FOREST PROTECTION AND LANDSCAPING

УДК 632.4:630*165.3

O. Yu. Baranov, PhD (Biology), leading researcher (Forest Research Institute of the NASB);
S. V. Panteleyev, PhD (Biology), senior researcher (Forest Research Institute of the NASB);
V. A. Yarmalovich, PhD (Biology), assistant professor (BSTU);
M. O. Romanenko, PhD student (BSTU)

MOLECULAR GENETIC ASPECTS OF DIAGNOSTICS AND IDENTIFICATION OF PHOMA BLIGHT AGENTS

Molecular phytopathological monitoring of forest nurseries revealed nine major *Phoma* species, which caused needle blight of pine and spruce (*Ph. pomorum*, *Ph. macrostoma*, *Ph. herbarum*, *Ph.* sp. 1-6). Comparative analysis of diagnostic loci identified major types of species differences, which are represented by mononucleotide substitutions, oligonucleotide deletions and insertions. Genus specific rDNA primers were designed for the subsequent diagnosis of pathogens in plant material and soil by PCR-assay.

Introduction. Phoma blight is an infectious disease, which attacks agricultural and forest plant species and caused by *Phoma* spp. fungi [1]. A considerable number of coniferous and deciduous plants are susceptible to the pathogen. *Phoma* fungi can attack different plant parts of fir, spruce, pine, larch, Douglas fir, maple, ash, walnut, birch and some other tree species. Phoma blight is most harmful to young plants including seedlings and saplings are cultivated in forest nurseries [2]. Up to now in Belarus only coniferous seedlings have been diagnosed with Phoma blight [3].

This infectious disease reaches its development peak in rainy seasons or excessive soil moisture periods [4]. The fungal spores are able to maintain their germinability for a long time (several years) in the upper soil layers or forest floor. The outbreak of Phoma blight agent spores is usually registered in the spring season. Either the apical buds get infected first (then the mycelium invades the stem of the plant) or the coniferous plant is infected through its needles which touch the forest floor [5]. The outbreak is also facilitated by heavy rains or spatters in cases of artificial watering. As a result: cones are formed around the seedlings or the seedlings become fully covered by the soil and catch the infectious spores from the forest floor. Thereafter the fungus spreads along the stem and up to its apical part subsequently causing defoliation and death of the apical bud. Infected leaves of plant are characterized by chlorosis, in particular those, which are touching the forest floor. As the disease progresses, the photosynthetic potential of the plant gets destroyed, which leads finally to death. The seedlings growing in soils with excessive calcium or iron content are extremely susceptible to Phoma blight [1].

The main external signs of seedlings Phoma blight are the following: the needles of current year first acquire goldish-brown color, then they are discolored to brown, turn ash-grey, dry out and fall. The early stage of the disease is characterized by a declining current increment, and then the seedling perish completely. Two years and older plants may be damaged only partially where only offshoots of the current year can dry out together with their leaves. The offshoots often get twisted [5-8].

This disease is widely spread all over the world, though for Belarus it is quite new. Therefore we can talk about only limited number of literature sources providing some data about the Phoma blight of tree species in this country. However, probably, the rare cases of Phoma blight - detection in Belarus can be explained by the fact that the disease is very difficult to diagnose by visual methods, particularly if only yellowing and drying-out of leaves are detected. Besides, no fungus spores are found on the surface of the attacked parts of the plant. For instance, brown discoloration and dying-off of the seedlings needles, which are typical signs of Phoma blight attack, are often confused with pine-leaf cast or blight.

Based on the above, the objective of this paper is to study species-specific features of diagnostic loci of Phoma blight agents in forest tree seedlings with the subsequent aim to elaborate molecular-genetic method of their early diagnostics and species identification. The scientific novelty of the obtained results involves determining the dominant *Phoma* species composition in the territory of Belarus, identifying and typing of species without mycological description, specifying systematic location of *Phoma* genus.

Research materials and methods. The experimental material was collected in 2011-2013 in 38 forest nurseries located in the Minsk, Mogilev, Grodno, Vitebsk and Brest regions. To carry out molecular-phytopathological diagnostics, samples of infected vegetative organs of forest planting stock were collected. The total number of the diagnosed infected samples was 1,120 pcs. The analyzed planting stock was represented by 11 forest tree species: pine, spruce, thuja, fir, larch, juniper, birch, oak, maple, linden, ash. To undertake phytopathological analysis of soil, some additional experimental material was collected in the territory of the forest nurseries representing 260 soil samples from the rhizosphere of the infected seedlings.

The DNA was extracted from plant tissue by the CTAB method, from soil – by the PEG method without preliminary isolation of cells of pathogens [9]. Samples of healthy tissue were analyzed as well for benchmarking purposes.

PCR analysis was carried out using DreamTaq™ Green PCR Master Mix (Fermentas) as prescribed by the user manual of the manufacturing company. During the research we used universal primers ITS1 and ITS4, which flank the rDNA region: ITS1 − 5,8S rRNA − ITS2 [10]. Electrophoretic assay of PCR-products was carried out in 1 % agarose gel High Efficiency of Separation (Pharmacia Biotech) for their efficient separation and typing. To identify the species composition of fungi, all PCR-bands were cut out of gel and sequenced by genetic analyzer ABI Prism 310 (Applied Biosystems) with the utilization of BigDye Terminator Sequence Kit v.1.1 as prescribed by the manufacturing company. Nucleotide structure of the sequenced fungi loci was analyzed by BLAST software in GenBank NCBI database [11].

Results and discussion. During the DNA-analysis of microflora of the plants and soil a wide spectrum of micromycetes was identified. At the same time *Phoma* generic types [12] were identified only in the the conifers plants. It should be noted as well that among the identified pathogenic diseases of pine and spruce seedlings (during test period) Phoma blight was most prevalent with 34% of the infected plants being diagnosed with it.

In the infected plants *Phoma* spp. was present both as monoinfection and as pathogenic complexes composed of some ecologically alike fungi such as *Sphaeropsis*, *Epicoccum*, *Botrytis,* etc.

The molecular genetic benchmark analysis of all identified isolates showed that they belonged to nine species, three of which were identified as *Ph. pomorum*, *Ph. macrostoma*, *Ph. herbarum*, the rest six ones revealed no homology with the samples of NCBI genetic database (more than 100 species). At the same time the level of genetic differentiation of diagnostic loci of the isolates was more than 2%, that indicate viability of the species status [13-16].

The study of the genetic variation of the identified *Phoma* spp. isolates revealed a high level of conservation of species specific rDNA region that proves their functional importance as well as of cosmopolitan pattern of the species expansion during phylogenetic process. Thus, for instance, the *Ph. pomorum* isolate identified in a nursery of the Bykhov forestry showed 99% similarity of DNA sequence to the Mexican strain *Ph. pomorum* AY904062.1 and the Korean strain *Phoma* sp. FJ950743. The *Phoma* sp. 3 isolate from a nursery of the Miloshevichi forestry had 99% genetic similarity to *Phoma* sp. NRRL JN093264.1 (USA), *Phoma* sp. P48E6 JN207352 (Venezuela), *Phoma* sp. CPO 10.003 JQ388280 (Mexico), *Phoma herbarum* JQ936331.1 (Brazil), *Phoma herbarum* GU073116 (China) and *Phoma* sp. R79-10 AB693778 (Japan). The *Phoma* spp. isolates, identified in the Oktyabr forestry, were to 99% identical to those from Venezuela (JN207352), the USA (HQ846581), China (HQ696085), Japan (AB693798) and Korea (HM008925), etc.

The detailed analysis of the rDNA loci structure of the identified *Phoma* spp. genotypes revealed species specific features of nucleotide sequence represented deletion/insertion of oligomeric fragments in the second internal transcribed spacer. The identified deletion include duplicated motifs (-GCGC- and -AGGC-). Besides, the detailed study of the spacer structure showed a large number of repeating sequences within ITS2 (CGT motif) that indicate the duplication processes of nucleotides sequences during phylogenesis and their subsequent species divergence [17]. The results of clustering (based on the aligment of nucleotide sequences) of the studied *Phoma* spp. isolates showed two different species clusters in a dendrogram, the genetic difference between their average samples being 2.2% (22 differences for 1000 nucleotides).

To specify taxonomic status of *Phoma* species we compared average genotypes of isolates with other representatives of Dotideomycete class – *Teratosphaeria*, *Epicoccum*, *Alternaria*, *Venturia*, *Cladosporium*, *Sphaeropsis* [12]. Based on the obtained coefficients of genetic differentiation it was determined that *Phoma* species is genetically close to the species of the *Pleosporaceae* family. Thus, the obtained genetic data can be used to relegate *Phoma* species to Pleosporous fungi. Moreover, the genetic difference between *Phoma* and *Epicoccum* doesn't exceed 1.5%. This factor can show the need of further revision of the two species and their subsequent unification.

Based on the determination of genus specific regions in the rDNA nucleotide sequence specific primers were developed to carry out molecular genetic di-

agnostics of the Byelorussian *Phoma* spp. isolates by classical PCR and real-time PCR methods.

Conclusion. The carried out molecular-phytopathological study of forest nurseries did it possible to identify nine species of *Phoma* fungi which were preliminary assessed as pathogenic to pine and spruce seedlings. The benchmark analysis of diagnostic pathogen loci revealed main types of generic differences. Based on the study of conservative (within *Phoma* genus) rDNA regions, primers were developed to diagnose Phoma blight agents in plants and soil by PCR method.

References

1. Davidson J. A. A new species of *Phoma* causes ascochyta blight symptoms on field peas (*Pisum sativum*) in South Australia // The Mycological Society of America. 2009. Vol. 101. P. 120–128.

2. Шевченко С. В., Цилюрик А.В. Лесная фитопатология. Киев: Вища школа, 1986. 382 с.

3. Молекулярно-генетическая диагностика грибных болезней в лесных питомниках / О. Ю. Баранов [и др.] // Лесное и охотничье хозяйство. 2012. № 6. С. 21–29.

4. Семенкова И. Г., Соколова Э. С. Фитопатология: учеб. для студ. вузов. М.: Издат. центр «Академия», 2003. 480 с.

5. James R. L. Lodgepole pine seedling chlorosis and mortality at the Bessey Nursery, Nebraska / Bio. Eval. R2-79-2. Denver, CO: U.S. Department of Agriculture, Forest Service, State and Private Forestry, Rocky Mountain Region. 1979. 10 p.

6. James R. L. Engelmann spruce needle and twig blight at the Coeur d'Alene Nursery, Idaho / U.S. Department of Agriculture, Forest Service, Northern Region. 1980. 7 p.

7. James R. L. Mortality of Mugo pine seedlings at the Fantasy Farms Nursery, Peck, Idaho / U.S. Department of Agriculture, Forest Service, Northern Region. 1983. 7 p.

8. Phoma blight of fir and Douglas-fir seedlings in a California nursery / J. T. Kliejunas [et al.] // Plant Disease. 1985. Vol. 69. P. 773−775.

9. Падутов В. Е., Баранов О. Ю., Воропаев Е. В. Методы молекулярно-генетического анализа. Минск: Юнипол, 2007. 176 с.

10. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics / T. J. White [et al.] // PCR Protocols: A Guide to Methods and Applications / M. A. Innis [et al.]. New York: Academic Press Inc., 1990. P. 315−322.

11. National Center for Biotechnological Information, NCBI [Electronic resource]. URL: http://www.ncbi.nlm.nih.gov (date of access: 14.09.2011).

12. Fungal Databases Nomenclature and Species Banks [Electronic resource]. URL: http:// www.mycobank.org (date of access: 14.09.2011).

13. Molecular diagnostics for fungal plant pathogens / H. Alastair [et al.] // Pest Management Science. 2003. Vol. 59. P. 129–142.

14. Berbee M. The phylogeny of plant and animal pathogens in the Ascomycota // Physiological and Molecular Plant Pathology. 2001. Vol. 59. P. 165–187.

15. Borman A. M. Molecular identification of pathogenic fungi // Journal of Antimicrobial Chemotherapy. 2010. Vol. 61. P. 7–12.

16. Дьяков Ю. Т. Общая и молекулярная фитопатология. М.: Общ-во фитопатологов, 2001. 301 с.

17. Levinson G., Gutman G. A. Slipped-strand mispairing: a major mechanism for DNA sequence evolution // Molecular Biology and Evolution. 1987. Vol. 4. P. 203–221.

Received 04.02.2014