AGRICULTURAL BIOLOGY,
2018, V. 53, № 5, pp. 1054-1061
(SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA)

ISSN 2412-0324 (English ed. Online)
ISSN 0131-6397 (Russian ed. Print)
ISSN 2313-4836 (Russian ed. Online)

Biocontrol

UDC 632.51:582.28:57.083.1

doi: 10.15389/agrobiology.2018.5.1054eng
doi: 10.15389/agrobiology.2018.5.1054rus

LIQUID FERMENTATION OF *Stagonospora cirsii* C-163, A POTENTIAL MYCOHERBICIDE FOR *Cirsium arvense* (L.) Scop.

S.V. SOKORNOVA, A.O. BERESTETSKIY

All-Russian Research Institute of Plant Protection, Federal Agency for Scientific Organizations, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail toxbiotech@vizr.spb.ru (✉ corresponding author), s.sokornova@spbu.ru
ORCID:
Sokornova S.V. orcid.org/0000-0001-6718-4818 Berestetskiy A.O. orcid.org/0000-0002-0612-6996
The authors declare no conflict of interests
Acknowledgements:
Supported financially by Russian Science Foundation (grant № 16-16-00085 «Technologies for production and use of mycobiocides against difficult-to-eradicate weeds»)
Received May 20, 2017

Abstract

The bioherbicides should exhibit stable effectiveness in the field, be specific and quick in action, compatible with other preparations and meet market demand. In many ways, the cost and quality of product is determined by the technology of obtaining infectious material. An infectious material is used as a mycelium and its modifications and as conidia as well. The extreme sensitive of the mycelium to drying is often referred as main disadvantage of using it as the basis of a formulation. At the same time, the technological process of obtaining conidia is often more complicated, and the efficiency in the field is less than the mycelium has. The phytopathogenic fungus *Stagonospora cirsii* J.J. Davis, which is causative agent of a leaf spot disease of creeping perennial weeds in the family *Asteraceae*, is considered a potential mycoherbicide of Canadian thistle *Cirsium arvense* (L.) Scop. However, the yield of *C. cirsii* C-163 mycelium on standard nutrient media is significantly lower than that used in biotechnology (3 g/l). Our paper is the first to report that manipulation with liquid nutrient medium allows a significant increase in *S. cirsii* mycelium pathogenicity and tolerance to exsiccation. The study is devoted to the optimization of liquid-phase deep fermentation parameters, as well as the duration of cultivation and composition of a nutrient medium, in order to obtain the *C. cirsii* C-163 mycelium with improved mycoherbicidal properties. This infection material is a good basis for development formulations that can be used both individually and jointly with other protective agents for perennial weed control. The advantage of the approach used in the work is that the resistance to drying, an important technological parameter which largely determines the success of the herbicides, was additionally considered, along with virulence and mycelial yield, during the optimization of fermentation parameters. The strain *C. cirsii* C-163 was used. The 10-day inoculum was obtained in Petri plates on potato dextrose agar medium. The mycelium was incubated in 250-ml Erlenmeyer flasks containing 50 ml of the medium at 130 rpm and 24±2 °C for 2-7 days. The base liquid nutrient media contained carbon source (20 g/l), organic (10 g/l) or inorganic (3.5 g/l) nitrogen source, yeast auto lysate (1 g/l), KH_2PO_4 (1 g/l), $MgSO_4$ (0.5 g/l). Dulcite, rhamnose, L-inositol, L-arabinose, D-sorbitol, glucose, trehalose, and sucrose were a source of carbon. Casein, soy peptone, enzyme peptone, soy flour, gelatin, lecithin, ammonium dihydrogen phosphate, ammonium chloride, ammonium sulfate, and sodium nitrate were a source of nitrogen was. The pH of all liquid nutrient media was adjusted to 6.0. To establish the optimum concentrations of sucrose and soy flour in a nutrient medium with yeast auto lysate (1 g/l), KH_2PO_4 (1 g/l), $MgSO_4$ (0.5 g/l), the amount of sucrose was changed from 10 to 70 g/l with a step of 10 g/l and the concentration of soy flour was changed from 5 to 20 g/l with a step of 2.5 g/l. The degree of leaf damage caused by disease was estimated by the necrosis area of leave disks or whole plants (5-6 true leaves). Drying of harvest mycelium, humidity 85-87 %, was carried out in a thin layer (1-2 mm) with flowing air at 30 °C without protectors for 3 hours. The highest yield of mycelium is when the carbon source in the nutrient medium is L-inositol. When inositol is substituted with sucrose or D-sorbitol, the biomass yield reduces by 25 %. At the same time, these nutrient media gave the most aggressive mycelium. Among nitrogen sources, the maximum yield of mycelium is in the case of casein, soy flour and enzymatic peptone. In the process of drying mycelium, loss of viability of propagules turned out to be significant. The mycelium obtained on sucrose-soy nutrient medium is the most resistant to drying. The most viable and aggressive mycelium was formed in the middle of the exponential growth phase which occurred on day 3 when cultivated in flasks on a soya-sucrose nutrient medium. Opti-

mization of the concentration of soybean flour (15 g/l) and sucrose (60 g/l) makes it possible to increase the yield and aggressiveness of mycelium 12 and 4 times, respectively, as compared to Czapek medium. Thus, the present study provides a method for the preparation of a mycelium having a high aggressiveness to the host-plant and a capability to remain viable during drying. The prospects of such a method of obtaining an infectious material are proved.

Keywords: phytopathogenic fungi, *Stagonospora cirsii* J.J. Davis, *Cirsium arvense* (L.) Scop., Canada thistle, submerged liquid cultivation, carbon source, nitrogen source, mycelium, mycoherbicide

Among more than 200 species of fungi and bacteria that were considered as potential bioherbicides of diverse action spectrum, by 2011 only 8.1% had become the basis for production of commercial formulations, 19.4% had been registered but not commercialized, and 72.5% had not confirmed their effectiveness [1]. The reason is that the commercial success of the bioherbicide is determined not only by the virulence for the target object, but also by its effectiveness in the field, the specificity and speed of action (aggressiveness), processability, the cost of the nutrient media used in the manufacturing cycle, the compatibility with other biological and chemical preparations, as well as the market demand [2, 3].

The technology of obtaining biological preparations is determined by the nature of the original infectious material. Most of the registered mycopesticides are developed on the basis of conidia, in some cases mycelium and its modifications are used for this purpose. The sensitivity of the mycelium to drying is often referred to as its main disadvantage. At the same time, the technological process of obtaining conidia is often more complicated, and the efficiency in the field is less stable than the mycelium has [3, 4].

The phytopathogenic fungus *Stagonospora cirsii* J.J. Davis is considered a potential mycoherbicide of Canadian thistle *Cirsium arvense* (L.) Scop. [5]. A leaf spot disease of creeping perennial weeds in the family *Asteraceae* can be caused by conidia as well as *S. cirsii* C-163 mycelium fragments. Strains of this species form conidia only under the influence of ultraviolet [5]. *S. cirsii* C-163 mycelium can cause disease of weeds under more severe temperature and humidity conditions than conidia [6]. The advantages of the use of preparations based on mycelium in the field are also shown for other potential mycoherbicides [6-9]. It is partly explained by the autoinhibition of the conidia development at their high number [10, 11]. The cases are known when phomoid micromycetes conidia obtained in vitro were avirulent [12]. In addition, the emergence of a synergistic effect in the co-use of mycelium and chemical herbicides in low doses provides more stable effectiveness of preparations based on mycelium in the field [13-15]. Therefore, *S. cirsii* mycelium is considered primarily as the infectious material for the development of a bioherbicide against Canadian thistle.

Compared to solid-phase cultivation, liquid-phase deep fermentation is a simpler and faster way to obtain infectious material [16-18]. The development of this technology includes the optimization of the nutrient medium for the viability and aggressiveness of the material [16]. For phytopathogenic micromycetes, it is shown that the aggressiveness of infectious material is determined by the nature of carbon and nitrogen sources, their ratio and absolute concentration [19-21], as well as the physiological state of propagules [6]. It is necessary to note that for phomoid micromycetes, which include *S. cirsii*, the comprehensive study to assess the impact of the cultivation duration, the nature, and concentration of carbon and nitrogen sources on the quality of the mycelium, obtained in the result of deep fermentation, has not been carried out.

This paper is the first to report that manipulation with liquid nutrient medium allows a significant increase in *S. cirsii* mycelium pathogenicity and tolerance to exsiccation.

The work objective was to optimize the composition of the nutrient medium (according to C, N sources) and the duration of deep liquid-phase cultivation to increase the yield of virulent mycelium of *Stagonospora cirsii* C-163.

Techniques. The strain *S. cirsii* C-163, which was stored at 5 °C in test tubes on potato-dextrose agar and at –80 °C in 10% glycerin, was used in the work. The inoculum was obtained on potato-dextrose agar.

The mycelium was incubated in 250-ml Erlenmeyer flasks containing 50 ml of the nutrient medium in the orbital shake-flask propagator (at 180 rpm). The carbon (dulcite, rhamnose, L-inositol, L-arabinose, D-sorbitol, glucose, trehalose, and sucrose) and nitrogen (casein, soy peptone, enzyme peptone, soy flour, gelatin, lecithin, ammonium dihydrogen phosphate, ammonium chloride, ammonium sulfate, and sodium nitrate) sources varied in liquid nutrient media. The following media composition was used: carbon source (20 g/l), organic (10 g/l) or inorganic (3.5 g/l) nitrogen source, yeast autolysate (1 g/l), KH_2PO_4 (1 g/l), $MgSO_4$ (0.5 g/l). The pH of liquid nutrient media was adjusted to 6.0 before autoclaving (taking into account the optimal pH 5-6 for the development of the *S. cirsii* mycelium) [8].

To establish the optimum concentrations of sucrose and soy flour in a nutrient medium with yeast autolysate (1 g/l), KH_2PO_4 (1 g/l), $MgSO_4$ (0.5 g/l), the amount of sucrose was changed from 10 to 70 g/l with a step of 10 g/l and the concentration of soy flour was changed from 5 to 20 g/l with a step of 2.5 g/l. The optimal cultivation time was determined in the range from 2 to 7 days at 25±2 °C on a nutrient medium (pH 6.0) of the following composition: soy flour (14 g/l), sucrose (60 g/l), yeast autolysate (1 g/l), KH_2PO_4 (1 g/l), $MgSO_4$ (0.5 g/l). The CFU, mycelium yield by dry weight, pH of culture liquid were determined by standard methods [22].

The aggressiveness of the mycelium against Canadian thistle was assessed with the area of damage of leaf disks or entire plants in the rosette phase. Disks with a diameter of 0.8 cm were cut with a Forstner bit from the leaves of the middle layer. They were placed in rows of 12 pcs with the adaxial side up in the leak-proof clear plastic containers on filter paper moistened with sterile water. Leaf disks were inoculated with fragments of *S. cirsii* C-163 mycelium (50 mg/ml) by applying an aqueous suspension (5 μl) to the center of the disc. In experiments on entire plants, they were sprayed with an aqueous suspension of the same concentration at a flow rate of 1.5 ml/plant. The aggressiveness of *S. cirsii* on the leaf disks was assessed 2 days after inoculation on the relative area of necrosis formed at a temperature of 25 °C and intermittent (12 h dark/12 h light) artificial light. The aggressiveness of *S. cirsii* on entire plants was determined by the relative area of leaf necrosis 7 days after inoculation.

Drying of mycelium (humidity 85-87%) was carried out in a thin layer (1-2 mm) with flowing air at 30 °C without protectors for 3 hours.

1. Yield and aggressiveness of 4-day *Stagonospora cirsii* C-163 mycelium at different C sources in the culture medium (*M*±SEM)

Carbon source	Biomass yield, g/l	Necrosis area, %
L-inositol	5.60±0.10	20±4
Sucrose	4.20±0.08	55±4
D-sorbitol	4.13±0.19	50±4
Rhamnose	3.79±0.13	23±2
L-arabinose	3.24±0.23	10±4
Trehalose	3.11±0.22	35±7
Glucose	3.08±0.08	25±4
Dulcite	2.37±0.13	5±2
LSD_{05}	0.29	9

The experiments were carried out in 4 replications. The results were subjected to dispersion analysis. Homogeneity of variances of the samples was checked with the help of Cochran's Q test. Standard deviations (±SEM) are given for the means (*M*). The significance of differences of mean values is determined with the criterion of least significant difference ($LSD_{0.05}$). Calculations were

performed in Microsoft Excel 2007.

Results. The first step in the cultivation conditions optimization for *S. cirsii* C-163 was to select a carbon source for the liquid culture medium. The variant with L-inosite as a carbon source provided the greatest yield. Substitution of L-inosite with sucrose and D-sorbitol resulted in the reduction of biomass yield by 25%. At the same time, the media with sorbitol and sucrose formed mycelium the most aggressive against thistle (Table 1). Due to this reason, and taking into account the commercial availability and stabilizing properties of sucrose [9], it was used as a carbon source when selecting a nitrogen source at the next optimization stage.

2. Yield and aggressiveness of 4-day *Stagonospora cirsii* C-163 mycelium at different N sources in the culture medium (*M*±SEM)

Nitrogen source	Biomass yield, g/l	Necrosis area, %
Casein	55.80±0.11	100±0
Soy flour	25.29±0.09	100±0
Enzyme peptone	21.24±0.13	96±5
Gelatin	18.12±0.14	100±0
Lecithin	12.01±0.08	75±12
Soy peptone	6.11±0.17	100±0
Ammonium dihydrogen phosphate	6.70±0.13	50±4
Ammonium sulfate	5.12±0.08	58±4
Sodium nitrate	4.50±0.08	45±7
Ammonium chloride	4.21±1.30	50±4
LSD_{05}	0.3	9

By varying N sources on the medium with sucrose, it was found that the yield of dry biomass on nutrient media with organic nitrogen (with the exception of soy peptone) was 12-55 g/l, more than by 3 times higher than in the standard Czapek medium with yeast extract (sodium nitrate as nitrogen source) (Table 2). Aqueous suspension based on mycelium fragments, obtained on nutrient media with casein, peptone, soy flour or gelatin, caused the death of disks from the leaves of thistle. The maximum yield of mycelium was found for casein, soy flour, and enzyme peptone (Table 2). Nutrient media of such composition were basic at the third stage of optimization.

3. Vability of 4-day *Stagonospora cirsii* C-163 mycelium at different N sources in the culture medium (*M*±SEM)

Nitrogen source	CFU, $\times 10^6$/g	
	before drying	after drying
Casein	0.4±0.1	0.05±0
Enzyme peptone	1.1±0.1	0.10±0.01
Soy flour	1.2±0.1	0.30±0.01
LSD_{05}	0.20	0.01

The possibility of successful stabilization of infectious material is formed at the very stage of cultivation [23, 24]. Therefore, the main selection criterion at the third stage of optimization of the nutrient medium composition was the stability of the mycelium obtained in different nutrient media to drying. When drying, the loss of propagules viability was substantial. The greatest stability to drying was shown by the mycelium obtained on the sucrose-soy nutrient medium, which was chosen for further optimization (Table 3). The obtained data on the low stability of the *S. cirsii* C-163 mycelium to drying are consistent with those published [3, 8]. In cases when the potential mycoherbicide forms sclerotia, they, taking into account greater thermal tolerance of this life-form, are used as the infectious material [25-27]. Phomoid micromycetes do not have such ability. Softer drying conditions are provided when receiving pesto- and alginate granules. Their use is considered promising in the mycoherbicides development on the basis of the mycelium of phomoid pathogens, since this allows reducing losses during drying, and the composition of such formulations may include additional active ingredients that improve effectiveness in the field [28-30].

At the fourth stage of nutrient medium optimization, the influence of the duration of fungus cultivation on the pathogenic properties of the mycelium and its yield was evaluated (Fig. 1). The maximum yield of mycelium (about 36 g/l)

corresponded to the beginning of the stationary phase of fungus growth and fell on days 4-5 of cultivation. At the same time, the 3-day mycelium showed the greatest activity against the thistle, which corresponds to the middle of the exponential phase of the fungus growth, characterized by the most active metabolic processes.

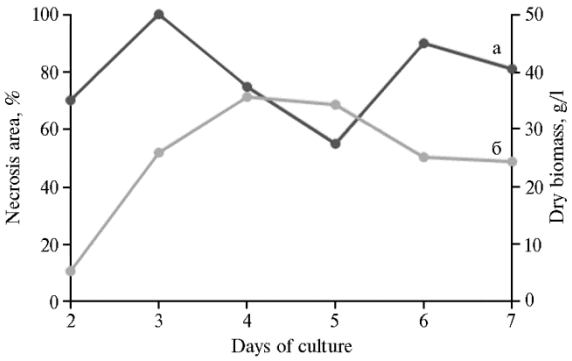

Fig. 1. Aggressiveness (a; LSD_{05} = 8.0) **and the yield of mycelium** (b; LSD_{05} = 0.9) **of *Stagonospora cirsii* C-163 depending on the time of cultivation on the sucrose-soy medium.**

The second peak of biological activity (day 6) was in the stationary phase of growth when the formation and accumulation of secondary metabolites occur usually. It is known that *S. cirsii* at stationary cultivation on the liquid nutrient Czapek medium produces stagonolide-similar toxins exhibiting phytotoxicity [31]. Substrate necrotization may accelerate the development of the disease, so the second peak of biological activity is associated with the beginning of toxin production [32].

Since the reduction of fermentation time becomes an important technological advantage in the deep liquid-phase cultivation, the concentrations of soy flour and sucrose for a 3-day mycelium were optimized in further work. The analysis of variance of the experimental data showed in all cases a statistically significant ($p < 0.001$) joint and individual influence of the concentrations of carbon and nitrogen sources in the liquid nutrient medium on the pathogenicity and mycelium yield. This result is fully consistent with the literature data on the influence of concentration and ratio of these components of the nutrient medium on the yield and pathogenicity of different types of infectious material, such as conidia of *Colletotrichum coccoides* [21], conidia, microsclerotia, and mycelium of *C. truncatum* [24, 27].

The highest yield of dry biomass (36 g/l) was at a concentration of sucrose 60 g/l and soy flour 15 g/l. A further increase in the soy flour amount reduced the yield of mycelium. Evidently, it was due to a decrease in the aeration of the nutrient medium due to an increase in its density. When treating the leaf disks with an aqueous suspension of *S. cirsii* C-163 mycelium (25 mg/ml), the maximum area of the necrosis was observed at sucrose concentration of 30 g/l and above and at a concentration of soy flour 12.5-17.5 g/l. The decrease in pathogenicity at high concentrations of soy flour was also associated with a decrease in aeration, leading to premature aging and degradation of the mycelium. As it can be seen from the graphs (Fig. 2), the range of the maximum values of the mycelium yield of the fungus lies within the range of the maximum values of the necrosis area. Therefore, the optimal concentrations of carbon and nitrogen were chosen according to the maximum yield of the *S. cirsii* biomass. The optimized sucrose-soy medium (pH 6.0) had the following composition: soy flour (15 g/l), sucrose (60 g/l), yeast autolysate (1 g/l), KH_2PO_4 (1 g/l), $MgSO_4$ (0.5 g/l). It is necessary to note that with an increase in the degree of aeration, the concentrations of sucrose and soy flour optimal for the greatest yield of aggressive mycelium may change and require correction [16]. The yield of dry mycelium highly aggressive against Canadian thistle on the optimized sucrose-soy medium for 3 days was 36 g/l. The yield of mycelium did not exceed 3 g/l on the initial Czapek medium with yeast extract.

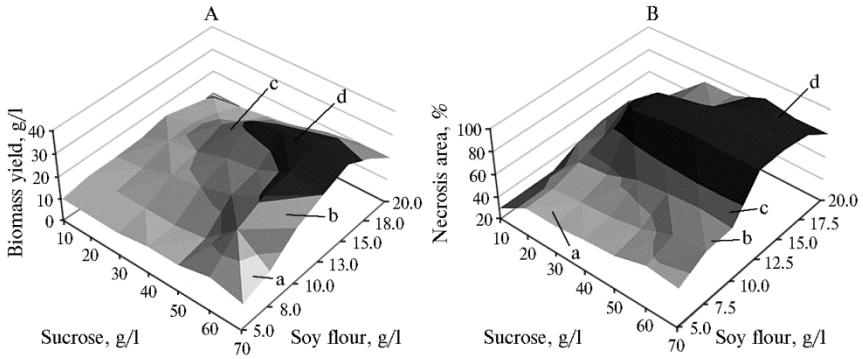

Fig. 2. Yield (A) **and aggressiveness** (B) of ***Stagonospora cirsii*** **mycelium depending on the concentration of sucrose and soy flour in the medium:** a — 0-10, b — 10-20, c — 20-30, d — 30-40 g/l, $LSD_{0.05}$ = 1.5 (A); a — 20-40, b — 40-60, c — 60-80, d — 80-100 g/l, $LSD_{0.05}$ = 7.0 (B).

Thus, optimization of parameters of liquid-phase deep fermentation provides more than 10-fold increase in the yield of virulent mycelium *Stagonospora cirsii* C-163 (to levels comparable with the accepted in the biotechnological practice). The duration of cultivation and the organic nature of nitrogen in the medium significantly affect *S. cirsii* mycelium properties. The proposed high-tech method for obtaining an infectious mycelium that retains viability during drying can be used in the manufacture of a biological preparation against Canadian thistle.

REFERENCES

1. Bailey K.L., Falk S. Turning research on microbial bioherbicides into commercial products — a *Phoma* story. *Pest Technology*, 2011, 5(1): 73-79.
2. Hershenhorn J., Casella F., Vurro M. Weed biocontrol with fungi: past, present and future. *Biocontrol Sci. Techn.*, 2016, 26(10): 1313-1326 (doi: 10.1080/09583157.2016.1209161).
3. Amsellem Z., Zidack N.K., Quimby P.C. Jr., Gressel J. Long-term dry preservation of viable mycelia of two mycoherbicidal organisms. *Crop Prot.*, 1999, 18(10): 643-649 (doi: 10.1016/S0261-2194(99)00070-8).
4. Berestetskiy A., Sokornova S. Production and stabilization of mycoherbicides. In: *Biological approaches for controlling weeds.* R. Radhakrishnan (ed.). TechOpen, 2018: 63-88 (doi: 10.5772/intechopen.76936).
5. Berestetskiy A.O., Kungurtseva O.V., Sokornova S.V. Can mycelial inoculum be an alternative to conidia in the case of *Stagonospora cirsii* J.J. Davis, a potential biocontrol agent of *Cirsium arvense*? *Proc. 13th EWRS Symposium «Current status and future prospects in bioherbicide research and product development»*. Bari, Italy, 2005: 7.
6. Sokornova S.V., Berestetskiy A.O. Production of virulent mycelial inoculum of *Stagonospora cirsii* Davis by liquid state fermentation. *Proc. XV Congress of European mycologists.* St. Petersburg, 2007: 204-205.
7. Amsellem Z., Zidack N.J., Charles P., Quimby Jr., Cohen B., Gressel J. Novel formulations of mycelia from liquid fermentation. *Proc. III International Weed Science Congress.* A. Légère (ed). Foz do Iguassu, Brazil, 2000: 379.
8. Dokken F. *Submerged fermentation of Colletotrichum truncatum for biological control of scentless chamomile. Master's thesis.* University of Saskatchewan, Saskatoon, 2007.
9. Makowski R.M.D. Effect of inoculum concentration, temperature, dew period, and plant growth stage on disease of round-leaved mellow and velvetleaf by *Colletotrichum gloesporioides* f. sp. *malvae*, *Phytopathology*, 1993, 83: 1229-1234 (doi: 10.1094/Phyto-83-1229).
10. Ng S.C., Kadir J., Hailmi M.S., Rahim A.A. Efficacy of *Exserohilum longirostratum* on barnyard grass (*Echinochloa crus-galli* spp. *crusgalli*) under field conditions. *Biocontrol Sci. Techn.*, 2011, 21(4): 449-460 (doi: 10.1080/09583157.2011.554972).
11. Heiny D.K., Templeton G.E. Effects of spore concentration, temperature and dew period on disease of field bindweed caused by *Phoma proboscis. Phytopathology*, 1991, 81: 905-909 (doi: 10.1094/Phyto-81-905).
12. Gasich E.L., Berestetskii A.O., Khlopunova L.B. *Mikologiya i fitopatologiya,* 2018, 3(52): 207-216 (in Russ.).
13. Jahromi F.G., Van De Ven R.J., Cother E.J., Ash G.J. The interaction between *Plectosporium alismatis* and sublethal doses of bensulfuron-ethyl reduces the growth of starfruit (*Damasonium*

minus) in rice. *Biocontrol Sci. Techn.*, 2006, 16(9): 929-940 (doi: 10.1080/09583150600828106).
14. Gressel J. Herbicides as synergists for mycoherbicides, and vice versa. *Weed Science*, 2010, 58(3): 324-328 (doi: 10.1614/WS-09-071.1).
15. Weaver M.A., Boyette C.D., Hoagland R.E. Rapid kudzu eradication and switchgrass establishment through herbicide, bioherbicide and integrated programmes. *Biocontrol Sci. Techn.*, 2016, 26(5): 640-650 (doi: 10.1080/09583157.2016.1141175).
16. Wraight S.P., Jackson M.A., de Kock S.L. Production, stabilization and formulation of fungal biocontrol agents. In: *Fungi as biocontrol agents. Progress, problems and potential.* T.M. Butt, C. Jackson, N. Magan (eds.). CAB International, NY, 2001.
17. Bailey K.L. The bioherbicide approach to weed control using plant pathogens. In: *Integrated pest management: current concepts and ecological perspective.* D.P. Abrol (ed.). San Diego, Academic Press, 2014: 245-266 (doi: 10.1016/B978-0-12-398529-3.00014-2).
18. Van Lenteren J.C., Bolckmans K., Köhl J., Ravensberg W.J., Urbaneja A. Biological control using invertebrates and microorganisms: plenty of new opportunities. *BioControl*, 2018, 63: 39-59 (doi: 10.1007/s10526-017-9801-4).
19. Hershenhorn J., Casella F., Vurro M. Weed biocontrol with fungi: past, present and future. *Biocontrol Sci. Techn.*, 2016, 26(10): 1313-1328 (doi: 10.1080/09583157.2016.1209161).
20. Boyette C.D., Hoagland R.E., Stetina K.C. Efficacy improvement of a bioherbicidal fungus using a formulation-based approach. *American Journal of Plant Sciences*, 2016, 7(16): 2349-2358 (doi: 10.4236/ajps.2016.716206).
21. Yu X., Hallet S.G., Sheppard J., Watson A.K. Effects of carbon concentration and carbon-to-nitrogen ratio on growth, conidiation, spore germination and efficacy of the potential bioherbicide *Colletotrichum coccoides*. *J. Ind. Microbiol. Biot.*, 1998, 20(6): 333-338 (doi: 10.1038/sj.jim.2900534).
22. *Metody eksperimental'noi mikologii* /Pod redaktsiei V.I. Bilai [Methods of experimental mycology. V.I. Bilai (ed.)]. Kiev, 1982 (in Russ.).
23. Jackson M.A., Cliquet S., Iten L.B. Media and fermentation processes for the rapid production of high concentrations of stable blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus*. *Biocontrol Sci. Techn.*, 2003, 13(1): 23-33 (doi: 10.1080/0958315021000054368).
24. Schisler D. A., Jackson M. A., Bothast R. J. Influence of nutrition during conidiation of *Colletotrichum truncatum* on conidial germination and efficacy in inciting disease in *Sesbania exaltata*. *Phytopathology*, 1991, 81(6): 587-590.
25. Aybeke M. Several pesta tablet trials with *Aspergillus alliaceus* Thom & Church for effective underground and aboveground *Orobanche* L. biocontrol. *Trakya University Journal of Natural Sciences*, 2016, 17(1): 65-70.
26. Tehranchian P., Adair R.J., Lawrie A.C. Potential for biological control of the weed Angled Onion (*Allium triquetrum*) by the fungus *Stromatinia cepivora* in Australia. *Australasian Plant Path.*, 2014, 43(4): 381-392 (doi: 10.1007/s13313-014-0279-6).
27. Schisler D.A., Jackson M.A. Germination of soil-incorporated microsclerotia of *Colletotrichum truncatum* and colonization of seedlings of the weed *Sesbania exaltata*. *Can. J. Microbiol.*, 1996, 42(10): 1032-1038 (doi: 10.1139/m96-132).
28. Glare T., Caradus J., Gelernter W., Jackson T., Keyhani N., Köhl J., Marrone P., Morin L., Stewart A. Have biopesticides come of age? *Trends Biotechnol.*, 2012, 30(5): 250-258 (doi: 10.1016/j.tibtech.2012.01.003).
29. Boyette C.D., Hoagland R.E., Weaver M.A., Stetina K.C. Interaction of the bioherbicide *Myrothecium verrucaria* and glyphosate for kudzu control. *American Journal of Plant Sciences*, 2014, 5: 394-395 (doi: 10.4236/ajps.2014.526413).
30. Duke S.O., Owens D.K., Dayan F.E. The growing need for biochemical bioherbicides. In: *Biopesticides: state of the art and future opportunities. ACS Symposium Series.* American Chemical Society, Washington, DC, 2014: 31-43 (doi: 10.1021/bk-2014-1172.ch003).
31. Yuzikhin O., Mitina G., Berestetskiy A. Herbicidal potential of stagonolide, a new phytotoxic nonenolide from *Stagonospora cirsii*. *J. Agr. Food Chem.*, 2007, 55(19): 7707-7711 (doi: 10.1021/jf070742c).
32. Duke S.O., Dayan F.E. Discovery of new herbicide modes of action with natural phytotoxins. In: *Discovery and synthesis of crop protection products. ACS Symposium Series.* American Chemical Society, Washington, DC, 2015: 79-92 (doi: 10.1021/bk-2015-1204.ch007).