

***In vitro* efficiency of bio-preparations against *Stewartia pseudocamellia* (Max.) pathogens**

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Conducted investigation aims at determination the typical composition of fungi occurred on diseased *Stewartia pseudocamellia* seedlings and the *in vitro* effect of biological preparations based on natural substances on the mycelium linear growth of fungi potentially pathogenic to *Stewartia* plants.

There were examined preparations Bioczos BR (B.A.S. garlic extract), Biochikol 020 PC (B. A. S. chitosan), Biosept 33 SL (33 % extract from grapefruit seeds and pulp), each at 3 concentrations. As standard fungicide Bravo 500 SC (B. A. S. chlorotalonil) was applied. Estimation of the preparation effect on the mycelium linear growth of dominant species isolated from the *Stewartia* seedlings was carried out using Kowalik and Krechniak method (1961).

In the laboratory experiment from root and stem bases with observed disease symptoms 14 fungi species and 15 bacteria were isolated. The dominant fungi were *Alternaria alternata*, *Cylindrocarpon radicum*, *Fusarium oxysporum*, *Fusarium avenaceum*, *Phomopsis thea*.

Received results showed that all the preparations taken under consideration significantly reduced mycelium linear growth of tested fungi. The effect of preparations was diversified and depended on concentration and the type of preparation.

Key words: chitosan, chlorotalonil, fungi, grapefruit extract, garlic extract, seedlings, *Stewartia pseudocamellia*.

Introduction. In the last few years dynamic development of decorative nursery was observed in Poland. Many little known or completely new species of ornamental shrubs have appeared. *Stewartia pseudocamellia* Max. belongs to them. In case of reproduction of *Stewartia pseudocamellia* from seed, sprouts, and seedlings can be destroyed by the fungi occurred in the soil. The soil-born pathogens can also attack slightly elder plants, quicksets during taking root and perennial shrubs as well. The following fungi were most frequent isolated from the sore seedlings: *Fusarium* spp., *Cylindrocarpon* spp., *Alternaria* spp., *Phytophthora* spp., *Rhizoctonia* spp., and *Verticillium* spp. (Gajda et al., 2002).

The most common method of plant protection against phyto-pathogens is chemical method, which is based on fungicide application to seeds and shoot dressing or shoot spraying. However, work for limitation of pesticides application and replacement them with biological preparations based on antagonistic microorganisms, organic compounds, plant's extracts is observed (Orlikowski, Skrzypczak, 2003; Pięta et al., 2006; Rose et al., 2003; Wojdyła, 2004). Among biological preparations, the effective influence showed: Bioczos BR, Biochikol 020 PC and Biosept 33 SL.

Biosept 33 SL containing 33 % of grapefruit seeds and pulp extract has a direct influence on pathogenic factor and as well induces plants' immunity to some pathogens (Orlikowski et al., 2002). Mentioned activity shows 7-geranoxycumarin enclosed in grapefruit extract (Orlikowski, Skrzypczak, 2003). According to Caccioni et al. (1998), among many components of this extract aliphatic aldehyde, monoterpenes and nectone dominate. Discussed substances can show synergetic effect in limitation of defined factor development or stimulate spores' germination. Protective Biosept 33 SL activity is connected with endogenous flavonoids, glycosides, citrate and limonene (Saniewska, 2002) existence in the mentioned preparation.

In plant protection against fungal and bacterial diseases Bioczos BR based on garlic pulp has a great weight. Antibiotic garlic activity is assigned of alliin existence, which under the influence of lyase enzyme and after mechanical tissue damage is born from allin. The alliin is an acetyl-Co A synthetase inhibitor and in this way it blocks production of many different substances as fatty acid, sterols and the others. It was found that the ajoene, product of alliin transformation, shows stronger antifungal activity than alliin (Wolski, Ludwiczuk, 2002).

Biochikol 020 PC preparation contains chitosan as biological active substance and may show antiviral, antibacterial and antifungal effect. Followed to Rożpiński (1997), chitosan induces immunity of many plants through cell wall lignification, phytoalexins production and synthesis of proteinase inhibitors. The treatment of the cells with chitosan stimulates them to intensification through production of additional structures and accumulation of phenol substances, which are harmful to fungi.

The aims of presented research work was determination of fungi occurred on diseased *Stewartia pseudocamellia* seedlings and the *in vitro* effect of Bioczos BR (B. A. S. garlic extract), Biochikol 020 PC (B. A. S. chitosan), Biosept 33 SL (33 % extract from grapefruit seeds and pulp) on the mycelium linear growth of fungi pathogenic to *Stewartia* plants. As standard fungicide Bravo 500 SC (B. A. S. chlorotalonil) was applied.

Object, methods and conditions. The experiment was carried out on material from dr. hab. Muras collection in Tomaszówce-Wielkie hills. The samples of one-year-old diseased *Stewartia* seedlings were taken for mycological analysis at second decade of October 2006. After washing under running water and soaking in distilled, sterile water, diseased root and stem bases were disinfected in 75 % ethyl alcohol solution for 10 min, then again rinsed in sterile, distilled water. After cutting them to 0.5 cm fragments they were dried in sterile blotting paper and put on Petri dishes with solidified PDA medium (5 pieces for one dish). Inoculation was in room temperature. Colonies, which grew, were inoculated on PDA slants. Isolated fungi were identified by using mycological keys and monographs (Domsch et al., 1980; Gerlach, Nirenberg, 1982; Nelson et al., 1983). The pathogenicity of *Alternaria alternata*, *Cylindrocarpon radicola*, *Fusarium oxysporum*, *F. avenaceum* and *Phomopsis theae* in relation to *Stewartia* seedling were investigated in separate examination.

The Kowalik and Krechniak (1961) method was used to evaluate the *in vitro* effect of discussed preparations on tested pathogens mycelium linear growth. Each of examined preparations were applied at three different concentrations: Bioczos BR at concentrations 1.0 %, 2.0 %, 3.0 %; Biochikol 020 PC at concentrations: 2.0 %

4.0 %, 6.0 %; Biosept 33 SL: 0.05 %, 0.1 %, 0.2 %; Bravo 500 SC in doses: 0.025 %, 0.05 %, 0.1 %.

During the test preparations were introduced directly into liquid and slightly cooled PDA agar. After thorough mixing with the agar, obtained suspension was poured into Petrie dishes of 90 mm in diameter. Afterwards, an agar disk with mycelium of examined fungi was placed centrally into each dish for each combination. A medium without amendments and with a disk of appropriate fungus was used as the control for each combination. The test was carried out in five repetitions for each combination. Measurement started when first increase of mycelium in control dishes was observed and was conducted till the complete overgrowth of mentioned dishes was noted down (2–3 weeks). The percent of inhibition of mycelium linear growth on medium with amendments of appropriate preparation compared to the growth of fungi on control Petri dish was used to measure the preparations activity (Kowalik and Krechniak, 1961). Received results were subjected to statistical analysis using analysis of variance. The multiple t-Duncan test was used for estimation of the differences between mean values at significance level $\alpha = 0.05$.

Results. From *Stewartia pseudocamellia* Max. root and stem bases with observed disease symptoms the 55 isolates belonging to 14 fungi species and 12 genera and 15 bacteria were isolated (Table).

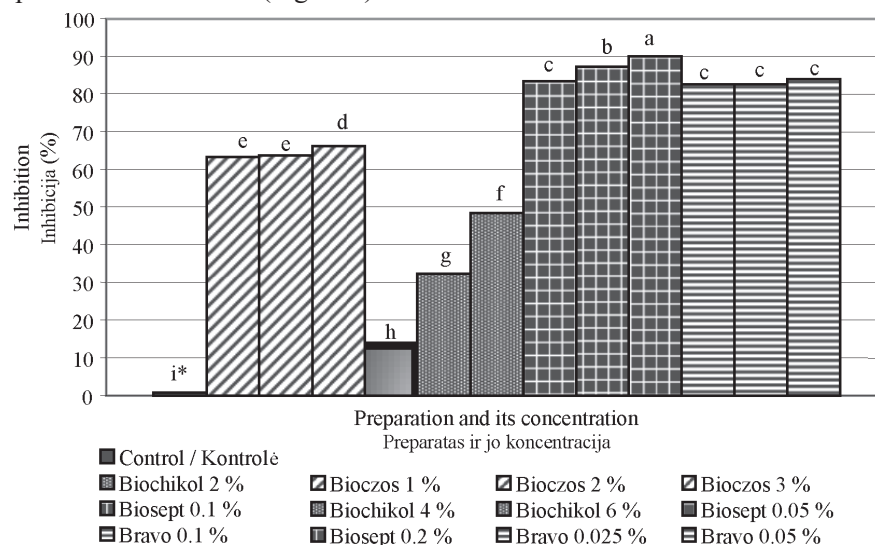
Table. Fungi isolated from root and stem base of diseased *Stewartia* seedlings
Lentelė. Grybai išskirti iš ligotų *Stewartia pseudocamellia* daigų šaknų ir stiebų

Species of fungus Grybų rūšys	Number Skaičius	Percent Procentai
<i>Alternaria alternata</i> (Fr.) Keissler	7	12.7
<i>Cylindrocarpon radicicola</i> Wollenweber	6	10.9
<i>Fusarium oxysporum</i> Schlecht.	6	10.9
<i>Fusarium avenaceum</i> (Fr.) Sacc.	5	9.0
<i>Phomopsis thea</i> Peth.	4	7.3
<i>Humicola fuscoatra</i> var. <i>fuscoatra</i> Traaen	4	7.3
<i>Phoma eupyrena</i> Sacc.	4	7.3
<i>Cladosporium cladosporioides</i> (Fres.) de Vries	4	7.3
<i>Cylindrocladium parvum</i> P. J. Anderson	3	5.5
<i>Penicillium canescens</i> Scopp	3	5.5
<i>Trichotecium roseum</i> (Pers.) Link ex Gray	3	5.5
<i>Phytophthora cactorum</i> (Leb. & Cohn) Schröt.	2	3.6
<i>Rhizoctonia solani</i> Kühn	2	3.6
<i>Verticillium albo-atrum</i> Reinke & Berthold	2	3.6
Total Bendrai	55	100
Bacteria Bakterijos	15	

The dominant fungi were: *Alternaria alternata* – 12.7 %, *Cylindrocarpon radicicola*, *Fusarium oxysporum* – each 10.9 %, *Fusarium avenaceum* – 9.0 %, *Phomopsis*

thea, *Phoma eupyrena*, *Humicola fuscoatra* var. *fuscoatra* and *Cladosporium cladosporioides* – each 7.3 % (Table). Besides, above-mentioned fungi *Phytophthora cactorum*, *Rhizoctonia solani* and *Verticillium albo-atrum* (potentially pathogenic fungi) in slightly less percentage settled stewartia seedlings (each 3.6 %) – Table.

According to the received results it was found that all investigated preparations (with exception of Biochikol 020 PC applied at concentrations 2.0 % in relation to *F. avenaceum*) significantly inhibited mycelium linear growth of tested fungi in comparison to the control (Fig. 1–5).



* Means followed by the same letter do not differ at 5 % level of significance (Duncan's multiple range test)

* Reikšmės, pažymėtos tomis pačiomis raidėmis, nesiskiria 5 % patikimumo lygyje (Dunkano testas)

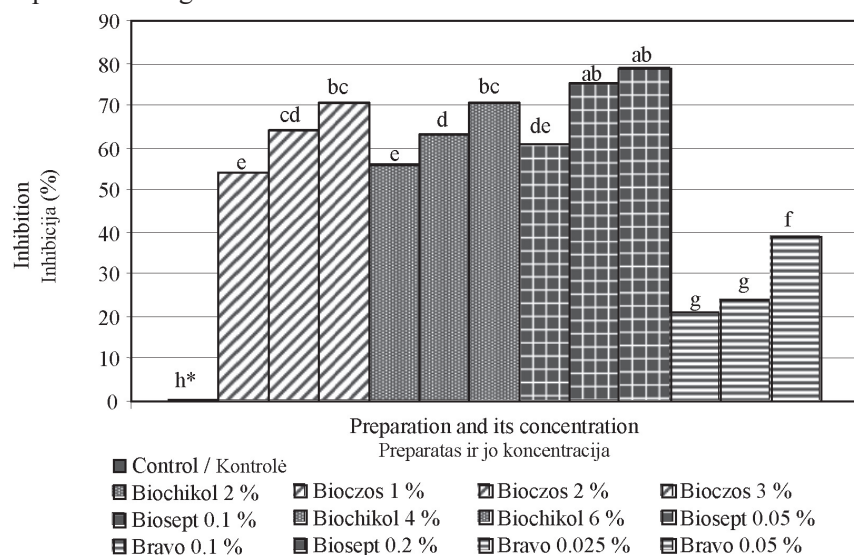
Fig. 1. Percent of inhibition of *Alternaria alternata* mycelium linear growth
1 pav. *Alternaria alternata* grybienos linijinio augimo inhibicijos procentas

Figure 1 represents the percent of inhibition of *A. alternata* mycelium linear growth. Among preparations under examination, Biosept 33 SL in all applied doses have shown the best inhibitory effect of mycelium linear growth of mentioned species. The percent of inhibition of mycelium linear growth appropriately amounted from 83.4 % to 90.0 % (Fig. 1).

Similar Biospet 33 SL reaction was observed in combination with *C. radicola* (Fig. 2) and *P. thea* (Fig. 5). The percent of inhibition of mycelium linear growth of above-mentioned fungi appropriately amounted from 60.1 % to 78.8 % (Fig. 2) and from 92.9 % to 96.0 % (Fig. 5). Biospet 33 SL in all tested concentrations showed the best effect of mycelium linear growth inhibition also in combination with *F. oxysporum* (from 80.0 % to 85.0 %) – Fig. 3. Similar result in relation to *F. oxysporum* was found in combination where Bravo 500 SC was used. The percent of inhibition of mycelium linear growth appropriately amounted from 80.9 % to 85.0 % – Fig. 3.

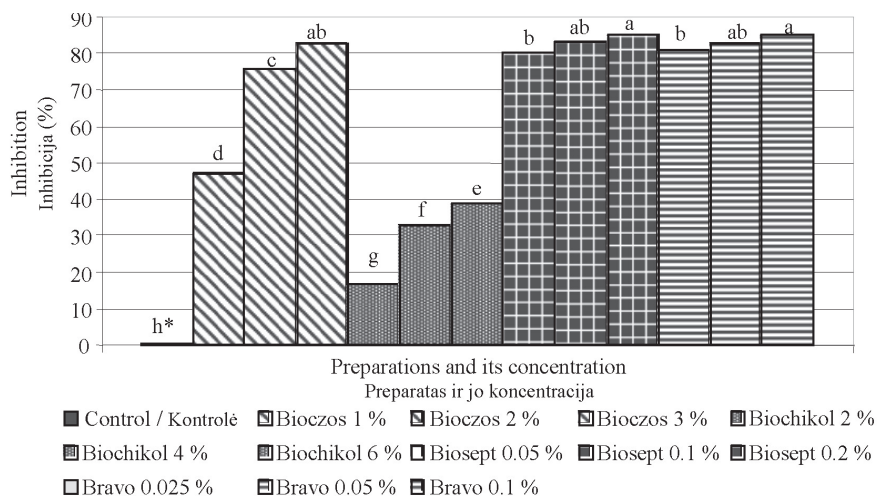
In case of *F. avenaceum*, it was found that, the most important effect on mycelium linear growth inhibition of discussed pathogen was obtained after Bravo 500 SC application (from 69.5 % to 75.6 %) – Fig. 4. Under *in vitro* condition bio-preparation

Bioczos has also demonstrated comparable high efficiency. Mentioned preparation limited tested fungi mycelium linear growth at slightly less percentage than Biosept 33 SL – Fig. 1–5.



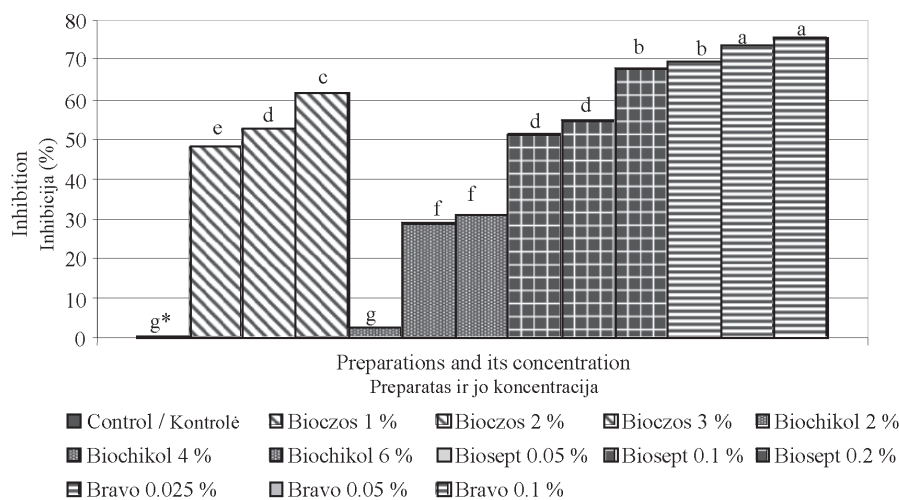
*Means followed by the same letter do not differ at 5 % level of significance (Duncan's multiple range test)
 * Reikšmės, pažymėtos tomis pačiomis raidėmis, nesiskiria 5 % patikimumo lygyje (Dunkano testas)

Fig. 2. Percent of inhibition of *Cylindrocarpon radicicola* mycelium linear growth
 2 pav. *Cylindrocarpon radicicola* grybienos linijinio augimo inhibicijos procentas



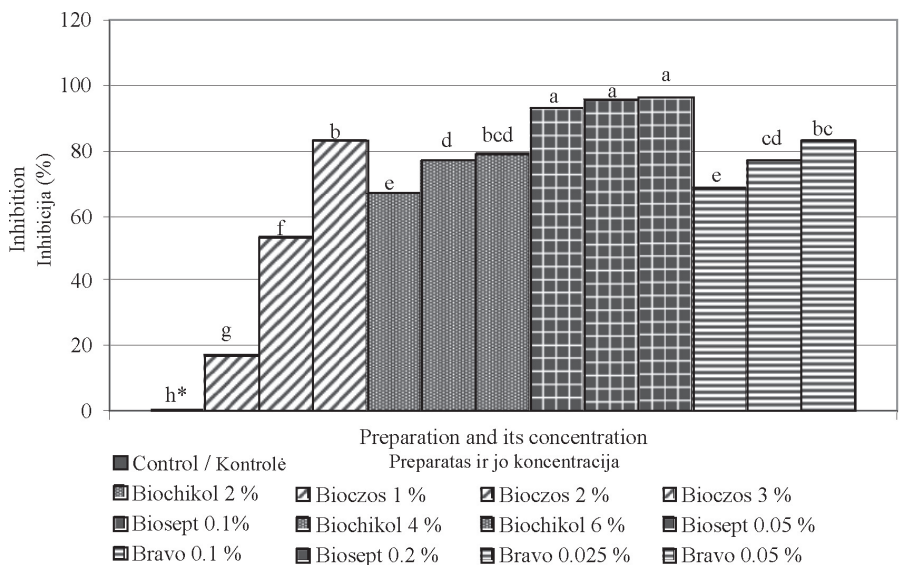
* Means followed by the same letter do not differ at 5 % level of significance (Duncan's multiple range test)
 * Reikšmės, pažymėtos tomis pačiomis raidėmis, nesiskiria 5 % patikimumo lygyje (Dunkano testas)

Fig. 3. Percent of inhibition of *Fusarium oxysporum* mycelium linear growth
 3 pav. *Fusarium oxysporum* grybienos linijinio augimo inhibicijos procentas



* Means followed by the same letter do not differ at 5 % level of significance (Duncan's multiple range test)
 * Reikšmės, pažymėtos tomis pačiomis raidėmis, nesiskiria 5 % patikimumo lygyje (Dunkano testas)

Fig. 4. Percent of inhibition of *Fusarium avenaceum* mycelium linear growth
4 pav. *Fusarium avenaceum* grybienos linijinio augimo inhibicijos procentas



* Means followed by the same letter do not differ at 5 % level of significance (Duncan's multiple range test)
 * Reikšmės, pažymėtos tomis pačiomis raidėmis, nesiskiria 5 % patikimumo lygyje (Dunkano testas)

Fig. 5. Percent of inhibition of *Phomopsis thea* mycelium linear growth
5 pav. *Phomopsis thea* grybienos linijinio augimo inhibicijos procentas

The least effect on mycelium linear growth inhibition of tested pathogens was found in combinations where Biochikol 020 PC was applied. However, the activity of this preparation (with the exception of the lowest concentration in relation to *F. avenaceum*) was statistically important in comparison to control Fig. 1–5.

Discussion. Among fungi isolated from root and stem bases of diseased *Stewartia* seedlings the most numerous group constituted species considered as pathogenic for plants. The most dominating species (over 50 % of isolated colonies) were: *Alternaria alternata*, *Cylindrocarpon radicum*, *Fusarium oxysporum*, *F. avenaceum*, *Phomopsis thea* and fungi from genera: *Phytophthora*, *Rhizoctonia* and *Verticillium*. Similar results were obtained by Gajda et al. (2002). According to Werner (1998) fungi *C. radicum*, *F. oxysporum* and *F. avenaceum* may cause wilting and decay of plants. *C. radicum* affect roots and root neck and *Fusarium oxysporum* destroy conductive wisps. Infected young plants die quickly. In case of older plants, disease develops slowly, and shrubs die gradually. Moreover, followed to Orlikowski (2000), fungi from *Phytophthora* genera, wilting and dying of ornamental leafed shrubs can cause other fungi – *Fusarium avenaceum* and *F. oxysporum*.

Findings from conducted investigations under *in vitro* conditions showed, that all applied preparations (with the exception of Biochikol 020 PC at concentration 2.0 % in relation to *F. avenaceum*) significantly reduced mycelium linear growth of the tested *Stewartia* pathogens. Among preparations under consideration, the strongest inhibition of examined pathogens mycelium linear growth (with the exception of *F. avenaceum*) was found in combination where Biosept 33 SL was used. Similar results were received by co-author in previous examinations (Kurzawińska et al., 2007). According to Orlikowski et al. (2001) Biosept 33 SL added to medium where pathogenic fungi grow, strongly inhibited mycelium expansion. High efficiency of discussed preparation was confirmed other investigations conducted by Orlikowski et al. (2001, 2002), Wojdyła (2004). Biosept 33 SL added to the ground significantly reduced number of *Phytophthora* spp., *Pythium* spp., *F. oxysporum* (Orlikowski, Skrzypczak, 2003; Orlikowski et al., 2001, 2002). Bioczol BR preparation was also efficient in inhibiting mycelium linear growth of the tested fungi. Mentioned preparation limited mycelium linear growth at slightly less percent than Biosept 33 SL. Followed to Saniewska (2002) garlic extract has an effect on over 100 fungi species. Besides, more of them show total growth inhibition. Author claims that content of 0.75 % garlic extract in medium strongly limiting growth of *Phoma narcissii* mycelium and some form specials of *F. oxysporum* *Phytophthora cryptogea*, *Phyllosticta antirrhini*, *Rhizoctonia solani*, *Botrytis cinerea*, *Botrytis tulipae* (Saniewska, 2000). The least *in vitro* effect on mycelium linear growth inhibition of fungi under consideration was found in combinations where Biochikol 020 PC was used. The effect of the mentioned preparation was statistically important in comparison with control (with the exception of concentration 2,0 % in combination with *F. avenaceum*). Followed to Orlikowski et al. (1998) *in vitro* activity of chitosan is rather poor. Results obtained from later studies conducted by this author have shown that discussed substance does not inhibit mycelium growth and spore germination under *in vitro* conditions. However, according to Połpiuszny (1997), chitosan inhibits growth of several fungi and bacteria but not all of them. Chitosan activity to pathogens mostly takes place through direct and long-lasting contact of both factors.

Conclusions. 1. The root and stem bases of diseased *Stewartia* seedlings were colonized mostly by species: *Alternaria alternata*, *Cylindrocarpon radicum*, *Fusarium oxysporum*, *Fusarium avenaceum* and *Phomopsis thea*.

2. Among all the tested preparations under *in vitro* conditions the most effective in reduction of above mentioned pathogens mycelium linear growth (with the exception of *F. avenaceum*) turned to be Biosept 33 SL bio-preparation. The *in vitro* response of tested pathogens depended on the fungi species, type of preparation and its concentration.

4. Bravo 500 SC, chemical, standard preparation, showed the most effective influence on *F. avenaceum* mycelium linear growth inhibition.

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Bio-preparatų veiksmingumas prieš *Stewartia pseudocamellia* (Max.) patogenus *in vitro*

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Santrauka

Tyrimų tikslas buvo nustatyti tipinius grybus, gautus nuo sergančių *Stewartia pseudocamellia* daigų ir biologinių preparatų, susidedančių iš natūralių medžiagų, poveikį potencialiai šiems augalams patogeniškų grybų micelio linijiniam augimui.

Buvo tirti šie preparatai: Bioczoz BR (B. A. S. česnako ekstraktas), Biochikol 020 PC (B. A. S. chitosanas), Biosept 33 SL (33 % ekstraktas iš greipfrutų sėklų ir minkštimo). Kiekvieno preparato tirtos 3 koncentracijos. Fungicidas Bravo 500 SC (B. A. S. chlorotalonilas) buvo panaudotas kaip kontrolė. Preparatų poveikis linijiniam micelio augimui buvo įvertintas naudojant Kowalik ir Krechniak metoda (1961).

Laboratorinio eksperimento sąlygomis nuo šaknų ir stiebo, remiantis pastebėtais ligų simptomais, išskirta 14 rūšių grybų ir 15 rūšių bakterijų. Dominavo šie grybai: *Alternaria alternata*, *Cylindrocarpon radicola*, *Fusarium oxysporum*, *Fusarium avenaceum*, *Phomopsis thea*.

Gauti rezultatai rodo, kad visi naudoti preparatai žymiai sumažino testuotų grybų micelio linijinį augimą. Preparatų poveikis buvo įvairus ir priklausė nuo koncentracijos ir preparatų tipo.

Reikšminiai žodžiai: chitosanas, chlorotalonilas, česnaku ekstraktas, daigai, greipfrutų ekstraktas, *Stewartia pseudocamellia*.