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EFFECT OF DISINFECTANTS AND EM PREPARATIONS ON THE GROWTH INHIBITION OF *Lecanicillium fungicola* OCCURRING IN CULTIVATION OF BUTTON MUSHROOM (*Agaricus bisporus*)

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ABSTRACT

Background. The dry bubble disease, caused by *Lecanicillium fungicola*, is the most common fungal disease of white button mushroom (*Agaricus bisporus*). The aim of this study was to estimate the effectiveness of two chemical agents based on periacetic acid and biopreparations Effective Microorganisms (EM) in inhibiting the growth of *L. fungicola* isolated from infected fruiting bodies of button mushrooms.
Material and methods. The material was the pathogen *L. fungicola* isolated from bottom mushrooms, treated with disinfectants: Agrosteril (in concentration 1, 1.5, 2, 2.5, 3, 3.5%), Larasept (in concentration 0.2%, 0.5%, 1%) and various configurations with biopreparations based on effective microorganisms (EM). The activity of the chemical preparations was assessed in a plate (on PDA medium) and pot experiment (in infected casing soil). The fungicidal efficacy of EM preparations was performed only in vitro.
Results. In a plate experiment Agrosteril disinfectant caused the complete inhibition of pathogen development at each tested concentration. In the case Lerasept the lack of mycelium growth was obtained only after the application of the highest dose of 1%. Biopreparation EM-5 and its combination with EM-NA in a dose of 100 mg·cm^{-3} of the medium turned out to be the most effective of EM preparations in inhibiting the development of *L. fungicola*. Those preparations also caused a significant decrease in sporulation of the pathogen. Chemical preparations applied in the form of sprinkling to the casing infected with the fungi turned out to be less effective. The highest decrease in the pathogen number was obtained on 7th day from inoculation of the casing.
Conclusion. Tested disinfecting preparations were characterized by strong fungicidal properties *in vitro*. Based on the pot experiment it was found that chemical preparations used for disinfecting the casing, applied in doses recommended by the producer, caused only a partial inactivation of *L. fungicola*.

Key words: disinfectant preparations, effective microorganisms (EM), *Lecanicillium fungicola*, mushroom cultivation

INTRODUCTION

Lecanicillium fungicola (Preuss) Zare and Games, formerly known as *Verticillium fungicola* (Preuss) Hassebrauk is one of the most serious fungal pathogens in mushroom cultivation (Zare and Gams 2008). Dry bubble induced by this species infects commonly cultivated mushroom *Agaricus bisporus*

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and, less frequently, *Agaricus bitorquis*. This disease can result in a decrease in yield even by 25 % (Gea *et al.*, 2003; Szumigaj-Tarnowska *et al.*, 2011). It happens that the symptoms are unnoticeable during harvest and can be observed on fruiting bodies only when going on sale, which causes high losses in mushroom trade. Mushrooms infected by *L. fungicola* do not decay but remain in the crop for some time. First symptoms after infection with spores of *Lecanicillium* are visible after about 7 days and the causes of development of the disease and the ways of pathogen infection and elimination from cultivation were investigated (Largeteau and Savoie, 2008, Berendsen *et al.*, 2010, 2012; Nunes *et al.*, 2017).

Prevention and providing the optimal growing conditions are of the utmost importance in mushroom cultivation. Application of crop protection preparations is necessary. The proper dose of a disinfectant and the sufficient time of its contact with the material should be provided (Zhao *et al.*, 2007; Potočnik *et al.*, 2008; Mehrparvar *et al.*, 2013).

It should be stressed that chemical preparations are sometimes not effective enough against dry bubble, and additionally they pose a risk of accumulating toxic substances in the fruiting bodies (Savoie and Largeteau, 2004). Partial withdrawing of chemical agents forced search for alternative crop protection preparations. Currently action is being taken to develope effective methods for crop protection using agents of natural origin. This opens new prospects for disease and pest control with preparations based on effective microorganisms and extracts from plants. Maintaining a high cultivation hygiene, by means of using chemical and biological disinfecting preparations, should be the basic element of cultivation in order to obtain a field of high quality (Boligłowa and Gleń, 2008; Potočnik *et al.*, 2015).

The aim of this study was to estimate the effectiveness of selected chemical agents and EM microbiological preparations in inhibiting development of the pathogenic fungus *Lecanicillium fungicola*, taking into consideration different preparation doses under *in vitro* conditions and in a pot experiment.

MATERIAL AND METHODS

The fungus *Lecanicillium fungicola* was isolated from the casing used in cultivation of *Agariqus bisporus* from a mushroom farm located near Piła (northen Poland). Systematic classification of the fungus was determined based on diagnostic and taxonomic analyses, using mycological keys (Gilman, 1980; Domsch *et al.*, 1990). The effectiveness of inhibiting the development of pathogenic fungus was evaluated for two chemical agents:

– Agrosteril at concentrations of 1%, 1.5%, 2%, 2.5%, 3%, 3.5% (active ingredient 110 g of peracetic acid per 1000 g preparation),
– Lerasept® Spezial at concentrations of 0.2%, 0.5%, 1% (active ingredient 50 g of peracetic acid and 260 g of hydrogen peroxide per 1000 g preperation).

The effectiveness of natural preparations based on effective microorganisms from the firm Greenland Technologia EM SP ZOO Company was determined for:

1) EM Naturally Active (EM-NA) containing bacteria of the genus *Azotobacter*, lactic acid bacteria, photosynthetic bacteria, yeasts and sugar cane molasses. It was used in two doses 50 and 100 $mg \cdot cm^{-3}$ of medium,
2) EM-5 (a composition of fermented natural components of garlic, paprika, wine vinegar, sugar cane molasses with Effective Microorganisms) at doses of 5.50 and 100 $mg \cdot cm^{-3}$ of medium,
3) combination of EM-NA and EM-5 together in two concentrations (EM NA 100 $mg \cdot cm^{-3}$ + EM-5 100 $mg \cdot cm^{-3}$, EM-NA 50 $mg \cdot cm^{-3}$ + EM-5 50 $mg \cdot cm^{-3}$).

Potato dextrose agar – PDA (Merck, Germany) was used for isolation and cultivation of fungi and for plate tests. In the plate experiment a medium prepared with an addition of tested preparations was inoculated with a 7-day culture of *L. fungicola*. For inoculation 5 mm discs cut with a sterile cork borer from the edge part of mycelium were used. Analyses were carried out in 6 replications for each tested preparation and concentration. The plates with the medium inoculated with mycelium without fungicidal preparations were used as a control. Cultures were incubated at 23°C without the access of light. The growth of mycelium was measured after 3, 5, 8, 11 and 14 days in two perpendicular directions, and then their diameter was calculated. The sporulation of *L. fungicola* on the medium with addition of preparations EM and the control was determined after 14 days of the

experiment. Concentration of spore suspension was measured using a hemocytometer (Thom's chamber), and their number (S) in cm^3 was calculated according to the formula:

$$S = \frac{x \cdot 1000}{0.004}$$

where:

x – average number of spores from randomly selected large squares.

Effectiveness of preparations in the *in vitro* experiment was determined comparing the colony diameter of fungi and the numbers of spores in suspension in relation to the control culture.

The next stage of the study was the pot experiment where the casing from the mushroom farm. The casing was sterilized 3 times in an autoclave at 24-hour intervals. Then it was inoculated with homogenized mycelium, which was obtained from the previously multiplied culture of *L. fungicola*. After even mixing, infected material was transfer red to pots with a volume of 500 ml and chemical preparations Agrosteril and Lerasept were added in appropriate doses. Preparations were applied gradually. First a part was sprinkled over 200 ml of the casing, then the pots were filled and sprinkled again with the rest of the preparation. The control was the casing inoculated with mycelium with an addition of sterile water in the same amount as the tested preparations. The pots were incubated at 23°C without the access of light.

The effectiveness of disinfecting preparations in the pot experiment was assessed after 2, 7 and 14 days from establishment. From each pot 10 g of coat was collected and weighed. Then a series of 10-fold dilutions in Ringer's solutions was prepared. From each dilution a deep inoculation was made in 4 replications, using the PDA liquid medium. The plates were incubated at 23°C for 10 days. Based on macro- and microscopic observations the number of grown colonies (cfu) of *L. fungicola* from each pot was determined.

Obtained results of the experiment were statistically worked out with the variance analysis method, and the significance of differences between the means was evaluated by Student's t-test (P = 0.05) in the STATISTICA 12 PL program (StatSoft Poland).

RESULTS

Based on the results obtained in the plate experiment, a high effectiveness of disinfecting agents in inhibiting the growth of *Lecanicillium fungicola* mycelium was observed. Agrosteril disinfectant showed a fungistatic effect in all the doses used (Table 1). Complete inhibition of mycelial growth was observed throughout the duration of the experiment. The mycelium diameter in the control culture reached 41.5 mm on the 14th day after inoculation. For the preparation Lerasept spezial, only at a concentration of 1% the complete lack of mycelium growth was observed (Table 2). It was found that the growth of pathogen mycelium significantly decreased along with an increase in a dose of Lerasept. At the concentration of 0.5% the inhibition of mycelium growth after 3 days from inoculation was 100%, and after 14 days decreased to 74.7% in relation to the control. In the case of the lowest dose (0.2%) the inhibition of mycelium growth after 3 and 14 days of the experiment was 31.8% and 26.5%, respectively.

Microbiological preparations EM also showed a strong inhibition of the development of *L. fungicola* on PDA medium. The size of inhibition zone depended on the kind of the EM preparation applied and the dose (Table 3). EM-5 turned out to be the most effective preparation. The complete inhibition of the pathogen growth was achieved after the application of EM-5 at a concentration of 100 $mg \cdot cm^{-3}$ PDA and of the combination EM-5 + EM-NA (each at a concentration of 100 $mg \cdot cm^{-3}$). High effectiveness was also found under the influence of a lower dose – 50 mg EM-5, applied separately to the medium and together with EM-NA (50 mg). The inhibition of mycelial growth with this amount of EM-5 on the 8th day of cultivation was 70.6% and on the 14th day – 77.1%. For the combination of EM-5 + EM-NA, the results were 100% and 75.9%, respectively. EM-NA at the tested concentrations, on day 14, inhibition was 42.2% relative to control.

Table 1. Effect of Agrosteril on the growth of *Lecanicillium fungicola* mycelium during 14-days *in vitro* culture

Agrosteril concentration %	Number of days after medium inoculation									
	3		5		8		11		14	
	MD mm	I %	MD mm	I %	MD mm	I %	MD mm	I %	MD mm	I %
1.0	0.0	100	0.0	100	0.0	100	0.0	100	0.0	100
1.5	0.0	100	0.0	100	0.0	100	0.0	100	0.0	100
2.0	0.0	100	0.0	100	0.0	100	0.0	100	0.0	100
2.5	0.0	100	0.0	100	0.0	100	0.0	100	0.0	100
3.0	0.0	100	0.0	100	0.0	100	0.0	100	0.0	100
3.5	0.0	100	0.0	100	0.0	100	0.0	100	0.0	100
Control	11.0	–	16.5	–	25.5	–	33.5	–	41.5	–

MD – mycelial growth; I – inhibition

Table 2. Effect of Lerasept Spezial on the growth of *L. fungicola* during 14-days *in vitro* culture

Lerasept concentration %	Number of days after medium inoculation									
	3		5		8		11		14	
	MD mm	I %	MD mm	I %	MD mm	I %	MD mm	I %	MD mm	I %
0.2	7.5 b*	31.8	12.5 c	24.2	16.0 c	37.3	22.0 c	34.3	30.5 c	26.5
0.5	0.0 a	100	5.5 b	66.7	6.0 b	76.5	7.0 b	79.1	10.5 b	74.7
1.0	0.0 a	100	0.0 a	100	0.0 a	100	0.0 a	100	0.0 a	100
Control	11.0 c	–	16.5 d	–	25.5 d	–	33.5 d	–	41.5 d	–

MD – mycelial growth; I – inhibition; *means in columns followed by the same letter did not differ significantly ($p = 0.05$) acc. to t-Student's test

Table 3. Effect of effective microorganisms (EM) on the growth of *L. fungicola* during 14-days *in vitro* culture

EM preparation concentration ($mg \cdot cm^{-3}$ medium)	Number of days after medium inoculation									
	3		5		8		11		14	
	MD mm	I %	MD mm	I %	MD mm	I %	MD mm	I %	MD mm	I %
EM-NA 50	5.0 b*	54.5	9.0 b	45.5	14.5 c	43.1	22.0 d	34.3	29.0 d	30.1
EM-NA 100	6.5 b	40.1	7.5 b	54.5	13.0 c	49.0	18.0 c	46.3	24.0 c	42.2
EM-5 5	11.0 c	0.0	15.0 c	9.1	21.0 d	17.6	27.5 e	17.9	35.0 e	15.7
EM-5 50	6.5 b	40.1	7.0 b	57.6	7.5 b	70.6	8.0 b	76.1	9.5 b	77.1
EM-5 100	0.0 a	100	0.0 a	100	0.0 a	100	0.0 a	100	0.0 a	100
EM-NA+EM-5 (50 + 50)	0.0 a	100	0.0 a	100	0.0 a	100	6.0 b	82.1	10.0 b	75.9
EM-NA+EM-5 (100 + 100)	0.0 a	100	0.0 a	100	0.0 a	100	0.0 a	100	0.0 a	100
Control	11.0 c	–	16.5 c	–	25.5 e	–	33.5 f	–	41.5 f	–

MD – mycelial growth; I – inhibition; *means in columns followed by the same letter did not differ significantly ($p = 0.05$) acc. to t-Student's test

Effective Microorganisms reduced sporulation of *L. fungicola* (Fig. 1). For the most tested preparation concentrations, a significantly smaller number o spores was obtained as compared with the control. Complete lack of spores was observed under the influence of the highest doses of combination EM-5 + EM-NA. Only in the culture where the smallest amount of EM-5 (5 $mg \cdot cm^{-3}$ PDA) was added, no significant decrease in spore number was observed.

In the pot experiment, chemical agents added to the casing infected with the pathogen *L. fungicola* showed a moderate fungicidal effect. The largest decrease in the $cfu \cdot g^{-1}$ in the casing was observed after the application of 3% Agrosteril (Fig. 2). It was found that the highest concentration (3.5%) of this preparation inhibited the development of the pathogen the most in the first term, because on the next day, doses of 3% and 2.5% showed higher efficacy. The number of CFU of mushrooms obtained from the vases with the lowest concentrations of Agrosteril 0.5%, 1%, 2% did not differ significantly from the values obtained for the control.

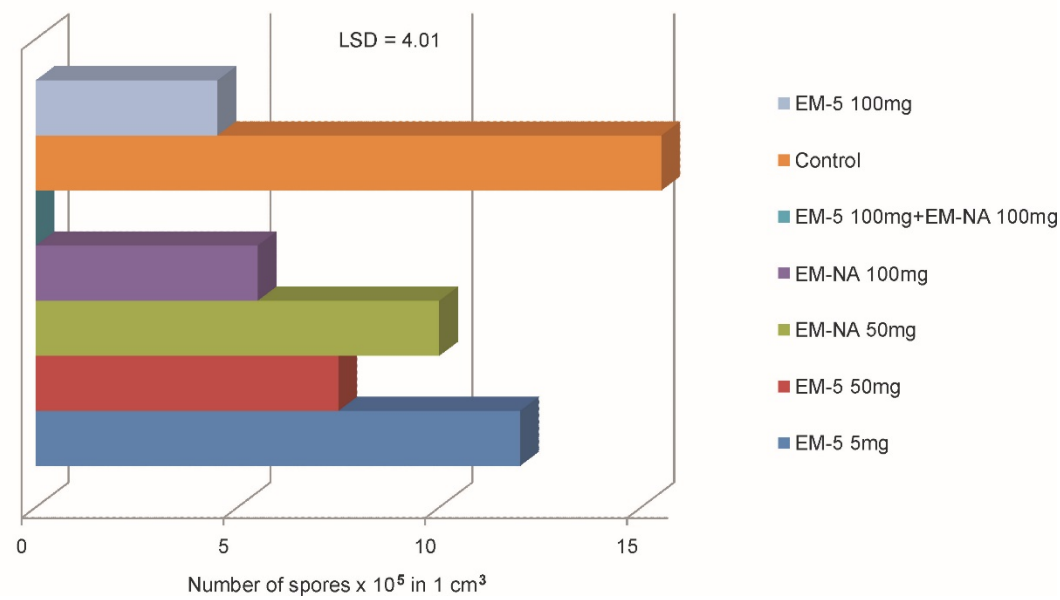

Fig. 1. Concentration of spores *L. fungicola* on PDA medium with addition of EM biopreparations, LSD- Least Significant Difference

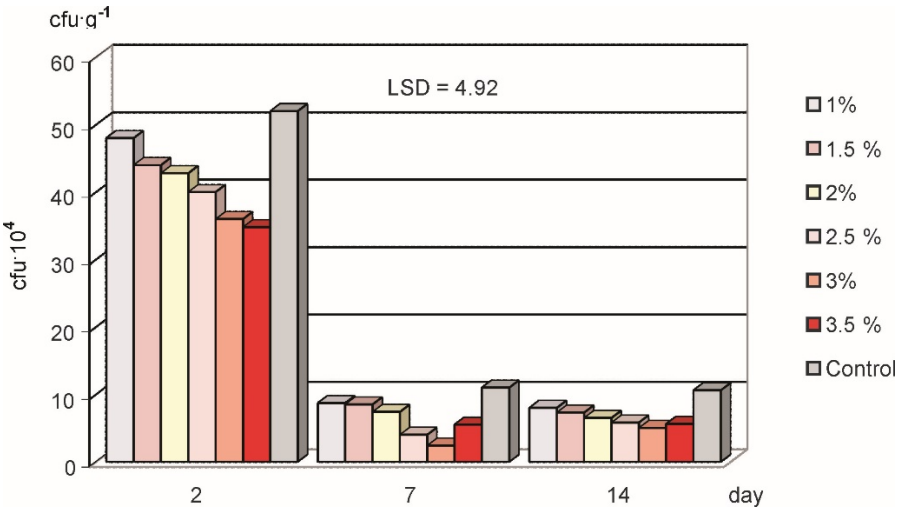

Fig. 2. Efficacy of Agrosteril in reducing the occurrence of *L. fungicola* in infected casing soil (cfu·g^{-1} of casing)

In case of Lerasept spezial is concerned, the highest fungicidal effectiveness was obtained at the concentration 1% (Fig. 3). After two days from application the preparation at all the tested doses, 0.2, 0.5% and 1%, significantly contributed to a decrease in the number of pathogen in the casing. However, after 14 days only at the highest concentration of Lerasept effectively inhibited the development of *L. fungicola*. The fungal cfu number obtained at that date from pots with an addition of 0.2 and 0.5% of Lerasept was higher or the same as for the control.

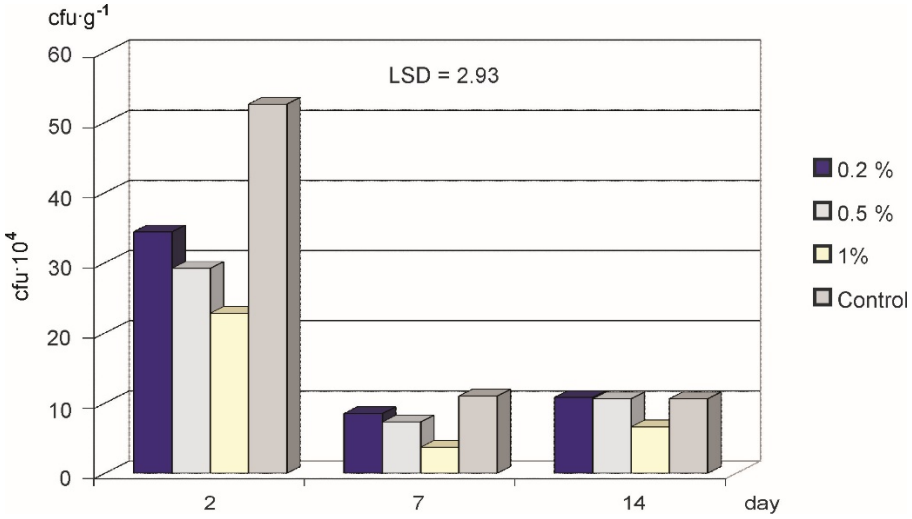

Fig. 3. Efficacy of Lerasept Spezial in reducing the occurrence of *L. fungicola* in infected casing soil (cfu·g^{-1} of casing)

DISCUSSION

The results obtained prove that the effectiveness of disinfecting agents Agrosteril and Lerasept spezial against dry bubble depended on conditions of the carried out experiment. Preparations showed a definitely higher fungicidal effectiveness in the plate culture than after their addition to the casing infected with the fungi.

The current state of knowledge concerning the species *Lecanicillium fungicola*, its biology, symptoms of disease, the source of infection and transmission was known so well that definitely effective methods for preventing and eliminating dry bubble should be expected (Farrag-Rasha *et al.*, 2009; Berendsen *et al.*, 2010; Largeteau and Savoie, 2010). According Gea *et al.* (2003), mistakes in cultivation practices made by the producer, e.g. carelessly disinfected casing, create favourable conditions for development of fungi pathogenic for *Agaricus bisporus*.

In plate tests each dose of Agrosteril completely inhibited the development of *L. fungicola* mycelium. In contrast, in the experiment on the casing the effect was definitely less satisfactory. Similar situation was observed after the application of Lerasept. Based on the results obtained from the pot experiment, it was found that the development of pathogen in the casing was highly inhibited by Agrosteril at a concentration of 3%. Such a concentration is also recommended by the producer of preparation for disinfecting the mushroom farm. According to the producer, the preparation prevents developing fungal and bacterial diseases of cultivated mushrooms (www.implus.pl). In the study conducted by Pastuszewska and Gryń (2009), Agrosteril turned out to be the most effective of 11 chemical agents subjected to evaluation. Its disinfecting effect was observed on the most of the tested surfaces at a concentration of 0.5%.

Protection recommendations, concerning the dates of hygienic treatments and the agents applied, change very often and new fungicidal preparations are preferred each year (Gea *et al.*, 2005; Zhao *et al.*, 2007; Mehrparvar *et al.*, 2013). For a few years, a tendency has been observed, partially forced by legal regulations, to replace chemical agents with preparations of natural origin. Dangerous chemical agents are party withdrawn from the market and replaced with those safe for the environment. It is possible to fight pathogens with the use of natural preparations, not disturbing the natural environment homeostasis. There are more and more scientific works in the literature concerning the use and effectiveness of preparations based on effective microorganisms (EM) in plant protection (Valarini *et al.*, 2003; Khaliq *et al.*, 2006; Boligłowa and Gleń, 2008).

On the basis of the conducted plate experiment, a high effectiveness of EM in inhibiting mycelium development and sporulation of *L. fungicola* was observed. Of the tested EM preparations, the pathogen development was completely inhibited by EM-5 alone

and together with EM-NA in an amount of 100 mg·cm^{-3} medium. Also the number of the fungus spores significantly decreased after the application of appropriate doses of EM. To confirm the effectiveness of the tested EM preparations in eliminating *L. fungicola* there is a need to carry out the research directly on the casing and at the mushroom farm. Then it will be possible to fully evaluate the fungicidal effect of those preparations. The results obtained by other authors (Górski and Góra, 2009; Breza-Boruta *et al.*, 2015) confirm the fungicidal effect of preparations EM-5 and EM-NA. Górski and Góra (2009) indicated strong effect of those preparations on reduction in growth of *Trichoderma harzianum*, which causes the green mould in mushrooms. EM preparations used in doses of 100 and 50 mg·cm^{-3} of the medium caused strong inhibition of mycelium growth of *T. harzianum* from 100% to 59.7% and a reduction in sporulation from 100% to 77.6%. The authors of these studies obtained the highest effectiveness of EM-5 and EM-NA applying them together, similarly to the present study. Thus the results obtained in plate analyses for EM preparations are promising. However, there are also negative opinions and doubts concerning the quality and effectiveness of EM. According to Martyniuk (2011), those biopreparations do not meet many requirements for products used in crop protection and cultivation in respect of microbiology. One of the objections to EM is, for instance, that the numbers of individual groups of microorganisms and their species composition are unknown. Moreover, the repetitiveness of cultures contained in those preparations is low (Wielgosz *et al.*, 2010). However, there are studies where the authors describe the effective protection of winter wheat against such pathogens as *Septoria nodorum*, *Drechslera tritici-repentis*, or winter rape against *Pucinia recondita* after the application of effective microorganisms (Boligłowa and Gleń, 2008). Another example of positive effect of the EM preparation was using it in the form of sprinkling on field pea, which considerably limited the development of *Fusarium* spp. (Okorski and Majchrzak, 2007). According to Soković and van Griensven, (2006), it is necessary to give up the application of pesticides both against diseases and pests, following the assumptions of health food production. It should be taken into account that chemical preparations pose a threat of accumulating toxic substances in fruiting bodies of *Agaricus bisporus*. Using natural preparations for eliminating the pathogenic fungi of mushroom, *Mycogone perniciosa* and *L. fungicola*, was presented in the study by Gea *et al.* (2013), Regnier *et al.*, (2010) and Tanović *et al.*, (2009). In this study, those pathogens responded to the tested essential oils from aromatic and medicinal plants with the complete inhibition of growth. The effectiveness depended first of all on the concentration of oils. The oil obtained from oregano appeared to be the most effective (Tanović *et al.*, 2009).

Dry bubble controlled with different chemical agents for years has developed resistance to many of them. New possibilities appeared of reducing losses by disinfection carried out during mushroom cultivation. According to Zied *et al.* (2015), *L. fungicola* is an indicator of the level of disease control in the mushroom farm. Tested chemical preparations based on periacetic acid used for casing disinfection in doses recommended by the producer did not cause the complete inactivation of *L. fungicola* and higher doses of Agrosteril and Lerasept spezial not always resulted in a significant inhibiting of the pathogen development.

CONCLUSIONS

1. Tested disinfecting preparations were characterized by strong fungicidal properties *in vitro*. The preparation Agrosteril caused the complete lack of mycelium growth in *Lecanicillium fungicola* at all the applied concentrations, whereas Lerasept Spezial at a concentration of 1%.
2. Preparations based on Effective Microorganisms showed a high effectiveness in inhibiting mycelium growth and reducing sporulation of *L. fungicola*. The greatest effectiveness was observed for the highest doses of preparation EM-5 and for the combination of EM-5 with EM-NA.
3. Tested chemical agents were less effective in eliminating dry bubble after application to the infected casing. Increasing the dose of Agrosteril not always resulted in a significant inhibiting of the phytopathogen development.
4. The preparations used for casing disinfection in doses recommended by the producer did not cause the complete inactivation of *L. fungicola*.

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WPŁYW ŚRODKÓW DEZYNFEKCYJNYCH I PREPARATÓW EM NA OGRANICZENIE WZROSTU *Lecanicillium fungicola* WYSTĘPUJĄCEGO W UPRAWIE PIECZARKI DWUZARODNIKOWEJ (A*garicus bisporus*)

Streszczenie

Celem badań było określenie skuteczności środków chemicznych Agrosteril i Lerasept oraz preparatów na bazie efektywnych mikroorganizmów EM w hamowaniu rozwoju patogenicznego grzyba *Lecanicillium fungicola* wyizolowanego z uprawy pieczarek. W doświadczeniu płytkowym preparat Agrosteril w każdym testowanym stężeniu (1%, 1,5%, 2%, 2,5% 3%, 3,5%) powodował całkowite zahamowanie rozwoju patogena. Natomiast pod wpływem Leraseptu brak wzrostu grzybni stwierdzono tylko po zastosowaniu najwyższej dawki 1%, zaś w niższych koncentracjach 0,2% i 0,5% jego działanie było słabsze. Spośród preparatów EM najbardziej skutecznym okazał się EM-5 i jego kombinacja z EM-NA w dawce 100 mg·cm^{-3} pożywki. Preparaty te również powodowały istotny spadek zarodnikowania. Na podstawie doświadczenia wazonowego stwierdzono, że użyte do odkażania okrywy preparaty chemiczne powodowały tylko częściową inaktywację *L. fungicola*.

Słowa kluczowe: efektywne mikroorganizmy (EM), *Lecanicillium fungicola*, preparaty dezynfekcyjne, uprawa pieczarek