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Biostimulants Managed Fungal Phytopathogens and Enhanced Activity of Beneficial Microorganisms in Rhizosphere of Scorzonera (*Scorzonera hispanica* L.)

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Abstract: The principles of good agricultural and horticultural practice, considering both environmental protection and high yielding of plants, require modern cultivation methods. In modern agriculture, it is possible to use biostimulants that protect the soil against degradation and plants against phytopathogens and stress. The purpose of 3-year field and laboratory studies was to determine the effect of *Trichoderma harzianum* T-22 and other biostimulants on the health status of scorzonera (*Scorzonera hispanica* L.) plants and microorganism populations in the rhizosphere of this plant. For this purpose, Biosept Active (a.s.—grapefruit extract), Timorex Gold 24 EC (based on tea tree oil), Trianum P (spores of *Trichoderma harzianum* Rifai T-22) and Zaprawa Nasienna T 75 DS/WS fungicide (a.s.—tiuram) were applied for the pre-sowing seed dressing of scorzonera cv. "Duplex". The number of seedlings and the health status of scorzonera plants were determined during three growing seasons. In each year of the study, mycological analysis of seedling roots and roots after scorzonera harvest was conducted to establish the quantitative and qualitative composition of fungi colonizing these parts. Moreover, microbiological analyses of scorzonera rhzisphere soil were conducted and served as the basis to determine the total population of fungi and bacteria (including *Pseudomonas* sp. and *Bacillus* sp.). Antagonistic activity of rhizosphere bacteria *Pseudomonas* sp., *Bacillus* sp. and fungi was determined based on laboratory tests on selected scorzonera soil-borne fungal pathogens (*Alternaria scorzonerae, Fusarium culmorum, Fusarium oxysporum,* and *Rhizoctonia solani*). The experiments showed that Trianum P most effectively protected the roots of scorzonera against infection by *Alternaria alternata, A. scorzonerae*, *Neocosmospora solani*, *Fusarium* spp., *Sclerotinia sclerotiorum*, *Rhizoctonia solani,* and *Botrytis cinerea.* The rhizosphere population of *Bacillus* sp. and *Pseudomonas* sp. in the treatments with Trianum P or Zaprawa Nasienna T 75 DS/WS was larger than in the other experimental treatments. A reverse relationship was observed in the population of rhizosphere fungi. The application of grapefruit extract, tea tree oil and *Trichoderma harzianum* T-22 increased antagonistic activity of *Pseudomonas* sp., *Bacillus* sp. and selected saprotrophic fungi against soil-borne fungal pathogens, especially *Alternaria* sp., *Rhizoctonia* sp., and *Fusarium* sp. In summary, Biosept Active, Timorex Gold 24 EC and Trianum P can be recommended as plant biostimulants in *Scorzonera hispanica* cultivation.

Keywords: scorzonera; organic farming; biostimulants; beneficial soil microorganisms; antagonistic bacteria and fungi

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1. Introduction

High human awareness of the need to lead a hygienic lifestyle, including healthy eating, and the necessity to protect the environment, require modern agriculture and horticulture to apply sustainable farming and plant cultivation methods. Too intensive cultivation practices reduce soil fertility and its biodiversity [1–3]. They lead to the accumulation of harmful microorganisms, including phytopathogens and pesticides and

their derivatives in soils and plants [4,5]. Suitable conditions for the growth and development of plants can be achieved by maintaining high activity and biodiversity of beneficial soil microorganisms, by more complex agricultural management, and by applying biological plant protection [6–9]. In modern agriculture, it is possible to use biostimulants that protect the soil against degradation and plants against phytopathogens and stress.

Scorzonera (*Scorzonera hispanica* L.), is a little known root vegetable that belongs to the family Asteraceae, and it is rarely cultivated worldwide [10,11]. Wild-growing forms of scorzonera occur in Southern Germany and France, Spain, and the Caucasus [10]. These include the following species: *Scorzonera humilis* L., *Scorzonera purpurea* L. and *Scorzonera rosea* W.K. In Turkey, *Scorzonera sandrasica* Hartvig and Strid, *Scorzonera pisidica* Hub.-Mor., *Scorzonera gokcheoglui* Ünal and Göktürk and *Scorzonera longiana* Sümbül can be encountered [12]. The cultivated species *Scorzonera hispanic*a L. deserves to be disseminated among consumers and producers due to its high biological value [10,12]. It can successfully enrich the assortment of fresh vegetables in the early spring and winter [10]. In addition to the extremely valuable inulin, the roots of scorzonera contain carbohydrates, glycosides (choline, asparagine, lactucine and coniferin), polyphenolic acids, vitamins (vitamin C, riboflavin, thiamine and niacin), and minerals (magnesium, calcium, iron, phosphorus, potassium, and sodium) [10,11,13]. Scorzonera and its products are effective in the prevention and treatment of cardiovascular, gastrointestinal, kidney, diabetes, and even cancer diseases [11,14].

These numerous health-promoting and healing properties of scorzonera should encourage farmers to cultivate this plant species. This is associated with the necessity to obtain a large and good-quality root yield [10,15]. The health-promoting properties of root vegetables, including scorzonera, is determined, among others, by physical and biochemical properties of the arable environment [10,16] and biodiversity of soil microorganisms, especially the presence of soil-borne pathogens [6,17,18]. Despite the relatively rare cultivation of scorzonera, information about infectious agents that threaten this species is available in the literature. According to Loerakker [19], alternariosis of *Scorzonera hispanica* is caused by *Alternaria scorzonerae.* Downy mildews of the genera *Plasmopara* and *Bremia* may occur on the above-ground plant organs [20]. Soil-borne fungi such as *Alternaria alternata, Fusarium oxysporum, F. culmorum, Neocosmospora solani,* and *Rhizoctonia solani* infect seedling roots as well as the roots of older scorzonera plants [21,22]. Proper soil microbiological activity promotes the growth and development of scorzonera, at the same time protecting its roots from contamination by soil-borne pathogens. Therefore, appropriate methods of growing *Scorzonera hispanica* should be used, which would limit its infestation by phytopathogens. Such conditions can be obtained by using biostimulants, PGPMs (plant growth-promoting microorganisms) and PGRs (plant growth regulators) in plant cultivation [6,23,24]. They protect the soil from degradation and plants from phytopathogens and stress [23,25].

According to Ricci et al. [26], a plant biostimulant is: "A product stimulating plant nutrition processes independently of the product's nutrient content, with the aim of improving one or more of the following characteristics of the plant: Nutrient use efficiency, tolerance to abiotic stress, crop quality traits or availability of confined nutrients in the soil and rhizosphere". Moreover, high effectiveness of biostimulants (especially those based on *Trichoderma*) was demonstrated by many authors in plant protection against pathogens [27,28]. Biostimulants increasing the tolerance of plants to abiotic and biotic stress [27–29] also include microorganisms that induce plant resistance to pathogens and modify the composition of soil microorganisms and microorganisms colonizing underground plant organs [25,28,30]. The use of natural preparations is particularly important due to the need to minimize the harmful effects of chemization and to preserve the biodiversity of agroecosystems.

The main active substances used in such preparations are beneficial fungal and bacterial agents, protein hydrolysates, fulvic and humic acids, salicylic acid, seaweed extracts, and compounds containing nitrogen [20–32]. Useful microorganisms applied as

biostimulants include, among others, bacteria: *Pseudomonas* spp., *Bacillus* spp., *Arthrobacter* spp., *Enterobacter* spp., *Rhodococcus* spp., *Ochrobactrum* spp., *Serratia* spp. [23,33–35] and fungi: *Trichoderma harzianum*, *Trichoderma reesei*, *Trichoderma atroviride*, *Heteroconium chaetospira*, *Claroideoglomus etunicatum,* and *Glomus intraradices* [6,25,36–38]. These microorganisms, inhabiting the rhizosphere soil and colonizing plant roots, belong to the PGPM (plant growth-promoting microorganisms) group [6,23,24,33–35]. Among them, we can distinguish PGPR (plant growth-promoting rhizobacteria) [23,24,33] and PGPF (plant growth-promoting fungi) [6,25,38]. PGPMs also have the ability to limit the growth and development of phytopathogens.

Phytopathogenic bacteria and fungi can be effectively reduced in the soil environment by PGPRs and PGPFs, which produce substances toxic to various pathogens. These include antibiotics, siderophores and hydrolytic enzymes (chitinases, β-1,3-glucanases, β-1,6-glucanases, proteases, cellulases) [39–43]. These substances break down the hyphae and spores of various phytopathogens [44,45]. Such abilities were demonstrated, among others, by *Pseudomonas* spp., *Bacillus* spp., *Clonostachys* spp., *Myrothecium* spp., or *Trichoderma* spp. [6,40–42]. The antagonistic activity of *Trichoderma* sp. towards other microorganisms is based on antibiosis, competition, and mycoparasitism [43–47].

Beneficial microorganisms and natural substances are used in the production of commercial preparations recommended for the cultivation of various plant species [6,7,27.28]. Such preparations include, among others, Biosept Active (a.s.—grapefruit extract), Trianum P (containing *Trichoderma harzianum* Rifai T-22 spores) and Timorex Gold 24 EC (based on tea tree oil) [6,48,49]. Tea tree oil is an extract obtained from *Melaleuca alternifolia* L. [48–50]. Its components (1,8-cineole, gamma-terpinene, terpinen-4-ol, p-cymene and sesquiterpenes) have antiseptic properties [50,51]. Tea tree oil breaks down cell membranes and organelles of phytopathogenic bacteria and fungi [48,52]. It effectively controls fungi of the genera *Botrytis*, *Fusarium*, *Penicillium*, *Aspergillus*, *Colletotrichum,* and *Alternaria* [53,54]. Extract from grapefruit pulp and seeds (*Citrus paradisi* Macfad.) contains glycosides (mainly naringin), endogenous flavonoids, coumarins, furanocoumarins, and terpenes [49,55]. They have antiviral, antibacterial and antifungal properties. They inhibit spore germination, growth of infectious hyphae, and mycelium development [52,56]. Grapefruit extract exhibits high efficiency in protecting ornamental plants and vegetables from fusariosis mold (*Fusarium* spp., *Fusarium oxysporum* f. sp. *cyclaminis*), alternariosis (*Alternaria* spp.), phytophtoriosis (*Phytophthora cinnamomi*, *P. cryptogea*, *P. infestans*), and gray mold (*Botrytis cinerea*) [7,48,57–60].

The aim of the study was to establish the effect of biostimulants (grapefruit extract, tea tree oil and *Trichoderma harzianum* Rifai T-22) on the antagonistic activity of microorganisms in the rhizosphere soil of scorzonera (*Scorzonera hispanica* L.) and on the health status of this plant in field cultivation.

2. Materials and Methods

2.1. Field Experiment

The experiments were carried out in 2014–2016 in south-eastern Poland (Lublin region; 51°23′ N, 22°56′ E), on Haplic Luvisol soil formed from silty medium loams. The subject of the research was scorzonera (*Scorzonera hispanica* L.) cv. "Duplex" cultivated on ridges. The experiment involved scorzonera cultivation after winter wheat (forecrop). Disking was performed after wheat harvest, and deep ploughing before winter (about 25 cm). Before scorzonera sowing, the soil contained 1.06–1.15% of humus in the 0–20 cm depth and was characterized by slightly acidic (pH in 1 M KCl—5.76–5.90). The amount of available phosphorus, potassium, and magnesium was as follows: P—146.8; K—111.5; Mg—102.9 mg/kg soil. NPK mineral fertilization was applied in the spring in the amount of: 100:50:100 kg/ha. Cultivator treatment and harrowing was performed after the application of mineral fertilizers. The experiment was set up as a completely randomized block design in 4 replicates. The area of each experimental plot was 14 m^2. The experi-

ment was established in the first 10-day period of May. Scorzonera seeds were sown to a depth of 3 cm, in rows every 50 cm, in the amount of 12 kg/ha. The plants were harvested in the second half of October.

Biostimulants and a fungicide were used for pre-sowing treatment of scorzonera seeds. These were the following preparations: Biosept Active (based on grapefruit seed and pulp extract) produced by Cintamani Poland; Timorex Gold 24 EC (based on essential tea tree oil) produced by Biomor Israel Ltd., Katzerin, Israel; Trianum P (containing spores of *Trichoderma harzianum* Rifai T-22) produced by Koppert BV, Veilingweg, Netherlands and the fungicide Zaprawa Nasienna T 75 DS/WS (a.s.—tiuram 75%) produced by Organika-Azot in Jaworzno, Poland. Untreated seeds were considered controls. The preparations were applied according to the manufacturers' recommendations: Biosept Active—10 mL/kg seeds, Trianum P—50 g/kg seeds, Timorex Gold 24 EC—150 mL/kg seeds and fungicide Zaprawa Nasienna T 75 DS/WS—5 g/kg seeds. During three growing seasons both scorzonera plants and their rhizosphere soil were analyzed. The number of scorzonera plants on individual plots and the proportion of plants with visible disease symptoms were determined in each year of the study at the BBCH 14–15 stage (4–5 leaf stage).

Both scorzonera plants with necrosis symptoms on the roots and the infected roots obtained after scorzonera harvest were subjected to laboratory mycological analysis. This analysis, described in detail in Section 2.2, was performed in accordance with the methods used in mycological and phytopathological research. Mycological analysis allowed to determine the quantitative and qualitative composition of the fungi colonizing scorzonera roots. In parallel, microbiological analysis of the rhizosphere soil, i.e., soil directly adhering to the roots of the tested plants, was performed. This analysis is described in detail in Section 2.3 and it was also performed in accordance with the methods applied in mycological and phytopathological research. The number of rhizospheric populations of fungi and bacteria, including *Pseudomonas* and *Bacillus* bacteria, was determined based on the microbiological analysis of the soil. Moreover, the isolated rhizosphere fungi were identified to the genus and species. The next stage of the research were laboratory tests, which determined the antagonistic effect of rhizobacterial isolates (*Pseudomonas* sp. and *Bacillus* sp.) and the influence of selected saprotrophic rhizosphere fungal species on selected fungi pathogenic for scorzonera. The studied saprophotophic fungi included the following species: *Acremonium rutilum*, *Albifimbria verrucaria, Penicillium chermesinum*, *Penicillium decumbens*, *Penicillium simplicissimum*, *Clonostachys rosea, Talaromyces flavus*, *Trichoderma* sp. and *Trichothecium roseum*. The tested pathogenic fungi included *Alternaria scorzonerae, Fusarium culmorum, Fusarium oxysporum* and *Rhizoctonia solani*. These laboratory tests determining the antagonistic activity of the studied rhizosphere fungi and bacteria were performed in accordance with the methodology used in phytopathological studies. These methods are described in detail in sections 2.4 (antagonistic activity of rhizosphere fungi) and 2.5 (antagonistic activity of rhizosphere bacteria).

2.2. Mycological Analysis of Plants

In each growing season, the health status of scorzonera plants and roots was determined. According to the method described for *Daucus carota*, 50 scorzonera seedlings (BBCH 14-15) with disease symptoms were randomly collected for laboratory mycological analysis from each variant of the experiment. After scorzonera harvest, 50 randomly selected roots (BBCH 49) with necrotic and etiological signs were also analyzed. The mycological analysis was conducted according to the method described by Patkowska et al. [6] for carrot. On this basis, the species and quantitative composition of fungi infecting scorzonera roots was determined at different development stages, i.e., BBCH 14-15 and BBCH 49.

Mycological analysis: The infected scorzonera roots were rinsed for 30 min under running tap water, subsequently disinfected in 1% sodium hypochlorite. Surface-disinfected plant material was rinsed three times for three minutes in sterile distilled

water. Three-millimeter fragments were cut from the thus prepared plant material and placed in 9-cm sterile Petri dishes on SNA (selective nutrient agar) medium with the following composition: 38 g saccharose, 0.7 g NH_4NO_3, 0.3 g KH_2PO_4, 0.3 g $MgSO_4 \times 7H_2O$, 20 g agar and trace quantities of $FeCl_3 \times 6\ H_2O$, $ZnSO_4 \times 7\ H_2O$, $CuSO_4 \times 7\ H_2O$ and $MnSO_4 \times 5\ H_2O$ In each of the experimental variants, 100 fragments of infected scorzonera roots were examined. After 10–12 days, fungal cultures were transferred to sterile Petri dishes with PDA (potato dextrose agar) medium and incubated at 20–22 °C, with 12 h light/12 h dark cycles. After 14–24 days, fungal colonies were identified to the genus and species level (morphological structures: Mycelium, conidiophores and conidia) under a microscope, based on the keys and monographs listed by Patkowska and Krawiec [61]. Moreover, fungi of the genus *Penicillium* were identified on Czapek-Dox and Malt media. SNA and PDA media were used for *Fusarium* sp. The number and percentage of occurrence of the recovered fungal species were calculated.

2.3. Microbiological Analysis of Rhizosphere Soil

Microbiological laboratory analysis of rhizosphere soil was performed according to the method described by Patkowska et al. [6] for carrot, and by Czaban et al. [62] for winter wheat. For this purpose, ten weeks after sowing scorzonera seeds, 10 plants were dug out of each experimental plot, i.e., 40 plants in each variant of the experiment. The soil directly adhering to scorzonera roots (i.e., rhizosphere soil) was shaken off into sterile Petri dishes. Under sterile laboratory conditions, 10 g of the soil was weighed from each soil sample for further analysis (4 replicates for each experimental treatment).

Soil solutions were prepared in laboratory conditions from 10 g weighed amounts with dilutions from 10^{-1} to 10^{-7}, according to the method described by Patkowska [63]. Martin's medium (10 g glucose, 5 g peptone, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \times 7H_2O$, 30 mg streptomycin, 30 mg bengal rose, 15 g agar) was used to determine the total number of fungi. The total size of bacterial population and the abundance of bacteria from the genera *Pseudomonas* and *Bacillus* were determined on nutrient agar (Pseudomonas agar F and Tryptic soy agar, respectively). For *Bacillus* sp. isolation, soil dilutions were heated for 20 min at 80 °C. After incubation (2–5 days at 20–22 °C), the number of fungal and bacterial colonies was determined and converted into CFU/g soil DW (colony forming units/g soil dry weight). The obtained fungal colonies were transferred to sterile Petri dishes with PDA medium and incubated for the next 2–3 weeks at 20–22 °C. After that time, the fungi were microscopically determined to the genus and species based on the keys and monographs. The number of obtained species of fungi was calculated.

2.4. Antagonistic Activity of Rhizosphere Fungi from Scorzonera Cultivation

Antagonistic effect of selected saprotrophic fungi isolated from the scorzonera rhizosphere (*Acremonium rutilum*, *Albifimbria verrucaria*, *Penicillium chermesinum*, *Penicillium decumbens*, *Penicillium simplicissimum*, *Clonostachys rosea*, *Talaromyces flavus*, *Trichoderma* sp., and *Trichothecium roseum*) against selected pathogenic fungi for this plant (*Alternaria scorzonerae*, *Fusarium culmorum*, *Fusarium oxysporum* and *Rhizoctonia solani*) was determined based on the methods described by Jamiołkowska et al. [64] and Patkowska and Błażewicz-Woźniak [65]. For this purpose, laboratory tests were carried out *in vitro* using Petri dishes with sterile PDA (potato dextrose agar) medium. In the central part of the dish, two 3-mm fungi inocula were grafted 2 cm apart. Colonies of the studied fungi grown from one 3-mm inoculum, grafted in the middle of the dish served as controls. The cultures were grown in an incubator at 24 °C. The biotic effect was established after 10 days of growth. Each experimental variants included 4 dishes, which were treated as replicates.

The antagonistic activity is expressed as the individual biotic effect (IBE), i.e., the effect of one isolate of a given species on pathogens. IBE multiplied by the species frequency gives the general biotic effect (GBE), considered as the effect of all isolates on the pathogen. The summary biotic effect (SBE) is obtained after adding all GBEs. The SBE

was a measure of antagonistic activity of saprotrophic fungi against the studied pathogens in the scorzonera rhizosphere. It was calculated for each variant of the field experiment.

2.5. Antagonistic Activity of Pseudomonas sp. and Bacillus sp. Rhizobacteria from Scorzonera Cultivation

The antagonistic activity of rhizobacteria was determined using research methods applied for *Daucus carota* and described by Patkowska and Błażewicz-Woźniak [65]. Considering the variants of the field experiment, isolates of *Pseudomonas* sp. and *Bacillus* sp. obtained from the scorzonera rhizosphere were tested each year of the study against frequently isolated fungi pathogenic for this plant, such as *Alternaria scorzonerae, Fusarium culmorum, Fusarium oxysporum,* and *Rhizoctonia solani*. The experiments were carried out in Petri dishes with PDA (potato dextrose agar) medium, onto which, individual bacterial isolates were inoculated in the central part of the dish in the form of two parallel lines, 4 cm apart. The fungus was inoculated in the center of the dish after two days of incubation of bacteria at 22 °C. The inoculum consisted of 3-mm mycelium-overgrown agar discs cut from 14-day-old fungal cultures grown on PDA at 24 °C. The controls were fungal colonies growing in Petri dishes with PDA medium without the bacterial isolate. Four replicates were included for each bacterial isolate and control. Further incubation was conducted at 24°C. After 10 days of co-culture, the inhibition zone and diameter of the fungus colony were measured (in mm) and the percentage of growth inhibition relative to the control colony was calculated.

The antagonistic effect of bacteria was determined on the basis of a five-point scale, taking into account the inhibition zone of fungal colony growth. These were: 0°—no inhibition zone, 1°—1–2 mm inhibition zone, 2°—3–5 mm inhibition zone, 3°—6–10 mm inhibition zone, and 4°—inhibition zone over 10 mm. The second factor determining the antagonistic abilities of bacteria was the inhibition of fungal colony growth. A five-point scale was also used for this purpose: 0°—no fungus growth inhibition, 1°—colony growth inhibited to 20%, 2°—colony growth inhibited to 50%, 3°—colony growth inhibited to 80%, 4°—colony growth inhibited to 100%. The value of the individual antagonistic activity (A) of each bacterial genus multiplied by the number of isolates was defined as general antagonistic activity (B). The total antagonistic activity of *Pseudomonas* sp. and *Bacillus* sp. against *Alternaria scorzonerae, Fusarium culmorum, Fusarium oxysporum,* and *Rhizoctonia solani* was calculated for each variant of the field experiment.

2.6. Statistical Analysis

Results concerning the density of scorzonera plants, health status and the number of rhizosphere fungi and bacteria were statistically analyzed. The means were compared to the use of the least significant differences based on the Tukey's test ($p \leq 0.05$). Statistical calculations were carried out using Statistica, version 7.1 (StatSoft, Krakow, Poland).

3. Results

The emergence and health of scorzonera seedlings after the application of biostimulants was significantly better than in the control (Table 1). The number of scorzonera seedlings in all experimental treatments in individual years of the study ranged from 28.5 to 56 plants/m^2. The highest number of seedlings was found after the application of Trianum P and the fungicide Zaprawa Nasienna T 75 DS/WS (52.2 and 46.5 plants/m^2, respectively). The lowest number of plants grew on the control plots (32.3 plants/m^2). Biostimulants and the fungicide significantly reduced the occurrence of diseased scorzonera plants. The highest number of infected seedlings was found in control, and the lowest after the application of Trianum P and Zaprawa Nasienna T 75 DS/WS.

Table 1. Field stand of scorzonera seedlings.

Experimental Treatment	Field Stand Per 1 m²			
	2014	2015	2016	Mean
Biosept Active	38.5 b	47.0 b	46.5 b	44.0 b
Timorex Gold 24 EC	36.0 b	44.5 b	43.0 b	41.2 b
Trianum P	48.0 a	52.5 a	56.0 a	52.2 a
Zaprawa Nasienna T 75 DS/WS	40.5 b	50.0 a	49.0 a	46.5 b
Control	30.0 c	38.5 b	28.5 c	32.3 c

Values in columns followed by the same letter do not differ significantly at $p \leq 0.05$.

Young scorzonera plants with symptoms of root necrosis were present in all experimental combinations (Figure 1). Similar necrosis symptoms and etiological signs in the form of hyphae, spores or sclerotia were observed on scorzonera roots after harvest (Figures 2–4).

Figure 1. Necrosis on the roots of scorzonera seedlings; (photo by E. Patkowska).

Figure 2. Scorzonera roots after harvest; (photo by E. Patkowska).

Figure 3. Mycelium and conidia of *Penicillium* sp. on the scorzonera root (scale 3:1); (photo by E. Patkowska).

Figure 4. Mycelium and sclerotia of *Sclerotinia sclerotiorum* on the scorzonera root (scale 3:1); (photo by E. Patkowska).

As a result of mycological analysis, 1289 colonies of fungi belonging to 13 genera were isolated from the infected scorzonera seedlings (Table 2). After applying Trianum P or the fungicide, more than twice as much fungi was obtained than from the control plants. Slightly more fungi were isolated when Biosept Active or Timorex Gold 24 EC biostimulants were applied. Among the fungi considered to be pathogenic, *Fusarium oxysporum* and *Rhizoctonia solani* were predominant, and their proportion was 18.9% and 15.1%, respectively (Table 2). Moreover, scorzonera seedlings were colonized mainly by *Alternaria scorzonerae* (8.6% all isolates), *Alternaria alternata* (6.6%), *Fusarium culmorum* (9.3%), *Neocosmospora solani* (6.3%), and saprotrophic species such as *Penicillium janczewskii* (4.5%) and *Talaromyces flavus* (4.8%).

Table 2. Fungi isolated from diseased scorzonera seedlings (sum 2014–2016).

Fungi	Number of Isolates/Experimental Treatment					
	I *	**II**	**III**	**IV**	**V**	**Total (%)**
Acremonium rutilum W. Gams	5	7	3	4	9	28 (2.2)
Alternaria alternata (Fr.) Keissler	17	21	8	11	27	84 (6.6)
Alternaria scorzonerae (Aderh.) Loer.	22	28	10	15	36	111 (8.6)
Botrytis cinerea Pers.	6	9	2	4	11	32 (2.5)
Cladosporium herbarum (Pers.) Link	7	9	3	5	12	36 (2.8)
Cylindrocarpon didymum (Harting) Wollenw.	10	12	4	7	16	49 (3.8)
Clonostachys rosea (Link) Schroers, Samuels, Seifert	2	2	3	-	-	7 (0.5)
Fusarium culmorum (W.G.Sm.) Sacc.	23	29	14	18	36	120 (9.3)
Fusarium oxysporum Schl.	46	54	32	38	74	244 (18.9)
Fusarium graminearum Schwabe	7	9	4	5	10	35 (2.7)
Neocosmospora solani (Mart.) L. Lombard and Crous	16	21	6	10	27	80 (6.3)
Hyalocylindrophora rosea (Petch) Réblová and W. Gams	9	11	4	6	14	44 (3.4)
Penicillium janczewskii K.W. Zaleski	12	14	6	8	18	58 (4.3)
Penicillium simplicissimum (Oudem.) Thom	7	9	3	5	10	34 (2.6)
Penicillium verrucosum Dierckx	6	8	4	5	10	33 (2.6)
Rhizoctonia solani J.G. Kühn	37	42	27	31	57	194 (15.1)
Talaromyces flavus (Klöcker) Stolk and Samson	12	15	6	9	19	61 (4.8)
Trichoderma sp.	12	12	15	-	-	39 (3.0)
Total isolates	256	312	154	181	386	1289 (100)

I *—Biosept Active, II—Timorex Gold 24 EC, III—Trianum P, IV—Zaprawa Nasienna T 75 DS/WS, V—control.

After harvest, 1664 colonies of fungi belonging to 14 genera were obtained from scorzonera roots with disease symptoms (Table 3). Trianum P and Biosept Active most effectively protected the roots of scorzonera against soil-borne pathogen infections. Timorex Gold 24 EC showed a slightly lower effectiveness. *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Fusarium culmorum,* and *Alternaria scorzonerae* were most commonly isolated from diseased scorzonera roots, and their percentage was 21.4%, 18.8%, 11.9%, 8.2%, and 7.9%, respectively (Table 3). The roots of the studied plants were colonized to a lesser extent by *Penicillium aurantiogriseum*, *Penicillium canescens*, *Cladosporium cladosporioides*, *Neocosmospora solani* and *Trichothecium roseum*. Additionally, fungi of the genera *Botrytis*, *Aureobasidium*, *Cylindrocarpon,* and *Rhizopus* were identified. Biostimulants also contributed to the colonization of scorzonera roots by *Clonostachys rosea* and *Trichoderma* sp. These microorganisms, known for their antagonistic properties, were not obtained from control or fungicide-protected plants.

Table 3. Fungi isolated from diseased scorzonera roots after harvest (sum 2014–2016).

Fungi	Number of Isolates/Experimental Treatment					
	I *	**II**	**III**	**IV**	**V**	**Total (%)**
Alternaria alternata (Fr.) Keissler	9	11	5	7	13	45 (2.7)
Alternaria scorzonerae (Aderh.) Loer.	26	32	13	21	40	132 (7.0)
Aureobasidium pullulans (de Bary and Löwenthal) G. Arnaud	6	8	2	4	10	30 (1.8)
Botrytis cinerea Pers.	8	11	3	4	16	42 (2.5)
Cladosporium cladosporioides (Fresen.) G.A. de Vries	11	15	3	6	21	56 (3.4)
Cylindrocarpon didymum (Harting) Wollenw.	7	10	3	5	12	37 (2.2)
Clonostachys rosea (Link) Schroers, Samuels, Seifert	4	3	5	-	-	12 (0.7)
Fusarium culmorum (W.G.Sm.) Sacc.	27	32	17	22	38	136 (8.2)
Fusarium oxysporum Schl.	59	71	41	49	92	312 (18.8)
Neocosmospora solani (Mart.) L. Lombard and Crous	9	11	4	6	15	45 (2.7)
Penicillium aurantiogriseum Dierckx	15	18	8	11	25	77 (4.7)
Penicillium canescens Sopp	11	13	5	8	16	53 (3.2)
Rhizoctonia solani J.G. Kühn	38	46	24	31	60	199 (11.9)
Rhizopus stolonifer (Ehrenb.) Vuill.,	6	10	1	2	14	33 (2.0)
Sclerotinia sclerotiorum (Lib.) de Bary	68	79	50	57	102	356 (21.4)
Trichoderma sp.	15	13	15	-	-	43 (2.5)
Trichothecium roseum (Pers.) Link	10	15	4	6	21	56 (3.4)
Total isolates	329	398	203	239	495	1664 (100)

I *—Biosept Active, II—Timorex Gold 24 EC, III—Trianum P, IV—Zaprawa Nasienna T 75 DS/WS, V—control.

The number of colonies of scorzonera rhizosphere microorganisms isolated in laboratory conditions on selective media varied. Both the biostimulants and fungicide reduced the population of rhizosphere fungi. Their average number in these variants of the experiment ranged from 3.82×10^3 to 6.30×10^3 CFU/g soil DW (Table 4). However, it was statistically significantly smaller than in control (9.38×10^3 CFU/g soil DW). Trianum P and Zaprawa Nasienna T 75 DS/WS were most effective in reducing the occurrence of rhizospheric fungi. Biosept Active and Timorex Gold 24 EC were slightly less effective in limiting the development of the fungal population, as their abundance in the scorzonera rhizosphere was on average 6.31×10^3 and 6.60×10^3 CFU/g soil DW, respectively. These values were statistically significantly different from control. The number of colonies of rhizobacteria in these variants of the experiment ranged from 2.36×10^6 (control) to 8.12×10^6 CFU/g soil DW (Zaprawa Nasienna T 75 DS/WS). *Pseudomonas* sp. bacteria colonized scorzonera roots to a lesser extent compared to *Bacillus* sp. The population size of the genus *Pseudomonas* ranged on average from 0.53×10^6 to 2.95×10^6 CFU/g soil DW, and the bacteria of the genus *Bacillus* ranged from 0.48×10^6 to 4.55×10^6 CFU/g soil DW. Biostimulants, especially Trianum P and Biosept Active, favored the development of the studied rhizobacteria. The number of these microorganisms after the application of biostimulants was statistically significantly higher than in control.

Table 4. Number of fungi and bacteria isolated from the rhizosphere of scorzonera.

Experimental Treatment	Total CFU of Fungi (10^3/g Soil DW)				Total CFU of Bacteria (10^6/g Soil DW)				CFU of *Pseudomonas* sp. (10^6/g Soil DW)				CFU of *Bacillus* sp. (10^6/g Soil DW)			
	2014	**2015**	**2016**	**Mean**	**2014**	**2015**	**2016**	**Mean**	**2014**	**2015**	**2016**	**Mean**	**2014**	**2015**	**2016**	**Mean**
Biosept Active	6.38 b	5.92 b	6.64 b	6.31 b	5.93 b	4.26 b	5.30 b	5.16 b	1.16 b	1.90 a	1.86 b	1.64 bc	2.14 b	2.00 b	3.06 b	2.40 b
Timorex Gold 24 EC	6.84 b	6.04 b	6.92 b	6.60 b	5.22 b	4.12 b	5.12 b	4.82 b	1.12 b	1.82 a	1.55 b	1.50 c	1.95 b	1.88 b	2.85 b	2.22 b
Trianum P	4.25 c	3.16 c	4.06 c	3.82 c	8.02 a	7.45 a	8.32 a	7.93 a	2.94 a	2.46 a	3.00 a	2.80 a	4.15 a	3.92 a	4.91 a	4.32 a
Zaprawa Nasienna T 75 DS/WS	4.34 c	3.28 c	4.15 c	3.92 c	8.14 a	7.68 a	8.54 a	8.12 a	3.05 a	2.66 a	3.14 a	2.95 a	4.38 a	3.96 a	5.32 a	4.55 a
Control	9.55 a	8.82 a	9.76 a	9.38 a	2.68 c	2.05 c	2.35 c	2.36 c	0.94 b	0.14 b	0,50 c	0.53d	0.62 c	0.28 c	0.53 c	0.48 c

Values in columns followed by the same letter do not differ significantly at $p \leq 0.05$.

Species and quantitative composition of rhizosphere fungi was different and depended on the biostimulants or fungicide used (Table 5). A total of 960 colonies of pathogenic or saprotrophic fungi were isolated from the scorzonera rhizosphere. They belonged to 22 genera. Fungi considered pathogenic were most numerous in the rhizosphere of control plants. The smallest number of them was found after the application of the Trianum P or Zaprawa Nasienna T 75 DS/WS. The rhizosphere soil of control plants was characterized by the greatest biodiversity of microorganisms, from which a total of 295 isolates were obtained. Nevertheless, the number of fungi with antagonistic properties was the lowest in this experimental combination. Timorex Gold 24 EC, Trianum P and Biosept Active reduced the occurrence of fungi in the scorzonera rhizosphere, especially those considered pathogenic. From these experimental combinations, 250, 157 and 144 isolates were obtained, respectively. Various species of the genera *Albifimbria*, *Alternaria*, *Aspergillus*, *Chaetomium*, *Cladosporium*, *Clonostachys*, *Fusarium*, *Penicillium, Rhizoctonia*, *Rhizopus, Sclerotinia*, *Talaromyces*, *Trichothecium,* and *Trichoderma* were frequently isolated. *Fusarium oxysporum*, *Rhizoctonia solani*, *Penicillium* spp. (including *P. chermesinum*, *P. decumbens* and *P. simplicissimum*) and *Trichoderma* sp. were dominant and their proportion was 21%, 12.4%, 10.5% and 8.4% of all isolates, respectively (Table 5). Moreover, the scorzonera rhizosphere was inhabited to a lesser extent by *Rhizopus stolonifer*, *Sclerotinia sclerotiorum*, *Albifimbria verrucaria*, *Cladosporium herbarum*, *Fusarium culmorum,* and *Clonostachys rosea*.

Table 5. Fungi isolated from the rhizosphere of scorzonera (sum 2014–2016).

Fungi	Number of Isolates/Experimental Treatment					
	I *	II	III	IV	V	Total (%)
Acremonium rutilum W. Gams	1	3	3	2	5	14 (1.4)
Albifimbria verrucaria (Alb. and Schwein.) L. Lombard and Crous	7	13	16	4	1	41 (4.3)
Alternaria alternata (Fr.) Keissler	1	2	1	-	3	7 (0.7)
Alternaria scorzonerae (Aderh.) Loer.	3	4	1	3	7	18 (1.9)
Arthrinium phaeospermum (Corda) M.B. Ellis	-	-	-	-	4	4 (0.4)
Aspergillus fumigatus Fresen.	2	4	-	-	8	14 (1.4)
Botrytis cinerea Pers.	1	2	-	-	5	8 (0.8)
Chaetomium piluliferum J. Daniels	6	10	3	4	15	38 (4.0)
Cladosporium cladosporioides (Fresen.) G.A. de Vries	-	2	-	-	5	7 (0.7)
Cladosporium herbarum (Pers.) Link	7	11	2	4	16	40 (4.2)
Clonostachys rosea (Link) Schroers, Samuels, Seifert	4	12	16	3	1	36 (3.8)
Dipodascus geotrichum (E.E. Butler and L.J. Petersen) Arx	1	3	-	-	7	11 (1.2)
Fusarium avenaceum (Fr.) Sacc.	-	2	-	-	4	6 (0.6)
Fusarium culmorum (W.G.Sm.) Sacc.	8	9	4	6	12	39 (4.1)
Fusarium graminearum Schwabe	-	1	-	-	3	4 (0.4)
Fusarium oxysporum Schl.	37	43	25	31	66	202 (21.0)
Mucor hiemalis Wehmer	-	5	-	4	14	23 (2.4)
Neocosmospora solani (Mart.) L. Lombard and Crous	1	4	-	1	9	15 (1.6)
Penicillium chermesinum Biourge	6	14	11	8	8	47 (4.9)
Penicillium decumbens Thom	5	15	11	6	3	40 (4.2)
Penicillium simplicissimum (Oudem.) Thom	-	4	3	-	7	14 (1.4)
Rhizoctonia solani J.G. Kühn	22	28	12	16	41	119 (12.4)
Rhizopus stolonifer (Ehrenb.) Vuill.	10	14	4	6	20	54 (5.6)

Sarocladium kiliense (Grütz) Summerb.	-	1	-	-	3	4 (0.4)
Sclerotinia sclerotiorum (Lib.) de Bary	8	11	4	6	15	44 (4.6)
Talaromyces flavus (Klöcker) Stolk and Samson	-	3	-	1	7	11 (1.1)
Torula herbarum (Pers.) Link	-	-	-	-	3	3 (0.3)
Trichoderma sp.	11	25	34	8	2	80 (8.4)
Trichothecium roseum (Pers.) Link	3	5	7	1	1	17 (1.8)
Total isolates	144	250	157	114	295	960 (100)

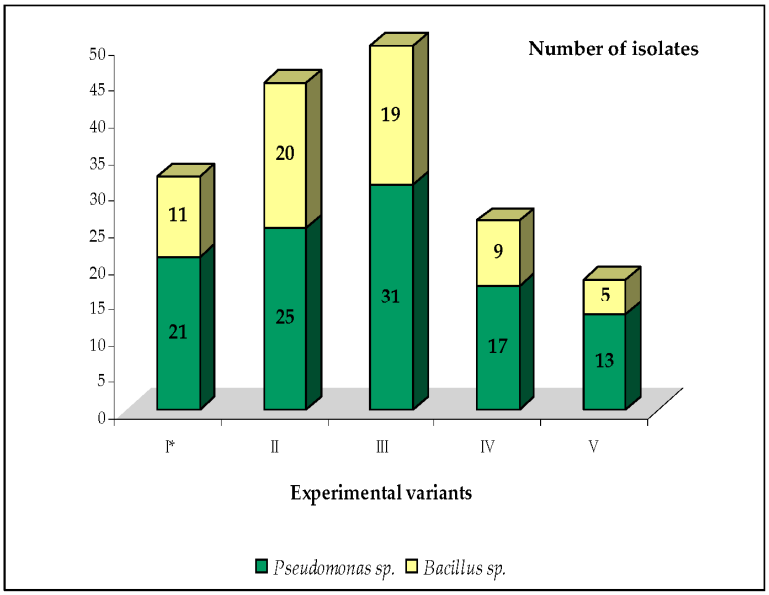
I *—Biosept Active, II—Timorex Gold 24 EC, III—Trianum P, IV—Zaprawa Nasienna T 75 DS/WS, V—control.

In vitro tests (Figure 5) allowed to determine the number of antagonistic rhizosphere bacteria (*Pseudomonas* sp. and *Bacillus* sp.) and fungi (*Acremonium rutilum*, *Albifimbria verrucaria*, *Penicillium* spp., *Clonostachys rosea*, *Talaromyces flavus*, *Trichoderma* sp., and *Trichothecium roseum*) against the fungi pathogenic for scorzonera (*Alternaria scorzonerae*, *Fusarium culmorum*, *Fusarium oxysporum*, and *Rhizoctonia solani*). Biostimulants, especially Timorex Gold 24 EC and Trianum P, stimulated the development of antagonists in the rhizosphere of the tested plant. The most antagonistic bacteria and fungi were obtained after their application (Figure 6a–d). Slightly less antagonists were found after the application of Biosept Active (*Pseudomonas* sp.—21 isolates, *Bacillus* sp.—11 isolates, total number of fungi—37 isolates). The lowest number of antagonistic *Pseudomonas* sp. (13 isolates) and *Bacillus* sp. (5 isolates) were obtained from the rhizosphere of the control plants. Among the antagonistic bacteria, *Pseudomonas* sp. was dominant, and among fungi: *Trichoderma* sp., *Penicillium* spp., *Clonostachys rosea* and *Albifimbria verrucaria* (Figure 6a,c). The lowest number of these fungi were obtained after using the fungicide.

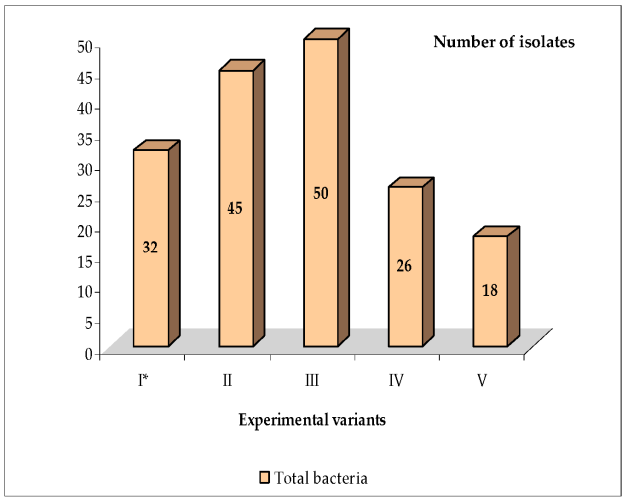
(**a**)

(**b**)

Figure 5. 10-day-old colonies of fungi on the potato dextrose agar (PDA) medium: (**a**) On the left—*Fusarium culmorum*, on the right—*Fusarium culmorum* with *Clonostachys rosea*; (**b**) on the left—*Fusarium culmorum*, on the right—*Fusarium culmorum* with *Trichoderma* sp.; (photo by E. Patkowska).



(**a**)



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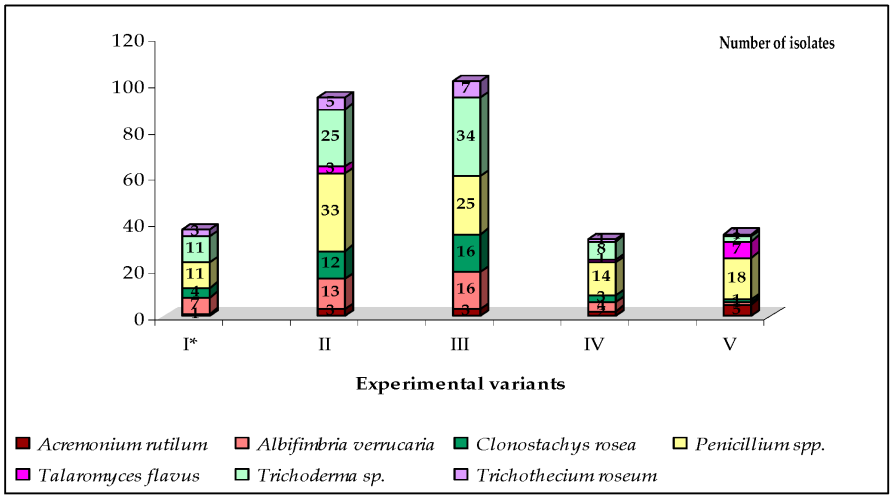
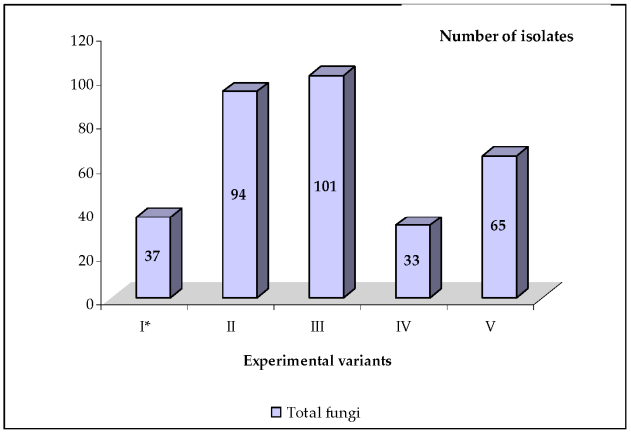


(**c**)



(**d**)

Figure 6. Antagonistic microorganisms in the rhizosphere of scorzonera: (**a**) *Pseudomonas* sp. and *Bacillus* sp.; (**b**) total bacteria; (**c**) antagonistic fungi; (**d**) total fungi. I*—Biosept Active, II—Timorex Gold 24 EC, III—Trianum P, IV—Zaprawa Nasienna T 75 DS/WS, V—control.

Rhizosphere fungi and bacteria showed different antagonistic activity against the tested pathogens (Tables 6 and 7). The lowest antagonistic activity of fungi was found in control, and the highest after Trianum P and Timorex Gold 24 EC application (Table 6). Saprotrophic fungi inhibited the growth of *Fusarium culmorum* and *Fusarium oxysporum* the least. The highest antagonistic activity was recorded against *Alternaria scorzonerae*. The summary biotic effect (SBE) for this species, after Timorex Gold 24 EC and Trianum P application, was +470 and +583, respectively. Biosept Active showed slightly lower effectiveness in stimulating antagonists to limit the growth of *Fusarium oxysporum, Fusarium culmorum*, *Alternaria scorzonerae* and *Rhizoctonia solani*, because the summary biotic effect against these fungi was: +150, +168, +200 and +276, respectively. In the case of the fungicide, these values were also lower compared to biostimulants, but higher than in control. Among the tested preparations, Timorex Gold 24 EC and Trianum P biostimulants were the most effective in stimulating the tested saprotrophic fungi to limit the growth and development of *Fusarium culmorum*, *Rhizoctonia solani,* and *Fusarium oxysporum*. Among all the tested saprotrophic fungi, the highest individual biotic effect (IBE) was recorded for *Trichoderma* sp., *Clonostachys rosea*, *Trichothecium roseum* and *Albifimbria verrucaria*. *Trichoderma* sp. fungi demonstrated the highest antagonistic activity against all pathogenic fungi tested (IBE = +8).

Table 6. Antagonistic activity of selected fungi isolated from the scorzonera rhizosphere towards pathogenic fungi.

Fungi	Average Number of Isolates (2014–2016)	*Alternaria scorzonerae*		*Fusarium culmorum*		*Fusarium oxysporum*		*Rhizoctonia solani*	
		IBE*	GBE**	IBE*	GBE**	IBE*	GBE**	IBE*	GBE**
Biosept Active									
Acremonium rutilum W. Gams	1	+4	+4	+3	+3	+2	+2	+2	+2
Albifimbria verrucaria (Alb. and Schwein.) L. Lombard and Crous	7	+6	+42	+4	+28	+3	+21	+4	+28
Clonostachys rosea (Link) Schroers, Samuels, Seifert	4	+7	+28	+5	+20	+4	+16	+4	+16
Penicillium chermesinum Biourge	6	+2	+12	+1	+6	+1	+6	+2	+12
Penicillium decumbens Thom	5	+1	+5	+1	+5	+1	+5	+1	+5
Trichoderma sp.	11	+8	+88	+8	+88	+8	+88	+8	+88
Trichothecium roseum (Pers.) Link	3	+7	+21	+6	+18	+4	+12	+5	+15
Number of isolates	37								
SBE***			+200		+168		+150		+276
Timorex Gold 24 EC									
Acremonium rutilum W. Gams	3	+4	+12	+3	+12	+2	+6	+2	+6
Albifimbria verrucaria (Alb. and Schwein.) L. Lombard and Crous	13	+6	+78	+4	+52	+3	+39	+4	+52
Clonostachys rosea (Link) Schroers, Samuels, Seifert	12	+7	+84	+5	+60	+4	+48	+4	+48
Penicillium chermesinum Biourge	14	+2	+28	+1	+14	+1	+14	+2	+28
Penicillium decumbens Thom	15	+1	+15	+1	+15	+1	+15	+1	+15
Penicillium simplicissimum (Oudem.) Thom	4	+3	+12	+1	+4	+1	+4	+1	+4
Talaromyces flavus (Klöcker) Stolk and Samson	3	+2	+6	+2	+6	+1	+3	+1	+3
Trichoderma sp.	25	+8	+200	+8	+200	+8	+200	+8	+200
Trichothecium roseum (Pers.) Link	5	+7	+35	+6	+30	+4	+20	+5	+25
Number of isolates	*94*								
SBE***			+470		+393		+349		+381
Trianum P									
Acremonium rutilum W. Gams	3	+4	+12	+3	+9	+2	+6	+2	+6
Albifimbria verrucaria (Alb. and Schwein.)	16	+6	+96	+4	+64	+3	+48	+4	+64

L. Lombard and Crous									
Clonostachys rosea (Link) Schroers, Samuels, Seifert	16	+7	+112	+5	+80	+4	+64	+4	+64
Penicillium chermesinum Biourge	11	+2	+22	+1	+11	+1	+11	+2	+22
Penicillium decumbens Thom	11	+1	+11	+1	+11	+1	+11	+1	+11
Penicillium simplicissimum (Oudem.) Thom	3	+3	+9	+1	+3	+1	+3	+1	+3
Trichoderma sp.	34	+8	+272	+8	+272	+8	+272	+8	+272
Trichothecium roseum (Pers.) Link	7	+7	+49	+6	+42	+4	+28	+5	+35
Number of isolates	101								
SBE***			+583		+492		+443		+477
Zaprawa Nasienna T 75 DS/WS									
Acremonium rutilum W. Gams	2	+4	+8	+3	+6	+2	+4	+2	+4
Albifimbria verrucaria (Alb. and Schwein.) L. Lombard and Crous	4	+6	+24	+4	+16	+3	+12	+4	+16
Clonostachys rosea (Link) Schroers, Samuels, Seifert	3	+7	+21	+5	+15	+4	+12	+4	+12
Penicillium chermesinum Biourge	8	+2	+16	+1	+8	+1	+8	+2	+16
Penicillium decumbens Thom	6	+1	+6	+1	+6	+1	+6	+1	+6
Talaromyces flavus (Klöcker) Stolk and Samson	1	+2	+2	+2	+2	+1	+1	+1	+1
Trichoderma sp.	8	+8	+64	+8	+64	+8	+64	+8	+64
Trichothecium roseum (Pers.) Link	1	+7	+7	+6	+6	+4	+4	+5	+5
Number of isolates	33								
SBE***			+148		+123		+111		+124
Control									
Acremonium rutilum W. Gams	5	+4	+20	+3	+15	+2	+10	+2	+10
Albifimbria verrucaria (Alb. and Schwein.) L. Lombard and Crous	1	+6	+6	+4	+4	+3	+3	+4	+4
Clonostachys rosea (Link) Schroers, Samuels, Seifert	1	+7	+7	+5	+5	+4	+4	+4	+4
Penicillium chermesinum Biourge	8	+2	+16	+1	+8	+1	+8	+2	+16
Penicillium decumbens Thom	3	+1	+3	+1	+3	+1	+3	+1	+3
Penicillium simplicissimum (Oudem.) Thom	7	+3	+21	+1	+7	+1	+7	+1	+7
Talaromyces flavus (Klöcker) Stolk and Samson	7	+2	+14	+2	+14	+1	+7	+1	+7
Trichoderma sp.	2	+8	+16	+8	+16	+8	+16	+8	+16
Trichothecium roseum (Pers.) Link	1	+7	+7	+6	+6	+4	+4	+5	+5

Number of isolates	35			
SBE***	+110	+78	+62	+72

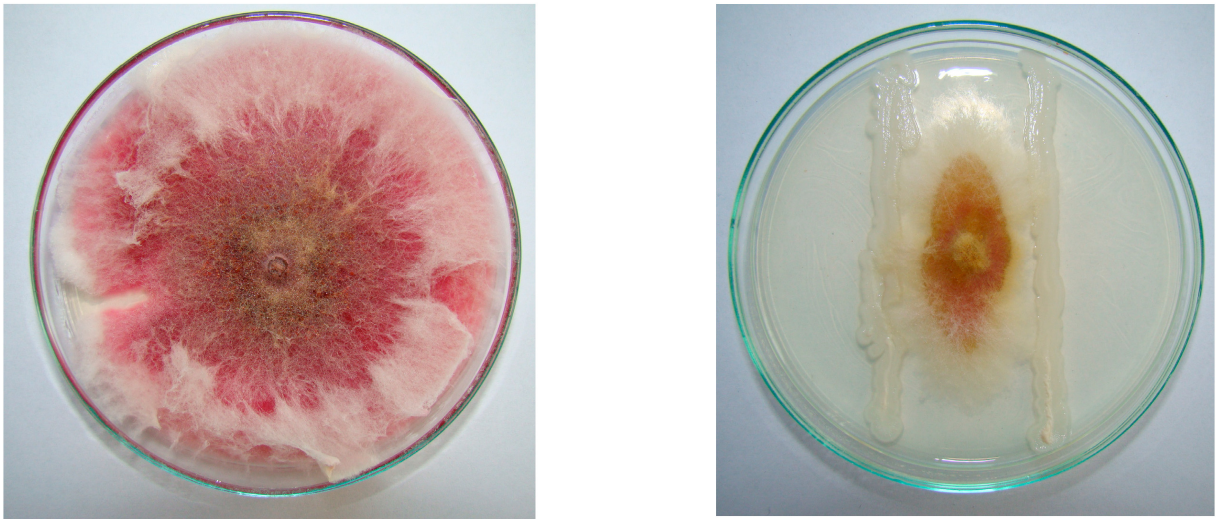
IBE*—individual biotic effect; GBE**—general biotic effect; SBE***—summary biotic effect

Table 7. Antagonistic activity of bacteria isolated from the scorzonera rhizosphere towards pathogenic fungi.

Genus of Bacteria	Number of Antagonistic Isolates (2014–2016)	*Alternaria scorzonerae*		*Fusarium culmorum*		*Fusarium oxysporum*		*Rhizoctonia solani*		Total Antagonistic Activity
		A*	B*	A	B	A	B	A	B	
Biosept Active										
Pseudomonas sp.	21	4	84	3	63	3	63	3	63	273
Bacillus sp.	11	3	33	2	22	2	22	3	33	110
Total antagonistic activity			117		85		85		96	383
Timorex Gold 24 EC										
Pseudomonas sp.	25	3	75	2	50	2	50	3	75	250
Bacillus sp.	20	2	40	2	40	2	40	2	40	160
Total antagonistic activity			115		90		90		115	410
Trianum P										
Pseudomonas sp.	31	5	155	4	124	3	93	4	124	496
Bacillus sp.	19	4	76	3	57	2	38	3	57	228
Total antagonistic activity			231		181		131		181	724
Zaprawa Nasienna T 75 DS/WS										
Pseudomonas sp.	17	2	34	2	34	1	17	2	34	119
Bacillus sp.	9	2	18	1	9	1	9	1	9	45
Total antagonistic activity			52		43		26		43	164
Control										
Pseudomonas sp.	13	2	26	2	26	1	13	1	13	78
Bacillus sp.	5	2	10	2	10	1	10	1	10	40
Total antagonistic activity			36		36		23		23	118

A*—individual antagonistic activity; B*—general antagonistic activity; (B* = number of antagonistic isolates × A*).

The antagonistic activity of bacteria *Pseudomonas* sp. and *Bacillus* sp., isolated from the scorzonera rhizosphere, towards the analyzed pathogenic fungi *Alternaria scorzonerae, Fusarium culmorum, Fusarium oxysporum,* and *Rhizoctonia solani* was different (Table 7 and Figure 7). *Pseudomonas* sp. showed a greater individual and total antagonistic activity than *Bacillus* sp. The applied preparations effectively simulated rhizobacteria to limit the growth of pathogenic fungi. Trianum P and Timorex Gold 24 EC showed the greatest effectiveness. The total antagonistic activity of the tested bacteria against all analyzed fungi, after the application of these biostimulants, was 724 and 410, respectively (Table 7). Biosept Active was slightly less effective. The lowest total antagonistic activity of *Pseudomonas* sp. and *Bacillus* sp. was recorded after the application of Zaprawa Nasienna T 75 DS/WS and in control. *Bacillus* sp. and *Pseudomonas* sp. inhibited the growth and development of *Alternaria scorzonerae* most effectively, *Fusarium culmorum* and *Rhizoctonia solani* slightly less, and *Fusarium oxysporum* the least effectively. The general antagonistic activity of the tested rhizobacteria against *Alternaria scorzonerae* ranged from 36 (control) to 231 (Trianum P). These values for *Fusarium culmorum* and *Rhizoctonia solani* were similar and ranged from 36 to 181 and from 23 to 181, respectively. The general antagonistic activity of *Pseudomonas* sp. and *Bacillus* sp. bacteria against *Fusarium oxysporum* was the lowest and ranged from 23 (control) to 131 (Trianum P) (Table 7).



(**a**) (**b**)

Figure 7. 10-day-old colonies of microorganisms on the potato dextrose agar (PDA) medium: (**a**) *Fusarium culmorum*; (**b**) *Fusarium culmorum* with *Bacillus* sp.; (photo by E. Patkowska).

4. Discussion

Biostimulants applied in scorzonera cultivation, namely Biosept Active (grapefruit extract), Timorex Gold 24 EC (tea tree oil) and Trianum P (spores of *Trichoderma harzianum* T-22) had a positive effect on the health status of plants of the tested species. They effectively protected the roots of scorzonera against soil-borne pathogens. Tea tree oil and grapefruit extract turned out to be slightly less effective than *Trichoderma harzianum* T-22. Nevertheless, all the biostimulants used in the field experiment improved seed germination, emergence and root health of scorzonera in comparison with the control plants. Other studies confirmed the stimulating effect of pre-sowing seed treatment with grapefruit extract on the emergence, health status and yielding of plants from the family Fabaceae (such as pea, soybean, runner bean, and common bean) [59,60,66,67]. Tea tree oil stimulated seed germination and growth of carrot plants [6], while *Trichoderma harzianum* T-22 stimulated the germination and growth of maize and carrot plants [6,68].

Trichoderma harzianum T-22 spores are able to germinate and grow in various soils and different pH values (4–8.5) [69]. These spores, when present on the surface of seeds, can accelerate their germination and subsequently stimulate the growth of seedlings and older plants. *Trichoderma harzianum* T-22 and grapefruit extract most effectively protected scorzonera roots against infection by soil-borne pathogens. Tea tree oil showed a slightly lower effectiveness. All biostimulants limited scorzonera root colonization by polyphages *Alternaria alternata*, *Alternaria scorzonerae*, *Fusarium culmorum*, *Neocosmospora solani, Sclerotinia sclerotiorum*, *Fusarium oxysporum,* and *Rhizoctonia solani*. On the other hand, they increased root colonization by beneficial saprotrophic fungi (*Clonostachys rosea*, *Trichoderma* sp., and *Talaromyces flavus*). Earlier studies demonstrated that *Trichoderma harzianum* T-22 and tea tree oil considerably improved the health status of carrot plants [6].

In the current study, the applied preparations modified fungal and bacterial communities in the scorzonera rhizosphere. *Trichoderma harzianum* T-22 and the fungicide significantly decreased the population of rhizospheric fungi. The efficiency of grapefruit extract and tea tree oil in limiting the development of rhizospheric fungi was slightly lower. Earlier studies also confirmed high effectiveness of *Trichoderma harzianum* T-22 and tea tree oil in limiting the population of soil-borne fungi in carrot cultivation [6]. Grapefruit extract and *Trichoderma harzianum* T-22 inhibited the development of pathogenic fungi of the genera *Fusarium*, *Alternaria*, *Rhizoctonia*, *Sclerotinia*, while they promoted the growth of antagonistic fungi of the genera *Trichoderma*, *Trichothecium*, *Clonostachys*, *Albifimbria* in the scorzonera rhizosphere. Many authors have reported the ability of various species of the genus *Trichoderma*, including *Trichoderma harzianum* T-22, to eliminate pathogenic fungi from the soil environment [6,25,45–47,68]. These fungi have the ability to grow rapidly, sporulate abundantly and survive in unfavorable conditions [70]. *Trichoderma* spp. colonize plant root surfaces similarly to mycorrhizal fungi [70]. Moreover, they are resistant to flavonoids, phytoalexins, phenols and terpenoids secreted by plants during infection [70]. They significantly modify the rhizosphere by affecting other soil microorganisms through competition, antibiotics and mycoparasitism [25,44–47]. They synthesize siderophores, lytic enzymes and secondary metabolites such as antibiotics [44,45,47,71]. They are able to lyse the cell walls, hyphae, and spores of harmful soil microorganisms [44,45]. *Trichoderma* spp. can produce volatile metabolites, including 6PAP (6-n-pentyl-6H-pyran-2-on, 6PP) [35]. This compound displays antifungal properties [72,73] and affects plant growth and leads to the development of systemic resistance [74,75]. According to Harel et al. [76], *Trichoderma harzianum*, when introduced to the soil, induced *Fragaria ananassa* resistance to powdery mildew caused by *Podosphaera aphanis*.

In the present study, *Trichoderma harzianum* T-22 and grapefruit extract more strongly stimulated the development of rhizobacteria populations, including antagonistic *Pseudomonas* sp. and *Bacillus* sp. than tea tree oil. The stimulating effect of grapefruit extract on the populations of these rhizobacteria was previously observed in common bean [63] and soybean cultivation [8]. Tea tree oil, chitosan, and *Trichoderma harzianum* T-22 increased the abundance of soil *Pseudomonas* sp. and *Bacillus* sp. in carrot cultivation [6]. These bacteria belong to the PGPR group and are also used as bacterial inoculants in organic farming [77,78]. These bacteria eliminate harmful microorganisms from the soil by producing substances toxic to pathogenic fungi (hydrolytic enzymes—chitinases, cellulases, proteases, various antibiotics and siderophores to competitively acquire ferric ion) [39,40,43]. A positive role of PGPR was observed in the cultivation of various horticultural plants [33,35,78]. *Bacillus pumilus* and *Bacillus subtilis* bacteria acted as biofertilizers in the cultivation of blackberry [35]. *Pseudomonas fluorescens* Pf5, introduced into the soil of highbush blueberry cultivation stimulated plant growth [79].

The biostimulants applied in scorzonera cultivation, especially *Trichoderma harzianum* T-22, induced antagonistic activity of saprotrophic rhizosphere bacteria and fungi (*Pseudomonas* sp., *Bacillus* sp., *Trichoderma* sp., *Clonostachys* sp., *Albifimbria*

verrucaria, and *Trichothecium roseum*) against the studied pathogenic fungi (*Alternaria scorzonerae, Fusarium culmorum, Fusarium oxysporum,* and *Rhizoctonia solani*). The antagonistic activity of various species of the genus *Trichoderma* against pathogenic microorgamisms has been described by many authors [25,44,46,70]. *Trichoderma* spp. (including *T. harzianum* T-22, *T. longibrachiatum* and *T. atroviride*) inhibited the growth and development of *Alternaria alternata*, *Sclerotinia sclerotiorum*, *Fusarium* spp., *Rhizoctonia solani*, *Colletotrichum* spp., and *Diaporthe* spp. [6,48,80]. Rhizospheric *Trichoderma virens* T2 and *Trichoderma longibrachiatum* T4 were antagonistic to *Fusarium oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *ciceris* causing Fusarium wilt of tomato and chickpea [46]. *Albifimbria verrucaria* was effective as a biocontrol agent (BCA) against gray mold (*Botrytis cinerea*) [81]. *Trichothecium roseum* limited the development of *Phakopsora pachyrhizi* [82]. According to Hinarejos et al. [83], the rhizosphere may be a common selective source of *Bacillus* species that are useful in the biocontrol of phyllospheric and soil-borne pathogenic fungi. The effectiveness of rhizospheric *Pseudomonas* sp. in limiting the development of harmful soil microorganisms has been also confirmed by many authors [6,84,85]. The species *Pseudomonas fluorescens, P. putida, P. cepacia*, and *P. chlororaphis* showed high antagonistic activity against polyphagous fungi [84,85]. *Pseudomonas fluorescens* BRZ63 inhibited mycelium growth of *Rhizoctonia solani*, *Colletotrichum dematium*, *Sclerotinia sclerotiorum,* and *Fusarium avenaceum* [86].

The present study demonstrated the favorable effect of biostimulants on the antagonistic activity of beneficial microorganisms in the rhizosphere soil of scorzonera. *Trichoderma harzianum* T-22 and tea tree oil increased the population and activity of antagonists (*Pseudomonas* sp., *Bacillus* sp., *Trichoderma* sp., *Clonostachys* sp., *Albifimbria verrucaria,* and *Trichothecium roseum*) against phytopathogenic fungi to a greater extent than grapefruit extract. The fungicide Zaprawa Nasienna T 75 DS/WS was not conducive to the development of antagonistic bacteria and fungi. The effectiveness of tea tree oil and grapefruit extract could result from their antiseptic, bactericidal [50,87] and fungicidal [49,52–54,88] properties. Natural essential tea tree oil contains a maximum content (15%) of 1, 8- cineole and a minimum content (30%) of terpinen-4-ol, which is the main active constituent of the oil [49]. This biostimulant was effective in controlling plant-pathogenic fungi in numerous crops [4,49,89]. Tea tree oil showed high antagonistic activity against fungi such as *Aspergillus fumigatus*, *Botrytis cinerea*, *Penicillium chrysogenum*, *Chaetomium globosum*, *Fusarium graminearum, F. oxysporum*, *F. culmorum*, *Ascochyta rabiei, Pyrenophora graminea, Colletotrichum lindemuthianum*, *Alternaria radicina*, *A. dauci,* and *Drechslera avenae* [49,52–54,88,89]. The effectiveness of grapefruit extract in the elimination of harmful soil and phyllospheric microorganisms has been previously confirmed by many authors [7,56–60]. Its antiseptic properties result from the presence of biologically active compounds such as aliphatic aldehydes, monoterpenes and nutkaton [46]. According to Woedtke et al. [90], triclosan, 7-geranoxycoumarin, and benzetonine chloride present in grapefruit extract could inhibit the growth of bacteria and fungi. The positive influence of grapefruit extract on the composition of soil microorganisms was confirmed in the cultivation of plants from the family Fabaceae [59].

5. Conclusions

The current study showed the beneficial effect of biostimulants on the antagonistic activity of microorganisms in the rhizosphere soil of scorzonera, and thus on the health status of this plant in field cultivation. The biostimulants Biosept Active (grapefruit extract), Timorex Gold 24 EC (tea tree oil), and particularly Trianum P (*Trichoderma harzianum* Rifai T-22), exerted a positive influence on microbal communities in the rhizosphere. They reduced the population of pathogenic soil-borne fungi infecting scorzonera roots, while increasing the population of antagonistic bacteria and fungi, including *Pseudomonas* sp., *Bacillus* sp., *Trichoderma* sp., *Clonostachys* sp., *Albifimbria verrucaria,* and *Trichothecium roseum*. These microorganisms were characterized by high antagonistic activity towards the studied fungi pathogenic to scorzonera (*Alternaria scorzonerae,*

Fusarium culmorum, Fusarium oxysporum, and *Rhizoctonia solani*). The biostimulants significantly improved the phytosanitary condition of the soil, and thus effectively protected the roots of scorzonera plants against infection by polyphagic soil-borne fungi. Therefore, they can be recommended in *Scorzonera hispanica* cultivation.

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