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CULTURAL AND MORPHOLOGICAL CHARACTERISTICS OF FUNGUS *PHOMA* SP.1 – PHOMA BLIGHT CAUSING AGENTS IN CONIFEROUS TREE SEEDLINGS

Phoma blight of coniferous seedling − a new and poorly understood disease in forest nurseries of the country. The main causing agentsis a pathogenic species *Phoma* sp.1, for the moment, has no taxonomy description. The basic morphological and cultural characteristics of the fungal strain *Phoma* sp.1 are investigated in this paper.

It was found that the according to the optimum temperature (22°C) the fungus belongs to the mesophilic group. It was found that the culture of *Phoma* sp.1 have been capable of forming a vegetative resting spores − chlamydospores.The process of formation began with the separation of protoplasm fragments, then the mycelium septated and covered with a dense shell. Chlamydospores formation was the most rapid agar medium at +4°C for 7 days cultivation. Pycnidia of the fungus formed deep in the nutrient media only after prolonged low-temperature storage of mycelium on agar media, but the spores have not been ripen.

The influence of pH on the relative fungus it is acidophilic. Maximizing biomass productivity rate was into the 4.3–4.9 range, but the growth of the fungus *Phoma* sp.1 is can be carried out in a wide pH range – from 2.5 to 8.5 depending on the composition of the nutrient medium. In the process of life fungus alters the reaction medium at a different number of pH units, bringing it closer to optimal for it development. The pH of the medium also affected cellular and colony morphology of *Phoma* sp.1.

More favorable conditions for the fungus *Phoma* sp.1 observed at constant medium aeration – the formation of the typical mycelium with proportional growth and stable biomass accumulation.

Key words: *Phoma* sp.1, Phoma blight, mycelium morphology, colonies, nutrient medium, growth rate, chlamidospories, acidity, biomass.

Introduction. Phoma blight of conifers in forest nurseries in Belarus is a new, but already widespread disease [1]. First symptoms appear in early May – the lower needles of seedlings and saplings become golden brown, then turn dark brown and begin to die. Then the pathogen spreads over the young stem, causing the death of side shoots of the current year and the apical bud. As a result, the plant is far behind in the growth and the end of summer fully dries. Despite the high losses from this disease, its pathogens (fungi of the genus *Phoma* Sacc.) have practically not been studied in forest tree species, although a significant amount of Phoma blights of crops is known [2].

The fungus *Phoma* sp.1 is a selected isolate that most often stood out from the affected planting material of coniferous species in laboratory diagnostics. It should be noted that the fungi of the genus *Phoma* are complex in their taxonomy because of the absence of sexual reproductive organs, in most species, the high morphological variability in vivo and polyphyly [3, 4].

Currently this fungus has no specific name and taxonomic description in the international gene data bank NCBI [5].

Fungi p. *Phoma* are known for their increased resistance to hot water and fungicide substances, they can be stored for a long time in the environment: in the soil, in a forest litter, which promotes their proliferation in susceptible plants [6].

One of the stages of the inclusion of the fungus in the taxonomic database and assigning it a specific name is a description of its vegetative and reproductive structures in vivo and in vitro, as well as the study of the conditions of growth of mycelium and formation of sporophores.

In this work we have studied the cultural-morphological characteristics of the fungus *Phoma* sp.1 (the appearance of colonies, the rate of growth on nutrient media of different composition, the effect of pH of nutrient media and aeration of the culture medium on the biomass accumulation, and others).

Main part. The immediate object of study, a pure culture of the fungus *Phoma* sp.1, was isolated from diseased planting material of Norway spruce with typical symptoms of Phoma blight taken from the permanent forest nursery SFI "Ivatsevichi Forestry". Confirmation of the fungus belonging to a popular strain of *Phoma* sp.1 was made by analyzing its DNA, performed by employees of SSI "Institute of Forest" NAS of Belarus (O. Y. Baranov, S. V. Panteleev). Comparison of the nucleotide sequence of the object of research with the data of NCBI gene bank showed that the most closely related species is *Phoma glomerata* (Corda) Wollenw. & Hochapfel (97% level of similarity of genetic material).

The study of cultural-morphological characteristics of isolate of the fungus *Phoma* sp.1 was con-

ducted according to the protocol Boerema G. H. [3] using the following nutrient media: starvation agar, mash agar medium, oat agar, potato-carrot agar, agar medium Capek-Dox and standard medium Malt Extract Agar.

The evaluation of the effect of temperature on the growth processes were carried out under controlled conditions at its next values: +4; +15; +22; +28; +36°C.

To determine the degree of the effect of pH on biomass accumulation of the pathogenic fungus two different liquid nutrient media were used: potato dextrose and mash medium. Different pH rates of environment were created by adding the required volume of 1n. of the solute of NaOH or HCl. The evaluation of pH solutes were carried out with a pH-meter HI 8314 (membrane pH-meter). At the end of the experiment (on day 15), the mycelium was filtered, washed, dried at $t = 103 \pm 2$°C till the final weight loss that was controlled on torsion scales TW. The ability of the fungus to change the acidity of the nutrient medium was also evaluated by comparing pH value of the experimental variants with the control variant (without mycelia).

The aboveground and deep cultivations of mycelium (temperature – +22°C, the initial pH value of the mash environment – 4.5) in versions with mycelium growing duration of 7, 10, 14 and 21 days were carried out in order to study the effect of aeration on the growth processes of the strain. Morphological parameters of the mycelium were described, the rate of increase of its mass was determined.

All experiments were performed in 3 replications for each variant of the experiment, the data processing is done in the statistical part of the Excel spreadsheets.

The polymorphism of the pathogen was determined according to the colony morphology, according to the growth rate of mycelium, and according to the peculiarities of formation of chlamydospores.

Among the morphological features of the growth of mycelium in different agar nutrient media the following should be noted – at the initial stages of their growth the hyphae are at first light or light gray, then they are gradually darkening. Eventually, the mycelium becomes dark gray or dark ocher, and only the edge of the colony remains light. Certain isolates had cream mycelium marking after prolonged storage, the darkening of mycelium was observed only in the center of inoculum seeding. Often liquid droplets can be observed on the surface of the culture. Distinctive concentric zoning appears on a standard nutrient medium Malt Extract Agar, which later disappears.

The data on the effect of temperature on the growth of colony of the fungus *Phoma* sp.1 and on the morphology of mycelium on various nutrient media are presented in Table 1.

As a result of an experiment on the effect of temperature on the growth processes of *Phoma* sp.1 it was revealed that the highest growth rate was observed at a temperature of 22ºC in the following nutrient media: potato-carrot agar, mash environment and Malt Extract Agar – fungal colony grows in diameter on average 11.4 ± 0.5; 11.2 ± 0.4 and 10.2 ± 0.3 mm/day respectively. Lowering and raising the temperature have a certain fungistatic effect. At high temperatures (above 36°C) mycelium completely stopped its growth (died) within 12 hours after the beginning of the experiment.

Table 1

Morphological characteristics and growth rate of mycelium *Phoma* sp.1 on different nutrient media at different temperatures

Nutrient medium	The speed of grow thon the mycelium in the diameter of the colony, mm/day, at a cultivation temperature, °C				Time of the emergence of chlamydospores day, at +4°C	The appearance of a colony
	+4	+15	+22	+28		
Mash-agar	10.3 ± 0.3	3.2 ± 0.2	11.2 ± 0.4	4.2 ± 0.4	18−21	Slightly velvety, light gray, sometimes dark ocher, the edge of the colony is lighter
Potato-carrot agar	1.2 ± 0.3	3.3 ± 0.3	11.4 ± 0.5	4.11 ± 0.4	15−21	Velvety, rough texture, the color is gray in the center, the edge of the colony is lighter
Chapeck-Docs	1.3 ± 0.3	3.0 ± 0.4	9.8 ± 0.3	5.1 ± 0.4	15−21	Velvety, almost flat, light gray
Malt Extract Agar	1.8 ± 0.3	3.9 ± 0.4	10.2 ± 0.3	5.3 ± 0.3	25−30	Slightly woolly, with concentric zoning, gray-ocher
Starvation agar	0.6 ± 0.2	1.9 ± 0.3	6.8 ± 0.3	2.1 ± 0.2	6−10	Flat, translucent, dark gray
Oat agar	1.1 ± 0.2	3.0 ± 0.3	9.5 ± 0.2	4.3 ± 0.2	15−21	Coated, gray and light gray

Table 2

The influence of the nutrient medium on the biomass of *Phoma* sp.1

Potato-dextrose medium			Mash medium		
pH		The mass of mycelium, g/l	pH		The mass of mycelium, g/l
At the beginning of the experiment	At the end of the experiment		At the beginning of the experiment	At the end of the experiment	
2.5	2.5	–	2.5	3.0	0.58 ± 0.12
3.5	3.4	0.51 ± 0.01	3.5	3.8	1.10 ± 0.11
4.5	4.3	2.52 ± 0.12	4.5	4.8	3.45 ± 0.03
5.5	5.1	0.82 ± 0.05	5.5	4.9	1.58 ± 0.07
6.5	6.0	0.61 ± 0.08	6.5	5.8	1.18 ± 0.11
7.5	6.7	0.50 ± 0.01	7.5	6.1	1.07 ± 0.10
8.5	7.9	0.21 ± 0.02	8.5	7.4	0.69 ± 0.05
9.5	–	–	9.5	–	–

Resting stage of the fungus in the form of chlamydospores forms quicker at low temperatures. Thus, the time of occurrence of chlamydospores at +4°C ranges from 6 to 30 days. On starvation medium chlamydospores appeared already on the 7th day after the start of cultivation, while on the standard nutrient medium Malt Extract Agar chlamydospores appeared only after exhaustion/drying of the nutrient medium, about a month after the start of the experiment. The formation pycnidia occurred in the thickness of the nutrient medium, but the spores did not ripen (probably due to the lack of specific nutrients in the environment or the need to create certain lighting conditions).

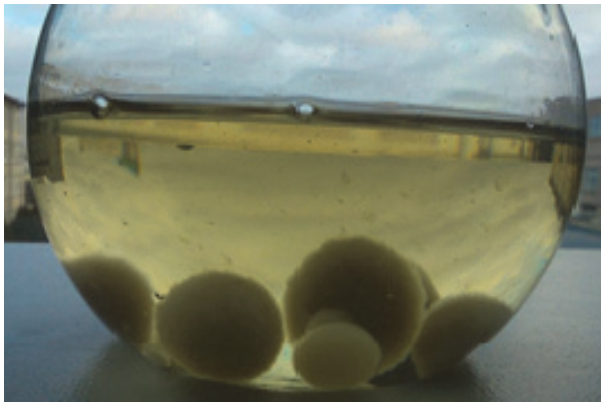
After prolonged storage (about one year at a temperature from 0 to +4°C) fugal culture easily reseeded to the fresh medium, forming colonies of the above-described appearance. The results of the effect of the acidity of the nutrient medium on the growth of mycelium *Phoma* sp.1 are given in Table 2.

These data show that the growth of the strain on a potato-dextrose medium is possible in the pH range from 3.5 to 8.5, on mash medium – from 2.5 to 8.6.

The narrowing of the acidity range for the growth of *Phoma* sp.1 on potato dextrose medium is probably due to the fact that the lack of batteries significantly reduces the tolerance of the fungus to the high alkaline and strongly acidic conditions.

The highest accumulation of the biomass of mycelium on mash medium took place in the variant of pH 4.8–4.9; on potato dextrose – pH 4.3. At such pH values there is a significant accumulation of biomass – 3.45 ± 0.03 and 2.52 ± 0.12 g/l, respectively. Consequently, in the absence of nutrients, the optimum acidity for the growth processes of the strain *Phoma* sp.1 is pH. 4.3–4.9.

The acidity of the nutrient medium is actively regulated by the strain. It has been registered that during its life the fungus alters the reaction of the medium at different pH values, bringing it closer to optimal for the development. Observations of the nature of the growth of *Phoma* sp.1 showed that the acidity of the medium affects the morphology of the fungus colonies. In liquid media with pH 4.3–5.8 fungus develops its typical structure of the mycelium (Figure).



The formation of specific areas
of the mycelium at deep cultivation
of *Phoma* sp.1 on potato dextrose medium

On acidic media (pH 3.5) the fungus colonies are located in dense separate islands in the medium depth of the nutrient medium. In alkaline media the colony is a loose, translucent mass. Differences in the accumulation of biomass under various conditions of aeration of the liquid medium were discovered (Table 3).

Under the conditions of low oxygen content the mycelium of the fungus *Phoma* sp.1 was a translucent loose white mass, located on the edge of the flask or creeping on the surface, the formation of chlamydospores was practically not observed.

The appearance of 3–4 fluffy cotton-like lumps was observed at the bottom of the flask on the second day of cultivation under constant aeration of the nutrient medium. The number of spheres of the mycelium is probably connected to the number of fragments that are separated from the inoculum. The growth rate of mycelium begins to slow down after two weeks of the experiment; it's probably because of the depletion of the medium and the accumulation of its metabolites of the pathogen.

Table 3

The influence of different circumstances of the aeration of nutrient medium on the accumulation of the biomass of *Phoma* sp.1

Method of cultivation	The weight of air-dry mycelium, g/l The average speed on of the accumulation of biomass, g/l·day			
	accounting days			
	7	10	14	21
Fixed (without aeration)	0.49 ± 0.07 0.07	0.51 ± 0.06 0.05	1.47 ± 0.21 0.10	2.15 ± 0.16 0.10
Shaking (with aeration)	1.76 ± 0.26 0.25	2.76 ± 0.01 0.28	4.99 ± 0.44 0.36	5.68 ± 0.52 0.27

Conclusion. The strain of pathogenic fungus *Phoma* sp.1 has a pronounced polymorphism, which is manifested in various conditions of growth and development.

The fungus *Phoma* sp.1 does not require special selective media with a complex composition for its growth and development. The optimal nutrient medium for the cultivation of the fungus is a standard mash-agar medium. Potato-carrot and Malt Extract Agar media also suit for the growth of the fungal mycelium. Mycelium can be stored on these media for a long time (one year or more) without loss of its properties. However, for quick vegetative spores (chlamydospores) it is recommended to grow the fungus on a starvation agar at a temperature of about +4°C. For the formation of asexual spores (pycnidia with conidia) specific culture conditions that are to be found out are required.

The mycelium on agar nutrient media acquires a typical color, texture and density. The morphology colonies can by of three types: flat, woolly, velvety. All gray colonies have a different shade, sometimes buffy. The pigmentation of nutrient medium is observed. For the development the fungus prefers weakly acidic medium (pH 4.3–4.9), but it can exist in acidic and strongly alkaline environments. Its degree of plasticity towards acidity depends on the richness of the nutrient medium.

The polymorphism is also present in different conditions of aeration of the medium. The average rate of accumulation of biomass of the fungus under constant shaking conditions in a liquid medium is 3–4 times greater than it is without aeration.

References

1. Yarmolovich V. A., Baranov O. Yu., Dishuk N. G., Seredich M. O. *Fomoz posadochnogo materiala v lesnykh pitomnikakh. Lesnoe i okhotnich'ye khozyaystvo* [Forest and hunting economy], 2013, no. 3, pp. 18–24 (In Russian).

2. Aveskamp M. M., De Gruyter J. and Crous P. W. Biology and recent development in the systematic of *Phoma*, a complex genus of major quarantine significance. Fungal Diversity 38, 2008, pp. 1–18.

3. Boerema G. H., Gruyter J., Noordeloos M. E. *Phoma* identification manual. CABI Publishing, Cambridge, 2004, pp. 1–479.

4. Aveskamp M. M. Phylogeny and DNA-based identification in *Phoma* and related genera, Wageningen. 2014, pp. 18−24.

5. National Center for Biotechnological Information, NCBI [Electronic resource]. Available at: http://www.ncbi.nlm.nih.gov (accessed: 14.09.2015).

6. James R. L. Effects of fumigation on soil pathogens and beneficial microorganisms. Paper presented at the intermountain forest nursery association annual meeting, Bismarck, North Dakota, august 14–18, 1989.

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