Вавиловский журнал генетики и селекции. 2022;26(5):422-430
DOI 10.18699/VJGB-22-52

Original Russian text www.bionet.nsc.ru/vogis/

Improving the efficacy of potato clonal micropropagation by inoculation with the rhizosphere bacteria *Azospirillum baldaniorum* Sp245 and *Ochrobactrum cytisi* IPA7.2

K.Yu. Kargapolova[1], O.V. Tkachenko[1] ✉, G.L. Burygin[1, 2], N.V. Evseeva[2], A.A. Shirokov[2], L.Yu. Matora[2], S.Yu. Shchyogolev[2]

[1] Saratov State Vavilov Agrarian University, Saratov, Russia
[2] Institute of Biochemistry and Physiology of Plants and Microorganisms – Subdivision of the Saratov Federal Scientific Centre of the Russian Academy of Sciences, Saratov, Russia
✉ oktkachenko@yandex.ru

Abstract. Sustainable development of agriculture depends on the provision of quality seeds to the market. Inoculation with plant-growth-promoting rhizobacteria in *in vitro* culture can be used to improve the growth efficacy and performance of microplants. We examined the effect of *in vitro* inoculation of microplants of the cultivars Nevsky and Kondor with the strains *Azospirillum baldaniorum* Sp245 and *Ochrobactrum cytisi* IPA7.2 separately and in combination. We examined the morphological variables of plant growth in *in vitro* culture and under *ex vitro* adaptation conditions; we also investigated the growth and performance of the plants in the greenhouse. The dependence of the inoculation efficacy on potato genotype, growth stage, and inoculum composition was ascertained throughout the experiment. *In vitro*, *A. baldaniorum* Sp245 alone and in combination with *O. cytisi* IPA7.2 promoted the formation of roots on the microplants of both cultivars and the growth of Nevsky shoots. During plant growth *ex vitro*, all growth variables of the Nevsky microplants were promoted by *O. cytisi* IPA7.2 alone and in combination with *A. baldaniorum* Sp245. In both cultivars grown in the greenhouse, shoot growth was promoted in most inoculation treatments. The survival ability of the Nevsky microplants in the greenhouse increased 1.7-fold under the effect of simultaneous inoculation. Inoculation of microplants with a combination of *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2 increased the number of Nevsky minitubers 1.5-fold and the number of Kondor minitubers 3.5-fold. Inoculation with the tested strains can be used to promote the growth of microplants and increase the yield of minitubers in potato seed breeding for the production of healthy planting material.
Key words: *Solanum tuberosum* L.; *Azospirillum baldaniorum* Sp245; *Ochrobactrum cytisi* IPA7.2; plant-microbe associations, clonal micropropagation; plant growth efficacy; adaptability; *in vitro*; *ex vitro*.

For citation: Kargapolova K.Yu., Tkachenko O.V., Burygin G.L., Evseeva N.V., Shirokov A.A., Matora L.Yu., Shchyogolev S.Yu. Improving the efficacy of potato clonal micropropagation by inoculation with the rhizosphere bacteria *Azospirillum baldaniorum* Sp245 and *Ochrobactrum cytisi* IPA7.2. *Vavilovskii Zhurnal Genetiki i Selektsii = Vavilov Journal of Genetics and Breeding*. 2022;26(5):422-430. DOI 10.18699/VJGB-22-522

Повышение эффективности клонального микроразмножения картофеля при инокуляции ризосферными бактериями *Azospirillum baldaniorum* Sp245 и *Ochrobactrum cytisi* IPA7.2

К.Ю. Каргаполова[1], О.В. Ткаченко[1] ✉, Г.Л. Бурыгин[1, 2], Н.В. Евсеева[2], А.А. Широков[2], Л.Ю. Матора[2], С.Ю. Щёголев[2]

[1] Саратовский государственный аграрный университет им. Н.И. Вавилова, Саратов, Россия
[2] Институт биохимии и физиологии растений и микроорганизмов – обособленное структурное подразделение Федерального исследовательского центра «Саратовский научный центр Российской академии наук», Саратов, Россия
✉ oktkachenko@yandex.ru

Аннотация. Устойчивое развитие сельского хозяйства зависит от обеспечения рынка качественными семенами. Инокуляция растений рост-стимулирующими ризобактериями в культуре *in vitro* может быть использована для повышения эффективности роста и продуктивности микрорастений при получении оздоровленного посадочного материала картофеля. Изучено влияние инокуляции *in vitro* штаммами *Azospirillum baldaniorum* Sp245 и *Ochrobactrum cytisi* IPA7.2 по отдельности и в консорциуме на микрорастения сортов Невский и Кондор. Оценены морфологические параметры роста растений в культуре *in vitro*, в условиях адаптации *ex vitro*, а также показатели роста и продуктивности растений в грунтовой теплице. На протяжении всего опыта была установлена зависимость эффективности бактеризации от генотипа картофеля, этапа культивирования и состава инокулята. Методом иммунофлуоресцентного анализа показано, что оба штамма бактерий успешно вступают во взаимодействие с клетками растений без антагонистического взаимного влияния. В культуре *in vitro A. baldaniorum* Sp245 и консорциум штаммов стимулировали образование корней на микрорастениях обоих сортов и

рост побегов сорта Невский. На этапе культивирования *ex vitro* на все ростовые показатели микрорастений сорта Невский положительно влияла инокуляция *O. cytisi* IPA7.2 и консорциум штаммов. При выращивании в теплице в большинстве вариантов инокуляции стимулировался рост побегов обоих сортов. Приживаемость растений сорта Невский в теплице повысилась под действием одновременной коинокуляции в 1.7 раза. Инокуляция микрорастений консорциумом штаммов *A. baldaniorum* Sp245 и *O. cytisi* IPA7.2 увеличивала количество мини-клубней у сорта Невский в 1.5 раза, а у сорта Кондор – в 3.5 раза. Инокуляция изученными штаммами может быть использована для стимулирования роста микрорастений и повышения урожайности мини-клубней в системе семеноводства картофеля при получении оздоровленного посадочного материала.
Ключевые слова: *Solanum tuberosum* L.; *Azospirillum baldaniorum* Sp245; *Ochrobactrum cytisi* IPA7.2; растительно-микробные ассоциации; клональное микроразмножение; эффективность роста растений; адаптационная способность; *in vitro*; *ex vitro*.

Introduction

In the production of seeds of many vegetatively propagated crops, *in vitro* clonal micropropagation methods have been widely used (Rajasekharan, Sahijram, 2015). In the clonal micropropagation of various plant species, rhizobacteria of different taxonomic groups can be used (Orlikowska et al., 2017; Soumare et al., 2021). Among herbaceous plants, orchid (Castillo-Pérez et al., 2021), sugarcane (Oliveira et al., 2002), and some other species (Dias et al., 2009) predominate as bacterization objects. Bacterial strains capable of promoting potato microclonal growth *in vitro*, adaptation to *ex vitro* conditions, and minituber productivity have been isolated (Oswald et al., 2010). Proper selection of bacterial associates is crucial (Wang et al., 2016). Our preliminary work has shown that pure cultures of the associative rhizobacteria *Azospirillum baldaniorum* Sp245 and *Ochrobactrum cytisi* IPA7.2 promote the growth of potato microplants *in vitro* and *ex vitro* (Tkachenko et al., 2015; Burygin et al., 2019; Kargapolova et al., 2020).

Some authors have pointed out that joint inoculation of plants with two or more strains of rhizospheric plant-growth-promoting bacteria (PGPR) can be more effective than inoculation with pure cultures (Thomas et al., 2010). When using consortia of strains for inoculation, one has to see that the component cultures are compatible (Yegorenkova et al., 2016). We have previously found that for strains with different characteristics, the inoculation of microplants during growth *in vitro* may be important (Burygin et al., 2018).

Here we examined the efficacy of inoculation of potato (*Solanum tuberosum* L. cvs. Nevsky and Kondor) microplants with pure cultures of *Azospirillum baldaniorum* Sp245 and *Ochrobactrum cytisi* IPA7.2 and with their mixture. The specific aim was to use clonal micropropagation to improve the production efficacy for seeds of healthy planting material.

Materials and methods

Growing of potato microplants *in vitro*. We used microplants of two middle early potato cultivars, Kondor and Nevsky. The cultivars had been obtained from the *in vitro* collection of the Department of Plant Breeding, Selection, and Genetics of the Faculty of Agronomy at Saratov State Vavilov Agrarian University (Saratov) and had been produced by isolation of apical meristems. The Nevsky and Kondor cultivars were used as material for study because the State Register for Selection Achievements Admitted for Usage (National List) (https://gossortrf.ru/gosreestr/) recommends them to be grown in the Lower Volga zone. Nevsky is a domestically bred cultivar (Russian Potato Research Center, Russia), whereas Kondor is a foreign-bred one (AGRICO U.A., Netherlands).

Microcuttings with one leaf and one bud were placed in a hormone-free liquid nutrient Murashige–Skoog medium (Murashige, Skoog, 1962). Plants were grown for 30 days at a temperature of 24 °C, a humidity of 60 %, a light intensity of 60 μM/($m^2 \cdot s$), and a day length of 16 h. The shoot and root morphometric variables examined were shoot length (mm), number of nodes per shoot, average root length (mm), and number of roots per shoot.

Inoculation of microcuttings. We used two rhizospheric bacterial strains – *A. baldaniorum* Sp245 (Baldani et al., 1983) and *O. cytisi* IPA7.2 (Burygin et al., 2017, 2019). Both strains were from the IBPPM RAS Collection of Rhizosphere Microorganisms (Saratov; http://collection.ibppm.ru/). Cultures were grown at 35 °C on a rotary shaker with a stirring intensity of 120 rpm until the end of the exponential growth phase (18 h) in a liquid malate medium composed as follows (g/l): Na malate, 5.0; KH_2PO_4, 0.4; NaCl, 0.1; $MgSO_4$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.02; $Na_2MoO_4 \cdot 2H_2O$, 0.002; NH_4Cl, 1.0, pH 6.8–7.0 (Döbereiner, Day, 1976). The cells were sedimented by centrifugation at 3000 g under sterile conditions and were resuspended in 0.12 M phosphate buffer (pH 7.2) containing (g/l): KH_2PO_4, 0.43; Na_2HPO_4, 1.68; NaCl, 7.2. Centrifugation was repeated twice in phosphate-buffered saline. For inoculation, 0.1 ml of cell suspension (10^8 cells/ml) was added to the tubes with plants, each tube containing 10 ml of the Murashige–Skoog medium. The final cell density in the medium was 10^6 cells/ml.

Bacteria were inoculated separately [*A. baldaniorum* Sp245 on day 0 of growth (microcuttings) and *O. cytisi* IPA7.2 on day 15 of growth] and in combination [simultaneously on day 15 of growth or successively (*A. baldaniorum* Sp245 on day 0 of growth and then *O. cytisi* IPA7.2 on day 15 of growth)]. The control was microplants grown without bacteria.

Growing of potato microplants *ex vitro*. Microplants were adapted to *ex vitro* conditions in a phytochamber in soil-filled vessels for 20 days (temperature, 24 °C; humidity, 60 %; light intensity, 60 μM/($m^2 \cdot s$); day length, 16 h). The morphometric variables analyzed were shoot length, leaf number, and leaf area.

Next, the plants were transferred to a soil-based greenhouse covered with agrotextile and were planted in a pattern of 0.4 × 0.4 m. The temperature and humidity in the greenhouse

were not regulated and depended on the weather; therefore, they were stressful for the plants (the daytime temperature could rise as high as 30 °C, and humidity could drop below 60 %). The plants were watered as needed (every 3–5 days on average). Three weeks after planting and at the beginning of the budding and flowering phase, we recorded the percentage of surviving plants, the height of the plants, the numbers of shoots and leaves, and the area of the leaves. Minitubers were dug out after the vines wilted. The number and weight of minitubers per plant and the weight and diameter of each tuber were recorded.

Immunofluorescence analysis. Bacteria on plant roots were identified by immunofluorescence analysis on day 30 after inoculation, by using strain-specific antibodies (Shelud'ko et al., 2010). The controls were uninoculated and inoculated roots treated with nonspecific antibodies. Nonspecific antibody sorption was blocked by 2-h incubation of root sections at room temperature in 0.05 % polyethylene glycol solution (MW 20000) in phosphate buffer. The primary antibodies were strain-specific rabbit antibodies to the LPS of *A. baldaniorum* Sp245 and to the LPS of *O. cytisi* IPA7.2 (concentration, 50 μg/ml); the secondary antibodies were tetramethylrhodamine isothiocyanate (TRITC)-labeled goat antirabbit antibodies (Abcam, USA; concentration, 1 μg/ml).

The inoculated roots of the microplants were observed with a TCS SP5 confocal microscope (Leica Microsystems, Germany) at the Simbioz Center for the Collective Use of Research Equipment in the Field of Physical-Chemical Biology and Nanobiotechnology (IBFRM RAS, Saratov).

Statistics. The experiment was repeated twice. In each experiment, three replicates of 10 plants each were used in each experimental treatment, with a total of 30 plants per treatment in each experiment. Data from all experiments were subjected to two-way analysis of variance (ANOVA) and were evaluated for a significance level p of 0.05. To test the null hypothesis, we calculated the F-test statistic (F_{fact}) and then determined the least significant difference ($LSD_{0.05}$) between experimental treatments. Means from each experimental treatment were subjected to multiple comparison by Duncan's test. The program package used was AGROS, a package for statistical and biometrical-genetic analysis in plant breeding and selection (version 2.09).

Results

Effect of bacteria on growth and development of potato microclones *in vitro*

For all variables examined *in vitro*, except for average root length, Kondor microplants lagged behind in growth, as compared with Nevsky microplants (Fig. 1). The shoot length of Nevsky microplants was promoted by all inoculation treatments (see Fig. 1, *a*). The microplants inoculated with *A. baldaniorum* Sp245 alone were 18.9 % taller than the control – the maximum height among the treatments examined. In Kondor, all inoculation treatments suppressed shoot length, except for the treatment with *A. baldaniorum* Sp245, in which the plants did not differ from the control.

In Nevsky, all inoculation treatments increased the number of nodes per shoot (see Fig. 1, *b*), except for inoculation with *O. cytisi* IPA7.2, when plant length did not differ from that in the control. The Nevsky microplants inoculated with *A. baldaniorum* Sp245 had 11.6 % more nodes than did the controls. The microplants inoculated sequentially with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15) had 5 % more nodes on their shoots than did the controls. The microplants inoculated simultaneously with *A. baldaniorum* Sp245 (day 15) and *O. cytisi* IPA7.2 (day 15) had 10.5 % more nodes than did the controls. The values for the Kondor microplants inoculated with *A. baldaniorum* Sp245 alone and sequentially with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15) were at the control level. The other inoculation treatments decreased the number of nodules on Kondor microplants.

In Nevsky, the average root length (see Fig. 1, *c*) increased by 4 % with *A. baldaniorum* Sp245 and by 3.7 % with *O. cytisi* IPA7.2, as compared with the control. However, sequential inoculation with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15) suppressed root length – it was 4.3 % lower than the control value. In Kondor, all inoculation treatments inhibited root length.

In both cultivars, the number of roots (see Fig. 1, *d*) increased after both inoculation with *A. baldaniorum* Sp245 alone and sequential inoculation with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15). With *A. baldaniorum* Sp245, both Nevsky and Kondor had 12.5 % more roots than did the control. The Nevsky microplants inoculated sequentially with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15) had 6.3 % more roots than did the control. The Kondor microplants inoculated sequentially with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15) had 26.7 % more roots than did the control.

Thus, inoculation with *A. baldaniorum* Sp245 alone and sequential inoculation with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15) had positive effects on the Nevsky microplants. The shoot length, the number of nodes per shoot, and the number of roots increased, whereas the average root length decreased.

Identification of bacteria on roots of potato microplants *in vitro*

Immunofluorescence analysis of the Nevsky roots by using confocal microscopy showed that both strains interacted successfully with plant cells (Fig. 2).

Both strains were detected on roots after both inoculation with pure cultures and coinoculation. Both strains were present in coinoculation treatments, which indicates that there was no antagonism between them and that neither strain had any advantage over the other in interacting with root cells.

Effect of bacteria on adaptation of potato microclones *ex vitro*

The survival ability of the microplants formed *in vitro* in soil-filled vessels under phytochamber conditions (*ex vitro* stage) was high (more than 80 %) (Fig. 3, *a*). In Nevsky, the survival ability decreased by 6 %, as compared with the control, only after inoculation with *O. cytisi* IPA7.2 alone. In Kondor, the survival ability decreased by 11 % after coinoculation with *A. baldaniorum* Sp245 (day 15) and *O. cytisi* IPA7.2 (day 15) and by 14 % after inoculation with *O. cytisi* IPA7.2 alone.

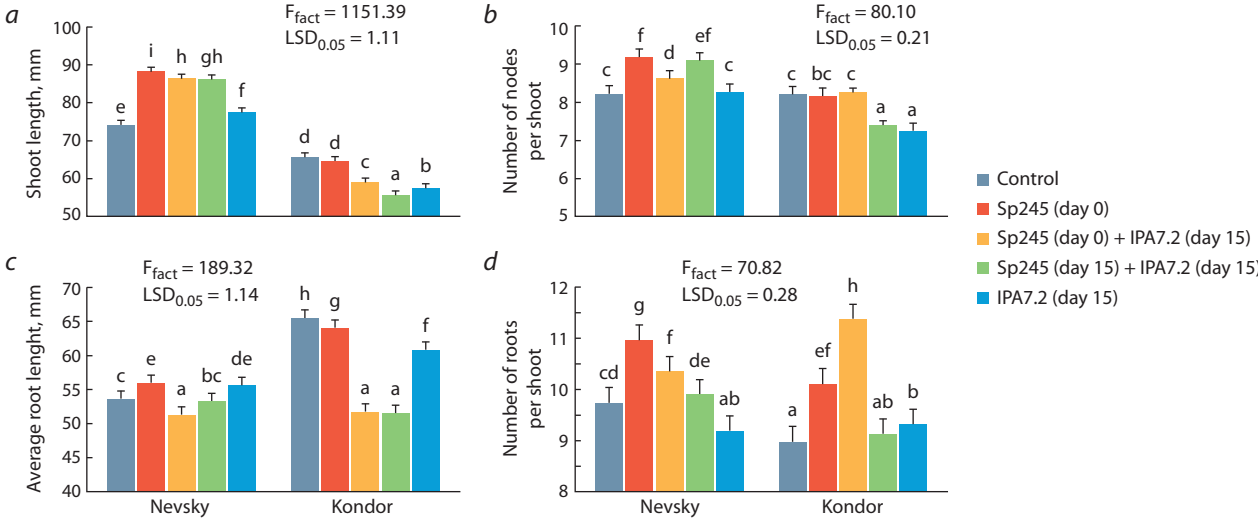

Fig. 1. Effect of *in vitro* inoculation with *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2 on morphological variables of potato microplants: *a*, shoot length; *b*, number of nodes per shoot; *c*, average root length; *d*, number of roots per shoot.

Here and in the Figures 3–5: for all variables, a significance level *p* of 0.05 ($n = 30$) was used. Different Latin letters (a, b, c, etc.) indicate values from treatments that differ significantly according to multiple comparison by Duncan's test.

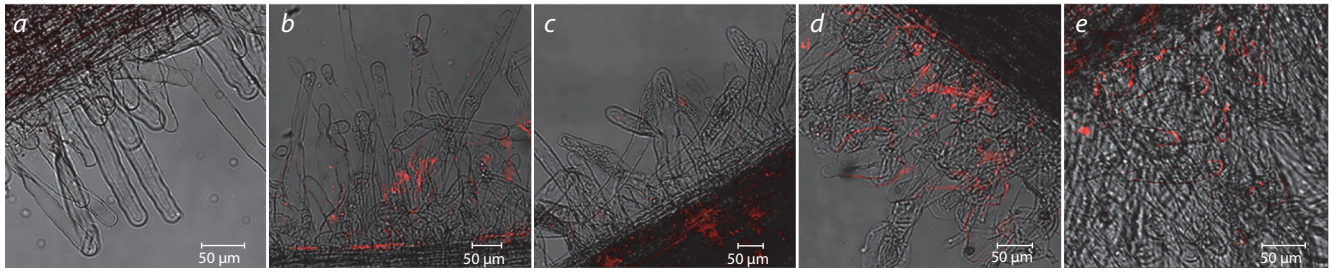

Fig. 2. Identification of bacteria on roots of potato microplants by immunofluorescence confocal microscopy: *a*, control (no inoculation), antibodies to *A. baldaniorum* Sp245 and antibodies to *O. cytisi* IPA7.2; *b*, inoculation with *A. baldaniorum* Sp245, antibodies to *A. baldaniorum* Sp245; *c*, coinoculation with *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2, antibodies to *A. baldaniorum* Sp245; *d*, coinoculation with *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2, antibodies to *O. cytisi* IPA7.2; *e*, inoculation with *O. cytisi* IPA7.2, antibodies to *O. cytisi* IPA7.2.

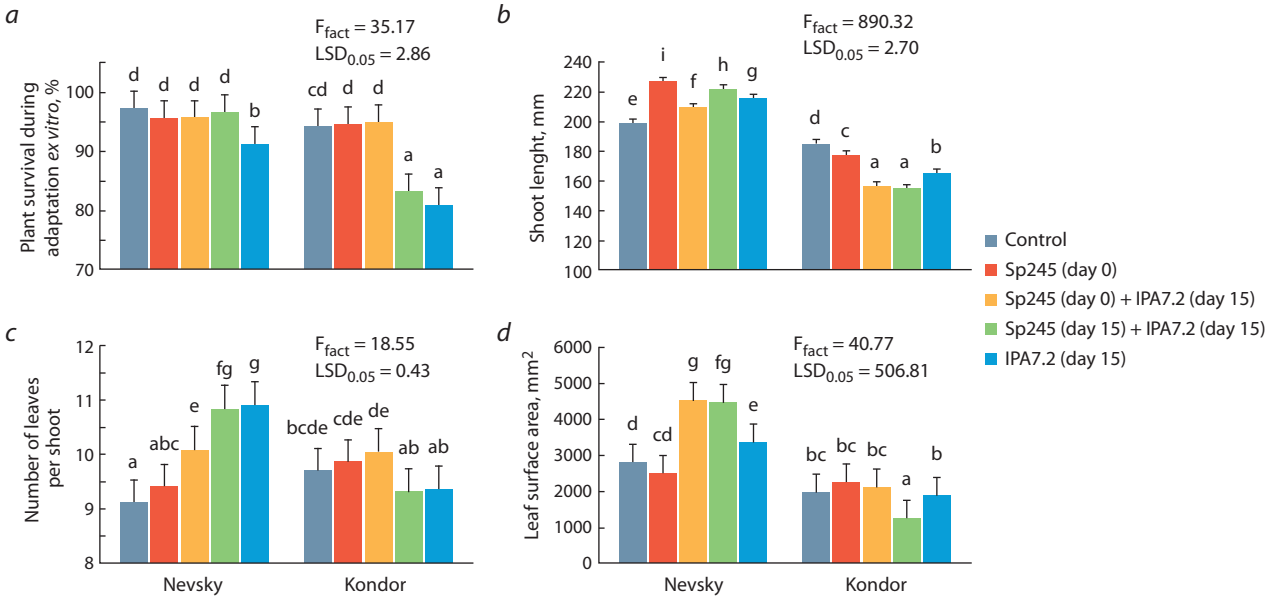

Fig. 3. Effect of inoculation with *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2 on morphological variables of potato microplants during plant adaptation *ex vitro*: *a*, plant survival during adaptation *ex vitro*; *b*, shoot length; *c*, number of leaves per shoot; *d*, leaf surface area.

Under *ex vitro* conditions, we found significant genotype effects on all variables examined. The Nevsky cultivar formed larger shoots with more large leaves than the Kondor cultivar (see Fig. 3).

In Nevsky, all inoculation treatments promoted shoot length (see Fig. 3, *b*). With *A. baldaniorum* Sp245 alone, shoot height increased by 14 %, as compared with the control; with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day15) inoculated sequentially, by 5 %; with *A. baldaniorum* Sp245 (day 15) and *O. cytisi* IPA7.2 (day 15) inoculated simultaneously, by 11.5 %; with *O. cytisi* IPA7.2 alone, by 8 %. In Kondor, all inoculation treatments suppressed shoot length (by 4 to 16 %).

The number of leaves per shoot (see Fig. 3, *c*) did not differ from the control value in any of the experimental treatments in Kondor. In Nevsky, on the contrary, all inoculation treatments promoted this variable, except for the use of *A. baldaniorum* Sp245 alone, in which no significant differences from the control were found. The Nevsky plants inoculated sequentially with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15) formed 10.5 % more leaves than did the control plants. The Nevsky plants inoculated simultaneously with *A. baldaniorum* Sp245 (day 15) and *O. cytisi* IPA7.2 (day 15) formed 18.7 % more leaves than did the control plants. The Nevsky plants inoculated with *O. cytisi* IPA7.2 alone had 19.4 % more leaves than did the control plants.

In Kondor, the leaf surface area (see Fig. 3, *d*) did not differ from the control value in any treatment except the simultaneous inoculation with *A. baldaniorum* Sp245 (day 15) and *O. cytisi* IPA7.2 (day 15), in which a 36.6 % negative effect was recorded. In Nevsky, the leaf surface area was promoted with *O. cytisi* IPA7.2 alone and with *A. baldaniorum* Sp245 coinoculated with *O. cytisi* IPA7.2 (in both coinoculation treatments, the leaf surface area was 60 % larger than that in the control). The Nevsky plants inoculated with *O. cytisi* IPA7.2 alone had larger leaves (by 19 %) than did the control plants.

Thus, the effects of microplant inoculation under *in vitro* conditions and during *ex vitro* adaptation depended significantly on the plant genotype. In Nevsky, all variables were promoted with *O. cytisi* IPA7.2 alone and in combination with *A. baldaniorum* Sp245. In Kondor, the effect was negative, or the plants did not differ from the control ones.

Effect of bacteria on microplant growth in the soil-based greenhouse and on minituber yield

The survival ability of plants in the soil-based greenhouse was significantly lower than that in the vessels under controlled conditions (Fig. 4, *a*), because environmental factors were uncontrolled and depended on the surrounding milieu. In Nevsky, survival ranged from 30 to 64 %; in Kondor, it was even lower – 18.33 to 25 %. In Nevsky, plant survival in the soil-based greenhouse was promoted by inoculation with *O. cytisi* IPA7.2 alone (by 1.5 times) and with *O. cytisi* IPA7.2 combined with *A. baldaniorum* Sp245 (by 1.2 and 1.7 times). In Kondor, the inoculation results did not differ from the control values.

As in the previous stages, the Kondor cultivar had significantly less green matter than did the Nevsky cultivar. Under greenhouse conditions, the positive effect of inoculation was stronger than in the previous growing stages (see Fig. 4). In Kondor, shoot length was suppressed by 11 % only after inoculation with *O. cytisi* IPA7.2 alone. In Kondor, no significant effects were observed in two experimental treatments: *A. baldaniorum* Sp245 alone (shoot length) and *A. baldaniorum* Sp245 (day 0) combined with *O. cytisi* IPA7.2 (day 15) (leaf area). In the other treatments, inoculation led to positive effects.

In both cultivars, shoot length (see Fig. 4, *b*) was promoted by sequential inoculation with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15) (by 57.1 and 27.5 %, respectively) and by simultaneous inoculation (day 15) (by 60.6 and 13.8 %, respectively).

In both cultivars, the leaf number (see Fig. 4, *c*) was promoted the most after simultaneous inoculation with *A. baldaniorum* Sp245 (day 15) and *O. cytisi* IPA7.2 (day 15) (by 80.5 and 51.1 %, respectively).

In both cultivars, the leaf surface area (see Fig. 4, *d*) increased in most inoculation treatments, but the increase was greatest with *O. cytisi* IPA7.2 alone (by 71.0 % in Nevsky and by 41.0 % in Kondor).

Tuber size was promoted the most with *O. cytisi* IPA7.2 alone (in Kondor) and with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15) inoculated sequentially (in both cultivars) (Fig. 5*a*, *b*). In Kondor, the larger minituber diameter was increased the most with *O. cytisi* IPA7.2 alone (by 41.9 %), and in Nevsky, it was increased the most with *A. baldaniorum* Sp245 (day 15) and *O. cytisi* IPA7.2 (day 15) inoculated simultaneously (by 12.5 %).

In most experimental treatments, the weight of minitubers (see Fig. 5, *b*) did not differ from that in the control. In Nevsky, tuber weight was suppressed after inoculation with *A. baldaniorum* Sp245 alone (by 70.5 %) and after simultaneous inoculation with *A. baldaniorum* Sp245 (day 15) and *O. cytisi* IPA7.2 (day 15) (by 20.5 %). In Kondor, tuber weight increased by 48.7 % after inoculation with *O. cytisi* IPA7.2 alone (day 15). Thus, in Kondor, both size and weight of minitubers were promoted by *O. cytisi* IPA7.2.

In Kondor, the minituber yield was lower than that in Nevsky, in agreement with the morphometric variables in all previous stages (see Fig. 5, *c*). Yet, in the higher-yielding Nevsky cultivar inoculation increased the minituber yield to a lesser extent than in the lower-yielding Kondor cultivar. The positive effect of microplant inoculation *in vitro* was maximal after sequential inoculation with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15). In this inoculation treatment, the yield of minitubers per square meter increased by 11.1 % in Nevsky and 6.8-fold in Kondor.

In these experiments, between 2.67 and 9.33 tubers were obtained per plant (see Fig. 5, *d*). The number of tubers per plant did not differ significantly between cultivars. The sequential inoculation with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15) affected Nevsky and Kondor 3.5- and 1.5-fold, respectively. In Nevsky, the number of minitubers per plant was also increased with *A. baldaniorum* Sp245 alone. In Kondor, the number of minitubers per plant was also increased with *O. cytisi* IPA7.2 alone (1.9-fold).

Thus, sequential inoculation *in vitro* of microplants with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15)

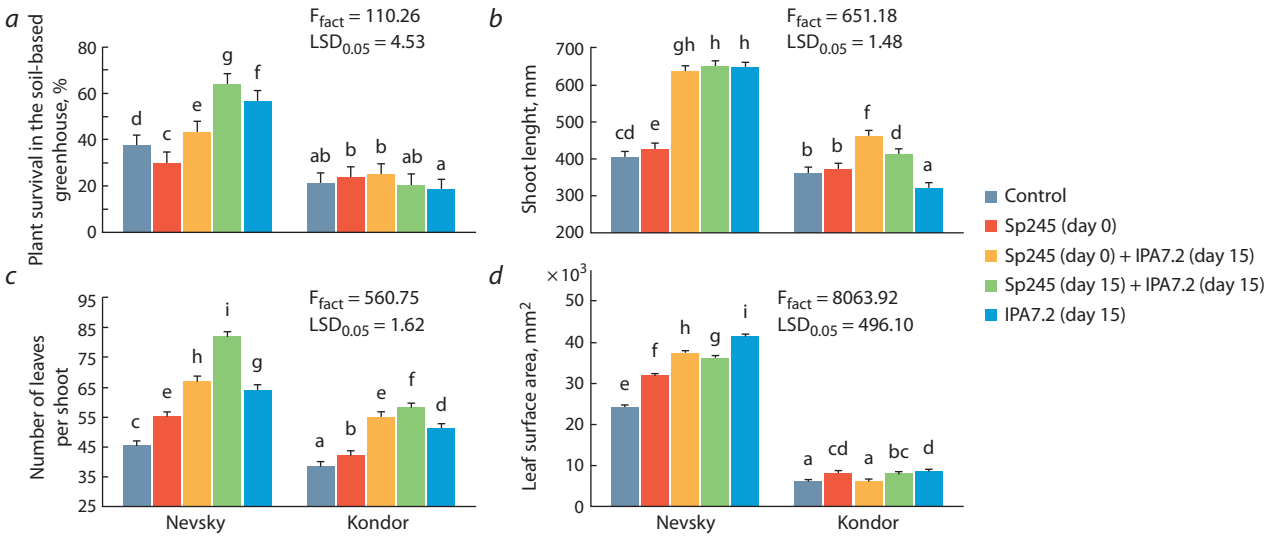

Fig. 4. Effect of inoculation with *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2 on variables of potato microplants grown in the soil-based greenhouse: *a*, plant survival in the soil-based greenhouse; *b*, shoot length; *c*, number of leaves per shoot; *d*, leaf surface area.

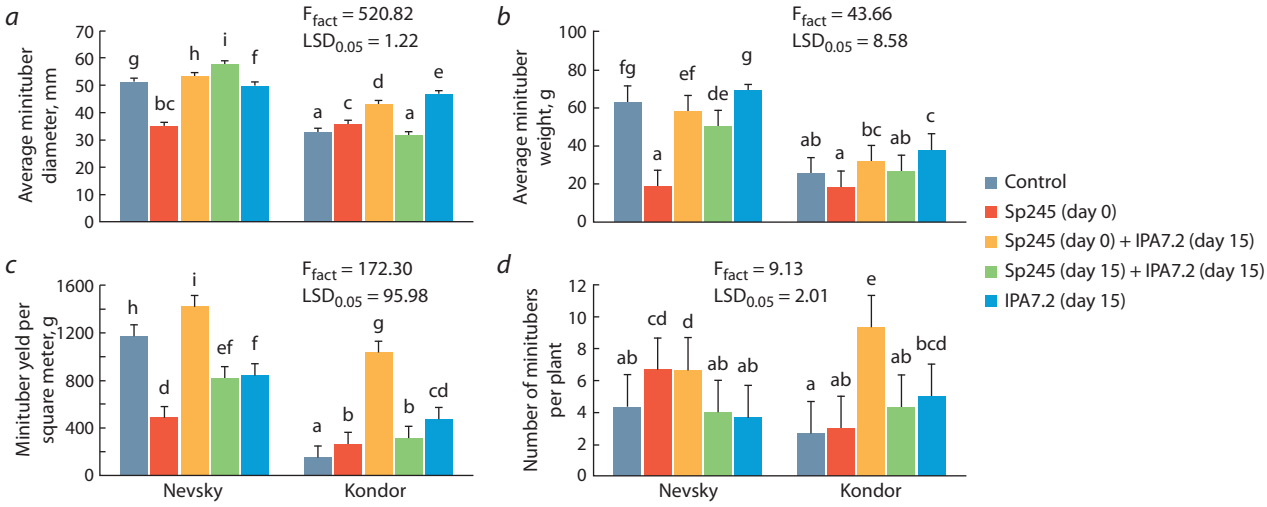

Fig. 5. Effect of inoculation with *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2 on minituber yield in potato plants grown in the soil-based greenhouse: *a*, average minituber diameter; *b*, average minituber weight; *c*, minituber yield per square meter; *d*, number of minitubers per plant.

significantly increased the weight and number of minitubers, which constitute healthy and unconventional planting material.

Discussion

Generation of healthy planting material is important in potato production technology. The clonal micropropagation of pathogen-free plants by growing apical meristems *in vitro* is obligatory in potato seed production. The effectiveness of this method can be increased by using rhizosphere bacteria. The data in the literature indicate that bacteria promote plant growth at all stages, including growth *in vitro* and *in vivo*, and they also promote the adaptive ability of microplants planted in nonsterile settings *ex vitro* (Oswald et al., 2010; Belimov et al., 2015; Santiago et al., 2017; Soumare et al., 2021).

Our previous studies have shown that the associative rhizospheric bacteria *A. baldaniorum* Sp245 (Tkachenko et al., 2015) and *O. cytisi* IPA7.2 (Burygin et al., 2019) can be used to promote the growth of potato microplants *in vitro* and *ex vitro*. The ability of *Azospirillum* bacteria to promote potato growth and productivity, including in the seed production system, is well known (Naqqash et al., 2016; Kargapolova et al., 2020; Tkachenko et al., 2021). The efficacy of use of these bacteria is higher *in vitro* (optimal conditions) but is lower when plants are grown in the field (Bacilio et al., 2017).

Our results also show that as compared to the other treatments, inoculation with *A. baldaniorum* Sp245 alone better stimulated the growth of Nevsky microplants *in vitro* (optimal conditions) than it did *ex vitro* or in soil under greenhouse conditions. *A. baldaniorum* Sp245 was isolated from wheat roots (Baldani et al., 1983; Ferreira et al., 2020) and is a model for many studies. Our data show that this strain has a high ability to produce the plant hormone indole-3-acetic

acid, which explains its promotion of the growth of microplant roots (Kargapolova et al., 2020).

O. cytisi IPA7.2, which we isolated directly from potato roots and which is native to the soils of Saratov Region, is more resistant to stress than *Azospirillum* (Burygin et al., 2017, 2019). This strain withstands large fluctuations in temperature and high salt and herbicide, which explains its ability to protect plants from stress, including osmotic stress (Evseeva et al., 2019).

The effect of bacterial inoculation on the formation and linear growth of plant organs (shoots and roots) depends on the hormonal balance existing in the plant at the moment. This balance is determined by genetic features, environmental factors, and the changes that specific strains cause in it (Arkhipova et al., 2020). Therefore, the promotion of shoot growth does not always coincide with that of root growth, and the effect of different strains may differ for different plant genotypes.

Combining different strains (e. g., azospirilla with other microsymbionts) for plant inoculation is considered promising owing to the possible synergistic effect and to the greater stability of the multicomponent system (Panahyan-e-Kivi et al., 2016; Trdan et al., 2019; Gavilanes et al., 2020). But in coinoculation, the compatibility of different strains and their ability to coinhabit plants without causing antagonism is important (O'Brien, Harrison, 2021). The efficacy of inoculation depends on plant genotype, development stage, and external and internal conditions (Andreote et al., 2010).

Previous work by us has found that the inoculation stage depends on the characteristics of the strain used (Burygin et al., 2018). *A. baldaniorum* Sp245 cannot grow independently on a nutrient growth medium for microplants and therefore can be used for inoculation at any stage of microplant growth *in vitro*. *O. cytisi* IPA7.2 can grow intensely on a nutrient growth medium for microplants and therefore can be used for inoculation only in the second half of the culturing period. Therefore, we examined two options for coinoculating microplants with *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2: simultaneous inoculation (day 15 of growth) and sequential inoculation (*A. baldaniorum* Sp245 on day 0 and *O. cytisi* IPA7.2 on day 15 of growth). Our results show that *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2 can be simultaneously present on potato roots without being antagonistic to each other (see Fig. 2). Synergistic effect, however, was not observed in all treatments, and it depended on the growth stage and the potato cultivar. Under *in vitro* conditions (see Fig. 1), the effects of the coinoculation treatments on most variables were not greater than the effect of each strain used separately, including *A. baldaniorum* Sp245, which was a good promoter of the growth of the Nevsky microplants.

In coinoculation, the adaptation ability of the microplants under favorable laboratory conditions at the stage of planting *ex vitro* (see Fig. 3) remained at the control level or at the level of the effects produced by the strains separately. But under the stressful conditions of the soil-based greenhouse, including poorly controlled environmental factors, the protective effect of inoculation was more pronounced (see Fig. 4), at least in Nevsky. In particular, after simultaneous inoculation, the survival ability of the Nevsky plants increased the most (by 71 %) – an effect greater than the positive effect of inoculation with *O. cytisi* IPA7.2 alone by almost 20 %.

Positive effect of coinoculation on the number and area of leaves was observed for the Nevsky cultivar during adaptation *ex vitro* (see Fig. 3), and the effect on the leaf area was synergistic. The promoting effect of inoculation was maximal under unfavorable greenhouse conditions (see Fig. 4), in agreement with the data of Cesari et al. (2019), who reported increased plant tolerance to stress under the influence of inoculation, including coinoculation with a bacterial consortium containing azospirilla. Coinoculation promoted the growth variables of both cultivars at the same level or even greater than did inoculation with *O. cytisi* IPA7.2 alone.

The efficacy of the whole technology of production of healthy potato planting material ultimately depends on the yield of minitubers. In seed breeding, it is not so much the weight of minitubers that matters as it is their number on plants, because the number of minitubers per plant determines the coefficient and rate of seed multiplication. The effect of inoculation on minituber production was particularly strong and was evident in both cultivars (see Fig. 5). The average minituber size changed nonsignificantly, but the number of minitubers on the plants increased significantly after sequential inoculation with *A. baldaniorum* Sp245 (day 0) and *O. cytisi* IPA7.2 (day 15). In Nevsky, the yield of minitubers was increased 1.5-fold; in Kondor, 3.5-fold. In Kondor, the sequential inoculation had a synergistic effect, as compared with the effects of the strains used separately. Similar synergistic effect on the yield of minitubers per square meter was noted in both cultivars in the same inoculation treatment.

The effects of sequential inoculation with *A. baldaniorum* Sp245 (microcuttings, day 0) and *O. cytisi* IPA7.2 (day 15) differed from those of simultaneous inoculation (day 15) at different stages of microplant growth. However, considering the promoting effect of *A. baldaniorum* Sp245 *in vitro* and the final yield of minitubers, sequential inoculation can be regarded as preferable.

Conclusions

Analysis of the experimental data shows that the bacterial strains *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2, both individually and in combination, had a positive effect on potato microplants. This effect manifested itself differently at different stages of plant growth. The maximal positive effect of inoculation *in vitro* was that on the number of adventitious roots; the number and area of leaves (during plant adaptation *ex vitro*); and the weight of minitubers and all variables for the vegetative portion of shoots (during plant growth in the soil-based greenhouse). The two strains were not antagonistic to each other. The growth-promoting effect of the bacteria depended significantly on the potato genotype. The positive effect of the interstrain interaction was maximal when plants were grown in the open ground. The strains *A. baldaniorum* Sp245 and *O. cytisi* IPA7.2, separately and in combination, can be recommended as inoculants for *in vitro*-grown potato microplants in potato clonal micropropagation to produce healthy planting material.

References

Andreote F.D., Rocha U.N., Araújo W.L., Azevedo J.L., van Overbeek L.S. Effect of bacterial inoculation, plant genotype and developmental stage on root-associated and endophytic bacterial communities in potato (*Solanum tuberosum*). *Antonie Leeuwenhoek*. 2010; 97(4):389-399. DOI 10.1007/s10482-010-9421-9.

Arkhipova T.N., Evseeva N.V., Tkachenko O.V., Burygin G.L., Vysotskaya L.B., Akhtyamova Z.A., Kudoyarova G.R. Rhizobacteria inoculation effects on phytohormone status of potato microclones cultivated *in vitro* under osmotic stress. *Biomolecules*. 2020;10(9): 1231. DOI 10.3390/biom10091231.

Bacilio M., Moreno M., Lopez-Aguilar D.R., Bashan Y. Scaling from the growth chamber to the greenhouse to the field: demonstration of diminishing effects of mitigation of salinity in peppers inoculated with plant growth-promoting bacterium and humic acids. *Appl. Soil Ecol*. 2017;119:327-338. DOI 10.1016/j.apsoil.2017.07.002.

Baldani V.L.D., Baldani J.I., Döbereiner J. Effects of *Azospirillum* inoculation on root infection and nitrogen incorporation in wheat. *Can. J. Microbiol.* 1983;29(8):924-929. DOI 10.1139/m83-148.

Belimov A.A., Dodd I.C., Safronova V.I., Shaposhnikov A.I., Azarova T.S., Makarova N.M., Davies W.J., Tikhonovich I.A. Rhizobacteria that produce auxins and contain 1-amino-cyclopropane-1-carboxylic acid deaminase decrease amino acid concentrations in the rhizosphere and improve growth and yield of well-watered and water-limited potato (*Solanum tuberosum*). *Ann. Appl. Biol*. 2015; 167(1):11-25. DOI 10.1111/aab.12203.

Burygin G.L., Kargapolova K.Y., Evseeva N.V., Tkachenko O.V. Peculiarities of plant inoculation with rhizosphere bacteria as a factor increasing the efficacy of potato microclonal propagation. *Vestnik Biotehnologii i Fiziko-Khimicheskoy Biologii im. Y.A. Ovchinnikova = Yu.A. Ovchinnikov Bulletin of Biotechnology and Physical and Chemical Biology*. 2018;14(2):12-16. (in Russian)

Burygin G.L., Kargapolova K.Y., Kryuchkova Y.V., Avdeeva E.S., Gogoleva N.E., Ponomaryova T.S., Tkachenko O.V. *Ochrobactrum cytisi* IPA7.2 promotes growth of potato microplants and is resistant to abiotic stress. *World J. Microbiol. Biotechnol.* 2019;35(4):55. DOI 10.1007/s11274-019-2633-x.

Burygin G.L., Popova I.A., Kargapolova K.Y., Tkachenko O.V., Matora L.Y., Shchyogolev S.Y. A bacterial isolate from the rhizosphere of potato (*Solanum tuberosum* L.) identified as *Ochrobactrum lupini* IPA7.2. *Agric. Biol.* 2017;52(1):105-115. DOI 10.15389/agrobiology.2017.1.105eng.

Castillo-Pérez L.J., Martínez-Soto D., Fortanelli-Martínez J., Carranza-Álvarez C. Asymbiotic seed germination, in vitro seedling development, and symbiotic acclimatization of the Mexican threatened orchid *Stanhopea tigrina*. *Plant Cell Tissue Organ Cult*. 2021;146: 249-257. DOI 10.1007/s11240-021-02064-9.

Cesari A.B., Paulucci N.S., López-Gómez M., Hidalgo-Castellanos J., Plá C.L., Dardanelli M.S. Performance of *Bradyrhizobium* and *Bradyrhizobium–Azospirillum* in alleviating the effects of water-restrictive conditions during the early stages of *Arachis hypogaea* growth. *J. Plant Growth Regul*. 2019;38:1362-1374. DOI 10.1007/s00344-019-09939-4.

Dias A.C.F., Costa F.E.C., Andreote F.D., Lacava P.T., Teixeira M.A., Assumpção L.C., Araújo W.L., Azevedo J.L., Melo I.S. Isolation of micropropagated strawberry endophytic bacteria and assessment of their potential for plant growth promotion. *World J. Microbiol. Biotechnol*. 2009;25:189-195. DOI 10.1007/s11274-008-9878-0.

Döbereiner J., Day J.M. Associative symbioses in tropical grasses: characterization of microorganisms and nitrogen-fixing sites. In: Proceedings of the 1st Int. Symp. on Nitrogen Fixation. Washington State Univ., 1976;518-538.

Evseeva N.V., Tkachenko O.V., Denisova A.Y., Burygin G.L., Veselov D.S., Matora L.Y., Shchyogolev S.Y. Functioning of plant-bacterial associations under osmotic stress in vitro. *World J. Microbiol. Biotechnol*. 2019;35:195. DOI 10.1007/s11274-019-2778-7.

Ferreira N.D.S., Sant'Anna F.H., Reis V.M., Ambrosini A., Volpiano C.G., Rothballer M., Schwab S., Baura V.A., Balsanelli E., Pedrosa F.O., Passaglia L.M.P., de Souza E.M., Hartmann A., Cassan F., Zilli J.E. Genome-based reclassification of *Azospirillum brasilense* Sp245 as the type strain of *Azospirillum baldaniorum* sp. nov. *Int. J. Syst. Evol. Microbiol.* 2020;70(12):6203-6212. DOI 10.1099/ijsem.0.004517.

Gavilanes F.Z., Andrade D.S., Zucareli C., Horácio E.H., Yunes J.S., Barbosa A.P., Alves L.A.R., Cruzatty L.G., Maddela N.R., Guimarães M.F. Co-inoculation of *Anabaena cylindrica* with *Azospirillum brasilense* increases grain yield of maize hybrids. *Rhizosphere*. 2020;15:100224. DOI 10.1016/j.rhisph.2020.100224.

Kargapolova K.Y., Burygin G.L., Tkachenko O.V., Evseeva N.V., Pukhalskiy Y.V., Belimov A.A. Effectiveness of inoculation of in vitro-grown potato microplants with rhizosphere bacteria of the genus *Azospirillum*. *Plant Cell Tissue Organ Cult*. 2020;141:351-359. DOI 10.1007/s11240-020-01791-9.

Murashige T., Skoog G. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant*. 1962;15(3):473-497. DOI 10.1111/j.1399-3054.1962.tb08052.x.

Naqqash T., Hameed S., Imran A., Hanif M.K., Majeed A., van Elsas J.D. Differential response of potato toward inoculation with taxonomically diverse plant growth promoting rhizobacteria. *Front. Plant Sci*. 2016;7:144. DOI 10.3389/fpls.2016.00144.

O'Brien A.M., Harrison T.L. Host match improves root microbiome growth. *Nat. Microbiol.* 2021;6(9):1103-1104. DOI 10.1038/s41564-021-00957-1.

Oliveira A.L.M., Urquiaga S., Döbereiner J., Baldani J.I. The effect of inoculating endophytic N_2-fixing bacteria on micropropagated sugarcane plants. *Plant Soil*. 2002;242:205-215. DOI 10.1023/A:1016249704336.

Orlikowska T., Nowak K., Reed B. Bacteria in the plant tissue culture environment. *Plant Cell Tissue Organ Cult*. 2017;128:487-508. DOI 10.1007/s11240-016-1144-9.

Oswald A., Calvo V.P., Davila D.Z., Pineda J.A. Evaluating soil rhizobacteria for their ability to enhance plant growth and tuber yield in potato. *Ann. Appl. Biol*. 2010;157(2):259-271. DOI 10.1111/j.1744-7348.2010.00421.x.

Panahyan-e-Kivi M., Raei Y., Hassanpanah D. Study the effect of growth promoting bacteria (GPRB) on number and weight of mini-tubers of *Solanum tuberosum* cultivars in greenhouse conditions. *J. Fundam. Appl. Sci.* 2016;8(2S):28-38. DOI 10.4314/jfas.v8i2s.556.

Rajasekharan P.E., Sahijram L. Plant biology and biotechnology. In: Plant Biology and Biotechnology. Vol. II. Plant genomics and biotechnology. New Delhi: Springer, 2015:417-443. DOI 10.1007/978-81-322-2283-5_30.

Santiago C.D., Yagi S., Ijima M., Nashimoto T., Sawada M., Ikeda S., Asano K., Orikasa Y., Ohwada T. Bacterial compatibility in combined inoculations enhances the growth of potato seedlings. *Microbes Environ*. 2017;32(1):14-23. DOI 10.1264/jsme2.ME16127.

Shelud'ko A.V., Shirokov A.A., Sokolova M.K., Sokolov O.I., Petrova L.P., Matora L.Y., Katsy E.I. Wheat root colonization by *Azospirillum brasilense* strains with different motility. *Microbiology.* 2010;79(5):688-695. DOI 10.1134/S0026261710050140.

Soumare A., Diédhiou A.G., Arora N.K., Al-Ani L.K.T., Ngom M., Fall S., Hafidi M., Ouhdouch Y., Kouisni L., Sy M.O. Potential role and utilization of plant growth promoting microbes in plant tissue culture. *Front. Microbiol*. 2021;12:649878. DOI 10.3389/fmicb.2021.649878.

Thomas J., Ajay D., Kumar R.R., Mandal A.K.A. Influence of beneficial microorganisms during in vivo acclimatization of in vitro-derived tea (*Camellia sinensis*) plants. *Plant Cell Tissue Organ Cult*. 2010;101:365-370. DOI 10.1007/s11240-010-9687-7.

Tkachenko O.V., Evseeva N.V., Boikova N.V., Matora L.Y., Burygin G.L., Lobachev Y.V., Shchyogolev S.Y. Improved potato microclonal reproduction with the plant-growth promoting rhizobacteria *Azospirillum*. *Agron. Sustain. Develop*. 2015;35:1167-1174. DOI 10.1007/s13593-015-0304-3.

Tkachenko O.V., Evseeva N.V., Terentyeva E.V., Burygin G.L., Shirokov A.A., Burov A.M., Matora L.Y., Shchyogolev S.Y. Improved production of high-quality potato seeds in aeroponics with plant-growth-promoting rhizobacteria. *Potato Res*. 2021;64:55-66. DOI 10.1007/s11540-020-09464-y.

Trdan S., Vučajnk F., Bohinc T., Vidrih M. The effect of a mixture of two plant growth-promoting bacteria from Argentina on the yield of potato, and occurrence of primary potato diseases and pest – short communication. *Acta Agric. Scand. B Soil Plant Sci.* 2019;69(1): 89-94. DOI 10.1080/09064710.2018.1492628.

Wang X., Yam T., Meng Q., Zhu J., Zhang P., Wu H., Wang J., Zhao Y., Song X. The dual inoculation of endophytic fungi and bacteria promotes seedlings growth in *Dendrobium catenatum* (Orchidaceae) under in vitro culture conditions. *Plant Cell Tissue Organ Cult*. 2016;126(3):523-531. DOI 10.1007/s11240-016-1021-6.

Yegorenkova I.V., Tregubova K.V., Burygin G.L., Matora L.Y., Ignatov V.V. Assessing the efficacy of co-inoculation of wheat seedlings with the associative bacteria *Paenibacillus polymyxa* 1465 and *Azospirillum brasilense* Sp245. *Can. J. Microbiol*. 2016;62(3):279-285. DOI 10.1139/cjm-2015-0647.

ORCID ID

K.Yu. Kargapolova orcid.org/0000-0002-6040-9401
O.V. Tkachenko orcid.org/0000-0001-8327-6763
G.L. Burygin orcid.org/0000-0001-8031-9641
N.V. Evseeva orcid.org/0000-0002-3973-6766
A.A. Shirokov orcid.org/0000-0003-4321-735X
L.Yu. Matora orcid.org/0000-0001-5654-8292
S.Yu. Shchyogolev orcid.org/0000-0002-1084-312X

Acknowledgements. This work was supported by the Russian Foundation for Basic Research (grant No. 19-016-00116).

Conflict of interest. The authors declare no conflict of interest.

Received November 12, 2021. Revised March 10, 2022. Accepted April 28, 2022.