

***Bacillus pumilus* – a new phytopathogen of Scots pine – Short Communication**

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ABSTRACT: Large bleeding lesions on stem and branches of pine trees, wet black spots and depressed cancer wounds of different sizes at the base of knots, dying off the bark around knots and sapwood exposure over a large area, wilting, yellowing and shedding of needles on the individual branches and whole crown may indicate the presence of pathogenic bacteria. Monitoring the health of pine plantations in Lviv and Minsk regions showed the spread of diseases that can be caused by bacteria. In order to identify the pathogenic bacteria associated with pine vascular diseases, we collected needles, bark and sapwood from symptomatic trees. Ten potential pathogenic isolates were prepared from diseased tissues. All isolates were found pathogenic and identified as Gram-positive, rod-shaped and spore-forming. The isolates were identified as *Bacillus pumilus*, based on morphological and biochemical characteristics, and also on 16S rRNA gene sequence analysis. Pathogenicity studies of different *B. pumilus* isolates revealed that they have a potential to cause the soft rot disease in pine seedlings and symptoms of wetwood disorder in young Scots pine trees.

Keywords: *Pinus sylvestris*; bacteriosis; phytopathogenic bacteria; soft rot

Scots pine (*Pinus sylvestris* L.) is one of the most important forest tree species in Eurasia. Therefore, various diseases of pine trees can lead to significant losses in the forestry sector. To date, several hundred of pine pathogens have been identified worldwide and the majority of them are of fungal aetiology. Notably, bacterial diseases of conifers are not so well studied, because their impact on the forest has been underestimated for a long time. It was difficult to identify the causative agents and to prove the pathogenicity of isolated bacteria. So far, it has been known that conifer diseases such as wetwood, fire blight, canker and tumour-like bacteriosis can be caused by bacteria (UROSEVIC 1968; RYBALKO, GUKASYAN 1986; WESTCOTT 2001).

Bacterial wetwood is a widespread systemic bacterial disease, which usually proceeds in a chronic

form and affects the central core of many forest trees. Common symptoms of the wetwood disorder include the presence of dark-brown to black water-soaked areas in wood, large bleeding lesions on trunk and branches and depressed cancer wounds of different sizes at the base of knots. Furthermore, the tissues of wood are moist and macerated, as a result of destruction of the middle lamella. Needles of diseased trees are light yellow and the development of chlorotic and necrotic areas on the surface can be detected. The chronic pathogenesis is accompanied by dieback, blight and death of the tree. Several bacteria, including the species of *Enterobacter*, *Klebsiella* and *Pseudomonas*, are often associated with wetwood. The bacteria *Erwinia multivora* Scz.-Parf. (SCZERBIN-PORFINENKO 1963) and *Erwinia cancerogena* Urosevic (UROSEVIC 1968)

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were isolated from the conifers, and found to have similar characteristics to *Erwinia nimipressuralis*, which is associated with wetwood in elm trees (CARTER 1945).

Another vascular disease of Scots pine is fire blight. The main symptoms are yellowing and shedding of needles, dead shoots with abundant resin secretion. During the acute course of the disease, inner bark and xylem are damaged, which causes rapid death of trees. It has been demonstrated that bacterial blight is caused by the bacterium *E. amylovora* var. *lignifilla* (CHERPAKOV 2011).

It is necessary to point out that the bacterial diseases of Scots pine are not well studied. Some sporadic studies were conducted in the Siberia region (RYBALKO, GUKASYAN 1986) and no research on bacterial diseases of pine trees in Ukraine and Belarus has been carried out to date. Visual inspection, which we conducted in natural stands of pines and forest plantations in 2012, revealed that approximately 10% of pine trees exhibit symptoms of bacterial diseases.

The aim of this study was to isolate and identify phytopathogenic bacteria associated with vascular diseases of Scots pine.

MATERIAL AND METHODS

Field studies were conducted in a middle-aged pine plantation located at Sudova Vyshnya forestry (49°48'19.0"N 23°22'27.9"E) of Lviv region (Ukraine) and young natural stands in the north-west of Minsk region near Lake Svir (54°48'38.0"N 26°27'13.7"E) in the Republic of Belarus in May and August 2012. The field diagnostics was performed by close examination of trees for obvious external symptoms including large bleeding lesions on stems and branches; dying off the bark and depressed cancer wounds around knots; resinosis, cancer on the trunk; water-soaked wood areas with sour smell; wilting, yellowing and shedding of needles. During the survey, we collected 39 symptomatic samples of needles, bark and sapwood from 35 diseased trees. Each sample had a part of visually healthy tissue along with the diseased one. Collected samples were placed in paper bags, kept cold and delivered to the laboratory within 24 h.

For a microbiological analysis, small pieces of tissues at the border of visually healthy and diseased parts were cut from collected samples. The samples were sterilized by a few washing steps under running water and then with sterile water and ethanol.

Bacteria from plant material were isolated in two ways: (i) soft tissue (needles, bark) was ground in

a sterile mortar and the homogenate was diluted 5 times with sterile water and plated onto potato agar; (ii) pieces of wood were placed on the agar surface for bacterial overgrowth. Then, colonies of grown bacteria were re-plated to obtain pure cultures.

Single bacterial colonies were picked up at random from the plates, checked for purity and grouped according to colony morphology, cell shape, growth rate and Gram reaction. Biochemical characteristics of the isolates were tested by using standard methods (BREED et al. 1974). All of the bacterial isolates were tested for the following properties: oxidative/fermentative test (O/F); hydrolysis of gelatin and starch; protease, oxidase and catalase production; presence of fluorescent pigment; nitrate reduction; fermentation with production of acid or acid and gas of galactose, glucose, lactose, maltose, rhamnose, sucrose, fructose, xylose, glycerol, mannitol and sorbitol; pectin digestion using a sodium polypectate; cellulose utilization using carboxymethyl cellulose, salt tolerance using a nutrient broth supplemented with different concentrations of NaCl (2, 5, 7%). To screen for antibiotic susceptibility, the isolates were grown on nutrient agar with a range of antibiotics, including ampicillin (50 µg·ml⁻¹), streptomycin (50 µg·ml⁻¹), tetracycline (5 µg·ml⁻¹), kanamycin (15 µg·ml⁻¹), rifampicin (10 µg·ml⁻¹) and nalidixic acid (20 µg·ml⁻¹). To test whether the isolates were pathogenic to plants, we assessed plant responses after the infiltration of bacterial suspensions into the leaves of tobacco (*Nicotiana benthamiana*) plants. For comparison, the reference strains of *Bacillus* sp. 1–15, *Pectobacterium carotovorum* JN42, *Erwinia amylovora* E2 from our own collection and *Erwinia amylovora* 1/79 (Germany, Cotoneaster sp.), were used.

Genomic DNA of isolates was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden) by following the manufacturer's instructions. Amplification of the 16S rRNA gene was carried out using universal primers 8F and 1492R as described in WEISBURG et al. (1991). The resulting PCR products were used as templates in sequencing reactions. DNA sequencing was performed on an ALFexpress automatic sequencer II (Amersham, Little Chalfont). The 16S rDNA sequences of all tested strains were analysed with NCBI BLASTN 2.2.27. Minimum correction of sequences was performed using the BIOEDIT v. 7.0.5.3 programme. A phylogenetic tree based on 16S rDNA sequences of the isolates was constructed using the neighbour-joining algorithm in the MEGA version 4.0 software (Sudhir Kumar et al., Tempe).

PCR-RAPD analysis was carried out using decamer oligonucleotide primers: CUGEA-1, CUGEA-2,

C1, C4, C15, OL21, OL25, OL44, D1. The amplification reaction was carried out in 15 µl and contained 25–50 ng·ml⁻¹ of genomic DNA, 10 × PCR buffer (100 mM Tris–HCl, pH 8.3, 250 mM KCl, 1.5 mM MgCl₂), 0.2 mM of each dNTPs, 0.2 U of Taq DNA polymerase (Fermentas) and 10 pmol primer. The amplified PCR products were separated by agarose gel electrophoresis and visualized in a UV transilluminator. The gel was photographed and analysed visually. Binary data, based on the presence or absence of bands, were directly computed from the Jaccard coefficient using the PAST Software Package (HAMMER et al. 2001). A phenogram was obtained by the analysis of RAPD data in order to study the relationship of isolated bacterial strains.

To test the pathogenicity, *Bacillus pumilus* isolates P10, P140, P142 and P144 were cultivated in a Luria-Bertani (LB) liquid medium for 24 h at 37°C in dark. Cells were collected, suspended in sterile water and adjusted to 1.0 × 10⁹ cfu·ml⁻¹. Generated bacterial suspensions were used in pathogenicity tests and sterile water was applied as a control.

Pathogenicity trials were performed on 4-years-old Scots pine potted trees grown in the greenhouse (early spring 2013) and 14-days-old pine seedlings. In this study, we tested four *B. pumilus* isolates P10, P140, P142 and P144. Five Scots pine trees were inoculated with each isolate and the same number of trees served as controls. The same trees were used for both needle and stem inoculations. Before the treatment, the sites of inoculation were sterilized with 70% ethanol.

In the first test, 20 needles of each tree were punctured with a sterile toothpick into 1 µl of bacterial suspension (10⁸ cfu·ml⁻¹) that was previously applied on the surface of the needle in the centre. The inoculated needles were located at three shoots of previous year from the bottom, middle and top of the tree and were marked with labels. Control trees were inoculated with sterile water. The outcome of the study was analyzed 15 days post inoculation (dpi). The lesions on the needles were evaluated using the following scale: (□) – no necrosis, (+) – up to 4 mm necrosis, (++) – 4–10 mm long necrosis, (+++) – necrosis more than 10 mm long.

In the second test, 100 µl of bacterial suspensions (10⁸ cfu·ml⁻¹) were injected under the surface-sterilized bark of the upper part of the stem of each tree using a sterile syringe. Control trees were injected with the same amount of sterile water. Each inoculation site was covered with Parafilm. Three weeks later, the areas of injections were examined for signs of infection.

In another test, seeds of *Pinus sylvestris* were surface sterilized in 30% H₂O₂ for 10 min with

agitation and then washed thoroughly with sterile distilled water. To germinate, seeds were placed on sterile filter paper which was pre-moistened with 5 ml sterile water in Petri dishes at 22°C in dark. 14-days-old seedlings were disinfected with 50% ethanol, washed in sterile distilled water and transferred with sterile forceps on filter paper pre-moistened with distilled water. Then, a 20 µl drop of bacterial suspension (10⁸ cfu·ml⁻¹) was applied to the rosette of cotyledons without damaging the surface. Pathogenicity of each isolate was tested on 20 seedlings, which were incubated for seven days at 22°C. The experiment was performed in triplicate.

In all tests, bacteria were re-isolated from the border of the healthy and diseased tissue and their morphology and physiological properties were compared with those used in the first round of the pathogenicity test (HOLT et al. 1994). The identity of bacterial isolates was verified by PCR-RAPD.

Means and standard errors of experimental data were calculated using Microsoft Office Excel. Student's *t*-test was used to evaluate the statistical significance of the obtained results.

RESULTS AND DISCUSSION

To identify pathogenic bacteria causing pine vascular diseases, we isolated 145 bacterial strains from 39 samples of symptomatic sapwood, bark and needles. No fungi were isolated from all tested samples. Potentially phytopathogenic isolates were chosen by the following criteria: the ability to hydrolyze cellulose and pectin, as well as the ability to cause a hypersensitivity reaction on tobacco leaves. Celluloses and pectate lyases are pathogenicity factors in phytopathogenic bacteria. A hypersensitivity reaction manifested by a local cell death at the site of bacterial inoculation occurs in plants that are not specific hosts for the tested phytopathogen. In this study, only ten isolates: P10, P107, P109, P113, P115, P123, P135, P140, P142, P144 met these three criteria. P10, P107, P123 and P140 were isolated from yellowing and drying needles of young pine trees (Fig. 1c). Yellowing of individual needles was observed on current-year shoots, while resinosis and cancers were found on stems and branches. P109, P115, P144 were isolated from pitch-soaked inner bark around canker on shoots (Fig. 1b). The isolation source of P113 was water-soaked, dark inner bark around a twig of a 40-years-old tree. P135 was isolated from wood of a wilting current-year shoot on 15-year-old tree with pitch flow on



Fig 1. Sources of isolated bacterial strains: (a) bleeding lesion on the trunk of a 40-years-old pine (sapwood was sampled using the increment borer); (b) bark and exudate from the current-year shoot of a 15-years-old tree; (c) needles from a 20-years-old pine

branches. P142 was isolated from water-soaked sapwood behind a bleeding lesion on the trunk (Fig. 1a).

These bacterial strains exhibited similar morphological, physiological and biochemical characteristics. They were Gram positive, rod-shaped, motile, endospore formers. Positive tests were evident for catalase and protease activities. All bacterial isolates utilized galactose, glycerol, maltose, mannitol, xylose, saccharose and fructose, but they did not utilize lactose. In addition, they were able to grow in a nutrient broth containing 5% NaCl. All isolates had a negative reaction for starch hydrolysis, nitrate reduction and were sensitive to ampicillin, streptomycin, kanamycin and tetracycline, rifampicin and nalidixic acid. Identification trials suggest that all ten isolates are *Bacillus pumilus* as reported by SHAAD et al. (2001).

To confirm these results and to clarify the taxonomic position of the isolates we carried out the sequence analysis of PCR products of rRNA genes. The 16S DNA sequencing results revealed 99% similarity between all samples tested in this study. Obtained sequences were compared with those available in the GeneBank, using BLASTN analysis to find the closest relatives. The database search showed that partial 16 rRNA gene sequences of

our isolates had 97–99% identity with *Bacillus pumilus* DW3 GenBank: (gb| JQ319538.1), *Bacillus pumilus* 7–5 (gb|EU912555.1) and *Bacillus pumilus* strain MW-1 (gb|HM027879.1), which was confirmed by the phylogenetic analysis (Fig. 2). These strains were isolated from soil and marine water, indicating that the 16S DNA sequences of *Bacillus pumilus* from different sources have high similarity.

Since 16S DNA sequences of our isolates were identical, the genetic diversity among them was studied using PCR-RAPD, which is a very efficient tool to define strains of the same bacterial species. RAPD data also confirmed the high similarity of the isolated strains. Based on the Jaccard similarity coefficient level, genetic similarity among them ranged from 95% to 100% (Fig. 3).

The isolated *Bacillus pumilus* strain represents a great interest as the causative agent of shoot/needle necrosis of *Pinus sylvestris* L. This is an interesting finding, since *Bacillus pumilus* is a naturally occurring bacterium in the soil and is regarded as an integral part of the normal epiphytic microflora of plants. Generally, *Bacillus pumilus* is ubiquitous both in the terrestrial and marine ecosystems. This bacterium is highly resistant to extreme environmental conditions, such as low or no nutrient avail-

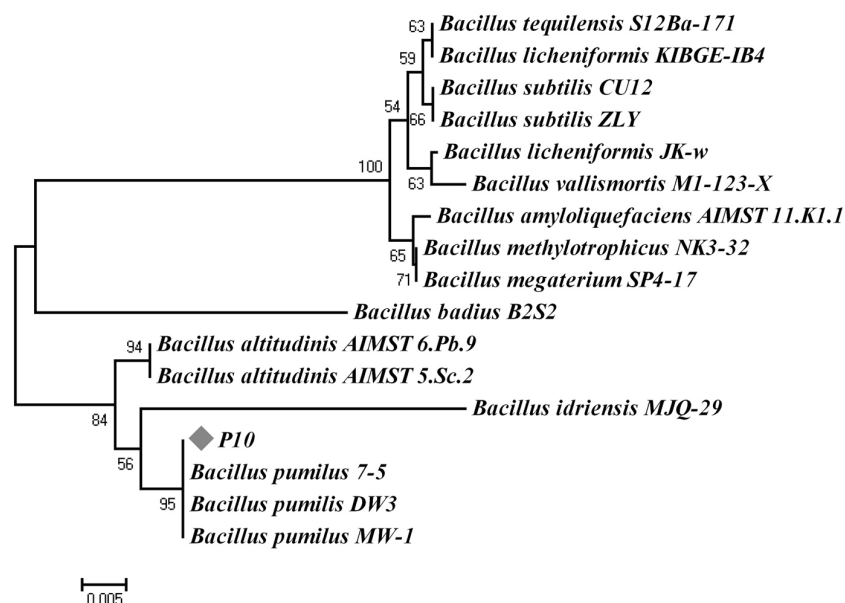


Fig. 2. The phylogenetic tree shows the taxonomic location of *B. pumilus* bacteria isolated from pine trees, based on 16S rRNA gene sequences

P10 indicates the position of all ten isolates obtained in this study. (Scale bar, 0.005 substitutions per nucleotide position)

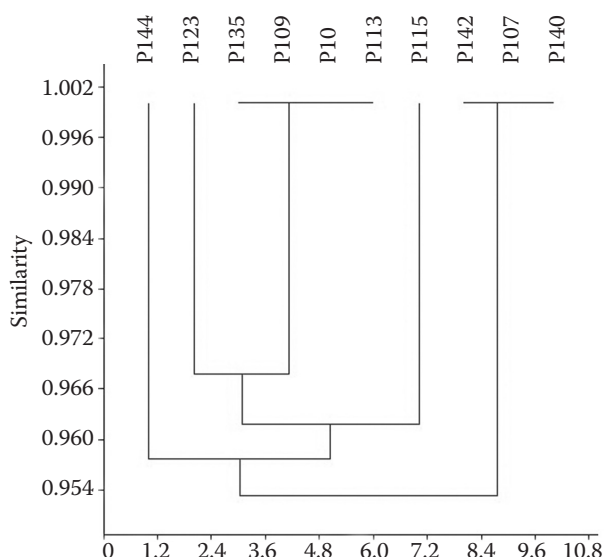


Fig. 3. Phenogram generated from the RAPD data by PAST using the Jaccard coefficient to estimate genetic distances

ability, desiccation, irradiation and chemical disinfections. Notably, *Bacillus pumilus* is also used as an antagonist to pathogenic agents of different bacterial and fungal diseases. However, there are several reports of *Bacillus pumilus* causing diseases in pears, apples, peaches, mangoes and olives (SALEH et al. 1997; GALAL et al. 2006; LI et al. 2009).

We tested the pathogenicity of the *Bacillus pumilus* strains P10, P140, P142 and P144 isolated from different sources and regions. P10 was isolated from the needles of a 20-years-old pine tree (Lake Svir in Minsk region) with yellow needles on individual branches and cankers on the stem. The other three strains P140, P142 and P144 were isolated from needles, sapwood and bark, respectively, of symptomatic pine trees (Lviv region, Ukraine).

Notably, the isolates showed different virulence in inoculation tests. In the test on pine needles, we observed the appearance of brown necrotic and chlorotic zones of various sizes 15 days after the inoculation. Interestingly, isolates P140 and P144 also caused significant drying of needles. We also found that isolate P140 caused evident disease symptoms

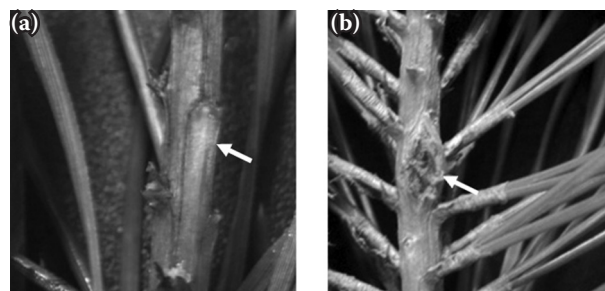


Fig. 4. The stems of the 4-years-old Scots pine trees inoculated with sterile water (a) and with isolate P10 (b); arrows indicate the sites of inoculation

in 47% of inoculated needles, while P142 affected only 1% of needles (Table 1).

In the test on the stem of 4-years-old Scots pine trees, the first symptoms of the pathological process were found on 10 dpi. We observed bloating bark and wet spots at the site of inoculation with isolate P10. After two weeks, similar symptoms were found on trees inoculated with the other tested *B. pumilus* isolates. After three weeks, we observed the occurrence of cankers, flowing out exudate from punctures and the descent of bark at the site of inoculation. In control trees, no pathological changes on the bark were detected at various post-inoculation times (Fig. 4). Interestingly, we found that all five trees inoculated with isolate P10 had cancer-like formations. In the case of the isolate P142, only one tree showed similar symptoms. In cross-sections at the site of inoculation, we found that isolates P10, P140 and P144 caused significant degradation of the bark. It is important to note that in the case of the strain P10, pathological changes spread in the xylem (Fig. 5). The tissues at the inoculation site were wet and there was an efflux of exudate. Isolate P142 induced the deformation of the bark and caused less intensive pathological changes in comparison with the other tested strains.

In the test on 14-days-old seedlings, we found that all tested isolates caused soft rot which led to the death of young plants (Fig. 6). High virulence was

Table 1. Pathogenicity of *Bacillus pumilus* isolates on *Pinus sylvestris* plants

Isolate	Source	Test on 4-years-old trees				Test on 14-days-old seedlings (percentage of seedlings with visible disease symptoms)	
		needle inoculation					stem inoculation number of trees with canker
		number of needles with visible necrosis					
		□*	+	++	+++		
P10	needle	7	38	45	10	5	7.8 ± 1.2
P140	needle	10	9	34	47	3	86.7 ± 6.1
P142	sapwood	6	55	38	1	1	72.3 ± 6.4
P144	bark	6	19	42	33	3	45.1 ± 7.5

*visible necrosis categories are: (□) – no necrosis, (+) – up to 4 mm necrosis, (++) – 4–10 mm long necrosis, (+++) – necrosis more than 10 mm long

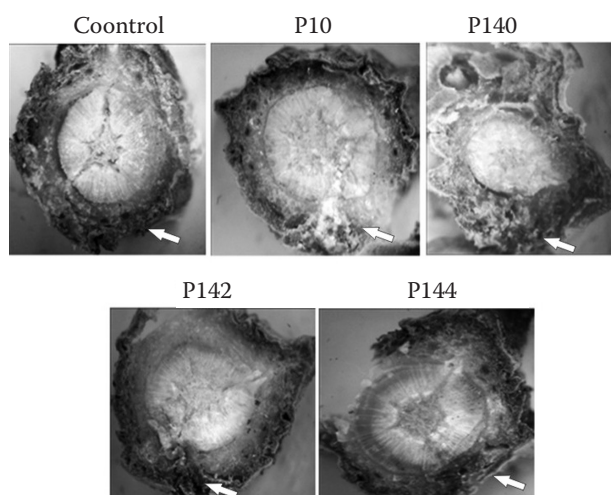


Fig. 5. Cross-sections of the Scots pine stem at the site of inoculation with different *Bacillus pumilus* isolates (21 dpi); arrows indicate the sites of inoculation

found for isolates P140 and P142, as they caused rot in 86.7% and 72.3% of seedlings, respectively. The isolate P144 was less aggressive, as disease symptoms were observed in 45% of the tested seedlings. The isolate P10 exhibited the lowest virulence: soft rot developed only in 7% of the inoculated seedlings, which had the deformation of cotyledons (Fig. 6a). In previous studies, *Bacillus pumilus* was isolated from healthy Scots pine seeds, but the authors identified them as saprotrophs (ROZENFELD 2008). The data presented in this study provide the evidence that *Bacillus pumilus* can cause soft rot and the death of pine seedlings. Similar results were obtained in

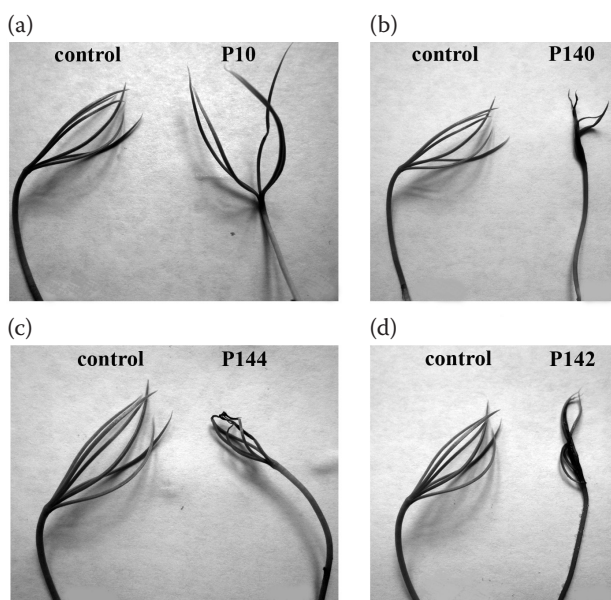


Fig. 6. The Scots pine seedlings inoculated with *Bacillus pumilus* isolates by a drop method (7 dpi): (a) P10; (b) P140; (c) P144; (d) P142; control – seedlings inoculated with sterile water

forest nurseries of Central Siberia, where bacteria of the genus *Bacillus* were shown to infect the root system and the base of the stem, leading to wilting and lodging of conifer seedlings (GRODNITSKAYA, GUKASYAN 1999).

Presented data clearly indicate that all tested *Bacillus pumilus* isolates have the potential to cause pathogenic symptoms in Scots pine trees. We found that isolates P10, P140 and P144 showed higher virulence, compared to P142 on 4-years-old trees (Table 1). Furthermore, no relationship between genetic variability and the pathogenicity among tested isolates was revealed. Thus, according to the RAPD analysis the similarity between P140 and P142 is 100%, but their pathogenicity was significantly different. At the same time, the test on seedlings showed high virulence of both strains.

In conclusion, this study demonstrates for the first time that *Bacillus pumilus* has the potential to cause soft rot in pine seedlings and the symptoms of wetwood in young Scots pine trees.

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