Article

Antifungal Activity of Industrial *Bacillus* Strains against *Mycogone perniciosa* the Causative Agent of Cultivated Mushrooms Wet Bubble Disease

Irina Novikova [1] and Julia Titova [1, *]

[1] Federal State Budget Scientific Institution "All-Russian Research Institute of Plant Protection" (FSBSI VIZR), 3, Podbelskogo shosse, St. Petersburg-Pushkin, 196608, Russia; irina_novikova@inbox.ru

* Correspondence: juli1958@yandex.ru; Tel.: +79112309063

Abstract: Microbiocontrol of *Mycogone perniciosa* is focused on casing soil antagonists use. Since no industrial producer strains of polyfunctional biologics were used in previous studies, research goal was to characterize *Bacillus subtilis* B-10 and M-22 effect on mycopathogen and reveal its control possibilities. Interactions between producer strains and *M. perniciosa* were evaluated using well method and spraying. Mycoparasite colony area and its lysis zone were determined. Biological efficacy was characterized by lysis zone proportion in total area occupied by the micromycete mycelium. Statistical processing included *W*-test, *ANOVA*, means (*M*) and standard errors (±*SEM*) calculation. The significance was assessed by *t*-test. *M. perniciosa* development suppressing by producer strains was established, indicates a prolonged B-10 and M-22 effect on mycopathogen. High biological efficacy of both strains at the early stages of mycopathogen development by introducing into the wells and spraying was shown: B-10 – 50.9–99.6 % and M-22 – 57.5–99.2 % respectively ($p \leq 0.05$). Significant differences between producer strains were not revealed, although during the first exposure day to developed *M. perniciosa* colonies M-22 has shown greater activity. Preventive treatment high efficiency when producer strains completely suppressed mycoparasite development, permit recommending them both to introduce when preparing casing for *M. perniciosa* control.

Keywords: *Mycogone perniciosa*; *Bacillus subtilis*; industrial *Bacillus* strains; microbiocontrol; antifungal activity; producer strains; biological efficacy; multirecycled spent mushroom substrates; multirecycled SMS aqueous extracts; submerged multibiorecycled biologics

1. Introduction

White button mushroom *Agaricus bisporus* (J.E. Lange) Imbach has been artificially cultivated for over 450 years, and has become one of the major crop [1–3]. The main problem of its farming is the yield instability. Even when keeping every approved technological rule, the grower often cannot predict with complete certainty the optimal yield. One this phenomenon reasons is the crop diseases which harmfulness in intensive cultivating technologies is yet high [4, 5]. In this regard, the study of organisms responsible for white button mushroom yield losses isn't losing relevance for the scientifically motivated measures development to their control [6]. The science and practice of mushroom farming has developed certain preventive measures to reduce the harm caused to the mushroom culture. The main of them, used both in our country and abroad: compliance with the compost and casing soil preparative technologies, hygienic requirements keeping when performing work in mushroom houses; disease-resistant *A. bisporus* strains use; regular pre-sawing and inter-cycling preventive measures use in cultivation facilities, nutrients and hormones introducing; compliance with microclimate technological parameters at all stages of culture development; chemicals limited set using to protect mushrooms [7, 8]. Nevertheless, the full measures complex implementing often does not lead to the desired results, in the mushroom production process the optimal and stable yield cannot be ensured precisely because of the crop diseases caused mainly by micromycetes. The main representative of the white button mushroom mycopathogenic complex is

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the wet bubble causative agent *Mycogone perniciosa* Magn. [9, 10]. The disease manifests in the basidiomata deformation with the subsequent bacteria development on them as a secondary infection, causing intense fruit body rotting [4, 9, 10]. The disease occurs in the industrial mushroom-growing farms almost everywhere when conditions are favorable for its emergence and development: temperature 16–20° C, humidity over 90 % [5, 7].

M. perniciosa has a high viability. In culture, the micromycete retained its vital functions for 18 months; in nature, its spores retain viability for up to 3 years; when thermally treated, they withstand a temperature of 60° C for more than 2 h; the mycelium dies within 30 min at this temperature. *M. perniciosa* and *A. bisporus* develop in almost the same temperature and pH ranges, but they are somewhat wider for the mycoparasite (20–28° C, pH=5–7) than for the host (22–24° C, pH=5.5–5.6) [11].

Most often, the disease occurs at the end of the growing cycle, when the main crop is weakened and depleted, when mushroom beds are no longer adequately maintained, ventilation deteriorates, and the number of pathogens insect vectors increases [4]. Wet bubble is the most harmful disease of cultivated mushrooms. Its appearance often leads to the entire crop loss in the cultivation chamber [4]. In its metabolism, *M. perniciosa* acidifies the habitat, which helps it develop insensibility to chemicals, particularly benomyl [12, 13].

M. perniciosa is an optional biotroph [14]. Its spores germinate on the *A. bisporus* mycelium in the casing, and from there the mycopathogen's hyphae penetrate into the fruit body anlage and thus into basidioma base. Mycopathogen spores can also germinate on already formed fruit bodies, but in this case, they do not cause serious damage to the host cells. On dead *A. bisporus* mycelium *M. perniciosa* spores germinate well and the micromycete actively develops; thus, the primary infection occurs through mycelial strands, especially through dead ones left after basidiomata harvesting. From there, the parasite mycelium passes to the living mycelial pulls and then to the generative formations – that is sclerodermoid infestation form. The common one (infestating formed basidiomata) does not cause fruit bodies deformation, they stayed covered with a white *M. perniciosa* mycelium. Such infestation form is very rare. It is important to note the lack of correlation between the basidiomata resistance and their mycelium antagonistic activity to *M. perniciosa*. The infection persists in the casing as the chlamydospores [15, 16]. Mycopathogen conidia and chlamydospores usually do not persist in compost because temperatures reach 70–80° C during its processing, but the source of infection can also be compost if phytosanitary rules are violated during its preparation and transportation [17].

The microbiological control of white button mushroom diseases has not yet found wide application in its industrial cultivation, despite the natural protective mechanisms use manifesting in regulating pest populations number by their antagonists has recently become a priority in the agricultural crops' protection. Agroecosystems phytosanitary optimizing involves the use of microorganisms with high ecological plasticity and various biologically active substances (BAS) complexes as polyfunctional biologics producer strains with complex biological activity. *Bacillus* bacteria, unpretentious with respect to growth conditions, high-tech, capable of producing many BAS and regulating the pathogen populations density when introduced into the agrobiocenosis for a long time, meet such requirements [18–20].

Bacillus subtilis strains B-10, M-22 highly effective against a wide range of various cultivated plants phytopathogens have been deposited and passported at the FSBSI VIZR Microbiocontrol laboratory [20]. The research-industrial technologies for polyfunctional biologics preparing on their basis were developed and biologics included in the State catalog of pesticides and agrochemicals permitted for use in Russian Federation territory (2023) [21]. These biologics have high biological efficacy (60–90 %) against pathogens of the most harmful diseases, their formulation include live cells and metabolome that determine the antagonistic activity, as well as polypeptide antibiotics of original structure [22]. From the above, the research goal was to characterize the effect of *B. subtilis* B-10 and M-22 producer strains on *M. perniciosa* and evaluate the possibility of wet bubble control using antagonist microbes, the basis of polyfunctional biologics. To achieve this goal, the following tasks were solved: to compare the effect of *B. subtilis* B-10 and M-22 producer strains on 1-day- and 7-day-old *M. perniciosa* colonies when applied by spraying and into the agar wells (methods simulating

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basidioma and casing soil treatments); to identify the most active antagonist to *M. perniciosa*; to evaluate the ability of the studied producer strains to suppress the wet bubble disease development.

2. Materials and Methods

2.1. Materials

The research was carried out at the FSBSI VIZR Microbiocontrol laboratory and at its edible mushrooms' pilot farm. Materials used for research were the spent mushroom substrates (SMS) after industrial cultivating *Lentinula edodes* (Berk.) Pegler (SMSLe) – shiitake mushroom, *Pleurotus ostreatus* (Jacq.) P. Kumm. (SMSPo) – oyster mushroom which were kindly provided by the edible mushrooms' farms "Prinevskoe" Ltd. and "Digris" Ltd. (Russia). There were used multirecycled SMS left after double edible mushrooms' cultivating on the same substrate (SMSLePo). Such substrate containing *L. edodes* 4080 and *P. ostreatus* NK-35 mycelium's derivates were used as aqueous extracts for biologics producer strains liquid culture obtaining [23]. The SMS was preliminarily disintegrated and 1 h boiled in 200 g/l volume, filtered, restored to its original volume. Sterilization mode was 30 min at 81.1 kPa in autoclave 5075ELVPV D (Tuttnauer Europe BV, Netherlands) [24]. Inoculation was carried out with submerged strains' cultures in sterility conditions. Incubation was carried out at 26–28° C. The trials in vitro were performed using Czapek-Dox agar (HiMedia Laboratories, India) [23, 24].

2.2. Objects

The research objects were strains *Bacillus subtilis* (Ehren.) Cohn B-10 and M-22 – industrial polyfunctional biopesticides' producers having antifungal activity, deposited and maintained in the State Collection of Microorganisms Pathogenic for Plants and Their Pests FSBSI VIZR. The Collection was registered on 28.01.1998 No. 760 in the World Federation for Culture Collections, World Data Center for Microorganisms (WFCC WDCM, Japan). The research objects were as well *B. subtilis* B-10 and M-22 liquid cultures obtained by the submerged fermenting in SMSLePo aqueous extracts. The production titers liquid cultures were: *B. subtilis* B-10 – 3.2×10^{12} CFU/ml (colony forming units per 1 ml) and B. subtilis M-22 – 1.2×10^{12} CFU/ml.

As a research object *M. perniciosa* was isolated from casing soil at the 2[d] fructification wave of *A. bisporus* X-22 in the farm "Prinevskoe" Ltd. (Russia) (Figure 1).

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

Figure 1. *M perniciosa* pure culture (**a**) and *A. bisporus* fruit bodies infection (**b**).

2.3. Methods

The following standardized [25] as well as authors modified research microbiological methods [24] were used. The following spore productivity calculated parameter is presented: titer as viable colony-forming units per ml (CFU/ml) [24, 25]. Liquid-phase producer strains' inoculum was grown at 27–28° C in 3 days with aeration (150 rpm, New Brunswick ™ Innova® 44 incubator shaker, Eppendorf, Germany). The quantity and quality of producer strains viable CFU/ml in the produced

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inoculums (titers), the producer strains spore productivity was determined by serial dilutions method on agar media by inoculating and spawning on: Czapek-Dox agar (HiMedia Laboratories, India); peptonic Czapek's and Dried nutrient agar (Microgen Co. Ltd., Russia) [25].

M. perniciosa 1-day- and 7-days-old lawns were treated by submerged cultures of strains *B. subtilis* B-10 and M-22 using well method and spraying with a laboratory sprayer use. *M. perniciosa* colony area as well as micromycete's lysis zone under producer strains treatment were determined using the circle area formula: where radius is the colony or lysis zone radius. The antagonist microbes' biological effectiveness (%) was determined by the mycelial lysis zone proportion in the total area occupied by the micromycete mycelium.

Statistical obtained results processing using the Microsoft Excel 2010 and Statistica 10.0 software packages (StatSoft, Inc., USA) included checking the analyzed data normal distribution with the help of Shapiro-Wilk's *W*-test, analysis of variance (ANOVA), calculating the means (*M*) as well as standard errors (±*SEM*) calculation. The statistical differences significance in options pairwise comparison was assessed by Student's *t*-test [26, 27].

3. Results

3.1. Antifungal activity of B. subtilis strains against 1-day-old M. perniciosa mycelium

After spraying 1-day-old *M. perniciosa* lawn with *B. subtilis* B-10 and M-22 liquid cultures, no mycopathogen development was observed; on the 4th day intensive antagonist microbes' development forming the biologics basis was detected. By the 8th cultivation day, the strain-producer lawn and complete *M. perniciosa* growth absence were observed (Figure 2).

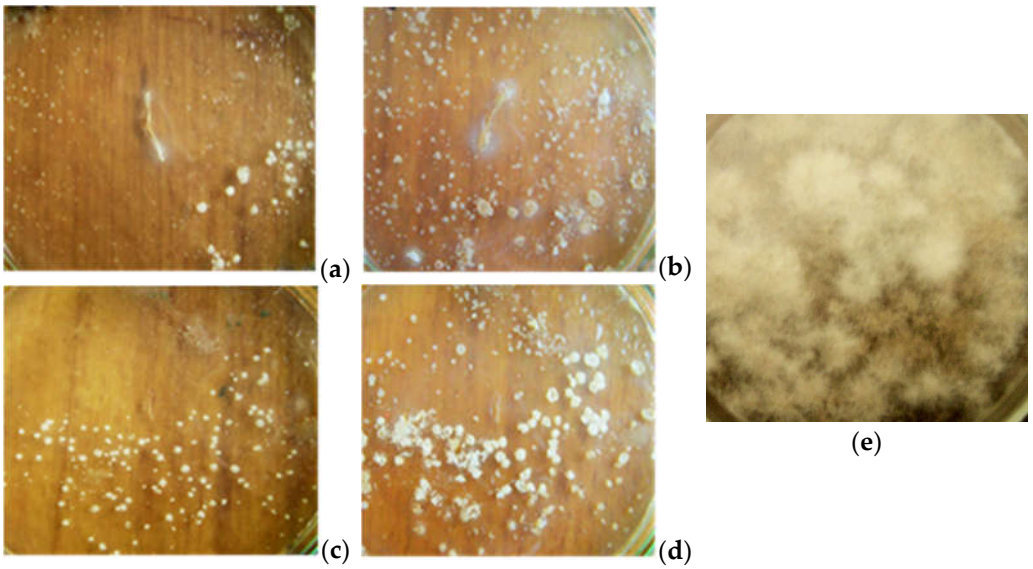

Figure 2. *M. perniciosa* development after inoculating and simultaneous spraying with *B. subtilis* B-10 (**a**), (**b**) and M-22 (**c**), (**d**) producer strains liquid cultures: (**a**), (**c**) – 4th cultivation day; (**b**), (**d**) – 8th cultivation day; (**e**) – control *M. perniciosa* without treatment, 8th cultivation day.

3.2. Antifungal activity of B. subtilis strains against 7-day-old M. perniciosa mycelium

When spraying 7-d-old *M. perniciosa* colonies with *B. subtilis* B-10 and M-22 liquid cultures, weak micromycete development was observed only during 3 days after treatment, and *M. perniciosa* colony lysis manifestation was registered on the 4th day. By the 8th cultivation day, the producer strains developing in the free part of the nutrient medium and *M. perniciosa* lysis zone increase was observed (Figure 3).

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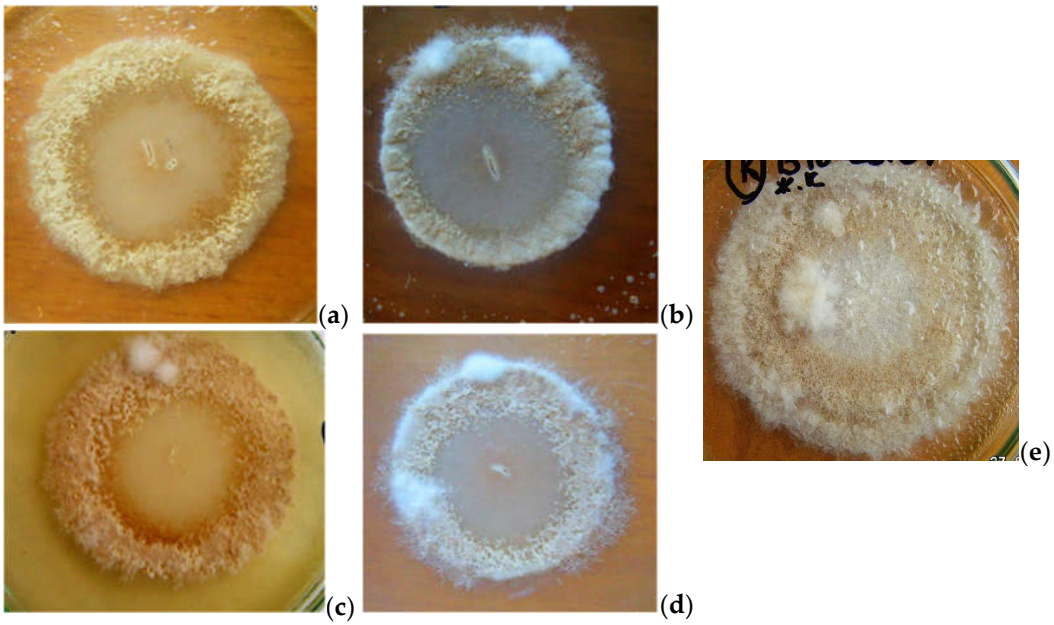

Figure 3. *M. perniciosa* development after spraying its 7-day-old colonies with *B. subtilis* B-10 (**a**), (**b**) and M-22 (**c**), (**d**) producer strains liquid cultures and (**e**) sterile water (control): (**a**), (**c**) – 4 days after treatment; (**b**), (**d**), (**e**) – 8 days after treatment.

The average rate of micromycete colony area increase in the control without treatment with antagonists was almost 30-fold higher than that after spraying 7-d-old colonies with *B. subtilis* B-10 liquid culture and 16-fold higher after spraying with *B. subtilis* M-22 strain. Moreover, treatment with *B. subtilis* B-10 strain produced an almost two-fold decrease in *M. perniciosa* growth rate compared to the decrease caused by *B. subtilis* M-22 spraying (Figure 4 a). Hereat the average rate of *M. perniciosa* mycelial lysis increase under *B. subtilis* B-10 effect was reliably ($p \leq 0.1$) 1.2 times higher than that caused by the *B. subtilis* M-22 (Figure 4 c). These indices dynamics during 15 days mycopathogen cultivating after its 7-d-old colonies spraying with *B. subtilis* B-10 and M-22 liquid cultures is presented in Figure4 b, d, respectively. Antagonist microbes' introducing already after one day leads to *M. perniciosa* growth retardation, abundant sporulating in the colony marginal zone, and later to complete growth cessation, sterile mycelial roll forming and a barrage zone as well located behind it. Subsequently, these processes lead to extensive micromycete's mycelial lysis zone forming and its increasing in time cultivation (Figure 3 b, d; 4 b, d). The higher integral values of the lysis area increase rate function when exposed to *B. subtilis* B-10 are noteworthy, although the rate of *M. perniciosa* lysis area increase when exposed to *B. subtilis* M-22 has a peak exceeding by 1.7 times the same function value for *B. subtilis* B-10 (Figure 4 d). This phenomenon biological value is that *B. subtilis* M-22 application causes a rapid and vast degradation *M. perniciosa* response, expressed in the rapid micromycete mycelium death and the impossibility of its development in the antagonist space. Exposure to *B. subtilis* B-10 leads to permanent growth retardation and *M. perniciosa* mycelium lysis, providing greater efficiency in suppressing the micromycete over time (Figure 4 d).

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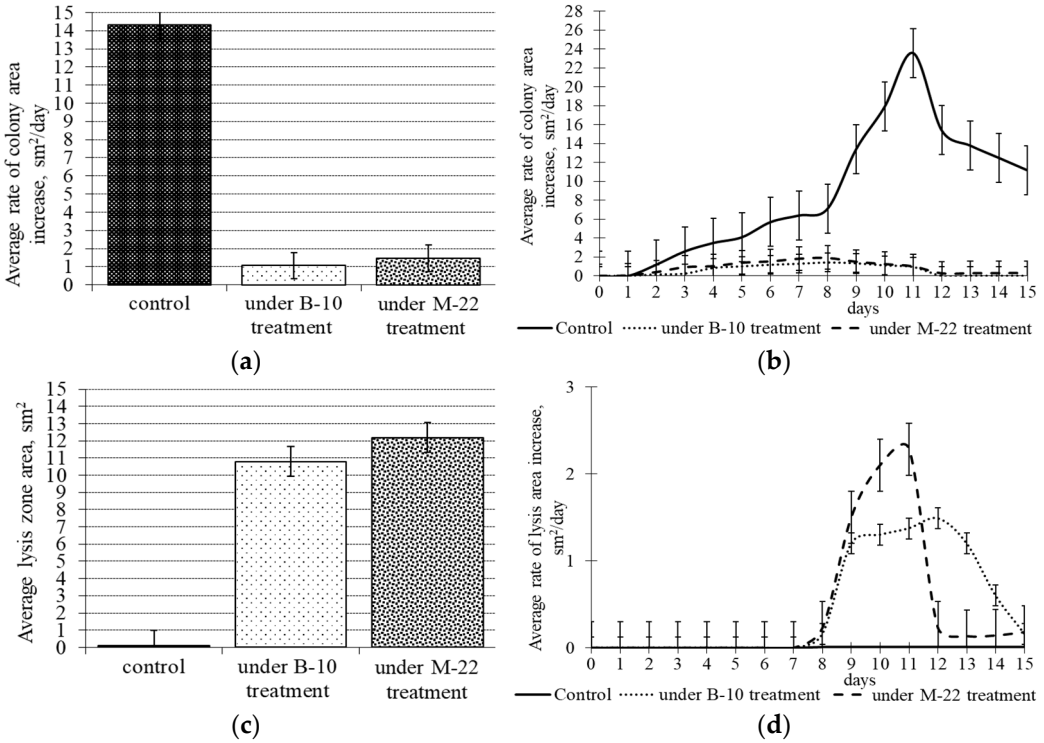

Figure 4. Average increase rate of *M. perniciosa* colony area (**a**) and its mycelium lysis zone (**c**), these parameters dynamics (**b**), (**d**) respectively during 15 days cultivating when 7-day-old colonies were sprayed with *B. subtilis* B-10 and M-22 producer strains liquid cultures.

3.3. Antifungal activity of B. subtilis strains secondary metabolites against M. perniciosa

The producer strains introducing into the wells on 1-day-long *M. perniciosa* lawn resulted in weak micromycete development as thin web-like mycelium with very few chlamydospores. By 8th day the maximum zone of *M. perniciosa* lysis was formed around the wells on the poorly developed lawn (Figure 5 a, d). The lysis proportion in the total area occupied by mycelium was reliably ($p \leq 0.01$) 2.3 times higher than that when antagonists' secondary metabolites were exposed to the formed *M. perniciosa* colonies. The differences in *B. subtilis* B-10 or M-22 secondary metabolites affect were within the measurements error (Figure 5 a, d). When producer strains were introduced into the wells onto the developed *M. perniciosa* lawn, their growth was observed directly in the wells, without coming on the developed *M. perniciosa* lawn. No lysis zones were observed, but the producer strains continued to develop in the wells of the *M. perniciosa* lawn (Figure 5 b, c, e, f).

Any studied producer strains liquid forms spraying on *M. perniciosa* colonies at very early stages of mycopathogen development led to its development complete slowing and death. The mycocidal activity against the pathogen was 61.7 % for *B. subtilis* B-10 and 50.1 % for *B. subtilis* M-22 respectively. One-week delay in treatment by spraying reduced mycocidal activity of both producer strains by more than 3 times. Antagonists local application by introducing into wells at early stages of *M. perniciosa* development revealed producer strains secondary metabolites mycocidal biological activity level for *B. subtilis* B-10 – 68.6 % and *B. subtilis* M-22 – 59.0 % respectively. The differences in the antagonists' activity were insignificant and reliable: *B. subtilis* M-22 more actively suppressed the mushroom wet bubble pathogen. *B. subtilis* B-10 and M-22 local application by introducing into wells on the sporulating developed *M. perniciosa* lawn was ineffective.

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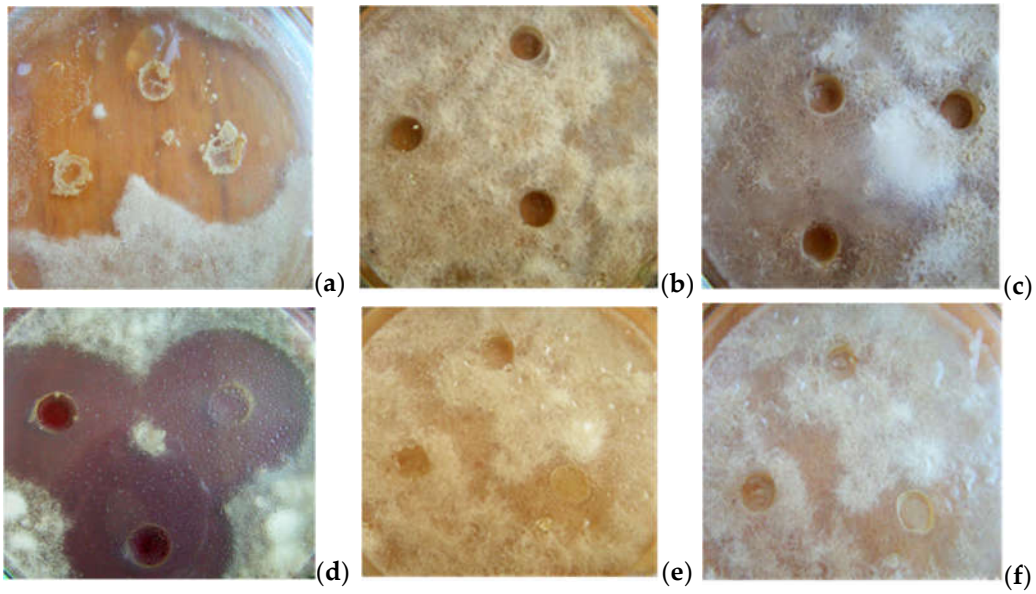

Figure 5. *M. perniciosa* mycelium lysis zone development after putting into agar wells *B. subtilis* B-10 (**a–c**) and M-22 (**d–f**) producer strains liquid culture: (**a**), (**d**) – simultaneously with *M. perniciosa* inoculating; (**b**), (**c**), (**e**), (**f**) – on 7-day-old *M. perniciosa* lawn; (**b**) – B-10; 4 days after putting into wells; (**a**), (**c**) – B-10, 8 days after putting into wells; (**e**) – M-22, 4 days after putting into wells; (**d**), (**f**) – M-22, 8 days after putting into wells.

4. Discussion

The biomethod for cultivated white button mushroom diseases control has not yet found wide application in industrial cultivating. Biologics based on *Bacillus* strains are mainly used in modern *A. bisporus* growing to control its competitive microbiota [28–31]. Just some approved chemicals are used to control mycoparasite *M. perniciosa* [32–34]. The search for microbiocontrol of wet bubble pathogen is focused on the casing soil antagonists isolating and use to which *M. perniciosa* shows sensitivity [19–35]. These studies do not use highly active industrial producer strains of polyfunctional biologics with fungicidal activity, which are *B. subtilis* B-10 and M-22 [20]. The interaction between the above producer strains and well-developed *A. bisporus* mycelium in the transition stage (pop-corn like generative forms appearing) does not negatively affect morphogenesis [22].

5. Conclusions

The perspective use of *B. subtilis* B-10 and M-22 producer strains to control the *M. perniciosa* development, the white button mushroom wet bubble disease causative agent has been revealed. Significant differences in the micromycete colonies growth rate suppression in terms of its mycelium lysis zone area were detected. Moreover, in all trials' variants a positive trend in *M. perniciosa* development suppressing was established, which indicates a prolonged *B. subtilis* B-10 and M-22 effect on *A. bisporus* wet bubble disease causative agent. The high biological efficacy of both producer strains against *M. perniciosa* at the early stages of its development by introducing into the wells and spraying was shown: *B. subtilis* B-10 – 50.9–99.6 % and *B. subtilis* M-22 – 57.5–99.2 % respectively ($p \leq 0.05$). Significant differences between the producer strains were not revealed, although during the first day of exposure to the developed *M. perniciosa* colonies *B. subtilis* M-22 has shown greater activity. The discovered preventive treatment high efficiency when the producer strains completely suppressed the *M. perniciosa* development, made it possible to recommend the *B. subtilis* B-10 and M-22 introducing when preparing casing to control mycopathogen development.

Supplementary materials: Not applicable.

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