

Antagonistic activity of microorganisms isolated from chernozem against plant pathogens

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The aim of the work was to establish the antagonistic activity spectrum of microorganisms of different taxonomic groups isolated from Calcic Chernozem, against plant pathogens. Antagonistic activity of the soil microorganisms against phytopathogens was checked by diffusion in agar, taking into account the diameter of the growth inhibition zones around the blocks. The strains of phytopathogenic bacteria *Xanthomonas ascampestris* 8003b, *Pectobacterium carotovorum* 8982, *Pseudomonas syringae* pv. *atropaciens* 8254, *Pseudomonas syringae* pv. *lachrymans* 7595 and fungi *Fusarium oxysporum* 54201, *Fusarium culmorum* 50716, *Cladosporium herbarum* 16878, *Alternaria alternata* 16 and *Aspergillus niger* 25 were used as the test cultures. Soil isolates were screened to determine the spectrum and level of their antagonistic activity in relation to test cultures of phytopathogens. The most active micromycetes isolates were *Trichoderma lignorum* 14, which exhibited antagonism against four of the five fungal species and three of the four bacteria, and *Trichoderma longibrachiatum* 17, active against all the tested plant pathogens. Among the actinobacteria, the most active isolates belonged to the genus *Streptomyces*: *Streptomyces* sp. 31 inhibited the growth of all tested plant pathogens, and *Streptomyces* sp. 35 showed antagonism against all phytopathogenic fungi and three of the four bacterial species. The most active bacterial isolates identified as *Bacillus megaterium* 3 and *Bacillus brevis* 6 inhibited the growth of all phytopathogenic cultures tested. All active antagonist isolates had no phytotoxic effect on spring barley seedlings, on the contrary, *T. longibrachiatum* 17 significantly increased the number of germinated seeds, and *Streptomyces* sp. 35 had a positive effect on shoot growth. Selected soil cultures can be used for the creation of plant protection products against fungal and bacterial disease.

Keywords: Soil microorganisms; Calcic chernozem; Phytopathogens; Antagonism; Plant protection

Introduction

The problems of land degradation and desertification, and soil dehumification are a negative consequence of the constantly increasing human impact on natural landscapes (Prudnikova & Savin, 2015; Chibrik et al., 2016; Savosko et al., 2018), as well as on biological diversity in natural habitat (Klymenko et al., 2017; Pirko et al., 2018) and artificially created communities of plants (Khromykh et al., 2018; Lykholat et al., 2019a, 2019b) and animals (Brygadyrenko, 2015). In Ukraine, the resource for expanding the arable land of Calcic Chernozems, which are the most fertile soils, is almost exhausted today. The plowing degree of chernozems is 85% in the forest-steppe zone and more than 90% in the steppe zone, ensuring the production of most of the fruit crop (Lykholat et al., 2019a, 2019b) and grain crops (Nazarenko et al., 2018), sugar beets, sunflowers and other crops (Pozniak, 2016). Intensive use of chernozem soils gradually leads to a deterioration in their physical and chemical properties and weakening of environmental functions (buffer capacity, aggregation, fertility and other indicators). The regional features of soil and the level of anthropogenic transformation in general determine the rate of soil degradation (Pakhomov et al., 2009; Pokhylenko et al., 2019; Savosko et al., 2019).

Currently, all highly productive systems for growing crops on Calcic Chernozems are based on the intensive use of chemical plant protection products against the diseases and pests (Nazarenko et al., 2019), since the yield depends on the biological potential of the crop variety and elimination of the negative impact of pests (Iutynska & Ponomarenko, 2010). Pathogens of such diseases as fusarium, pyriculariosis, alternariosis and aspergillois, which can reduce the yield of grain crops by more than 40%, are of particular danger (Trenozhnikova, 2018). Phytopathogenic fungi can produce mycotoxins (fusaric acid, trichothecenes, zearalenone, fumonisin, aflatoxin) that accumulate in affected plant tissues (Basler, 2016) and can pose a significant risk to human and animal health if they enter food and feed (Shi et al., 2016; Oldenburg et al., 2017; Sarrocco et al., 2019). A number of negative environmental consequences accompanies the widespread use of chemical plant protection products, since most of the pesticides enter the soil and become a source of pollution of the environment, feed and food (Iutynska & Ponomarenko, 2010). Pesticides can affect non-target and beneficial microorganisms and upset the ecological balance, which negatively affects the stability of agricultural systems. Intensive use of fungicides increases the risk of resistant strains (Trenozhnikova, 2018; Fan et al., 2019). Most chemicals show only fungicidal effects and do not have antibacterial activity (Patyka & Pasichnyk, 2014).

In integrated plant protection systems, a special place belongs to the products based on the strains of microorganisms and their metabolites, which provide control of phytopathogens, induce plant resistance to diseases and stimulate plant growth. Antagonists of the phytopathogenic bacteria and fungi are common among the different groups of microorganisms: micromycetes, actinobacteria and bacteria. Inhibition of the phytopathogenic microorganism growth is characteristic of fungi of the genera *Aspergillus*, *Trichoderma*, *Ampelomyces*, *Trichothecium* (Van Bohemen et al., 2016; Yang et al., 2018), *Penicillium* (Sarrocco et al., 2019), *Stachybotrys* (Ribeiro et al., 2018), and *Fusarium* (Ibrahim, 2017; Abro et al., 2019). Actinobacteria synthesize about 40–45% of all biologically active metabolites that have found practical use. The championship belongs to representatives of the genus *Streptomyces*, which produce 7,500 biologically active compounds from 10,000 known metabolites of actinobacteria. Secondary metabolites of these microorganisms have antibacterial, antifungal, herbicidal, antiparasitic activity, so they may be considered as the potential biological agents for plant protection (Iutynska & Ponomarenko, 2010; Shakeel et al., 2018; Qi et al., 2019). Speaking about the antagonists of phytopathogenic microorganisms, it is also worth noting bacteria of the genus *Bacillus*, which produce various antimicrobial substances (Meena & Kanwar, 2015; Irkitova et al., 2018), lytic enzymes (Bodhankar et al., 2017) and chitinases (Rishad et al., 2017), and induce an increase in the plants resistance to pathogens (Khong et al., 2013; Yamamoto et al., 2015).

The demand for effective microbiological plant protection products determines the need for a constant search for phytopathogenic antagonists among soil microorganisms, especially those that have a wide range of biological activity. The aim of the work was a comprehensive assessment of the antagonistic activity of different taxonomic group of microorganisms isolated from Calcic Chernozem with respect to phytopathogenic bacteria and fungi, as well as verification of their possible phytotoxic effect.

Material and Methods

Soil sampling

Soil samples were taken in a relatively clean ecological zone (Kirovograd province, Petrove village, 48°20' N, 33°16' E) on the plots of land that were not subjected to mechanical or chemical treatment. According to the international classification (IUSS Working Group WRB, 2015), the soil in the studied areas was assigned to Calcic Chernozem. The vegetation in the plots included such steppe and meadow species as *Poa angustifolia* L., *Elytrigia repens* (L.) Nevski, *Koeleria cristata* (L.) Pers., *Euphorbia seguierana* Neck., *Stachys recta* L., *Salvia nemorosa* L. Soil sampling was carried out at a depth of 5–10 cm according to generally accepted methods. An averaged soil sample, which consisted of five samples weighing 100–200 g each, was investigated.

Microbiological research

Streptomyces were isolated from averaged samples of soil and cultured on the Gauze mineral medium. Bacteria were cultivated on meat-peptone agar, and fungi grown on Chapek's medium according to method of Tepper et al. (2004). Antagonistic activity against phytopathogenic microorganisms was tested by agar diffusion method (Egorov, 2004), taking into account the diameter of growth retardation zones around blocks with antagonist cultures.

The test cultures were the strains of phytopathogenic bacteria *Xanthomonas campestris* 8003b, *Pectobacterium carotovorum* 8982, *Pseudomonas syringae* pv. *atrofaciens* 8254, *Pseudomonas syringae* pv. *Lachrymans* 7595 from the collection of the Department of phytopathogenic bacteria of the Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine (IMB). The Department of Physiology and Systematics of Micromycetes of the IMB provided the strains of phytopathogenic fungi *Fusarium oxysporum* 54201, *F. culmorum* 50716, *Cladosporium herbarum* 16878. In addition, the strains of *Alternaria alternata* 16 and *Aspergillus niger* 25 from the collection of the Department of Microbiology, Virology and Biotechnology of Oles Honchar Dnipro National University were used. Phytopathogenic bacteria grown on meat-peptone agar and phytopathogenic fungi were grown on potato agar with the addition of 1% glucose. Identification of the isolated microorganisms was carried out using determinants for micromycetes (Dighton, 2003; Domsh et al., 2007), and Bergi's determinant (Buchanan & Gibbons, 1974; Holt et al., 1994) for the identification of actinobacteria and bacteria.

Phytotoxicity test

The phytotoxic effect of the isolated microorganisms was studied on the seedlings of spring barley (Kristallia cultivar). To do this, barley seeds (100 seeds for each experiment variant) were soaked in the culture fluid (1 : 10 dilution) for two hours for bacteria and actinobacteria, or 24 hours for micromycetes, after which the seeds germinated on wet filter paper in Petri dishes. The number of germinated seeds and the length of the roots and shoots of the barley seedlings were determined on the fourth day after treatment with a culture liquid of bacteria and actinobacteria and on the sixth day after treatment with a culture liquid of micromycetes. The barley seeds soaked in distilled water served as a control samples.

Statistical processing The research results were statistically processed using a descriptive statistical procedure. The significance of differences between the samples was determined using ANOVA procedures according to the Fisher test.

Results

The total number of soil isolates of micromycetes was 27 samples, among which 15 isolates showed antagonistic activity against the test cultures of different phytopathogenic organisms. Most isolates had a low or medium level of activity with respect to a narrow spectrum of phytopathogens. High antagonistic activity was detected only in four isolates (Figure 1).

Isolate No. 1 antagonized three species of pathogenic fungi and one species of bacteria. Isolate No. 14 was active against four species of fungi and three species of bacteria. Isolate No. 17 was highly active against all tested cultures (width of the growth inhibition zones varied from 14.7 to 38.3 mm). Isolate No. 21 inhibited the growth of only three species of phytopathogenic fungi.

Identification of the most active isolates based on morphological and cultural characters made it possible to attribute isolate No. 1 to the genus *Trichoderma*. Isolate No. 14 was identified as *Trichoderma lignorum*, isolate No. 17 as *Trichoderma longibrachiatum*, and isolate No. 21 – as *Fusarium sambucinum*. In our work, only 14 actinobacterial isolates among 35 obtained samples show antimicrobial activity, and only four of them (nos. 11, 31, 35, and 36) were characterized by a wide spectrum of their action (Figure 2).

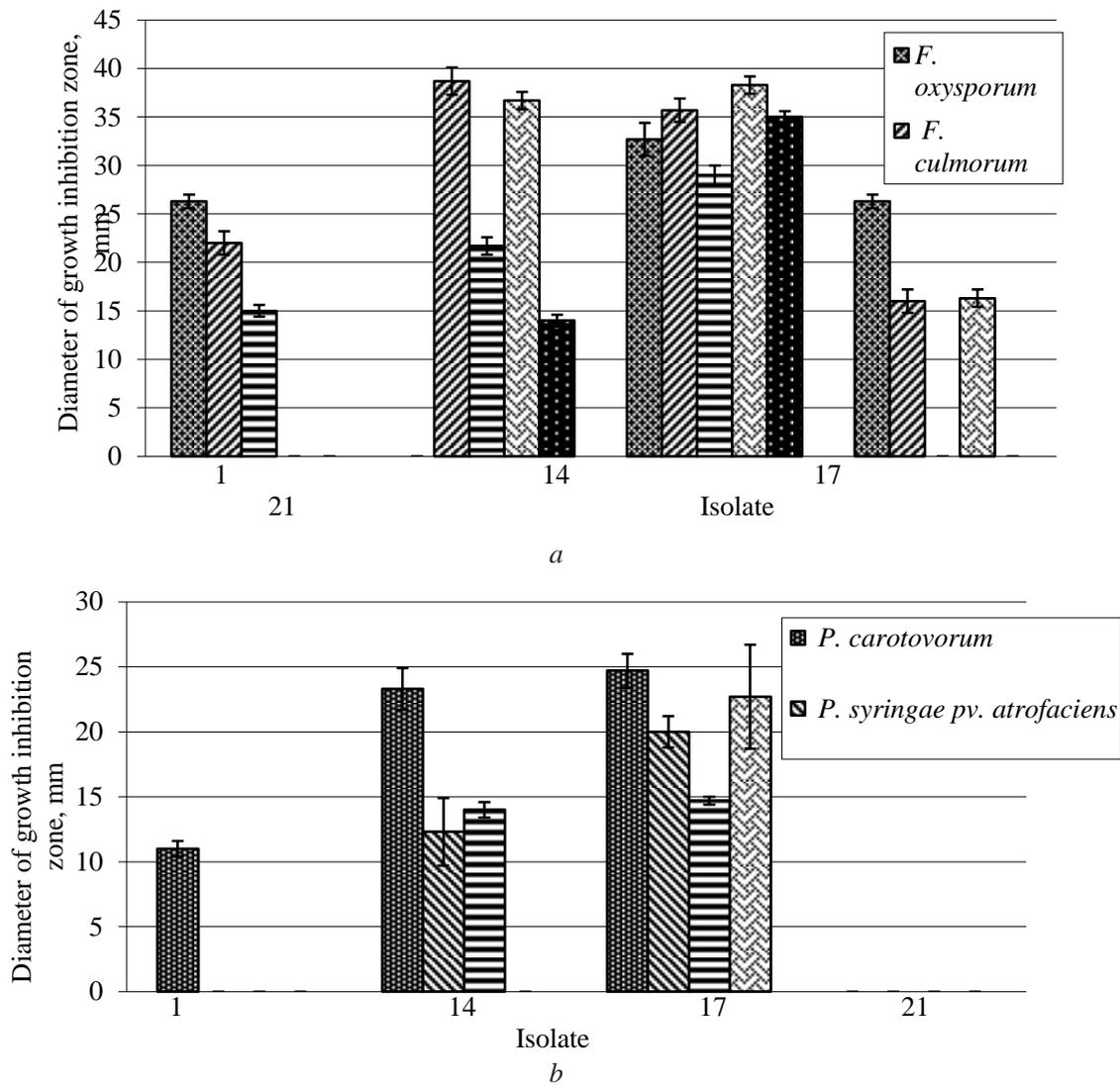
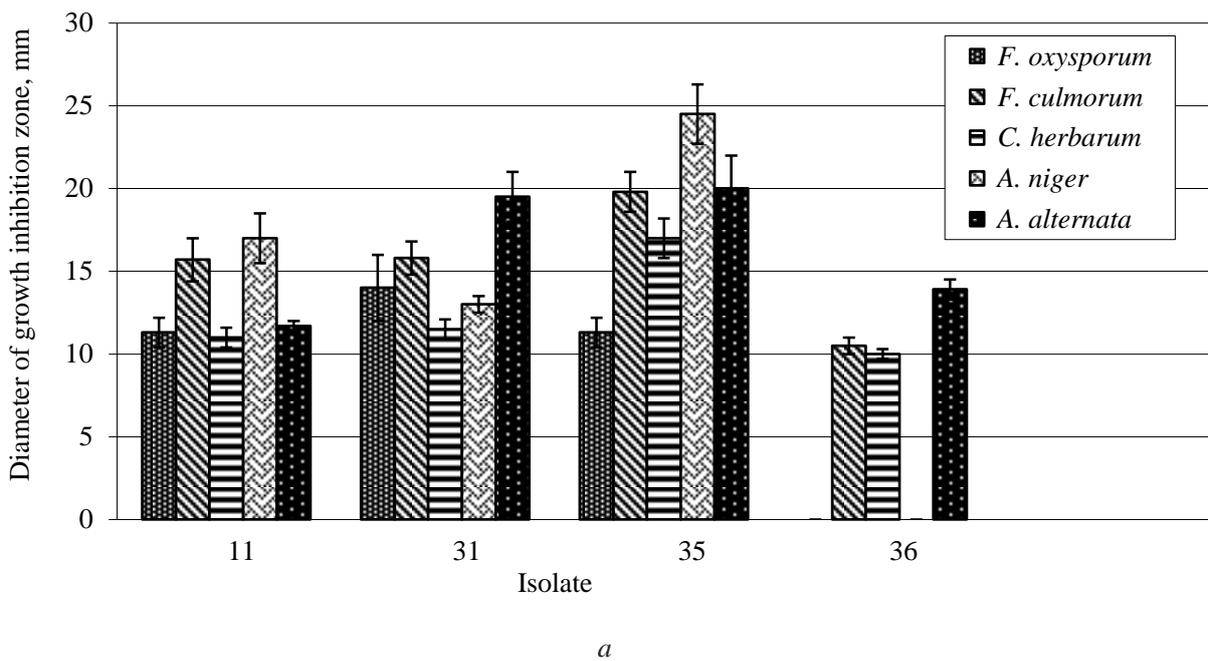
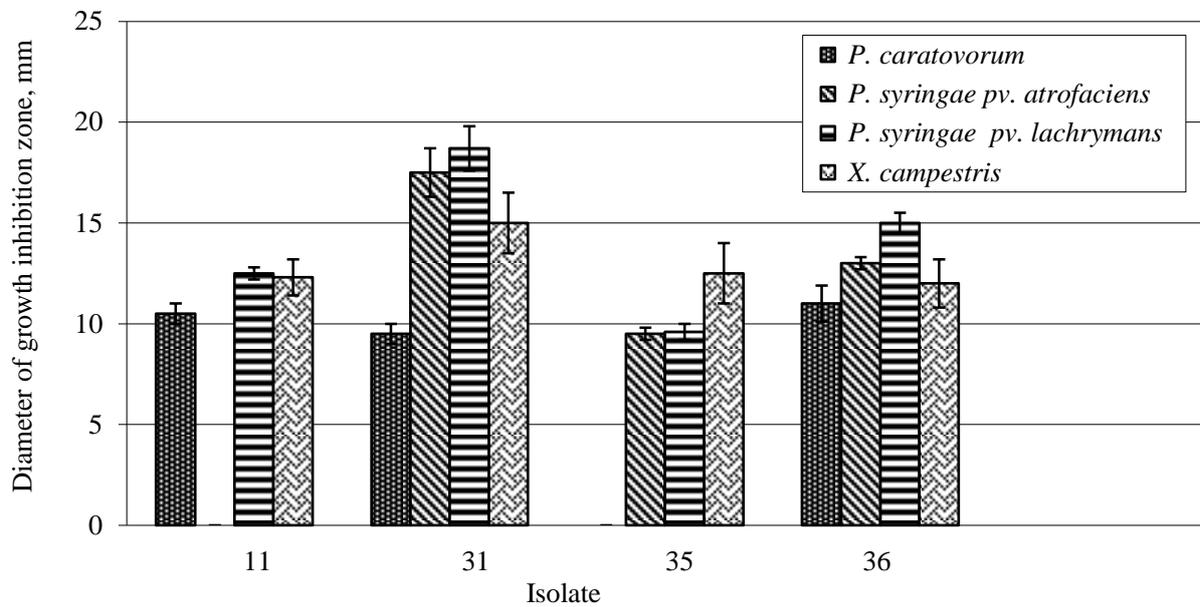


Figure 1. The spectrum of antagonistic action of the most active isolates of micromycetes against phytopathogenic fungi (a) and bacteria (b).



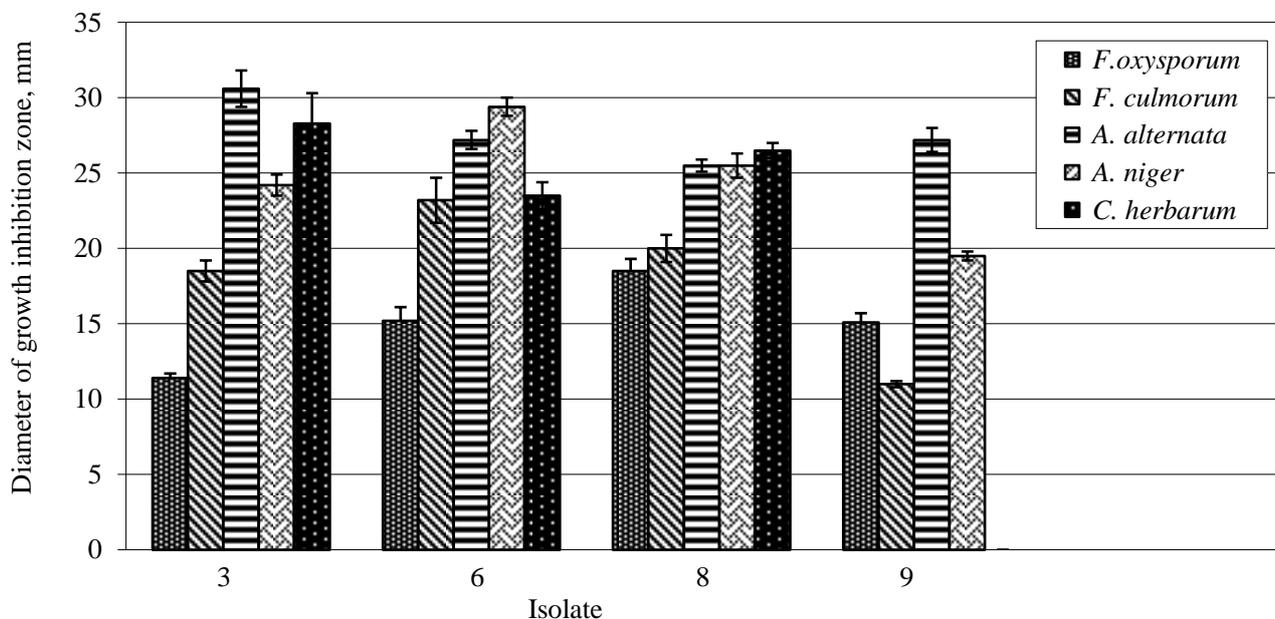


b

Figure 2. The spectrum of antagonistic action of the most active isolates of actinobacteria against phytopathogenic fungi (a) and bacteria (b).

The most active against phytopathogenic fungi was actinobacteria isolate No. 35 (the width of the growth inhibition zone from 11.3 to 24.5 mm). The greatest antagonist of phytopathogenic bacteria was isolate No. 31 (diameter of the growth inhibition zone of 9.5–18.7 mm). Isolates No. 11 and No. 36 showed a wide spectrum of antimicrobial activity, but low activity level.

Based on the identification of actinobacteria by morphological, cultural, physiological and biochemical characteristics, actinobacterial isolates nos. 11, 31, 35, and 36 were assigned to the genus *Streptomyces*. Among the streptomycetes that we isolated, only the *Streptomyces* sp. 35 possessed a rather high activity against *F. culmorum* 50716 (the width of the zone of inhibition of growth of about 20 mm), and less activity with respect to the strain *F. oxysporum* 54201. The total number of isolates of soil bacteria was 23 samples, among which 15 isolates showed antagonistic activity against different phytopathogens. Of these, only four isolates were characterized by a wide spectrum of action. The greatest antagonists were the isolates No. 3 and No. 6, which inhibited the growth of all tested cultures of phytopathogens (Figure 3).



a

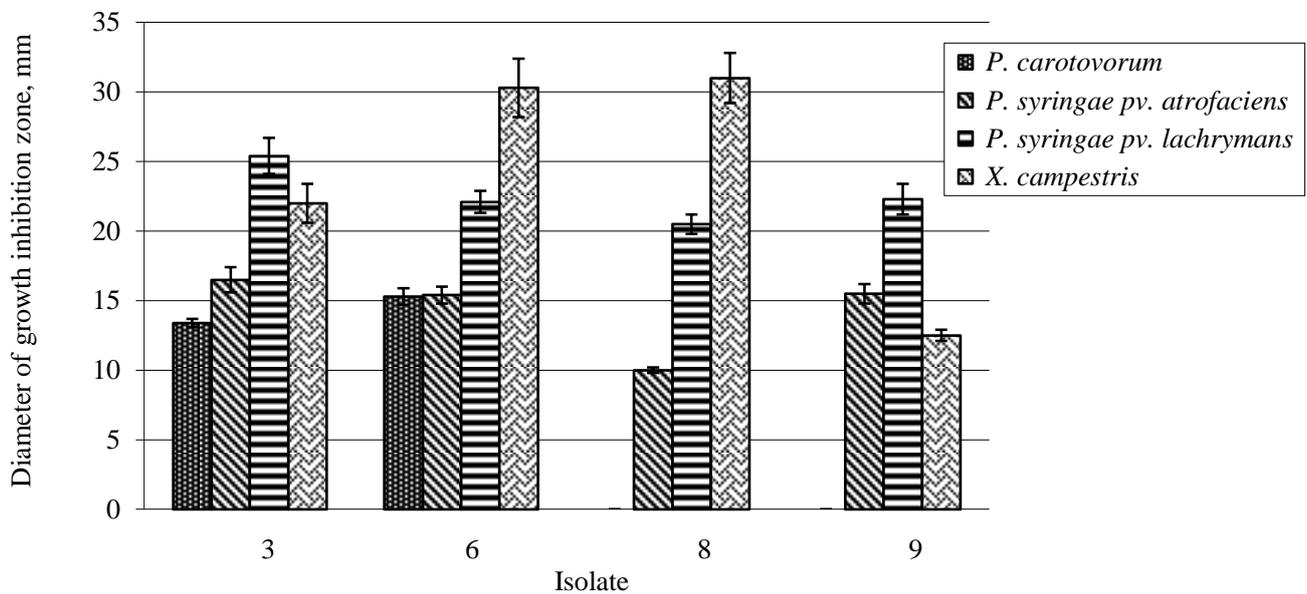


Figure 3. The spectrum of antagonistic action of the most active isolates of bacteria against phytopathogenic fungi (a) and bacteria (b).

A comparative analysis of the morphological, cultural, physiological and biochemical properties of the obtained isolates made it possible to identify them by a set of characters and assign isolate No. 3 to the species *Bacillus megaterium*, isolates No. 6 and No. 9 to the species *Bacillus brevis*, and isolate No. 8 to the species *Bacillus firmus*.

The selection of microorganisms that may be promising for the control of phytopathogens requires the verification of selected strains for phytotoxicity. In our work, the treatment of spring barley seeds with culture fluids of active antagonistic microorganisms showed the absence of their phytotoxic effect (Table 1).

Thus, the treatment of barley seeds with *T. longibrachiatum* 17 culture fluid significantly increased the number of germinated seeds ($P \leq 0.05$), while the effect of *Streptomyces* sp. 35 led to increased growth of shoots of seedlings ($P \leq 0.05$). Differences of all other indicators from the control were unreliable.

Discussion

Studied isolates from the Calcic Chernozem sampled in the conditionally clean zone contained various microorganisms, including micromycetes, actinobacteria, and bacteria. However, only a small number of isolates showed high antagonistic activity against a wide range of phytopathogens. Among them, representatives of the genera *Trichoderma* and *Fusarium* from micromycetes, representatives of the genus *Streptomyces* from actinomycetes, and microorganisms of the genus *Bacillus* from bacteria.

An analysis of the antagonistic spectra of micromycete isolates showed that high activity against different groups of phytopathogens was characteristic mainly of representatives of the genus *Trichoderma*. Our results indicate the ability of the studied strains of the genus *Trichoderma* to produce metabolites with antifungal and anti-microbial effects. Most of all, this concerns the strain *T. longibrachiatum* 17, which showed a wide spectrum of activity in relation to all tested cultures of phytopathogens.

Table 1. The effect of the active antagonists' culture fluid on seedlings of spring barley cultivar "Kristallia" ($M \pm SE$, $n=100$)

Processing option	Germinated seeds share, %	Shoots length, mm	Shoots length, % towards control	Roots length, mm	Roots length, % towards control
Micromycetes					
<i>T. lignorum</i> 14	85.0 ± 4.2	70.7 ± 4.9	106.8	487.8 ± 16.1	111.6
<i>T. longibrachiatum</i> 17	90.0 ± 3.2*	75.2 ± 3.3	113.5	478.6 ± 43.8	109.5
Control	78.0 ± 6.0	66.2 ± 4.0	100.0	437.2 ± 30.3	100.0
Streptomyces					
<i>Streptomyces</i> sp. 31	85.0 ± 3.7	41.4 ± 3.3	93.5	367.1 ± 14.0	94.4
<i>Streptomyces</i> sp. 35	82.0 ± 4.1	50.9 ± 4.0*	135.0	431.4 ± 15.8	111.0
Control	80.0 ± 3.5	42.9 ± 1.6	100.0	388.6 ± 26.9	100.0
Bacteria					
<i>B. megaterium</i> 3	91.0 ± 1.9	38.8 ± 2.0	90.1	361.0 ± 6.3	98.6
<i>B. brevis</i> 6	84.0 ± 6.6	38.3 ± 2.8	88.9	346.7 ± 18.4	93.2
Control	95.0 ± 2.2	43.1 ± 2.9	100.0	364.7 ± 26.4	100.0

* – Significant differences from control ($P \leq 0.05$). Seedlings morphometric indicators under the influence of micromycetes were determined on the sixth day; under the influence of bacteria and streptomyces – on the fourth day of the experiment.

It is known that the antagonistic activity of the genus *Trichoderma* fungi is due to their ability to produce a variety of metabolites with antimicrobial properties. Among these metabolites are harzianic acids, 6-pentyl- α -pyrone, alamethicin, tricholine, gliotoxin, gliovirin, viridin, heptelidic acid (Alimova, 2006; Asad et al., 2014), and peptaiboles (Van Bohemen et al., 2016). In addition to

antibiosis, some strains of the genus *Trichoderma* are able to show mycoparasitism in relation to phytopathogenic fungi. Parasitism occurs through physical contact and the synthesis of various hydrolytic enzymes such as proteinases, β -1,3-glucanases and chitinases (Alimova, 2006). Although most of the data known today relates to the antifungal action of representatives of the genus *Trichoderma*, there are also reports of their antibacterial activity (Kaushal et al., 2013).

As for representatives of the genus *Fusarium*, the most attention should be paid to the strain *F. sambucinum* 21, which showed antifungal activity against *F. oxysporum* 54201, *F. culmorum* 50716 and *A. niger* 25, but did not inhibit the growth of the studied phytopathogenic bacteria. Recently, Zhang et al. (2019) reported that the culture of *F. sambucinum* TE-6L contains alkaloids that were active against a wide range of fungi, and inhibited the growth of pathogenic human bacteria and one phytopathogenic bacterium.

In our work, most of the studied isolates of streptomycetes did not show high antagonistic activity against a wide range of phytopathogens. Isolate No. 35 had the most significant antifungal effect, while isolate No. 31 showed the highest antibacterial activity. In the recent years, streptomycetes have attracted attention not only as producers of medical antibiotics, but also as producers of substances that can inhibit the growth of phytopathogenic microorganisms. For example, a strain of *Streptomyces netropsis* IMBAC-5025, which had the activity of a relatively wide spectrum of phytopathogenic bacteria and fungi, was isolated. This strain most significantly inhibited the growth of *Alternaria alternata* and some phytopathogenic bacteria that belong to the genera *Pseudomonas* and *Xanthomonas* (Belyavskaya et al., 2016). Our results also indicate a rather high degree of antagonism of the strain *Streptomyces* sp. 31 regarding the listed pathogens.

Literature data on the antifungal activity of streptomycetes indicate that a small number of highly active isolates against toxigenic fungi of the genus *Fusarium* are known today. For example, of the 133 isolates tested by Colombo et al. (2019), only six samples inhibited the growth of fungi of this genus, while a high degree of inhibition was noted only against *F. culmorum*. The results of our studies are consistent with these observations.

Among the studied 15 isolates of soil bacteria that showed antagonistic activity against phytopathogens, only four samples were characterized by a wide spectrum of action, and only two isolates were able to inhibit the growth of all phytopathogens. According to the results of our work, all active bacteria-antagonists belonged to the genus *Bacillus*. Literature data indicates that bacteria of the genus *Bacillus* are capable of producing various antimicrobial agents. Among these secondary metabolites, cyclic lipopeptides are often mentioned. Mycosubtilin, fengycins *A* and *I*, and iturin exhibit antifungal effects, while surfactin has a wide spectrum of antimicrobial activity (Khong et al., 2013; Meena & Kanwar, 2015).

Conclusion

The composition of microorganisms isolated from black soil selected in an ecologically clean zone included 27 isolates of micromycetes, 35 isolates of actinobacteria and 23 isolates of bacteria. Screening of the inhibitory activity of isolates against phytopathogenic fungi and bacteria revealed samples with a wide spectrum of antagonistic action.

The most active isolates of micromycetes are classified as *Trichoderma lignorum* 14, which showed antagonism against four of five fungi species and three of four species of bacteria, and *Trichoderma longibrachiatum* 17, which was active against all tested phytopathogens. Among actinobacteria, the most active isolates are assigned to the genus *Streptomyces*, including *Streptomyces* sp. 31, active against all phytopathogens, and *Streptomyces* sp. 35, which depressed the growth of all fungi and three of the four phytopathogenic bacteria. The most active bacterial isolates were identified as *Bacillus megaterium* 3 and *Bacillus brevis* 6, which were antagonists of all pathogenic test cultures. All active antagonist cultures showed no phytotoxic effect on spring barley seedlings. At the same time, the strain *T. longibrachiatum* 17 significantly increased the proportion of sprouted seeds of barley, and the strain *Streptomyces* sp. 35 increased the growth of shoots of seedlings. Active cultures of antagonistic microorganisms isolated from Calcic Chernozem can be used as the components of drugs to protect cultivated plants from the bacterial and fungal pathogens.

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