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A RESPONSE OF *RHIZOCTONIA SOLANI* KÜHN. TO BIOTECHNICAL PREPARATIONS

Summary

The aim of the present study was to examine the effect of natural substances such as garlic extract, grapefruit extract, vermicompost extract, and chitosan on mycelial growth, sclerotia germination, and biological activity of Rhizoctonia. solani. It was found that the tested substances inhibited mycelial growth and sclerotia germination of R. solani. At the lowest experimental concentration, all biological substances, contrary to the fungicide, had a positive effect on the relationship between R. solani and Trichoderma viride.
***Key words**: Rhizoctonia solani, wyciągi roślinne, substancje naturalne*

REAKCJA GRZYBA *RHIZOCTONIA SOLANI* KÜHN. NA PREPARATY BIOTECHNICZNE

Streszczenie

Celem pracy było zbadanie oddziaływania substancji takich, jak: wyciąg z czosnku, wyciąg z grapefruita, wermikompost i chitozan na wzrost grzybni, kiełkowanie sklerocjów i aktywność biologiczną grzyba Rhizoctonia solani. Stwierdzono, że badane substancje hamowały wzrost grzybni i kiełkowanie sklerocjów R. solani. W najmniejszym badanym stężeniu wszystkie substancje biologiczne, w przeciwieństwie do fungicydu, działały korzystnie na relacje między R. solani i Trichoderma viride.
***Słowa kluczowe**: Rhizoctonia solani, plant extracts, natural substances*

1. Introduction

Rhizoctonia solani Kühn is a soilborne fungal opportunistic pathogen that infects a wide variety of plant species and is among the most common pathogens of crops. *R. solani* infects members of the families Poaceae (e.g., maize, wheat, barley, oat, and rice), Fabaceae (e.g., soybean, dry bean, alfalfa, chickpea, lentil, and field pea), Solanaceae (e.g., potato and tobacco), Amaranthaceae (e.g., sugar beet), Brassicaceae (e.g., canola), Rubiaceae (e.g., coffee), Malvaceae (e.g., cotton), Asteraceae (e.g., lettuce), Moraceae (e.g., ficus), and Linaceae (e.g., flax). Symptoms of *R. solani* infection in diverse hosts include seed rot, root rot, hypocotyl rot, crown rot, stem rot, limb rot, pod rot, stem canker, black scurf, seedling blight, and pre- and post-emergence damping off [1, 2]. Agrotechnical and chemical methods are used for protection against canker disease. Because of the increasing demand of consumers to have healthy and safe foods, researchers have focused their attention on the possibility of using natural substances to combat *R. solani* infection. In the past decade, antimicrobial agents containing biologically active substances of natural origin have been included in the list of plant protection products [3]. At present, because of high cost of registration, such antimicrobial agents are used as supplementary products for crop cultivation [4].

Natural products have the potential for interesting applications in crop cultivation, particularly in organic farming where there is a lack of effective tools for managing biotic diseases. Plant disease control in organic farming, especially those diseases caused by fungal and bacterial pathogens, is currently based on treatment with copper-containing compounds [5]. However, the development of an ecological alternative is required because of the toxic environmental effects related to the use of this heavy metal. Thus, natural products could represent an innovative eco-friendly strategy for managing plant diseases and replacing copper or reducing its use.

The present study aimed at examining the effect of natural substances such as garlic extract, grapefruit extract, vermicompost extract, and chitosan on mycelial growth, sclerotia germination, and biological activity of *R. solani*.

2. Materials and methods

The research material consisted of fungal isolates obtained from the collection of the Department of Agricultural Environment Protection, Agriculture University of Kraków: *Rhizoctonia solani* Kühn. and *Trichoderma viride* Pers. ex Gray. The effect of natural substances (Table 1) such as chitosan, grapefruit extract, garlic extract and vermicompost extract was studied at their concentrations of 1, 10, 100 ppm ($mg \cdot kg^{-1}$). The chemical agent thiophanate-methyl was used as a chemical standard.

The *in vitro* effect of the substances on *R. solani* linear growth was examined with the poisoned medium method [6]. Potato dextrose agar (PDA) was prepared with the addition of respective substances. The media were inoculated with agar disc (5 mm in diameter) overgrown with 2–week–old culture of *R. solani*. Control combination consisted of medium without substances. The results obtained were expressed as the inhibition coefficient of linear fungal growth, calculated according to Abbott's formula [7].

To investigate the effect of the natural substances on sclerotial germination of *R. solani*, batches of ten sclerotia were each placed on four replicate PDA plates (9 cm diam) with the addition of respective substances. Germination of sclerotia was determined after 72 h incubation at 25°C by viewing the outgrowing hyphae under a stereo binocular microscope at 45 magnification.

Table 1. List of natural substances used in the experiment
Tab. 1. Wykaz substancji naturalnych wykorzystanych w doświadczeniu

Active substance	Content of the active substance	The trade name of the preparation	Producer
chitosan (ß-1,4-D-glukozaminy poly-d-glucosamine) obtained from the exoskeletons of marine crustaceans dissolved in a mixture of lactic acid and succinic acids	20 g·dm^{-3}	Beta-chicol 020 PC	Poli-Farm® Sp. z o.o Łowicz
vermicompost extract produced by *Eisenia fetida*	20%	Wspomag	**HOST International® Sp. z o.o., Przedsiębiorstwo Rolno- Ekologiczne Cedry Małe Kolonia**
polish garlic extract		Bioczos płynny	Himal Łódź
grapefruit extract	33%	Biosept Active	BIOSEPT sp. z o. o. Sp. K. Piaseczno
thiophanate-methyl (chemical standard)	500 g	Topsin M 500 WP	Sumi Agro Poland Sp. z o.o.

Source: own work / Źródło: opracowanie własne

A sclerotium was considered to have germinated when outgrowing hyphae were equal to or greater than the diam of the sclerotium. The sclerotia germination was calculated into percentages.

The results of the experiments were verified statistically with variance analysis assumed for two–factor experiments (factor A – studied preparations, factor B – concentration of the preparations). Significance of differences was verified with Duncan's test.

The biotic correlations between *R. solani* pathogen and *T. viride* antagonistic fungus were defined with the biotic series method following Mańka [8]. The analyzed fungi were inoculated at a distance of 2 cm one from another in a central part of Petri plate with PDA medium supplemented with the analyzed substances at concentrations of 1, 10 or 100 ppm. After 10 days of incubation, each combination was assessed on a scale, regarding three parameters: extent to which one fungal colony was surrounded by the other, inhibition zone and colony diminishing. The highest mark on the 8-point scale denoted a complete lack of fungal growth. A "+" sign (positive effect) was used in the case of *T. viride* domination, a "–" sign (negative effect) for the domination of *R. solani* fungus, and "0" was given if no prevalence of any colony could be observed. The values obtained provided jointly an individual biotic effect (IBE) illustrating the influence of *T. viride* isolate on the growth of *R. solani*.

All the above experiments were carried out in 4 replicates.

3. Results and discussion

It was found that the tested substances inhibited mycelial growth of *R. solani* (Fig. 1, 2). However, they had significantly weaker effect than the chemical standard thiophanate-methyl. Among the tested natural substances, vermicompost extract showed the strongest inhibitory effect on the growth of mycelium. This preparation significantly inhibited mycelial growth at the concentration of 1 ppm. The vermicompost solution showed high antifungal efficacy at the concentration of 100 ppm, with almost 70% mycelial growth inhibition; this result was the highest among the results obtained for the tested biosubstances. Grapefruit extract at 100 ppm concentration also significantly inhibited *R. solani* growth. However, garlic extract at 10 and 100 ppm concentrations reduced *R. solani* growth to a lesser extent. Moreover, even at its highest concentration, chitosan was unable to inhibit the mycelial growth to a considerable extent.

All the tested substances inhibited sclerotia germination of *R. solani* (Fig. 3). Thiophanate-methyl completely inhibited sclerotia germination. However, the tested biological substances had markedly weaker effect than the chemical agent. Vermicompost and grapefruit extracts showed the strongest inhibitory effect on sclerotia germination. Sclerotia germination was completely abolished by vermicompost extract at 10 and 100 ppm concentrations. Grapefruit extract also completely inhibited sclerotia germination at 100 ppm concentration. Garlic extract had less pronounced effect, while chitosan had the lowest inhibitory effect on sclerotia germination. It was observed that with an increase in the concentration of the tested substance, the number of germinating sclerotia was reduced.

The tested natural substances caused changes in biotic relationship between *R. solani* and *Trichoderma viride* (Fig. 4). At the lowest experimental concentration, all biological substances, contrary to the fungicide, had a positive effect on the relationship between *R. solani* and *T. viride*. Biological agents added to the culture medium favored the development of the antagonistic fungus *T. viride*, which inhibited the growth of the pathogenic *R. solani*. The most positive effect on the relationship of *R. solani* and *T. viride* was shown by vermicompost extract, which at 1 ppm concentration increased *T. viride* antagonism by 4 units. At this concentration, vermicompost extract caused *R. solani* to develop weakly and to be heavily restricted in growth by its antagonistic partner *T. viride*. However, at the concentration of 100 ppm, all substances, except chitosan, decreased individual biotic effect IBE.

The present study demonstrated that vermicomopst extract limited mycelial growth and sclerotia germination of *R. solani* to the greatest extent. Moreover, this extract had a positive effect on *R. solani* and *T. viride* relationship, thereby increasing the effect of the antagonistic fungus *T. viride*.

Literature indicates that Biokal 1, Biokal 2, and Biojodis agents containing biohumus aqueous extracts influenced the reduction of colonization of barley seeds by the fungi *Drechslera*. Although the preparations applied to seeds did not reduce the counts of *Fusarium*, *Alternaria*, and *Penicillium*, they improved the health of barley seedling roots [9]. In a study on the effect of green composts on fusarium wilt in melon plants, it was found that biotic and abiotic components of the composts were responsible for their biopesticide effect on *Fusarium oxysporum*. The main fungal and bacterial isolates from the composts in vitro had a suppressive effect on *F. oxysporum*.

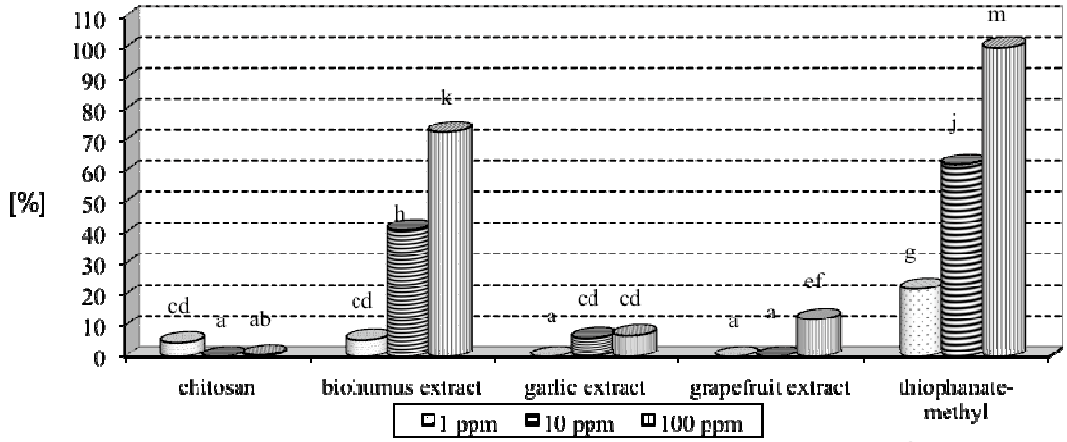

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Fig. 1. Effect of preparations on inhibition of mycelium development of *Rhizoctonia solani* – % - compared to control without preparation (*columns marked with different letters differed significantly according to Duncan's test at p = 0.05)

Rys. 1. Wpływ preparatów na zahamowanie rozwoju grzybni Rhizoctonia solani - % - w porównaniu do kontroli bez preparatu (rubryki oznaczone odmiennymi literami różniły się znacząco zgodnie z testem Duncana przy p=0,05)*

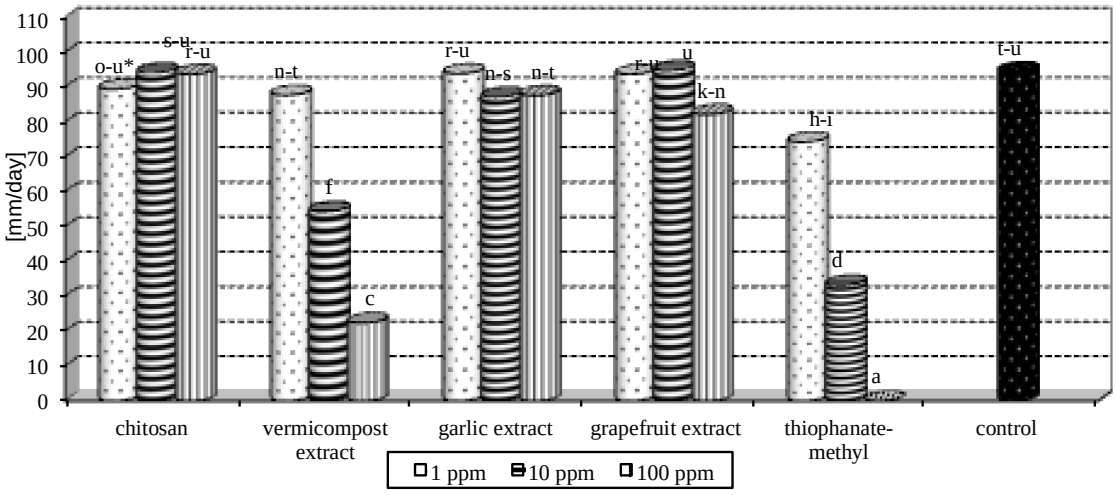

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Fig. 2. Growth rate of *Rhizoctonia solani* exposed to preparations (*columns marked with different letters differed significantly according to Duncan's test at p = 0.05)

Rys. 2. Tempo wzrostu Rhizoctonia solani poddanego działaniu preparatów (rubryki oznaczone odmiennymi literami różniły się znacząco zgodnie z testem Duncana przy p=0,05)*

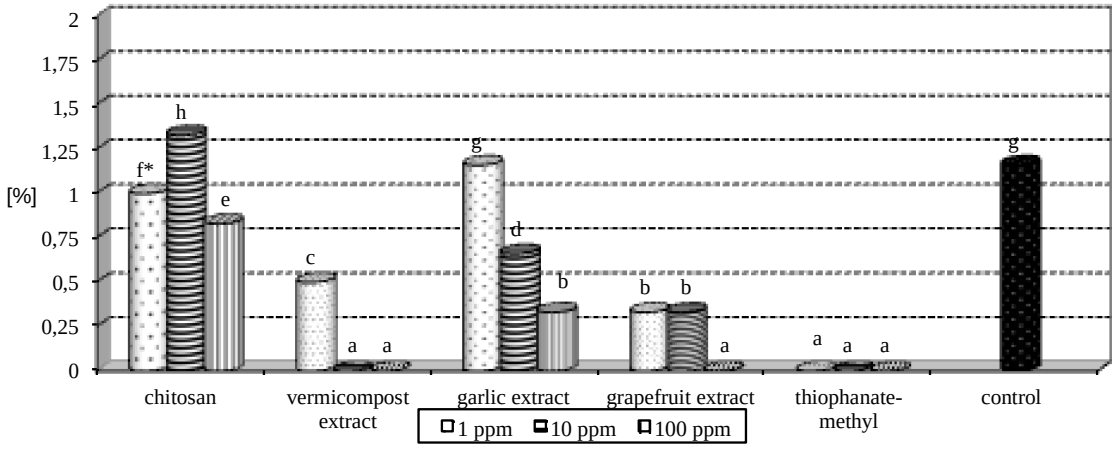

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Fig. 3. Effect of preparations on sclerotium germination of *R. solani* (*columns marked with different letters differed significantly according to Duncan's test at p = 0.05)

Rys. 3. Wpływ preparatów na kiełkowanie sklerocjum R. solani (rubryki oznaczone odmiennymi literami różniły się znacząco zgodnie z testem Duncana przy p=0,05)*

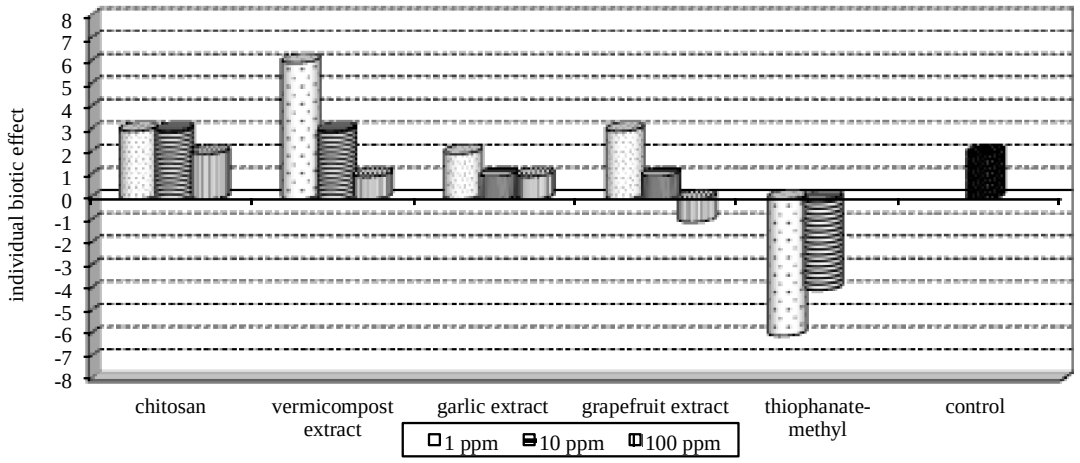

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Fig. 4. Effect of preparations on biotic relations between *R. solani* and *Trichoderma viride* (control – without preparation)
Rys. 4. Wpływ preparatów na relacje biotyczne między R. solani a Trichoderma viride (kontrola bez preparatu)

The fungal isolates showed a greater degree of biological control against the pathogen than the bacterial isolates [10]. Moreover, Szczech [11] reported that vermicompost significantly inhibited the infection of tomato plants by *F. oxysporum* f. sp. *lycopersici.* Vermicompost strongly inhibited the growth of *F. oxysporum* on agar medium. Microscopic observations showed that hyphae removed from fungal plates treated with vermicompost were completely destroyed and colonized by microbes. Bacterial and fungal isolates from the vermicompost formed clear zones of growth inhibition or overgrew the pathogen's mycelium on the plates [11].It has been indicated that vermicompost extract introduces compounds that are capable of restricting growth and germination of fungi in the environment, apart from numerous microorganisms and their metabolites found in biohumus. The inhibitory effect of compost extracts has been explained by their direct effect on spore germination and growth of fungal germ hyphae and immunity induction in plants [12].

Grapefruit extract at 100 ppm concentration significantly reduced mycelial growth and completely abolished sclerotia formation in *R. solani*, and at 1 ppm concentration, it increased the IEB value.

Some advantageous effects of grapefruit extract on plant health and pathogen control were noted. This extract can effectively protect germinating seeds of bean and pea as well as seedlings from soilborne fungi, which present a considerable risk, and especially from species such as *Alternaria alternata*, *Botrytis cinerea*, *Fusarium culmorum*, *F. oxysporum*, *F. solani*, *Pythium irregulare*, *Phoma exigua*, and *R. solani* [13]. Grapefruit extract limited leaf infestation by *Bipolaris sorokiniana* and *Drechslera teres* [14]. Similarly, the extract significantly reduced seed infestation with *Alternaria radicina* [15]. It was also found that grapefruit extract severely limited the linear growth of the mycelia of pathogens such as *A. alternata*, *Cylindrocarpon radicicola*, *F. oxysporum*, and *Phomopsis theae* [16]. Furthermore, 33% grapefruit extract inhibited the linear growth of five isolates of *Phomopsis sojae* under in vitro conditions. The pathogen's mycelial growth under the effect of grapefruit extract was pale white, and the mycelial hyphae formed a fairly loose structure that was distinct from the more compact mycelium of control colonies. Complete degradation of mycelial hyphae was observed in microscopic preparations [17]. Grapefruit extract at 100 ppm concentration strongly restricted *B. cinerea* growth, limiting it by 50%, and inhibited its conidia germination [18]. Sapiecha-Waszkiewicz et al. [19], however, reported that *B. cinerea* was rather resistant to grapefruit extract. The growth of the fungus was completely inhibited at a concentration that was fivefold higher than the recommended amount and concentration, but only at the initial stage of culturing. Furthermore, no sclerotia were formed on media containing the extract. On the other hand, grapefruit extract stimulated the development of the antagonistic species *Gliocladium roseum* [14]. Laboratory studies revealed that the extract contributed to the increase in the number of antagonistic bacteria (*Bacillus* spp. and *Pseudomonas* spp.) and fungi (*Gliocladium* spp. and *Trichoderma* spp.) in the rhizosphere of runner bean [20]. Furthermore, antagonistic microorganisms dominated the rhizosphere of pea plants grown from the seeds treated with grapefruit extract at 200 $g \cdot dm^{-3}$ concentration [21].

The advantage of plant extracts is that they frequently contain a mixture of chemicals that may synergistically act to inhibit the growth of phytopathogenic fungi. Many plant extracts also contain more than one antifungal compound. If these compounds have different mechanisms of antifungal activity, it may lead to a decrease in the development of resistance. Therefore, the use of plant extracts may prevent the development of resistance against antimicrobial compounds. Grapefruit extract directly affects pathogenic factors and induces resistance to certain pathogens in plants [22]. 7-Geranoxicumarin, which is found in grapefruit juice, shows this effect [22, 23]. Aliphatic aldehydes, monoterpenes, sesquiterpenes, and nutcaton dominate among the numerous compounds present in grapefruit extract. These diverse compounds may act synergistically to inhibit the growth of a specific pathogen [24]. According to Saniewska [25], the protective effect of grapefruit extract is related to the presence of endogenous flavonoids, glycosides, citrate, and limone in the preparation. Moreover, grapefruit extract can also act as a scavenger of free oxygen radicals, which can be components of host defence against pathogen penetration [26]. One approach for antifungal treatment would be to identify new antifungal compounds from plants, but the other possibility is that a plant extract with a complex mixture of different antifungal compounds can be used. The latter approach has the advantage of reduced development of resistance if the different antifungal compounds in an extract target different receptors. There is,

however, a disadvantage compared to using a single chemical product in terms of ensuring good quality control and variation in activity according to genetic or environmental factors [22].

In the present study, garlic extract at 10 and 100 ppm concentration reduced the growth rate and sclerotia germination in *R. solani*; yet, it had a markedly limited effect as compared to vermicompost and grapefruit extracts.

Garlic extract most effectively inhibited the growth of *F. oxysporum*, *B. cinerea*, and *R. solani* [27], and *Alternaria brassisicola*, *Magnaporthe grisea*, and *Fusarium tabacinum* [28]. Garlic extract also showed good inhibitory effect on the mycelial growth of isolates of *Colletotrichum* spp. [29]. Extract from *Allium* sp. showed an intermediate level of inhibition of mycelial growth of *Verticillium dahliae* [30]. Lower incidence, lower disease severity, and the highest percentage control of anthracnose caused by *Colletotrichum musae* were also observed after using essential oil of *Allium sativum* [31]. Allicin is present in garlic and could inhibit the growth of both *Armillaria gallica* and *A. mellea* in vitro. The effect was more pronounced at higher allicin concentrations of 50 and 100 mg/l [32]. Moreover, garlic extracts decreased the sporulation of *F. oxysporum* with an increasing concentration, and cultures grown on extract-supplemented agar plates remained viable [33].

The fungistatic properties of garlic extract are primarily associated with allin present in garlic, which is metabolized to allicin and other sulfur derivatives such as diallyl sulfide, ajoene, and other derivatives [34-37]. It has also been indicated that ajoene exhibits stronger fungistatic activity than allicin [28, 38]. Analysis of garlic extract with HPLC revealed that the major active ingredients were 3-vinyl-1,2-dithiacyclohex-5-ene and 3-vinyl-1,2-dithiacyclohex-4-ene. Changes observed in membrane permeability and protein leakage by scanning electron microscopy suggested that the antimicrobial activity of garlic extracts may be due to disintegration of the structural integrity of cell membranes, leading to cell death [39]. It has been pointed out that garlic extract causes cytomorphological changes consisting in the accumulation of fatty bodies in the cells, decrease in the thickness of cellular walls, and corrugation of the cell membrane. These changes are similar to those occurring in fungal cells after treatment with synthetic fungicides [38].

In the present study, chitosan even at its highest concentration did not have a significant inhibitory effect on the mycelial growth, and it reduced sclerotia germination of *R. solani* to a small degree. Moreover, it did not affect the antagonism of *T. viride* on *R. solani*.

It is assumed that the effect of chitosan is more fungistatic than fungicidal in nature [40]. In general, chitosan applied at a concentration of 1 mg/mL can reduce the in vitro growth of a number of fungi and oomycetes except Zygomycetes, which have chitosan as a component of their cell wall [41]. The mechanism by which chitosan protects rice from *R. solani* was attributed to the direct destruction of the mycelium, as evidenced by scanning and transmission electron microscopy observations and pathogenicity testing; indirect induced resistance was demonstrated by changes in the activities of the defence-related enzymes such as phenylalanine ammonia lyase, peroxidase, and polyphenol oxidase in rice seedling [42].

Generally, chitosan has been reported to be very effective in inhibiting spore germination, germ tube elongation, and radial growth [40]. Spores were clearly more sensitive to chitosan than hyphae. The affected conidia showed retraction of the cytoplasm, but still retained their structure [43]. Chitosan oligomers diffuse inside hyphae and interfere with the enzyme activity responsible for the fungus growth [40]. Studies of the ultrastructure of fungi treated with chitosan also showed changes in cell walls in the form of their relaxation, vacuolization, and in the final stage of disintegration of the protoplasm. These changes may be due to the effect of inhibition of chitin synthesis and appearance of higher amounts of chitosan in the cellular membrane. It is likely that chitosan externally supplemented to fungi stimulates deacetylation of the fungal chitin into chitosan and disturbs the balance of the proportion of these components in the cell membrane, thereby leading to its relaxation [44]. In contrast, other authors have shown that chitosan does not inhibit mycelial growth and spore germination in in vitro cultures, but it induces the systemic immunity of plants to certain pathogens [41, 45].

The present study found that the tested biological substances at the concentration of 1 ppm favored the development of the antagonistic fungus *T. viride*, which in turn inhibited the growth of the pathogen *R. solani*.

Gliocladium spp. and *Trichoderma* spp., as antagonistic fungi, were most abundant in the rhizosphere soil of soybean after the application of chitosan (Biochikol 020 PC) and grapefruit extract (Biosept 33 SL) [20]. Chitosan is a compound that stimulates the growth and development of antagonistic microorganisms, especially *Trichoderma* spp. [46].

Continued research, including the use of plant-based products, is required to provide effective biological products that are cheap, less toxic, and effective. Pathogen control by using plant-based products may offer relief in the fight against fungal plant diseases [22].

4. Summary

It was observed that the tested substances inhibited mycelial growth and sclerotia germination of *R. solani.* Vermicompost extract showed the strongest inhibitory effect on the growth of mycelium and sclerotia germination. The most positive effect on the relationship of *R. solani* and *T. viride* was shown by vermicompost extract at 1 ppm concentration. Pathogen control by using plant-based products may offer relief in the fight against fungal plant diseases in organic farming.

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