, in both study periods, the presence of EPF of the genera *Beauveria*, *Metarhizium* and *Cordyceps* was found.

2. Entomopathogenic fungi of the genus *Metarhizium* spp. dominated in the soil constituting wasteland, while the genus *Beauveria* spp. produced the most CFU in the object where the cultivated plant was *Medicago satvia* L. When comparing the research objects where the crop plant was *Zea mays* L., it was observed that the addition of the biostimulant increased the number of fungi of the genus *Metarhizum* spp., which created more CFU in both study dates than in the object without the addition of the biostimulant.

4. By assessing the average density of infectious units of identified types of EPF in both study dates on arable land, it was found that fungi of the genus *Metarhizium* spp. formed more infectious units in the spring study date, whereas fungi of the genera *Beauveria* spp. and *Cordyceps* spp. formed more infectious units in the autumn study date and these differences were statistically significant.

5. The analysis of simple correlation coefficients between the identified genera of EPF and the content of available forms of P, K and Mg as well as pH of the tested soils did not reveal any statistically significant correlations.

6. Research conducted so far indicates that entomopathogenic fungi are an important factor in reducing insect pests in cultivated soils. Therefore, it seems important to conduct further research related to the assessment of the impact of soil biostimulants, which are increasingly used in agriculture, on the generic composition and the intensity of the occurrence of entomopathogenic fungi in the context of the diversity of cultivated plant species and their protection.

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