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Prospects of *Serratia plymuthica* strain 23B78/1 as a biocontrol agent for tomato protection

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Abstract. Tomato protection from diseases is necessary to obtain high yields of quality fruits. In protected soil conditions, tomatoes bear fruit for up to 265 days, while the fruits are harvested 1 or 2 times a week starting from 60 days after seed germination for early varieties and 100 days for late varieties. When growing tomatoes, especially during the fruiting period, it is optimal to use biological pest control agents, which, unlike chemical ones, are harmless to humans and do not accumulate in the fruits. Existing biological products are not effective enough against the entire range of tomato diseases. It is necessary to look for new strains of microorganisms. This paper presents the results of the study of strain 23B78/1 *Serratia plymuthica* with the aim of exploring the prospects for its use as a biocontrol agent. Pesticides based on this species are not registered in the Russian Federation. Species identification was determined by 16S gene sequence analysis and biochemical profiling. Antagonistic activity against phytopathogenic fungi was assessed *in vitro* using double culture method. Phytotoxicity testing was carried out on germinating seeds of tomato. Evaluation of antagonistic effect revealed effectiveness against the following phytopathogenic fungi: *Alternaria solani*, *Botrytis cinerea*, *Colletotrichum truncatum*, *Fusarium citri*, *F. incarnatum*, *F. duofalcatisporum*, *F. incarnatum*, *F. oxysporum*, *Globisporangium ultimum*, *Sclerotinia sclerotiorum*. The maximum antagonistic effect was observed during paired fusion with fungus *B. cinerea*, mycelium of which grew strictly in the opposite direction from the bacterium. Germination of tomato seeds in presence of strain 23B78/1 did not reveal any inhibitory effect on seed germination and development of young tomato plants. The conducted research shows that the strain *Serratia plymuthica* 23B78/1 is promising for creation of biofungicide for protecting tomato plants.

Keywords: phytopathogens, biological plant protection, bioproducts, biofungicides

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Chudinova E.M. — study concept and design, writing the manuscript. All authors have read and approved the final version of the manuscript.

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Introduction

Tomato is an extremely flexible crop, grown everywhere both in open and protected ground. In protected ground conditions, tomatoes bear fruit for up to 265 days, with the fruits being harvested 1 or 2 times a week [1]. Tomatoes are susceptible to diseases. During epiphytotic years, fruit yield losses can reach 80…90% [2]. To obtain high yields of high-quality fruits, it is necessary to protect tomatoes from diseases. However, tomato fruits are eaten fresh, which imposes significant restrictions on the use of chemicals, primarily due to the long waiting period. A study of insecticides and fungicides used to protect tomatoes showed that residual amounts of these substances are present in the fruits in high concentrations for 6…8 days after treatment [3].

A possible solution to the problem may be the use of biological products for which the waiting period is absent or does not exceed 7 days. These products are based on living organisms or natural biologically active compounds produced by organisms. Biological agents are more environmentally friendly and do not accumulate in the environment. The catalog of pesticides and agrochemicals permitted for use in the territory of the Russian Federation for protecting tomatoes from diseases includes the following products: *Bacillus subtilis, B. amyloliquefaciens, Pseudomonas asplenii, P. aureofaciens, Lactobacillus plantarum, Trichoderma harzianum, T. reesei, T. asperellum, T. atroviride, T. longibrachiatum, T. viride*. However, products containing these microorganisms in live form or products of their vital activity are not effective enough against the whole range of tomato diseases. It is necessary to search for new strains of microorganisms. Bacteria belonging to the genus *Serratia* are of great interest as potential biocontrol agent. There are no products based on these bacteria registered in Russia. Abroad, bacteria of this genus are considered promising for use as biocontrol agents. It has been shown that *S. ureilytica* strain ILBB 145 protects tomato plants well from pythium rot [4]. Experiments with the ETR1 strain of *S. marcescens* showed good results for protecting tea plants [5]. *S. marcescens* (strain C8) showed an inhibitory effect on growth of phytopathogenic fungi under laboratory conditions [6]. Strain MM *S. plymuthica* showed a high degree of antagonism towards *Fusarium oxysporum* isolated from watermelon [7]. An immunostimulating effect was also revealed: treatment of tomato

plants with a bioagent based on the C2 strain of *Serratia* sp. increased resistance to the PVY virus and osmotic stress [8].

We investigated the antagonistic activity and phytotoxicity of *Serratia plymuthica* strain 23B78/1 **in order** to evaluate its use as a biofungicide for the control of fungal diseases of tomato.

Materials and methods

The strain *Serratia plymuthica* was isolated from mycelium of fungus *Aspergillus ochraceus* (strain 23TaPT78), isolated from a potato tuber grown in Tajikistan. The species of the bacterium was determined by sequencing the universal species-specific sequence of the 16S ribosomal RNA gene using primers (27f/1492r 5′-AGAGTTTGATCCTGGCTCAG-3′/5′-CTACGGCTACCTTGTTACGA-3′) [9].

The biochemical profile was studied using reagent kit No. 1 "Paper indicator systems for the identification of microorganisms" (JSC NPO Mikrogen). The bacteria were tested *in vitro* for antagonistic activity against 11 phytopathogens (Table 1) using the double culture method as described in [10] with minor modifications. An agar block (5 × 5 mm) with fungal mycelium was placed in a Petri dish with potato glucose agar (PGA). Bacteria were streaked at 20 mm from the agar block (Fig. 1). As a control, fungal strains were placed in the center of a free Petri dish, which was incubated under the same conditions as the dishes with paired adhesion. The dishes were incubated in the dark at 25 °C for 7 days, after which the growth of the fungal colony was assessed. Antagonistic activity was assessed by width of zone of inhibition of mycelial growth between fungal colony and bacteria. All experiments were conducted in three replications. To test the antagonistic activity, pure cultures of fungi from the RUDN collection were used: *Alternaria solani* s.l., *Botrytis cinerea* Pers., *Colletotrichum truncatum* (Schwein.) Andrus & W.D. Moore, *Fusarium citri* M.M. Wang, Qian Chen & L. Cai, *F. incarnatum* (Desm.) Sacc., *F. duofalcatisporum* J.W. Xia, L. Lombard, Sand.-Den., X.G. Zhang & Crous, *F. incarnatum* (Desm.) Sacc., *F. oxysporum* Schltdl., *Globisporangium ultimum* (Trow) Uzuhashi, Tojo & Kakish. (=*Pythium ultimum*), *Sclerotinia sclerotiorum* (Lib.) de Bary (Table 1).

Table 1

Fungal strains used in the study

Strain	Species	Source of strain isolation	Sample collection location
23MLTF87	*Alternaria solani*	Tomato fruit	Mali
T129_22MOVTL2	*Botrytis cinerea*	Tomato fruit	Moscow Region, Russia
23MLTF62	*Colletotrichum truncatum*	Tomato fruit	Mali
20UgTF2	*Fusarium citri*	Tomato fruit	Uganda
20UgTF3	*F. incarnatum*	Tomato fruit	Uganda

Ending tabl. 1

Strain	Species	Source of strain isolation	Sample collection location
20UgLaTF7	*F citri*	Tomato fruit	Uganda
23MLTF61	*F. duofalcatisporum*	Tomato fruit	Mali
23MLTF88A	*F. incarnatum*	Tomato fruit	Mali
20UgLaTF4	*F. oxysporum*	Tomato fruit	Uganda
Pyth	*Globisporangium ultimum*	Potato tuber	Minsk Region, Belarus
21KTOP2	*Sclerotinia sclerotiorum*	Jerusalem artichoke stem	Kostroma Region, Russia

Source: compiled by V.A. Platonov, M.B.E. Nzhoya, A.S. Elansky, D.N. Skokov, S.N. Elansky, E.M. Chudinova.

We consider pre-planting seed treatment to be a promising period for application of bioproducts based on the tested bacterium *Serratia plymuthica*, so the phytotoxicity assessment was performed on germinating seeds of the tomato cv. 'Spely Banan'. Tomato seeds were placed in a Petri dish on filter paper moistened with 10 ml of a bacterial suspension at a concentration of 10^3, 10^5 and 10^7 CFU/ml. Sterile water was used for control. The dishes were incubated under a photoperiod of 16/8 day/night at 25 °C for 7 days, after which root and shoot length was measured.

The calculation of confidence interval of mean μ was performed as follows:

$$\overline{X} - t\frac{s}{\sqrt{n}} \leq \mu \leq \overline{X} + t\frac{s}{\sqrt{n}},$$

where X — mean; S — standard deviation; n — number of observations; t — t-test constant for a significance level of 0.05. All calculations were performed in Excel 2010.

Results and discussion

Ten bacterial strains of different species were isolated from mycelium of various phytopathogenic fungi. They were tested for antagonistic activity against four fungal strains of species: *A. solani, C. truncatum, F. citri*, and *F. oxysporum* (see Table 1). Strain 23B78/1 was the only one that had inhibitory effect on growth of all tested fungi, and, therefore, was selected for further studies.

Determination of species affiliation of strain 23B78/1 by the 16S gene sequence (NCBI PQ675617) showed that it is 100% identical to strains C1 (CP053398), SWSY-3.47 (AP035790), and 3Re4–18 (CP01209) of *Serratia plymuthica*.

To confirm the species diagnosis, a biochemical profile analysis of the strain was performed. According to the biochemical profile, the strain fully corresponded to the species *S. plymuthica* (Table 2) [11]. Based on the results of both tests, it was decided to classify isolate 23B78/1 as *S. plymuthica.*

Table 2

Biochemical properties of strain 23B78/1

Compound	Sucrose	Glucose	Maltose	Lactose	Mannose	Inositol	Mannitol	Indole formation
Biochemical reaction	+	+	+	+	+	+	+	–

Enzyme	Urease	Ornithine decarboxylase	Lysine decarboxylase	Arginine dehydrolase	Oxidase
Biochemical reaction	–	–	–	–	–

Source: compiled by V.A. Platonov, M.B.E. Nzhoya, A.S. Elansky, D.N. Skokov, S.N. Elansky, E.M. Chudinova.

The antagonistic activity was re-evaluated on a wider range of strains of phytopathogenic fungi, including *B. cinerea*, *F. incarnatum*, *F. duofalcatisporum*, *F. incarnatum*, *S. sclerotiorum* and the oomycete *G. ultimum* in addition to the previously tested *A. solani*, *C. truncatum*, *F. citri*, *F. oxysporum*. Strain 23B78/1 successfully inhibited the growth of all analyzed phytopathogenic fungi (Table 3). The strain had the most effective effect on *B. cinerea*, mycelium of which grew strictly in opposite direction from the bacterium, so distance from the fungal colony to the bacterium was the maximum possible (Figs. 1, 2). A strong effect on the growth of *A. solani*, *C. truncatum*, *F. oxysporum* was also noted. The control test for fungal mycelium growth in bacteria-free dish showed rapid growth of most strains. Colonies of *F. citri*, *F. oxysporum*, *G. ultimum*, *S. sclerotiorum* occupied the entire surface of agar medium. *Botrytis cinerea*, *F. incarnatum*, *F. duofalcatisporum*, *F. incarnatum* also occupied almost the entire area of the dish. *Alternaria solani* and *Colletotrichum truncatum* demonstrated slower growth (Table 3).

Table 3

Influence of strain 23B78/1 on growth of phytopathogenic fungi

Species name	Strain name	Disease caused by the pathogen	Width of zone of mycelial growth inhibition, mm	Diameter of colonies in the control, mm
Alternaria solani	23MLTF87	Alternaria leaf spot, fruit rot	5*	55*
Botrytis cinerea	22MOVTL2	Gray rot of fruits and other organs	20	68
Colletotrichum truncatum	23MLTF62	Anthracnose (cankers on fruits, stems, spots on leaves)	4	58
Fusarium citri	20UgTF2	Dry rot of fruits, wilting	5	80
F. incarnatum	20UgTF3		4	71
F citri	20UgLaTF7		5	80
F. duofalcatisporum	23MLTF61		2	75
F. incarnatum	23MLTF88A		3	70

Ending tabl. 3

Species name	Strain name	Disease caused by the pathogen	Width of zone of mycelial growth inhibition, mm	Diameter of colonies in the control, mm
F. oxysporum	**20UgLaTF4**	**Wilting, root rot**	**5**	**80**
Globisporangium ultimum	**Pyth**	**Root rot**	**3**	**80**
Sclerotinia sclerotiorum	**21KTOP2**	**White rot of fruits and stems**	**7**	**80**

Note: * — average of 3 measurements. Averaged to whole number.

Source: compiled by V.A. Platonov, M.B.E. Nzhoya, A.S. Elansky, D.N. Skokov, S.N. Elansky, E.M. Chudinova.

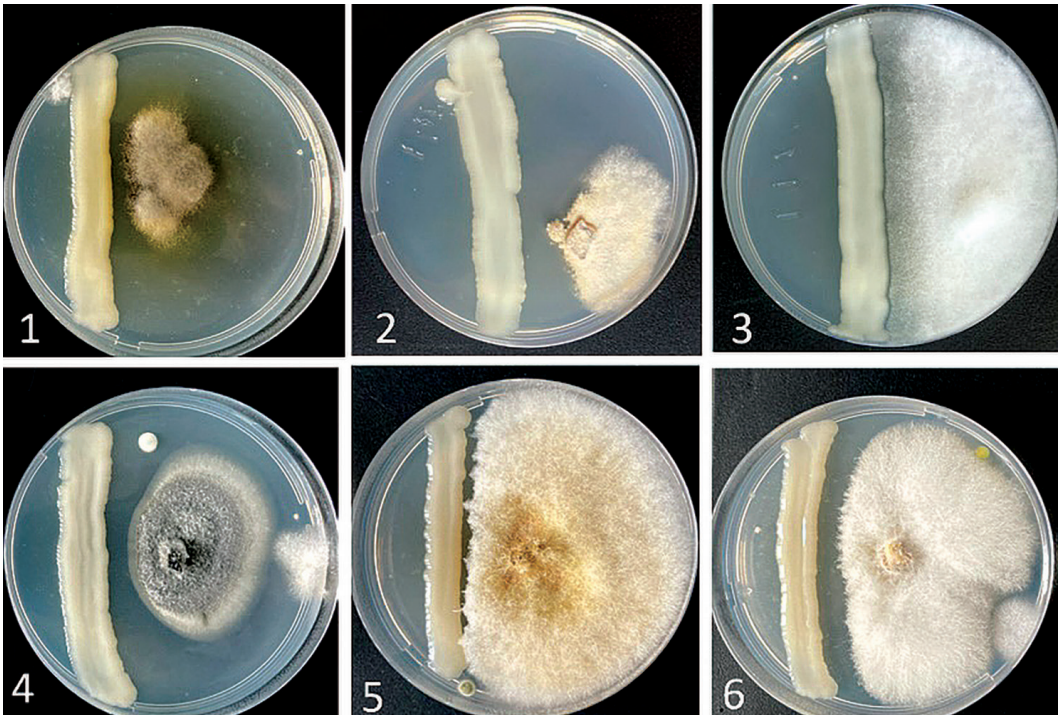

Fig. 1. Evaluation of antagonistic activity of strain 78/1: 1 — *Alternaria solani;* 2 — *Botrytis cinereal;* 3 — *Pythium ultimum;* 4 — *Colletotrichum truncatum;* 5 — *Fusarium citri* (20UgLaTF7); 6 — *Fusarium oxysporum*

Source: compiled by E.M. Chudinova.

Phytotoxicity assessment on tomato seeds showed that presence of bacteria in different concentrations does not inhibit germination and growth of tomato seeds. As can be seen in Fig. 1, length of roots and shoots is approximately the same and does not have statistically significant differences either in the control variant or in the presence of bacteria, even at their sufficiently high concentration (10^7 CFU/ml) (see Fig. 1).

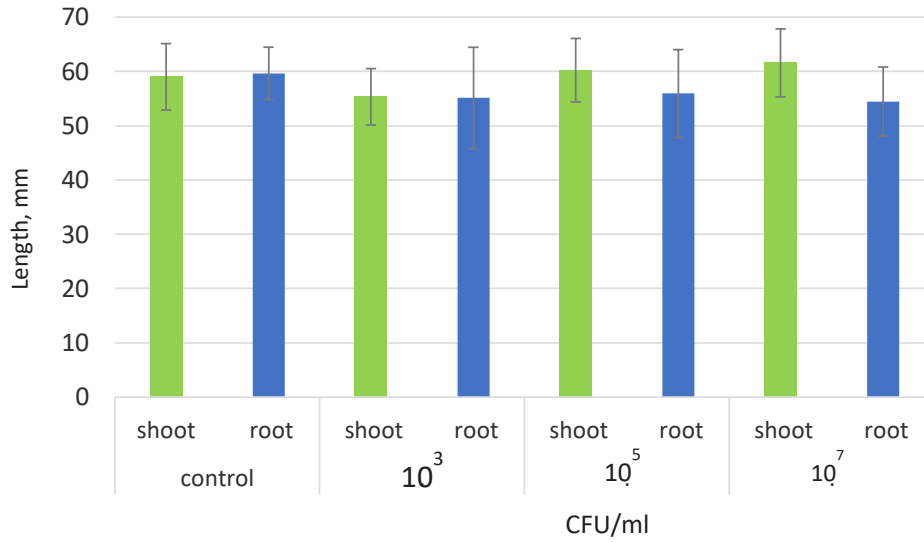

Fig. 2. Length of tomato shoot and root, mm, 7 days after sowing seeds in presence of bacteria strain 78/1 at a concentration of 10^3, 10^5 and 10^7 CFU/ml and without bacteria (control). Error bars show the confidence interval of the mean at a significance level of 0.95

Source: compiled by V.A. Platonov, M.B.E. Nzhoya, A.S. Elansky, D.N. Skokov, S.N. Elansky, E.M. Chudinova.

Bacteria of *Serratia* genus are increasingly considered as biocontrol agents and growth-promoting organisms [12]. It is noted that they can synthesize plant hormones, phytosiderophores, which help plants absorb mineral elements, produce secondary metabolites that inhibit growth of fungi, insects and phytopathogenic bacteria [13–15]. In future studies, we plan to test the effectiveness of strain 23B78/1 on plants in protected ground and on field plots.

Conclusion

The strain *Serratia plymuthica* 23B78/1 showed antagonistic activity against pathogens of significant tomato diseases in *in vitro* tests and did not have negative effect on germination of tomato seeds and young plants, which allows us to consider this strain as a potential agent for controlling fungal diseases of tomato. The search for new strains for plant protection will make agriculture less dependent on the use of chemical pesticides and will increase the environmental sustainability of plant production.

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Перспективы штамма 23B78/1 *Serratia plymuthica* как агента биоконтроля для защиты томата

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Аннотация. Защита томата от болезней необходима для получения высоких урожаев качественных плодов. В условиях защищенного грунта томат плодоносит до 265 дней, при этом сбор плодов производят 1 или 2 раза в неделю начиная с 60 суток после прорастания семечки у ранних сортов и 100 суток у поздних. При выращивании томата, в особенности в период плодоношения, оптимально применение биологических средств защиты, которые в отличии от химических безвредны для человека и не накапливаются в плодах. Существующие биопрепараты недостаточно эффективны против всего комплекса болезней томата, в связи с чем необходимо искать новые штаммы микроорганизмов. Приведены результаты исследования штамма 23B78/1 *Serratia plymuthica* с целью изучения перспективы его использования в качестве агента биоконтроля. Препараты на основе данного вида в Российской Федерации не зарегистрированы. Видовая идентификация определена в результате анализа видоспецифичной последовательности гена 16S рибосомной РНК и по биохимическому профилю. Антагонистическую активность в отношении фитопатогенных грибов оценивали *in vitro* методом двойных культур. Проверку фитотоксичности проводили на прорастающих семенах томата. Оценка антагонистического действия выявила эффективность в отношении фитопатогенных грибов *Alternaria solani, Botrytis cinerea, Colletotrichum truncatum, Fusarium citri, F. incarnatum, F. duofalcatisporum, F. incarnatum, F. oxysporum, Globisporangium ultimum, Sclerotinia sclerotiorum*. Максимальный антагонистический эффект отмечен при попарном сращивании с грибом *B. cinerea*, мицелий которого рос строго в противоположную сторону от бактерии. Проращивание семян томата в присутствии штамма 23B78/1 не выявило угнетающего действия на прорастание семян и развитие молодых растений томата. Проведенная работа показывает, что штамм *Serratia plymuthica* 23B78/1 перспективен для создания биопрепарата с фунгицидным действием для защиты томата.

Ключевые слова: фитопатогены, биологическая защита растений, биопрепараты, биофунгициды

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