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The Effect of Humic Substances on the Colony Growth and Conidial Germination of Entomopathogenic Fungi from the Genus *Metarhizium*

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Abstract: The development of sustainable agricultural production involves the use of new plant protection products, with low toxicity to non-target organisms and high biodegradability. The aim of this study was to investigate the effect of commercially available preparations containing humic substances, in comparison with pure humic acids, on the growth and germination of spores of entomopathogenic fungi (EPFs) from the genus *Metarhizium* in vitro. AmiAGRA, HumiAGRA, AlgoHUM (recommended field dose) and pure humic acids extracted from peat, brown coal and spent mushroom substrate were added to Sabouraud's culture substrate. Observation of the growth of the colonies of the tested species of EPFs was carried out every 5 days until day 20, measuring their diameter (mm). In the second stage of the experiment, the culture medium with the addition of preparations and pure humic acids was applied in a thin layer to the surface of glass slides and an aqueous solution with spores was introduced. Observation was carried out after 24 and 48 h, and the results obtained were expressed as percentages in relation to the control. The conducted research showed that on the 20th day of the culture (on average), preparations containing humic substances had a stimulating effect, while pure acids limited the growth of the colonies of the tested isolates slightly. The growth of *M. anisopliae* fungal colonies was most strongly stimulated by AlgoHUM, and *M. flavoviride* by HumiAGRA. The share of germinated spores after 48 h of contact with the substrate was higher than after 24 h, and more spores germinated on substrates with the addition of preparations containing humic substances than on pure humic acids.

Keywords: *Metarhizium* spp.; colony growth rate; conidia germination; preparations containing humic substances; pure humic acids

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1. Introduction

In the European Union countries, the European Green Deal and the Farm to Fork Strategy have been introduced into agricultural practice to minimize the use of chemicals in favor of biological products, including beneficial microorganisms. As a result, numerous bioproducts have been tested and implemented in regard to crop production toward sustainable agriculture [1,2]. They are based on natural biological materials, such as plant extracts, polysaccharides and humic substances. Products containing humic substances stimulate the decomposition of organic matter introduced into the soil and, as a result, the released nutrients become available to plants more quickly [3]. Additionally, humic acids can be used as adjuvants to increase the effectiveness of biocontrol agents [4] and as carriers for microorganisms [5]. Humic substances extracted from lignite or peat are most often applied to lighter soils [6,7]. Humic acids are considered to be biocompatible and environmentally friendly [8,9].

Bioproducts containing beneficial bacteria and fungi are also used to protect plants against pests [10]. Due to their ability to directly penetrate the body of insects through the

epidermis, EPFs are relatively highly effective when integrated into pest control strategies. Their conidia adhere to the insect epidermis, germinate and penetrate the host, which eventually dies [11–13]. After the death of the insect, hyphae emerge from its corpse and produce conidiophores and conidia, infecting new hosts through horizontal transmission [12]. There has been increasing interest in microbiological products among farmers due to the decreasing number of chemical pesticides approved by the EU [14]. Mycoinsecticides, currently available in Poland, are based on three EPFs: *Beauveria bassiana* strain ATCC 74040, *Metarhizium anisopliae* var. *anisopliae* strain F52 and *Isaria fumosorosea* (=*Cordyceps fumosorosea*) strain Apopka 97. The first one has been registered to control wireworms, but only in the cultivation of eggplants, bell peppers and tomatoes grown under cover. In turn, *M. anisopliae* var. *anisopliae* strain F52 is used to control vine weevils, a species of beetle, in the cultivation of, among others, currants and strawberries. PreFeRal containing the Apopka 97 strain is used to stop whitefly from attacking plants grown under cover [15,16].

The aim of this study was to determine the effectiveness of commercially available products containing humic substances, in comparison with the effectiveness of pure humic acids, on colony growth and spore germination of EPFs from the genus *Metarhizium*.

2. Materials and Methods

2.1. Fungal Isolates

The effect of preparations containing humic substances and pure humic acids on EPF colony growth and on spore germination was investigated in laboratory conditions. Two selected species from the *Metarhizium* genus were used in the research: the fungus *M. anisopliae* (Metschn.) Sorokin (M05-UPH), isolated from the soil of a cultivated field with *Galleria mellonella* larvae in Chodów (Mazowieckie Voivodeship, Poland); and *M. flavoviride* W. Gams and Rozsypal (M06-UPH), isolated from the soil of a cultivated field with *Galleria mellonella* larvae in Samowicze (Lubelskie Voivodeship, Poland). The cultures of the fungi used in this experiment were deposited in the fungal collection of the Institute of Agriculture and Horticulture, the University of Siedlce, Poland, and stored in an SDA medium at 4 °C. They were identified macroscopically using standard keys [17,18], and their systematic affiliation was also confirmed by molecular identification. The ITS marker, proposed as a universal DNA code marker for fungi, was chosen for identification [19]. Molecular identification of isolates was carried out in the mycological laboratory at the Biological and Chemical Research Centre of the University of Warsaw, using Qiagen and Blirt tools (DNA isolation kits, PCR kit and cleaning kit). The PCR reaction was carried out according to the procedure provided by Kovač et al. [20]. Sanger sequencing was used with single ITS2, ITS3, ITS4 and ITS5 primers and the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Waltham, MA, USA), containing fluorescently labeled dideoxynucleotide triphosphates (ddNTPs), deoxynucleotide triphosphates (dNTPs), Taq-FS polymerase and buffer. After sequencing, product cleaning was conducted by molecular filtration on columns with Sephadex G-50, and the reading of the result was entrusted to Genomed (Warsaw). The sequences were compared to those available in the NCBI database using the BLASTN 2.2.2 algorithm [21].

2.2. Preparations and Pure Humic Acids Used

Preparations containing humic substances including AmiAGRA, HumiAGRA and AlgoHUM (recommended field dose) and humic acids extracted from peat, lignite and spent mushroom substrate (SMS) were used in the experiment. Based on the content of the humic substances in the AmiAGRA preparation, the dose of pure humic acids was calculated. Fractions of organic matter from peat, lignite and SMS were obtained on the basis of sequential fractionation carbon compounds. The sequential fractionation of carbon compounds was performed based on the Schnitzer method. The pure humic acids were calculated on an ash-free mass [22–24]. The characteristics of the preparations and pure humic acids used are presented in Table 1.

Table 1. Characteristics of preparations containing humic substances and pure humic acids used in the experiment.

Preparation	Chemical Composition	Farm Crop Dose
AmiAGRA Agraplant	pH—7.0–9.0, humic substances—60%, amino acids—70%, azot (N)—6%	soil application: 0.5–1 kg ha^{-1} in 250–300 L of water; foliar application: 100 g ha^{-1} in 250–300 L of water
HumiAGRA Agraplant	pH—8.0–10.0 humic substances—60%, potassium oxide (K_2O)—8%	soil application: 0.5–1 kg ha^{-1} in 250–300 L of water; foliar application: 100 g ha^{-1} in 250–300 L of water
AlgoHUM Agraplant	pH—7.8–9.8, humic substances—28% including 50% humic acids, sea algae—50%	100 g ha^{-1} in 250–300 L of water
Peat	Humic acids extracted from low peat—Liwiec river valley, pH—4.5–5.0	1.2 g on dm^3 of water
Lignite	Humic acids extracted from lignite—the Bełchatów mine, pH—4.5–5.0	1.2 g on dm^3 of water
SMS	Humic acids extracted from spent mushroom substrate—large-area mushroom farms, Siedlce district, pH—4.5–5.3	1.2 g on dm^3 of water

In the experiment, Sabouraud dextrose agar (SDA) produced by bioMérieux was used as a culture medium, with casein enzymatic hydrolyzate—5.0 g, hydrolyzed animal tissues—5.0 g, glucose—40 g and agar—15.0 g. They were all sterilized using a steam-pressure autoclave at 121 °C, under a pressure of 1 atmosphere. Preparations containing humic substances and pure humic acids were added to the culture medium prepared in this way. Then, they were transferred to sterile plastic Petri dishes, with a diameter of 90 mm.

2.3. Effect on Colony Growth

In the first part of the experiment, the selected fungal isolates were grown on the SDA medium at 21 ± 1 °C. A fragment of mycelium from 10-day-old cultures was sampled with a preparation needle, to be inoculated centrally in to the solid SDA medium. The plates with the inoculated isolates were placed in incubators, protected from light, at 22 ± 1 °C. Colony growth observations were carried out every 5 days until day 20, by measuring the colony diameter in mm. The experiment was performed in four repetitions. The control consisted of cultures grown on substrates without the addition of preparations and pure humic acids. The results are presented as the size of the colony diameter expressed in mm and in the case of the 20th day of the culture as a percentage in relation to the control.

2.4. Effect on Spore Germination

In the second part of the experiment, the effect of the preparations containing humic substances and pure humic acids on the germination of spores of the fungal isolates was investigated. Spores from 3-week-old colonies were transferred with a scalpel to an aqueous solution. Under a microscope at 400× magnification, the titer (concentration) of the spores in the solution was determined using a Fuchs–Rosenthal chamber. The spore solution was diluted to 1.0×10^7 conidia/mL, which made it easier to observe the germinating spores. The number of spores in the field of view did not exceed 20–30. Approximately 1 mL of spore solution was pipetted onto the Sabouraud medium. The slides with the medium and spores were placed in incubators, at the temperature ranges outlined above. Germination observations were carried out after 24 and 48 h. A drop of lactophenol was

added to the medium with spores, and it was covered with a cover glass. Then, in the field of view, the number of germinating spores per 100 observed conidia was counted. For each combination, i.e., EPF isolate–preparation, three replications were performed.

2.5. Statistical Analyses

The obtained results were analyzed statistically using the Statistica 13.3 TIBCO Software Inc. program. A one-way analysis of variance (ANOVA) and post-hoc Tukey test were performed. The calculated means were combined into homogeneous groups at the significance level $\alpha = 0.05$. The standard deviation was calculated (SD).

3. Results

The present research on the development of the *Metarhizium anisopliae* fungus showed that products containing humic substances added to a culture medium, according to the recommended field dose, stimulated colony growth in relation to the control on the 10th, 15th and 20th day of observation (Table 2). On each date, statistically significant differences between the size of the colonies grown on the culture media with the products added and the control colonies were found. In the initial stage (day 5), only AmiAGRA stimulated colony growth, and the results were statistically significant. HumiAGRA and AlgoHUM limited the growth only slightly. However, on the 20th day of observation, the colonies of the *M. anisopliae* fungus were the largest when grown on the culture medium with the addition of AlgoHUM, but no statistically significant differences were found between the effects of the products. On the 5th day of observation, pure humic acids limited both the growth of the control colonies and the colonies grown on the culture medium with the addition of preparations containing humic substances. In the present research, the smallest size of the strain colony was recorded when grown on the culture medium with the addition of pure humic acids extracted from lignite. During each observation, a limiting effect of humic acids extracted from peat and added to the culture medium was found on the growth of the *M. anisopliae* fungus. Compared to the control, the larger sizes were observed on the culture medium with pure humic acids extracted from spent mushroom substrate, but this difference was not statistically significant.

Table 2. Colony diameter of the fungus *Metarhizium anisopliae* during culture on media with the addition of preparations containing humic substances and pure humic acids.

Preparation	Diameter of Fungus Colony (mm)			
	5th Day	10th Day	15th Day	20th Day
Preparations containing humic substances				
AmiAGRA	18.5 ± 1.00 a	43.2 ± 0.95 a	52.5 ± 1.73 a	69.7 ± 0.50 a
HumiAGRA	15.0 ± 2.94 b	40.0 ± 0.96 b	54.3 ± 0.47 a	69.3 ± 0.47 a
AlgoHUM	14.4 ± 1.91 cd	37.3 ± 0.94 b	54.0 ± 0.83 a	70.3 ± 1.25 a
Pure humic acids				
Peat	14.5 ± 0.57 cd	30.0 ± 0.82 c	45.2 ± 1.25 d	59.7 ± 4.91 c
Lignite	13.2 ± 1.25 d	32.3 ± 0.47 c	47.6 ± 0.47 c	62.6 ± 1.88 bc
SMS *	13.7 ± 0.95 cd	31.0 ± 0.82 c	49.6 ± 0.47 b	67.0 ± 1.87 b
Control	15.3 ± 0.51 b	31.2 ± 1.47 c	46.4 ± 2.53 cd	63.7 ± 1.93 bc
p-value	0.00	0.00	0.00	0.00
F-value	19,903.56	14,337.38	103,343.70	282,408.00

* SMS—spent mushroom substrate; abcd—means within columns with the same lowercase letters are not significant at $\alpha = 0.05$, Tukey's HSD; ± standard deviation (SD).

The results of the present study indicated that when products containing humic substances were added to the culture medium, they stimulated the growth of *Metarhizium flavoviride* fungal colonies in relation to the control in each of the observation periods (Table 3). These differences were statistically significant. Growth was stimulated in the

strongest way by HumiAGRA, with colonies reaching 67.7 mm on the 20th day of observation. In the present research, on day 5 of observation, it was found that pure humic acids added to the culture medium slightly stimulated the growth of *M. flavoviride* colonies compared to the control, but without any statistically significant effect. At each observation period, *M. flavoviride* colonies grown on the culture medium with pure humic acids extracted from peat were larger than the control colonies and, also, larger than those grown on the culture media with the addition of lignite or spent mushroom substrate.

Table 3. Colony diameters of the fungus *Metarhizium flavoviride* during culture on media with the addition of preparations containing humic substances and pure humic acids.

Preparation	Diameter of Fungus Colony (mm)			
	5th Day	10th Day	15th Day	20th Day
Preparations containing humic substances				
AmiAGRA	17.2 ± 0.95 ab	34.5 ± 0.58 b	48.2 ± 2.21 c	61.5 ± 1.73 c
HumiAGRA	17.5 ± 1.29 a	36.5 ± 2.38 a	52.7 ± 2.98 a	67.7 ± 3.30 a
AlgoHUM	16.2 ± 1.50 bc	33.5 ± 1.73 b	50.0 ± 1.41 b	64.3 ± 3.29 b
Pure humic acids				
Peat	15.5 ± 0.50 cd	35.2 ± 0.95 ab	51.2 ± 1.50 b	62.5 ± 1.00 c
Lignite	15.2 ± 1.00 cd	29.0 ± 1.15 d	42.0 ± 0.81 e	53.6 ± 1.25 e
SMS *	15.2 ± 0.50 cd	31.7 ± 2.36 c	44.2 ± 3.77 d	55.0 ± 4.32 e
Control	15.0 ± 0.63 d	29.5 ± 1.37 d	44.4 ± 3.38 d	58.0 ± 1.90 d
p-value	0.00	0.00	0.00	0.00
F-value	31,142.96	55,751.52	160,742.40	276,171.30

* SMS—spent mushroom substrate; abcde—means within columns with the same lowercase letters are not significant at $\alpha = 0.05$, Tukey's HSD; ± standard deviation (SD).

The strongest limitation of the colony growth in relation to the control was on the culture medium with the addition of pure humic acids extracted from lignite, with statistically significant differences on the 15th and 20th days of observation.

On the 20th day of observation, it was found that products containing humic substances stimulated the growth of *M. anisopliae* fungal colonies more than that of *M. flavoviride* ones, and this difference was statistically significant. Pure humic acids limited the growth of the isolates, and *M. flavoviride* turned out to be a more sensitive species (Figure 1).

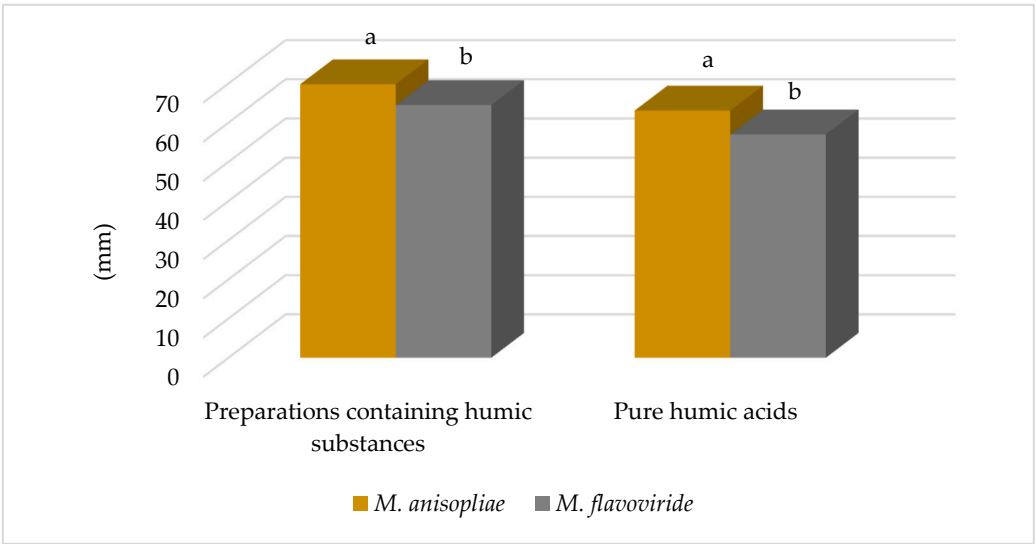

Figure 1. Average colony size on the 20th day of cultivation on media with the addition of preparations containing humic substances and pure humic acids. Notes: a,b—significant at $\alpha = 0.05$, Tukey's HSD.

The proportion of EPF germinated spores after 48 h on the culture medium was higher than after 24 h (Table 4). In a statistically significant way, pure humic acids reduced spore germination more than products containing humic substances (Table 4, Figure 2). AlgoHUM after 24 h and HumiAGRA after 48 h reduced spore germination of the strains to the smallest extent. Pure humic acids extracted from peat after 24 h and from lignite after 48 h, on the culture medium, significantly lowered the number of germinated spores of the *M. anisopliae* fungus compared to the control. The germination of *M. flavoviride* spores after 24 h was most strongly inhibited by lignite humic acids added to the culture medium.

Table 4. Germination of fungal spores on media with the addition of preparations containing humic substances and pure humic acids (in % in relation to the control).

Species/ Preparation	*Metarhizium anisopliae*		*Metarhizium flavoviride*	
	after 24 h	**after 48 h**	**after 24 h**	**after 48 h**
Preparations containing humic substances				
AmiAGRA	90.5 b	94.6 b	90.2 c	94.8 c
HumiAGRA	91.3 b	96.6 b	91.8 b	96.3 b
AlgoHUM	92.2 b	94.8 b	92.3 b	96.2 b
Pure humic acids				
Peat	76.3 c	84.2 c	70.4 e	85.7 d
Lignite	74.7 c	86.2 c	76.4 d	83.5 d
SMS *	77.1 c	88.6 c	78.3 d	84.1 d
Control	100 a	100 a	100 a	100 a
p-value	0.00	0.00	0.00	0.00
F-value	1,059,559	693,375.00	913,424.60	817,020.10

* SMS—spent mushroom substrate; abcde—means within columns with the same lowercase letters are not significant at $\alpha = 0.05$, Tukey's HSD.

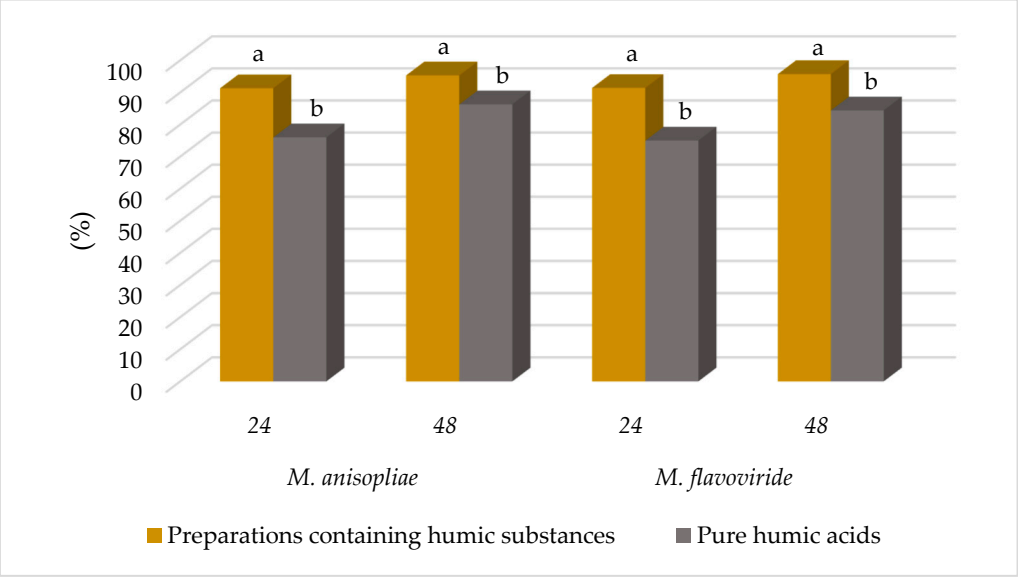

Figure 2. Germination of spores on media with the addition of preparations containing humic substances and pure humic acids. Notes: a,b—significant at $\alpha = 0.05$, Tukey's HSD.

4. Discussion

The development of sustainable agricultural production involves the use of new plant protection products, with low toxicity to non-target organisms and high biodegradability. Resulting in a reduction in environmental damage and human health risks, agrotechnical and biological control methods are currently the safest alternative to chemical plant protection products [11,25–27]. In addition to reducing the number of pests, EPFs can also

penetrate the tissue of plants, positively affecting their growth and development [2,16]. Some studies indicate that mycoinsecticides are more effective if they are used with the addition of appropriate excipients [2,28]. So far, humic substances have been studied mainly to determine their effects on bacterial and plant growth [29–31], while their effects on fungi and, in particular, on entomopathogenic fungi, have not been investigated enough [2,32,33].

Fedoseeva et al. [34] reported that humic substances act directly on living cells, with a wide range of biological effects, stimulating or inhibiting fungal growth. Tomaszewski et al. [35] and de Melo et al. [36] found that the addition of humic substances to a culture medium promoted an increase in fungal stability and resistance to stress. Raypuria et al. [37] reported that adjuvants added to preparations containing the *M. anisopliae* complex may increase the stability of its effectiveness. Testing the effect of three biofertilizers containing humic substances on the growth of the fungus *M. anisopliae*, Majchrowska-Safaryan and Tkaczuk [2] found that on the 20th day of observation all the biofertilizers limited the vegetative growth. However, there were no statistically significant differences in the sensitivity of the fungus *M. anisopliae* to the biofertilizers. Studying the effect of organic acids on the growth and development of EPFs, Li Holdom [38] concluded that they limit the vegetative growth of *M. anisopliae* mycelium. Hirose et al. [39] investigated the in vitro effect of three biofertilizers (EM-4, Multibion and Supermagro) on the *M. anisopliae* fungus and recorded a significant reduction in the vegetative growth and spore germination. The application of Multibion, one of the biofertilizers, resulted in the maximum negative effect on the vegetative growth of the fungus (43.59%), but no negative effects were found when the Supermagro biofertilizer was added to the culture medium.

Majchrowska-Safaryan and Tkaczuk [2] reported that the Rosahumus biofertilizer, added to a culture medium at the recommended field dose, on days 10 and 15 of observation did not limit the growth of the *M. flavoviride* fungus, but even stimulated it. Felizatti et al. [40] investigated the effect of various biopolymers, including humic acids, on the growth and germination of *B. bassiana* spores, and found no inhibitory effects. According to Fedosova et al. [34], the addition of humic substances to a culture medium stimulated *A. alternata* conidia production, with parallel inhibition of colony growth. When introduced into soil in the form of a biofertilizer, humic substances stimulate the release of organic acids from plant roots, and they become nutrients for beneficial microorganisms. This, in turn, enhances the growth of plant roots and their colonization by microorganisms, which is beneficial for plant and soil health [4,5,41]. Felizatti et al. [40] indicate that the inhibitory effect of humic acids is probably caused by a decrease in the pH value of the growth medium. Hirose et al. [39] found that Multibion and EM-4 significantly reduced the germination of *M. anisopliae* spores. Similarly, Gonazalez et al. [42] found that neem oil reduced the germination of *M. anisopliae* spores by 17.3%. In vitro studies conducted by Kaiser et al. [43] indicated the high potential of humic substances (above 90%) to protect *B. bassiana* spores against UV radiation, a physical factor limiting EPF conidia germination [44,45]. In addition, a significant increase in *B. bassiana* spore survival compared to the control was observed in field conditions 7 and 14 days after products containing humic acids were used [43].

The effect of humic substances and pure humic acids on the growth and germination of EPF spores has not been reported in the literature to any great extent. However, they are both increasingly being used as fertilizers, as they improve the condition of arable soils. Soils with high humic substances support plant growth, making them more tolerant to stress, healthier and able to produce higher yields with superior nutritional quality of the harvested food and feed [46]. In turn, EPFs inhabiting the soil environment form very complex relationships with other microorganisms and with plants, including crops [47–49].

In our own research, the commercial preparations used that contained humic substances had a positive effect on the growth of colonies and the germination of spores of the tested species of EPF. These preparations were characterized by higher pH values and also contained macroelements (N, K) and microelements, compared to pure humic acids [50]. The pH of pure humic acids was in the range of 4.5–5.3, which could have contributed to the

acidification of the culture medium. Sellamuthu and Govindaswamy [51], when examining the effect of different doses of humic acids with potassium on the microbiology of the soil where sugar cane was grown, found that these treatments did not affect the growth of the bacterial population, but the total amount of fungi and actinomycetes increased. This was found to the greatest extent in the root rhizosphere, by as much as 150%.

The sustainable use of plant protection products is an increasingly important issue in crop production, especially in the context of restrictions on the amount of chemicals that can be used against pests. The present research indicates that environmentally compatible products that do not harm the natural environment, such as biofertilizers containing humic substances, could be applied simultaneously with EPFs, increasing their potential effectiveness in biological pest control [2,4,52,53]. Currently, microbiological preparations containing, among others, EPFs, constitute only 2% of the entire market of plant protection products in Poland [54]. However, it is expected that their range will expand due to the legal regulations introduced by the EU in 2022 regarding registration procedures for biopesticides containing microorganisms [55,56] and the opportunity for farmers to receive subsidies under the "Biological Pest Control" program introduced from 2023 [4,14].

5. Conclusions

Research on the impact of the combined use of humic substances with isolates of EPFs for the control of insects or mites is of great practical importance because it allows the use of selected strains that can be used together with commercially available biofertilizers in plant cultivation and protection toward sustainable agriculture. The conducted research showed that on the 20th day of culture (on average), preparations containing humic substances had a stimulating effect, while pure acids slightly limited the growth of the colonies of the tested isolates. The growth of *M. anisopliae* fungal colonies was most strongly stimulated by AlgoHUM, and *M. flavoviride* by HumiAGRA. The share of germinated spores after 48 h of contact with the substrate was higher than after 24 h, and more spores germinated on substrates with the addition of preparations containing humic substances than on pure humic acids.

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