Microbiology, Vol. 73, No. 4, 2004, pp. 383–388. Translated from Mikrobiologiya, Vol. 73, No. 4, 2004, pp. 455–460.

EXPERIMENTAL ARTICLES

Regularities in the Germination of Conidia of Phytopathogenic Fungi

L. M. Polyanskaya*, T. E. Tolstikhina*, G. A. Kochkina, N. E. Ivanushkina**, and D. G. Zvyagintsev***

**Faculty of Soil Science, Moscow State University, Vorob'evy gory, Moscow, 119899 Russia*

***Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia*

Received August 7, 2003; in final form, January 26, 2004

Abstract—The autoregulation of conidium germination in phytopathogenic micromycetes of the genera *Fusarium, Botrytis*, and *Bipolaris* was studied. It was shown that *Trichoderma longibrachiatum* was less competitive than *Fusarium oxysporum* after their simultaneous inoculation but inhibited the phytopathogen growth in the case of earlier introduction. In the latter case, no autoinhibition of the germination of *F. oxysporum* conidia occurred; moreover, a cooperative effect was observed, i.e., the number of germinated *F. oxysporum* conidia increased with an increase in their density.

Key words: micromycetes, phytopathogens, conidia, germination, autoregulation, competition, inhibition.

Phytopathogenic microorganisms are widespread concomitants in agricultural practice; many diseases are caused by various micromycetes [1]. Much attention has been paid by researchers to biological features of phytopathogenic fungi and infection mechanisms [2]; however, data on the ecology of phytopathogen populations are scarce. Modern agriculture requires optimization of the means of plant protection and resolution of the contradiction between the activity of preparations, their environmental safety, and profitability [3]. Of special importance is the study of the reasons for the explosive increase in the number of phytopathogens on certain substrates and the elucidation of mechanisms controlling their abundance. Fungi, antagonists of phytopathogens, such as representatives of the genus *Trichoderma*, are finding expanding application in agricultural practice [4–7]. It is known that *Trichoderma* species are widespread within microbial communities of healthy plants [8].

The effect of environmental factors on the behavior of microbial populations has been extensively studied; it was shown that intrapopulational interactions are of particular ecological importance. There is evidence for the role of autoregulation in the activation of resting forms of microorganisms [9–11]. The study of autoregulation of resting form germination in pure and enrichment cultures of micromycetes is essential for the elucidation of their ecological strategies and prediction of microbial interactions in nature.

The aim of this work was to study intrapopulational regulation of conidium germination in phytopathogenic micromycetes and its role in the competition between these micromycete populations and fungi of the genus *Trichoderma.*

MATERIALS AND METHODS

Strains of phytopathogenic micromycetes, typical representatives of the genera *Fusarium, Botrytis*, and *Bipolaris*, were obtained from the All-Russia Collection of Microorganisms (VKM): *Fusarium oxysporum* Schlechtendal emend. Snyger et Hansen FW-387, *Botrytis cinerea* Persoon: Fries FW-401, and *Bipolaris sorokiniana* (Saccardo) Schoemaker VKM F-3045. Strain *Trichoderma longibrachiatum* Rifai FW-829 was isolated by us from the rhizoplane of rose plants grown in a greenhouse [8].

To obtain conidia, micromycetes were cultivated at 28°C for 7 days on agar medium containing (g/l) peptone, 5; glucose, 10; KH_2PO_4, 1; $MgSO_4$, 0.5; agar, 20. The conidia were washed off the agar, centrifuged, washed thrice with water, and subjected to ultrasonic treatment on a UZDN-1 disintegrator at 22 kHz and 0.44 A for 30 s to obtain a suspension of single spores. Microconidia of *F. oxysporum* FW-387 were obtained by filtration of the spore suspension through a filter with pore size 10 μm.

Viability of conidia was determined routinely [12]; monoconidium suspension (0.04 or 0.02 ml in the case of phytopathogenic micromycetes and *Trichoderma* strains, respectively) was placed on a thoroughly degreased microscope slide and distributed evenly with a loop over an area of 4 cm^2. For each sample, 12 specimens were prepared and 50 microscope fields were

0026-2617/04/7304-0383 © 2004 MAIK "Nauka/Interperiodica"

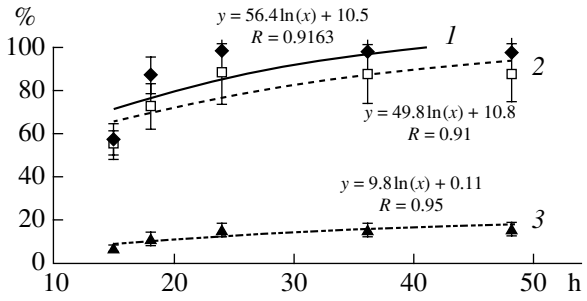

Fig. 1. Time courses of germination of the *F. oxysporum* conidia as dependent on the average distance between them: (*1*) 50, (*2*) 35, and (*3*) 13 μm. *R* is the correlation coefficient.

examined. The number of conidia per milliliter of suspension (population density) was calculated by the formula

$$N = S_1an/vS_2,$$

where N is the number of conidia per milliliter of suspension, S_1 is the specimen area (μm^2), a is the number of conidia per microscope field (averaged over all specimens), n is the dilution of the suspension, v is the volume of the drop placed onto the slide (ml), and S_2 is the area of the microscope field (μm^2).

Population density and the absence of conidium germination were determined by microscopic examination of specimens fixed and stained immediately after their preparation. To estimate the germination capacity of conidia, specimens, prior to drying, were incubated in a humid chamber at 28°C and 100% humidity for 6, 15, 18, 24, and 39 h. Then the specimens were air-dried, fixed, and stained with Calcofluor White [13]. Conidia were counted under a LYuMAM I-3 luminescent microscope; the microscope field area was 9800 μm^2. The average distance between conidia ($r = \sqrt{1/4m}$, where r is distance and m is the population density calculated per unit area) was used as the abundance index. Conidium numbers (a) of 0.1, 1, 2, 5, 10, 15, 20, and 40 per microscope field corresponded to population densities of 1×10^5, 1×10^6, 2×10^6, 5×10^6, 1×10^7, 1.5×10^7, 2×10^7, and 4×10^7 conidia per milliliter with an average distance between conidia of 156, 50, 35, 22, 15, 13, 11, and 8 μm, respectively.

Standard deviations (δ_{n-1}) for conidium numbers did not exceed 10%.

RESULTS AND DISCUSSION

The time course of conidium germination in the micromycete *F. oxysporum* is shown in Fig. 1. The germination of conidia in the humid chamber depended on their abundance; by the 15th hour of incubation, the proportion of germinated conidia comprised 50 and 5.7% in specimens containing 1–5 and 15 conidia per microscope field, respectively, with an average distance between them of 50–22 and 13 μm, respectively. By the 18th hour of incubation, the proportion of germinated conidia increased 1.5-fold in all specimens. By the end of incubation, conidium germination was almost complete or comprised 90% in specimens containing one and five spores per microscope field, respectively. In specimens containing 15 conidia per microscope field, the proportion of germinated conidia reached 16% by the 24th hour of incubation and then remained unchanged. No conidium germination was observed in specimens containing 20 conidia per microscope field with an average distance between them of 11 μm. It should be noted that both micro- and macroconidia germinated in specimens with low population densities (Figs. 2a and 2b), whereas only macroconidia germinated at the density of 15 conidia per microscope field (Figs. 2c and 2d). In all cases, conidium germination could be described by logarithmic regression equations with rather high correlation coefficients (R), ranging from 0.91 to 0.95.

A different character of conidium germination was observed in another phytopathogenic micromycete, *Bot. cinerea* (Figs. 2e, 2f, and 3); in specimens containing one spore per microscope field, conidium germination reached 50% only by the 18th hour and increased to 95% at the end of incubation. When the population density was five conidia per microscope field, the number of germinated conidia was 11–13% within 15–18 h of incubation, increased threefold by the 24th hour, and then remained unchanged. Thus, the number of germinated conidia at the density of five spores per microscope field was less than half that in specimens containing one conidium per microscope field. In this case, conidium germination could be also described by logarithmic regression equations with high correlation coefficients (R ranged from 0.91 to 0.98). Thus, conidium germination in *Bot. cinerea* appeared to be more sensitive to population density than that in *F. oxysporum.* The proportion of germinated conidia decreased threefold and by 10%, respectively, with increasing spore density from one to five conidia per microscope field. No germination of *Bot. cinerea* conidia was observed in specimens containing 15 spores per microscope field.

The character of conidium germination in *Bip. sorokiniana* specimens containing one spore per microscope field was similar to that in the fungi described above; within 15 h of incubation, germinated conidia amounted to 50%. In specimens containing 0.1 conidia per microscope field with an average distance between spores of 156 μm, about 70% of conidia germinated by the 15th hour of incubation. In both variants, 100% (or almost 100%) germination of conidia was observed by the 18th hour of incubation. It should be noted that conidia of *Bip. sorokiniana* could not be stained with Calcofluor White; a dark conidium with luminescent germ tubes is visible against a slightly bright background. By the 15th hour of incubation, germ tubes in *Bip. sorokiniana* could reach 400–500 μm in length, whereas the length of germ tubes in *F. oxysporum* and *Bot. cinerea* never exceeded 50 μm. At the end of incu-

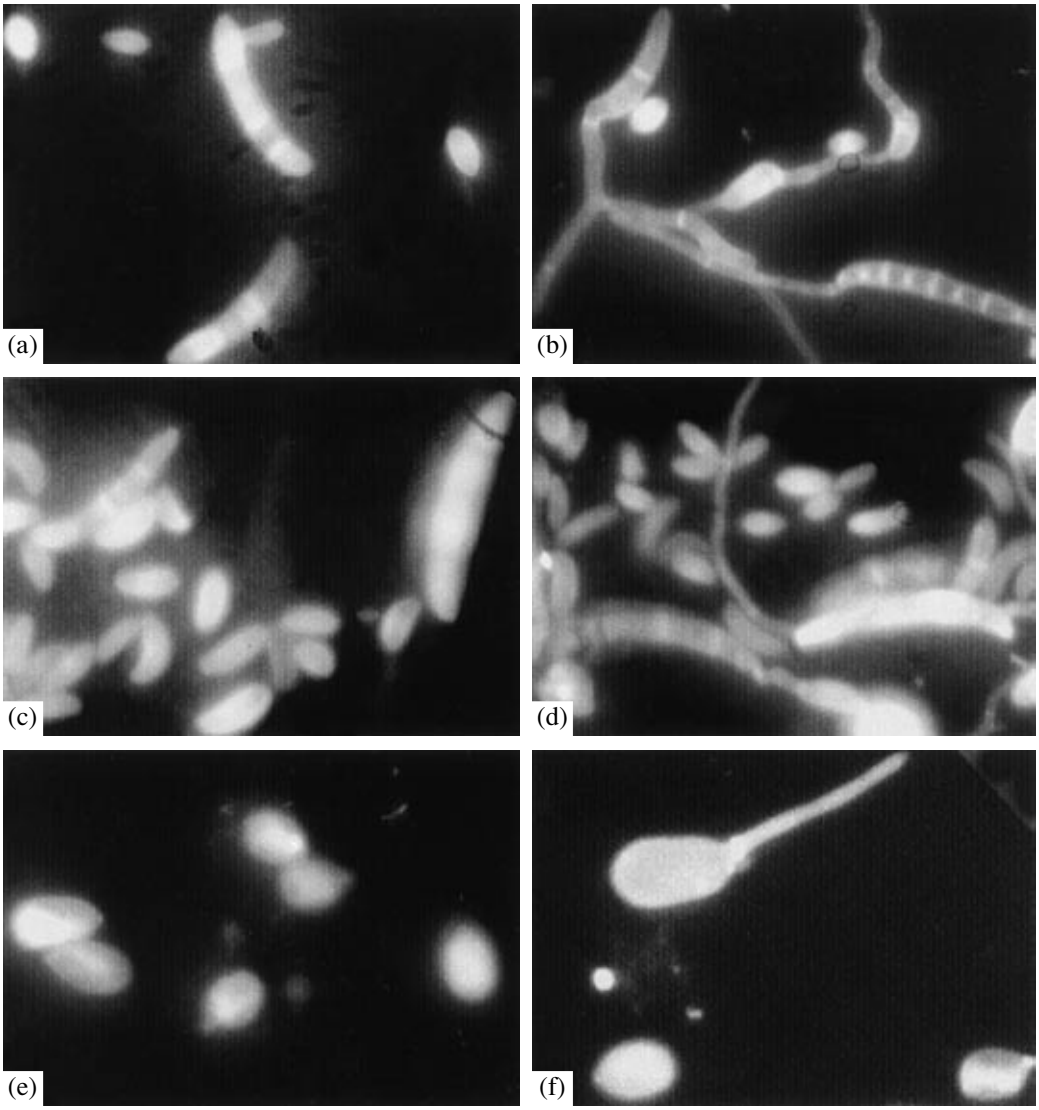

Fig. 2. Germination of *F. oxysporum* conidia as dependent on their density. (a, b) At low population density, both micro- and macroconidia germinated; (c, d) at a density of over 15 conidia per microscope field, only macroconidia germinated. (e, f) Germination of *Bot. cinerea* conidia. Magnification, 1000×.

bation, clearly defined microcolonies were formed. Growth curves satisfied logarithmic regression equations, much like those obtained for the *F. oxysporum* specimens containing one conidium per microscope field.

The dependence of conidium germination on the population density in the micromycetes studied is demonstrated in Fig. 4. For all micromycetes, the preferable distance between conidia for their germination was 50 μm; in the case of *F. oxysporum* specimens, a distance of 30 μm was also favorable, and the proportion of germinated conidia reached 80–95%. The behavior of micromycete populations was different; in the case of *F. oxysporum* and *Bot. cinerea*, the proportions of germinated conidia increased with increasing distances between them more sharply than in *Bip. sorokiniana.*

Thus, intrapopulational regulation of conidium germination is a common property of two micromycetes (*F. oxysporum* and *Bot. cinerea*) out of the three genera studied.

Taking into account the complicated interactions between micro- and macroconidia in *F. oxysporum*

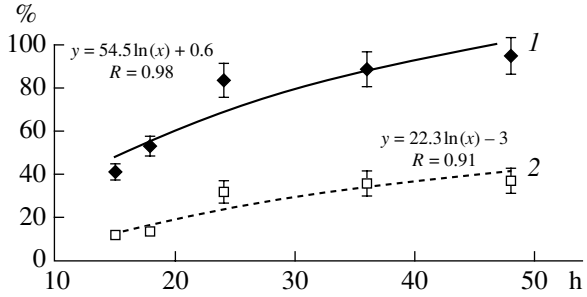

Fig. 3. Time courses of germination of the *Bot. cinerea* conidia as dependent on the average distance between them (μm). Curve designations are as in Fig. 1.

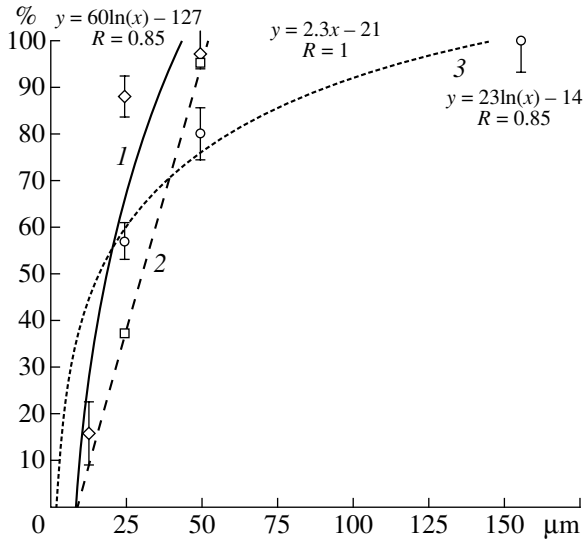

Fig. 4. Relative amounts of germinated conidia as functions of the average distances between them (μm): (*1*) *F. oxysporum,* (*2*) *Bot. cinerea*, and (*3*) *Bip. sorokiniana*.

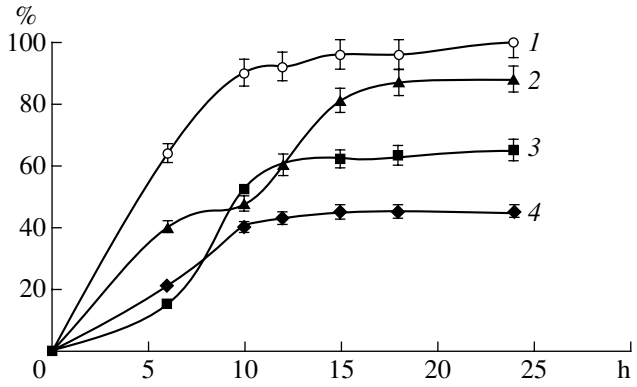

Fig. 5. Time courses of the germination of the *F. oxysporum* microconidia as dependent on the average distance between them: (*1*) 35, (*2*) 15, (*3*) 13, and (*4*) 8 μm.

(Fig. 2), the germination of microconidia in this fungus was studied in more detail. The effect of population density on the germination of microconidia in *F. oxysporum* is shown in Fig. 5. In all variants, microconidium germination started by the sixth hour of incubation. In specimens containing two conidia per microscope field with an average distance between them of 35 μm, germinated microconidia comprised over 50% by the sixth hour and almost 100% at the end of incubation. The autoinhibition of microconidium germination was observed in specimens containing 10 and 15 conidia per microscope field with average distances between them ranging from 15 to 13 μm; about 80% of microconidia germinated by the 18th hour; conidium germination almost ceased by the 15th hour of incubation. In specimens containing 40 conidia per microscope field with an average distance between them of 8 μm, spore germination ceased earlier, by the 12th hour of incubation; germinated microconidia comprised about 40%. It can be concluded that distances between conidia of 35 μm and above were most favorable for microconidium germination.

Our earlier studies on the rate of conidium germination in fungi of the genus *Trichoderma* [11] did not give sufficient grounds for the assumption that control of a phytopathogen population involved a competition mechanism [9, 15]. Modeling experiments on the interactions between phytopathogens and representatives of the genus *Trichoderma* were carried out.

To evaluate the competitive abilities of fungi belonging to the genera *Fusarium* and *Trichoderma*, their conidia were placed together onto a microscope slide. Specimens contained five conidia of *T. longibrachiatum* and two and ten microconidia of *F. oxysporum* per microscope field. As seen from Fig. 6 (columns *1*), combined incubation of conidia of the two fungi showed no effect on the time course of microconidium germination in *F. oxysporum*; the autoinhibition of microconidium germination prevailed. When microconidia of *F. oxysporum* were incubated with partly germinated (by 20%) *T. longibrachiatum* conidia, germination of the former was inhibited at both spore densities tested (Fig. 6, column *2*).

It is noteworthy that the competitive ability of *F. oxysporum* was higher at an increased density of microconidia, which indicates the appearance of a cooperative effect instead of autoinhibition.

Thus, experiments modeling initial micromycete colonization of such substrates as root-surrounding zones and plant roots allowed the study of conidium germination in micromycetes of the genus *Trichoderma*, widely applied in agricultural practice, and phytopathogenic fungi of the genera *Fusarium, Botrytis*, and *Bipolaris.*

The autoregulation of the germination of resting forms (conidia) was revealed in all the micromycetes studied. Phytopathogenic micromycetes appeared to be more active colonizers than the fungi of the genus *Trichoderma*; conidia of fungi belonging to the genera *Fusarium, Botrytis*, and *Bipolaris* germinated at higher rates than did those of *Trichoderma.* On combined incubation, *T. longibrachiatum* conidia did not inhibit the germination of *F. oxysporum* microconidia; however, when *F. oxysporum* microconidia were incubated with the developed *T. longibrachiatum* culture, an inhibitory effect of the latter was revealed. The optimal strategy for the practical application of *Trichoderma* cultures seems to be presowing seed treatment [16].

It is of interest that, under competition between *T. longibrachiatum* and *F. oxysporum*, microconidium germination in the latter increased with increasing cell density, which indicates the appearance of a cooperative effect instead of autoinhibition. In the future, it is

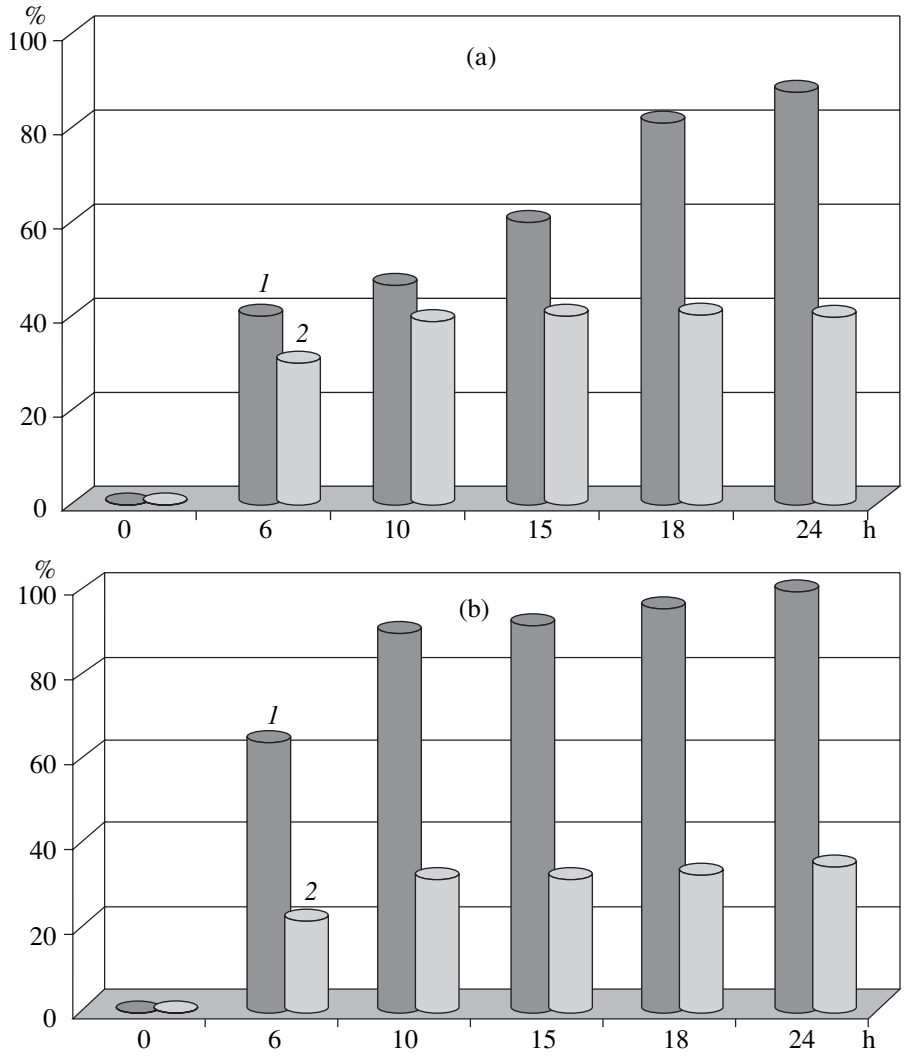

Fig. 6. Germination of *F. oxysporum* microconidia incubated together with *T. longibrachiatum* conidia that were (*1*) nongerminated or (*2*) germinated for 24 h. The population density of *F. oxysporum* was (a) two conidia per microscope field with an average distance between them of 35 μm and (b) 10 conidia per microscope field with an average distance between them of 15 μm. In both variants, the population density of *T. longibrachiatum* was five conidia per microscope field.

worthwhile to define conditions for the operation of mechanisms responsible for autoinhibitory and cooperative effects on the development of various micromycetes; such studies are of both fundamental and practical importance.

REFERENCES

1. Monastyrskii, O.A., Fungi Start a Hunt for Humans, *Nezavisimaya Gazeta*, 2001, no. 3, p. 4.
2. D'yakov, Yu.T., Ozeretskovskaya, O.L., Dzhavakhiya, V.G., and Bagirova, S.F., *Obshchaya i molekulyarnaya fitopatologiya* (General and Molecular Phytopathology), Moscow: Obshchestvo fitopatologov, 2001.
3. Ricard, J.L. and Ricard, T.J., The Ethics of Biofungicides—a Case Study: *Trichoderma harzianum* AATC 20476 on Elsanta Strawberries against *Botrytis cinerea* (gray Mold), *Agric. Human Values*, 1997, vol. 14, pp. 251–258.
4. Tronsmo, A., *Trichoderma* Used as a Biocontrol Agent against *Botrytis cinerea* Rots on Strawberry and Apple, *Sci. Rep. Agric. Univ. Norway*, 1986, vol. 65, no. 17, pp. 1–22.
5. Robinson, P., McKee, N., Tompson, I., Harper, D., and Hamilton, J., Autoinhibition of Germination and Growth of *Geotrichum candidum, Mycol. Res*, 1989, vol. 93, pp. 214–222.
6. Tronsmo, A., Biological and Integrated Controls of *Botrytis cinerea* on Apple with *Trichoderma harzianum, Biol. Control*, 1991, vol. 1, pp. 59–62.
7. Roco, A. and Perez, L.M., *In Vitro* Biocontrol Activity of *Trichoderma harzianum* on *Alternaria alternata* in the Presence of Growth Regulators, *Electronic J. Biotechnol.* [online], 15 August 2001, vol. 4, no. 2:

http://www.ejbiotechnology.into/content/vol2/issue3/full/3/index.html. ISSN 0717-3458.

8. Polyanskaya, L.M., Ozerskaya, S.M., Kochkina, G.A., Ivanushkina, N.E., Golovchenko, A.V., and Zvyagintsev, D.G., The Abundance and Structure of the Root-Associated Microbial Complexes of Two Greenhouse Rose Cultivars, *Mikrobiologiya*, 2003, vol. 72, no. 4, pp. 554–562.
9. Tronsmo, A. and Hjeljord, L.G., Biological Control with *Trichoderma* Species, *Plant-Microbe Interactions and Biological Control*, Boland, G.J. and Kuykendall, L.D., Eds., New York: Marcel Dekker, 1998, pp. 111–126.
10. Triger, E.G., Polyanskaya, L.M., and Kozhevin, P.A., Microbial Interactions in Soil as Exemplified by Certain Populations of Streptomycetes and Bacteria, *Mikrobiologiya*, 1990, vol. 59, no. 4, pp. 688–694.
11. Polyanskaya, L.M., Tolstikhina, T.E., Kochkina, G.A., Ivanushkina, N.E., Ozerskaya, S.M., Vedina, O.T., and Zvyagintsev, D.G., Autoregulation of Conidium Germination in Micromycetes of the Genus *Trichoderma, Mikrobiologiya*, 2004, vol. 73, no. 1, pp. 79–83.
12. *Metody pochvennoi mikrobiologii i biokhimii* (Methods of Soil Microbiology and Biochemistry), Moscow: Mosk. Gos. Univ., 1991.
13. Polyanskaya, L.M., Direct Microscopic Count of Fungal Spores and Mycelium in Soil, *Tez. konf. "Izuchenie gribov v biogeotsenozakh"* (Abstracts of the Conference "Investigation of Fungi in Biogeocenoses), 1988, p. 30.
14. Schirmbock, M., Lorito, M., Wang, Y.L., Hays, C.K., Arisan-Atac, I., Scala, F., Harman, G.E., and Kubicek, C.K., Parallel Formation and Synergism of Hydrolytic Enzymes and Peptabiol Antibiotics, Molecular Mechanisms Involved in the Antagonistic Action of *Trichoderma harzianum* against Phytopathogenic Fungi, *Appl. Environ. Microbiol.*, 1994, vol. 60, pp. 4364–4370.
15. Sivan, A. and Chet, I., The Possible Role of Competition between *Trichoderma harzianum* and *Fusarium oxysporum* in Rhizosphere Colonization, *Phytopathology*, 1989, vol. 79, pp. 198–203.
16. Taylor, A.G. and Harman, G.E., Concepts and Technologies of Selected Seed Treatments, *Ann. Rev. Phytopathol.*, 1990, vol. 28, pp. 321–339.