

Identification and pathogenicity of *Raoultella* and *Pseudomonas* species associated with roots of *Taraxacum kok-saghyz*

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Abstract

Natural rubber (NR) is a critical raw material essential for the production of thousands different rubber and latex products. In most cases, it cannot be replaced by synthetic rubber alternatives. At the present time, NR is produced solely from a single species, *Hevea brasiliensis*, which is grown in tropical regions. Several important reasons including the danger that South American Leaf Blight disease might spread to Southeast Asia stimulate the search for alternative rubber producers. One of them, *Taraxacum kok-saghyz*, attracts particular attention. In this study, performed in an aeroponic phytotron, as well as *in vitro* culture, we identified two bacteria associated with root rot of the NR-producing plant *T. kok-saghyz* - *Pseudomonas putida* and *Raoultella terrigena*. According to the literature, interaction of these bacteria with plants is described as symbiosis. However, our data suggest that under certain conditions well-characterized endophytic bacteria can act as pathogens. We showed that plants, cured of phytopathogens, demonstrate fast growth rates, even in at high summer daytime temperatures. *T. kok-saghyz* continued to grow (defoliation did not occur) and to build up a biomass, which may lead to an increase in the accumulation of NR and inulin in the roots. Our research demonstrates that aeroponic cultivation is a promising way to grow *T. kok-saghyz*, for production of NR and inulin. In this study we show that plant-associated microorganisms are an important factor influencing plant responses to changes in cultivation conditions.

Introduction

Fundamental study of physiological and biochemical mechanisms underlying the functioning of biological systems is the basis of modern biotechnology. Today, controlled biosynthesis provides production of many target products (substances for pharmacological preparations, human and animal nutrition components, enzymes, fuels etc.) with the necessary quantitative and qualitative properties. New methods for production of natural rubber (NR) from alternative sources are also a strategically important goal of biotechnological community.

NR is a critical raw material essential for the production of more than 50,000 different rubber and latex products. In most cases, it cannot be replaced by synthetic rubber alternatives (Cherian et al., 2019). It possesses unique properties such as elasticity, resiliency, shock resistance, effective heat dispersion, and ability to maintain plasticity at low temperature (Eng & Ong, 2000; McIntyre et al., 2000; Puskas, 2001; Gronover et al., 2011). NR is an isoprenoid because its basic backbone structure composed exclusively of 1,4-polymers of the isoprene unit (C_5H_8) with a *cis*-double bond (Tanaka, 2001; Yamashita & Takahashi, 2020). More than 2500 plant species produce NR (Cornish, 2001); however, only few of them are able to synthesize a high-quality NR with molecular mass about 10^6 Da (Mooibroek & Cornish, 2000; van Beilen & Poirier, 2007ab). At the present time natural rubber is produced solely from a single species, the rubber tree (*Hevea brasiliensis*), which is grown as genetically similar clones in tropical regions. According to forecasts by the Association of Natural Rubber Producing Countries (ANRPC), total NR consumption is expected to reach 16.7 million tons by 2026

(<https://www.reportlinker.com/dataset/228401ad9f7e5cafa9fcca28f25d7f286b8dba1a>),

and there is no doubt that demand will continue to increase. Therefore, it cannot be ruled out that humanity will face a shortage of NR in near future. Moreover, even regardless of growing demands, NR production from *H. brasiliensis* is at risk, because only a very few closely related clones were used to establish the NR plantations (Cornish, 2017). Thus, a lack of genetic diversity puts hundreds of thousands of hectares of plantations in danger. Indeed, genetically almost identical trees are exposed to numerous phytopathogenic infections, including fungal South American Leaf Blight (SALB), caused by *Microcyclus (Pseudocercospora) ulei* (Guyot et al., 2008; Guyot & Guen, 2018). This disease is lethal for *H. brasiliensis*. It prevented NR production in Brazil, the country of origin of this species. Currently, the work on developing SALB-resistant plants is in progress, but it will take at least 25 years to introduce high NR yielding, SALB resistant trees (Cornish, 2017). Also, it should be noted that Hevea latex products cause severe allergic reactions because of proteins naturally associated with the rubber particles (Siler et al., 1996; Bousquet et al., 2006). Therefore, biodiversification of the NR sources is an essential goal that attracts increasing attention from scientific community.

Several rubber-producing species are under development to find a reliable alternative source of NR. These are Rubber dandelion (*Taraxacum kok-saghyz*) (Arias et al., 2016), Guayule (*Parthenium argentatum*) (Rousset et al., 2021), and undeservedly forgotten Tau-saghyz (*Scorzonera tau-saghyz*) (Buranov & Elmuradov, 2010). One of them, *T. kok-saghyz*, fits to obvious requirement to effective NR producer better than others. It has a very short life-cycle (6–8 months) relative to other plants, such as *H. brasiliensis*, and is amenable to genetic manipulations. Moreover, its rubber phenotype can be meaningfully analyzed after only ten weeks of cultivation (Amerik et al., 2018; Amerik et al., 2021). Remarkably, the agricultural ranges of *T. kok-saghyz* covers most of the regions of the world, and the plant shows little dependence on the climatic conditions of the growing zone. So, it was logical to further investigate this crop in order to make it more attractive for commercial cultivation (Cornish, 2017).

Numerous studies on the physical and chemical properties of the dandelion NR clearly demonstrated that it has an outstanding quality. For instance, tiers, made from the *T. kok-saghyz* NR are as resilient as those made from *H. brasiliensis* polymer (Araujo-Morera et al., 2021). Notably, molecular mass of dandelion NR is very high. It was reported that it can be as high as 2.2×10^6 Da (Mooibroek & Cornish, 2000; Hallahan & Keiper-Hrynko, 2004; Cornish, 2017). The only potential problem is a large number of associated proteins, similarly to Hevea NR (van Beilen & Poirier, 2007a). Therefore, people who are sensitized to *H. brasiliensis* NR, may also develop an allergic reaction to *T. kok-saghyz* NR (Siler et al., 1996; Bousquet et al., 2006). Thus, NR from dandelion should not be used in medical industry. It should be considered for conventional dry rubber application such as tire production (<https://www.continental.com/en/press/press-releases/20220927-forschungsnetzwerk-neuer-zuechter>). The principal tasks of improving the characteristics of this potential alternative source of NR are as follows: acceleration growth and development of plants, formation of a large root biomass, increasing the content of target products, and selection of forms resistant to phytopathogens.

To date, industrial cultivation of *T. kok-saghyz* in the field is complicated by the low productivity of plants, even under protected ground conditions, high vulnerability to bacterial and fungal pathogens, and the necessity to create special technologies for collecting and processing rubber-containing roots. Development of methods for the selective destruction of weeds is also a difficult task. Other complications include the danger of over-pollination, uneven germination of seeds, high sensitivity of plants to fluctuations in weather conditions, etc. The time frame is also important. In the field conditions, *T. kok-saghyz* is cultivated for 2 years.

The development of a new technology for growing *T. kok-saghyz* plants under controlled phytotron conditions (Fig. 1) would speed up the solution of many of these problems. The use of the aeroponic cultivation method with optimization of all factors affecting the rate of plant growth and development may lead to improvement of the biosynthesis of target products. Potentially, this approach has obvious commercial prospects.

As noted above, among several factors limiting NR production from Hevea fungal disease SALB is the most prominent one because it has a devastating impact on NR production (Guyot et al., 2008; Guyot & Guen, 2018). SALB wiped out the large-scale *H. brasiliensis* plantations in Brazil and it has not been possible to restart production since that catastrophe. The present production of NR in Brazil is the marginal 1% of world production. Accidental spread of SALB to Southeast Asia would have a severe impact on the NR industry including reduction of production by millions of tons, serious shortage, and, as a result, to drastically higher prices (Cornish, 2017).

Unfortunately, similar problems arose during the adaptation of *T. kok-saghyz* plants to the conditions of aeroponic cultivation. Plants grew normally for 60–70 days of cultivation. Then many specimens showed signs of vascular bacteriosis (withering). Darkening and maceration were observed in plants in the root area, and, finally, roots fell off the shoots (Fig. 2). It was assumed that this was happening due to increased humidity in the root zone in a phytotron. However, other processes also might be involved including intensification of plant cultivation. It is also likely that latent infection was present initially in seeds, then in plants grown from them. This led to the infected root culture of *T. kok-saghyz*. Remarkably, the "hairy roots" (HR) cultures after some time of cultivation showed clear signs of vascular bacteriosis (Fig. 3).

Here we describe isolation of pathogens from infected plants, their identification and biochemical characterization. We also discuss the results of our studies demonstrating the influence of plant growth conditions (*in vitro*, in a phytotron and in soil) on interaction of endophytic microorganisms with host plants.

Materials and Methods

Plants used in this study

To perform this study, a collection of *T. kok-saghyz* plants consisting of various population samples was created in the laboratory. Seeds and plants were received from the Vavilov Institute of Plant Genetic Resources (Saint Petersburg, Russian Federation) and from the Institute of Plant Biology and Biotechnology (Almaty, Republic of Kazakhstan). From this collection, highly productive plants with a high content of NR and inulin (the second valuable product that increases commercial value of *T. kok-saghyz*) were selected. Subsequently, with the help of chemotherapy and thermotherapy of seeds (Grondeau et al., 1994; Sanna et al., 2022), we created a collection of *T. kok-saghyz* plants cured of phytopathogens *in vitro* (Fig. 4). These plants were subsequently used to obtain a culture of transformed roots and as a planting material for adaptation and cultivation in an aeroponic phytotrons.

Manifestations of bacterial infection obviously depended on the quality of asepticization of plant explants. However, as subsequent experiments demonstrated, neither effective asepticization of plant explants nor compliance with aseptic rules guaranteed the absence of bacterial infections in *in vitro* cultures. Root rot appeared in all variants of cultivation of *T. kok-saghyz* plants - under *in vitro* conditions, in an aeroponic phytotron, in a culture of HR, as well as when in soil grown plants.

Growing plants in an aeroponic phytotron

As noted, for controlled growing of plants, automatic devices (phytotrons) have been developed (Fig. 1). Remarkably, several critical parameters including temperature, composition of the gas environment with adjustable CO₂ concentration, spectral composition and intensity of the light flux are under strict control in phytotrons (Martirosyan et al., 2023). The major principle of the aeroponic cultivation is to provide plants with mineral components without use of soil or soil substitutes. Thus, growing plants in artificial climatic conditions leads to the creation of the most favorable environment for their growth and development (Martirosyan et al., 2020; Martirosyan et al., 2023). *T. kok-saghyz* plants, grown from treated seeds *in vitro* in the phase of 3–4 true leaves (days 19–20), were adapted and planted in aeroponic phytotrons. Plants received mineral nutrition components by finely dispersed spraying a nutrient solution directly into the root system zone, balanced in composition for each phase of *T. kok-saghyz* growth. In a phytotron, plants reached full size and high NR content (up to 7–10%), in about 120 days of vegetation period (Martirosyan et al., 2023).

Creation of the root culture with the HR phenotype and *T. kok-saghyz* composite plants

It was important to investigate whether the biosynthesis rate and concentration of NR and inulin would increase with increasing root biomass. For this purpose, we transformed the *T. kok-saghyz* root culture with bacterium *Agrobacterium rhizogenes* (strain R1000). This led to the so-called HR phenotype. The culture of transformed roots is a convenient system that is often used for studying the metabolic processes of plants (Thwe et al., 2016; Khazaei et al., 2019). The resulting root cultures will be examined for NR and inulin content. Subsequently, these cultures were used for obtaining the composite plants. Specifically, roots had a pronounced HR phenotype, while the aerial parts looked like the original wild-type plants.

Vegetative growth of plants and manifestation of pathogenesis

In the process of curing *T. kok-saghyz* of the internal infection that affected the root system, two types of bacteria were initially isolated (below). Subsequently, the infectivity of the resultant bacterial cultures was analyzed. *T. kok-saghyz* roots were treated with the bacteria, and pronounced symptoms of bacteriosis were observed. The symptoms were identical to those initially observed in growing *T. kok-saghyz* plants under aeroponic conditions, as well as *in vitro*. Remarkably, the same pattern of damage was seen during vegetation experiments in soil mixture (peat, sand and soil, 2:1:1). To avoid the influence of other microorganisms on growth processes the soil was pre-autoclaved twice. Plants were grown outdoors in natural climatic conditions of Moscow region for 180 days, from May to October (Table 1). Plants were divided into 3 groups: 1 control and 2 experimental. Each group consisted of 10 vegetation vessels with plants. Experiments were repeated three times. Thus, 90 vessels were analyzed. Specifically:

1st group. Control, intact plants *in vitro*. Grown from seeds that have undergone thermotherapy and chemotherapy, without bacterial infection.

2nd group. Intact plants grown from seeds that have undergone chemotherapy and thermotherapy, infected with isolated bacteria during the vegetation season.

3rd group. Intact plants grown from seeds that have not undergone chemotherapy and thermotherapy, infected with isolated bacteria during the vegetation season.

Bacterial cultures isolated as described below were grown in a rich liquid nutrient medium. (g/l: peptone 10; yeast extract 5; NaCl 10). Cultures were incubated for 72 hours on an orbital shaker, 200 rpm, 28°C. For inoculation, plants, before the onset of the budding phase, were watered with a diluted (1:20) suspension of bacteria (approximately 100 ml per plant).

Isolation of the pathogenic microorganisms

Isolation of bacterial cells was carried out as follows. Infected plants were washed with sterile water and dried in air. Next, at the junction of the root collar with the caudex, where the symptoms of necrosis were most pronounced, transverse sections (3–5 mm thick) were made with a sterile scalpel. For surface asepticization, fragments of *T. kok-saghyz* infected roots were placed in sterile 100 ml conical flasks containing 50 ml 70% ethanol. Flasks were shaken on a rotary shaker for 120 sec. To remove residual ethanol, the plant material was washed 5 times with sterile distilled water. Then, the root fragments were transferred to sterile flasks containing 50 ml 5% sodium hypochlorite (NaClO), for 60 s. To remove residual NaClO, the plant material was washed 10 times with sterile distilled water. Finally, the plant material was placed into the 50 ml 6% H₂O₂ solution for 60 sec. To remove H₂O₂ the root fragments were where washed 5 times with 100 ml sterile H₂O. The surface asepticization procedure was done in a laminar flow hood.

Approximately 100 mg of treated roots of each sample were cut into small pieces and placed in sterile 2 ml microtubes containing sterile stainless-steel balls, (5mm diameter), for further homogenization in TissueLyser LT (Qiagen).

Next, the roots, crushed into a pulp, were centrifuged (1 min, 2500g) and 1 ml of the supernatants was transferred into sterile 15 ml tubes, then 10 ml of sterile distilled water was added. The tubes were hermetically sealed and placed on a rotary shaker (150 rpm) at 25°C for 2 h. 100 µl of each sample was plated in triplicate onto Petri dishes with LB medium and incubated at 28°C for 120 hours. Colonies were selected from each plate based on their color, texture, and morphology. To evaluate the effectiveness of surface sterilization, 100 µl of liquid after the last wash (for each analyzed sample) was plated on Luria nutrient agar.

Biochemical identification of the plant pathogens (Ksz-1, Ksz-2)

For identification of isolated bacterial cultures, named Ksz-1 and Ksz-2, biochemical approaches were employed. The API 20 NE (Biomérieux, France) system facilitates identification of non-fastidious Gram-negative rod bacteria not belonging to the *Enterobacteriaceae*. The API 20 NE strip consists of microtubes containing dehydrated media and substrates. The media microtubes containing conventional tests were inoculated with a bacterial suspension which reconstituted the media. After incubation, the metabolic end products were detected by indicator systems or addition of reagents. The substrate microtubes contained assimilation tests and were inoculated with a minimal medium. If bacteria were capable of utilizing the corresponding substrate, then they would grow. Briefly, 5ml of distilled water was added to the honeycombed wells of the tray of an incubation box to create a humid atmosphere. Then API 20 NE strip was placed in the box. 1–4 colonies of identical morphology were resuspended in 2ml of API 0.85% saline in a sterile test tube. All tests (GLU, ADH, URE, ESC, GEL, PNPG, NO₃, TRP etc.) were performed as described in the manufacturer's manual. The APIWEB™ software was employed for identification of the organisms.

API 10 S (Biomérieux, France) is a qualitative standardized system intended of identification of *Enterobacteriaceae* and other Gram-negative rod bacteria. The protocol API 10 S is very similar to the protocol described above. All tests were done according to the manufacturer's manual.

Molecular-biological identification of the *T. kok-saghyz* pathogens, PCR and sequencing

For the preliminary identification of the bacterial species PCR amplification and sequencing of the variable 16S rDNA (Yamamoto & Harayama, 1998) region of Ksz-1 and Ksz-2 were performed using conservative primers 8f – agagtttgatcctggctcag and 926r – ccgtcaattccttagagttt. The amplification was conducted as follows: denaturation at 95°C for 3 min followed by 35 cycles of 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 1 min 30 s; with a final extension at 72°C for 5 min. Partial nucleotide sequences of the 16S rRNA genes were determined directly from the PCR fragments according to the method of Edwards (Edwards et al., 1989).

For further characterization of the *Pseudomonas* species (Ksz-1), PCR amplification of the *gyr B* and *rpo D* variable gene fragments (Yamamoto & Harayama, 1998; Yamamoto et al., 2000). was performed using conservative pairs of primers ggagcagtagcatcaaggacga/ ggggccatgggtgggtttccacA and cctccgaaggtggccgtctgtc/ ACATGCGCAGGAAGTCGGCACG for *gyr B* and *rpo D* respectively. *gyrB* encodes subunit B of DNA gyrase, *rpoD* – sigma factor of RNA polymerase. The amplification was conducted as described above. Amplified DNA fragments were gel purified using QIAquick Gel Extraction Kit (Qiagen) and cloned into pAL2-T plasmid (Eurogen). Nucleotide sequences of the cloned DNAs were determined using plasmid specific SP6 – ATTTAGGTGACACTATAGAATACT and T7 – GTAATACGACTCACTATAGGG primers. Identification of the *Raoultella* species (Ksz-2) was conducted similarly.

The BLASTn software was employed to compare determined sequences to GenBank.

Inoculation of a culture of isolated HR and plants *in vitro* with bacteria isolated from the roots of *T. kok-saghyz*

An inoculation media was prepared from bacteria grown on Petri dishes with LB media for 24 hours. Bacteria were resuspended in 10 mM MgCl₂, Silwet L-77 was added at a final concentration of 200 ml l⁻¹. OD 600 of inoculation media was 0.05 (approximately 2.5 × 10⁷ colony forming units per ml (CFU ml⁻¹). The fragments of healthy HR and *in vitro* intact plants were placed in 100 ml conical flasks containing 50 ml of inoculation media. Flasks were placed in a vacuum chamber and vacuuming was carried out at 0.8 atm for 120 sec. Next, HR cultures and plants were washed three times with 100 ml sterile distilled water. Washed HR-infected cultures were placed on Petri dishes, and plants were placed in phytocontainers on agar with minimal MS medium (Murashige-Skoog), which does not contain carbohydrates. So, the only source of carbon was the roots of *T. kok-saghyz*. Incubation of the dishes was carried out at 28 °C for 10 days, phytocontainers - for 3 days. Number of repetitions – 10. Then the dishes were placed on light racks (light intensity of 100 μmol/m²s) for 25 days. Phytocontainers were placed on light racks for 32 days. The development of symptoms of bacteriosis (darkening) of the roots were monitored. Dishes with uninfected HR roots on MS medium and with bacterial cultures on carbohydrate-free MS medium were used as a control. Phytocontainers with healthy plants of an appropriate age in complete MS medium served as another control.

Results

Molecular biological and biochemical identification of the *T. kok-saghyz* pathogens

As noted, cultures designated as Kcz-1 and Kcz-2 were isolated from microbiota of infected roots. They were grown on L-agar and minimal medium with pectin, respectively.

Remarkably, Gram staining showed that these bacteria are gram-negative (Fig.S1)

PCR-amplification and sequence analysis (comparison to GenBank database) of the variable fragments of 16S rDNA revealed that Ksz-1 belongs to the systematic groups: *Bacteria*; *Proteobacteria*;

Gammaproteobacteria; *Pseudomonadales*; *Pseudomonadaceae*; *Pseudomonas*. Five species of *Pseudomonas* show highest levels of identity to the 16S rDNA Ksz-1 fragment. These species are: *Pseudomonas putida*, *Pseudomonas plecoglossicida*, *Pseudomonas taiwanensis*, *Pseudomonas monteilii*, and *Pseudomonas fluorescens*.

Similarly, the 16S rDNA sequence of Ksz-2 was also compared to the GenBank database. It was shown that Ksz-2 belongs to the systematic groups: *Bacteria*; *Proteobacteria*; *Gammaproteobacteria*; *Enterobacteriales*; *Enterobacteriaceae*; *Klebsiella/Raoultella group*; *Raoultella*. Sequence analysis suggested that Ksz-2 is likely *Raoultella terrigena*, *Raoultella ornithinolytica*, or *Klebsiella aerogenes*.

These bacteria are differed slightly in their gene sequences. So, identification of Ksz-1 and Ksz-2 is difficult at the 16S rDNA level. In an attempt to identify bacterial species, we relied on biochemical testing as the types of biochemical reactions of each organism act as a thumbprint for its identification (Brenner et al., 2005). We used API 20 NE and API 10 S tests for biochemical strain characterization (Tables S1, S2). Kcz-1 demonstrated close similarity to the *P. putida* species (the ability to grow on mannitol, maltose, mannose and acetylglucosamine as the only carbon sources), but, on the other hand, unlike *P. putida*, Kcz-1 produced indole from tryptophan, lacked arginine dihydrolase activity, and assimilated arabinose. Several features of Ksz-2, such as absence of gelatinase activity, indole formation during growth on a medium containing tryptophan, absence of the urease activity and high ornithine decarboxylase activity, as well as the ability to grow at 10°C and at 41°C suggest that this species is *R. terrigena*. However, it was reported that all species of *Raoultella*, lacked pectinase activity (Hansen et al., 2004; Sekowska, 2019), while corresponding activity of the Kcz-2 strain was relatively high. Interestingly, the Ksz-2 strain grew equally efficiently on media containing apple and citrus pectin and on a medium with polygalacturonic acid as the sole carbon source. By contrast, the Kcz-1 strain was capable of growing only on the last indicated medium (data not shown). Perhaps, this isolate lacks the esterases pectin methylesterase and pectin acetylesterase, suggesting that it lacks pectin esterase activity. Thus, biochemical approaches, as well as analysis of the 16S rDNA sequences failed to identify *T. kok-saghyz* pathogenic species.

To distinguish between these species, we PCR-amplified and sequenced fragments of the *gyrB* and *rpoD* genes that have been successfully used for analysis of the *Pseudomonas spp* (Yamamoto & Harayama, 1998; Yamamoto et al., 2000). Comparison to the GeneBank database convincingly demonstrated that Ksz-1 is *P. putida* (Kivisaar, 2020) (99.79% and 98.79% of identity for *gyrB* and *rpoD* fragments respectively). Sequences of the *P. putida gyrB* and *rpoD* genes are available in the GenBank database (accession numbers CP046872 and LR135071). Primers, corresponding to the conserved regions of the *P. putida gyrB* gene, were successfully used for amplification of the Ksz-2 fragment. The identity level of this fragment to the *R. terrigena gyrB* gene was 99.07%. Interestingly, *rpoD* primers have failed to amplify the *R. terrigena* gene, but non-specific PCR have led to amplification of the *era* gene encoding GTP-binding protein (March, 1992). A very high identity level of the Ksz-2 *era* gene (99.53%), combined with the *gyrB* data, clearly demonstrate that Ksz-2 is *R. terrigena* (Appel et al., 2021). Sequences of the *R.*

terrigena gyrB and *era* genes are available in the GenBank data base (accession numbers LR134253.1 and LR13127.1).

The influence of the environment on the development of pathogenesis in *T. kok-saghyz*

Vascular bacteriosis of roots of *T. kok-saghyz* cultivated by plantation methods was previously described (below). We performed similar experiments in Moscow region (moderate climate, Table 1). At the beginning of the vegetation season, the plants developed evenly and bloomed profusely, and seeds were set. In the beginning of May, the weather was moderate, the average daily air temperature ranged from +15 °C to 21°C. The plants from all three groups developed evenly, without signs of bacterial infection, and basically all specimens bloomed vigorously. In June-July, daytime temperatures rose to 30 °C, and on certain days up to 33°C. A pronounced period of high daytime air temperatures lasted 8–10 days. Those plants that were grown from cured material *in vitro* (group 1) were not affected by heat and continued to actively grow and develop (Fig. 5). In experimental groups, the plants gradually began to wither, and eventually the leaves dried out and fell off (Fig. 6, 7). After the onset of moderate daytime temperatures favorable for the growth of *T. kok-saghyz* (15–20°C), plants from the experimental group 2 began to recover new, green leaves grew without signs of bacterial infection. In the group 3, plants initially showed signs of recovery, but both bacterial isolates caused extensive damage to above-ground tissue, resulting in plant death approximately 47–50 days after inoculation (not shown). Thus, bacterial infection affects resistance of *T. kok-saghyz* to elevated temperature.

P. putida and *R. terrigena* are causative agents of *T. kok-saghyz* root rot

The pathogenicity of *P. putida* and *R. terrigena* was tested on a culture of isolated *T. kok-saghyz* roots with the HR phenotype and on plants *in vitro*. Both of bacteria were introduced into *T. kok-saghyz* HR cultures and plants *in vitro* by vacuum infiltration. This led to characteristic disease-like symptoms such as black necrotic areas initially at the root tips. Remarkably, after 30 days of growth the root biomass became completely brown (Fig. 8).

Plants infected with *P. putida* and *R. terrigena* *in vitro* showed gradually increasing symptoms of infection. Initially infection affected roots and fully developed leaves located at the base of the plant (in 6–7 days after inoculation). Then infection started to spread to the upper part of the plant in approximately 14 days after inoculation. Finally, in 35 days after introduction of bacteria into *T. kok-saghyz* tissue, plant died.

The severity of disease symptoms did not change in plants with cut root tips compared to uncut roots, even though bacterial plant pathogens typically require a wound or natural opening to enter the tissue. In addition to *in vitro* studies, we tested the ability of isolates to infect soil-grown *T. kok-saghyz* plants in growing vessels. Both bacterial isolates caused extensive damage to above-ground part of the plants, resulting in plant death in approximately 37–40 days after penetration of inoculates into the soil, immediately surrounding the root system. Both bacterial isolates also caused plant death when penetrating into the leaves (data not shown).

Discussion

As noted in the introduction, controlled biosynthesis provides unique opportunity to obtain target products with the necessary quantitative and qualitative characteristics. However, the results, in addition to establishing the optimal parameters of the bioprocess, critically depend on the properties of the starting biological material. During *in vitro* cultivation of plants, the long-term and asymptomatic presence of endophytic bacteria is due to the suppression of their growth by factors that influence the processes of cultivation of plant explants (low temperature and light intensity, the presence of immune activators, antibiotics and other plant protection chemicals in the culture media). On the other hand, plants generate a sufficient amount of root exudates to support bacterial growth and activity. Bacteria associated with plant tissues in *in vitro* culture play an important role in various physiological and pathogenetic processes. Their identification helps to understand how these microorganisms affect plant growth and development, disease resistance and productivity (Trivedi et al., 2020).

In this study, performed in an aeroponic phytotron, as well as *in vitro* culture of plants and HR, we identified two bacteria associated with root rot of the NR-producing plant *T. kok-saghyz* - *P. putida* and *R. terrigena*. We also investigated their pathogenicity. Remarkably, the relationships between these bacterial species and other plant species have been described in the literature (Kivisaar, 2020; Mengistu, 2020; Martínez-García & de Lorenzo, 2024). It is noteworthy that several studies demonstrated that *P. putida* species are involved a wide range of processes, ranging from plant growth stimulation and bioremediation to pathogenicity (Kivisaar, 2020). It was also shown that some endophytic strains of *P. putida* and *R. terrigena* have the ability to stimulate plant growth in a natural ecosystem (Costa-Gutierrez et al., 2022; Zakharchenko et al., 2024).

Interestingly, three strains of *P. putida*, were characterized in a gnotobiotic system, with a specific microbial community. A clear inhibitory effect on pea growth under different environmental conditions was demonstrated. Moreover, these strains affected the root morphology, which led to a decrease in root biomass (Berggren et al., 2001). It is also important to note that some bacterial isolates previously identified as growth promoters may have detrimental effects on plant growth if the titer is too high (Glick et al., 1998; Reed & Glick, 2005; Premachandra et al., 2016).

Interaction of the bacteria *P. putida* and *R. terrigena* with plants is usually described as symbiosis (Hardoim et al., 2015). However, our data suggest that under certain conditions well-characterized endophytic bacteria can act as pathogens. To prove that, in fact, the two bacterial species we isolated can be pathogens of *T. kok-saghyz*, we used them to infect HR cultures and reinfect *in vitro* plants of *T. kok-saghyz* that had previously have been cured of these bacteria.

The high adaptability of *P. putida* is based on many features, including high genetic plasticity and broad metabolic, transport, signaling and regulatory capabilities (Nikel & de Lorenzo, 2018). Interestingly, *Pseudomonas* species can use various chemical compounds, simple and complex, as the only source of carbon depending on their living conditions (Song et al., 2015). Thus, *P. putida* strain mt-2 isolated from soil in Japan is able to grow on meta-toluate as the sole carbon source (Vercellone-Smith & Herson,

1997). Sequencing of its genome has shown that it contains multiple genes encoding proteins responsible for degradation of plant-derived chemicals, including various methoxylated aromatic acids, hydroxylated aromatic acids, and other compounds (Castillo et al., 2008). Remarkably, genome also contains genes encoding proteins responsible for degradation of fructose (Velazquez et al., 2004) and glucose (Castillo et al., 2008), which are the most common sugars present in plant root exudates (Kamilova et al., 2006).

Some diseases of *T. kok-saghyz* cultivated by plantation methods were previously described. These diseases are typically caused by soil bacteria, fungi, and oomycetes that penetrate roots and enter xylem, where they interfere with the transport of water and minerals. Among them, vascular bacteriosis of roots. The causative agent of the disease is a bacterium *Pseudomonas campestris* (Tscheremissinov, 1951). Seedlings and young plants die. The disease is more common in soils with a very high humidity. Diseased plants appear wilted in the second half of the day. Yellowing or browning of the central cylinder is clearly visible. As a result, leaves wither and die. This can lead to damage to the entire plant and ultimately to the plant death (Tscheremissinov, 1951). Similar changes can be caused by increased temperature and drought, and together they affect the composition, abundance or activity of microbial communities associated with plants. It should be noted that similar symptoms can appear in plants despite the presence of sufficient water in the soil, which the plant cannot absorb (Martirosyan L. et al., personal communication). We observed similar phenomena during vegetation experiments (above).

The summer drought caused the *T. kok-saghyz* plants to shed leaves and flower buds. This phenomenon was called “summer dormancy” (Scarth et al., 1947). Originally it was assumed that the reason was adverse conditions such as drought. However, currently “summer dormancy” is defined as an intrinsic trait independent of environmental conditions (Volaire & Norton, 2006). Conceivably, the reason of summer dormancy is a consortium of bacteria and their antagonism towards the host plant. We assume that some of endophytic bacteria, upon the onset of unfavorable conditions for them, synthesize plant growth inhibitors, in particular, abscisic acid, which triggers plant defense mechanisms from drought (Chen et al., 2020). When such mechanisms are launched, additional watering does not lead to an improvement in the condition of the plants, but on contrary, some bacteria from the consortium are activated, and destruction of plant tissue occurs, primarily in the area where the roots join the caudex.

Obviously, optimal growth conditions are not reachable in most environments and, therefore, plants exposed to numerous abiotic and biotic stresses are prone to diseases.

For example, an auxin-secreting strain of *P. putida* negatively affects the tolerance of *Arabidopsis thaliana* to water stress, while promoting plant growth under normal watered conditions. Plants subjected to water stress and simultaneously inoculated with *P. putida* showed signs of wilting and decreased moisture content compared to plants subjected to water stress without inoculation. Certainly, the severity of the reaction depended on the amount of inoculum (Shah et al., 2017).

It has been demonstrated that environmental factors are key components influencing the development of plant diseases (Keane & Kerr, 1997; Brader et al., 2017). These include temperature, precipitation (i.e.,

dew, rainfall, and snow) intensity and duration, wind, and aerial pollution (including CO₂ and ozone content). Soil parameters such as organic compound, pH, nutrient content, and content of toxic components (e.g., metals, salt, and pesticides) are also critical (Brader et al., 2017). Moreover, quantity and quality of light required for plant immunity (Hua, 2013). Interestingly, temperature stress signal pathways overlap with multiple pathways responsible for the expression of disease-resistance proteins and defense mechanisms against fungi, nematodes, and viruses (Hua, 2013). Also, resistance to pathogen attack can be enhanced by ozone and UV stresses. This phenomenon is called cross-tolerance (Bowler & Fluhr, 2000). Noteworthy, humidity plays a pivotal role in disease development (Gent et al., 2013; Giaque & Hawkes, 2013). Biotic factors also play an important role in infection process. The most crucial is certainly microbiota containing endophytes and microbes of rhizosphere (Mortier et al., 2012; Brader et al., 2017). In the natural environment, bacteria face a variety of stress conditions, such as carbon starvation, suboptimal nutrient balance (e.g., lack of N or P sources), changes in hydration conditions, desiccation, lack of suitable terminal electron acceptors, and suboptimal pH and temperature (Ramos et al., 2015).

Thus, suggestion that environment plays a key role in pathogenicity led to numerous models that shed light on the mechanistic insides of plant diseases (Garrett et al., 2022; Juroszek et al., 2022). The influence of climatic factors, including increases in temperature and CO₂ levels, and extreme fluctuations in humidity levels, on the interaction of plants and microorganisms is actively being studied (Juroszek & Von Tiedemann, 2011; Velasquez et al., 2018). As noted, in most cases, under these conditions, endophytic microorganisms have a beneficial effect on plants. However, endophytic microorganisms associated with plants react to some environmental changes in a very unique way. Specifically, catabolic processes are activated. This stimulates pathogenesis in the plant-microorganism relationship (Tanaka et al., 2006; Eaton et al., 2011). Overall, pathogens trigger a cascade of reactions in plants that lead to the synthesis of stress metabolites, including H₂O₂, phytoalexins, and stress signals such as abscisic acid, jasmonic acid, and salicylic acid (Lichtenthaler, 1998; Fan et al., 2009; Bharath et al., 2021).

The pathogenicity of opportunistic endophytes depends on the triggering of a cascade of regulatory reactions in metabolic processes. These bacteria use a number of genetic tools to cause diseases in their hosts. Significant progress has been made in recent years in identifying virulence factors of opportunistic bacteria. For example, a disease known as Fern Deformation Syndrome (FDS) results from latent infections with harmful fluorescent pseudomonads, which cause damage when a threshold population is reached (Kloepper et al., 2013).

Catabolic processes in plant cells play a key role in the life of bacteria. As a result of activation of the plant catabolic systems by bacteria, the rate of breakdown of complex molecules is accelerated and the energy necessary to maintain the vital activity of bacterial cells is released. Bacteria use this energy for synthesis of substances necessary for their own needs. Conceivably, under stress conditions (e. g. heat or light intensity, starvation etc.), the activity of catabolic processes may increase, allowing bacterial cells to mobilize the necessary resources for survival and recovery. The activity of the catabolic systems of endophytic bacteria depends on availability of nutrients such as carbohydrates, fats and proteins. A

lack of essential macro- and microelements may slow down the catabolic processes. Plant growth regulators, such as abscisic acid, ethylene, gibberellic acid etc. may potentially affect catabolic processes. Infection by phytopathogens or pests (e. g. insects) may also significantly change the rate of catabolism. We suggest that in *T. kok-saghyz* plants, the so-called "summer dormancy" may develop due to high ambient temperatures. Suboptimal, high temperatures in the root zone of plants may initiate the activity of bacterial enzymes. They, in turn, can accelerate metabolic processes, including catabolic ones. In addition, these factors may synergistically interact with each other, affecting the overall activity of catabolic systems.

The size of potential pathogen population is also important for disease development. Leatherleaf fern (*Rumohra adiantiformis*) is a valuable ornamental plant used in cut flower arrangements. The disease known as FDS (above) was reported in 1980s. It causes distortions of ferns and other prominent symptoms (Kloepper et al., 2010). Interestingly, the severity of FDS correlates with the size of populations of opportunistic endophytic fluorescent *Pseudomonas* inside rhizomes of plants with distorted fronds (Kloepper et al., 2010). It was noted that distribution of FDS coincided with the areas of widespread use of Benlate systemic fungicide (Mills et al., 1996). Conceivably, Benlate act as an inducing factor for FDS. It was shown that 24 months after treatment with Benlate pronounced FDS symptoms were present along with increased endophytic populations of fluorescent pseudomonads inside rhizomes (Kremer et al., 1996).

Under aeroponic growing conditions, plant metabolism changes significantly. Specifically, fluctuations in moisture levels in the root zone and on the leaf surface have a significant impact on concentration, osmolarity and other characteristics of the components of the nutrient solution, which, in turn, affect their availability for adsorption and uptake by root hairs (Chiaranunt & White, 2023). During the period of growth and development of *T. kok-saghyz* in a phytotron, the physical and chemical parameters of the environment constantly change as the root system and leaves grow from the juvenile stage to the aging stage. The morphology and anatomical features of tissues and organs also change. Some substances accumulate, while the biosynthesis of others slows down or stops altogether. These changes certainly affect the physiology and biochemistry of the consortium of bacteria, which are always present both on the surface of plants and in the intercellular space of plant tissues [Martirosyan et al., 2023]. The results of antagonistic interactions between opportunistic endophytic bacteria and their plant hosts depend on the virulence factors of the induced bacteria, i.e. bacteria that have emerged from the dormant state in response to changes in the host's metabolic products. Nevertheless, much remains to be discovered about the causes and mechanisms of triggering pathogenesis processes by opportunistic endophytes.

A characteristic feature of growth in a phytotron is that endophytic bacteria living in plants are forced to exist in a constantly changing physical and chemical environment. The life and activity of endophytes depend on the type and quantity of plant assimilates. These parameters are important for the stable life of bacteria. Likewise, the quantity and composition of these species-specific plant assimilates can control the structure of endophytic communities and regulate their activity (Gorke & Stulke, 2008). Under the influence of the controlled environment and artificial light in a phytotron, plant organs, and

consequently the bacteria inhabiting them, undergo various transformations. This leads to corresponding changes in the metabolism of endophytic bacteria (Kremer et al., 2024). Most bacteria can selectively utilize substrates from a mixture of different carbon sources. The presence of preferred carbon sources prevents the expression of genes and the corresponding activities of catabolic systems that allow the use of alternative substrates. Normally, the presence of preferred carbon sources does not lead to activation of pathogenesis. The lack of these substrates forces endophytic bacteria to radically rearrange their interaction with the host plant cells. The ability of bacteria to adapt to various environmental changes is determined genetically and depends on certain signals. Recognition of such signals and the transformation of this information into specific transcriptional responses play a key role in signal transduction (Parkinson & Kofoed, 1992). Remarkably, most bacteria have a two-component system responsible for this process (Celine et al., 1999). Phosphorylation is an important mechanism of signal transduction since addition of phosphoryl groups affects functional activities of proteins (Stock et al., 1989). This may stimulate synthesis of pectolytic, cellulolytic and other related enzymes (Deutscher et al., 2006).

Most endophytes act as commensals with no known effect on their host plant, but numerous bacteria and fungi establish mutualistic relationships with plants. However, some microbes act as pathogens. The outcome of these plant-microbe interactions depends on biotic and abiotic environmental factors, as well as the genotype of the host and interacting microorganism. In addition, endophytic microbiota and the numerous interactions between members, including pathogens, have a profound effect on plant function and pathobiome development. Collectively, these events may lead to pathogenesis. Interestingly, it has been shown that bacterial strains belonging to a known pathogenic species of a particular host plant can even exert a growth-promoting effect on another plant (Reiter et al., 2002; Coombs & Franco, 2003). The effects attributed to endophytes in healthy plants may change if host plants are grown under less favorable or even stressful conditions. Remarkably, it is difficult to reliably distinguish a non-pathogenic endophyte from a pathogen and that properties such as pathogenicity or mutualism may depend on many factors, including plant and microbe genotype, microbe number and quorum sensing, or environmental conditions (Hardoim et al., 2015).

The interaction of plants and bacteria is a complex and multifaceted process, where positive and negative effects may depend on specific environmental conditions, plants and microorganisms. The obvious suggestion for changes in the behavior of endophytic bacteria associated with cells/tissues of the *T. kok-saghyz* plants is the influence of plant cultivation conditions. Conceivably this may have an impact on plant hormonal systems and, via adjustment of metabolic pathways of carbohydrate exchange, activate specialized enzymes of endophytic bacteria that destroy plant tissue. The distinction between endophytes and pathogens is not always obvious, as both live on and within plant tissues, where opportunities for recombination often arise following horizontal gene transfer, a process that imparts new phenotypic traits (or sets of traits) to bacteria sharing an ecological habitat (Berg et al., 2005).

Therefore, virulence factor genes can move between species of bacterial phylogenetic groups in the rhizosphere or within plants, and virulence factor expression can be linked to bacterial strain population density. Several virulence factors have been shown to be regulated by bacterial cell density through quorum sensing. These include the tobacco hypersensitivity testing system, which has been used to determine the phytopathogenic potential of plant-associated bacteria (Mathesius et al. 2003), indole acetic acid production (Preston, 2004), and biosynthesis of cell wall degrading enzymes including pectinase (Berg et al., 2005; Laasik et al., 2006). In addition, quorum sensing regulates both horizontal gene transfer and bacterial colonization of host plants (Berg et al., 2005).

Conclusion

In conclusion, this study shows that plant-associated microorganisms are an important factor affecting plant responses to changes in cultivation conditions. It led to identification of two bacterial species associated with root rot of the NR-producing plant *T. kok-saghyz* - *Pseudomonas putida* and *Raoultella terrigena*. According to the literature, interaction of these bacteria with plants is described as symbiosis. However, our data clearly demonstrate that under certain conditions well-characterized endophytic bacteria can act as pathogens. Moreover, we showed that plants, cured of phytopathogens, demonstrate significant increase in fitness. Plants exhibit resistance to high ambient temperatures, which is not typical for these species. *T. kok-saghyz* continued to build up a biomass, which may lead to an increase in accumulation of NR and inulin in the roots. In addition, our research showed that aeroponic cultivation is a promising way to grow *T. kok-saghyz*, for production of NR and inulin. Free access to the root zone allows rapid assessment of the conditions of the roots and their biochemical composition directly during the process of plant growth and development.

Our results indicate the need for further research for understanding the mechanisms of biochemical and physiological interactions between microbes and plants. New developments in high-throughput technologies such as next-generation sequencing enable the exploration of complex microbiomes and facilitate in-depth studies of the largest possible number of microbial communities. Genomic analysis of individual microbial strains and metagenomic studies of entire microbial communities will provide insights into the composition and physiological potential of plant-associated microorganisms (Knief, 2014). Conceivably, in the future, with more information on the microbiome of *T. kok-saghyz* and its growth under different cultivation conditions available, it will be possible to achieve maximum productivity of this NR-producing plant.

Declarations

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Data Availability All data generated or analyzed during this study are included in this manuscript

Ethics approval consent This study did not involve human participants or animals, and therefore ethical approval was not required.

Conflict of interest The authors have neither conflict of interests nor competing interests to declare

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Table

Table 1 is available in the Supplementary Files section.

Figures

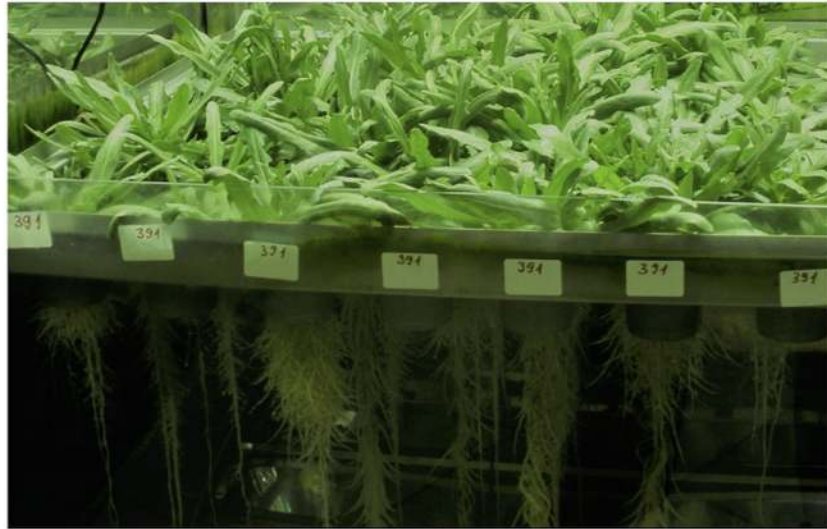


Figure 1

Figure 1

T. kok-saghyz plants growing in an aeroponic phytotron - enclosed research facility used for studying interactions between plants and the environment. It provides controlled conditions of light irradiation (intensity and spectral composition), temperature, humidity, mineral nutrition, and gas composition of the environment.



Figure 2

Figure 2

Root rot is a disease that primarily affects the roots of plants, causing them to decay and die.

Symptoms can include stunted growth, yellowing or browning leaves, and even wilting and death. The symptoms of root rot disease in *T. kok-saghyz* plants grown in an aeroponic phytotron. Healthy roots (A). Roots affected with the disease (B). The symptoms appear after 60-70 days of cultivation. The disease begins with softening in the root collar area, at the junction of the root base with the caudex.

A**B**

Figure 3

Figure 3

The symptoms of root rot disease in the HR cultures. Healthy culture (A). Culture affected with the disease (B). Manifestations appear during long-term (70 days or more) cultivation, despite the fact that the nutrient solution is periodically changed.

A



B



Figure 4

Figure 4

T. kok-saghyz plants (A) and roots (B) cured of phytopathogens. Chemotherapy and thermotherapy approaches were employed. Both thermotherapy and chemotherapy are techniques used to eliminate pathogens from plants, particularly in the context of *in vitro* culture and propagation. Thermotherapy involves heat treatment of seeds to kill pathogens, while chemotherapy utilizes specific chemicals. These methods can be used alone or in combination to enhance pathogen eradication success.



Figure 5

Figure 5

T. kok-saghyz plants, grown from seeds in vegetative vessels in the soil, cured of pathogens using chemo- and thermotherapy, grow actively at high outside air temperatures (30-33°C). They were not subjected to secondary infection with bacteria isolated from the roots of *T. kok-saghyz*(control plants, group 1).



Figure 6

Figure 6

Secondary infection with bacteria isolated from the roots of *T. kok-saghyz* causes plant sensitivity to high outside air temperatures (30-33°C);(experimental plants cured of pathogens, group 2). At lower temperatures (20-22°C) plant growth is restored.



Figure 7

Figure 7

Plants grown from seeds containing endogenous pathogens subjected to secondary infection with bacteria isolated from *T. kok-saghyz* roots. They are very sensitive to high outside air temperatures (30-33°C); (experimental plants, group 3). At lower temperatures (20-22 °C), plant growth is not restored.

A



B



Figure 8

Figure 8

HR root culture (A) and *in vitro* plants (B) subjected to secondary infection with *P. putida* and *R. terrigena* bacteria isolated from *T. kok-saghyz* roots. Infection leads to the development of the root rot disease symptoms.

Supplementary Files

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