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Article

Beauveria Bassiana Water Extracts Effect on the Growth of Wheat

Dawid J. Kramski[1,4], Daria Nowinski[2,4], Kaja Kowalczuk[4], Piotr Kruszyński[4], Jagoda Radzimska[4], Beata Greb-Markiewicz[3,*]

[1] Department of Advanced Material Technology, Faculty of Chemistry, Wroclaw University of Science and Technology
[2] Department of Organic and Organic and Medicinal Chemistry, Faculty of Chemistry, Wroclaw University of Science and Technology
[3] Department of Biochemistry, Molecular Biology and Biotechnology, Faculty of Chemistry, Wroclaw University of Science and Technology
[4] Students Science Association Bio-Top, Faculty of Chemistry, Wroclaw University of Science and Technology
* Correspondence: beata.greb-markiewicz@pwr.edu.pl

Abstract: Entomopathogenic fungi perform important functions in the ecosystem as natural antagonists of insects, which can be used in agriculture. Interestingly, recent studies showed a significant promotion of tested plants growth in the presence of fungi. We hypothesize that some of various compounds produced by entomopathogenic fungi can positively affect plant development. To test this hypothesis, fungal extracts of entomopathogenic fungus *Beauveria bassiana*) were prepared at different conditions of temperature and pH. In addition to determination of the ammonium nitrogen content, the composition of extracts was analyzed by elemental ICP-OES. Then, their effect on the wheat germ growth was studied using various extract concentrations. After experiments, tested plants were measured, weighed, and the chlorophyll content was determined. Finally, the impact of extracts on the selected G+ and G- bacteria growth was examined to exclude the possibility of interference with soil microorganisms. The highest length of the wheat shoot was obtained for the use of 10-times diluted extract (10%) at pH 10 obtained at 20°C. In contrast, addition of 10% extract (pH 10) obtained at 75°C resulted in the shortest shoot. Generally, the extracts obtained at 75°C showed phytotoxic properties leading to lower values of shoot length and fresh weight in comparison to the control group. Our preliminary results are the first confirming the potential of fungal water extracts as factors promoting plant growth. Further detailed study should be carried out to confirm the effects in real environment conditions. Also, the consistency of the plant growth stimulation across different entomopathogenic fungi, and agriculturally used plant species should be tested.

Keywords: sustainability; entomopathogenic fungi; food security; fertilizer; micronutrients; organic matter; plant growth biostimulants;

1. Introduction

The United Nations stated in the 2021 progress report [1] that its climate goals set to be achieved by 2030 will not be met. It's imperative to fight climate change on all fronts, including the agricultural sector, which in Europe consumes 3.2% of total energy consumption [2]. The chemical fertilizers can make up to 50% of total energy used to produce wheat [3] or sugar beet [4]. This energy is mostly extracted from fossil fuels. To reduce the dependence on nonrenewable energy sources, a search for alternatives has begun, one being entomopathogenic fungi.

The term endophyte, used for the first time by De Bary (1866), defines microorganisms such as bacteria, fungi that occur within the plant tissues without causing any harmful symptoms to the plants. Importantly, endophytes play an important role in the plant's growth process and allows plants to better adapt to environmental conditions [5].

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Currently, it is known that fungal endophytes are ubiquitous in the world and have been detected in various plant species. Endophytic fungi (such as *Dothideomycetes* spp., among others) and tomatoes can be an interesting example of this relationship. Fungi supporting plant development are also involved in mechanisms related to survival under stress conditions [6].

Entomopathogenic fungi are usually known as exerting an adverse effect on insects and plant pathogens. For this reason, intensive studies were performed and the entomopathogenic fungi were proposed as an alternative for chemical insecticides. Despite some limitations caused by susceptibility to ultraviolet light and low moisture, some of them are currently commercially used. Highly surprising was the discovery that some species of the entomopathogenic fungi can also form symbiotic and mutualistic interactions with plants [7, 8] and can be considered as endophytes. Vega *et al.* [9] reported the presence of 16 different entomopathogenic endophytes isolated from coffee plants in several countries. Isolated fungi belong to five different genera: *Acremonium*, *Beauveria*, *Cladosporium*, *Clonostachys*, and *Paecilomyces*. It is especially interesting that *Beauveria* can also form endophytic interaction with plants, because it is one of most widely used entomopathogenic fungus in commercially available preparations. In addition to endophytic interactions, entomopathogenic fungi can also form mycorrhiza-like interactions with various plants. In general, mycorrhizal interaction aids plants to withstand stress (both biotic and abiotic) and absorb water and nutrients [8]. Both endophytic and mycorrhiza-like interactions can be very beneficial for plants, as was shown by Dara *et al.* [10] during the experiment using cabbage. Interaction with fungi not only had a positive impact on survival, but also health and growth of tested plants.

1.1. Entomopathogenic fungi as plant disease control agents

Importantly, it was shown that endophytic relations between fungi and plants are important not only in the context of pest control. Usually, fungi act as a plant bodyguard causing strong and negative effects on herbivore insects [11]. However, greenhouse experiments performed in the absence of insects, revealed a significant growth promotion of plants in the presence of fungi in normal conditions. Interestingly, increased growth was also observed in plants inoculated under salt stress conditions [12]. Entomopathogenic fungi were shown to influence the metabolism of plants that improved resistance to diseases. For example, a cauliflower plant treated with *B. bassiana* spores, presented induced resistance to *Plutella xylostella*. Larvae were not able to develop on treated leaves, despite having no preference in laying eggs on treated and non-treated leaves. Chromatographic and spectroscopic methods revealed the difference between profiles of secondary metabolites secreted by treated and untreated cauliflower, supporting the hypothesis that presence of interacting fungus leads to changes in plant metabolism [13]. It is worth to mention that *B. bassiana* can produce a wide range of secondary metabolites presenting cytotoxic, antibacterial, and antifungal activities like: beauvericin, bassianolides, oosporein, cyclosporin A and oxalic acid [14]. Also, antibacterial, and antifungal properties presented some of the identified volatile compounds of melon and cotton induced in the presence of *B. bassiana* strains: benzaldehyde, (*2Z,13E*)-octadeca-2,13-dien-1-ol, and hexadecan-4-yl 2,2,2-trifluoroacetate. This suggested the major role of fungus in the context of plant protective factors against parasites and linked to them diseases [15]. Strains of *B. bassiana* were also applied on tomato and cotton seed. In addition to successfully protecting these plants from *Rhizoctonia solani* and *Pythium myriotylum*, they caused the release of seedlings and root rot. Interestingly, the degree of protection was correlated with population density of fungi conidia on seeds [16]. Other tests carried out on maze seed coated with conidia from *B. bassiana* showed between 24-44% reduction in *Fusarium graminearum* rot root symptoms [17]. Also, promising data were obtained by Hwi-Geon Yun et. al. [18] which revealed that isolates from *Beauveria bassiana* and *Metarhizium anisopliae* display activity against *Myzus persicae* and *Botrytis cinerea*.

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Plants as living organisms exhibit various mechanisms of defence against parasites and pathogens, which can be described as age-related resistance, organ specific resistance and induced resistance (IR), which is a resistance triggered by activation of genetically programmed pathways inside plants to diminish the effect of consecutive pathogens attack [19]. Plants exhibit two forms of induced resistance: systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR can be triggered by exposing plants to both biotic and abiotic stressors, such as virulent microbes or certain chemicals, which cause accumulation of pathogenesis-related proteins and salicylic acid throughout the plant. Salicylic acid then activates the SAR gens preparing the plant for future infections. On the other hand, ISR is potentiated by non-pathogenic microbes. In comparison to SAR, ISR does not involve protein accumulation and relies on pathways regulated by jasmonate and ethylene instead of salicylic acid [20].

Genome-wide expression analysis of *A. thaliana*, whose roots were dipped in *B. bassiana* conidial suspension, provided evidence for transcriptional reprogramming, also explaining observed resistance against phytopathogen *Sclerotinia sclerotiorum*. Root colonisation caused by *B. bassiana* strains caused strain-specific changes in expression of genes related to pathogenesis, phytoalexins, jasmonate and salicylic acid pathways [21]. Changes in jasmonate and salicylic acid production were also observed in *Metarhizium brunneum* inoculated cabbage. The results indicated an increase in the production of these compounds in some plant parts colonised by the fungus [22]. These studies documented that entomopathogenic fungi can influence expression of genes regulating pathogenesis.

Based on presented results, few mechanisms of interference with plant diseases have been proposed. First mechanism involves production of antifungal and antibacterial agents (both by plants [15] and entomopathogenic fungi themselves [12, 14]]) which inhibit bacterial and fungal growth. Interestingly, some researchers suggested that the competition for space and nutrients between microorganisms and entomopathogenic fungi could lead to the suppression of plant disease. Finally, entomopathogenic fungi can lead to induction of host defence mechanisms [12]. Considering antagonistic activity of entomopathogenic fungi against insects and ability to antagonize plant diseases it would be highly beneficial to utilize fungi as dual control agents.

1.2. Entomopathogenic fungi as herbivores repellent agents

Entomopathogenic fungi can also indirectly affect herbivore populations. Through induced systemic resistance, antibiotics and other metabolites, fungi can render plants less favorable to herbivores thus contributing to plants health, longevity, and fecundity [8]. When *B. bassiana* was applied directly to the soil, strawberries colonized by fungus were healthier and were able to withstand peach aphid (*Myzus persicae*) attacks better than untreated plants [23]. Sweet pepper (*Capsicum annum*) roots drenched with *B. bassiana* and *M. brunneum* conidial suspension reduce development and fecundity of both 1st and 2nd generation of *M. persicae*. The negative effect on aphids also included prolonged development time, delayed reproduction, and reduced birth rate. In the same experiment the relationship between entomopathogenic fungi and aphid endoparasitoid *Aphidius colemani* was tested. Conducted study shows no effect on *A. colemani* – both percentage mummification and adult emergence was not affected by fungi. Also, neither differences in development time, nor percentage of females and adult longevity were observed, which leads to the conclusion that entomopathogenic fungi can be used simultaneously with parasitoids for aphid control [24].

1.3. Entomopathogenic fungi as plant growth improving agents

In addition to the previously shown functions, the ability of entomopathogenic fungi to improve nutrient uptake and plant growth was also presented [8]. Results of a study conducted on maze treated by *B. bassiana* revealed that coating maze seeds by fungi promoted plant growth: both above-ground and roots biomass was higher. This effect was only observed when nutrients were abundantly available [25]. Also, inoculation of bean

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seeds in conidial suspensions of *M. robertsii* and *B. bassiana* caused improvement in plants' growth in comparison to control plants. Treatment improved not only plants' height, but also number of leaves and fresh and dry weight of roots [26]. Another interesting experiment carried out on chive shows that plants exposed to *B. bassiana* recorded greater total alkaloid in comparison to control plants. This study demonstrates that fungi can improve yield of active chemicals in cultivated medicinal plants besides being used as growth promoting agents [27].

In most plants, nitrogen is obtained through symbiotic relationship between nitrogen-fixing bacteria and microbial decomposition process. Though nitrogen constitutes 78% of the atmosphere it is unavailable for plans, unless it is fixed by microbial symbionts or free-living bacteria, causing nitrogen to become a limiting nutrient in many natural and agricultural settings. Given the importance of nitrogen and ability of entomopathogenic fungi to form endophytic associations with many plants, a hypothesis was formed that fungi can translocate nitrogen acquired from insects' material to plants. This hypothesis was confirmed in study based on ^{15}N-labelled nitrogen. This data is evidence that entomopathogenic fungi play an important role in the nitrogen cycle [28].

Iron is essential for plant growth. The lack of iron can lead to iron deficiency – a serious disorder that affects sensitive plants. Iron ions are components of many enzymatic systems and take part in major processes, such as photosynthesis. Studies show that endophytic *B. bassiana* and *M. brunneum* can improve iron availability, increase leaf chlorophyll content, and increase root length [29].

Calcium and magnesium are also vital elements for the development of plants. The tissue analysis of grapevine infected by *B. bassiana* revealed significantly higher contents of those two minerals, than in untreated plants [30]. Study conducted on two different kinds of wheat - *Triticum durum* and *Triticum aestivum* growing on unsterilized, zinc-poor soil showed that direct application of fungi (*M. brunneum*) to the soil increased manganese uptake and grain its concentration in *T. durum*. They also observed significantly increased uptake of zinc in *T. aestivum* growing on the soil with the lowest zinc content [31].

One can also note other tests conducted on *Phaseolus vulgaris* (haricot bean) and *M. robertsii* showed that positive relationship between plants and fungi works both ways. By using $^{13}CO_2$, researchers were able to prove that carbon is translocated from plants to fungi. This evidence reveals that host plants are providing carbon to the fungus, likely in exchange for insect-derived nitrogen. That gives us more insight into this tripartite interaction between plants, fungi, and insects [32].

In this study we asked a question how interaction between the fungus *B. bassiana* and wheat can impact this plant growth and quality. We chose wheat as a model plant presenting high agricultural significance. We performed evaluation of microbial activity of extracts isolated from liquid fungal culture, followed by experiments verifying the influence of fungal inoculation for wheat germination, and the effect of obtained extracts on the composition of chlorophylls in cultured plants.

2. Results and Discussion

2.1. Multi-elemental Composition of Fungal Extracts

For the proper growth and reproduction, plants need adequate amounts of micro and macro elements, which are necessary for the life cycle and balanced plant metabolism. The required micro and macro elements, including such elements as nitrogen, calcium potassium, phosphorus, sulphur, magnesium, iron, boron, manganese, zinc, copper, nickel, and molybdenum can be delivered from the soil [33]. The effects of plant nutrient deficiencies can be manifested in various ways: inhibition of the plant's growth dynamics, deformation of the plant, the appearance of pigmentation, or complete inhibition of plant growth [34]. Importantly, the excessive delivery of nutrients to the soil can also have a negative effect on the condition of the plant [35]. The chemical composition of the tested extracts is shown in Table 1. It is worth noticing that the content of potassium (about 400 mg/L), phosphorus (over 30 mg/L) and sulphur (over 20 m/L) in all tested extracts is very high.

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Table 1. The elemental composition of *B. bassiana* extracts. The table presents the content of micronutrients, macronutrients, and toxic metals in the tested extracts (E3 - aqueous extract obtained at 20°C pH=3, EB3 - aqueous extract obtained at 75°C pH=3, E7 - aqueous extract obtained at 20°C pH=7, EB7 - aqueous extract obtained at 75°C pH=7, E10 - aqueous extract obtained at 20°C pH=10, EB10 - aqueous extract obtained at 75°C pH=10).

Element	E3	EB3	E7	EB7	E10	EB10
	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
Macroelements						
Ca	5.317	5.140	6.613	6.923	3.863	7,047
K	411.3	389.1	463.1	484.6	470.1	409,9
Mg	11.11	14.18	8.469	15.21	12.41	15,02
Na	9.875	7.057	12.40	10.22	9.927	9,520
P	34.33	45.56	27.26	52.04	50.76	44,23
S	23.54	20.35	27.00	30.88	29.35	22,43
Microelements						
B	0.275	0.119	0.159	0.082	0.050	0.048
Co	0.0015	0.0065	0.0092	< LOD	< LOD	< LOD
Cr	0.031	0.271	0.026	0.524	0.025	0.022
Cu	0.190	0.179	0.197	0.202	0.275	0.236
Fe	0.789	1.329	0.605	2.651	0.753	0.759
Mn	0.018	0.043	0.030	0.053	0.020	0.031
Mo	0.025	< LOD	0.038	0.062	0.081	0.032
Ni	0.112	0.228	0.091	0.320	0.051	0.072
Zn	0197	0.165	0.308	0.096	0.116	0.115
Toxic metals						
As	< LOD	< LOD	0.109	0.310	< LOD	< LOD
Cd	0.0043	0.0101	0.0080	0.0101	0.0071	0.0117
Pb	0.093	0.045	0.057	< LOD	< LOD	0.064

* < LOD below the Limit of Detection

Phosphorus stimulates the root growth. It also participates in the transport and storage of energy and improves the general condition of plants and increases their resistance to adverse climatic conditions. Phosphorus is also essential in the formation of organic compounds and the proper course of photosynthesis. Potassium is involved in the regulation of water management and the transport of reserve substances of the plant. It has a positive effect on photosynthesis. Potassium improves the plant's ability to withstand adverse conditions. In Table 2, the ammonium nitrogen content of the studied extracts is presented, ammonium nitrogen is an easily assimilable form of nitrogen for plants, nitrogen in plant growth is extremely important and affects many processes during plant growth [36].

Table 2. The ammonia nitrogen content of B. bassiana extracts. The table presents the content of ammonia nitrogen in the tested extracts (E3 - aqueous extract obtained at 20°C pH=3, EB3 - aqueous extract obtained at 75°C pH=3, E7 - aqueous extract obtained at 20°C pH=7, EB7 - aqueous extract obtained at 75°C pH=7, E10 - aqueous extract obtained at 20°C pH=10, EB10 - aqueous extract obtained at 75°C pH=10).

Sample	**N-NH_3 mg/kg**
E3	133.07

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EB3	27.22
E7	139.60
EB7	85.53
E10	146.17
EB10	64.13

2.2. Evaluation of Antibacterial Activity of B. bassiana Extracts

Microorganisms are one of the most important components of the soil ecosystem. A wide variety of microorganisms with beneficial, neutral, or pathogenic effects occur in the soil. They participate in providing plants with nutrients [37], stimulating plant growth and controlling or inhibiting the activity of plant pathogens [38]. Microorganisms also contribute to the improvement of soil structure by aggregating soil particles, improving soil fertility, porosity, agronomic productivity by affecting plant germination and root growth. The ability of soil bacteria to bioremediate contaminated soils, i.e., mineralization of organic pollutants, is also known [39, 40]. While bacteria and plants grow in the soil at the same time, the activity of microorganisms affects the physicochemical properties of the soil and thus affects plant growth. The introduction of antibacterial substances into the soil together with fertilizers could lead to an imbalance between the plant and soil bacteria, which could result in the deterioration of plant growth and development. To test possible interference between microorganisms, we evaluated activity of *B. bassiana* extracts against gram-positive (*Bacillus subtilis*) and gram-negative (*Escherichia coli*) bacteria strains using the agar disc diffusion method. The activity of the extracts was assessed based on the presence of the growth inhibition zones. After 24 hours of incubation no inhibition zone was observed, showing the lack of antibacterial activity of the tested extracts.

2.3. Agricultural Properties of Fungal Extracts

Previous studies related to the effects of entomopathogenic fungi on plant growth have shown promising results demonstrating the beneficial effects of entomopathogenic fungi on plant growth (shoot growth, more leaves, higher fresh weight) and their protection against infection. However, to date entomopathogenic fungi extracts were not tested as potential biostimulants for plant growth. This study aimed to test the influence of extracts obtained from *B. bassiani* on the total shoot height, dry weight, and chlorophyll content in the cultivated wheat. In the experiments, we used different dilutions (0.5%, 2.5% or 10%) of the crude extract obtained under different conditions of pH (3, 7 or 10) and temperature (25°C or 75°C). Vegetative growth enhancement may be related to the composition of the extracts, which are a rich source of macro- and microelements (Ca, K, Mg, Na, P, S, B, Cu, Fe, Zn), or amino acids and vitamins, which affect the cellular metabolism of plants, leading to increased growth and yield [41, 42, 43].

2.3.1. Total Height of Cultivated Wheat

The effect of the fungal extracts was evaluated by the wheat germination test (Fig. 1). After 14 days, the plants from each group were harvested and weighed, and then the length of the seedling was measured. For each extract (obtained at 25 and 75°C pH 3, 7, 10), plant length (N=20 from each group) was determined for all three dilutions (0.5%, 2.5% and 10%) (Fig. 2).

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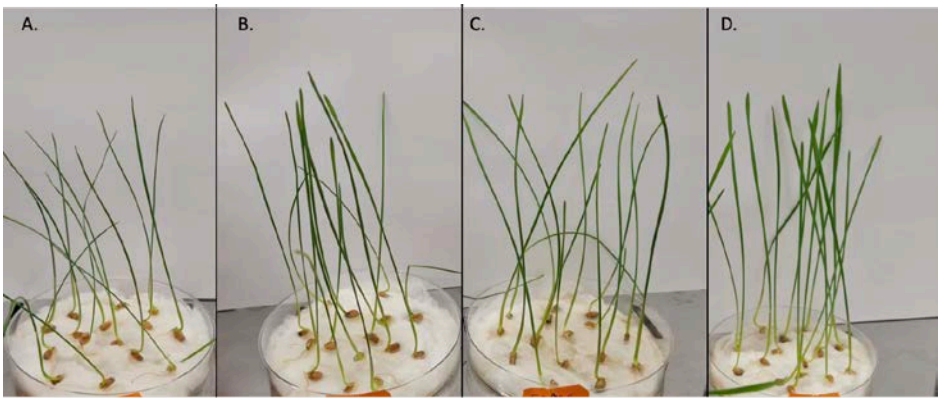

Figure 1. Wheat plants after 14 days of incubation. The control group (A) was watered only with water, while the other groups were treated with water fungal extracts obtained at 75°C: (B) pH=7, concentration 0.5%. (C) pH=3, concentration 2.5%. (D) pH=10, concentration 0.5%.

For E3, E10 extracts (E3 - aqueous extract obtained at 20°C pH=3, E10 - aqueous extract obtained at 20°C pH=1), plant height increased with increasing extract concentration. For E7, EB3, EB7, EB10 extracts (E7 - aqueous extract obtained at 20°C pH=7, EB3 - aqueous extract obtained at 75°C pH=3, EB7 - aqueous extract obtained at 75°C pH=7, EB10 - aqueous extract obtained at 75°C pH=10), treatment using the highest concentration (10%) resulted in a reduction of the plants' height. The use of EB10 extract had a negative effect on plant growth giving a lower plant height than the control group (plant length lower by 32%). The largest increase in plant height was obtained by applying E3 and E10 extract in a concentration of 10% (43% and 51% higher, respectively, compared to the control group). The increased height of treated plants is comparable to those of arbuscular mycorrhizal fungal [44], those of *Pseudomonas fluorescens* [45] or marine yeast [46]. A similar effect on wheat growth was shown in a study mitigating the effects of heat stress on wheat. In the tests 3% moringa leaf extract (MLE) along with 0.075% sorghum water extract (SWE), 0.01% salicylic acid and distilled water were used. The results of study showed that the application of stimulants such as MLE and SWE maximally improved yield factors, i.e., spike length, number of grains per spike and 1000 grain weight. Moreover, the maximum number of productive tillers and biological yield were obtained using SA and MLE [47].

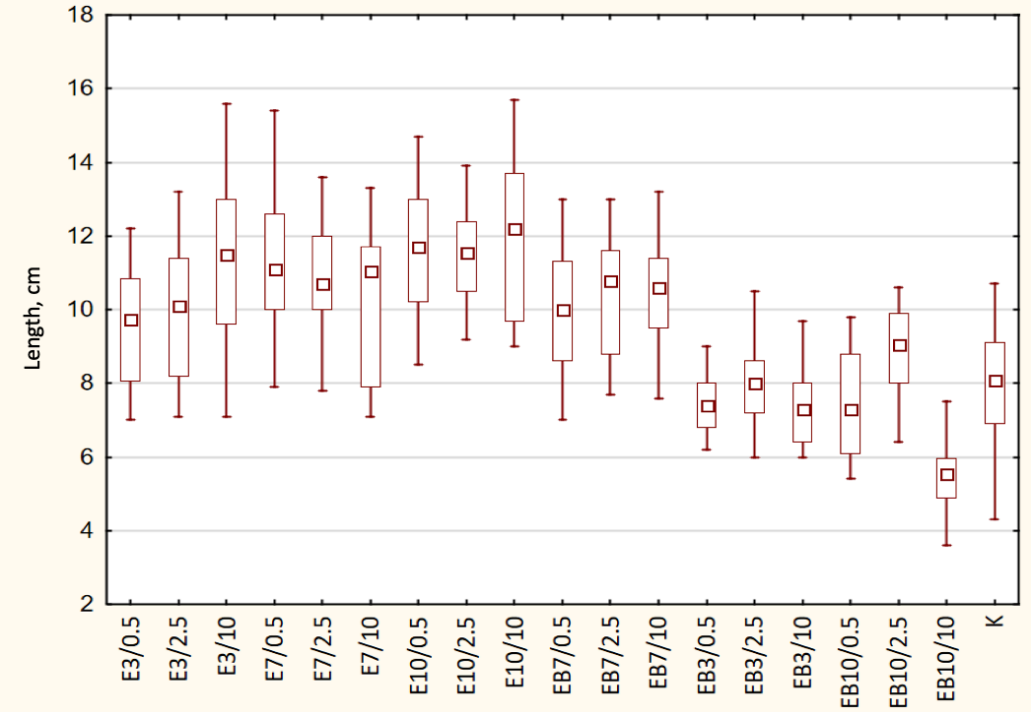

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Figure 2. Wheat length comparison - whiskers represent the maximum and minimum, the box represents interquartile range (25-75%), square denotes the median for the entire sample. K - control, E3 -group treated with the aqueous extract obtained at 20°C pH=3, E7 - group treated with the aqueous extract obtained at 20°C pH=7, E10 - group treated with the aqueous extract obtained at 20°C pH=10, EB3 - group treated with the aqueous extract obtained at 75°C pH=3, EB7 - group treated with the aqueous extract obtained at 75°C pH=7, EB10 - group treated with the aqueous extract obtained at 75°C pH=10. 0.5-concentration 0.5%, 2.5-concentration 2.5%, 10 - concentration 10%.

2.3.2. Weight of the Cultivated Wheat

In the present study, the fresh weight of plants treated with E3, E7, E10, EB7 extracts was similar and/or higher in relation to the control group. In the case of EB3 and EB10 extracts, the weight obtained was significantly lower in relation to the control group (weight lowered by 65% - 72%). The two-fold increase in dry weight of wheat sample E3/10 is comparable to those of wheat samples inoculated with arbuscular mycorrhizal fungi [48], phosphorus-solubilizing *Penicillium bilaji* [49] and in *rhizobium* spp. [50]. A study investigating the influence of *Ulva linza* and *Corallina officinalis* various concentration extracts (5,10,15,20 and 30%) on wheat, showed that concentration of used extract is highly important for obtaining satisfactory results. Researchers showed that the most effective 20% extract of *U. linza* increased germination (by 18,07%), total plant length (33,04%), total fresh weight (53,35%) and total dry weight (75,82%) of wheat seedlings. In contrast, in the case of *C. ofcinalis*, though the 20% extract increased germination by 17.23%, total plant length, total fresh weight and total dry weight were increased respectively 28.63, 34.52 and 37.91%, (compared to the control) by the 15% concentration of extract.

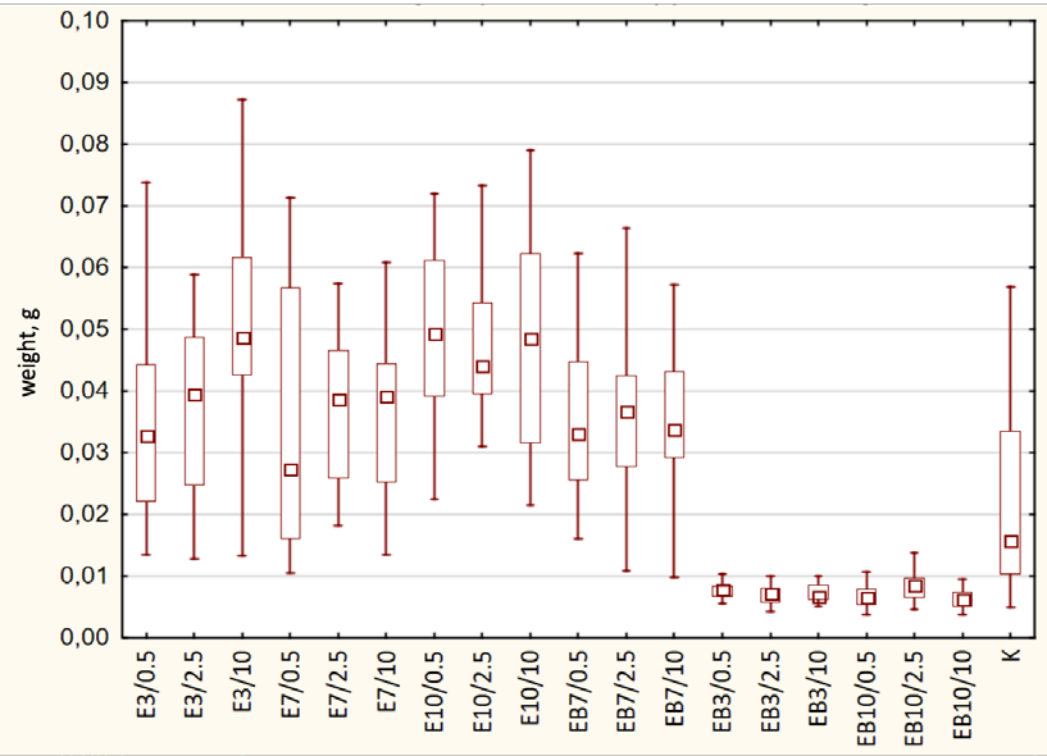

Figure 3. Wheat weight comparison - whiskers represent the maximum and minimum, the box represents interquartile range (25-75%), square denotes the median for the entire sample. K - control, E3 -group treated with the aqueous extract obtained at 20°C pH=3, E7 - group treated with the aqueous extract obtained at 20°C pH=7, E10 - group treated with the aqueous extract obtained at 20°C pH=10, EB3 - group treated with the aqueous extract obtained at 75°C pH=3, EB7 - group treated with the aqueous extract obtained at 75°C pH=7, EB10 - group treated with the aqueous extract obtained at 75°C pH=10. 0.5-concentration 0.5%, 2.5-concentration 2.5%, 10 - concentration 10%.

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2.3.3. Chlorophyll Content in the Cultivated Wheat

Chlorophyll is an important photosynthetic pigment involved in the photosynthetic reaction. The photosynthetic reaction has been the most important source of energy for the plant to grow and develop [51]. The photosynthesis reaction is carried out in 3 steps 1- primary reaction, 2- electron transport and phosphorylation, and 3- carbon assimilation. *Chl a* and *Chl b*, are necessary for the initial reaction and are both involved in the absorption of solar light at different wavelengths. Therefore, it is concluded that the sum of *Chl a* and*Chl b* as well as the ratio of *Chl a*/*Chl b* are directly related to the plant's ability to carry out photosynthesis [52, 53].

The chlorophyll content of the plants studied is shown in Table 4. For all dilutions of EB3, EB7 and EB10 extracts, the higher values of total chlorophyll *Chl a* and *Chl b* were observed. Considering the concentrations of the extracts, the chlorophyll content increased with the concentration of the respective extract (0.5<2.5<10%). In most of the cases examined, chlorophyll content was obtained higher than the control group, except for plants treated with E3 at a concentration of 10%. The high values of chlorophylls in plants from the EB3, EB7 and EB10 groups may be due to the high content of Mg and Fe in these extracts, which can affect chlorophyll synthesis.

Table 4. Evaluation of chlorophylls in the tested wheat. The table shows the chlorophyll content of the green part of the tested plants treated with the fungal extracts. C - control, E3 -group treated with the aqueous extract obtained at 20°C pH=3, E7 - group treated with the aqueous extract obtained at 20°C pH=7, E10 - group treated with the aqueous extract obtained at 20°C pH=10, EB3 - group treated with the aqueous extract obtained at 75°C pH=3, EB7 - group treated with the aqueous extract obtained at 75°C pH=7, EB10 - group treated with the aqueous extract obtained at 75°C pH=10. 0.5-concentration 0.5%, 2.5-concentration 2.5%, 10 - concentration 10%.

Sample	*Chl a*	*Chl b*	*Chl tot*	*Chl a/Chl b*
	mg/L	mg/L	mg/L	-
K	631.2	256.5	887.5	2.46
E3/0.5	971.3	361.6	1333	2.69
E3/2.5	750.3	270.1	1020	2.78
E3/10	562.5	227.2	789.5	2.48
E7/0.5	1026	320.5	1346	3.20
E7/2.5	1084	414.7	1498	2.61
E7/10	1329	510.6	1839	2.60
E10/0.5	959.2	409.3	1368	2.34
E10/2.5	821.1	316.3	1137	2.60
E10/10	582.2	262.2	844.2	2.22
EB3/0.5	2334	825.3	3159	2.83
EB3/2.5	2624	860.5	3483	3.05
EB3/10	2851	934.7	3785	3.05
EB7/0.5	1038	399.5	1437	2.60
EB7/2.5	810.7	288.3	1099	2.81
EB7/10	805.3	279.1	1084	2.89
EB10/0.5	1767	560.0	2327	3.16
EB10/2.5	2517	863.7	3379	2.91
EB10/10	1575	553.9	2128	2.84

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The role of *Chl a* in photosynthesis is preeminent over *Chl b*, which has a supporting pigment role. In all test plants, the value is above 2, indicating a good ratio of both chlorophylls [53].

3. Materials and Methods

3.1. Chemicals

All the reagents used in the experiment were an analytical grade purity. Ammonia solution, nitric acid, methanol, ethanol, acetone was purchased from POCH SA (Poland).

3.2. Fungi cultivation

The *B. bassiana* fungus was cultivated on PDB medium. First, a pre-culture was made by transferring the spores with a piece of agar (1 cm^2 slice) into a Erlenmeyer flask with 100 ml PDB liquid medium. Then, culture was cultivated for 7 days on rotary shaker (PSU-21, Biosan, Latvia) using 150 rpm at 25°C. After 7 days, 4 ml of inoculum were transferred to other flasks and cultures were cultivated in the same conditions (150 rpm, 25°C). After 13 days of cultivation, the biomass was separated from the medium by filtration using a Büchner funnel. The biomass was washed three times with 100 ml of deionized water and then dried at 50°C for 78 h.

3.3. Extracts preparation

Extraction processes were performed in two ways based on the modified procedure described by Godlewska *et al.* [54]. In the first method, the dried and milled fungal biomass (0.2 g), was added to 10 ml of deionized water with pH 3, 7, 10 respectively (marked as extract (E) E3, E7, E10). To set appropriate pH (using pHmeter Seven Multi; Mettler Toledo) ammonia solution and nitric acid were used. Next, the flasks were incubated on a magnetic stirrer for 1 hour at 25°C. The second method was a variation of previous one with the addition of heating step - 0.2g dried and milled fungal biomass was added to 10 ml deionized water with pH set to 3, 7, 10 (extracts marked respectively as EB3, EB7, EB10). Then, samples were incubated for 1 hour on a magnetic stirrer at 75°C. After extraction, all samples were filtered using Nylon 0.2 mm filters. The obtained supernatants were set as a 100% fungal liquid extract.

3.4. Multi-elemental composition of fungal extracts

Sample mineralization was done by microwave digestion system (Start D, Milestone) followed by multi-elemental analysis with inductively coupled plasma optical emission spectrometer (ICP- OES iCAP Duo Thermo Scientific, USA). Content of N-NH_4^+ was determined according to EPA 350.2 method.

3.5. Antibacterial Assays

Extracts were tested for their antibacterial activity against both gram-positive (*Bacillus subtilis*), and gram-negative (*Escherichia coli*) bacteria. To evaluate the antimicrobial activity of extracts, the agar disc diffusion test (diffusion method) was performed on model microorganisms. *E. coli* (ATCC 25922) and *B. subtilis* (ATCC:6633) were grown on Mueller-Hinton agar plates for 24 hours. Then, biomass from one or two bacterial colonies was collected and suspended in a tube containing sterile saline. The turbidity of the bacterial suspensions (expressed as optical density, OD) was measured using an optical spectrophotometer (Varian Cary 50 Conc. Instrument, Victoria, Australia) (λ = 600 nm) and adjusted to 0.25 with sterile saline. Fifty µl of the bacterial cell suspension was dispensed and spread onto agar plates. Then, filter paper discs (6 mm in diameter) were individually impregnated with 10 µl of the tested extracts. After 2 hours of drying on air, the discs were placed on the plates with bacterial cultures. After 24 hours of incubation at 37°C, the influence of the fungal extracts on the growth of both tested bacteria was assessed. A toxic effect after exposure of the bacteria to the tested extracts was determined by measuring in

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millimeters the diameter of the zone of inhibition. The experiment was carried out in triplicate.

3.6. Germination tests

The phytotoxicity of the different concentration of fungal extracts was evaluated by the wheat germination test. For each group, tests in standardized conditions using Jacobsen apparatus according to the International Seed Testing Association (ISTA) were performed. Twenty seeds were aseptically placed at equal intervals on sterile Petri dishes lined with cotton wool. After seed stratification (24 h, 4°C) each Petri dish was moistened with 10 mL distilled water. After 3 days, all dishes were treated with 5 mL different concentrations of tested extracts (samples) or water (control). The seed germination was run for 14 days at 22°C in the day-night mode (16h:8h). After two weeks of growth, each individual seedling was weighed and the root and shoot length of cucumber plants in all test groups was measured. The tests were run in three replicates. The effect of fungal extracts on plants was determined by measuring the plant's length, weight, and chlorophyll a, b, and total chlorophyll content.

3.7. Chlorophyll Content in Cultivated Plants

To test the impact of fungal extracts on the chlorophyll content, the fresh green parts of the wheat cultivated without extracts addition (control) and with addition of different extracts (samples) were used. The total chlorophyll (*Chl tot*), chlorophyll a (*Chl a*) and chlorophyll b (*Chl b*) level/amount in the extracts from the fresh green parts of wheat was determined by measuring the absorbance at the 663 nm and 645 nm wavelengths using UV-Vis spectrophotometer (Varian Cary 50 Conc. Instrument, Victoria, Australia). The extracts of chlorophylls were prepared in 80% acetone. The chlorophyll content was calculated from equations [55]:

$$\text{Chl tot}=8.02\cdot\text{Abs}663+20.2\cdot\text{Abs}(645) \quad (1)$$

$$\text{Chl a}=12.7\cdot\text{Abs}663-2.69\cdot\text{Abs}(645); \quad (2)$$

$$\text{Chl b}=22.9\cdot\text{Abs}645-4.68\cdot\text{Abs}(663) \quad (3)$$

3.8. Statistical Analysis

The results were elaborated statistically by Statistica ver 13 (StatSoft Polska Sp. Zoo, Poland). Normality of the experimental results distribution was assessed by the Shapiro-Wilk test. For distribution other than normal, the Kruskal-Wallis test was used. If distribution was normal the homogeneity of variance was tested by means of the Brown-Forsythe test, for more than two groups the difference was investigated with the Tukey test, which compares all pairs of means following on-way ANOVA). Results were considered significantly different when $p<0.05$.

4. Conclusions

Entomopathogenic fungi, such as *B. bassiana*, represent a unique source to search for alternatives of chemical fertilizers. In the present study, the stimulating effect of aqueous extracts from *B. bassiana* on the growth of tested plants was demonstrated. Importantly, we documented, that extracts contained micro and macro elements indispensable for the plant growth and expansion. Interestingly, tested extracts presented no antibacterial activity, which suggests that they would not exert an adverse effect on the soil bacteria and environment. Importantly, our study presents for the first time the positive impact of fungal extracts on the plant growth. Previous tests were performed with *B. bassiana* conidia suspension added to soil or hydroponic culture, or alternatively, used in seed coating methods with fungal spores [56, 57]. The use of aqueous extracts of *B. bassiana* biomass,

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may be a positive alternative that will not cause public concern about the presence of spores in the plant and possible effects on human health [58, 59]. Additional research needs to be carried out to optimize the extract dissolution ratio, confirm the effects in real environment conditions and to check the consistency of the plant growth stimulation across different plant species.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

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