ISSN 0026-2617, Microbiology, 2010, Vol. 79, No. 6, pp. 865–870. © Pleiades Publishing, Ltd., 2010.
Original Russian Text O.K. Strunnikova, N.A. Vishnevskaya, I.A. Tikhonovich, 2010, published in Mikologiya i Fitopatologiya, 2010, Vol. 44, No. 2, pp. 160–168.

ARTICLES FROM THE RUSSIAN JOURNAL
MIKOLOGIYA I FITOPATOLOGIYA
(MYCOLOGY AND PHYTOPATHOLOGY)

Colonization of Barley Roots by *Fusarium culmorum* and Influence of *Pseudomonas fluorescens* on the Process

O. K. Strunnikova[1], N. A. Vishnevskaya, and I. A. Tikhonovich

All-Russian Research Institute of Agricultural Microbiology, Russian Academy of Agricultural Sciences, sh. Podbel'skogo 3, Pushkin-8, St. Petersburg, 196608 Russia

Abstract—The stages of barley root colonization by *Fusarium culmorum* were studied in sterile vermiculite by the method of fluorescent antibodies. The influence of the antagonistic bacterium *Pseudomonas fluorescens* on the process of root colonization by *F. culmorum* was demonstrated. In vermiculite inoculated with *F. culmorum*, the fungus density on the roots increased gradually. In the case of joint inoculation of vermiculite with the fungus and the bacterium, the *F. culmorum* density on the roots changed abruptly. It was shown that the site of primary colonization of the roots by the fungus was mainly the zone of root hairs. When *Pseudomonas fluorescens* was present on the roots, *F. culmorum* colonized not only root hairs, but also the elongation zone, during the first two days. Introduction of *Pseudomonas fluorescens* into vermiculite resulted in lower intensity of barley root rot.

Keywords: *Fusarium culmorum*, barley root colonization, colonization sites, influence of *Pseudomonas fluorescens*.

DOI: 10.1134/S0026261710060251

Root colonization by a phytopathogenic fungus is an important stage of interaction between a plant and a pathogen, which may affect both the intensity of disease severity and the increase of the phytopathogen density in soil. At the same time, studies devoted to assessment of root colonization by phytopathogenic fungi in space and in time have been developed only in recent years due to the use of fungal strains labeled with fluorescent proteins [1–7] or *gus*-marked fungal strains [8, 9]. The possibility of simultaneous monitoring of the development on roots of fungal strains with different pathogenicity [3, 5, 6] or of a phytopathogenic fungus and an antagonistic bacterium [2] was demonstrated. Marked strains made it possible to study the initial stages of tomato root colonization by *Fusarium oxysporum* f. sp. *radicis-lycopersici* [1, 2, 5].

The colonization ability of *F. culmorum*, a causative agent of spike disease and root and stem rots of cereals and other crops, has not been studied sufficiently as yet. Kang and Buchenauer [10] examined the colonization of wheat heads by *F. culmorum* by light and electron microscopy over eight days and found differences in the growth of the fungus on the inner and outer surfaces of lemma and glume. Colonization of rice roots by pathogenic and nonpathogenic strains of *F. culmorum* was investigated [11]. The researchers showed that the pathogenic isolate penetrated into the stele, while nonpathogenic isolates colonized only the cells of epidermis and cortex. The quantitative assessment of *F. culmorum* in roots was carried out by plating on a nutrient medium.

The development of *F. culmorum* and *Pseudomonas fluorescens* on barley rots was studied and the interaction between the fungus and the bacterium was established [12]. The studies were performed in nonsterile soil; therefore, a decrease or increase in the density of microorganisms under study could probably result from the mediated effect of soil microflora. It was interesting to assess the colonization abilities of the fungus and the bacterium and determine their interactions in the absence of aboriginal microflora.

The goals of the present work were to study the development and find out the sites of barley roots colonization by *F. culmorum*, to compare the density of mycelium on root surface with the intensity of development of barley root rot, and to estimate the influence of the bacterium on these processes.

MATERIALS AND METHODS

The strain *Fusarium culmorum* 30 was isolated from a diseased barley plant in the Leningrad region. The inoculum was prepared by growing the strain in Czapek medium on a rotary shaker (160 rpm) for three days. The mycelium was filtered through two layers of dense nylon tissue. Macroconidia were precipitated from the filtrate by centrifugation. The pellet containing macroconidia was resuspended in sterile water and again precipitated by centrifugation. Macroconidia were once more resuspended in sterile water and added to vermiculite.

1 Corresponding author; e-mail: olgastrunnikova@rambler.ru

The strain *Pseudomonas fluorescens* 2137 isolated from wheat roots was kindly provided by L.V. Kravchenko and N.M. Makarova (All-Russian Research Institute of Agricultural Microbiology). The *Gus* gene was introduced into the genome of *P. fluorescens* 2137 by Tn5 tranposon mutagenesis [13]. The *Gus*-labeled strain suppressed the formation of *F. culmorum* mycelium and macroconidia during joint incubation of the fungus and the bacterium on membrane filters in soil [14, 12]. Before the experiment, the strain 2137gus was grown for 24 h on agarized potato medium (pH 7.0). The cells were suspended in sterile distilled water and precipitated by centrifugation (4000 rpm, 10 min); the procedure of washing and centrifugation was repeated.

The colonization ability of *F. culmorum* was evaluated in the vegetation experiment. Sterile vermiculite was used as a substrate for plant growth. In all experimental variants, the nutrient mixture was added as 250 ml of solution per 100 g of vermiculite. The nutrient mixture contained the following (g/l): $Ca(NO_3)_2 \cdot 4H_2O$, 1.18; KNO_3, 0.5; KH_2PO_4, 0.136; and $MgSO_4 \cdot 7H_2O$, 0.48. The suspension of *F. culmorum* macroconidia (3×10^5/ml) was added to vermiculite in one of the variants, the suspension of bacterial cells (5×10^7/ml) was added in the second variant, and the fungal and bacterial suspensions were added in the third variant.

Inari barley seeds were sterilized 24 h before the beginning of the experiment: they were flooded with 0.1% $AgNO_3$ solution for 30 min, washed with sterile water many times, and put into sterile petri dishes on wet filter paper. Such a procedure made it possible to eliminate seed infection and to select the germinating seeds that would provide bursting emergence of the seedlings.

Barley was grown for 17 days: in 500-ml pots in the first five days of the experiment and in 900-ml pots in the following days. During the experiment, the plants were sampled ten times daily in the first six days. Eight plants were sampled from each variant in the first two days, six plants were sampled on days 3–10, and five plants were sampled on days 12 and 17. The roots were carefully separated from the vermiculite, submerged in water for the final removal of the substrate particles, and examined under an ×8 magnifier in order to detect the rot symptoms. Root rot lesion was accounted by the procedure developed at the All-Russian Research Institute of Plant Protection [15].

The roots were dried at room temperature. Dry roots were macerated in water for 2 h, stained with 1% neutral red solution in water for 20–30 min depending on root diameter (for autofluorescence quenching), washed with water two to three times, and placed on filter paper to remove excessive moisture. The roots were then sequentially submerged for 20 min in solutions of species-specific antibodies diluted 1 : 64 and anti-rabbit immunoglobulins labeled with fluorescein isothiocyanate. After each staining procedure, the roots were washed two to three times with buffered saline, pH 7.6. No cross-reaction of immunofluorescence of the antibodies to *F. culmorum* diluted at a rate of 1 : 64 was detected with the cultures of *F. oxysporum, F. solani, F. verticillioides, F. heterosporum, F. sambucinum, F. semitectum, F. nivale, F. equiseti*, and *F. aquaeductum*.

The procedure for obtaining the antibodies to *F. culmorum*, isolation and purification of immunoglobulins and their specificity testing was described previously [16].

All roots of each plant were carefully put on slides, fixed with cover glasses, and examined in an Imager A1 fluorescence microscope (Carl Zeiss, Germany) at a magnification of ×400. Each root was examined throughout the length with recording of the number of a visual field containing fungal colonies and the serial number of the beginning and end of each zone: root collar, root hairs, apex, and elongation. Previously, a scale of correspondence of visual fields to the millimeter of root length was drawn up. For this purpose, the length of straight root sections was measured and then the number of visual fields at this section was counted in the microscope at a magnification of ×400. As a result, it was shown that one visual field of the microscope at a magnification of ×400 corresponded to 0.3 mm of root length. On this basis, the length of root zones and the number of *F. culmorum* colonies in each zone and on the root as a whole were counted.

On days 12 and 17 of the analysis, the roots of three plants only were completely examined. By that time, the total length of roots in each variant was 140–150 cm.

Statistical treatment of the data obtained was carried out using the Stat Soft Statistica v. 6.0, 1995, software package.

RESULTS AND DISCUSSION

In the case of vermiculite inoculation with *F. culmorum*, the fungus density on barley roots increased gradually (Fig. 1). A reliable decrease in mycelium density on root surface was observed only on day 8, but on day 10 the fungal density was already recovered.

In the case of joint inoculation of the substrate, when *P. fluorescens* was present on the roots, considerable fluctuations in the *F. culmorum* density were noted (Fig. 1). For example, on day 1, a considerable increase in fungus density on the roots was observed. The density of *F. culmorum* on the roots decreased on day 2 but increased again on day 4; the fluctuations in fungal quantity were observed until the end of the experiment.

Previously we have also noted an increase in the density of the strain *F. culmorum* 30 on barley roots in the presence of *P. fluorescens* 2137gus in the earliest periods of root colonization by the fungus [12]. In the previous work, we have suggested possible explana-

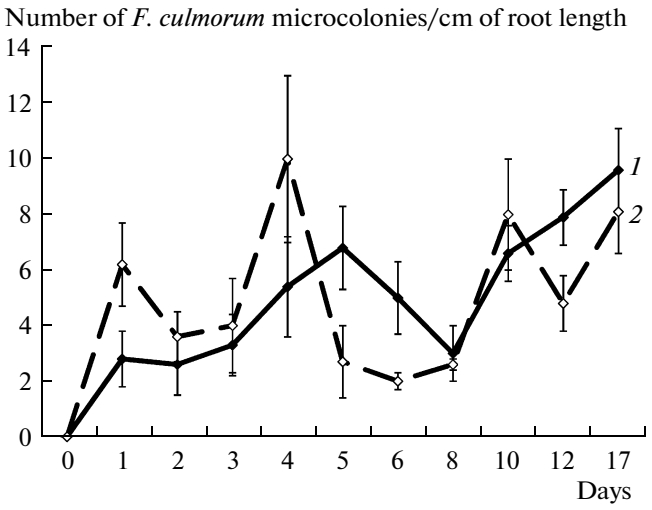

Fig. 1. Intensity of barley root colonization by *F. culmorum*. Inoculation of vermiculite by: *F. culmorum* (*1*) and by *F. culmorum* and *P. fluorescens* together (*2*). The same is for Fig. 3.

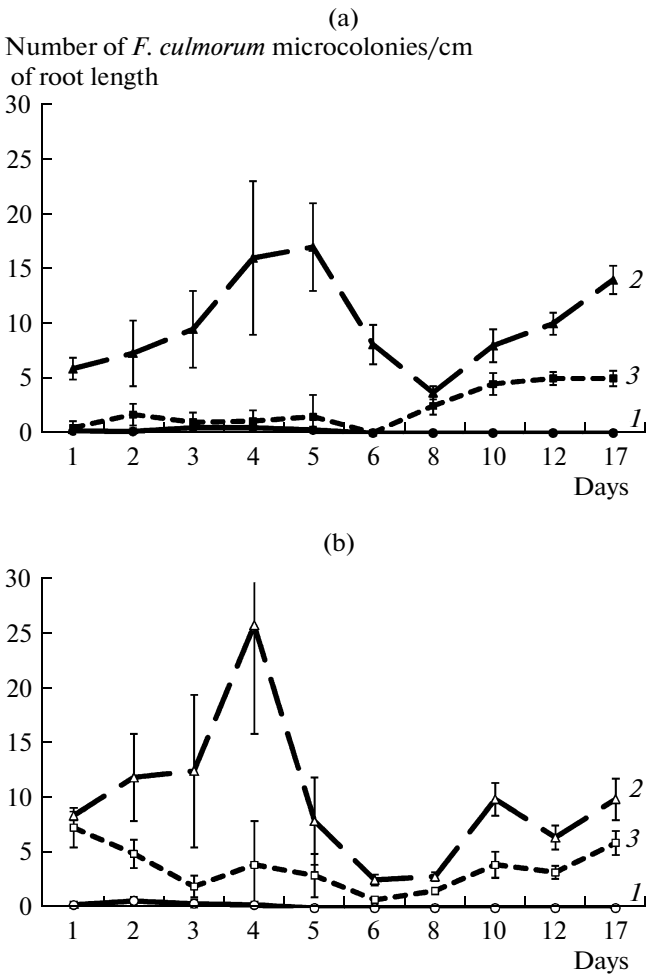

Fig. 2. Intensity of colonization of root zones by *F. culmorum* under inoculation of vermiculite by *F. culmorum* (a) and by *F. culmorum* and *P. fluorescens* together (b). Zones: root collar (*1*), root hairs (*2*), apex and elongation (*3*).

tions of this phenomenon. It is known that *Pseudomonas* strains can change the composition of root excretions [17]. Apparently, this is what attracts the fungus to the roots where the bacteria are present. It is also possible that the bacterial strain itself produces certain metabolites attracting the fungus. The increase in the fungus density on the roots observed in our experiment may be an evidence of a taxis.

It has been shown that primary colonization of the roots by *F. culmorum* occurs mainly in the zone of root hairs (Fig. 2). Single hyphae of the fungus were observed on root hairs already 24 h after the inoculation and were almost absent near the root collar. The elongation zone was colonized by the fungus by far less frequently than the zone of root hairs.

When assessing root rot symptoms under a magnifier, we often observed root tip die-back. In a fluorescence microscope, the tip tissues sometimes acquired green fluorescence; it was most often observed as a result of joint colonization of the roots by the fungus and the bacterium. Microscopic assessment of the roots only seldom revealed *F. culmorum* hyphae directly on the apex.

Comparison of our results with the literature data shows the character of *F. culmorum* distribution on the roots similar to that of *F. oxysporum* [1, 2, 5]. The possibility of *F. oxysporum* penetration into the roots through root hairs was shown in tomato plants by electron microscopy [8]. Obviously, the way of *F. culmorum* penetration into the root is the same as in *F. oxysporum*, because we have more than once observed the fungus in root hairs.

Our observations demonstrated that the roots were colonized by single fungal hyphae; such hyphae were observed near the root surface even on day 5. Based on these observations, it may be suggested that root colonization by *F. culmorum* is not of mass character and is time-expanded. Root colonization from the substrate probably occurs later as well; however, already on day 6 it was sometimes difficult to differentiate the fungal hyphae colonizing the root from the hyphae developed on its surface and eventually rising above the root.

After colonizing the zone of root hairs, the fungus generally remains within this zone (Fig. 2a). At the same time, the frequency of occurrence of mycelium during the whole experiment was higher in the zone of developed root hairs than in the younger part of the zone. On day 8 after inoculation with *F. culmorum*, fungal colonies were also observed in the elongation zone adjoining the apex. However, occurrence of the fungus in the apical zone is most likely explained by the movement of the pathogen toward the root tip during root growth rather than by direct colonization of this zone.

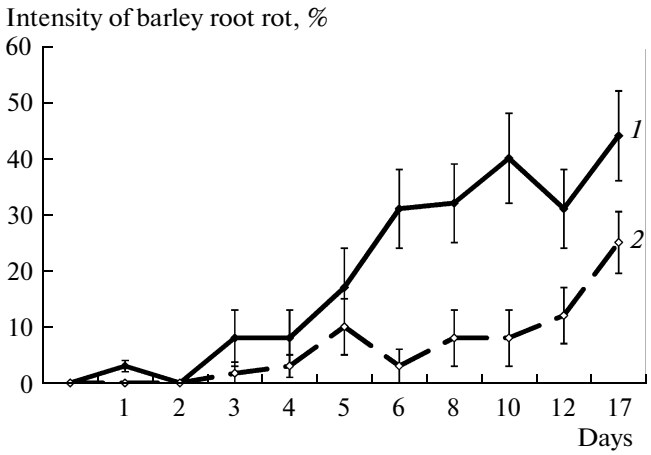

Fig. 3. Intensity of barley root rot.

When the bacterial strain was also present on the roots, *F. culmorum* in the first two days colonized not only the zone of root hairs but also the elongation zone (Fig. 2b). In the presence of the bacterial strain, the fungus more often occurred in the elongation zone on day 4 and 5 (Fig. 2b), in contrast to the conditions of colonization by the fungus alone (Fig. 2a). On days 5 and 6, fungal density in the zone of root hairs decreased considerably in the presence of the bacterial strain, while it was much higher in the variant without the bacterium.

On the roots, *F. culmorum* was represented mainly by mycelium, much more rarely by macroconidia and chlamydospores. Macroconidia were more often observed on the roots colonized by the fungus alone, while chlamydospores were most often formed in the presence of the bacterial strain.

Directly on the root surface, *F. culmorum* colonizing the root hairs of 1-day barley roots occurred only under conditions of joint inoculation by the fungus and the bacterium. In the variant of vermiculite inoculation by the fungus alone, the hyphae appeared directly on the root surface on day 2. On days 3 and 4, *F. culmorum* formed compact microcolonies of substrate mycelium occupying a very limited area of the root surface. During this period, root tissues in the site of colonization usually looked healthy. The colony gradually enlarged and the root area occupied by the fungus increased. Root tissues in the site of colony formation became dark as a result of incipient necrosis. Fungal hyphae of the large colony were located chaotically and in due course often rose above the root surface. Abundant growth of the fungus on the surface was usually observed on days 5–8, though not throughout the root length but locally. Under a microscope, the root in the site of location of such a colony looked dark-brown or black due to complete destruction of the tissues.

The fungus was often observed to expand along the root toward the apex. As the root grew, *F. culmorum* colonized the places of formation of lateral roots, which often resulted in their complete destruction. Eventually, the fungus was not revealed on the dead sites of the root.

No distinct correlation between the stages of fungal colonization of the roots and the periods of plant growth was revealed. It was probably due to a certain variability in the development of each plant. Although we tried to align the periods of emergence of the seedlings by incubating the seeds for 24 h in a moist chamber, as well as to provide more simultaneous growth of plants, still there were some differences in growth between the plants. It is likely that the colonization process itself is extended in time. In any case, both the beginning of the colonization process and establishment of the fungus on the surface followed by its spreading could be observed on the roots of different plants in the same variant.

None of the experiments showed such mass colonization of roots by fungal hyphae as has been shown in the works with *F. oxysporum* [1–3, 5, 8]. The researchers showed that the hyphae of *F. oxysporum* almost completely covered tomato roots. Such abundant root colonization by the fungus is explained by the sterility of the experimental conditions and, probably, by the species specificity of *F. oxysporum*. In our experiment, the average density of *F. culmorum* in sterile vermiculite was 4–6 colonies/cm of root, while in nonsterile soil it was 2–3 colonies/cm [12]. The hyphae of *F. culmorum* usually were not located on the root surface as those of *F. oxysporum* due to their concealed spread over the root and sometimes disappeared from the surface, apparently as a result of penetration into the epidermis cells. We believe that such concealed distribution of the fungus over the root is quite explainable, because free colonization of root surface by the hyphae is hardly possible under the natural conditions of severe competition with the rhizospheric microflora.

Simultaneous introduction of the bacterial strain into vermiculite together with the phytopathogen resulted in considerable decrease of the quantity of diseased plants (Fig. 3). In this variant, the fungus density on the roots was lower (except for the reliable increase in density in the first 24 h) than under colonization by the fungus alone (see Fig. 1). Microscopy of the roots colonized by both microorganisms occasionally showed the lysed fungal hyphae. Moreover, in the variant of joint colonization by the fungus and the bacterium formation of mycelial chlamydospores was observed. These structures were usually formed by *F. culmorum* during incubation with *Pseudomonas fluorescens* on membrane filters in soil in the period of active suppression and lysis of mycelium by the bacterial strain [12, 14]. These facts give evidence that *P. fluorescens* can suppress the fungus not only in soil but also on barley roots. Decrease in the density of *F. oxysporum* mycelium on tomato roots in the presence of pseudomonads was demonstrated by Dutch

researchers [2]. Nevertheless, the differences in fungal density on the roots colonized by *F. culmorum* separately and in combination with *P. fluorescens* were not very significant in our experiment. It is quite possible that the protective effect of the bacterial strain observed in our experiment was manifested by not only the decrease in fungal density on the roots but also a direct effect on the plant, which we have noted previously [12]. It is also possible that the bacterial strain influences fungal physiology, e.g., affecting production by the fungus of the toxins that provide its aggressiveness. For example, microscopy of four-day roots colonized by the fungus and the bacterial strain showed that root tissues in the site of *F. culmorum* localization looked healthy in spite of higher density of the fungus.

When assessing the fungus density on the roots, we found no dependence between the root length and the level of colonization. Shorter roots could be colonized by the fungus to a greater extent than the longer ones and vice versa. Dependence between the intensity of colonization of the roots of the same plant and of different plants was not revealed either. It is quite possible that root colonization by the fungus from the substrate in the absence of competitors and in the presence of sufficient nutrition for the pathogen is of random nature. Colonization proceeds as the root grows through vermiculite and depends on the zonality of pathogen localization. In the presence of the bacterium in the vermiculite, fungal colonization of the roots is directed, which may be due both to competitive displacement of the fungus from the substrate and to attraction of the fungus to the roots where the bacterial strain is located.

ACKNOWLEDGMENTS

The work was supported by the Russian Foundation for Basic Research (projects nos. 08-04-00356-a and 08-04-1367-ofi_c) and the Foundation of the President of the Russian Federation (NS-5399.2008.4).

REFERENCES

1. Lagopodi, A.L., Ram, A.F.J., Lamers, G.E., Punt, P.J., van den Hondel, C.A.M.J.J., Lugtenberg, B.J.J., and Bloemberg, G.V., Novel Aspects of Tomato Root Colonization and Infection by *Fusarium oxysporum* f. sp. *radicis-lycopersici* Revealed by Confocal Laser Scanning Microscopic Analysis and Using the Green Fluorescent Protein as a Marker, *Mol. Plant–Microbe Interact.*, 2002, vol. 15, pp. 172–179.
2. Bolwerk, A., Lagopodi, A.L., Wijfjes, A.H.M., Lamers, G.E.M., Chin-A-Woeng T.F.C., Lugtenberg B.J.J., and Bloemberg G.V., Interactions in the Tomato Rhizosphere of Two *Pseudomonas* Biocontrol Strains with the Phytopathogenic Fungus *Fusarium oxysporum* f. sp. *radicis-lycopersici, Mol. Plant–Microbe Interact.*, 2003, vol. 16, no. 11, pp. 983–993.
3. Bolwerk, A., Lagopodi, A.L., Lugtenberg, B.J.J., and Bloemberg, G.V., Visualization of Interactions Between a Pathogenic and a Beneficial *Fusarium* Strain During Biocontrol of Tomato Foot and Root Rot, *Mol. Plant–Microbe Interact.*, 2005, vol. 18, no. 7, pp. 710–721.
4. Oren, L., Ezrati, S., Cohen, D., and Sharon, A., Early Events in the *Fusarium verticillioides*-Maize Interaction Characterized by Using a Green Fluorescent Protein-Expressing Transgenic Isolate, *Appl. Environ. Microbiol.*, 2003, vol. 69, no. 3, pp. 1695–1701.
5. Olivain, C., Humbert, C., Nahalkova, J., and Fatehi, J., L'Haridon F., and Alabouvette C., Colonization of Tomato Root by Pathogenic and Nonpathogenic *Fusarium oxysporum* Strains Inoculated Together and Separately Into the Soil, *Appl. Environ. Microbiol.*, 2006, vol. 72, no. 2, pp. 1523–1531.
6. Nahalkova, J., Fatehi, J., Olivain, C., and Alabouvette, C., Tomato Root Colonization by Fluorescent-Tagged Pathogenic and Protective Strains of *Fusarium oxysporum* in Hydroponic Culture Differs from Root Colonization in Soil, *FEMS Microbiol. Lett.*, 2008, vol. 286, no. 2, pp. 152–157.
7. Macié-Vicente, J.G., Jansson, H.-B., Talbot, N.J., and Lopez-Llorca, L.V., Real-Time PCR Quantification and Live-Cell Imaging of Endophytic Colonization of Barley (*Hordeum vulgare*) Roots by *Fusarium equiseti* and *Pochonia chlamydosporia, New Phytol.*, 2009, vol. 182, no. 1, pp. 213–228.
8. Olivain, C. and Alabouvette, C., Process of Tomato Root Colonization by a Pathogenic Strain of *Fusarium oxysporum* f. sp. *lycopersici* in Comparison with a Non-Pathogenic Strain, *New Phytol.*, 1999, vol. 141, pp. 497–510.
9. Bao, J.R. and Lazarovits, G., Differential Colonization of Tomato Roots by Nonpathogenic and Pathogenic *Fusarium oxysporum* Strains May Influence *Fusarium* Wilt Control, *Phytopathology*, 2001, vol. 91, no. 5, pp. 449–456.
10. Kang, Z. and Buchenauer, H., Cytology and Ultrastructure of the Infection of Wheat Spikes by *Fusarium culmorum, Mycol. Res.*, 2000, vol. 104, no. 9, pp. 1083–1093.
11. Jaroszuk-Ściseł, J., Kurek, E., Winiarczyk, K., Baturo, A., and Łukanowski, A., Colonization of Root Tissues and Protection Against *Fusarium* Wilt of Rye (*Secale cereale*) by Nonpathogenic Rhizosphere Strains of *Fusarium culmorum, Biol. Control*, 2008, vol. 45, no. 3, pp. 297–307.
12. Strunnikova, O.K., Shakhnazarova, V.Yu., Vishnevskaya, N.A., Chebotar', V.K., and Tikhonovich, I.A., Interactions between *Fusarium culmorum* and *Pseudomonas fluorescens* in the Rhizosphere and Rhizoplane of Barley, *Mikol. Fitopatol.*, 2008, vol. 42, no. 1, pp. 70–77.
13. Chebotar, V., Nakayama, Y., Kang, U.G., El Gaali, E., and Akao, S., Use of Reporter *gus*-Gene to Study the Colonization of Rice Roots by *Azospirillum lipoferum, Soil Microorganisms*, 1999, vol. 53, pp. 13–18.
14. Strunnikova, O.K., Shakhnazarova, V.Yu., Vishnevskaya, N.A., Chebotar', V.K., and Tikhonovich, I.A., Development and Relations of *Fusarium culmorum* and *Pseudomonas fluorescens* in Soil, *Mikrobiologiya*, 2007,

vol. 76, no. 5, pp. 675–681 [*Microbiology* (Engl. Transl.), vol. 76, no. 5, pp. 596–602].

15. *Ekologicheskii monitoring i metody sovershenstvovaniya zashchity zernovykh kul'tur ot vreditelei, boleznei i sornyakov* (Ecological Monitoring and Methods for Improvement of Plant Protection against Pests, Diseases, and Weeds), St. Petersburg: VIZR, 2002, p. 31.
16. Strunnikova, O.K., Shakhnazarova, V.Yu., Vishnevskaya, N.A., and Muromtsev, G.S., Application of Membrane Filters and Immunofluorescent Staining for Monitoring Developments of Soil Micromycetes, *Mikol. Fitopatol.*, 1998, vol. 32, no. 2, p. 65–72.
17. Kamilova, F., Kravchenko, L.V., Shaposhnikov, A.I., Makarova, N., and Lugtenberg, B., Effects of the Tomato Pathogen *Fusarium oxysporum* f. sp. *radicis-lycopersici* and of the Biocontrol Bacterium *Pseudomonas fluorescens* WCS365 on the Composition of Organic Acids and Sugars in Tomato Root Exudates, *Mol. Plant–Microbe Interact.*, 2006, vol. 19, no. 10, pp. 1121–1126.