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IMPACT OF STRAINS OF ENTOMOPATHOGENIC FUNGI ON SOME MAIN GROUPS OF SOIL MICROORGANISMS

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Abstract: The influence of entomopathogenic fungal strains – one *Metarhizium anisopliae* (Metsch.) Sorok. and seven *Beauveria bassiana* (Bals.-Criv.) Vuill. on some main groups of soil microorganisms was studied after introduction of their conidia into the soil. The soil samples were analyzed for densities of bacteria, actinomycetes and fungi by decimal dilutions of soil suspensions grown on selective media. The presence of conidia of fungal strains in the soil after a month from introduction was proved by bait method. Three of the strains of *B. bassiana* were established to be of the highest persistence, this being expressed by mortality of *Galleria mellonella* L. (Lepidoptera, Pyralidae) larvae from 70% to 90%, followed by strains 224Re of *B. bassiana* and 31 of *M. anisopliae*.

The obtained results showed that examined strains of the entomopathogenic fungi manifested different in manner and varying in degrees of impact on density of the main groups of soil microorganisms. Relatively insignificant changes were established under the influence of the strains 224Re *B. bassiana* and 31 *M. anisopliae*. The other strains of *B. bassiana* caused alterations in microbial balance expressed in different manner – stimulation or suppression on density of free-living nitrogen-fixing microorganisms, mineral nitrogen utilizing bacteria, spore-forming bacteria, cellulose degrading microorganisms, actinomycetes, soil fungi. So each strain could be characterized by specific impact on examined groups of soil microorganisms.

The strain 412 of *B. bassiana* showed the most strongly manifested stimulation effect on the heterotrophic microorganisms, on the mineral nitrogen utilizing bacteria, on the free-living nitrogen-fixing microorganisms and the soil fungi – 14, 15, 7 and 30 times higher density of the microorganisms compared to the control treatments. The same strain caused high degree of suppression on densities of cellulose degrading microorganisms – 0.0064×10^6 compared to 0.0305×10^6 CFU/g in the control treatment. Densities of the soil spore-forming bacteria were not affected by the examined strains of *B. bassiana* and *M. anisopliae*. In the conducted experiments it has been established that the manifested impact on actinomycetes density by strains of the entomopathogenic fungi *B. bassiana* and *M. anisopliae* was different in manner and varying in degrees, on the contrary to the stimulation influence on density of the soil fungi.

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Key words: *Beauveria bassiana, Metarhizium anisopliae*, soil microorganisms, bacteria, actinomycetes, fungi, density

INTRODUCTION

The species *Beauveria bassiana* (Bals.-Criv.) Vuill., *Beauveria brongniartii* (Sacc.) Petch, *Metarhizium anisopliae* (Metsch.) Sorokin (anamorphic, Clavicipitaceae), *Paecilomyces fumosoroseus* (Wise) Brown & Smith and *Paecilomyces farinosus* (Holm.) Brown & Smith (anamorphic, *Trichocomaceae*) have been reported as the most applicable entomopathogenic fungi in biological control of the insect pests with soil dwelling developmental stages. The soil is a reservoir of these entomopathogens and they were often isolated from it (Vänninen 1996; Miętkiewski and Tkaczuk 1998; Vänninen *et al*. 2000; Bidochka *et al*. 2001, 2002; Keller *et al*. 2003; Sosnowska *et al*. 2004; Meyling and Eilenberg 2006; Meyling 2007). Introduction of their conidia into the soil could change the interactions among the microorganisms within soil communities (Shimazu *et al.*, 2002; Jung *et al.*, 2005). In the view of importance of microorganisms' role in soil biota for soil fertility, productivity and crop protection it is necessary to broaden investigations on safety of entomopathogenic fungal conidia in relation to conservation of soil biodiversity.

The aim of this preliminary study was to establish the influence of strains of *B. bassiana* and *M. anisopliae* on some main groups of soil microorganisms (bacteria, actinomycetes and fungi) after introduction their conidia into the soil.

MATERIALS AND METHODS

Seven *B. bassiana* strains and one strain of *M. anisopliae* used in the study were obtained from the Collection of Entomopathogenic Fungi maintained in the Department of Biological and Integrated Pest Control (Plant Protection Institute, Bulgaria). They were initially isolated from the following insect species: 3 representatives of Lepidoptera, Tortricidae – *Archips rosana* L. (strain 383 *B. bassiana*), *Cydia pomonella* L. (strain 412 *B. bassiana*), *Hedya nubiferana* Haw. (strains 416, 417 and 418 *B. bassiana*); 3 representatives of Coleoptera – from Chrysomelidae, *Leptinotarsa decemlineata* Say (strain 224 Re *B. bassiana*), from Scolytidae, *Ips sexdentatus* Boerner (strain 426 *B. bassiana*) and from Scarabaeidae, *Melolontha melolontha* L. (strain 31 *M. anisopliae*).

The strains were grown on SDAY (Sabouraud dextrose agar with yeast extract) in test tubes at 25 ± 1°C for 25 days. Obtained conidia were used for inoculation of Sabouraud dextrose broth with yeast extract – 10 ml of conidial suspension containing 1 x 10^6 conidia/ml per 50 ml broth in 500 ml flasks. After submerged cultivation on a reciprocal shaker (150 rpm) for 3 days the cultures were kept in horizontal position in flasks for 25 days – static growing at the same temperature conditions (25 ± 1°C) for conidia production. The obtained conidia after removing from flasks and air-drying were mixed with soil (1 : 500 w/w). Plastic pots (Ø10 cm, volume of 500 ml) with perforated walls were filled with the mixture and dug in the soil to a depth of 15 cm in the experimental field of the Department of Biological and Integrated Pest Control. The experiments were conducted in 3 replicates per strain. Control variant was not supplemented with fungal conidia. Soil type was chernozem smolnitsha after potato cultivation. The samples (20 g per repetition) were collected from the pots a month after digging the pots in the soil (January 2007).

The soil samples were analyzed for the occurrence of entomopathogenic fungi. Bait method was applied (Zimmermann 1986; Meyling 2007) using the fifth instar of larvae of *Galleria mellonella* L. (Lepidoptera: Pyralidae) (ten larvae per replicate) as bait insects. Dead larvae were placed into humid chamber to allow the reproductive structures of fungal pathogens to develop. Grown entomopathogenic fungi were identified according to their morphological characters observed on prepared durable specimens with lactophenol and aniline blue (Humber 1997).

The soil samples were analyzed for densities of the main groups of soil microorganisms (bacteria, actinomycetes and fungi). Analyses were confirmed by decimal dilution method using soil suspensions grown on selective media (Gushterov *et al*. 1970). The results were presented as mean of Colony Forming Units (CFU) per gram of soil.

Statistic method ANOVA was applied to determine the significance of differences in densities of the main groups of soil microorganisms after introduction of conidia of *B. bassiana* and *M. anisopliae* strains into the soil.

RESULTS AND DISCUSSION

It was proved by bait method that conidia of all studied strains of *B. bassiana* and *M. anisopliae* were present in the soil one month after their introduction. As it could be seen in Fig. 1 evidence of natural occurrence of entomopathogenic fungi in the samples was not stated, in control variant mortality by mycosis of bait insects was 0%. The strains 412, 383 and 416 of *B. bassiana* were found to exhibit the highest persistence in the soil followed by strains 224Re of *B. bassiana* and 31 of *M. anisopliae*. Mortality of *G. mellonella* larvae caused by mycosis in these experimental variants was from 60% to 90%. As the experiments were conducted during the winter the temperature of the soil could not be the reason of the decrease of fungal persistence in the variants with conidia of the strains 426 and 417 of *B. bassiana*.

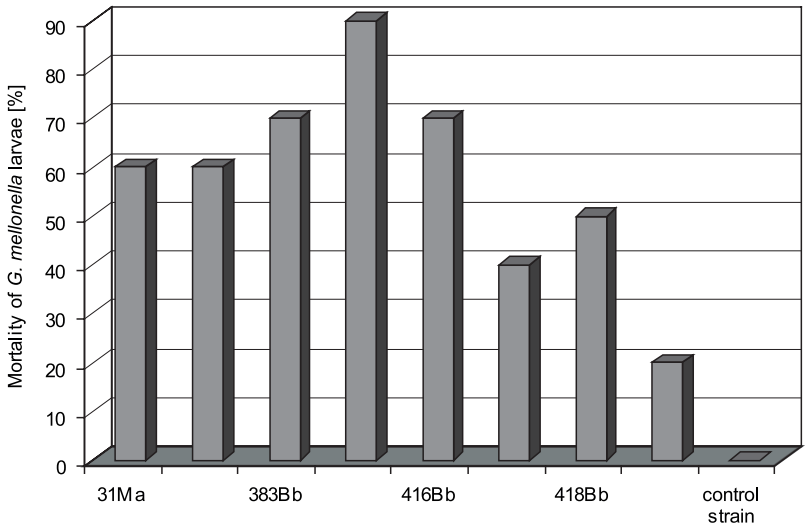

Fig. 1. Mortality of *G. mellonella* larvae in the soil samples a month after introduction of conidia of entomopathogenic fungi *B. bassiana* and *M. anisopliae*

McCoy *et al*. (2000) considering reports about factors affecting the survival of entomopathogenic fungi summarized that in the soil environment, soil temperatures higher than 30°C, soil moistures near saturation, and the presence of antagonists fungal persistence is reduced. Naturally occurring microbes, such as bacteria and actinomycetes and their secondary metabolites appear to function as antagonists under certain soil conditions, influencing both infectivity and survival of entomopathogenic fungi. This phenomenon has been demonstrated most clearly in numerous published comparative studies with natural and pasteurized soil or soil extracts. Studies with *B. bassiana* suggest that inhibition in natural soils is caused by fungistasis rather than a result of microbial competition. Shields *et al*. (1981) identified patulin, a water-soluble metabolite of *Penicillium uriticae*, which was inhibitory to conidial germination and growth of *B. bassiana*.

According to Popowska-Nowak *et al*. (2003) volatile metabolites produced by actinomycetes *Streptomyces flavescens* totally inhibited growth of all studied strains of *B. bassiana, Paecilomyces fumosoroseus* and *P. farinosus* while those produced by bacteria *Bacillus subtilis, Bacillus pumilus* and *Pseudomonas aurantiaca* had only a fungistatic effect.

Most soil fungi, bacteria and actinomycetes are heterotrophs, they rely on organic materials for carbon and energy needs. As it could be seen on Fig. 2 the strains of entomopathogenic fungi used in the investigation had a stimulating effect on the heterotrophic microorganisms. It was the most strongly manifested in the treatment with conidia of the strain 412 of *B. bassiana* where density of the heterotrophic microorganisms was 14-times higher than in the control variant – (102.80 ± 1.20) x 10^6 and (6.67 ± 1.10) x 10^6 CFU/g, respectively. Significant differences were found at p level < 0.05 between the mean values of the heterotrophs' density in different experimental variants with supplement of strains conidia compared to the control treatment and among

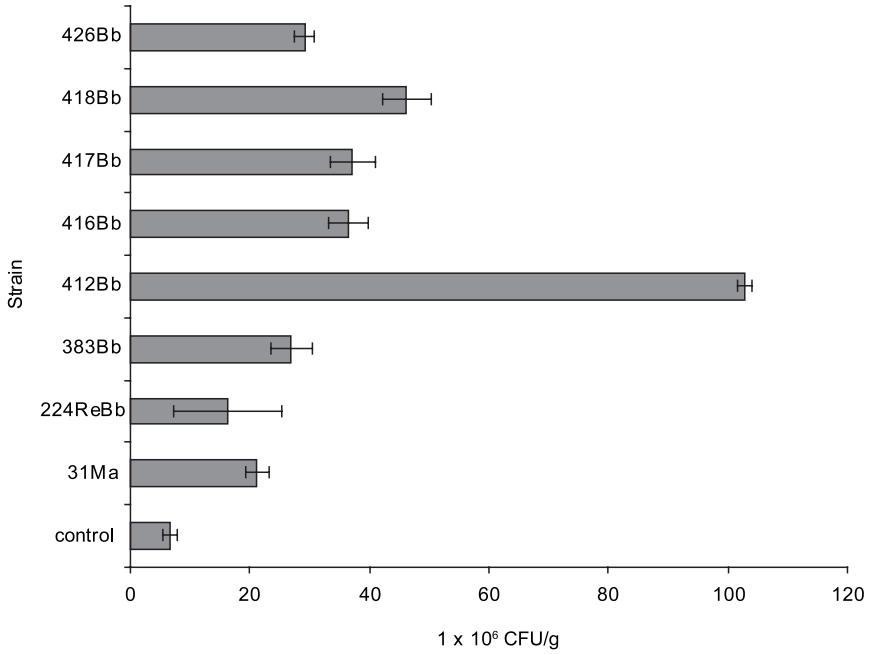

Fig. 2. Densities of heterotrophic microorganisms in soil samples a month after supplement of entomopathogenic fungi *B. bassiana* and *M. anisopliae*

different treatments with the exception of treatments in the pairs 224Re *B. bassiana* – 31 *M. anisopliae*, 416 *B. bassiana* – 417 *B. bassiana* and 426 *B. bassiana* – 383 *B. bassiana*. The studied strains of entomopathogenic fungi could be arranged according to decreasing stimulation effect as follows: 412 *B. bassiana* > 418 *B. bassiana* > 417 *B. bassiana* > 416 *B. bassiana* > 426 *B. bassiana* > 383 *B. bassiana* > 31 *M. anisopliae* > 224 Re *B. bassiana*.

Stimulation effect of all studied strains of entomopathogenic fungi on free-living nitrogen-fixing microorganisms and mineral nitrogen utilizing bacteria was proved at p level < 0.05, as well (Fig. 3 and Fig. 4). Supplement of the strain 412 of *B. bassiana*

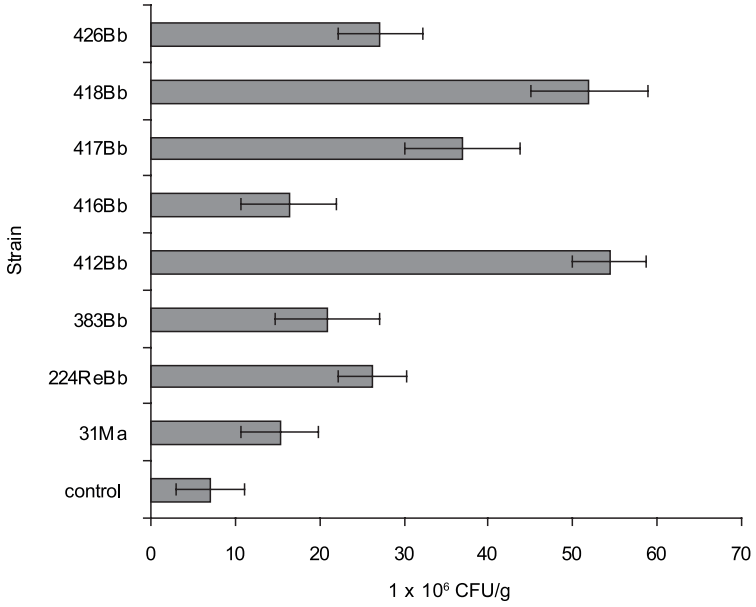

Fig. 3. Densities of free-living nitrogen-fixing microorganisms in soil samples a month after supplement of entomopathogenic fungi *B. bassiana* and *M. anisopliae*

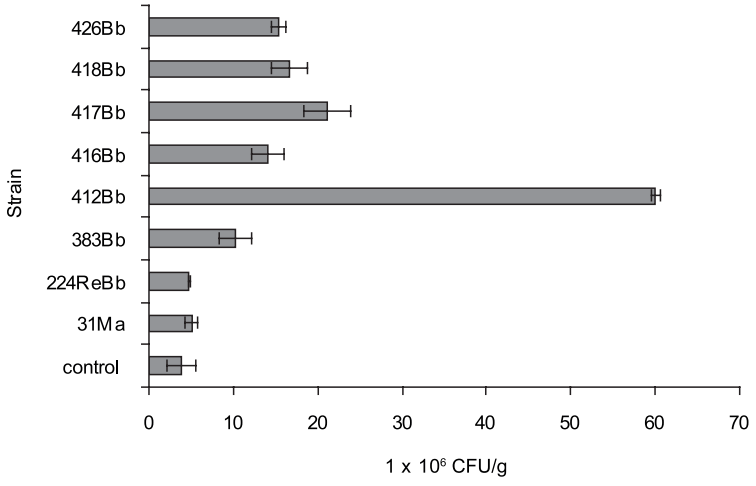

Fig. 4. Densities of mineral nitrogen utilizing bacteria in soil samples a month after supplement of entomopathogenic fungi *B. bassiana* and *M. anisopliae*

into the soil caused near 15 times increase of density of mineral nitrogen utilizing bacteria – (60.04 ± 0.48) x 10^6 CFU/g and over 7-fold increase of free-living nitrogen-fixing microorganisms – (54.33 ± 4.35) x 10^6 CFU/g compared to control treatments – (3.91 ± 1.72) x 10^6 and (7.06 ± 4.10) x 10^6 CFU/g, respectively. A linear arrangement of the strains according to decrease of the effect on both groups of microorganisms was the same as for the heterotrophic ones. In the view of active role of the free-living nitrogen-fixing microorganisms and mineral nitrogen utilizing bacteria in soil fertility the stimulation impact of the examined strains of the entomopathogenic fungi could improve the fertility status of the soil and contribute to plant growth.

In the conducted experiments it was established that the manifested impact on actinomycetes density by strains of the entomopathogenic fungi *B. bassiana* and *M. anisopliae* was different in manner and varying in degrees (Fig. 5). Stimulation effect was proved for strains 383 and 417 of *B. bassiana* – (2.28 ± 0.08) x 10^6 and (1.43 ± 0.38) x 10^6 CFU/g, respectively, compared to the control variant – (0.43 ± 0.19) x 10^6 CFU/g and suppression in low degree by strains 224Re and 412 *B. bassiana*. No effect was registered in variants with supplement of the strains 426 and 416 *B. bassiana*.

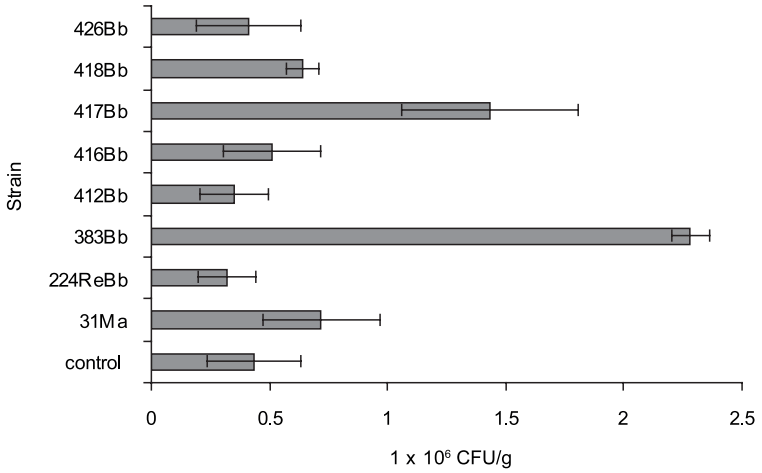

Fig. 5. Densities of actinomycetes in soil samples a month after supplement of entomopathogenic fungi *B. bassiana* and *M. anisopliae*

According to Shimazu *et al*. (2002) who studied density dynamics of the entomopathogenic fungus *B. bassiana* introduced into the soil and its influence on other soil microorganisms, the densities of bacteria were between 1 x 10^6 and 10^8 CFU/g, actinomycetes were between 10^6 and 10^7 CFU/g in both treated with *B. bassiana* and non-treated plots in the period up to one year. No statistical differences of bacteria and actinomycetes densities between treatment and non-treated control were found.

Some other results were presented by Augustyniuk-Kram *et al.* (2007). They found that between the 7th and the 14th day after introduction into the soil the population of *Streptomyces griseoviridis* in the association with *B. bassiana* increased 2–3 times compared to initial density.

Obtained results about different in manner and varying in degrees impact of strains of the entomopathogenic fungi on actinomycetes lead us to speculations about

specific characters of each strain to release or not to release substances with toxic or stimulation effect on actinomycetes.

Similar results were obtained concerning densities of spore-forming bacteria (Bacilli) in the soil samples a month after supplement of strains of *B. bassiana* and *M. anisopliae* into the soil (Fig. 6). As significant differences have not been found between the mean values of bacilli densities in control variant and inoculated variants, impact of the entomopathogenic fungi could not be proved.

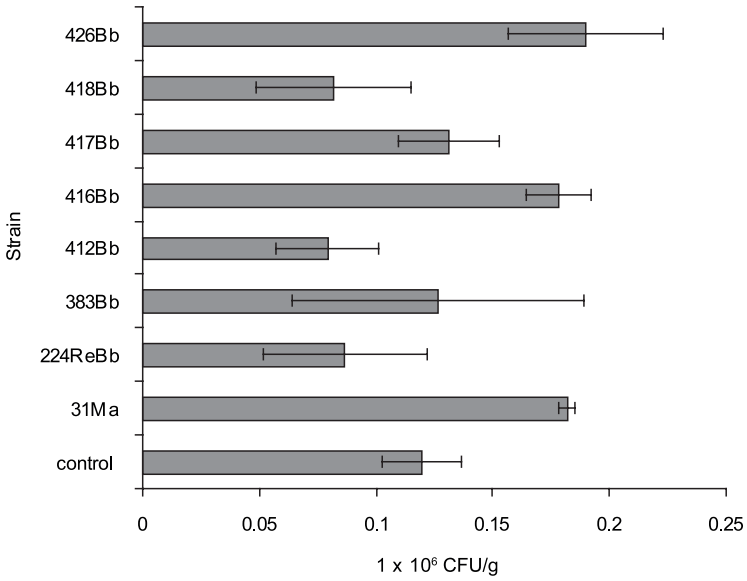

Fig. 6. Densities of spore-forming bacteria (Bacilli) in soil samples a month after supplement of entomopathogenic fungi *B. bassiana* and *M. anisopliae*

As it could be seen in Fig. 7 the strains of *B. bassiana* and *M. anisopliae* manifested suppression effect on density of cellulose-degrading microorganisms after a month from introduction of the entomopathogenic fungi into the soil. With the exception of the strains 417 and 426 of *B. bassiana* significant differences between variants with conidia supplement and control treatment were proved at p level < 0.05. The highest degree of suppression was stated in the experimental variant with the strain 412 of *B. bassiana* – $(0.0064 \pm 0.0033) \times 10^6$ compared to densities of cellulose degrading microorganisms in the control variant – $(0.0305 \pm 0.0024) \times 10^6$ CFU/g.

The examined strains of *B. bassiana* and *M. anisopliae* demonstrated stimulation impact on density of the soil fungi (Fig. 8). Significant differences were found at p level < 0.05 between variants inoculated with conidia of the strains 412 and 426 and control treatment compared to the other strains. Density of the soil fungi was established to be $(0.1216 \pm 0.0234) \times 10^6$ and $(0.2380 \pm 0.0703) \times 10^6$ CFU/g in the variants with strains 412 and 426 of *B. bassiana* compared to $(0.0040 \pm 0.0034) \times 10^6$ CFU/g in the control treatment.

Obtained by us results were in agreement with experiments conducted by other researchers as well. According to Shimazu *et al*. (2002) densities of general fungi in the non- treatment plot were approximately 1×10^5 CFU/g throughout the investigation

period. They increased markedly to 3–5 x 10^7 CFU/g in the treatment plot after mixing with *B. bassiana* and then started to decrease to 1/10 of density after 12 months. Densities of general fungi in the treatment plot were always greater than in the non-treatment plot.

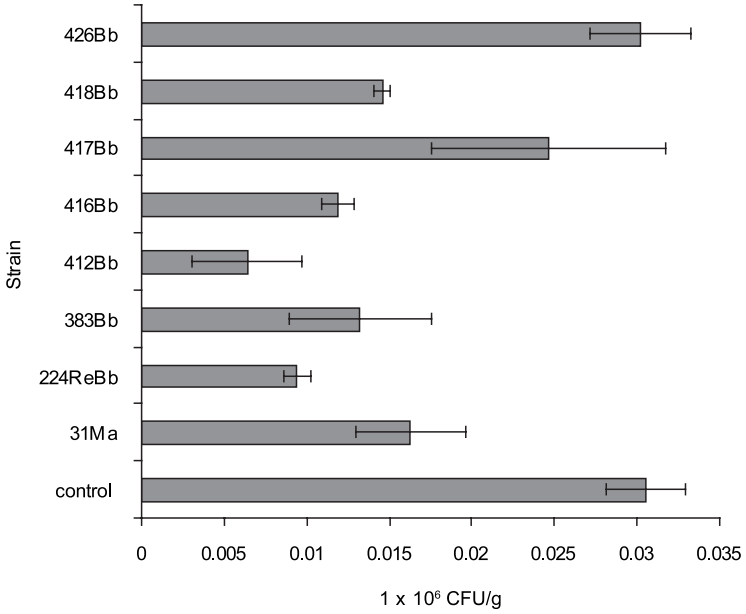

Fig. 7. Densities of cellulose degrading microorganisms in soil samples a month after supplement of entomopathogenic fungi *B. bassiana* and *M. anisopliae*

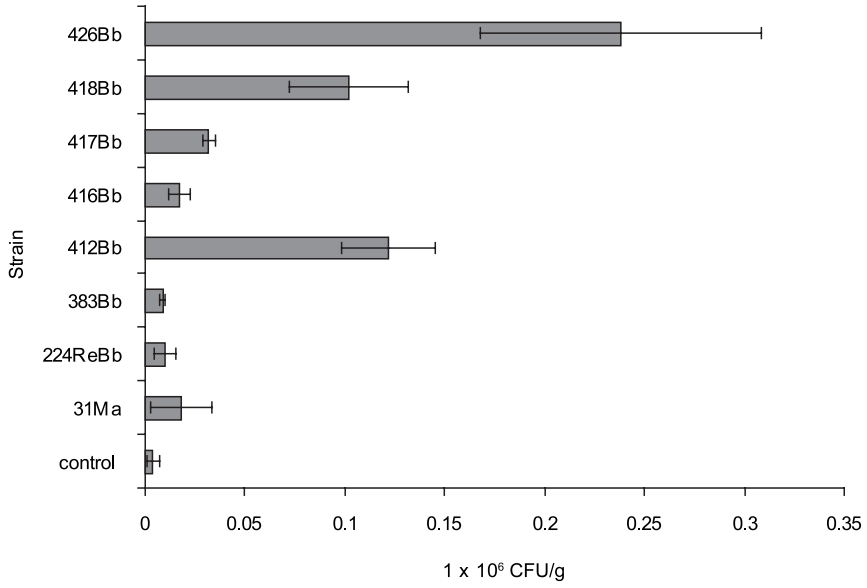

Fig. 8. Densities of fungi in soil samples a month after supplement of entomopathogenic fungi *B. bassiana* and *M. anisopliae*

CONCLUSIONS

It was experimentally proved that conidia of all 7 strains of *B. bassiana* and *M. anisopliae* were present in the soil a month after introduction. Evidence about natural occurrence of entomopathogenic fungi in the soil samples was not obtained. The strains 412, 383 and 416 of *B. bassiana* were found to exhibit the highest persistence in the soil followed by strains 224Re of *B. bassiana* and 31 of *M. anisopliae*.

Obtained results showed that the examined strains of entomopathogenic fungi *B. bassiana* and *M. anisopliae* manifested different in manner and varying degrees of impact on density of main groups of soil microorganisms (bacteria, actinomycetes and fungi). Relatively insignificant changes were found under the influence of the strains 224Re of *B. bassiana* and 31 of *M. anisopliae*. The other strains of *B. bassiana* caused alterations of microbial balance expressed in different manner – stimulation or suppression of density of free-living nitrogen-fixing microorganisms, mineral nitrogen utilizing bacteria, spore-forming bacteria, cellulose degrading microorganisms, actinomycetes, soil fungi. So each strain could be characterized by specific impact on examined groups of soil microorganisms.

The strain 412 of *B. bassiana* the most strongly manifested stimulation effect on the heterotrophic microorganisms, on the mineral nitrogen utilizing bacteria, on the free-living nitrogen-fixing microorganisms and the soil fungi – $(102.80 \pm 1.20) \times 10^6$, $(60.04 \pm 0.48) \times 10^6$, $(54.33 \pm 4.35) \times 10^6$ and $(0.1216 \pm 0.0234) \times 10^6$ CFU/g, respectively, that was 14, 15, 7 and 30 times higher than densities of the microorganisms compared to the control variants. The same strain caused high degree of suppression on densities of cellulose degrading microorganisms – $(0.0064 \pm 0.0033) \times 10^6$ compared to – $(0.0305 \pm 0.0024) \times 10^6$ CFU/g in the control variant.

Densities of the soil spore-forming bacteria have not been affected by the examined strains of *B. bassiana* and *M. anisopliae*.

In the conducted experiments it has been established that the manifested impact on actinomycetes density by strains of the entomopathogenic fungi *B. bassiana* and *M. anisopliae* was different in manner and varying in degrees, on the contrary to the stimulation influence on the density of the soil fungi.

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POLISH SUMMARY

WPŁYW ENTOMOPATOGENICZNYCH SZCZEPÓW GRZYBÓW NA NIEKTÓRE GŁÓWNE GRUPY DROBNOUSTROJÓW GLEBOWYCH

Badano wpływ grzybów entomopatogenicznych – jednego szczepu *Metarhizum anisopliae* (Metsch.) Sorok. oraz sześciu szczepów *Bauveria bassiana* (Bals.-Criv.) na niektóre główne grupy drobnoustrojów glebowych, po wprowadzeniu konidiów tych grzybów do ziemi. Analiza prób tej ziemi obejmowała gęstość występowania zasiedlających próby bakterii, promieniowców i grzybów wyhodowanych na selektywnych podłożach, przy wykorzystaniu metody dziesięciokrotnych rozcieńczeń. Obecność konidiów szczepów grzybów entomopatogenicznych stwierdzano stosu-

jąc metodę przynęty, miesiąc po ich wprowadzeniu do ziemi. Ustalono na podstawie śmiertelności larw *Galeria mellonella* L. (*Lepidoptera*, *Pyralidae*), wynoszącej od 70% do 90%, że trzy szczepy *B. bassiana*, w tym szczep 224 Re, a następnie szczep 31 *M. anisopliae*, wykazywały najwyższą zdolność przeżywania w ziemi.

Uzyskane wyniki wskazywały, że badane szczepy grzybów entomopatogenicznych miały różny sposób i nasilenie działania w odniesieniu do gęstości występowania głównych grup drobnoustrojów glebowych. Względnie nieistotne zmiany stwierdzono pod wpływem szczepów 224 Re *B. bassiana* oraz szczepu 31 *M. anisopliae*. Inne szczepy *B. bassiana* powodowały zmiany w równowadze biologicznej, co znajdowało odbicie w różnym sposobie działania – stymulacji lub ograniczeniu gęstości występowania wolno żyjących, wiążących azot drobnoustrojów, bakterii pobierających azot mineralny, zarodnikujących bakterii, bakterii rozkładających błonnik, promieniowców, grzybów glebowych. Tak więc każdy szczep mógł być scharakteryzowany na podstawie specyficznego wpływu na określone grupy drobnoustrojów glebowych.

Szczep 412 *B. bassiana* wykazywał najsilniejsze działanie stymulacyjne w stosunku do drobnoustrojów heterotroficznych, bakterii zużywających azot mineralny, wolno żyjących bakterii wiążących azot i grzybów glebowych, co wyrażało się 14, 15, 7 i 30 razy większą gęstością występowania w porównaniu do kombinacji kontrolnej. Ten sam szczep powodował wysoki stopień redukcji gęstości występowania drobnoustrojów rozkładających błonnik –0,0064 x 10^6 w porównaniu do 0,0305 x 10^6 w kombinacji kontrolnej.

Badane szczepy *B. bassiana* i *M. anisopliae* nie wpływały na gęstość występowania bakterii zarodnikujących. W przeprowadzonych doświadczeniach wykazano, że stwierdzony wpływ grzybów entomopatogenicznych *B. bassiana* i *M. anisopliae* na gęstość występowania promieniowców był zróżnicowany pod względem charakteru oraz nasilenia, w przeciwieństwie do stymulacyjnego wpływu na gęstość występowania grzybów.