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# PLANT HOST RANGE SPECIFICITY OF *BURSAPHELENCHUS MUCRONATUS* MAMIYA ET ENDA, 1979 TESTED IN THE LABORATORY EXPERIMENTS

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The host range of the *Bursaphelenchus mucronatus* (subspecies *kolymensis*) to seven tree species (conifers and deciduous) was tested. 20 cuttings of every plant species (not rootstocks) were used for infected and control plants; 200 nematode individuals per plant as an inoculum; 45 days at 20—22 °C. Parameters estimated after 45 days were: the final population density, percentage of wilted leaves or needles, size and color of the necrotic spot in the point of inoculation. The population density exceeded inoculum in *Pinus sylvestris* that was the native host of the nematode isolate. In other plant species the nematode abundance decreased. The immune response was expressed as the necrotic reaction of hypersensitivity: in *P. sylvestris* it was no necrosis reaction, weak necrosis in *Picea abies*, in deciduous plant species the immune reaction had the maximum expression. It was concluded that the plant host range of *B. mucronatus* was conditioned not only by its vector preferences, but own nematode adaptations to the natural plant host to overcome the immune response of the plant.

*Key words*: *Bursaphelenchus*, plant resistance, population dynamics, inoculation, pathogenicity test, host range, nematode-caused wilt.

# ЛАБОРАТОРНЫЕ ЭКСПЕРИМЕНТЫ ПО СПЕЦИФИЧНОСТИ СТВОЛОВЫХ НЕМАТОД *BURSAPHELENCHUS MUCRONATUS* MAMIYA ET ENDA, 1979 К РАСТЕНИЯМ ХОЗЯЕВАМ

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В лабораторном эксперименте тестирована специфичность стволовых нематод *Bursaphelenchus mucronatus* (подвид *kolymensis*) к семи видам хвойных и лиственных древесных растений. В качестве тестовых инфицированных и контрольных растений использованы черенки деревьев, а не саженцы; по 20 черенков, зараженных нематодами, и контрольных растений для каждого вида дерева; инокулюм составлял 200 экз. нематод на растение; продолжительность теста 45 дней при 20—22 °С. Через 45 дней исследованы параметры: численность нематод в черенке; процент листьев, почек или хвойных игл с признаками увядания (вилта), размер, цвет и контрастность пятна некротической реакции сверхчувствительности в точке инокуляции нематод в растение. Финальная численность нематод в растении превысила инокулюм лишь в природном хозяине изолята нематод: сосне *Pinus sylvestris*. В других видах растений численность нематод снижалась. Иммунный ответ растения на экспериментальную инфекцию выражался в виде пятна некротической реакции сверхчувствительности; в природном хозяине: сосне *P. sylvestris* некротическая реакция отсутствовала; слабый локальный некроз был выявлен для ели *Picea abies*, а в лиственных растениях некротическое пятно было выражено максимально. Сделан вывод о том что специфичность *B. mucronatus* к растениям хозяевам определяется не только предпочтениями вида жука переносчика нематод, но также собственными адаптациями вида нематод к преодолению иммунных барьеров их природного растения хозяина — сосны.

*Key words*: *Bursaphelenchus*, фитоиммунитет, популяционная динамика, инокуляция, тест на патогенность, специфичность, нематодный вилт.

Wood-inhabiting nematodes of the genus *Bursaphelenchus* may have pathogenic importance for forest and park plantations while some species of these roundworms are causative agents of woody plants wilt diseases. The most known species is the pinewood nematode (PWD) *Bursaphelenchus xylophilus*, that is the causative agent of wilt disease of pines in South Asia, and from 1990-thies also in South Europe and therefore included in the A2 list of the EPPO, 2017; another nematode pathogen which important for Carribean is *B. cocophilus*, that caused wilt of the coconut palms vectored by the palm weevil (see review in Ryss et al., 2005). Now there are new data on the possible role of *Bursaphelenchus* spp. as the causative agents of wilt symptoms in conifers and deciduous trees, e. g. *B. ulmophilus* may be a part of the pathogenic association of the

Dutch elm disease (DED) in the European Russia; this disease usually considered as the illness caused by the fungus *Ophiostoma novo-ulmi* vectored by bark beetles *Scolytus* spp., and with an establishment of the nematode role in the wilt expression, the DED may be characterized as the entomo-vectored mycosis associated with nematodes (Ryss et al., 2015).

The wood-inhabiting nematodes attract high attention because of forest export and transportation of large volumes of wood across Eurasia. Most of wood are cut in the native refugia of forest pathogens in taiga zone. The abnormal summer heat of 2010 when the daily temperature in July and August was eight Celsium degrees higher than the local climatic norm lead to appearance of large wilted forest areas in five regions of Russia where the nematodes *Bursaphelenchus* spp. were detected in ill trees (Ryss, Mokrousov, 2014).

An estimation of the risks for wilt disease expansion is a serious challenge of the applied forestry science. Simultaneously this is a research area for a phylogenetic analysis of the host parasite relations in part of the plant host ranges within the clades of the molecular phylogram of the genus *Bursaphelenchus* (Ryss, Subbotin, 2017).

The life cycle of wood-inhabiting nematodes of the genus *Bursaphelenchus* includes, besides of host tree, the beetle vector belonging to the fam. Cerambycidae or Curculionidae and the saproxylic fungus commonly belonging to the fam. Ophiostomataceae (Ryss, 2016).

The most selective associations among organisms participating in the life cycle of *Bursaphelenchus* spp., are between nematode species and insect vector taxa (Ryss et al., 2005; Kanzaki et al., 2014; Ryss, 2016; Ryss, Subbotin, 2017). For the phylogenetic species group (clade) within the genus *Bursaphelenchus* the vector range usually restricted to family or subfamily whereas every species of *Bursaphelenchus* is associated with single insect genus. For instance, vectors of the *B. mucronatus* are beetles of the genus *Monochamus: M. galloprovincialis* in Europe and *M. alternatus* and *M. saltuarius* in Asia (the vectors are listed in a table by Ryss et al., 2005). The plant host range within a phylogenetic nematode group is more wide covering conifers and deciduous plants of different families; every nematode species associated with the only plant family. For instance, *B. mucronatus* is associated with the plant species of *Pinus*, *Picea*, *Larix*, *Cedrus*, which are taxa of the fam. Pinaceae (records compiled by Ryss et al., 2005).

The associative relations of the nematode and plant taxa may be easily explained by the beetle vector preferences in feeding and oviposition in the target host plant; thus the possible evolutionary based combination of plant and nematode taxa is not necessary. However the vector is the latest chain among organisms involving in the life cycles of the nematodes of the fam. Aphelenchoididae. It follows from a comparison of the advanced *Bursaphelenchus* life cycle with the cycle of more primitive nematodes of the genus *Aphelenchoides*, which usually do not have any vector (Ryss, 2016). Therefore it may be supposed that bursaphelenchs still maintain some plant host range specificity as a heritage from their ancestors, and not only the association with vector species is the selective. This research is aimed to test whether the host range specificity of the species within *Xylophilus* clade does exist as the independent from insect vector preferences adaptive biological feature. To answer, the laboratory tests on pathogenicity and host specificity were carried out on the *Bursaphelenchus mucronatus* Mamiya et Enda, 1979.

## MATERIAL AND METHODS

For tests the following isolate was used: *Bursaphelenchus mucronatus* Mamiya et Enda, 1979, subspecies *Bursaphelenchus mucronatus kolymensis* Korentchenko, 1980. Host plant *Pinus sylvestris* L. with larval tunnels of vector *Monochamus galloprovincialis* (Olivier, 1795). Tomsk region of Russia, Tomskiy district, Timiryazevo settlement, forestry farm Temiryazevskoye. GPS: 56°27.597′ N 084°51.157′ E. Collection date 19.06.2015.

Nematodes were extracted from wood using the modification of the Baermann funnel technology (Ryss, 2015; Ryss, 2017). The extracted roundworms were multiplied in a sterile sporousless culture of the fungus *Botrytis cinerea* Pers. 1794 (Ascomycota); the teleomorph sexual form of the fungus is named *Botryotinia fuckeliana* (de Bary) Whetzel, 1945. The nematode multiplication was done at 21—22 °C during seven days until the nematodes had eaten all the mycelium white lawn and filled the 6 cm Petri dish space reaching number of 5000 (2500—10 000) specimens. The *B. mucronatus* isolate is maintained in the living collection section of the Zoological institute RAS.

As the experimental hosts cuttings of the following woody plant species have been used: fam. Pinaceae: *Pinus sylvestris* L., *Picea abies* (L.) H. Karst., deciduous, fam. Betulaceae: *Betula pendula* Roth., fam. Salicaceae: *Salix caprea* L. and *Populus nigra* L., fam. Sapindaceae: *Acer platanoides* L., fam. Ulmaceae: *Ulmus glabra* Huds. The list of the experimental hosts is given in table. The live branches for cuttings were collected in the park of the Saint Petersburg State Forest Technical University in the day of experimental infection or not earlier than 5 days before inoculation with nematodes.

As an inoculum for the experimental infection 200 nematode specimens per plant (cutting) were used. To prepare an inoculum dose, nematodes previously multiplied in the *Botrytis cinerea* culture were washed with 1 ml of distilled water into 1.5 ml Eppendorf tube. The suspension was shaken to distribute nematodes randomly and ten 20 µl-volumes were taken with a pipette and counted. From ten counts the total number of active nematodes of mixed stages in a who-

*Bursaphelenchus mucronatus*. Inoculation experiment in plant host cuttings

| Woody plant species | Final population, nematode specimens per plant | Wilt % | Immune response, value |
|---|---|---|---|
| *Pinus sylvestris* | 803.4 ± 96.3 [713.3—899.7] | 100 | 0 |
| *Picea abies* | 13.8 ± 11.7 [6.1—25.5][a] | 30 ± 7 (25—35) | 1 |
| *Betula pendula* | 28.0 ± 3.2 [25.0—31.1] | 0 | 2 |
| *Ulmus glabra* | 5.6 ± 1.5 [4.3—7.0][ab] | 0 | 2 |
| *Salix caprea* | 9.0 ± 2.9 [6.5—11.8][ac] | 0 | 2 |
| *Acer platanoides* | 3.5 ± 1.2 [2.5—4.7][bc] | 0 | 2 |
| *Populus nigra* | 0 | 0 | 2 |

Note. Final population densities, wilt expression as a percentage of wilted leaves and needles, and the immune response characterized by the necrosis spot in the point of inoculation. Populations values are given as mean± SD (confidence intervals, minimum — maximum) at $p < 0.05$. Non-parametric Tukey HSD Post Hoc test has been used (Error: Between MS = 4.66, df = 133.00). Final population means followed by the same uppercase letter are not significantly different at $p < 0.05$.

le tube was defined, and the volume that according to calculations contained 200 specimens as a mean value, was calculated.

Cuttings of 20—25 cm length and 0.5—1 cm diam. and not rootstocks were used in the experiments. Cuttings were prepared using a standard gardening procedure. A cutting must have at least four buds among which two or more buds in anterior part and two in posterior part; 20—25 needles (in conifers) or 1—5 small leaves were left only in anterior end of a cutting, removing all of them along the branch. The root end had to be cut obliquely at the level of a last bud retaining a «heel» of the previous year increment. The root end was slightly wounded at surface by longitudinal incisions and then powdered with a root stimulator «Kornevin» containing the plant hormone indole-3-butyric acid (Selhozservis, 2012). Before planting into loam soil the inoculation procedure was processed (see below). In 2—4 weeks after inoculation and planting the cuttings develop roots from posterior buds, and leaves and small branches from the anterior buds. In infected cuttings in case of wilt the leaves and buds were wilted to the end of the experiment (45 days). The use of cuttings and not rootstocks gives an opportunity of large samples for statistics with low costs for planting material and small experimental rooms and vegetation pots. Unlike forest pathology experiments with freshly cut wood log sections, in a case of cuttings one could be sure that a plant with live buds is definitely alive during experiment period. Additional advantage is redundancy of the daylight hours regulation while the cuttings may survive in dark, and the experiment requirements are only to maintain plant vitality during the time of experiment. Using this ability, the tests were done in a room with not artificially regulated daylight hours regime. For cuttings sampling a winter period was optimum, thus the astronomic daylight consisted from 6 to 8 hours during experiment period from January to April, 2016.

Inoculation. To inoculate a plant with a nematode suspension, the longitudinal 15—20 mm slit to be cut with the narrow blade of paper cutter in the distal part (internode) of cutting. The slit was expanded with a sterile screw, and a moistened cottonwool tampon was inserted into slit. The nematode suspension of 200 specimens was poured with a pipette into a tampon and a screw was removed. The place of incision was taped with Parafilm-M. A root end of cutting was powdered with the root stimulator «Kornevin», containing the plant hormone indole-3-butyric acid (Selhozservis, 2012) and paced into a plastic vegetation pot consisting of two parts: 1L cylindrical base with loam soil and upper conical part with an upper hole of 3 cm diam. used to water the plants with spray, twice a week. Each pot contained 10 cuttings. The control plants were cuttings with the same manipulations but the nematode suspension was substituted with equal volume of distilled water. For every plant species 20 infected plants and 20 control plants were used. The test duration was 45 days, this time was established in the preliminary experiments with 3-year pine seedlings (*Pinus sylvestris*) as sufficient to obtain wilt symptoms in all infected twenty plants *vs* 0 % wilt values in the control plants. Similar test time to cause wilt symptoms of conifers by *Bursaphelenchus* spp. nematodes (5—13 weeks) was defined in experiments of plant pathologists (Dayi, Akbulut, 2011).

The nematode extraction after 45 days was done using the modification of Baermann funnel technology (Ryss, 2015; Ryss, 2017). Time of extraction was 24 hours. Nematodes in every plant were counted using the method described above for inoculum calculations.

At the end of experiment, besides of the definition of the final population, two other parameters were characterized: i) a share of wilted leaves (or needles or buds) in % to total quantity; ii) a size and color of the necrotic spot as the expression of the immune response: 2-value: a dark contrast spot of 4 mm width from an inoculation slit; 1-value: a blurred weakly pigmented spot of 2 mm; 0-value: absence of spot around slit.

The differences between population means were processed with non-parametric one-way Anova, post-hoc analysis, Tukey HSD test with a preliminary transformation of a sample to normal distribution. The software Statistica 8.0, Statsoft 1984—2007, was used.

## RESULTS

The results of inoculation testing are given in table and figure.

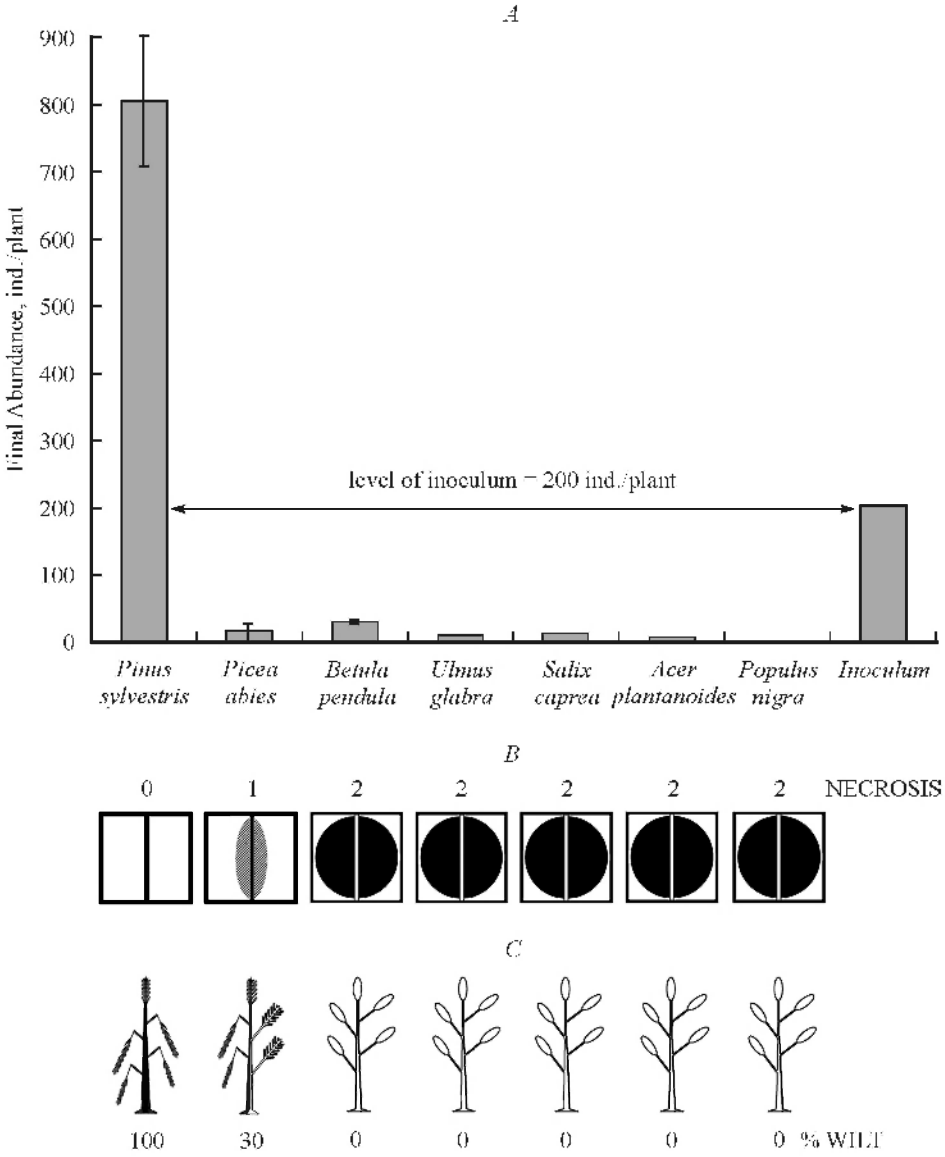
Among tested plant species, the population growth of *B. mucronatus* was revealed only in the native host, the pine *Pinus sylvestris*. Other hosts expressed the significant decrease of population comparing to inoculum. Nematodes caused 100 % wilt in pine cuttings whereas the spruce infection led to 30 % wilt. Wilt symptoms were absent in the deciduous plants. Control plants retained 100 % vegetation during 45 days, without wilt symptoms, an immune response was absent. Among infected plants the strong immune response was expressed in deciduous plants, no necrotic reaction was found in pine, and in spruce the reaction was weak (1-value).

According to test results, final population means of the following hosts were different at $p < 0.05$: *Pinus sylvestris* from all other hosts, *Picea abies* from *Betula pendula* and *Acer platanoides*; *Betula pendula* from *Ulmus glabra*, *Salix caprea*, *Acer platanoides*. In *Populus nigra* nematodes were not multiplied; after 45 days no nematodes were found in wood; however the poplar showed high immune response level in the point of the nematode inoculum input.

## DISCUSSION

Due to the need to develop the plant host range testing suitable for statistical processing of mass plant quantities, the standard laboratory test on seedlings was substituted to the original technology of the winter laboratory testing based on cuttings of woody plants.

The fact of the differential host suitability follows from the results of testing. The nematode multiplication abilities and pathogenicity symptoms in different hosts were the same as in natural habitats during forest monitoring (Ryss et al., 2005). The 45 days duration of the experiment until revealing the wilt symptoms corresponds to that detected in tests with other *Bursaphelenchus* species (Dayi, Akbulut, 2011). The wilt symptoms as indicated by Japanese scientists with water soluble stains, are conditioned by the nematode-caused destructions of xylem tracheal tissues, which lead to the slowdown of water transportation along the tree trunk (Kanzaki et al., 2011). The *B. mucronatus* nematodes studied in the tests showed the statistically reliable differences between plant hosts in population growth comparing to inoculum, wilt symptoms expressions and the




*Bursaphelenchus mucronatus*. Pathogenicity and host range specificity testing.

*A* — final nematode abundance, Y-axis: final nematode abundance, individuals per plant; X-axis: plant hosts. A horizontal line of 200-value is an inoculum dose (200 individuals per plant). The confidence intervals are given as horizontal bars for every final population mean (n = 20). *B* — the immune responses (in a form of necrotic reaction): 0 — no response; 1 — weak response with a blurred spot, 3 mm wide or less from an inoculation slit; 2 — strong immune response with a contract dark spot, 4 mm wide or more from an inoculation slit. *C* — the wilt values, %. Latin names of host plants are indicated below the diagram A; data for every plant species form a column across *A*, *B* and *C*.

immune responses of plants. It may be concluded that the plant host range is conditioned not only by the insect vector preferences in plant species but also by the inherited adaptations of nematodes themselves.

The probability of the vector switch is increased with global warming, because the distribution areas of south insect are expanded to the north. Worming le-

ads to a pulsation of the climatic barriers dividing species of insects and nematodes. For nematode species therefore a probability of plant host switch is increased. The host switch trend caused by the global warming and climate pulsation is already well known phenomenon (Brooks, Hoberg, 2007). Climate warming has changed two main climatic parameters responsible for the plant host resistance to pathogens: i) duration of the period with daily temperatures 25 °C and higher and ii) annual precipitation of 600 mm or less. Climatic changes make it necessary to monitor the regional wilted trees refugia (Ryss, Mokrousov, 2015). However, the nematode species selective adaptation not only to vectors but also to plant host should be a barrier restricting the nematode host switch.

From the comparison of the life cycles among species of the fam. Aphelenchoididae it may be concluded that the insect vector is the latest chain in evolution of the life cycles of wood-inhabiting nematodes (Ryss, 2009, 2016; Ryss, Subbotin, 2017). The important role of the latest chain in the nematode speciation is in contradiction with the coevolution hypothesis applied to host-parasite association. However the extraordinary selective abilities of nematodes in relation of vector taxa may be explained by the highest risks for the entomophilic phase of the nematode life cycle. If the parasitic nematode load will be too harmful and weakening for the insect, the latter will be not capable to the efficient transmission of the pathogen to a new uninfected tree. The results of the current research indicate on the selective processes which led to relatively highly selected host range also in the association nematode and plant, that are independent from selection in the nematode-vector associative links.

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