Effect of rhizosphere microorganisms on the adaptation of regenerated plants of apple clonal rootstocks to *ex vitro* conditions

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Abstract. In a vegetation experiment, the effect of bacterial preparations Extrasol, Fitosporin-M based on bacterial strains *Bacillus subtilis* and bacteria strains of the genus *Pseudomonas – P. chlororaphis* OV17, *P. protegens* 38a, *P. putida* O9-10 on the number of rhizosphere microorganisms, growth and development of regenerant plants of apple clone rootstocks in ex vitro conditions was studied. After 90 days of growing plants, the greatest number was found in the *P. protegens* 38a strain – 0.56 million CFU/g roots. Artificial inoculation of the roots of regenerant plants contributed to a significant increase in the total number of native microorganisms as compared to the control. The most diverse bacterial population in terms of cultural and morphological characteristics was revealed in the variant with the treatment of plants with the *P. putida* O9-10 strain. The introduced bacterial strains contributed to an increase in the adaptive capacity and had a phytostimulating effect on the development of plants. The plant survival rate in the process of adaptation to *ex vitro* conditions is most influenced by the *P. putida* 09-10 strain compared to the control. The bacterial preparation Fitosporin-M contributes to the improvement of biometric parameters of plant growth.

1 Introduction

The weak adaptive ability of regenerant plants to *ex vitro* conditions is a consequence of the heterotrophic type of nutrition of microplants in culture *in vitro*, low photosynthetic ability of plants, their dehydration due to weak activity of the stomatal apparatus due to prolonged exposure to cytokinins, the absence of second-order roots and root hairs in plants [1-4]. Controlling the cultivation parameters before and during the acclimatization period allows for the gradual adaptation of plants to external environmental factors. Pre-acclimatization of M9 apple rootstocks propagated *in vitro* promoted lower transpiration losses and led to higher plant survival after transplantation [4]. Cultivation of microplants in aseptic *in vitro* conditions, and the resulting weak colonization of root systems by associative microflora

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contributes to additional stress of regenerated plants caused by the impact of aboriginal microflora of non-sterile substrates during *post-vitro* adaptation. Various endophytic microorganisms are associated in plant tissues under *in vitro* conditions, performing both a positive effect and capable of limiting their growth and development [6-8]. Their role in the settlement of the rhizosphere and their importance in the further development of regenerant plants under *ex vitro* conditions have not been studied at present. High efficiency was demonstrated by artificial inoculation of the root systems of regenerant plants in order to adapt them to *ex vitro* conditions by various associative microorganisms based on arbuscular-vesicular fungi, ascomycete fungi of the genus *Trichoderma*, bacteria of the genera *Bacillus*, *Azospirillum*, *Pseudomonas*, etc. [4, 5, 9 -15]. At present, microbiological preparations based on *B. subtilis* Extrasol, Fitosporin-M have been developed and are used in biologized agricultural technologies for the cultivation of agricultural plants [16]. The strains of rhizosphere bacteria of the genus *Pseudomonas*, promising for the creation of biological products intended to protect and stimulate plant growth, which combine phytostimulating properties, resistance to heavy metals, and have the ability to biodegrade organic pollutants, have been obtained [14,15]. The positive effect of bacteria of the genus *Pseudomonas* on plants is to stimulate plant growth through the synthesis of phytohormones, improve mineral nutrition, and suppress the development of phytopathogenic fungi and bacteria [14].

Purpose of the work: to study the effect of inoculation of roots of regenerated plants of clonal rootstocks of apple trees 57-545 with bacteria *B. subtilis* Ch-13 (biological product Extrasol), *B. subtilis* 26 D (biological product Fitosporin-M), *P. chlororaphis* OV17, *P. protegens* 38a, *P. putida* O9-10 on the total number of rhizosphere microorganisms, plant growth and development in *ex-vitro* conditions.

2 Materials and methods

2.1 Conditions of plant growing

The studies were carried out in 2020-2021 in a vegetation experiment with plants-regenerants of apple rootstocks 57-545 after *in vitro* culture, in containers with a capacity of 0.5 kg of soil. A 3:1 peat-sand mixture was used as a nutrient substrate. The content of nutrients in the substrate before setting the experiment, mg/kg: alkaline hydrolysable N - 5.68 mg/100 g, mobile P_2O_5 - 6.65 mg/100 g, mobile K_2O - 11.53 mg/100 g, pH_{KCl} - 6, 7, pH of the aqueous extract - 6.8, N-NH_4 - 2.2 mg/100 g, N-NO_3 - 5.3 mg/100 g, CaO - 12.8 mg/100 g, MgO - 6.0 mg/100 g. Before planting, the roots of apple rootstock plants were kept for 3 hours in aqueous suspensions of microorganisms with a cell concentration of 107 CFU/ml. In each variant, 25 plants were used. The results were processed by the statistical method of analysis of variance using the MS Excel software package (v. 2016).

2.2 Bacterial strains

We used bacteria of the genera *Bacillus* and *Pseudomonas*, which have multiple positive effects on plant development. For the inoculation of plants with *B. subtilis* strains, the drugs Extrasol and Fitosporin-M were used, which are widely represented on the Russian market. The *B. subtilis* Ch-13 strain is active bioagent of the Extrasol drug. The biopreparation formulation is liquid, the cell and spore titer is not less than 100 million CFU/ml. The active ingredient of the biological product Fitosporin-M is *B. subtilis* strain 26 D. The biopreparation formulation is liquid, the titer of living cells and spores is not less than 1 billion CFU/ml. These biological products are designed to improve nutrition, accelerate plant growth and development, as well as to increase resistance to fungal and bacterial infections.

For inoculation of plants with rhizosphere strains of pseudomonads, we used pure cultures of bacteria with a number of at least 1 billion CFU/ml obtained by batch cultivation. Strains *P. chlororaphis* OV17 (BKM B-2391D), *P. protegens* 38a (BKM B-3228D), *P. putida* O9-10 (VKM B-2955D) synthesize antibiotic active compounds (phenazines - strain OV17, O9-10, pyoluteorin - strain 38a), hydrogen cyanide, auxins, surfactants, solubilize phosphates (O9-10, 38a), inhibit the growth of phytopathogenic fungi *Rhizoctonia solani*, *Gaeumannomyces graminis* var. *tritici*, *Fusarium graminearum* and *Pectobacterium wasabiae* bacteria. The *P. chlororaphis* OV17 strain contains the naphthalene biodegradation plasmid, which provides it with the ability to utilize naphthalene and salicylate as the only source of carbon and energy [14].

2.3 Cultivation conditions

The media below were used to grow the bacteria. Medium LB (Maniatis et al., 1984), containing in g/L: bacto-tryptone - 10.0, yeast extract - 5.0, NaCl - 10.0. King B medium (for isolation and differentiation of fluorescent bacteria), g/l: bacto-peptone - 20, glycerol - 10, K_2HPO_4 - 1.5, $MgSO_4 \times 7H_2O$ - 1.5. Pseudomonas Isolation Medium (PIA) (Pseudomonasisolationagar, Sigma, USA). The synthetic medium was M9 (Maniatis et al., 1984) containing in g, ml/L: Na_2HPO_4 - 6, KH_2PO_4 - 3, NaCl - 0.5, NH_4Cl - 1, 1M $MgSO_4$ - 2, 1M $CaCl_2$ - 1, pH 7.5. The last two solutions were sterilized separately and added to the prepared medium. To obtain agar media, 15 g/L agar agar (Difco, USA) was added.

Growth substrates used for the differentiation of pseudomonads - xylose, trehalose, mesoinositol, adipic, benzoic, phenylacetic, salicylic, anthranilic, hippuric acids - were added to M9 medium to a final concentration of 0.5 g/L. Water-insoluble aromatic acids were introduced into the growth medium in the form of sodium salts. Bacteria were grown at 30 ° C on agar media in Petri dishes or in liquid medium on a shaker (150 rpm) in Erlenmeyer flasks containing 200-250 ml of nutrient medium.

For carrying out vegetation experiments, strains of bacteria of the genus *Pseudomonas* were grown in LB medium diluted 2 times until the late logarithmic growth phase. The number of cells of the *P. chlororaphis* OV17 strain was 2×109 - 3×109 CFU/ml, *P. protegens* 38a - 5×109 - 6×109 CFU/ml, *P. putida* O9-10 - 1×109 - 3×109 CFU/ml. For the treatment of plant roots, cultures of strains 107 CFU/ml were used.

The number of introduced microorganisms in the rhizosphere of apple rootstocks was determined on day 90. For this purpose, the roots of plants were isolated, successive washings were made from their surface in physiological solution according to Tepper E.Z. (2004) and plated the corresponding dilutions on agar media TSA (trypticase-soy agar), MPA (meat-peptone agar), SAA (starch-ammonia agar), acidified Czapek's medium. Individual colonies were replicated onto King B, PIA, LB, and M9 media containing various carbon and energy sources. The experiment was carried out in triplicate.

3 Results and discussion

The *P. putida* 09-10 strain exerted the greatest influence on the survival rate of regenerated plants of apple-tree rootstocks 57-545 in the process of adaptation to *ex vitro* conditions in comparison with the control (Table 1). The biometric indicators of plant growth were mostly influenced by Fitosporin-M.

Table 1. Influence of inoculation on biometric indicators of plant development of apple rootstocks

Experiment variant	Plant survival, %	Plant height, cm	Main root length, cm	Number of leaves, pcs / plant
Control	27	6.0	2.5	20
Extrasol	28	5.0	4.7	29
Fitosporin-M	47	10.5	8.5	37
P. putida 09-10	57	5.7	6.7	36
P. protegens 38a	35	4.7	4.3	29
P. chlororaphis OV17	40	5.0	2.8	16
LSD_{05} (least significant difference 95%)	0.4	0.4	*Femp<**Fcrit	*Femp<**Fcrit

* empirical value of Fisher's F-test;
** critical value of Fisher's F-test.

Root samples were taken from the rhizosphere of plants treated with microorganisms, 90 days after inoculation, and from the corresponding dilutions were inoculated on agar media TSA, MPA, KAA, Czapek's medium. The total number of microorganisms in the variants with inoculation on all nutrient media was significantly higher compared to the control (table 2).

Table 2. Influence of bacterization on the number of cultivated microorganisms, million CFU/g roots

Experiment variant	Cultivation medium			
	TSA	MPA	SAA	Czapek's medium
Control	0.006	0.78	0.006	0.059
Extrasol	1.97	0.53	1.74	0.98
Fitosporin-M	1.18	1.31	1.64	0.99
P. putida 09-10	0.65	0.98	0.87	0.87
P. protegens 38a	1.14	1.13	1.25	0.84
P. chlororaphis OV17	0.76	1.23	1.06	0.51

For the variants of the experiment with the introduction of *Pseudomonas* strains, individual colonies differing in morphological characteristics were plated on TSA medium and then a pure culture of microorganisms was tested for the ability to grow on King B, PIA, M9 nutrient media containing various organic substrates as the only source of carbon and energy. Strains *P. chlororaphis* OV17, *P. protegens* 38a, and *P. putida* O9-10, which were used for plant inoculation, were used as controls.

These strains belong to the fluorescent group of pseudomonads. They produce pyoverdins, water-soluble fluorescent pigments that, when exposed to ultraviolet radiation, give bacterial colonies their characteristic blue-green color. In addition, the *P. chlororaphis* OV17 strain is characterized by bright orange colonies, which is associated with the production of phenazine-1-carboxylic acid hydroxy derivatives. The strains differ from each other in their ability to utilize some organic carbon sources - adipic, phenylacetic and salicylic acids. *P. putida* O9-10 does not grow on these compounds, *P. protegens* 38a utilizes adipate and phenyl acetate, and *P. chlororaphis* OV17 is capable of growing only on salicylate.

Several morphotypes of colonies were revealed on a complete TSA medium in each variant of treatment with rhizosphere strains. The most phenotypically diverse bacterial

population was found in variants with plant inoculation with *P. putida* O9-10 and *P. protegens* 38a strains - 7 and 6 morphotypes, respectively. Treatment of plants with the *P. chlororaphis* OV17 strain revealed two types of colonies. All selected isolates differed from the control strains in terms of cultural and morphological characteristics. Most of them, except for isolate 471-1, were able to grow on King B and PIA media, but did not produce green fluorescent pigment. The only exception was the strain 471-3, which completely corresponded to the *P. protegens* 38a strain in terms of the morphology and coloration of colonies, fluorescence on complete nutrient media, and the spectrum of utilized substrates. Its number in the rhizosphere of plants was 0.56 million CFU/g of roots. After 90 days of plant cultivation, it was not possible to detect the strains of *P. chlororaphis* OV17 and *P. putida* O9-10 according to the cultural and morphological characteristics.

4 Conclusion

Bacterial drugs Extrasol, Fitosporin-M based on bacterial strains *Bacillus subtilis* and rhizosphere strains of bacteria of the genus *Pseudomonas* - *P. chlororaphis* OV17, *P. protegens* 38a, *P. putida* O9-10 have a significant positive effect on the growth and development of regenerated plants under *ex vitro* during the first 90 days of adaptation. The plant survival rate in the process of adaptation to *ex vitro* conditions was most influenced by the *P. putida* O9-10 strain compared to the control. The bacterial preparation Fitosporin-M had a greater effect on biometric parameters of plant growth.

Artificial inoculation of the roots of regenerant plants contributed to a significant increase in the number of microorganisms cultivated on elective agar media in comparison with the control. In each variant of treatment with rhizosphere strains, several different colony morphotypes were identified. The most diverse in terms of cultural and morphological characteristics, the bacterial population was found in the variant with inoculation of plants with the *P. putida* O9-10 strain (7 morphotypes). All selected strains, with the exception of 471-1, were able to grow on King B and PIA media, but did not produce green fluorescent pigment. Isolate 471-3 corresponded to *P. protegens* strain 38a in terms of colony morphology and coloration, fluorescence on full-fledged nutrient media, and the spectrum of utilized substrates.

After 90 days of growing plants of apple rootstocks, the number of introduced strains of pseudomonads significantly decreased. We failed to isolate the *P. chlororaphis* OV17 and *P. putida* O9-10 strains by cultural and morphological characters, and the number of *P. protegens* 38a was 0.56 million CFU/g roots. Nevertheless, over 90 days of cultivation, the strains contributed to an increase in the adaptive capacity and had a phytostimulating effect on plant development.

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