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ANTAGONISTIC ACTION OF 26UF7 AND 35NG3 *STREPTOMYCES* SPECIES TO *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS* 10_2

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***Background**. Ecological pathways of decision the question of plants opportunism against bacterial cancer of tomatoes. **Aim**. Search for streptomycetes-antagonists against Clavibacter michiganensis subsp. michiganensis 10_2 and investigation of their influence on the infectious process in tomatoes in hothouse conditions. The first aim provided microbiological methods, the second required agricultural. **Results**. 36 (45%) soil streptomycetes from 80 had antagonistic activity against C. michiganensis 10_2. Selected Streptomyces sp. 26Uf7 and 35NG3 strains showed high and stable rates of activity against phytopathogen in vitro. The pre-sowing treatment with fermentation filtrates of researched streptomycetes doubled the seed germination rate of Cherry tomatoes infected with C. michiganensis 10_2. However, it has not contributed to the resistance of plants to the disease after mechanical infection of tomato leaves in the juvenile period. In general, metabolites of Streptomyces sp. 26Uf7 and 35NG3 strains increased the tomato yields, although they have not completely nullified the impact of infection in hothouse conditions. **Conclusions**. Metabolites of Streptomyces sp. 26Uf7 and Streptomyces sp. 35NG3 strains with high rates of in vitro antagonism to C. michiganensis 10_2 doubled the seed germination and the yield of Cherry tomatoes and had 75% efficiency against phytopathogen in hothouse conditions.*

Keywords: Clavibacter michiganensis subsp. michiganensis, bacterial cancer, antagonism of streptomycetes.

Clavibacter michiganensis subsp. *michiganensis* (Cmm) – aerobic gram-positive bacteria without the type III secretion system (T3SS), is developing in the intercellular space of tomatoes, is spreading through xylem vessels and blocks it. Course of disease is slow, 1.5–2 months. Pathogenicity factors – the *chp*/*tomA* region is localized in the chromosome (encodes proteases that destroy the plant cell wall/Tomatinase) and plasmids pCM1 (encodes β-1,4-endocellulase), pCM2 (encodes serine protease). The absence of any of the three factors is manifested by reduced colonization of phytopathogen without the disease symptoms on the plant, although the fruits remain infected [1, 2, 3].

Cmm is the causative factor of tomato bacterial cancer that decreases tomatoes yield by 20% in the field and up to 100% in the hothouse condition. The source of infection is mainly infected seeds, where bacteria are viable for up to 3–4 years, and mechanical damage of the plant during the growing season. Cmm does not overwinter in the soil, except in cases when parts of the plant are not decomposing in conditions of warm winter. Bacteria can grow at 16–28° C and die at a temperature above 48° C [2, 4, 5].

General methods of prophylaxis against phytopathogens are heating of seeds at 48–50° C for 20 min and treatment with 1% $KMnO_4$ [5, 6]. Today, the following are using against Cmm: fermentation of tomato pulp together with seeds, because acetic and lactic acids adversely affect bacteria; synthetic fungicides mancoceb and metalaxyl; recommended biological products haupsin, mycosan, phytosporin, phytolavin developed on basis of *Pseudomonas aureofaciens*, *Trichoderma, Bacillus subtilis, Streptomyces lavendulae* and *S. griseus*, respectively [2, 5, 7, 8]. Two last species are representatives of *Streptomyces* genus. As known, streptomycetes are an endless source of metabolites, so producers of known compounds and new ones are constantly searching among them [6, 9, 10].

The aim of the research was to analyze the antagonistic activity of soil isolates of streptomycetes against *C. michiganensis* 10_2, to select

productive and stable variants of antagonists against *C. michiganensis* 10_2 *in vitro* and to study the effect of their metabolites on the infectious process of tomatoes during cultivation in hothouse conditions.

Materials and methods. Objects of the study were metabolites of *Streptomyces* sp. 25Uf7 and 35NG3 strains. The subject of research was antagonistic activity of *Streptomyces* sp. 25Uf7 and 35NG3 strains metabolites to *C. michiganensis* 10_2. *C. michiganensis* 10_2 phytopathogenic bacterium obtained from the IMV NAS of Ukraine cultures collection was used as test culture, it was isolated from tomatoes in Kyiv region in 1958. *C. michiganensis* 10_2 strain is characterized by pathogenicity in hothouse conditions with a temperature optimum of 22–28° C [11]. *In vitro*: search for producers-antagonists to *C. michiganensis* 10_2 strain was performed among 80 soil streptomycetes isolates taken from the collection of the department. Experiments in the hothouse were carried out using self-pollinated Cherry tomatoes produced by "Seeds of Ukraine". Characteristics: 90–100 days early ripening variety, 25–45cm height, 10–15g fruit weight, 300–500g harvest from one bush. The soil in the hothouse was treated with quicklime one month before seeding of tomatoes. Additional fertilizers and stimulants were not added in the soil.

Streptomycetes, isolated from soil were grown on a corn-soy medium, (g/l): corn flour 10.0; soybean flour 5.0; NaCl 3.0; $CaCO_3$ 3.0; agar 15.0; pH 7.0. *C. michiganensis* 10_2 was grown in MPA medium: 100 ml of molten MPA was cooled to 40° C, 1 ml of resuspended phytopathogen suspension (10^8–10^9 CFUs) was added to agar and poured into Petri dishes. All cultures were grown in thermostat at 28° C.

The screening of 7-day streptomycetes isolates activity was carried out by imposing of the d= =10mm blocks on MPA agar. The result was analyzed after 2 days. Multilevel selection: suspensions of 7 days 26 and 35 isolates were treated by UV rays (100 J/m^2) or by N-methyl-N'-nitro-N-nitrosoguanidine (1.5 mg/ml) and were sown in dilutions. After 5 days growing, colonies of streptomycetes were poured around in molten MPA. After 2 days more active colonies against the phytopathogen were selected and the stability of their antagonistic properties was analyzed. Metabolites of 25Uf7 and 35NG3 variants were obtained by extraction with chloroform:acetone (2:1) and chloroform:ethyl acetate (2:1) after 7 days growing on a corn-soy agar and were tested for antagonism.

Seed of tomatoes were treated with the fermentation filtrate of researched strains after their cultivation in a liquid corn-soy medium for 3 days. Manipulations of the research are described in Table 1. Seed that down the bottom of solutions were sown in non-sterile soil in a hothouse in May 2018 and 2019. Seed germination and juvenile tomato growth were analyzed after 4 weeks. Yield analysis was done from 4 bushes in early August in 2018 and 2019. The statistical analysis was performed with Windows Software Excel 2007.

Table 1

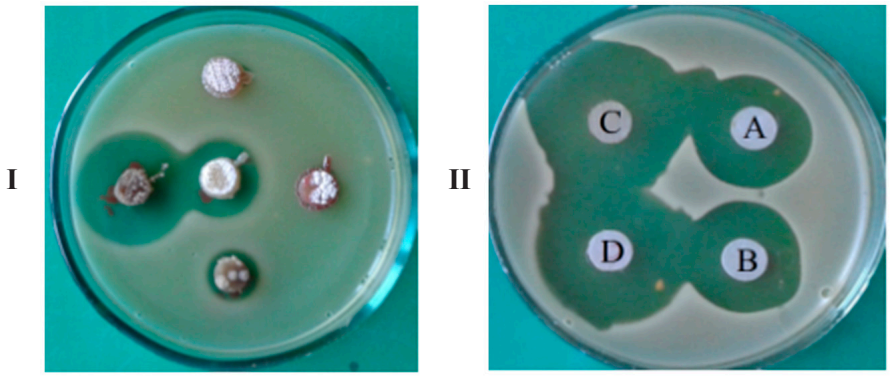
Seeds and leaves treatment during the experiment

Seed treatment in May	**1% $KMnO_4$, 1 h, pcs**	**Suspension of *C. mich* 10_2, 1 h, pcs**	**Fermentation filtrate, 1 h, pcs**	**Treatment of tomatoes leafs in June**	**Suspension of *C. mich* 10_2, 1 h, pcs**	**Fermentation filtrate, 1 h, pcs**
Variants				**Variants**		
Control 1	20	–	–		–	–
				/inf	8	–
Control 2	30	30	–		–	–
Experiment 1	50	50	26 Uf7, 50		–	–
				/inf	8	–
				inf/26Uf7	8	26Uf7, 8
Experiment 2	50	50	35 NG3, 50		–	–
				/inf	8	–
				/inf/35NG3	8	35NG3, 8

*Note** Variants: /inf – tomato leafs (4 weeks) mechanically damaged by injection and infected with *C. michiganensis* 10_2; /inf/26Uf7 and /inf/35NG3 – infected leaves that were treated with fermentation filtrate of 26Uf7 and 35NG3 strains, respectively.

Results. 80 streptomycetes isolated from soils of Ukraine with different ecological load were analyzed for antagonism to *C. michiganensis* 10_2. Nearly half out of 80 streptomycetes (36 isolates, 45%) had antagonistic activity against *C. michiganensis* 10_2 strain in laboratory conditions. Diameters of test culture growth inhibition zones, caused by streptomycetes, were from 20 to 45 mm (Fig. 1.I). 26 and 35 isolates were selected for subsequent studies because they demonstrated antagonism to the test culture and to other phytopathogens. The multilevel selection of these isolates made it possible to obtain a series of variants with increased synthesis of antagonistic metabolites. The stability of obtained variants properties was analyzed 5 times. 26Uf7 and 35NG3 productive variants were selected for further studies. Their fermentation filtrates and their extracts inhibited the growth of test culture (Fig. 1, II).

Determination of fermentation filtrates of *Streptomyces* sp. 26 Uf7 and 35 NG3 strains effect on seed germination and on juvenile growth of tomatoes after their treatment with *C. michiganensis* 10_2 was the next stage of the research (Table 2).



F i g. 1. Antagonistic activity to *C. michiganensis* 10_2: I – of some isolated streptomycetes; II – of fermentation filtrates of *Streptomyces* sp. 26Uf7 (A, C) and 35NG3 (B, D) strains extracts

T a b l e 2

The analysis of seed germination and growth of Cherry tomatoes in hothouse conditions, 4 weeks of growing, June 2018 and 2019

Variants	Seeding, pcs	Seed germination, pcs (%)	Shoots height, cm *
Control 1	20	19/20 (95/100)	15–20 / 15–21
Control 2	30	11/10 (35/33)	7–11 / 6–11
Experiment 1	50	31/33 (62/66)	8–20 / 10–22
Experiment 2	50	34/33 (68/66)	10–22 / 9–21

*Note** – the experiment error is up to 1.0 cm.

Germination of Control 2 seeds infected with *C. michiganensis* 10_2 was nearly three times lower than Control 1 uninfected seeds. Experiment 1 and Experiment 2 seeds germination were twice higher than Control 2, but on average was lower on 32 ± 2.5% than in Control 1. Although metabolites of *Streptomyces* sp. 26Uf7 and 35NG3 strains increased Cherry tomatoes seeds germination in hothouse conditions, they did not completely nullify the infectious process caused by *C. michiganensis* 10_2 strain. The appearance of leaves and shoots of all plants including infected were satisfactory after 4 weeks of growth. However, plants infected with *C. michiganensis* 10_2 developed more slowly than others and had 4 leaf blade with petiole, as opposed to 6 formed in Control 1. The variations of shoots height in Experiments 1 and 2 had wider limits than of Controls 1 and 2. Shoots height of some plants of these variants was higher than in Control 1 and in some plants it was similar to Control 2. The number of their leaf blades varied from 4 to 6, depending on the height of shoots. Thus, Cherry tomatoes from Experiments 1 and 2 had signs of both Controls after 4 weeks of growth in hothouse conditions.

Harvesting took place in early August, which corresponded to the characteristics provided by supplier. Fruit ripening was the same for all variants. The infected tomatoes fruits appearance corresponded to the description of the disease given in the literature: external small white "eye of bird" spots on the surface of the fruit and internal white stiff streaks [2, 5, 10]. Results of the harvest are presented in Table 3. The yield from one bush of Control 1 was in average 120 ± 14 g lower than noted by supplier, although the weight of individual tomatoes was 14 ± 0.7 g in average, that corresponds with the norm for this tomatoes variety. Visual signs of bacterial cancer were not detected in of Control 1 tomato fruits.

Control 1/inf (leaves of Cherry tomatoes infected with *C. michiganensis* 10_2 in the juvenile period) had the lowest yields among all researched variants. The mean fruits number was twice lower and the yield decreased by 60% than that of uninfected Control 1. Fruits had less weight 11 ± 0.5 g and 70% of them had visually signs of disease. Plants of Control 1/inf have given a partial reaction of hypersensitive response (HR) (Fig. 2A). The reaction was manifested by the death and drying of the leaf cells treated with phytopathogen, but has not stopped the spread of infection. The HR is the mechanism of plant defense in response to pathogenic effectors, it manifested in the death of infected host cells [12].

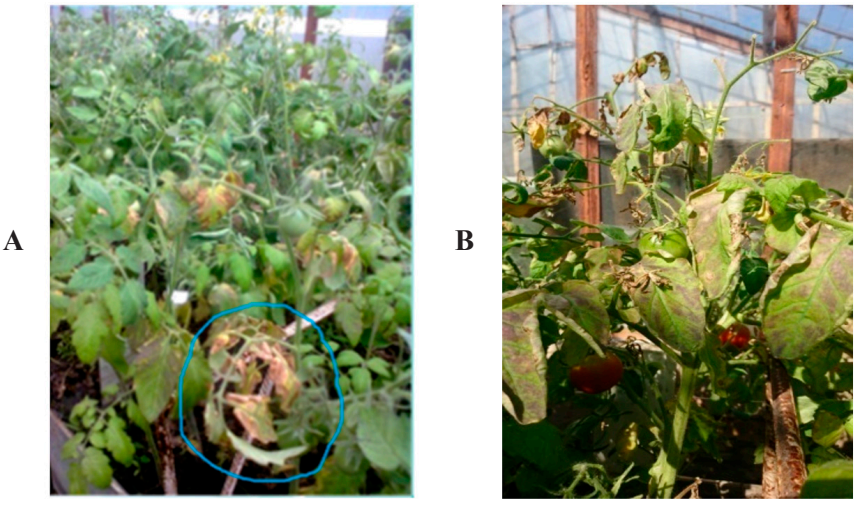
The yield of the Control 2 (infected seeds) was half that one in the Control 1. Fruits of plants of the Control 2 were small – 8 ± 0.5 g on average. 50–60% of fruits in this variant had "eye of bird" signs of the disease, but when fruits were cut off, we observed thin white streaks inside 100% of them. The plants did not show any signs of infection during growth, but the stems quickly dried out after fruiting. It is interesting that the amount of fruits in Control 2, Experiment 1 and 2 in average was the same as in Control 1.

Table 3

Yield analysis of Cherry tomatoes from 4 shrubs in hothouse conditions in 2018-2019 years

No	Variants	Mean fruits number, pcs	Mean weight: of fruits ± ± of one fruit, g	Signs of disease,% *
1	Control 1	34 ±3	475 ± 14	–
2	Control 1/inf	18 ± 2	198 ± 11	70 ± 2.8
3	Control 2	33 ± 3	264 ± 8	100
4	Experiment 1	38 ± 3	612 ± 16	24 ± 1.3
5	Experiment 1/inf/26Uf7	107 ± 5	1291 ± 12	25 ± 1.3
6	Experiment 2	33 ± 3	528 ± 16	17 ± 0.6
7	Experiment 2/inf/35NG3	63 ± 4	756 ± 12	23 ± 1.3

Note * Statistical errors of 2018 and 2019 years results were about 10% (n=4).



F i g. 2. The appearance of leaves of Control 1/inf tomatoes variant: 30 days after leaf processing (A), fruiting period (B)

The yield in Experiments 1 and 2 due to the higher average weight of the fruits (16 ± 0.8 g) was higher than in Control 1; 24% and 17% of fruits respectively had signs of tomatoes bacterial cancer infection. It should be noted that the data in the Table shown the average amount from 4 bushes, while individual plants of the experiments sometimes showed the same percentage of infection. On the other hand, even the most non-optimistic variant of the plant whose seed was treated with streptomycetes metabolites after phytopathogen infection did not exceed 25% for both variants. Therefore, metabolites of researched streptomycetes have 75% efficiency in cases of Cherry tomatoes seeds infected with *C. michiganensis* 10_2.

Plants from Experiments 1/inf and 2/inf were developing and giving yield in different ways. The most fruits had symptoms of the disease, although some were visually healthy. The response of HR to the mechanical infection of tomato leaves was varied from complete absence to similar Control 1/inf. Experiments are not presented due to the complexity of statistically processing of the results.

Leaves of Experiment 1/inf/26Uf7 and of Experiment 2/inf/35NG3 plants did not have any HR-response, they only had traces of injections with a syringe. Results of these variants were interesting because of the highest yield of 1294 ± 12 g and 756 ± 12 g, respectively. Fruits of these variants had the same average weight of 12 ± 0.6 g, which was lower compared to Control 1 (14 ± 0.7g) and Experiments 1 and 2 (16 ± 0.8 g). Also, the percentage of fruits infected with bacterial cancer in all the above variants was up to 25%. Thus, re-infection with metabolites of *Streptomyces* sp. 26Uf7 and 35NG3 strains treatment of Cherry tomatoes leaves in the juvenile period in the hothouse increased the yield in 1.5–2 times, a quarter of which was visually infected.

Discussion. Streptomycetes, isolates from soil have shown high antagonistic activity (45%) to *C. michiganensis* 10_2 *in vitro*. According to the literature, 37% of streptomycetes isolated from tomatoes rhizosphere (from one site) are active against Cmm [10]. That is, a high amount of streptomycetes-antagonists against Cmm are directly in the soil. In addition, Cmm don't have the T3SS secretion system, don't enter the cell and given the pressure of microorganisms in soil conditions are vulnerable [2]. However, germination rate of seeds infected with *C. michiganensis* 10_2 was only 33–35%, and 100% of Cherry tomatoes fruits from this seeds had disease signs in hothouse conditions. Probably, in research conditions there were no sufficient antagonistic metabolites in the soil to neutralize bacteria. Seeds treatment with researched streptomycetes metabolites enhanced germination rate of infected seeds to 62–68% and decreased the number of infected fruits to 25%. Thus, metabolites of *Streptomyces* sp. 26Uf7 and 35NG3 strains doubled the seed germination and contributed to 75% the visually healthy yield of Cherry tomatoes in hothouse conditions.

Mechanical infection in the juvenile period of Cherry tomato leafs caused the immune response (HR) in Control 1/inf variants and in some plants from Experiment 1/inf and Experiment 2/inf. The absence of HR in some plants of last two variants was more likely caused by plant immune system response against *C. michiganensis* 10_2. Again, according to the literature, some Cmm pathogens stimulate biosynthesis of the protective hormone ethylene by tomatoes, and only plants with deficiency of this hormone could be infected [3]. Increasing of yields in plants after researched streptomycetes metabolites using probably was due to synthesis of stimulating components, such as auxins, cytokinins and gibberellins. In general, the use of *Streptomyces* sp. 26Uf7 and 35NG3 strains metabolites improved the yield indices, but did not cure bacterial cancer of Cherry tomatoes in hothouse conditions.

It has been shown, that 36 (45%) from 80 soil streptomycetes had antagonistic activity against *C. michiganensis* 10_2. *Streptomyces* sp. 26Uf7 and 35NG3 variants with high level of activity against test culture *in vitro* were obtained. It was detected that metabolites of the streptomycetes doubled the seed germination of Cherry tomatoes infected with *C. michiganensis* 10_2, improved the yield and had 75% efficiency against phytopathogen in hothouse conditions. However, high rates of antagonism of researched strains to *C. michiganensis* 10_2 *in vitro* have not completely nullified the effect of infection in hothouse conditions.

АНТАГОНІЗМ ШТАМІВ *STREPTOMYCES* SPECIES 26UF7 ТА 35NG3 ДО *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS* 10_2

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Резюме

Актуальність роботи. Екологічні шляхи вирішення питання опортунізму рослин до бактеріального раку помідор. **Мета**. Пошук стрептоміцетів-антагоністів проти *Clavibacter michiganensis* subsp. *michiganensis* 10_2 та дослідження їх впливу на інфекційний процес у помідорів в умовах теплиці. **Результати**. 36 (45%) грунтових стрептоміцетів з 80 мали антагоністичну активність проти *C. michiganensis* 10_2. Відібрані варіанти *Streptomyces* sp. 26Uf7 та 35NG3 демонстрували високі та стабільні показники проти фітопатогеної бактерії *in vitro*. Передпосівна обробка фільтратом ферментації досліджуваних стрептоміцетів вдвічі підвищувала схожість інфікованого *C. michiganensis* 10_2 насіння помідорів сорту «Вишенька». Однак, вона не сприяла стійкості рослин до захворювання після механічного інфікування листків помідорів в ювенільному періоді. В загальному метаболіти штамів *Streptomyces* sp. 26 Uf7 та 35 NG3 сприяли підвищенню врожайності помідорів, хоча повністю не нівелювали вплив інфекції в умовах теплиці. **Висновки.** Показано, що метаболіти штамів *Streptomyces* sp. 26Uf7 та 35NG3 з високими показниками антагонізму проти *C. michiganensis* 10_2 *in vitro* вдвічі підвищували схожість насіння та врожайність помідор сорту «Вишенька» і мали 75% ефективність проти фітопатогенної бактерії в умовах теплиці.

Ключові слова: *Clavibacter michiganensis* subsp. *michiganensis*, бактеріальний рак, антагонізм стрептоміцетів.

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